ECOLOGY OF AMPHIBIAN-MICROBIAL SYMBIOSES

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ECOLOGY OF AMPHIBIAN-MICROBIAL SYMBIOSES

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Image: "*Litoria serrata*" by Ross A. Alford. This is one of the species studied by Bell et al., 2018 as part of this Research Topic.

The field of amphibian microbial ecology has greatly advanced in recent years. The work published to date has shown that amphibian skin bacterial communities can be influenced by host species, host life-history stage, environmental conditions, surrounding bacterial communities that serve as reservoirs and external biotic agents such as pathogens.

As the ecology of amphibian-microbial symbiosis is a relatively new field, there are still many unanswered questions. The aim of this Research Topic is to highlight recent research on amphibian microbiomes that addresses relevant questions on the ecology of amphibian-microbe interactions. The publications gathered in this

Research Topic have expanded our knowledge on the role of microbial symbionts of amphibians and have revealed novel insights that can direct the next set of research questions.

We suggest that soon the field will move from the basic (and necessary) descriptions of microbial communities to more experimental approaches that include the use of omics methods and a variety of novel analytic and multivariate approaches. In addition to providing more insights into the microbial and disease ecology of amphibians, these studies may lead to effective ways to manipulate the microbiome to achieve protection from diseases, such as chytridiomycosis.

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INTERACTIONS OF AMPHIBIANS' MICROBIAL COMMUNITY WITH *BATRACHOCHYTRIUM*

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EDITORIAL
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Editorial: Ecology of Amphibian-Microbial Symbioses

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Keywords: microbiota, amphibians, chytridiomycosis, symbiosis, antifungal bacteria

Editorial on the Research Topic

Ecology of Amphibian-Microbial Symbioses

Symbiotic interactions among microorganisms and animals are ubiquitous in nature. Recent studies on amphibians have identified bacterial communities that establish symbiotic relationships with their hosts. Specifically, skin bacteria are known to play a relevant role in protecting the host against pathogens such as *Batrachochytrium dendrobatidis* (Bd) and the more recently discovered *B. salamandrivorans* (Bsal). Many of these bacterial symbionts have been isolated in culture and have been shown to produce antifungal metabolites with the capacity to inhibit Bd growth *in vitro* and *in vivo*. These findings have led to the development of probiotics as a promising strategy against chytridiomycosis, the disease caused by Bd and Bsal, which has caused dramatic declines and extinctions of amphibian species worldwide. In this context, the protective role of the skin microbiota, in addition to and likely in concert with the host immune responses, may be fundamental to the survival of amphibians that encounter emerging infectious diseases.

The field of amphibian microbial ecology started in earnest about 12 years ago, and it has greatly advanced since the development of next generation sequencing technologies and bioinformatic tools. The work published to date has shown that skin bacterial communities can be influenced by host species, host life-history stage, environmental conditions, surrounding bacterial communities that serve as reservoirs and external biotic agents such as pathogens. In addition to the bacterial component, the skin microbiome is also composed of symbiotic fungi that may also be protective, but whose role largely remains to be determined. Moreover, amphibians harbor symbiotic microbiota within their digestive tract, and there is growing evidence that these microorganisms may also play an important role for their hosts' health.

As the ecology of amphibian-microbial symbiosis is a relatively new field, there are still many unanswered questions such as: How stable are skin communities over time, and how resilient are they to perturbations? How do hosts influence the structure and function of these communities? What are the interactions occurring within these communities? And how do skin microbiomes function to protect amphibian hosts from pathogens, and under what conditions do pathogens change microbial community structure? Are key microbial species adapted to live on amphibian skins?

The aim of this Research Topic is to highlight recent research on amphibian microbiomes that addresses relevant questions on the ecology of amphibian-microbe interactions. The 21 publications gathered in this Research Topic have expanded our knowledge on the role of microbial symbionts of amphibians and have revealed novel insights that can direct the next set of research questions.

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Many of the studies published in this Research Topic are based on field surveys. This is to be expected in a scientific discipline where the ability to characterize communities with culture-independent methods has only recently been possible. We are just now learning what amphibians' microbial communities look like and what may be affecting community structure. Studies that describe skin microbiota in natural settings aimed to evaluate how environmental factors, microhabitat conditions or host genetic traits influence the skin community diversity.

Specifically, Hernández-Gómez et al. evaluated how skin bacterial community structure of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*) varied across sites. The authors found a high OTU turnover with changes in latitude and elevation, suggesting that environmental factors have a strong effect on skin microbiota. In addition, they found that skin community divergence was positively correlated with host population genetic divergence. Bird et al. evaluated the effect of environmental bacteria and host phylogenetic relatedness on two terrestrial salamander genera from the family Plethodontidae. The authors found that environmental factors were likely playing a more significant role in salamander cutaneous microbiome assemblages than host-specific traits. On a broader geographic scale, Bletz et al. determined that the microhabitat (arboreal, terrestrial, or riparian) is the strongest predictor of skin bacterial community structure on 89 frog species across 30 sites in Madagascar, with host phylogeny explaining less but significant portions of the observed variation.

Additional studies contribute to the descriptions of skin microbiota in tropical amphibians: Abarca et al. found that skin bacterial community structure of the Cane Toad (*Rhinella marina*) differed between Costa Rica and Puerto Rico. Moreover, Abarca et al. described the skin microbiota of 12 species of frogs at La Selva Biological Station in Costa Rica and found clear differences in skin microbial composition among different host species. Varela et al. analyzed the skin microbiota of three dendrobatid species in Panama and found that bacterial structure and predicted function may be influenced by environmental variables such as soil pH and precipitation. Additionally, Familiar-López et al. analyzed the skin bacterial communities of the cryptic frog *Philoria loveridgei* in tropical Australia. The authors found a strong effect of temporal variation (two years of sampling under distinct precipitation regimes) on the bacterial community structure and on the presence of Bd. They identified several OTUs whose relative abundances were significantly correlated with the presence of the pathogen. These studies confirm that species-specific and site-specific factors as well as environmental factors are involved in shaping skin microbial communities.

Many studies in this Research Topic focused on analyzing host-pathogen-bacteria interactions through different approaches including field studies, bacterial culturing and experiments. In a field survey of three species of frogs in tropical Australia, Bell et al. found that the upper limit of Bd infection intensity was negatively correlated with the number of inhibitory bacteria suggesting a protective role of the bacterial community. Catenazzi et al. found in a study of 28 frog species in the Peruvian Andes that the proportion of anti-Bd isolates was negatively associated with susceptibility to Bd, again suggesting a protective role of an anti-Bd bacterial community. In an experimental study, Robak and Richards-Zawacki found that bioaugmentation with an inhibitory bacterial isolate led to longer survival of a Bd infection at 14°C, but not at 26°C, demonstrating the important role of temperature in the bacterial community's protective function. However, in an experimental study, Jani and Briggs found that the effect of Bd on the skin microbiome of *Rana sierrae* from the western US was greater than the effect of microbial community structure on Bd infection intensity. Thus, the skin microbiota can have a protective function, although if the pathogen Bd is able to achieve wide-spread infection, then Bd can alter bacterial community structure. In addition, the authors determined a clear effect of the host and the aquatic environment on skin microbial structure.

Anthropogenic factors in the environment may have a negative effect on the protective nature of the microbiota. Huang et al. found evidence that the gut microbiota of frogs in degraded habitats differed from the microbiota in a natural habitat, and these differences could be related to disease susceptibility. The effect of pesticides discussed in McCoy and Peralta will likely cause alterations on host-bacteria-pathogen interactions. Thus, this topic requires further investigations that will be particularly relevant to amphibian species that are exposed to human activities such as agriculture and cattle raising.

Several studies evaluated pathogen-bacteria or bacteria-bacteria interactions in order to inform probiotic development. Probiotics rely on our capacity to isolate in culture a high proportion of bacteria. In this respect, Medina et al. evaluated the proportion of culturable bacteria using different media and determined that a high proportion of the skin community on American toads (*Anaxyrus americanus*) is culturable and that these bacteria include antifungal bacteria independent of isolation media. In an experiment involving the gut microbiota of an Asian tropical treefrog (*Polypedates megacephalus*), Weng et al. obtained interaction networks from 16S amplicon sequencing data, and the interactions were validated through bacterial inoculations. The authors showed that as opposed to single strains, a combination of cooperative microbes yielded a higher relative abundance of probiotic bacteria and did not have negative effects on the acquired immune system. Muletz-Wolz et al. found that *Batrachochytrium* strains (including Bsal) and temperature interact to determine the ability of bacteria to inhibit the pathogen, again highlighting the importance of temperature as shown by Robak and Richards-Zawacki. Bacteria that inhibit the pathogens over a range of temperatures would be preferred probiotics. Bletz et al. also found that bacterial inhibition could be a function of Bd genotypes, but that some bacterial species showed broader spectrum inhibition against all tested Bd genotypes and that these OTUs would be better probiotic candidates. Kueneman et al. characterized skin-associated bacterial and micro-eukaryotic diversity and used a network analysis to identify species that inhibited Bd and thus could be probiotic candidates. Kearns et al. presented evidence that anti-Bd fungal species could be effective probiotics while not negatively affecting responses of the acquired and innate immune systems as some bacterial species

can do. These last two studies emphasize the need to study skin fungi as important microorganisms present in amphibian skin microbiomes.

Omics methods (metagenomics and transcriptomics) are starting to be used to study microbial function and will certainly expand our knowledge on host-microbe interactions. Rebollar et al. used a shotgun metagenomic approach and found that the skin of *Craugastor fitzingeri* contained bacteria with a wide variety of genes that code for secondary metabolites, which may be involved in bacterial-bacteria communication and bacteria-host interactions and which could in addition protect the host against fungal pathogens. Madison et al. used transcriptome sequencing of the antifungal skin resident, *Serratia marcescens*, and demonstrated that key genes were up- and down-regulated in response to Bd presence *in vitro*.

In summary, this Research Topic has compiled a variety of studies that address relevant questions about symbiotic microbiomes in amphibians with particular emphasis on their role in pathogen protection. These studies used distinct approaches to analyze skin and gut microbial diversity such as culturing, 16S and I8S amplicon sequencing, shotgun metagenomics and transcriptomics. Overall, it is becoming clear that different abiotic and biotic factors shape skin microbial communities and that host-bacteria-pathogen interactions are dependent upon many of these factors. We suggest that soon the field will move from the basic (and necessary) descriptions of microbial communities to more experimental approaches that include the use of omics methods and a variety of novel analytic and multivariate approaches. In addition to providing more insights into the microbial and disease ecology of amphibians, these studies may lead to effective ways to manipulate the microbiome to achieve protection from diseases.

AUTHOR CONTRIBUTIONS

ER and RH contributed equally to the proposal and editorial work of this Research Topic. Both ER and RH equally contributed to the writing of the Editorial.

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ORIGINAL RESEARCH
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Inferring Microbial Interactions in the Gut of the Hong Kong Whipping Frog (*Polypedates megacephalus*) and a Validation Using Probiotics

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The concerted activity of intestinal microbes is crucial to the health and development of their host organisms. Investigation of microbial interactions in the gut should deepen our understanding of how these micro-ecosystems function. Due to advances in Next Generation Sequencing (NGS) technologies, various bioinformatic strategies have been proposed to investigate these microbial interactions. However, due to the complexity of the intestinal microbial community and difficulties in monitoring their interactions, at present there is a gap between the theory and biological application. In order to construct and validate microbial relationships, we first induce a community shift from simple to complex by manipulating artificial hibernation (AH) in the treefrog *Polypedates megacephalus*. To monitor community growth and microbial interactions, we further performed a time-course screen using a 16S rRNA amplicon approach and a Lotka-Volterra model. Lotka-Volterra models, also known as *predator–prey equations,* predict the dynamics of microbial communities and how communities are structured and sustained. An interaction network of gut microbiota at the genus level in the treefrog was constructed using Metagenomic Microbial Interaction Simulator (MetaMIS) package. The interaction network obtained had 1,568 commensal, 1,737 amensal, 3,777 mutual, and 3,232 competitive relationships, e.g., *Lactococcus garvieae* has a commensal relationship with *Corynebacterium variabile*. To validate the interacting relationships, the gut microbe composition was analyzed after probiotic trials using single strain (*L. garvieae, C. variabile,* and *Bacillus coagulans*, respectively) and a combination of *L. garvieae*, *C. variabile*, and *B. coagulans*, because of the cooperative relationship among their respective genera identified in the interaction network. After a 2 week trial, we found via 16S rRNA amplicon analysis that the combination of cooperative microbes yielded significantly higher probiotic concentrations than single strains, and the immune response (interleukin-10 expression) also significantly changed in a manner consistent with improved probiotic effects. By taking advantage of microbial community shift from simple to complex, we thus constructed a reliable microbial interaction network, and validated it using probiotic strains as a test system.

Keywords: network, gut microbiota, artificial hibernation, probiotics, *Polypedates megacephalus*

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INTRODUCTION

Gut microbes and their hosts exist in a symbiotic relationship. Gut microbes contribute to important host functions, including fermenting unused energy substrates, training the immune system, preventing growth of pathogenic bacteria, and regulating gut development (Hooper et al., 2002; Xu and Gordon, 2003; Li et al., 2008; Perez et al., 2010; Ye et al., 2014). Although recent studies using 16S rRNA amplicon sequencing have emphasized the importance of microbes for their hosts (Manichanh et al., 2006; Peterson et al., 2009; Round and Mazmanian, 2009; Turnbaugh and Gordon, 2009; Turnbaugh et al., 2009; Arumugam et al., 2011), the functional roles of most gut microbes remain unknown. One approach to explore this question is through network inference. This approach has been widely used to explore interactions between various organisms (Newman, 2003; Proulx et al., 2005; Shafiei et al., 2014). To uncover hidden patterns beyond the animal world, the generalized Lotka-Volterra (gLV) equations have recently been adopted as a dynamic model for studying microbial communities (Faust and Raes, 2012; Stein et al., 2013). The gLV model uses non-linear differential equations, which govern prey-predator relations. The gLV equations have been used successfully to predict temporal dynamics of microbiota in the mouse intestine (Stein et al., 2013), and within a cheese-making environment (Mounier et al., 2008), by analyzing microbiome time-series data. Time-series data inherently contain information including the statistical dependency of observations as a function of time. When these features of time-series data are properly modeled, it is possible to gain substantial new insights into the behavior of the system under study. Some studies even suggested that the distribution of interaction pairs (also obtained using a gLV dynamic model) in an ecological system can be used to predict microbiota stability (Coyte et al., 2015).

Network inference methods are commonly distinguished into two groups. The first approach is similarity-based network inference, which assesses the co-occurrence and/or mutual exclusion pattern of two species over multiple samples, using a measure that quantifies the similarity of two species' distributions. However, pairwise relationships do not capture more complex forms of ecological interactions, in which one species is influenced by (or depends on) multiple other species (Faust and Raes, 2012). To infer these types of interactions, the second approach is to apply regression-based networks, in which the abundance of one species is predicted from the combined abundances of other organisms. The latter is more convincing and was used by Stein et al. (2013) to reanalyze the *Clostridium difficile* infection data generated by Buffie et al. (2012). Marino et al. (2014) also used the gLV equations to model population dynamics of the gut microbiota in mice. However, these works generated interaction networks but did not further validate the inferred relations. To explore how the biological outcomes are directly related to the specific inferred microbial interactions, a well-investigated *in vivo* system describing the effects of gut microbes on their host must be applied for validation. Conventionally, the prevention and control of aquaculture diseases has focused on the use of vaccines or antibiotics (Pasteris et al., 2009b). However, treating or feeding frogs with antibiotics may cause the development of resistant bacteria (Akinbowale et al., 2006). Further evidence has shown that antibiotics can cause a decrease in the biodiversity of gut bacteria and increase the risk of bacterial infections (Buffie et al., 2012; Taur et al., 2012). Antibiotics and their effects can also persist for several days after the end of treatment (Jernberg et al., 2007; Dethlefsen and Relman, 2011; Buffie et al., 2012). An alternative solution is the use of probiotics (Reid et al., 2003), which are able to inhibit gut colonization by pathogens and to exert inhibitory effects against undesired micro-organisms, as well as to support natural host microbial defense mechanisms (Hernandez et al., 2005). Thus, a wide range of Gram (+) and Gram (−) bacteria, yeast, microalgae, and bacteriophages have been evaluated as probiotics (Pasteris et al., 2009b).

In this study, we highlight the feasibility of conducting network inference at the genus level to decipher the possible interactions within the microbial ecosystem of the amphibian gut. We took advantage of a well-investigated probiotic system to validate the inferred microbial interactions. Compared to the conventional strategy that orally introduces specific single bacterial species as probiotics, we emphasize the power of inferred network by simultaneously applying interacting microbial partners that can be used together to enhance the growth and beneficial effects of target probiotics. In addition to the validation of the inferred network, this test is also the first attempt to use a combination of cooperative strains as probiotics in raniculture.

The innate immune system is the first line of an organism's defense against infection. Probiotics interact with immune cells, such as mono-nuclear phagocytes, polymorphonuclear leukocytes, and natural killer cells, to enhance innate immune responses. Studies have shown that probiotics can increase the numbers of erythrocytes, granulocytes, macrophages, and lymphocytes (Balcazar et al., 2006; Akhter et al., 2015). The most commonly used probiotics in amphibians are lactic acid bacteria (LAB). LAB produce a range of important molecules such as organic acids, hydrogen peroxide, diacetyl, antimicrobial peptides (AMPs), and bacteriocins (Verschuere et al., 2000; Küng et al., 2014). The characterization of these compounds explains the beneficial effects of LAB; thus, some bacterial species with similar functions have been introduced as probiotics to restore beneficial microbial populations (Balcázar et al., 2007a; Pasteris et al., 2009b; Ringø et al., 2010; Mendoza et al., 2012). In this study, we induced a community shift from simple to complex via artificial hibernation (AH) in the treefrog and performed 16S rRNA amplicon analysis on the gut microbiome. Through the use of MetaMIS, a package that employs the gLV model to infer microbial interactions (Shaw et al., 2016), we generated the microbial interaction data and constructed the interaction network. In order to validate the microbial interactions, instead of the conventional strategy that only introduces bacteria with similar functions as probiotics, we selected a target LAB, and selected bacteria of the genera *Corynebacterium* and *Bacillus* as functional partners based on the network analysis. Our results

Abbreviations: IIP, inferred interaction partner; AH, artificial hibernation.

showed that the combination of three representatives of these genera, *L. garvieae*, *C. variabile*, and *B. coagulans*, works more efficiently than any single strain, reflecting the reliability of the inferred microbial interaction.

MATERIALS AND METHODS

Sample Collection

Eighty adult treefrogs (*Polypedates megacephalus*) were collected from New Taipei City and Taichung City, Taiwan. All animals were housed in 240-l glass tanks at 23°C under a 8:16 h light:dark cycle. Turkestan cockroach nymphs (Finke, 2013) were fed to the treefrogs at a quantity of 10% of treefrog biomass twice a week. The treefrogs were acclimatized for 3 months prior to experiments.

Microbiome 16S rRNA Amplicon Analysis

Fecal samples were collected from treefrog guts within 20 min after euthanasia. To avoid cross-contamination, each sample was collected using a fresh pair of sterile tweezers. The contents of each gut were emptied into a sterile vial and immediately stored at −80°C. DNA was subsequently extracted with the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). The V4 region (292 bp) of 16S rRNA gene was PCR-amplified with 515F (5′−GTGCCAGCMGCCGCGGTAA−3′) and 12-base barcoded 806R (5′−GGACTACHVGGGTWTCTAAT−3′) primers (Caporaso et al., 2012). Following PCR, samples were gel extracted with the NucleoSpin Gel Extraction kit (Macherey-Nagel, Germany). The purified samples were pooled in equal concentrations and sequenced using an Illumina MiSeq (Illumina, San Diego, CA, USA) with a V2 PE500 cartridge (500 cycles). All datasets have been deposited in GenBank under the BioProject ID PRJNA341914 and BioSample ID SAMN05730167 and SAMN05730170.

All paired-end sequences were merged by FLASH (Magoč and Salzberg, 2011), and all merged sequences were further analyzed by conducting the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010b). In the QIIME analysis pipeline, the low-quality sequences (sequences that were <200 bp in length, had a quality score <25, contained ambiguous characters, had an unreadable barcode, or did not contain the primer sequence) were removed using the USEARCH quality filter (Edgar, 2010). The UCLUST (Edgar, 2010) function in QIIME was used to cluster the remaining sequences, with a minimum coverage of 99% and minimum sequence identity of 97%. The longest sequences from each phylotype were selected to perform sequence identification, and PyNAST (Caporaso et al., 2010a) and UCLUST were selected to perform the sequence alignment and taxonomy assignment.

Microbial Community Shift from Simple to Complex and the Generalized Lotka-Volterra Model

A concept for improvement of the investigation of the functional roles of gut microbiota is to explore the microbial world from a simple to complex state, from an initial stage to homeostasis. Hibernation is considered as a survival strategy designed to conserve energy when conditions are harsh. During hibernation, animals can adapt to temperature perturbations and extend their lifespan by slowing their heartbeat, reducing metabolic activity and their energy requirements (Book, 1974). At the same time, most microorganisms in the gut of a host decline in numbers due to extreme temperature and low nutrient supply (Gossling et al., 1982a). Hibernation of treefrogs, therefore, provides a natural model for monitoring microbial growth starting from inoculation (i.e., the beginning of a developing microbial interaction network) to homeostasis, and could provide an opportunity to shed light on our understanding of how microorganisms form their interaction networks.

To stimulate AH, we modified a program previously described in leopard frogs (*Rana pipiens*) (Gossling et al., 1982a). Fasting is known to reduce microbial complexity in amphibians, as well as fish, reptiles, birds, and mammals (Gossling et al., 1982b; Sonoyama et al., 2009; Costello et al., 2010; Kohl et al., 2014). Therefore, prior to stimulation of AH, food was withheld from the treefrogs kept in the same housing for 7 days to reduce overall diversity. All fasting treefrogs were then transferred into an incubator kept in constant darkness. AH was stimulated initially at 21°C with a relative humidity of 90%. During day 1, the temperature was maintained. During days 2–3, the temperature was gradually reduced to 4°C. Thereafter, treefrogs were housed in the same manner for 7 days. During days 11–12 (i.e., after 7 days of AH described above), the temperature was gradually increased to 21°C, and the treefrogs returned to their active status. Each active treefrog was fed with Turkestan cockroach nymphs (∼0.25–0.3 g). Post-feeding samples were collected within 2.5 days. All protocols were approved by the Academia Sinica Biosafety Committee and Institutional Animal Care and Utilization Committee.

To collect time series samples for generating the microbial interaction network, we collected the gut contents of the treefrogs over a total of 12 time points spanning 15 days over the AH period, including day 1 (fasting for 7 days before AH), day 11–11.75 (every 6 h), and days 12–15 (every 12 h). For each time point, three to five treefrogs were euthanized. Details of treefrog body mass, sampling size, and time points of fecal collection over the AH period are presented in **Supplementary Table 1**.

We used the Metagenomic Microbial Interaction Simulator (MetaMIS) with a user friendly interface (Shaw et al., 2016) to infer microbial relations by introducing a single time-series dataset of microbial composition containing 12 time points. MetaMIS is a tool based on the generalized Lotka-Volterra model (Bhargava, 1989), and designed to infer underlying microbial interactions according to metagenomic abundance profiles. Lotka-Volterra equations have been widely used to infer animal interactions in dynamic systems, and recently have been applied to reveal microbial interacting relationships between operational taxonomic units (OTUs). The detailed algorithms and equations were described by Bucci et al. (Stein et al., 2013). Due to the limitation of computing power (Interl® Core™ i7-4770 CPU @3.40 GHz processor and 32 Gb RAM), it is not feasible to infer interaction network at the species level. Therefore, we assumed that in general, all species in a genus identified in our amplicon study share similar functions. According to the compositional profiles among 12 time points at the genus level, MetaMIS can systematically examine interaction patterns, such as mutualism

or competition; only the top 25% of interacting relationships were considered in this study. We used the mfinder tool (Milo et al., 2002) to identify significant 3-node directed motifs that contain two directed edges pointing to the same node. This process was repeated 100 times and all relationships that passed the criteria described above at least 50 times (permutation cutoff > 0.5) were considered to be reliable interacting relationships.

Probiotic Selection and Culturing

There are several genera of bacteria characterized as probiotic in amphibians (Pasteris et al., 2009a,b; Dias et al., 2010; Becker et al., 2011; Mendoza et al., 2012; Bletz et al., 2013). LAB have been considered as the major probiotics for the treatment of raniculture (Dias et al., 2010; Mendoza et al., 2012). In this study, *L. garvieae* was selected as a probiotic strain because *Lactococcus* was present in at least 85% of all the samples with a relative abundance of 0.14 ± 0.39%, and *L. garvieae* has been identified as a biological control agent in bullfrogs (Mendoza et al., 2012). According to the interacting relationships inferred by MetaMIS, *Corynebacterium* showed beneficial effects on *Lactococcus*, while *Bacillus* was found to have a beneficial interacting relationship with *Corynebacterium*. We decided to use three representative species of the three mentioned genera for further validation. Therefore, *L. garvieae*, *C. variabile,* and *B. coagulans* were chosen to validate the inferred interacting relationships. These three species were selected by their abundance ranking within the genus according to 16S rRNA amplicon data. We reasoned that if the inferred interacting relations were correct, the cooperative combination would yield a higher probiotic concentration than using a single strain.

To prepare for oral administration, *L. garvieae* and *B. coagulans* were grown in Tryptic Soy broth at 30 and 55°C respectively. *C. variabile* was grown in Brain Heart Infusion broth at 30°C. All strains were grown overnight with agitation in a shaking incubator. After incubation, the cells were harvested by centrifugation at 2,500 × g for 20 min at 4°C. The cell pellets were washed twice with 0.9% saline and resuspended using the same buffer. The measured population level of bacteria in the test diet was 10^7 CFU g^{-1} (colony-forming unit).

Treefrogs were divided into five groups ($N = 8$ per group), and acclimated for 1 week before the start of the trial. The trial was conducted for a 2-week period. Each test group of treefrogs was dosed with *L. garvieae* (G_1), *C. variabile* (G_2), or *B. coagulans* (G_3) singly, or with a combination of the three strains (G_4) once per day by direct oral gavage. The control (G_5) was fed with 0.9% saline during the entire trial period. After the trial, four fecal samples were collected from four treefrogs in each group (**Supplementary Table 2**) for further 16S rRNA amplicon analysis, and the other four treefrogs were used to perform quantitative PCR for interleukin 10 (IL-10) expression as described in the following section.

Quantitative Real Time PCR for IL-10

In order to test the immune response after oral administration with probiotic, the expression level of IL-10 was measured after LPS (lipopolysaccharide) stimulation (Qi et al., 2015). To characterize the change of treefrog IL-10 expression after LPS stimulation, four treefrogs in each group were injected intraperitoneally (i.p.) with LPS (150 μg/100 g body weight). Animals were anesthetized and euthanized 24 h after injection. All fecal contents were removed and the remaining tissue samples of gut were collected (weight range 0.03 to 0.08 g) (**Supplementary Table 3**). All tissue samples were homogenized and total RNA was extracted from homogenized samples using Trizol reagent (Invitrogen, USA), quantified using a Nanodrop–1000 spectrophotometer, and reverse transcribed into cDNA using the Superscript II reverse transcription system (Invitrogen, USA) according to the manufacturer's instructions. Quantitative real time PCR was performed using Power SYBR green PCR Mastermix (Applied Biosystems) on a real-time instrument (ABI mode 7300 Sequence Detector) in 96-well reaction plates. The reaction mixture included 10 ml of Power SYBR green PCR Mastermix, 1 μl of forward and reverse primer (10 μM each) and 1 ml of cDNA, and then brought up to a final total volume of 20 μl with ultra pure water. The sequence of IL-10 in *P. megacephalus* was described in previous study (Huang et al., 2016) and the forward and reverse primers were designed by NCBI Primer-BLAST (Ye et al., 2012). β-actin was used as a housekeeping control. The primer sequences for amplification of IL-10 and β-actin were as follows: (F, 5′-ACGACCCTGCTCACGTTATG-3; R, 5′-TCCGGGATGGAGTAAGAGGG-3′) and (F, 5′-GGTC GCCCAAGACATCAG-3; R, 5′-GCATACAGGGACAACACA-3′) (Hamdan et al., 2016), respectively. The relative expression of IL-10 in gut tissue samples was normalized to the expression of β−actin. The change of gene expression was expressed as fold change (log base 2) and calculated as described (Qi and Nie, 2008; Qi et al., 2010, 2015). A paired Student's *t*-test was applied to analyze the significance and fold change (log base 2), with a *p*-value less than 0.05 considered to be statistically significant.

Statistical Analysis

To estimate the change of microbial complexity throughout the AH period, alpha-diversity was determined using the Shannon index, Simpson index, and the Inversed Simpson index. To determine the differences in bacterial community composition during AH, we used the Bray-Curtis similarity index (a taxonomic metric), which provides a measure of phylogenetic distance between communities from individual samples (Lozupone et al., 2007). To test the differences in richness and phylogenetic indices between time points, Student's *t*-test was used to determine the significance (p-value < 0.05) between time points. For comparison between each bacterial challenge and controls, we determined the fold-change in relative abundance to demonstrate a response to the stimulus relative to the background. Fold-change significantly higher than 2 or smaller than 0.5 was considered to be relevant. Differences between the two groups were analyzed for significance (p-value < 0.05) by Wilcoxon's test.

RESULTS

In this study, we conducted a continuous 15-day time series data collection through communities from simple to complex to infer interacting relationships of microbes in the gut of treefrogs. We obtained an average of 128,608 ± 32,367 high quality, classifiable 16S rRNA gene sequences, with an

average count per time point ranging from 91,908 ± 7,767 to 161,142 ± 45,301. At the numbers of reads generated for each sample, the numbers of genera were in the saturation phase (**Supplementary Figure 1**), indicating that genera from each sample had been sufficiently recovered in MiSeq sequencing. We observed that the phylogenetic indices, including the Shannon index and the Simpson Index, were significantly increased at all time points after day 12 (24 h after AH), compared with day 11.25 (6 h after AH). The Inverse Simpson Index was also significantly increased at days 12, 13.5, 14, 14.5, 15, compared with day 11.25 (**Table 1**). In addition, three predominant phyla were significantly altered in their relative abundance (Student's *t*-test, p-value < 0.05) from day 11.25 at all time points after day 13.5 (**Figure 1**). For example, the increased relative abundance of Bacteroidetes at days 13.5, 14, 14.5, and 15 were 23 ± 4.2%, 24 ± 2.5%, 18 ± 5.6%, and 31 ± 3.6%, compared with 1.7 ± 0.8% at day 11.25. The relative abundance of Firmicutes at day 11.25, 13.5, 14, 14.5, and 15 were 3.9 ± 1.3, 18 ± 3.6, 25 ± 6.5, 53 ± 8.2, and 20 ± 6.2%, while the relative abundance of Proteobacteria at day 11.25, 13.5, 14, 14.5, and 15 were 90 ± 1.6, 49 ± 6.6, 37 ± 6.3, 8.4 ± 1.3, and 34 ± 7.2%. We also found that other phyla were significantly increased, such as Tenericutes and Verrucomicrobia, or significantly decreased, such as Thermi, in relative abundance after day 13.5 compared to day 11.25. These observations suggest that AH successfully reduced the microbial complexity, which may enhance the accuracy of inferred interactions.

The Inferred Interacting Relations

To infer microbial interactions, we focused on the level of genera. Overall, 325 genera that were characterized via the QIIME pipeline were used to construct the interacting relationships network using MetaMIS. In each run, pairwise interacting relationships with defined criteria (such as commensalism or amensalism) were generated. We obtained interacting paired relationships for 26,320 to 26,327 pairs in 100 permutations.

TABLE 1 | Time-dependent phylogenetic diversity spanning 15 days over AH.

Time (day)	Richness	Shannon index	Simpson index	Inverse Simpson index
1	14 ± 1.47	1.32 ± 0.1	0.68 ± 0.03	3.17 ± 0.33
11	13.67 ± 1.45	0.94 ± 0.4	0.47 ± 0.21	2.56 ± 0.87
11.25	13.25 ± 0.25	0.41 ± 0.07[a]	0.18 ± 0.03[a]	1.22 ± 0.04[a]
11.5	12.67 ± 0.88	1.15 ± 0.2	0.63 ± 0.06[b]	2.81 ± 0.43
11.75	13.67 ± 0.67	0.3 ± 0.19	0.13 ± 0.09	1.18 ± 0.14
12	15 ± 1.15	1.36 ± 0.06[b]	0.69 ± 0.03[b]	3.32 ± 0.34[b]
12.5	17.33 ± 2.96	1.14 ± 0.12[b]	0.56 ± 0.08[b]	2.41 ± 0.42
13	19.33 ± 3.38	1.2 ± 0.17[b]	0.62 ± 0.09[b]	2.87 ± 0.57
13.5	11.75 ± 0.75	1.24 ± 0.05[b]	0.64 ± 0.04[b]	2.91 ± 0.31[b]
14	14 ± 0.41	1.32 ± 0.07[b]	0.69 ± 0.03[b]	3.36 ± 0.32[b]
14.5	14.25 ± 1.11	1.31 ± 0.14[b]	0.63 ± 0.07[b]	2.93 ± 0.49[b]
15	13.4 ± 1.21	1.31 ± 0.03[b]	0.68 ± 0.01[b]	3.17 ± 0.13[b]

Within each column, values not sharing superscripts (a and b) differ significantly (p-value < 0.05, Student's t-test). Values are expressed as mean values ± SD.

After analysis, 10,314 inferred interaction pairs (IIPs) passed the permutation criterion (cutoff > 0.5 described in Methods), including 1,568 commensal, 1,737 amensal, 3,777 mutual, and 3,232 competitive relationships (**Supplementary Table 4**). For reference, we also constructed the interaction network at the species level (**Supplementary Table 5**). Most inferred relations are consistent in both networks generated in the level of genera and species.

To choose probiotic bacteria for the validation of IIPs, we surveyed the literature and selected three genera, i.e., *Lactococcus*, *Lactobacillus*, and *Pediococcus* that have been commonly used in probiotic treatments in raniculture (Dias et al., 2010; Mendoza et al., 2012). In our dataset, there were 84, 64, and 60 IIPs correlated to *Lactococcus*, *Pediococcus*, and *Lactobacillus*, respectively. Therefore, we selected *Lactococcus* as the target probiotic. In order to select the commensal partners for *Lactococcus,* we further surveyed the genera that directly imposed beneficial effects on *Lactococcus* (inferred from the interaction network). *Corynebacterium* was selected due to the fact it had the highest beneficial effects in the network. Additionally, *Bacillus* imposed beneficial effects on *Corynebacterium* and was selected from 69 IIPs of *Corynebacterium.* Consequently, in this study, we performed oral administration using three representatives of these genera, i.e., *L. garvieae, C. variabile*, and *B. coagulans* to validate the inferred interacting relations in gut microbes of the treefrogs.

To further describe probiotic networks, we identified specific IIPs for *Lactococcus*, *Corynebacterium*, and *Bacillus* from 1,012 IIPs to pinpoint the genera that directly or indirectly interact with our selected bacteria (**Supplementary Table 6**). There were 84 IIPs interacting with *Lactococcus*, including one commensal, 41 mutual, one amensal, and 41 competitive. From the 84 interacting partners with *Lactococcus*, three of them were predominant genera (relative abundance on average was larger than 5%), *Bacteroides* (20.8 ± 16.2%), *Citrobacter* (6.6 ± 10.4%), and *Shewanella* (16.3 ± 24.1%), and were all inferred as competing partners, suggesting that a considerable population that colonized the treefrog intestine may intrinsically inhibit the genus *Lactococcus*. The number of IIPs with *Corynebacterium* and *Bacillus* were 69 and 60 respectively. It is worth noting that compared with *Lactococcus*, *Corynebacterium* had more mutual relations with other intestinal bacteria; there were no amensal relationships and only 21 of 69 IIPs were competitive. A small group of microbes that interact with *Lactococcus*, *Corynebacterium*, and *Bacillus* is shown in **Figure 2**.

Oral Administration of *L. Garvieae*, *C. variabile*, and *B. Coagulans*

From the inferred interactions, 84, 69, and 60 IIPs were correlated to *Lactococcus*, *Corynebacterium*, and *Bacillus*, respectively. After oral administration of the three representative probiotics of these genera (*L. garvieae, C. variabile*, and *B. coagulans*), 65, 57, and 52 IIPs of *Lactococcus*, *Corynebacterium*, and *Bacillus* were identified in the trial by 16S rRNA amplicon analysis.

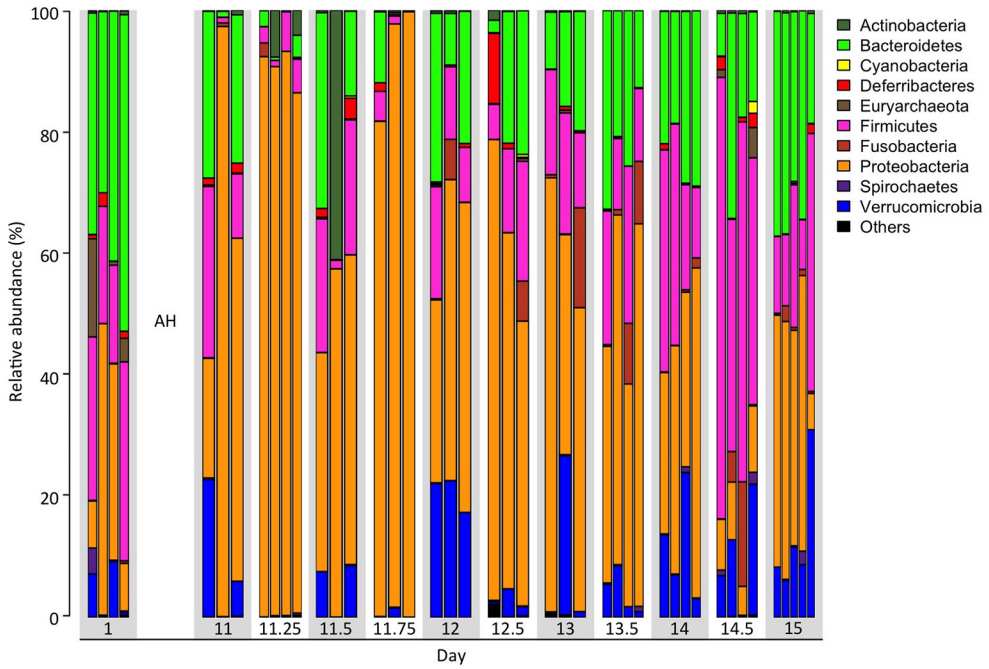

FIGURE 1 | Time dependent taxonomic composition spanning 15 days over AH. Taxonomic composition of fecal microbiota over 12 time points including pre- and post-artificial hibernation (AH) at the phylum level. Each bar represents one individual.

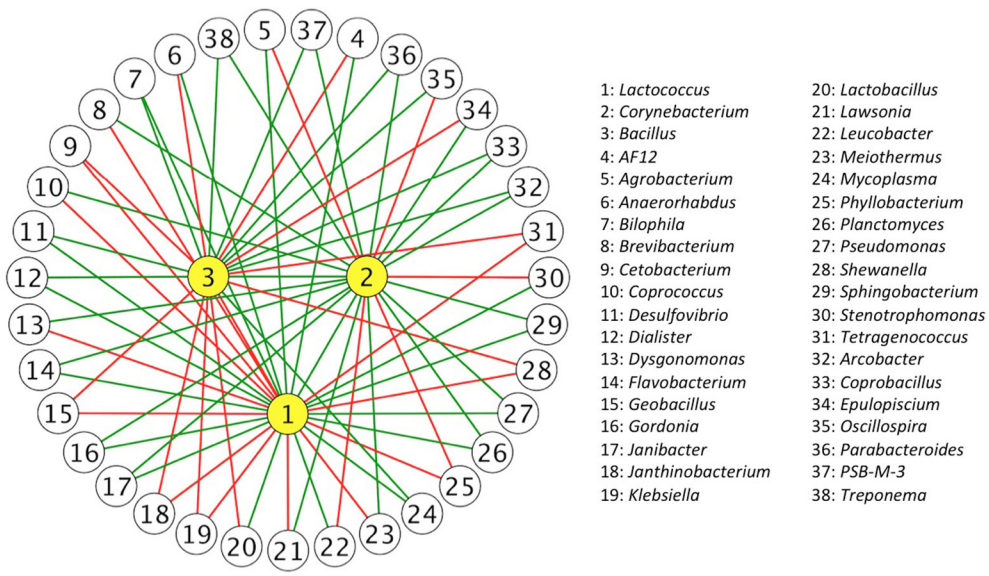

FIGURE 2 | Inferred interaction partners of *Bacillus, Corynebacterium,* and *Lactococcus*. Complex relationships were inferred from gut bacterial communities in the 15-day time series data. Each node represents an inferred interaction partner (IIP) and each edge represents an inferred interaction relation between them. The edges in green represent commensal or mutual interactions, and the edges in red represent amensal or competitive interactions. Only the IIPs that contained two inferred interaction relationships are shown.

The IIPs with abundance data of zero were excluded from the following analysis. Oral administration of three distinct bacterial species representative of each genus led to successful gut colonization, and led to a reasonable change in IIPs. In the G_1 treatment group (oral administration of *L. garvieae*), *Lactococcus* significantly increased in relative abundance 9.88 ± 2.1-fold compared with control (G_5 treatment group) (**Supplementary Table 6**). In the G_2 treatment group (oral administration of *C. variabile)*, *Corynebacterium* significantly increased in relative abundance 160.51 ± 58.54-fold (data

insufficient to test the change of *Lactococcus*). On the other hand, in the G_3 treatment group (oral administration of *B. coagulans)*, the relative abundance of *Bacillus* decreased 0.59 ± 0.49-fold, with no change in *Corynebacterium,* and a 1.91 ± 0.49-fold increase in *Lactococcus*. In the G_4 treatment group (oral administration of a combination of *L. garvieae, C. variabile*, and *B. coagulans*), as expected, *Lactococcus* significantly increased in relative abundance 26.17 ± 6.96-fold (Wilcoxon's test, p-value < 0.05), 2.64 times greater increase than see for *L. garvieae* alone, reflecting the positive effects of the commensal partners. Also in the G_4 treatment group, *Corynebacterium* increased 95.99 ± 9.57-fold in relative abundance (Wilcoxon's test, p-value $= 0.0625$), while *Bacillus* decreased 0.09 ± 0.04-fold in relative abundance in comparison with controls, reflecting the inhibitory effect of *Lactococcus*. **Figure 3** and **Supplementary Table 7** illustrated the relative abundances of microbes that interact with *Lactococcus*, *Corynebacterium*, and *Bacillus* after the two-week oral trials.

The inferred network describes the potential interactions among genera, in addition to the interactions mentioned above, more information remained to be discussed (**Figure 3**). For example, a recent *in vitro* experiment showed an inhibition of *L. garvieae* K2 against *Klebsiella pneumoniae* U11468 (Olaoye, 2016), and our network inference also suggests a similar interaction between these two species. To test the whole immune response after oral probiotic administration, IL-10, an immunoregulatory cytokine involved in immune response in amphibians, was used as an index. The frog IL-10 contains conserved amino acid residues and motifs that are essential for bioactivity. The same residues have been proved to be necessary for immunostimulatory function of human IL-10. Studies have been done using IL-10 expression to examine the host immune response to bacterial infection (Qi et al., 2015). In our study, we found that IL-10 expression variation was consistent with the level of *Lactococcus* in G_1 to G_4 treatment groups (**Table 2**). For example, IL-10 expression was increased in the G_1 treatment group, while the corresponding level of *Lactococcus* in G_1 was upregulated by 9.88, and the change was significant compared with controls (Wilcoxon's test, p-value < 0.05). In addition, IL-10 level in the G_4 treatment group was significantly higher than in controls, also reflecting the high *Lactococcus* level in G_4. These results also support the idea that a cooperative combination of *Lactococcus* triggered significantly higher expression of IL-10 than observed when using a single strain.

To validate the inferred interaction network, the corresponding changes of all IIPs were evaluated by calculating the fold changes between the test and control groups. In

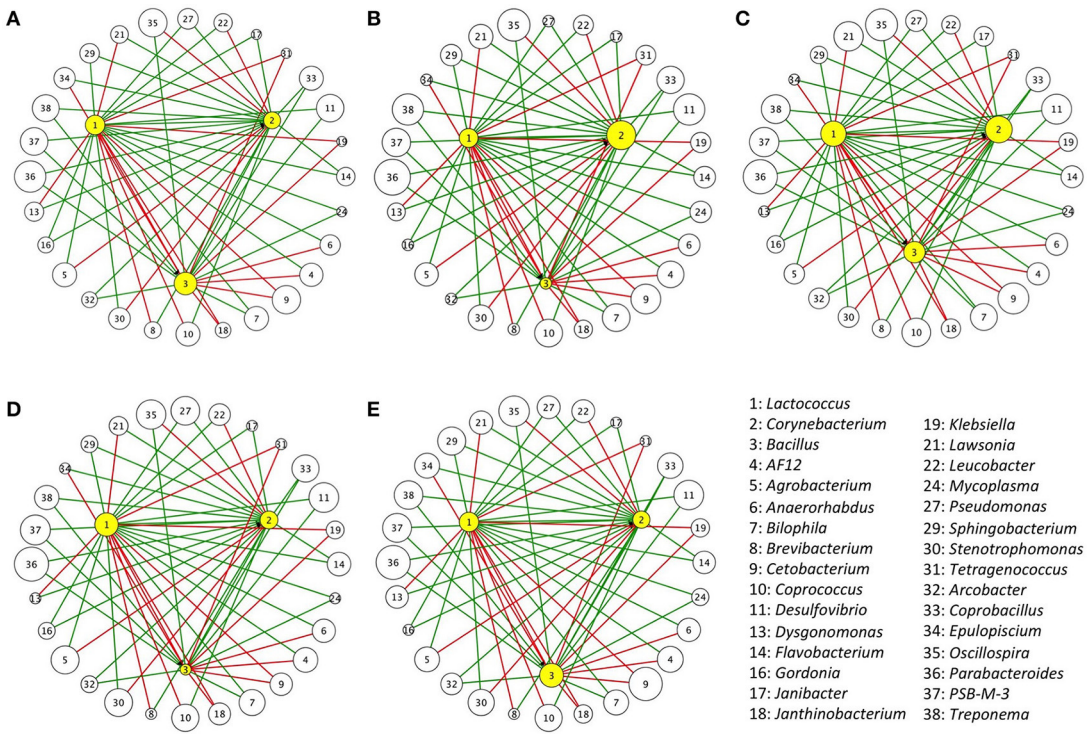

FIGURE 3 | Relative abundance of IIPs of *Bacillus, Corynebacterium,* and *Lactococcus* after two-week oral trials. Five oral administrations included **(A)** G_1: *L. garvieae* (10^7 CFU g^{-1}) in 0.9% saline, **(B)** G_2: *C. variabile* (10^7 CFU g^{-1}) in 0.9% saline, **(C)** G_3: *B. coagulans* (10^7 CFU g^{-1}) in 0.9% saline, **(D)** G_4: A combination of *B. coagulans*, *C. variabile*, and *L. garvieae* (each contains 10^7 CFU g^{-1}) in 0.9% saline, and **(E)** G_5: Control (0.9% saline). Each node represents an IIP that is correlated with *Bacillus*, *Corynebacterium*, or *Lactococcus*, and each edge represents an inferred interaction relation between them. The edges in green represent commensal or mutual interactions, and the edges in red represent amensal or competitive interactions. To better visualize the distribution, the size of each node represents the relative abundance of gut microbes in logarithmic scale.

TABLE 2 | Expression analysis of IL-10 and level of *Lactococcus*.

Treatment	*N*	Fold change of IL-10 (log base 2)	Fold change of *Lactococcus*
G_1	4	1.64 ± 1.64	9.88 ± 2.1*
G_2	4	1.48 ± 2.11	NA
G_3	4	2.34 ± 1.81*	1.91 ± 0.49
G_4	4	1.68 ± 1.22*	26.17 ± 6.96*

*Gut tissues sampled for quantitative PCR analysis. The expression levels of IL-10 were determined relative to β-actin. The expression changes of IL-10 were expressed as fold change (log base 2) relative to controls. p-values generated by paired sample Student's t-test between test groups and controls are shown (*p-values < 0.05). The relative abundance changes of Lactococcus by 16S rRNA amplicon analysis were expressed as fold change relative to controls. Values are expressed as means ± SD. NA, data insufficient for test. L. garvieae (G_1), C. variabile (G_2), B. coagulans (G_3), and a combination of the three strains (G_4).*

TABLE 3 | Validation of IIPs that correlate with *Lactococcus, Corynebacterium,* or *Bacillus*.

	Total IIPs	Treatment	Consistent IIPs	Conflict IIPs	No difference
Lactococcus	65	G_1	34 (52.3%)	15 (23.1%)	16 (24.6%)
		G_2	30 (46.2%)	14 (21.5%)	21 (32.3%)
		G_3	28 (43.1%)	19 (29.2%)	18 (27.7%)
		G_4	31 (47.7%)	16 (24.6%)	18 (27.7%)
Corynebcaterium	57	G_1	27 (47.4%)	18 (31.6%)	12 (21.1%)
		G_2	27 (47.4%)	13 (22.8%)	17 (29.8%)
		G_3	31 (54.4%)	17 (29.8%)	9 (15.8%)
		G_4	31 (54.4%)	13 (22.8%)	13 (22.8%)
Bacillus	52	G_1	24 (46.2%)	18 (34.6%)	10 (19.2%)
		G_2	24 (46.2%)	14 (26.9%)	14 (26.9%)
		G_3	23 (44.2%)	19 (36.5%)	10 (19.2%)
		G_4	27 (51.9%)	13 (25%)	12 (23.1%)

Values are number of IIRs that correlate with Lactococcus, Corynebacterium, or Bacillus in each treatment group. The value in parentheses corresponds to the ratio of consistent IIPs, conflict IIPs, and no difference. L. garvieae (G_1), C. variabile (G_2), B. coagulans (G_3), and a combination of the three strains (G_4).

the G_1 group, after oral administration of *L. garvieae* for 2 weeks, 52.3% (34 out of 65) of IIPs correlated with *Lactococcus* responded consistently with our inferred relations, including one commensal, 10 competitive, and 23 mutual interactions (**Table 3** and **Supplementary Table 6**). There were 47.4% (27 out of 57) and 46.2% (24 out of 52) of IIPs correlated with *Corynebacterium* and *Bacillus*, respectively, which also responded consistently with our inferred relations. However, there were 23.1% (15 out of 65), 31.6% (18 out of 57), and 34.6% (18 out of 52) IIPs that correlated with *Lactococcus*, *Corynebacterium*, and *Bacillus*, presenting a conflicting response with our inferred relations, respectively. Overall, in the G_1 treatment group, the ratio of IIPs that responded according to the inferred relationship was 48.9% (85 out of 174), the ratio of IIPs that showed a conflicting response to the inferred relationships was 29.3% (51 out of 174) (**Table 3**). In the evaluations for the G_2, G_3, and G_4 treatment groups, we found 43.1–54.4% of corresponding changes of IIPs were consistent with the inferred interaction (**Table 3**).

DISCUSSION

Recent advances in sequencing technology have created a new opportunity to explore population of microbes and their associations with environmental changes. Combining mathematical and computational models to infer the interacting networks reveals more details of microbe-microbe and microbe-host interactions. For example, Stein et al. (2013) extended generalized Lotka-Volterra equations to study the mechanism of *C. difficile* colonization in mice. They inferred that the genera *Akkermansia*, *Blautia*, and *Coprobacillus* had inhibitory interactions on *C. difficile*. In contrast, *Enterococcus* and *Mollicutes* could positively affect the growth of *C. difficile*, while the genus *Barnesiella* was predicted to inhibit growth of the genus *Enterococcus*. The results highlight a multi-layered sub-network associated with *C. difficile*. A study by Trosvik et al. (2010) also inferred microbial interactions in human infant gut using a dynamic systems modeling approach called time-dependent generalized additive models (GAM). They showed an agreement between predictions by dynamic interaction modeling and observed data of Firmicutes and Proteobacteria, suggesting that microbe-microbe interactions were sufficient to explain the growth patterns via modeling from time-series data. However, due to the complexity of microbial composition and difficulty in handling intestinal colonization, there was no experimental data to examine the inferred interactions (Mounier et al., 2008; Trosvik et al., 2010; Stein et al., 2013; Marino et al., 2014). To fill the gap, in this study, we used a user-friendly tool, MetaMIS (Shaw et al., 2016), to apply the Lotka-Volterra equations to infer the microbial interaction. The results were further validated by manipulating the gut bacteria and examine the corresponding changes in microbial composition.

Although MetaMIS is a versatile tool for predicting microbial relationships, Lotka-Volterra equations still have some weaknesses, for example, bias resulting from the complexity of microbiota. For data input, gLV equations require knowledge of the growth rates of all community members, and the complexity of microbiota may cause difficulties in determining the dynamic growth rate calculated from relative abundance, especially in the case of rare species (Pedrós-Alió, 2012). To improve the accuracy of MetaMIS, we implemented a time series data with the microbial communities from simple to complex for network inference. We firstly used AH in the treefrog to reduce the microbial diversity, and consequently less species were involved in the interaction network. After AH, increased temperature triggered bacterial turnover in composition, making it possible to reveal the evolving interactions between bacteria over time. The data also allowed us to collect time-series data after the perturbation to generate more dynamic fluctuations and provide deeper insights, compared with static communities (Holling, 1973; May, 1973, 1974; Ives and Carpenter, 2007). We therefore collected fecal samples in a 15-day time series, and increased the density of

sampling points after AH to monitor dynamic changes. The experimental design of the data input could lead to a low ratio of inconsistent IIPs.

To validate the interactions, we focused on a small group of microbes that interact with the probiotic strains, which have been well-studied and applied in raniculture (Dias et al., 2010; Mendoza et al., 2012). The well-known LAB, *Bacillus*, *Enterococcus*, and *Lactococcus*, have been described and applied in many organisms (Balcázar et al., 2007b; Pasteris et al., 2009a; Dias et al., 2010; Nayak, 2010; Mendoza et al., 2012). Their inhibitory mechanisms against pathogenic bacteria have also been discussed (Hyronimus et al., 1998; Payot et al., 1999; Kesarcodi-Watson et al., 2008; Pasteris et al., 2009a). Our results indicated that the inferred network is biologically significant, and the compositional change of probiotics and the immune responses consistently supported our inference. In addition, our inferred relations were supported by the literature. For instance, *Enterococcus* competed with *Citrobacter* and *Staphylococcus* according to the inferred interaction, and these relationships were supported by previous studies showing that *Enterococcus* spp. 334 maintained its inhibitory effect against *Citrobacter freundii* and *Staphylococcus epidermidis* in *Lithobates catesbeianus* (Mendoza et al., 2012). Another evidence also showed that *Enterococcus faecium* imposed an inhibitory effect against *C. freundii* in *Rana catesbeiana* (Pasteris et al., 2009a). In addition, *L. garvieae* was found to inhibit *C. freundii* by the production of organic acid (Mendoza et al., 2012), and this correlation between *Citrobacter* and *Lactococcus* is in agreement with our IIPs. Furthermore, the inferred network suggested that *Corynebacterium* and *Lactococcus* were mutually beneficial partners. This is consistent with the culture-based studies showing that the culturable microbiota of milk consists of primarily of LAB such as *Enterococcus* and *Lactococcus*, and were often accompanied by the presence of *Corynebacterium* (Coppola et al., 2008).

In this study we generated a microbial interaction network of gut microbiota in the treefrog, and sub-groups of inferred relations were also validated. The experimental approach using probiotic administration indicated that the inferred interactions were reliable, and the results were also supported by the literature. However, there are still some puzzles remaining. For example, in the G_2 treatment group, oral administration with *C. variabile* caused barely any beneficial effects on *Lactococcus* colonization. Although unexpected changes of gut probiotic levels were commonly observed after probiotic treatment (Hai, 2015; Ramos et al., 2015; Yang et al., 2015), we reasoned that more complex factors derived from other indirect IIPs also imposed various effects (commensalism, mutualism, amensalism, and competition) on these targets. The study is the first attempt to manipulate gut bacteria composition according to the inferred microbial interactions. We demonstrated the possibility that the gut microbiome can be changed accordingly. Moreover, our study provides a new potential strategy for the use of probiotics in raniculture.

AUTHOR CONTRIBUTIONS

DW and FW designed the analyses. YY, CW, and FW collected the data. FW performed the analyses. GS implemented gLV model. DW and FW wrote the paper. DW was the principle investigator and conceived the analyses. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00525/full#supplementary-material

Supplementary Figure 1 | Rarefaction analyses for the observed number of genera from 12 time points. The rarefaction curves for each sample of 12 time points were displayed by different colors.

Supplementary Table 1 | Summary of sample information of 12 time points spanning 15 days over AH. Values are expressed as mean values ± *SD*.

Supplementary Table 2 | Summary of sample information of oral administration for bacterial composition. Oral administration of five treatments was carried out for 2 weeks. G_1: *L. garvieae* (10^7 CFU g^{-1}) in 0.9% saline. G_2: *C. variabile* (10^7 CFU g^{-1}) in 0.9% saline. G_3: *B. coagulans* (10^7 CFU g^{-1}) in 0.9% saline. G_4: A combination of *B. coagulans*, *C. variabile*, and *L. garvieae* (each contains 10^7 CFU g^{-1}) in 0.9% saline. G_5:Control (0.9% saline). Values are expressed as mean ± *SD*.

Supplementary Table 3 | Summary of sample information of oral administration for quantitative PCR. Oral administration of five treatments was performed over 2 weeks. G_1: *L. garvieae* (10^7 CFU g^{-1}) in 0.9% saline. G_2: *C. variabile* (10^7 CFU g^{-1}) in 0.9% saline. G_3: *B. coagulans* (10^7 CFU g^{-1}) in 0.9% saline. G_4: a combination of *B. coagulans*, *C. variabile*, and *L. garvieae* (each contains 10^7 CFU g^{-1}) in 0.9% saline. G_5: Control (0.9% saline). Values are expressed as mean ± *SD*.

Supplementary Table 4 | Ten thousand and three hundred fourteen significant inferred interacting pairs (IIPs) identified by MetaMIS. 10,314 IIPs passed the permutation criterion (cutoff > 0.5 described in Methods), including 1,568 commensal relationships (represented by O), 1,737 amensal relationships (represented by A), 3,777 mutual relationships (represented by M), and 3,232 competitive relationships (represented by C).

Supplementary Table 5 | The raw data for the interaction network at the species level. 1,568 IIPs passed the permutation criterion (cutoff > 0.5 described in Methods), including 174 commensal relationships (represented by O), 211 amensal relationships (represented by A), 461 mutual relationships (represented by M), and 722 competitive relationships (represented by C).

Supplementary Table 6 | Fold change of the inferred relationships with *Lactococcus, Corynebacterium,* and *Bacillus*. Commensalism is represented by O, amensalism by A, mutualism by M, and competition by C. Fold changes are expressed as mean values ± *SD*. NA, data insufficient for test.

Supplementary Table 7 | Relative abundance of IIPs of *Bacillus, Corynebacterium,* and *Lactococcus* after 2-week oral trials. Five oral administrations included G_1: *L. garvieae* (10^7 CFU g^{-1}) in 0.9% saline. G_2: *C. variabile* (10^7 CFU g^{-1}) in 0.9% saline. G_3: *B. coagulans* (10^7 CFU g^{-1}) in 0.9% saline. G_4: A combination of *B. coagulans*, *C. variabile*, and *L. garvieae* (each contains 10^7 CFU g^{-1}) in 0.9% saline. G_5: Control (0.9% saline). Values are expressed as mean values ± *SD*.

REFERENCES

Akhter, N., Wu, B., Memon, A. M., and Mohsin, M. (2015). Probiotics and prebiotics associated with aquaculture: a review. *Fish Shellfish Immunol.* 45, 733–741. doi: 10.1016/j.fsi.2015.05.038

Akinbowale, O. L., Peng, H., and Barton, M. D. (2006). Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. *J. Appl. Microbiol.* 100, 1103–1113. doi: 10.1111/j.1365-2672.2006.02812.x

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., et al. (2011). Enterotypes of the human gut microbiome. *Nature* 473, 174–180. doi: 10.1038/nature09944

Balcazar, J. L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., and Muzquiz, J. L. (2006). The role of probiotics in aquaculture. *Vet. Microbiol.* 114, 173–186. doi: 10.1016/j.vetmic.2006.01.009

Balcázar, J. L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A. C., Márquez, I., et al. (2007a). Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *Br. J. Nutr.* 97, 522–527. doi: 10.1017/S0007114507432986

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., and Muzquiz, J. L. (2007b). Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunol. Med. Microbiol.* 51, 185–193. doi: 10.1111/j.1574-695X.2007.00294.x

Becker, M. H., Harris, R. N., Minbiole, K. P., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Bhargava, S. C. (1989). Generalized Lotka-Volterra equations and the mechanism of technological substitution. *Technol. Forecast. Soc. Change* 35, 319–326. doi: 10.1016/0040-1625(89)90068-1

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Book, S. G. (1974). *Amphibians: Guidelines for the Breeding, Care and Management of Laboratory Animals.* Washington, DC: National Academy of Sciences.

Buffie, C. G., Jarchum, I., Equinda, M., Lipuma, L., Gobourne, A., Viale, A., et al. (2012). Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to *Clostridium difficile*-induced colitis. *Infect. Immun.* 80, 62–73. doi: 10.1128/IAI.05496-11

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Coppola, S., Blaiotta, G., and Ercolini, D. (2008). "Dairy products," *Molecular Techniques in the Microbial Ecology of Fermented Foods,* eds L. Cocolin and D. Ercolini (Heidelberg: Springer), 31–90.

Costello, E. K., Gordon, J. I., Secor, S. M., and Knight, R. (2010). Postprandial remodeling of the gut microbiota in Burmese pythons. *ISME J.* 4, 1375–1385. doi: 10.1038/ismej.2010.71

Coyte, K. Z., Schluter, J., and Foster, K. R. (2015). The ecology of the microbiome: networks, competition, and stability. *Science* 350, 663–666. doi: 10.1126/science.aad2602

Dethlefsen, L., and Relman, D. A. (2011). Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl. Acad. Sci.* 108(Suppl. 1), 4554–4561. doi: 10.1073/pnas.1000087107

Dias, D. D. C., De Stéfani, M. V., Ferreira, C. M., França, F. M., Ranzani-Paiva, M. J. T., and Santos, A. A. (2010). Haematologic and immunologic parameters of bullfrogs, *Lithobates catesbeianus*, fed probiotics. *Aquac. Res.* 41, 1064–1071. doi: 10.1111/j.1365-2109.2009.02390.x

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Faust, K., and Raes, J. (2012). Microbial interactions: from networks to models. *Nat. Rev. Microbiol.* 10, 538–550. doi: 10.1038/nrmicro2832

Finke, M. D. (2013). Complete nutrient content of four species of feeder insects. *Zoo Biol.* 32, 27–36. doi: 10.1002/zoo.21012

Gossling, J., Loesche, W., and Nace, G. (1982a). Large intestine bacterial flora of nonhibernating and hibernating leopard frogs (*Rana pipiens*). *Appl. Environ. Microbiol.* 44, 59–66.

Gossling, J., Loesche, W., and Nace, G. (1982b). Response of intestinal flora of laboratory-reared leopard frogs (*Rana pipiens*) to cold and fasting. *Appl. Environ. Microbiol.* 44, 67–71.

Hai, N. V. (2015). Research findings from the use of probiotics in tilapia aquaculture: a review. *Fish Shellfish Immunol.* 45, 592–597. doi: 10.1016/j.fsi.2015.05.026

Hamdan, A. M., El-Sayed, A. F. M., and Mahmoud, M. M. (2016). Effects of a novel marine probiotic, *Lactobacillus plantarum* AH 78, on growth performance and immune response of Nile tilapia (*Oreochromis niloticus*). *J. Appl. Microbiol.* 120, 1061–1073. doi: 10.1111/jam.13081

Hernandez, D., Cardell, E., and Zarate, V. (2005). Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol.* 99, 77–84. doi: 10.1111/j.1365-2672.2005.02576.x

Holling, C. S. (1973). Resilience and stability of ecological systems. *Annu. Rev. Ecol. Syst.* 4, 1–23. doi: 10.1146/annurev.es.04.110173.000245

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259

Huang, L., Li, J., Anboukaria, H., Luo, Z., Zhao, M., and Wu, H. (2016). Comparative transcriptome analyses of seven anurans reveal functions and adaptations of amphibian skin. *Sci. Rep.* 6:24069. doi: 10.1038/srep24069

Hyronimus, B., Le Marrec, C., and Urdaci, M. (1998). Coagulin, a bacteriocin-like-inhibitory substance produced by *Bacillus coagulans* I. *J. Appl. Microbiol.* 85, 42–50. doi: 10.1046/j.1365-2672.1998.00466.x

Ives, A. R., and Carpenter, S. R. (2007). Stability and diversity of ecosystems. *Science* 317, 58–62. doi: 10.1126/science.1133258

Jernberg, C., Löfmark, S., Edlund, C., and Jansson, J. K. (2007). Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *ISME J.* 1, 56–66. doi: 10.1038/ismej.2007.3

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., and Gibson, L. (2008). Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. *Aquaculture* 274, 1–14. doi: 10.1016/j.aquaculture.2007.11.019

Kohl, K. D., Amaya, J., Passement, C. A., Dearing, M. D., and McCue, M. D. (2014). Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. *FEMS Microbiol. Ecol.* 90, 883–894. doi: 10.1111/1574-6941.12442

Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E., and Woodhams, D. C. (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS ONE* 9:e87101. doi: 10.1371/journal.pone.0087101

Li, M., Wang, B., Zhang, M., Rantalainen, M., Wang, S., Zhou, H., et al. (2008). Symbiotic gut microbes modulate human metabolic phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* 105, 2117–2122. doi: 10.1073/pnas.0712038105

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Magoč, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., et al. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut* 55, 205–211. doi: 10.1136/gut.2005.073817

Marino, S., Baxter, N. T., Huffnagle, G. B., Petrosino, J. F., and Schloss, P. D. (2014). Mathematical modeling of primary succession of murine intestinal microbiota. *Proc. Natl. Acad. Sci.U.S.A.* 111, 439–444. doi: 10.1073/pnas.1311322111

May, R. M. (1973). *Stability and Complexity in Model Ecosystems*. Princeton, NJ: Princeton University Press.

May, R. M. (1974). Biological populations with nonoverlapping generations: stable points, stable cycles, and chaos. *Science* 186, 645–647. doi: 10.1126/science.186.4164.645

Mendoza, G. M., Pasteris, S. E., Ale, C. E., Otero, M. C., Bühler, M. I., and Nader-Macías, M. E. F. (2012). Cultivable microbiota of *Lithobates catesbeianus* and advances in the selection of lactic acid bacteria as biological control agents in raniculture. *Res. Vet. Sci.* 93, 1160–1167. doi: 10.1016/j.rvsc.2012.05.007

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., and Alon, U. (2002). Network motifs: simple building blocks of complex networks. *Science* 298, 824–827. doi: 10.1126/science.298.5594.824

Mounier, J., Monnet, C., Vallaeys, T., Arditi, R., Sarthou, A.-S., Hélias, A., et al. (2008). Microbial interactions within a cheese microbial community. *Appl. Environ. Microbiol.* 74, 172–181. doi: 10.1128/AEM.01338-07

Nayak, S. (2010). Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol.* 29, 2–14. doi: 10.1016/j.fsi.2010.02.017

Newman, M. E. J. (2003). The structure and function of complex networks. *Siam Rev.* 45, 167–256. doi: 10.1137/S003614450342480

Olaoye, O. A. (2016). Antimicrobial activities of five strains of Lactococcus isolated from beef against indicator organisms of public health significance. *Turkish J. Agri. Food Sci. Technol.* 4, 887–892.

Pasteris, S. E., Pingitore, E. V., Babot, G. R., Otero, M. C., Bühler, M. I., and Nader-Macías, M. E. (2009a). Characterization of the beneficial properties of lactobacilli isolated from bullfrog (*Rana catesbeiana*) hatchery. *Antonie Van Leeuwenhoek* 95, 373–385. doi: 10.1007/s10482-009-9329-4

Pasteris, S. E., Roig Babot, G., Otero, M. C., Bühler, M. I., and Nader-Macías, M. E. (2009b). Beneficial properties of lactic acid bacteria isolated from a Rana catesbeiana hatchery. *Aquac. Res.* 40, 1605–1615. doi: 10.1111/j.1365-2109.2009.02261.x

Payot, T., Chemaly, Z., and Fick, M. (1999). Lactic acid production by *Bacillus coagulans*—kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme Microb. Technol.* 24, 191–199. doi: 10.1016/S0141-0229(98)00098-2

Pedrós-Alió, C. (2012). The rare bacterial biosphere. *Ann. Rev. Mar. Sci.* 4, 449–466. doi: 10.1146/annurev-marine-120710-100948

Perez, T., Balcazar, J. L., Ruiz-Zarzuela, I., Halaihel, N., Vendrell, D., de Blas, I., et al. (2010). Host-microbiota interactions within the fish intestinal ecosystem. *Mucosal Immunol.* 3, 355–360. doi: 10.1038/mi.2010.12

Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J. A., et al. (2009). The NIH human microbiome project. *Genome Res.* 19, 2317–2323. doi: 10.1101/gr.096651.109

Proulx, S. R., Promislow, D. E. L., and Phillips, P. C. (2005). Network thinking in ecology and evolution. *Trends Ecol. Evol.* 20, 345–353. doi: 10.1016/j.tree.2005.04.004

Qi, Z., and Nie, P. (2008). Comparative study and expression analysis of the interferon gamma gene locus cytokines in *Xenopus tropicalis*. *Immunogenetics* 60, 699–710. doi: 10.1007/s00251-008-0326-y

Qi, Z., Nie, P., Secombes, C. J., and Zou, J. (2010). Intron-containing type I and type III IFN coexist in amphibians: refuting the concept that a retroposition event gave rise to type I IFNs. *J. Immunol.* 184, 5038–5046. doi: 10.4049/jimmunol.0903374

Qi, Z., Zhang, Q., Wang, Z., Zhao, W., and Gao, Q. (2015). Cloning of interleukin-10 from African Clawed Frog (*Xenopus tropicalis*), with the finding of IL-19/20 homologue in the IL-10 locus. *J. Immunol. Res.* 2015:462138. doi: 10.1155/2015/462138

Ramos, M. A., Goncalves, J. F., Batista, S., Costas, B., Pires, M. A., Rema, P., et al. (2015). Growth, immune responses and intestinal morphology of rainbow trout (*Oncorhynchus mykiss*) supplemented with commercial probiotics. *Fish Shellfish Immunol.* 45, 19–26. doi: 10.1016/j.fsi.2015.04.001

Reid, G., Sanders, M. E., Gaskins, H. R., Gibson, G. R., Mercenier, A., Rastall, R., et al. (2003). New scientific paradigms for probiotics and prebiotics. *J. Clin. Gastroenterol.* 37, 105–118. doi: 10.1097/00004836-200308000-00004

Ringø, E., Løvmo, L., Kristiansen, M., Bakken, Y., Salinas, I., Myklebust, R., et al. (2010). Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquac. Res.* 41, 451–467. doi: 10.1111/j.1365-2109.2009.02339.x

Round, J. L., and Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* 9, 313–323. doi: 10.1038/nri2515

Shafiei, M., Dunn, K. A., Chipman, H., Gu, H., and Bielawski, J. P. (2014). BiomeNet: a Bayesian model for inference of metabolic divergence among microbial communities. *PLoS Comput. Biol.* 10:e1003918. doi: 10.1371/journal.pcbi.1003918

Shaw, G. T. W., Pao, Y.-Y., and Wang, D. (2016). MetaMIS: a metagenomic microbial interaction simulator based on microbial community profiles. *BMC Bioinformatics* 17:488. doi: 10.1186/s12859-016-1359-0

Sonoyama, K., Fujiwara, R., Takemura, N., Ogasawara, T., Watanabe, J., Ito, H., et al. (2009). Response of gut microbiota to fasting and hibernation in Syrian hamsters *Appl. Environ. Microbiol.* 75, 6451–6456. doi: 10.1128/AEM.00692-09

Stein, R. R., Bucci, V., Toussaint, N. C., Buffie, C. G., Rätsch, G., Pamer, E. G., et al. (2013). Ecological modeling from time-series inference: insight into dynamics and stability of intestinal microbiota. *PLoS Comput. Biol.* 9:e1003388. doi: 10.1371/journal.pcbi.1003388

Taur, Y., Xavier, J. B., Lipuma, L., Ubeda, C., Goldberg, J., Gobourne, A., et al. (2012). Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. *Clin. Infect. Dis.* 55, 905–914. doi: 10.1093/cid/cis580

Trosvik, P., Stenseth, N. C., and Rudi, K. (2010). Convergent temporal dynamics of the human infant gut microbiota. *ISME J.* 4, 151–158. doi: 10.1038/ismej.2009.96

Turnbaugh, P. J., and Gordon, J. I. (2009). The core gut microbiome, energy balance and obesity *J. Physiol.* 587, 4153–4158. doi: 10.1113/jphysiol.2009.174136

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., and Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* 1, 6ra14–16ra14. doi: 10.1126/scitranslmed.3000322

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671. doi: 10.1128/MMBR.64.4.655-671.2000

Xu, J., and Gordon, J. I. (2003). Honor thy symbionts. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10452–10459. doi: 10.1073/pnas.1734063100

Yang, G., Tian, X., Dong, S., Peng, M., and Wang, D. (2015). Effects of dietary *Bacillus cereus* G19, B. cereus BC-01, and *Paracoccus marcusii* DB11 supplementation on the growth, immune response, and expression of immune-related genes in coelomocytes and intestine of the sea cucumber (*Apostichopus japonicus* Selenka). *Fish Shellfish Immunol.* 45, 800–807. doi: 10.1016/j.fsi.2015.05.032

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:1. doi: 10.1186/1471-2105-13-S6-S1

Ye, L., Amberg, J., Chapman, D., Gaikowski, M., and Liu, W. T. (2014). Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *ISME J.* 8, 541–551. doi: 10.1038/ismej.2013.181

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Cutaneous Microbial Community Variation across Populations of Eastern Hellbenders (*Cryptobranchus alleganiensis alleganiensis*)

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Multicellular hosts maintain complex associations with microbial communities. While microbial communities often serve important functional roles for their hosts, our understanding of the local and regional processes that structure these communities remains limited. Metacommunity analyses provide a promising tool for investigating mechanisms shaping microbiome heterogeneity, which is essential for predicting functional variation between hosts. Using a metacommunity framework, we examined heterogeneity in the skin microbiome of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*). Hellbenders are broadly distributed throughout river systems in the eastern United States, but are present in specific environmental locations throughout their range. The large range of the species and history of population fragmentation suggest that local and regional processes contribute to the distribution of cutaneous symbiont diversity. Therefore, we characterized the skin and environmental bacterial communities at eight rivers throughout the range of the species. We observed variation among hellbender populations in skin microbial community diversity and proportion of shared operational taxonomic units (OTUs) between animal and river water communities. Among populations sampled, we noted significant clumped OTU turnover (i.e., Clementsian structure) resulting in unique cutaneous communities. In addition, we observed a significant positive correlation between skin community divergence and hellbender population genetic divergence. Host-population skin community dissimilarity did not correlate strongly with distance between sampling locations, indicating a weak spatial effect on the distribution of symbionts. These results suggest that species sorting mechanisms (i.e., local processes) structure local skin microbial communities in hellbenders. The variation in skin community composition observed among host populations foreshadows a similar pattern in important functional characteristics (e.g., resistance to dysbiosis). Future work should focus on investigating forces shaping microbiome structure in eastern hellbenders, examining functional variation among populations, and evaluating effectiveness of microbiome management recommendations.

Keywords: community ecology, host-associated bacteria, metacommunity, amphibian, conservation, range-wide

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INTRODUCTION

In animal and plant systems, symbiont microbes provide important functional services to host physiological processes including immune system activation, metabolic regulation, energy uptake, tissue differentiation, and pathogen defense (Turnbaugh et al., 2007; Kaplan et al., 2011; Costello et al., 2012; Grice and Segre, 2012). Importantly, functionality of the host's symbiont community can correlate with the overall diversity of the microbiome (Chang et al., 2008) or presence of key symbionts (Woodhams et al., 2007). For example, variation in the gut microbiome of mammals correlates with differences observed in digestive function between host species (Muegge et al., 2011; Phillips et al., 2012). In addition, *Actinobacteria* serve as keystone taxa in the gut of humans due to their high degree of ecological connectedness with other members of the microbiota (Trosvik and de Muinck, 2015). Given the associations between microbiota and host phenotype, understanding mechanisms shaping microbiome heterogeneity is essential to predict functional variation among hosts. While local processes (e.g., host selection) are known to influence microbial community assembly among individuals (Lindstrom and Langenheder, 2012), the contributions of regional processes (e.g., between host populations) to symbiont community structure are frequently overlooked (Mihaljevic, 2012). To provide a more comprehensive understanding of the microbiome's significance for hosts, knowing the relative importance of local and regional processes in driving microbiome heterogeneity is key.

The amphibian skin microbiome plays an important role in resistance against pathogens (Woodhams et al., 2007). For instance, host-level variation in microbial community composition is associated with differences in immunity to the fungal pathogen *Batrachochytrium dendrobatidis* (Lam et al., 2010; Becker et al., 2015). Consequently, recent studies have focused on characterizing compositional variation in the skin microbiome between amphibian species and populations, and the local processes that shape heterogeneity in the skin microbiome. For example, antimicrobial peptides on the amphibian skin likely act as a selective filter on the microbial community (Rollins-Smith et al., 2006). Furthermore, environmental characteristics (e.g., local reservoirs, habitat quality) are correlated with variability in skin community composition among populations of the same species (Kueneman et al., 2014; Loudon et al., 2014). Thus, it is likely that throughout the range of a host species there are important alternative evolutionary associations between symbionts and hosts (Zilber-Rosenberg and Rosenberg, 2008).

Evaluating changes in host–symbiont associations across spatial scales may help inform current conservation approaches in amphibians (Jiménez and Sommer, 2017). For example, host-associated microbial communities can provide guidance in planning translocations and captive-population management. Amphibian conservation programs benefit from captive propagation and translocation strategies as a method to combat declines due to habitat loss, pollution, and emerging infectious diseases (Gagliardo et al., 2008; Zeisset and Beebee, 2013). However, divergence in microbial community composition between source and supplemented populations can negate the intended effects of translocations (Redford et al., 2012; Bahrndorff et al., 2016). Symbionts derived from captive populations can introduce pathogenic or antibiotic-resistant traits to naïve wild communities (Woodford and Rossiter, 1994). In addition, environmental variation can induce changes in the incidence or abundance of microbial symbionts (dysbiosis) that negatively impact host health (Lokmer and Wegner, 2015). To this end, amphibian management and conservation strategies may benefit from: (1) quantifying the natural distribution of bacterial symbionts across different geographic regions; and (2) establishing the relative importance of dispersal and local environmental selection in the assembly of skin microbial communities (Jiménez and Sommer, 2017).

Metacommunity theory can predict microbial community responses to eco-evolutionary processes shaping diversity at local (e.g., among individuals) and regional scales (e.g., among populations; Leibold et al., 2004). Under metacommunity theory, amphibian hosts correspond to patches of suitable habitat connected through transmission or dispersal (Mihaljevic, 2012; Christian et al., 2015). Host characteristics (e.g., immunity) and environmental variables can create a gradient of suitable habitat for microbial symbionts. Along this gradient, variation in symbiont dispersal and physiological constraints/adaptive trade-offs can result in varying patterns of taxonomic turnover (i.e., species replacements) among patches. In addition, factors that influence host movement can also bestow barriers to the dispersal of microbes throughout the host metapopulation (Mihaljevic, 2012). Quantifying skin symbiont turnover can be useful to characterize barriers to symbiont dispersal and predict important functional associations between host and symbionts across spatial scales.

Eastern hellbenders (*Cryptobranchus alleganiensis alleganiensis*) serve as an ideal model to investigate community structure and distribution patterns of cutaneous microbes. This species has exhibited population declines and low levels of recruitment within the last 30 years (Wheeler et al., 2003). Hellbenders are habitat specialists restricted to lotic habitats and have a large range throughout the eastern United States (Nickerson and Mays, 1973). Their dispersal is approximately linear and influenced by flood events (Humphries, 2005) and/or changes in river architecture (Quinn et al., 2013). Recent population genetic assessments using microsatellite markers found two genetic demes (Routman et al., 1994; Sabatino and Routman, 2009; Tonione et al., 2011; Unger et al., 2013) restricted to the major watersheds where these salamanders are found (i.e., the Ohio River Drainage and the Tennessee River Drainage; Unger et al., 2013). This genetic information is currently implemented in the execution of conservation management actions (e.g., translocations). However, the expansive range of the species could lead to variance in environmental conditions among populations (e.g., water quality and temperature). Environmental effects combined with limited host dispersal can lead to variation among the skin microbial communities of individuals within the hellbender metapopulation. Thus, characterizing skin microbiome turnover among individual communities throughout the range of the eastern hellbender

may benefit current conservation approaches by providing an additional guide for translocations.

We implemented culture-independent microbiome characterization methods to identify cutaneous bacterial communities on the skin of eastern hellbenders and river water. We obtained skin and water samples from hellbender populations in different rivers throughout the range of the subspecies. We then tested for differences in skin community diversity and the proportion of shared microbes between water and skin communities among populations. Due to the large range of the species, we predicted that bacteria richness/diversity and the proportion of input from environmental reservoirs would differ among populations. We also evaluated metacommunity structure of hellbender skin communities to quantify species turnover among individuals. While we predicted positive turnover among individuals, we expected more prominent turnover among populations due to variation in environmental characteristics (e.g., elevation, latitude, land use). In addition, we assessed the influence of dispersal on local community structure. We predicted a positive correlation between geographic distance and community dissimilarity in response to limits to hellbender dispersal. Finally, we tested for differences in community composition between the two genetic demes and between hellbenders from each population (i.e., river). We expected to observe differentiation between both genetic demes and among each locality (species sorting paradigm) given that host dispersal and environmental heterogeneity can influence skin community assembly in amphibians.

MATERIALS AND METHODS

Field Methods

We sampled eastern hellbenders between June 18 and July 31, 2014. Our sampling occurred throughout five states (**Figure 1**; Indiana, West Virginia, North Carolina, Tennessee, and Georgia), making sure to include sites across the range for each of the two genetic demes described in Unger et al. (2013). We handled hellbenders following an approved protocol by the Purdue University Animal Care and Use Committee (PACUC protocol # 14060011094). We captured and sampled the microbiome of each individual following the protocol of Hernández-Gómez et al. (2017). We returned all hellbenders to their original location of capture within the river after swabbing was complete. From each population, we also collected two liters of water in sterilized Nalgene bottles 1–10 m upstream from where sampling began. We stored the bottles on ice for up to 2 h until filtration occurred in an aseptic environment. We chose to filter the water on a Whatman #1 11 μm filter paper (GE Healthcare, Chicago, IL, United States) due to the high siltation in several water samples. These filters helped to reduce clogging and filtering times. Given the size of the filters, we expected to have captured bacteria attached to particulate matter only, and not the whole planktonic community. We stored the filters in 15-mL centrifuge tubes, and placed them in liquid nitrogen before moving them to a −80°C freezer until DNA isolation occurred.

Environmental Data Collection

For each population sampled, we recorded GPS coordinates at the position where sampling began, and estimated linear distance between sampled sites *post hoc* using Google Earth (Google, Mountain View, CA, United States). We obtained land-use, latitudinal, and elevational data from publically available GIS databases. We chose to include these layers as they have been previously associated with stream microorganism biogeography (Lear et al., 2013). We used the USGS National Watershed Boundary Dataset to identify the HUC 12-level boundaries at each river sampled in ArcGIS 10.2 (Esri, Redlands, CA, United States). We applied these boundaries to characterize the percentage of land covered in forest using the National Land Cover Data from 2006 (Fry et al., 2011). Because we did not obtain sampling points for each hellbender, we randomly assigned geographic coordinates for each individual along the estimated sampling length within its river. At each pseudo-coordinate, we obtained elevation data from the USGS Elevation Point Query Service.

Laboratory Methods

We isolated DNA from skin swabs using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, United States) following the protocol described in Hernández-Gómez et al. (2017). We processed water filters using the PowerWater DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, United States) following manufacturer's instructions. We prepared the sequencing library using two sequential PCRs. The first PCR consisted of amplification of the 16S rRNA V2 region using primer pair 27F/338R (Fierer et al., 2008) with the attachment of connector sequences (Hernández-Gómez et al., 2017). We ran each sample in triplicate, and each reaction consisted of 5 μL of template DNA, 12.5 μL of MyTaq Master Mix (Bioline, Tauton, MA, United States), 1 μL of 10 mM forward and reverse primers, and 6.5 μL of water (MoBio Laboratories, Inc., Carlsbad, CA, United States) for a total of 25 μL per reaction. PCR conditions consisted of 95°C for 2 min, 30 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, followed by 72°C for 10 min. We pooled triplicates and cleaned the PCR products using the UltraClean PCR Clean-up kit (MoBio Laboratories, Inc., Carlsbad, CA, United States). The second PCR was performed to add-on dual-index barcodes connected to Illumina sequencing adaptors (Hernández-Gómez et al., 2017) to the ends of amplicons. The PCR consisted of 5 μL of clean amplicons, 12.5 μL of MyTaq Master Mix, 1 μL of forward and reverse barcode primers, and 6.5 μL of water for a total of 25 μL reactions. PCR conditions consisted of 95°C for 2 min, 5 cycles of 94°C for 45 s, 65°C for 60 s, and 72°C for 90 s, followed by 72°C for 10 min. We quantified the PCR products using a Qubit Fluorometer (Invitrogen, Corp, Carlsbad, CA, United States), and pooled the samples in equimolar amounts to be cleaned using the UltraClean PCR Clean-Up kit. The cleaned sample pool was sequenced on a MiSeq machine (Illumina, Inc., San Diego, CA, United States) utilizing the Reagent Kit V2 to produce 250 bp paired end reads.

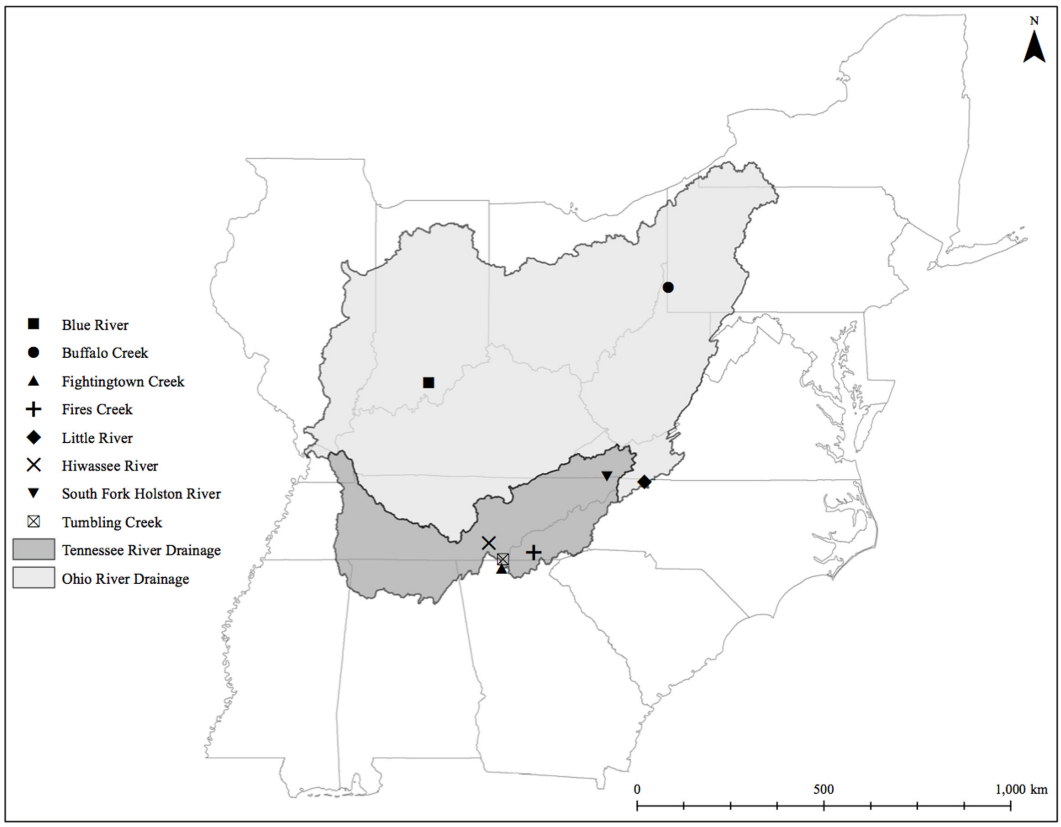

FIGURE 1 | Eastern hellbender cutaneous microbiome sampling sites across the subspecies range.

Sequence Analysis

We processed raw reads using Trimmomatic (Bolger et al., 2014) to remove adapter sequences, bases below threshold quality of phred-20 from both ends of reads, and any resulting reads under 30 bp. We paired reads that passed initial quality control using PANDAseq (Masella et al., 2012). Only reads that paired successfully were utilized in subsequent analysis. We used a custom Python script to remove quality scores from reads and re-name the reads with a name compatible with our chosen analysis pipeline. We processed the resulting read file using the Quantitative Insights Into Microbial Ecology version 1.8.0 (QIIME) pipeline (Caporaso et al., 2010b). We clustered reads at the standard 97% similarity using the open-reference protocol (Rideout et al., 2004) and the Greengenes 13_5 reference database (DeSantis et al., 2006). Reads that failed to cluster using the open-reference algorithm were clustered into *de novo* operational taxonomic units (OTUs) with UCLUST (Edgar, 2010). OTUs that were clustered using the Greengenes database retained the matched taxonomy, while *de novo* OTUs were assigned taxonomy using the RDP Classifier (Wang et al., 2007) at 80% confidence. We aligned representative sequences to the pre-aligned Greengenes reference using PyNAST (Caporaso et al., 2010a) and the alignment was used to produce a phylogenetic tree through FastTree (Price et al., 2010).

To avoid including OTUs generated by sequencing error (e.g., chimeras and base miscalls) we removed OTUs that were represented by fewer than 0.005% of the total read count (Bokulich et al., 2013). We also rarified sequencing depth to 8,800 reads per sample to standardize our samples. We calculated the core microbiome (defined as the set of detectable OTUs present in at least 80% of all samples) to derive the ubiquitous OTUs across the range of the eastern hellbender.

Data Analysis

We conducted a series of analyses to explore the skin microbiota of hellbenders. In brief, we evaluated differences in richness and diversity between the cutaneous microbial communities of hellbenders from different populations. In addition, we implemented metacommunity analysis to assess patterns in bacterial OTU distribution across the eastern hellbender metapopulation. Lastly, we assessed differences in community composition between the two genetic demes of eastern hellbenders and among the sampled populations. We performed all statistical analyses in R version 3.2.0 unless otherwise noted.

Richness and Diversity Comparison

We assessed differences in community richness/diversity between the two genetic demes and among populations, and compared

the proportion of shared OTUs between skin and water samples at each population. We calculated richness (observed OTUs) and Shannon Diversity Index values within each sample from the abundance-based OTU table in QIIME. We used richness or Shannon Diversity Index values for each hellbender as dependent variables, genetic deme assignment as a fixed factor, and population as a random factor in negative binomial generalized linear mixed models (richness) or linear mixed models (Shannon Diversity Index). In addition, we assessed pairwise differences in richness and Shannon Diversity Index between all populations using Tukey's honest significant difference (HSD) tests. We also calculated the proportion of OTUs shared between hellbenders and the corresponding river water samples. We used the proportion of shared OTUs as dependent variables, genetic deme assignment as fixed factor, and population as random factors in a generalized linear mixed model. Lastly, we tested pairwise differences in proportion of shared OTUs between all populations using Tukey's HSD tests.

Metacommunity Structure

We assessed the metacommunity structure as described in Leibold and Mikkelson (2002) and Presley et al. (2010) using the package metacom in R (Dallas, 2014). From the QIIME generated OTU table, we created an individual hellbender by OTU matrix. We used reciprocal averaging (i.e., correspondence analysis) to rank hellbenders so that those having similar OTU composition are close to each other, and OTUs were ordinated so that those having similar occurrence among the hellbenders were close to each other. OTU distribution patterns were derived by calculating the three elements of metacommunity structure (EMS): coherence, turnover, and boundary clumping. *Coherence* is a measure of the number of embedded species absences (gaps within a species range) within an ordinated community matrix. Negative coherence (more embedded absences within the matrix than expected by random chance) indicates a "checkerboard" metacommunity structure. Positive coherence indicates the response of species to a gradient of environmental variation, and denotes the need to evaluate species turnover and boundary clumping to identify structure (Leibold and Mikkelson, 2002). *Species turnover* represents the number of times that one species replaces another between sites. Negative turnover (denoted as lower replacements than expected by chance) is associated with a nested structure while positive turnover (larger replacements than expected by chance) is associated with Gleasonian or Clementsian structures (Leibold and Mikkelson, 2002). *Boundary clumping* is evaluated through the calculation of Morisita's index; a value greater than one corresponds with a Clementsian structure (i.e., clumped range boundaries), a value less than one corresponds with an even spaced structure (hyperdisplaced range boundaries), and a value equal to 1 corresponds with a Gleasonian structure (randomly distributed boundaries). We implemented the default 'r1' fixed-proportional null model to calculate the significance of the EMS indices calculated. The fixed-proportional model maintains the OTU richness within each locality while filling OTU ranges based on their marginal probabilities. Because we were working with a very large dataset, we restricted permutations to 99 and allowed null matrices to have empty rows and columns as recommended by Dallas (2014) to reduce computation time.

We chose to include all OTUs in the analysis even though a high proportion of OTUs in our dataset were rare (∼77.5% of OTUs had a total sequencing depth of less than 100 sequences). While metacommunity analysis was developed for macro-communities with fewer species, it has been effectively implemented to characterize the metacommunity structure of bacterial communities before (Heino et al., 2015). As such, we followed the methodology of Heino et al. (2015) in our approach to characterize the metacommunity of hellbender microbial communities. We also performed a trial analysis to compare the structure of the complete dataset and the community data without rare species. Because we did not find a difference in metacommunity structure between both approaches, we only present analysis/results on the complete dataset. We performed the metacommunity analysis three times. For the first analysis, we used an OTU-by-hellbender incidence matrix that included all skin samples throughout the range. To evaluate the effect of environmental variables on hellbender ordination, we performed Pearson correlation tests between sample ordination scores and HUC 12-level percent forest cover, elevation, and latitude. Upon performing the first analysis, we noted a pattern of clumped OTU turnover between two groups of samples (i.e., compartments). These compartments formed non-random groupings of hellbender populations; thus, we decided to evaluate the metacommunity structure of each compartment independently to evaluate species turnover as suggested in Presley et al. (2010). We split the data matrix on this boundary and performed the metacommunity structure analysis on each of the two compartments.

Microbial Community Structure

For all community composition comparisons, we transferred the OTU tables and corresponding Newick phylogenetic tree to R, and the package GUniFrac was used to build UniFrac distance matrices (unweighted and weighted; Lozupone et al., 2011). We performed Adonis and ANOSIM tests using the unweighted and weighted UniFrac distance matrices within the package vegan 2.2-1 (Oksanen et al., 2007) to partition the variation between groups. To assess the influence of genetic deme assignment on the microbiota of eastern hellbenders, we tested differences between skin samples assigned to each major drainage (i.e., Ohio or Tennessee). We used major watershed identity as a representative of genetic deme assignment as described in Unger et al. (2013). To evaluate the influence of population on the microbiota of eastern hellbenders across the range, we tested differences between skin samples assigned to each population. We visualized differences in community structure using unweighted and weighted UniFrac distances through a principal coordinate analysis (PCoA) generated through package phyloseq in R (McMurdie and Holmes, 2013).

To evaluate the effect of population-level genetic differentiation we conducted correlations between microbial community distances and genetic/geographic distances (Fst). To

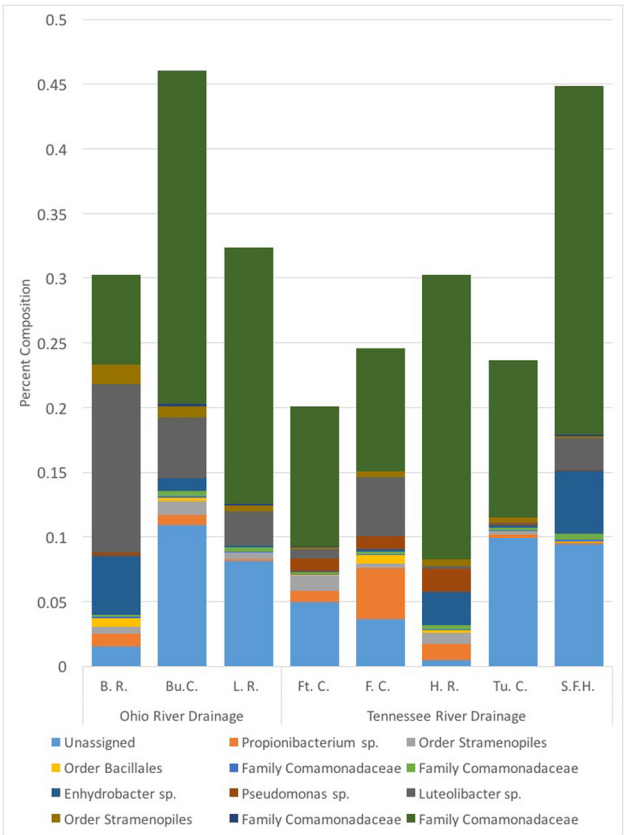

FIGURE 2 | Core skin microbial operational taxonomic units (OTUs – 80% prevalence) of eastern hellbenders and the mean relative abundance of those OTUs within each locality sampled. Core microbiome across the eastern hellbender range is dominated by similar taxa. (Localities: BuC – Buffalo Creek, WV; SFH – South Fork of the Holston, VA; TuC – Tumbling Creek, TN; HR – Hiwassee River, TN; FC – Fires Creek, NC; LR – Little River, NC; BR – Blue River, IN; FtC – Fightingtown Creek, GA).

evaluate population genetic distance, we obtained microsatellite genotype data from Unger et al. (2013) for each of the populations sampled in this study. Unger et al. (2013), did not sample the Little River, NC population; thus, we obtained genotypes for the closest population in the respective drainage system (South Fork of the New River). We calculated population genetic distances (Fst) using the package hierfstat in R (Goudet, 2005). We assessed correlations between Fst and UniFrac (unweighted and weighted) using a Mantel test. Lastly, we assessed the correlation between linear distance and community dissimilarity to evaluate dispersal barriers. We uploaded the pseudo-coordinates to QGIS 2.18 (Open Source Geospatial Foundation Project)[1], and calculated linear distances between all points. In R, we conducted a Mantel test using the pairwise spatial distance and community dissimilarity (1-Jaccard) matrices. The derived correlations were compared to correlations originated from 10,000 random permutations.

[1]http://qgis.osgeo.org

RESULTS

We collected samples from three populations within the Ohio River Drainage (Blue River, Indiana-BR, $n = 5$; Buffalo Creek, West Virginia-BuC, $n = 5$; and Little River, North Carolina-LR, $n = 8$) and five populations within the Tennessee River Drainage (Hiwassee River, Tennessee-HR, $n = 5$; Tumbling Creek, Tennessee-TC, $n = 3$; Fightingtown Creek, Georgia-FT, $n = 5$; South Fork of the Holston River, Virginia-SFH, $n = 8$; and Fires Creek, North Carolina-FC, $n = 5$) for a total of 43 hellbender and eight water samples (Supplementary Table S1). Amplicon sequencing resulted in more than 10 million reads with an average length of 344 base pairs. We deposited sequencing data into the NCBI Sequence Read Archive (accession numbers: SRR5479798-SRR5479789). A total of 1,656 OTUs were identified across all adult swabs and water samples after filtration and rarefication. Hellbender skin bacterial communities were dominated by a few abundant OTUs. The core microbiome of eastern hellbenders was dominated by nine OTUs belonging to the phyla Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria, and Verrucomicrobia (**Figure 2**). OTUs with the highest abundance on the skin of eastern hellbenders were assigned to the family Comanmonadaceae (mean relative abundance: 46.5%), order Stramenopiles (9.4%), and the genus *Proprionibacterium* (8.4%).

Richness/Diversity Comparisons

Hellbenders from the Ohio River Drainage possessed richer communities (observed OTUs values; mean ± SD: 491.28 ± 178.09) than hellbenders from the Tennessee River Drainage (mean ± SD: 333.77 ± 149.29; LRT = 3.75, $p = 0.053$). Shannon Diversity Index values did not differ between the two genetic demes (LRT = 1.25, $p = 0.263$). We found significant differences in skin community richness and diversity among sampling localities from *post hoc* multiple comparisons (**Figure 3**). With respect to the proportion of shared OTUs between individuals and river water, hellbenders from the Ohio River Drainage (mean ± SD: 0.29 ± 0.12) shared a higher proportion of OTUs with the river water compared to hellbenders from the Tennessee River Drainage (mean ± SD: 0.16 ± 0.10; LRT = 6.00, $p = 0.014$). Finally, we found significant differences among sampling localities in the proportion of shared OTUs between river water and the skin from *post hoc* multiple comparisons (**Figure 4**).

Metacommunity Analysis

The fixed-proportional null model in the EMS analysis revealed that the hellbender cutaneous bacterial metacommunities displayed positive coherence (absences: 44,120; simulated mean: 52,216 ± 668.0; $p < 0.001$) and positive turnover (replacements: 6,764,076; simulated mean: 552,825.38 ± 205,075.34; $p < 0.001$). In addition, we found significant positive boundary clumping in the distribution of OTUs (Morisita's index = 72.41, $p < 0.001$, df = 1,642). Together, these three values indicate a Clementisan pattern in skin microbial metacommunities throughout the eastern hellbender range. We noted significant correlations between hellbender sample ordination scores and elevation

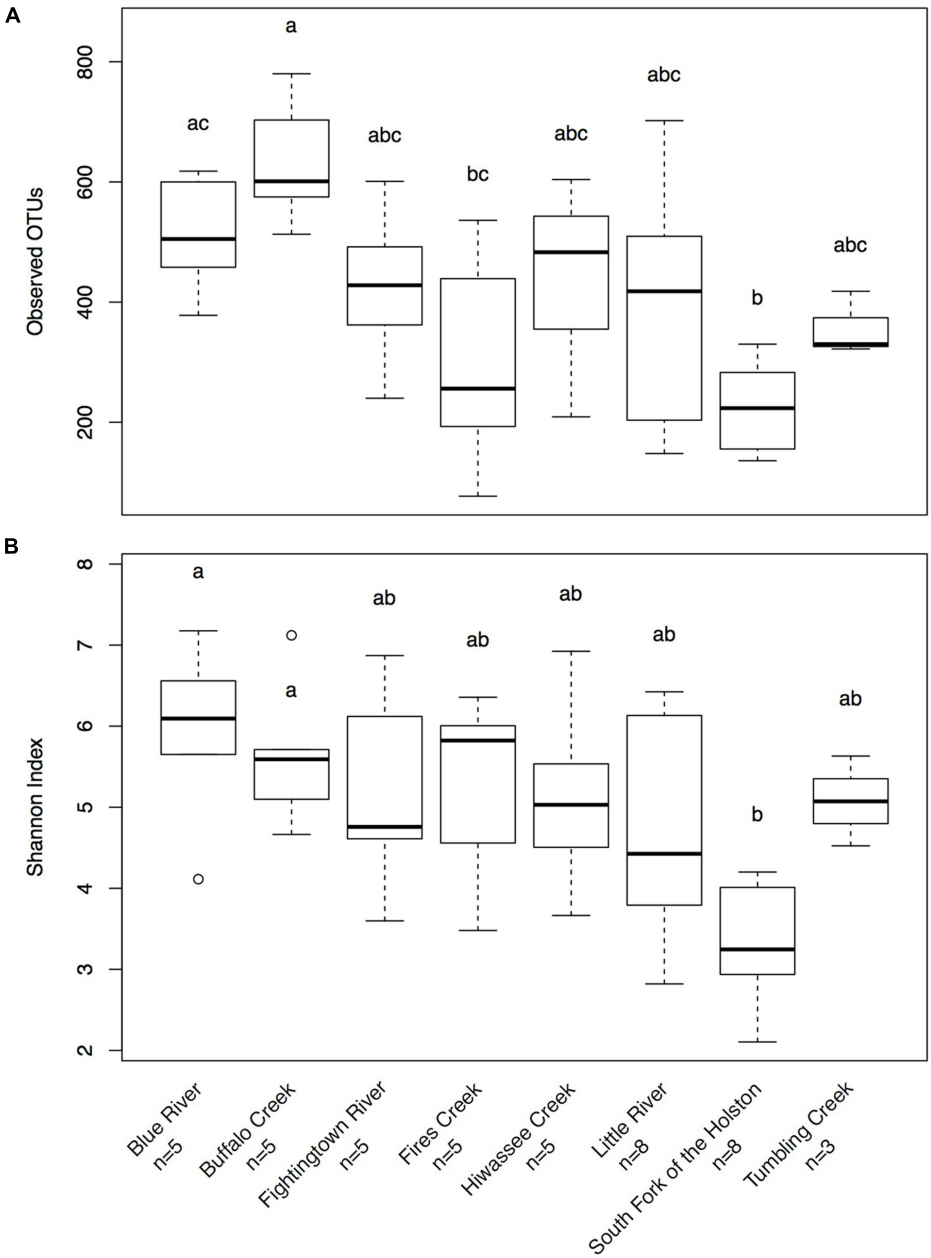

FIGURE 3 | Box-plot distributions of alpha diversity values **(A)**- Observed OTUs, **(B)**- Shannon Diversity Index for cutaneous microbial communities from eastern hellbenders at different localities in the subspecies' range. There is more variation in values of observed OTUs among populations over Shannon Diversity Index. Letters (a, b, c) indicate significant differences among hellbender populations using *post hoc* Tukey's HSD tests. Outliers are noted by circles.

($r = 0.58$, $p < 0.001$) and latitude ($r = -0.48$, $p = 0.002$). However, we did not note a significant correlation between sample ordination scores and HUC 12-level percent forest cover ($r = 0.25$, $p = 0.106$). There was a discernable clumping of OTU boundaries (**Figure 5**) that separated two major compartments of ordinated samples corresponding mostly to the Blue River, Buffalo Creek, and South Fork of the Holston River (compartment 1) and the Little River, Hiwassee River, Tumbling Creek, Fightingtown Creek, and Fires Creek (compartment 2). However, the boundary between these compartments did not align as expected between the two eastern hellbender genetic demes (**Figure 1**).

Within both compartments cutaneous bacterial metacommunities displayed Clementsian structure as evidenced by positive coherence (compartment 1: absences = 11,154, simulated mean = 15,127.41 ± 537.14, $p < 0.001$; compartment 2: absences = 22,558, simulated mean = 26,512.00 ± 622.17, $p < 0.001$), positive

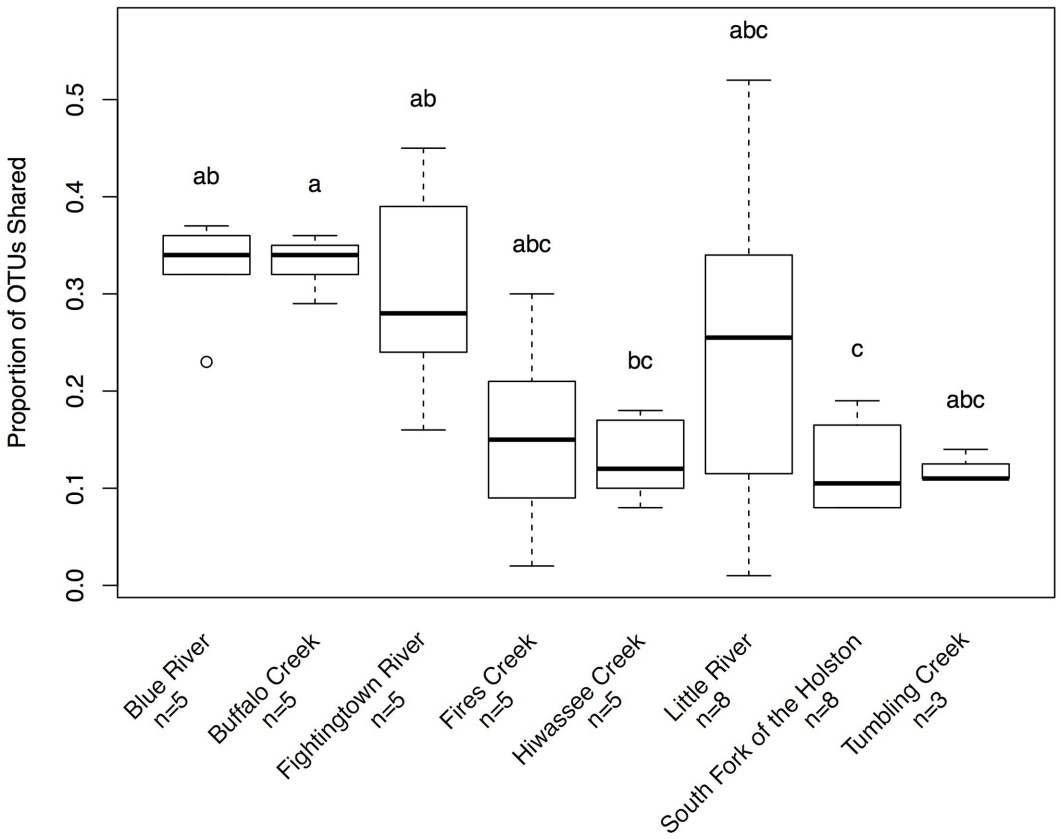

FIGURE 4 | Box-plot distributions of the proportion of OTUs shared between hellbenders and river water samples at different localities in the subspecies' range. Populations of eastern hellbenders vary in the proportion of OTUs shared between the skin and river water. Letters (a, b, c) indicate significant differences among hellbender populations using *post hoc* Tukey's HSD tests. Outliers are noted by circles.

turnover (compartment 1: replacements = 1,601,524, simulated mean = 268,412.29 ± 78,876.94, $p < 0.001$; compartment 2: replacements = 3,819,214, simulated mean = 411,109 ± 78,876.94, $p < 0.001$), and significant positive boundary clumping (compartment 1: Morisita's index = 38.59, $p < 0.001$; compartment 2: Morisita's index = 115.22; $p < 0.001$). Within compartment 1, sample grouping is noticeable between hellbenders from the Blue River/Buffalo Creek and the South Fork of the Holston River (**Figure 6A**). Within compartment 2, grouping is noticeable between hellbenders from Fightingtown Creek/Little River and Hiwassee River/Fires Creek/Tumbling Creek (**Figure 6B**). Samples from compartment 1 were collected from sites with higher latitudes (mean ± SE; 38.13 ± 0.35 vs. 35.50 ± 0.13°), lower elevation (392.56 ± 54.88 vs. 595.33 ± 43.23 m), and lower HUC 12-level percent forest cover (62.94 ± 1.61 vs. 75.39 ± 3.75%) than samples form compartment 2.

Microbial Community Structure

Composition of the hellbender skin microbiome varied more strongly by population than by genetic deme. Community composition between the two genetic demes varied moderately when based on presence/absence of OTUs alone (unweighted UniFrac: Adonis $R = 0.09$, $p < 0.001$; ANOSIM $R = 0.20$, $p = 0.001$). However, no variation was noted when abundance of OTUs was taken into account (weighted UniFrac: Adonis $R = 0.02$, $p = 0.285$; ANOSIM $R = 0.05$, $p = 0.023$). In contrast, a strong difference among sampling localities was noted when comparing microbiome communities using unweighted (Adonis $R = 0.42$, $p < 0.001$; ANOSIM $R = 0.56$, $p < 0.001$) and weighted UniFrac distances (Adonis $R = 0.40$, $p < 0.001$; ANOSIM $R = 0.37$, $p < 0.001$). PCoA graphs display clear partitioning of points based on population over genetic sub-population for unweighted UniFrac distances (**Figure 7**) and weak grouping for weighted UniFrac distances (**Figure 8**). There were significant positive correlations between skin community dissimilarity and genetic distances (unweighted UniFrac: $r = 0.39$, $p < 0.001$; weighted UniFrac: $r = 0.31$, $p < 0.001$). There was a weak, but significant, correlation between skin community dissimilarity and distance throughout the range of the species ($r = 0.12$; $p = 0.035$).

DISCUSSION

We implemented culture-independent microbiome characterization and microbial community analyses to describe

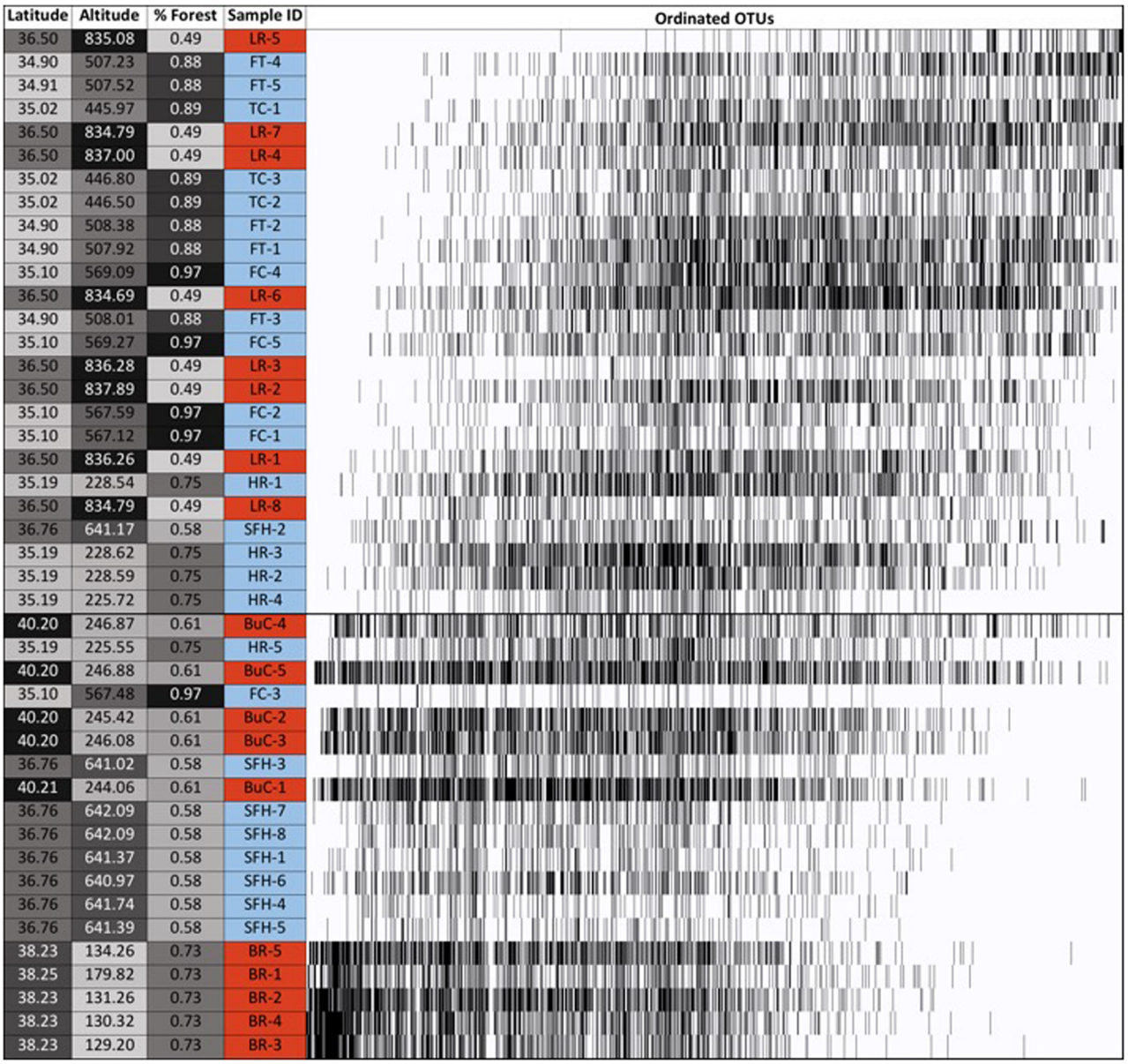

FIGURE 5 | Incidence matrix for skin bacterial communities from eastern hellbenders. Skin samples and operational taxonomic units (OTUs) were ordinated using reciprocal averaging as described in Leibold and Mikkelson (2002). Solid bars indicate each OTU's presence throughout the ordinated hellbenders. Sample labels correspond to rivers where hellbenders were sampled from: BuC – Buffalo Creek, SFH – South Fork of the Holston, TuC – Tumbling Creek, HR Hiwassee River, FC – Fires Creek, LR – Little River, BR – Blue River, and FtC – Fightingtown Creek. Sample label color corresponds with the two hellbender genetic demes (Ohio River Drainage – red, Tennessee River Drainage – blue). Latitude, altitude, and % forest cover data is presented and labeled in gray scale from lowest (light) to highest (dark) values. Metacommunity structure analysis indicated Clementsian structure corresponding with clumped OTU turnover. The horizontal line marks boundary of OTU boundaries generating two major compartments.

the eastern hellbender cutaneous microbiome across the range of the subspecies. We observed variation among populations in skin microbial community richness, diversity, and the proportion of shared OTUs between animal and environmental samples. The metacommunity analysis revealed Clementsian structure, which suggests clumped species turnover among groups of samples. We observed compartmentalization within the hellbender skin microbiome metacommunity that aligned with changes in latitude, elevation, and HUC 12-level percent forest cover. The effect of environmental heterogeneity on community assembly in the skin of hellbenders is further evident given that OTU turnover correlated weakly with linear distance. Finally, OTU turnover among populations resulted in phylogenetically distinct communities. Together, the results suggest there is a large amount of regional-level variation in the skin bacterial communities of eastern hellbenders. Large-scale variation is

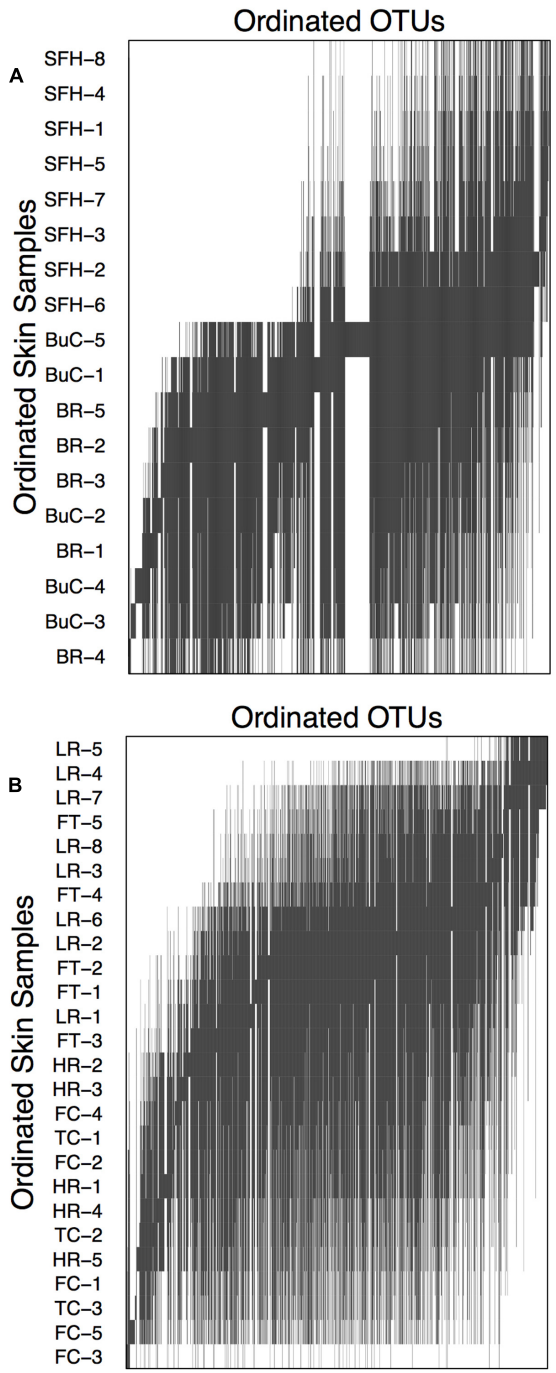

FIGURE 6 | Compartment incidence matrices for skin bacterial communities from eastern hellbenders. Compartments correspond to the species distribution boundary outlined in **Figure 5**. Skin samples and OTUs were ordinated using reciprocal averaging as described in Leibold and Mikkelson (2002). Solid bars indicate each OTU's range throughout the ordinated hellbender samples. Within both compartments, metacommunity structure corresponds with Clementsian structure. Sample labels correspond to rivers where hellbenders were sampled from BuC – Buffalo Creek, WV; SFH – South Fork of the Holston, VA; BR – Blue River, IN for compartment in **(A)** and TuC – Tumbling Creek, TN; HR Hiwassee River, TN; FC – Fires Creek, NC; LR – Little River, NC; and FtC – Fightingtown Creek, GA for compartment in **(B)**.

formed by symbiont turnover among hellbender populations in response to differences in local processes.

Eastern hellbenders occupy a large range that encompasses broad variation in environmental factors (e.g., stream order, habitat quality, temperature; Hanson et al., 2012); therefore, it is likely that OTU turnover among populations occurred in response to environmental differences. In our metacommunity analysis, we were able to detect significant bacterial OTU turnover with changes in latitude and elevation. While we lack real-time environmental data (e.g., salinity, pH, temperature) to evaluate OTU turnover against stream characteristics, we can infer that climatic variability may have an influence on the skin microbiome of hellbenders. In addition, we only evaluated overlap between particle-attached communities in the river water and the skin of the salamanders ignoring other possible environmental reservoirs (e.g., free-living, sediment, cover-rocks, alternative hosts). Furthermore, we did not test for *Batrachochytrium dendrobatidis* or other integumentary pathogens in this study. While evaluating the presence of specific pathogens was not within the scope of our study, the presence of these pathogens can alter the microbial communities of amphibians (Jani and Briggs, 2014). Identifying environmental variables, reservoirs, and biotic factors that influence the assembly of symbiont communities is pertinent to pinpoint factors that contribute to functional variation among populations or individuals. While we lacked an environmental component, we still implemented community structure patterns to draw conservative inferences regarding possible functional variation within the microbiome of this salamander.

Variation in Skin Community Richness/Diversity

We observed higher skin bacterial community richness in hellbenders from the Ohio River Drainage compared to hellbenders from the Tennessee River Drainage. In addition, bacterial communities varied in richness among hellbender populations. Differences in community richness likely result from different environmental conditions (e.g., temperature, stream order, habitat quality) between populations. Despite differences in richness, we did not observe significant variation in community diversity (i.e., Shannon Diversity Index) between the two genetic demes or among populations. An absence of variation in community diversity likely resulted from similar patterns of OTU abundances (skewed toward a few abundant OTUs) throughout the range.

Variation in community richness among populations can translate into differences in community functional diversity. Within macro- and microbial systems, it is common to observe a relationship between biodiversity and ecosystem functionality (Balvanera et al., 2006). More importantly, symbiont diversity may correlate with the ability of microbial communities to resist or recover from disturbances (i.e., dysbiosis) generated from environmental pressures or pathogen invasion (Girvan et al., 2005; Lozupone et al., 2012). Diversity is important to community stability as it increases the probability that functionally important bacteria are present in the environment, or can enhance

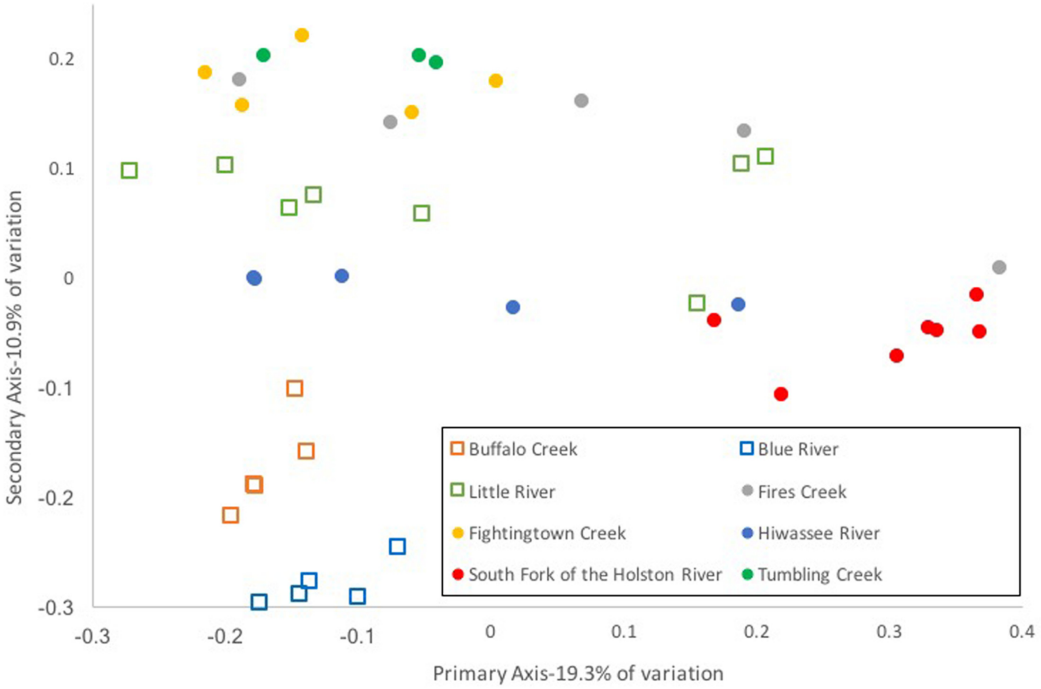

FIGURE 7 | Principal coordinate analysis of un-weighted UniFrac distance matrices from hellbender skin microbial communities at eight rivers in the species range. Each point represents the skin bacterial community of an individual hellbender; shape indicates genetic deme assignment (squares -Ohio River Drainage, circles -Tennessee River Drainage) and color indicates river locality. While there is no distinct clustering of points by genetic deme, clustering by river locality is visible.

synergistic effects (Loreau and Hector, 2001). In humans, loss of gut microbiome diversity due to recurrent antibiotic use is associated with recurrent *Clostridium difficile* infections (Chang et al., 2008). Within amphibians, skin community diversity is linked with the ability to suppress *Batrachochytrium dendrobatidis* growth (Rebollar et al., 2016; Piovia-Scott et al., 2017). While much of the research on amphibian microbial immunity is concentrated on the contributions by individual bacterial species, recent investigations show that defense might depend on overall community diversity (Lam et al., 2010; Jani and Briggs, 2014; Piovia-Scott et al., 2017). Therefore, within the range of the eastern hellbender, populations possessing richer skin communities may possess increased ability to resist dysbiosis induced by disease or environmental changes (e.g., translocation, disease, pollution) than those with low richness.

Clumped Skin Bacterial Lineage Turnover

In addition to diversity differences, bacterial turnover in skin communities among our hellbender samples may result in functional heterogeneity. Within amphibians, OTU turnover among populations of the same host species is not a novel finding (Lam et al., 2010; Kueneman et al., 2014; Rebollar et al., 2016). However, this study is the first to implement metacommunity structure analysis to quantify the magnitude of species turnover among individuals and populations. In our observations, clumped species turnover (i.e., Clementsian structure) resulted in unique communities among two major compartments (compartment 1: Blue River, Buffalo Creek, South Fork of the Holston River; compartment 2: Fightingtown Creek, Fires Creek, Hiwassee River, Little River, Tumbling Creek; **Figure 5**). This structure indicates similarities among groups of OTUs in their adaptive trade-offs or tolerances to local environmental factors (Clements, 1916; Presley et al., 2010). In addition, clumped turnover of bacterial skin symbionts to environmental pressures could indicate replacement of functionally important traits. For example, patterns of symbiont turnover are associated with loss/gain of anti-pathogen metabolite producing bacteria on the skin of amphibians (Lam et al., 2010). The complete replacement of symbionts among the observed compartments in the metacommunity could translate into major differences in community functionality. Integrating '-omic' approaches to characterize functionality of the skin communities may help in quantifying functional redundancy in the regional pool of hellbender skin symbionts. For example, metagenomics was successful in describing functional redundancy among gut microbiota in humans despite disparity in community composition (Ferrer et al., 2013). Belden et al. (2015) implemented high-performance liquid chromatography-mass spectrometry to characterize skin microbe metabolites, and identified a lack of differentiation among host species despite community dissimilarity. Thus, incorporating functional characterization in future assessments of hellbender skin will be useful to evaluate how OTU turnover influences important functional traits, such as pathogen defense

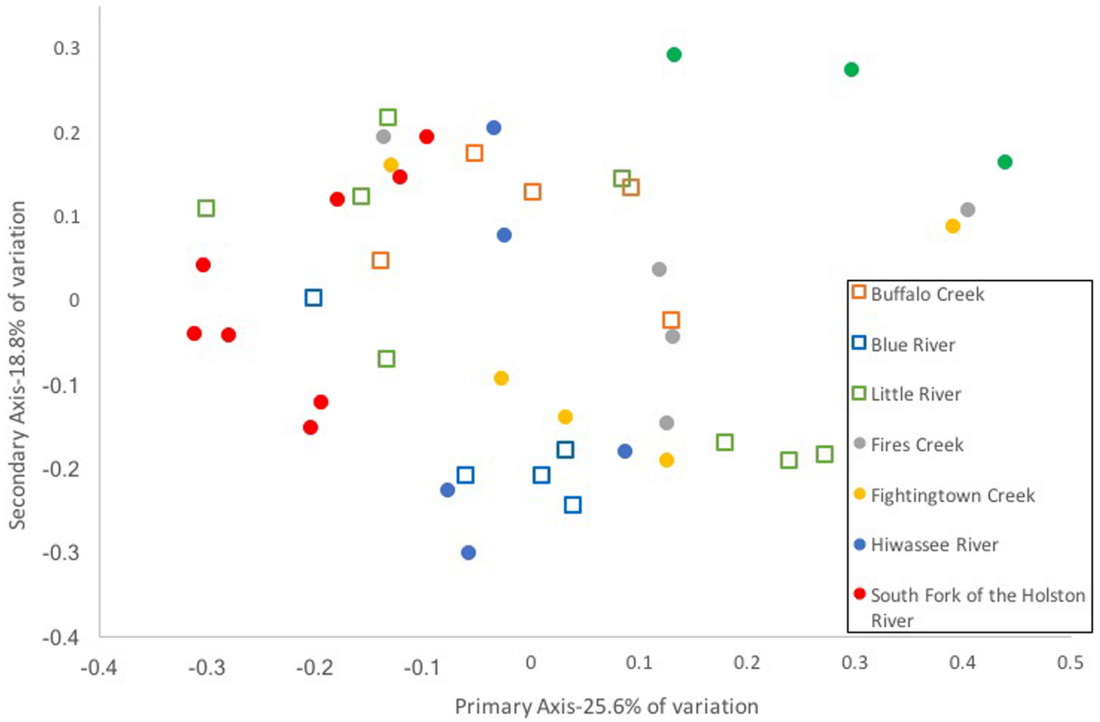

FIGURE 8 | Principal coordinate analysis of weighted UniFrac distance matrices from hellbender skin microbial communities at eight rivers in the species range. Each point represents the skin bacterial community of an individual hellbender; shape indicates genetic deme assignment (squares -Ohio River Drainage, circles -Tennessee River Drainage) and color indicates river locality. While there is no distinct clustering of points by genetic deme or river locality.

or community resistance to anthropogenic activities (Jiménez and Sommer, 2017).

Skin Community Compositional Differences

Implementing a phylogenetic based dissimilarity index to characterize beta diversity among hellbender skin communities allowed us to assess bacterial lineage turnover. We expected to observe segregation between samples of each genetic deme, given that historic host movement may have monopolized host bacterial lineages within each genetic deme (Mihaljevic, 2012). Eastern hellbender communities throughout the range were dominated by a few taxa. As such, we did not observe a strong pattern of differentiation from weighted UniFrac tests. However, we did observe clear partitioning by population using the unweighted UniFrac measure, corresponding to a pattern of bacterial lineage turnover among rare members of the skin microbiome. In addition, we observed a positive correlation between individual community dissimilarity and population-level genetic differentiation. These two patterns suggest the importance of host and symbiont adaptations to varying environments (Lozupone and Knight, 2005). It is interesting to consider how associations between hellbenders and the microbiome can vary at higher taxonomic levels. Considering variation in the hellbender skin microbiome might be useful to evaluate loss of immunocompetence in the eastern hellbender's sister subspecies, the Ozark hellbender. Within their limited range in Missouri and Arkansas, the endangered Ozark hellbender expresses a high frequency of chronic cutaneous wounds that often result in necrosis of tissues of the limbs and face (Wheeler et al., 2002; Hiler et al., 2005). A previous study evaluated the microbiome communities between the two hellbender subspecies, and characterized divergence in skin communities of both populations (Hernández-Gómez et al., 2017). However, that study only sampled one population of each subspecies; and thus, the differentiation patterns encountered could result from environmental rather than host-specific associations. Therefore, expanding characterization of the microbiome across distinct environments may be beneficial to observe whether the microbiome truly diverges between these two subspecies.

Considerations for Hellbender Conservation

Patterns of symbiont distribution in eastern hellbender skin metacommunities can inform current conservation management. Hellbender host characteristics (e.g., immunity) and environmental variables may create a gradient of suitable habitat for cutaneous microbial symbionts. Among populations of hellbenders, we have characterized variation in symbiont dispersal and possible physiological constraints/adaptive trade-offs that result in varying patterns of taxonomic

turnover (i.e., species replacements). Hellbenders have experienced population reductions due to habitat loss, water quality degradation, harvesting, and disease (Furniss et al., 2003; Nickerson and Briggler, 2007; Federal Register, 2011). Recently, hellbender conservation efforts have focused on captive breeding, captive rearing, and translocations in attempts to recover populations (Bodinof et al., 2012; Ettling et al., 2013). Captive hellbenders are currently reared in aseptic conditions that are absent of environmental sources of microbes (Ettling et al., 2013). In fact, divergent community composition has been documented between the skin microbiome of captive and wild hellbenders in Missouri (Hernández-Gómez, unpublished data) and among other amphibians (Antwis et al., 2014; Becker et al., 2014; Loudon et al., 2014). Eliminating natural sources of microbial symbionts can have consequences on the health, adaptability, and immunity of captive individuals (Redford et al., 2012).

It is important to evaluate whether captive eastern hellbenders maintain associations with the core microbes identified in this study (i.e., Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria, and Verrucomicrobia). Similar microbes are also described as core members of the skin microbiome of wild hellbenders and other amphibians (McKenzie et al., 2012; Kueneman et al., 2014; Hernández-Gómez et al., 2017), indicating important associations between these symbionts and hosts. Finding ways to assimilate the captive microbiome to naturally occurring communities in wild counterparts may increase re-introduction success. For skin communities, environmental reservoirs such as water, soil, or organic material can serve as a vehicle to introduce "wild" microbes in captivity. Both Loudon et al. (2014) and Walke et al. (2014) have described the use of environmental reservoirs (water and soil respectively) to colonize the communities on the skin of amphibians. While we did not sample all possible environmental reservoirs in the wild hellbender habitat (e.g., sediment, rocks, alternative hosts), we did find similarities between river water and the skin of hellbenders. Therefore, river water from future release sites can be used in controlled microbe exposures to alter the diversity of the skin microbiota of captive hellbenders. This technique also has the potential to be applied to captive individuals prior to release. Future investigations should focus on evaluating the effects of controlled microbial exposures in captivity, timing of assimilation of novel microbes into the skin, and how an assimilated microbiome can influence translocation success.

Conservation management can benefit by incorporating microbial barriers into translocation planning as well. Currently, translocation design is performed following recommendations from population genetics studies (Unger et al., 2013). However, we encountered barriers to microbial distributions at the genetic deme level. Translocating animals across metacommunity compartments can result in: (1) erosion of natural barriers to pathogen dispersal (Cunningham, 1996; Redford et al., 2012) and (2) alterations to the structure of the amphibian skin microbiome (dysbiosis) due to different environmental characteristics (Loudon et al., 2014). Movement of amphibians has contributed to the propagation of lethal amphibian pathogens throughout the globe (Daszak et al., 1999). In addition, the effects of exposure to numerous novel bacteria on the health of translocates is unknown. Environment-induced dysbiosis could also alter the protective phenotype of the host–microbe association resulting in increased susceptibility to pathogens (Willing et al., 2011; Woodhams et al., 2011). To limit dispersal of pathogens or exposure to novel microbes, biologists should limit heterogeneity in symbiont composition between source and supplemented populations. Therefore, conservation managers should consider symbiont boundaries in planning animal movement within each genetic deme. Increasing microbiome sampling efforts throughout each genetic deme may also increase the resolution of the boundaries that we observed.

AUTHOR CONTRIBUTIONS

OH-G, JH, and RW contributed in the research design. OH-G performed sample collection, laboratory work, and bioinformatics analysis. Statistical analysis was performed by OH-G with input from JH. The manuscript was written by OH-G with input from JH and RW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01379/full#supplementary-material

REFERENCES

Antwis, R. E., Haworth, R. L., Engelmoer, D. J. P., Ogilvy, V., Fidgett, A. L., and Preziosi, R. F. (2014). Ex situ diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). *PLoS ONE* 9:e85563. doi: 10.1371/journal.pone.0085563

Bahrndorff, S., Alemu, T., Alemneh, T., and Lund Nielsen, J. (2016). The microbiome of animals: implications for conservation biology. *Int. J. Genomics* 2016, 7. doi: 10.1155/2016/5304028

Balvanera, P., Pfisterer, A. B., Buchmann, N., He, J. S., Nakashizuka, T., Raffaelli, D., et al. (2006). Quantifying the evidence for biodiversity effects on ecosystem

functioning and services. *Ecol. Lett.* 9, 1146–1156. doi: 10.1111/j.1461-0248.2006.00963.x

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B., and Belden, L. K. (2014). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biol. Conserv.* 176, 199–206. doi: 10.1016/j.biocon.2014.05.029

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. Biol. Sci.* 282, 20142881. doi: 10.1098/rspb.2014/2881

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bodinof, C. M., Briggler, J. T., Junge, R. E., Mong, T., Beringer, J., Wanner, M. D., et al. (2012). Survival and body condition of captive-reared juvenile Ozark hellbenders (*Cryptobranchus alleganiensis bishopi*) following translocation to the wild. *Copeia* 2012, 150–159. doi: 10.1643/CH-11-024

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bolger, D., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., et al. (2008). Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. *J. Infect. Dis.* 197, 435–438. doi: 10.1086/525047

Christian, N., Whitaker, B. K., and Clay, K. (2015). Microbiomes: unifying animal and plant systems through the lens of community ecology theory. *Front. Microbiol.* 6:869. doi: 10.3389/fmicb.2015.00869

Clements, F. E. (1916). *Plant Succession: An Analysis of the Development of Vegetation*. Washington, DC: Carnegie Institution of Washington. doi: 10.5962/bhl.title.56234

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., and Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science* 336, 1255–1262. doi: 10.1126/science.1224203

Cunningham, A. A. (1996). Disease risks of wildlife translocations. *Conserv. Biol.* 10, 349–353. doi: 10.1046/j.1523-1739.1996.10020349.x

Dallas, T. (2014). metacom: an R package for the analysis of metacommunity structure. *Ecography* 37, 402–405. doi: 10.1111/j.1600-0587.2013.00695.x

Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E., and Speare, R. (1999). Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Dis.* 5, 735–748. doi: 10.3201/eid0506.990601

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microb.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Ettling, J. A., Wanner, M. D., Schuette, C. D., Armstrong, S. L., Pedigo, A. S., and Briggler, J. T. (2013). Captive reproduction and husbandry of adult Ozark hellbenders, *Cryptobranchus alleganiensis bishopi*. *Herpetoculture* 44, 605–610.

Federal Register (2011). Endangered and threatened wildlife and plants; endangered status for the Ozark hellbender salamander. *Fed. Commun. Comm.* 76, 61956–61978.

Ferrer, M., Ruiz, A., Lanza, F., Haange, S. B., Oberbach, A., Till, H., et al. (2013). Microbiota from the distal guts of lean and obese adolescents exhibit partial functional redundancy besides clear differences in community structure. *Environ. Microbiol.* 15, 211–226. doi: 10.1111/j.1462-2920.2012.02845.x

Fierer, N., Hamady, M., Lauber, C. L., and Knight, R. (2008). The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17994–17999. doi: 10.1073/pnas.0807920105

Fry, J., Xian, G., Jin, S., Dewitz, J., Homer, C., Yang, L., et al. (2011). Completion of the 2006 national land cover database for the conterminous united states. *Photogramm. Eng. Remote Sensing* 77, 858–864.

Furniss, L. L., Briggler, J. T., Rogers, S., Huang, Y., Mathis, A., Trauth, S., et al. (2003). *Conservation Assessment for Ozark Hellbender (Cryptobranchus alleganiensis bishopi Grobman)*. Rolla, MO: United States Department of Agriculture Forest Service, Eastern Region.

Gagliardo, R., Crump, P., Griffith, E., Mendelson, J., Ross, H., and Zippel, K. (2008). The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. *Int. Zoo Yearb.* 42, 125–135. doi: 10.1111/j.1748-1090.2008.00043.x

Girvan, M. S., Campbell, C. D., Killham, K., Prosser, J. I., and Glover, L. A. (2005). Bacterial diversity promotes community stability and functional resilience after perturbation. *Environ. Microbiol.* 7, 301–313. doi: 10.1111/j.1462-2920.2005.00695.x

Goudet, J. (2005). HIERFSTAT, a package for R to compute and test hierarchical F-statistics. *Mol. Ecol. Resour.* 5, 184–186. doi: 10.1111/j.1471-8286.2004.00828.x

Grice, E. A., and Segre, J. A. (2012). The human microbiome: our second genome. *Annu. Rev. Genomics Hum. Genet.* 13, 151–170. doi: 10.1146/annurev-genom-090711-163814

Hanson, C. A., Fuhrman, J. A., Horner-Devine, M. C., and Martiny, J. B. H. (2012). Beyond biogeographic patterns: processes shaping the microbial landscape. *Nat. Rev. Microbiol.* 10, 497–506. doi: 10.1038/nrmicro2795

Heino, J., Nokela, T., Soininen, J., Tolkkinen, M., Vistanen, L., and Virtanen, R. (2015). Elements of metacommunity structure and community-environment relationships in stream organisms. *Freshwater Biol.* 60, 973–988. doi: 10.1111/fwb.12556

Hernández-Gómez, O., Kimble, S. J. A., Briggler, J. T., and Williams, R. N. (2017). Characterization of the cutaneous bacterial communities of two giant salamander subspecies. *Microb. Ecol.* 73, 445–454. doi: 10.1007/s00248-016-0859-9

Hiler, W. R., Wheeler, B. A., and Trauth, S. E. (2005). Abnormalities in the Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) in arkansas: a comparison between two rivers with a historical perspective. *J. Arkansas Acad. Sci.* 59, 88–94.

Humphries, W. (2005). *Cryptobranchus alleganiensis* displacement by a flood. *Herpetol. Rev.* 36, 428.

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jiménez, R. R., and Sommer, S. (2017). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Kaplan, J. L., Shi, H. N., and Walker, W. A. (2011). The role of microbes in developmental immunologic programming. *Pediatr. Res.* 69, 465–472. doi: 10.1203/PDR.0b013e318217638a

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Lear, G., Washington, V., Neale, M., Case, B., Buckley, H., and Lewis, G. (2013). The biogeography of stream bacteria. *Glob. Ecol. Biogeogr.* 22, 544–554. doi: 10.1111/geb.12046

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., et al. (2004). The metacommunity concept: a framework for multi-scale community ecology. *Ecol. Lett.* 7, 601–613. doi: 10.1111/j.1461-0248.2004.00608.x

Leibold, M. A., and Mikkelson, G. M. (2002). Coherence, species turnover, and boundary clumping: elements of meta-community structure. *Oikos* 97, 237–250. doi: 10.1007/s00442-009-1341-x

Lindstrom, E. S., and Langenheder, S. (2012). Local and regional factors influencing bacterial community assembly. *Environ. Microbiol. Rep.* 4, 1–9. doi: 10.1111/j.1758-2229.2011.00257.x

Lokmer, A., and Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J.* 9, 670–682. doi: 10.1038/ismej.2014.160

Loreau, M., and Hector, A. (2001). Partitioning selection and complementarity in biodiversity experiments. *Nature* 413, 548–548. doi: 10.1038/35097128

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Lozupone, C., Lladser, M. E., Knights, D., Stombaugh, J., and Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *ISME J.* 5, 169. doi: 10.1038/ismej.2010.133

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G., and Neufeld, J. D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13:31. doi: 10.1186/1471-2105-13-31

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Mihaljevic, J. R. (2012). Linking metacommunity theory and symbiont evolutionary ecology. *Trends Ecol. Evol.* 27, 323–329. doi: 10.1016/j.tree.2012.01.011

Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., Gonzalez, A., Fontana, L., et al. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332, 970–974. doi: 10.1126/science.1198719

Nickerson, M. A., and Briggler, J. T. (2007). Harvesting as a factor in population decline of a long-lived salamander; the Ozark hellbender, *Cryptobranchus alleganiensis bishopi* Grobman. *Appl. Herpetol.* 4, 207–216. doi: 10.1163/157075407781268354

Nickerson, M. A., and Mays, C. E. (1973). *The Hellbenders: North American 'Giant Salamanders'*. Milwaukee, WI: Milwaukee Public Museum.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., and Stevens, M. H. H. (2007). *vegan. Community Ecology Package R Package Version* 2.4–1. Available at: https://github.com/vegandevs/vegan

Phillips, C. D., Phelan, G., Dowd, S. E., McDonough, M. M., Ferguson, A. W., Hanson, J. D., et al. (2012). Microbiome analysis among bats describes influences of host phylogeny, life history, physiology and geography. *Mol. Ecol.* 21, 2617–2627. doi: 10.1111/j.1365-294X.2012.05568.x

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., McKenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

Presley, S. J., Higgins, C. L., and Willig, M. R. (2010). A comprehensive framework for the evaluation of metacommunity structure. *Oikos* 119, 908–917. doi: 10.1111/j.1600-0706.2010.18544.x

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

Quinn, S. A., Gibbs, J. P., Hall, M. H., and Petokas, P. J. (2013). Multiscale factors influencing distribution of the eastern hellbender salamander (*Cryptobranchus alleganiensis alleganiensis*) in the northern segment of its range. *J. Herpetol.* 47, 78–84. doi: 10.1670/11-127

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibanez, R., and Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Redford, K. H., Segre, J. A., Salafsky, N., del Rio, C. M., and McAloose, D. (2012). Conservation and the microbiome. *Conserv. Biol.* 26, 195–197. doi: 10.1111/j.1523-1739.2012.01829.x

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., et al. (2004). Subsampled open reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ.* 2:e545. doi: 10.7717/peerj.545

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vrendenburg, V. T., Briggs, C. J., Nielsen, P. F., et al. (2006). Antimicrobial peptide defenses fo the mountain yellow-legged frog (*Rana muscosa*). *Dev. Comp. Immunol.* 30, 831–842. doi: 10.1016/j.dci.2005.10.005

Routman, E., Wu, R., and Templeton, A. R. (1994). Parsimony, molecular evolution, and biogeography: the case of the North American giant salamander. *Evolution* 48, 1799–1809. doi: 10.1111/j.1558-5646.1994.tb02215.x

Sabatino, S. J., and Routman, E. J. (2009). Phylogeography and conservation genetics of the hellbender salamander (*Cryptobranchus alleganiensis*). *Conserv. Genet.* 10, 1235–1246. doi: 10.1007/s10709-010-9538-9

Tonione, M., Johnson, J. R., and Routman, E. J. (2011). Microsatellite analysis supports mitochondrial phylogeography of the hellbender (*Cryptobranchus alleganiensis*). *Genetica* 139, 209–219. doi: 10.1007/s10709-010-9538-9

Trosvik, P., and de Muinck, E. J. (2015). Ecology of bacteria in the human gastrointestinal tract-identification of keystone and foundation taxa. *Microbiome* 3, 44. doi: 10.1186/s40168-015-0107-4

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C., Knight, R., and Gordon, J. I. (2007). The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* 449, 804–810. doi: 10.1038/nature06244

Unger, S. D., Rhodes, O. E., Sutton, T. M., and Williams, R. N. (2013). Population genetics of the eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) across multiple spatial scales. *PLoS ONE* 8:e74180. doi: 10.1371/journal.pone.0074180

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wheeler, B. A., McCallum, M. L., and Trauth, S. E. (2002). Abnormalities in the Ozark hellbender, *Cryptobranchus alleganiensis bishopi*. *J. Arkansas Acad. Sci.* 56, 250–252. doi: 10.1371/journal.pone.0028906

Wheeler, B. A., Prosen, E., Mathis, A., and Wilkinson, R. F. (2003). Population declines of a long-lived salamander: a 20+-year study of hellbenders, *Cryptobranchus alleganiensis*. *Biol. Conserv.* 109, 151–156. doi: 10.1016/S0006-3207(02)00136-2

Willing, B. P., Russell, S. L., and Finlay, B. B. (2011). Shifting the balance: antibiotic effects on host–microbiota mutualism. *Nat. Rev. Microbiol.* 9, 233–243. doi: 10.1038/nrmicro2536

Woodford, M. H., and Rossiter, P. B. (1994). "Disease risks associated with wildlife translocation projects," in *Creative Conservation*, eds P. J. S. Olney, G. M. Mace, and A. T. C. Feistner (New York, NY: Springer), 178–200.

Woodhams, D. C., Bosch, J., Briggs, C. J., Cashins, S., Davis, L. R., Lauer, A., et al. (2011). Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. *Front. Zool.* 8:8. doi: 10.1186/1742-9994-8-8

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Zeisset, I., and Beebee, T. J. C. (2013). Donor population size rather than local adaptation can be a key determinant of amphibian translocation success. *Anim. Conserv.* 16, 359–366. doi: 10.1111/acv.12003

Zilber-Rosenberg, I., and Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiol. Rev.* 32, 723–735. doi: 10.1111/j.1574-6976.2008.00123.x

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Host Ecology Rather Than Host Phylogeny Drives Amphibian Skin Microbial Community Structure in the Biodiversity Hotspot of Madagascar

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Host-associated microbiotas of vertebrates are diverse and complex communities that contribute to host health. In particular, for amphibians, cutaneous microbial communities likely play a significant role in pathogen defense; however, our ecological understanding of these communities is still in its infancy. Here, we take advantage of the fully endemic and locally species-rich amphibian fauna of Madagascar to investigate the factors structuring amphibian skin microbiota on a large scale. Using amplicon-based sequencing, we evaluate how multiple host species traits and site factors affect host bacterial diversity and community structure. Madagascar is home to over 400 native frog species, all of which are endemic to the island; more than 100 different species are known to occur in sympatry within multiple rainforest sites. We intensively sampled frog skin bacterial communities, from over 800 amphibians from 89 species across 30 sites in Madagascar during three field visits, and found that skin bacterial communities differed strongly from those of the surrounding environment. Richness of bacterial operational taxonomic units (OTUs) and phylogenetic diversity differed among host ecomorphs, with arboreal frogs exhibiting lower richness and diversity than terrestrial and aquatic frogs. Host ecomorphology was the strongest factor influencing microbial community structure, with host phylogeny and site parameters (latitude and elevation) explaining less but significant portions of the observed variation. Correlation analysis and topological congruency analyses revealed little to no phylosymbiosis for amphibian skin microbiota. Despite the observed geographic variation and low phylosymbiosis, we found particular OTUs that were differentially abundant between particular ecomorphs. For example, the genus *Pigmentiphaga* (Alcaligenaceae) was significantly enriched on arboreal frogs, *Methylotenera* (Methylophilaceae) was enriched on aquatic frogs, and *Agrobacterium* (Rhizobiaceae) was enriched on terrestrial frogs. The presence of shared bacterial OTUs across geographic regions for selected host genera suggests the presence of core microbial communities which in Madagascar, might be driven more strongly by a species' preference for specific microhabitats than by the physical,

physiological or biochemical properties of their skin. These results corroborate that both host and environmental factors are driving community assembly of amphibian cutaneous microbial communities, and provide an improved foundation for elucidating their role in disease resistance.

Keywords: host-associated microbiota, 16S rRNA illumina sequencing, amphibians, community assembly, bacteria

INTRODUCTION

Mucosal environments of vertebrate hosts are inhabited by diverse microbial assemblages (Bäckhed et al., 2005; Rosenthal et al., 2011; Krediet et al., 2013; Brune and Dietrich, 2015; Colombo et al., 2015; Jiménez and Sommer, 2016). These communities often play critical roles in host development and in maintaining host health (Stecher and Hardt, 2008; Robinson et al., 2010; Engel and Moran, 2013; Sanford and Gallo, 2013; Fraune et al., 2014). With the advent of next generation sequencing, it is possible to study host microbiota in intricate detail, and numerous host microbiotas have been characterized (Caporaso et al., 2011). Most studies to date, however, concentrate on human and other mammalian systems, and our ecological understanding of host microbiota from a diverse host range is still in its infancy (Robinson et al., 2010; Fierer et al., 2012).

Amphibian skin hosts one of the best-studied wildlife microbiotas due to the role of these cutaneous microbial communities in meditating defense against the lethal pathogen, *Batrachochytrium dendrobatidis* (*Bd*) (Belden and Harris, 2007; Bletz et al., 2013; Jiménez and Sommer, 2016). These microbial communities provide a first line of defense against invading pathogens, such as *Bd* (Becker and Harris, 2010). This fungal pathogen causes the disease chytridiomycosis, which is responsible for amphibian declines around the world, particularly in Central America, Australia, and the western US (Berger et al., 1998; Lips et al., 2006; Cheng et al., 2011). Bacterial symbionts isolated from amphibian skin can inhibit *Bd* growth through the production of anti-fungal compounds (Harris et al., 2006; Brucker et al., 2008a,b; Flechas et al., 2012; Woodhams et al., 2015), and population survival has been linked to the proportion of amphibians with *Bd*-inhibitory bacteria in the western United States (Lam et al., 2010). Microbial therapies have been proposed as a possible disease mitigation strategy for combating chytridiomycosis (Bletz et al., 2013; Walke and Belden, 2016; Woodhams et al., 2016), and thus, investigation of the basic ecological principles dictating skin microbial community structure on amphibians can inform the development and application of probiotic therapies.

The extent to which host factors versus environmental factors structure skin microbial communities of amphibians as well as those of other hosts is not fully understood. Furthermore, no studies to date have explored the role of host phylogeny (i.e., do amphibian skin microbiotas exhibit phylosymbiosis)? (Brooks et al., 2016) or the role of host ecology in shaping amphibian skin microbial communities on a large scale. While multiple studies have demonstrated that amphibian cutaneous microbiotas vary among species (McKenzie et al., 2012; Kueneman et al., 2014; Belden et al., 2015), most studies are limited to a few host species and often focus on hosts with distinct host ecologies (e.g., arboreal versus terrestrial). Physical and chemical properties of the skin ecosystem likely differ between amphibian species. For example, amphibian species produce different suites of antimicrobial peptides (Woodhams et al., 2006; Conlon, 2011), and alkaloids are synthesized or sequestered by particular amphibian species (Erspamer, 1994; Daly, 1995). Species may also differ in the mucins and glycoproteins present on their skin (Austin, 2000; Wells, 2007). Factors such as these, all may play a role in shaping the cutaneous microbiota of amphibians. On the other hand, species also differ in their ecology, and thus spend time in different micro-habitats, exposing them to different microbial reservoirs or pathogenic stressors. This variation in the microbes available for colonization and/or the pathogenic stressors could also be a strong force dictating community composition.

Madagascar is an amphibian biodiversity hotspot, home to over 400 endemic frog species (Vences et al., 2009; Vieites et al., 2009). Multiple locations are known to have over 100 co-occurring species, which is ideal for investigating the primary drivers of microbial community assembly on amphibian skin on a large scale and teasing apart how symbiotic microbiota are influenced by environmental factors, host-produced factors, and host ecology. Therefore, using this system, we explored the factors structuring the cutaneous microbial communities of amphibians in Madagascar, by investigating the following main questions: (1) what are the primary drivers of microbial community structure and diversity?, and more specifically, (2) what is the role of host phylogeny *versus* host ecology in shaping microbial community structure and diversity?

MATERIALS AND METHODS

Field Sampling

Field sampling occurred during three field visits: 14 August – 12 September 2013, 4 January – 9 February 2014, and 5 November – 15 December 2014. In total, 1021 microbial samples (989 frog skin swabs, 32 environmental samples) were collected from 10 locations (30 sites) and 96 host species (**Figure 1** and Supplementary Tables 1, 2).

Amphibians were captured during day and night surveys with clean nitrile gloves and placed in sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI, United States). For skin microbe sampling, individuals were removed from the bag with a clean pair of nitrile gloves and rinsed with 50 ml of sterilized water. After rinsing, each individual was swabbed with a single sterile rayon swab (MW113, Medical Wire Equipment & Co. Ltd., Corsham,

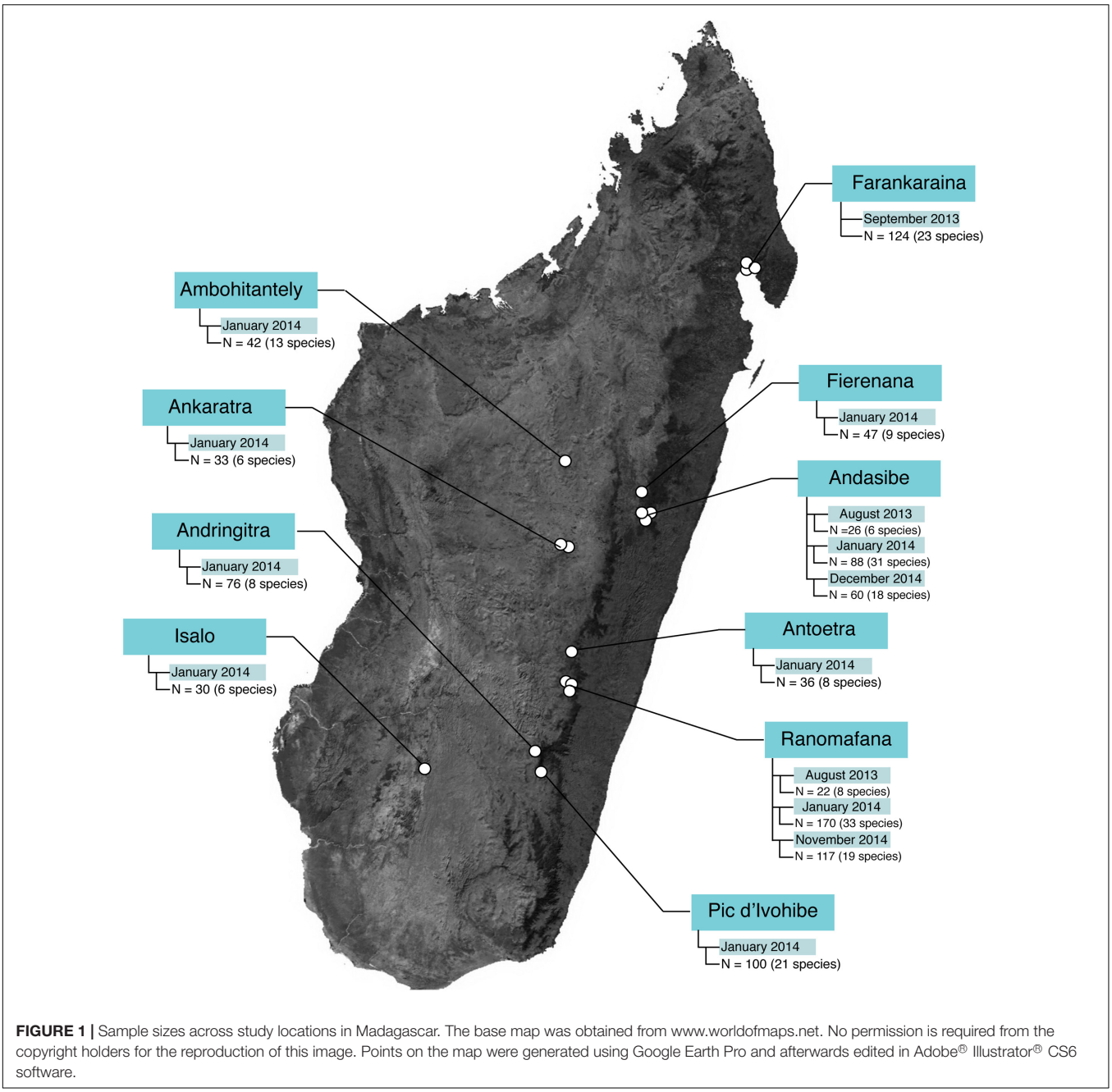

FIGURE 1 | Sample sizes across study locations in Madagascar. The base map was obtained from www.worldofmaps.net. No permission is required from the copyright holders for the reproduction of this image. Points on the map were generated using Google Earth Pro and afterwards edited in Adobe® Illustrator® CS6 software.

United Kingdom), applying 10 strokes on the ventral abdomen, 5 strokes on each ventral thigh, and 5 strokes on each foot. Swabs were stored in microcentrifuge tubes and transported on ice until transfer to a −20°C freezer. Environmental samples were collected from amphibian-associated habitat, including soil, water, and leaves. For soil samples, 1–2 g of soil were collected; for water samples, 60–120 ml of water were hand-pumped through a 0.22 μm filter; for leaf samples, the surface was swabbed with 30 strokes. Environmental samples were stored in 2 ml tubes and transported on ice until transfer to a −20°C freezer. This study was approved by the Institutional Animal Care and Use Committee of James Madison University (protocol #A01-15), and necessary research and access permits were obtained from the Malagasy Direction Générale des Forêts (DGF) and Madagascar National Parks for all sampling.

DNA Extraction and PCR

Bacterial DNA was extracted using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, United States) following the manufacturer's protocol with minor modifications to increase DNA yield. The V4 region of the 16S rRNA gene was PCR-amplified in triplicate with barcoded primers (515f/806r) following Kueneman et al. (2014). Amplicon concentration was quantified with Quant-iT PicoGreen dsDNA

Assay kit (Thermofisher, Waltham, MA, United States). Equal concentrations of each sample were pooled, and the pooled amplicons were cleaned using MoBio UltraClean PCR Clean-up kit (MoBio Laboratories, Carlsbad, CA, United States). The pooled barcoded amplicons were sequenced using 2 × 150 pair-end technology on an Illumina MiSeq platform at the BioFrontiers Institute at the University of Colorado.

Sequence Processing

Sequence reads were filtered and pre-processed in Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). Forward reads were demultiplexed and filtered with the following criteria to retain only high quality reads: no Ns within the sequence, no barcode errors, and a minimum of three consecutive low-quality base pairs (minimum $q = 10$) before read truncation. Only forward reads were used because reverse reads typically suffer from lower quality (Kwon et al., 2013). Quality filtered sequences were clustered into operational taxonomic units (OTUs) using the deblur workflow[1]. Deblur is a new sub-operational taxonomic unit (sOTU) approach for amplicon sequencing that incorporates known Illumina error profiles and uses Hamming distances along with a greedy algorithm (Amir et al., 2017). Within this workflow, sequences were trimmed to 150 bp, and sOTU clusters (hereafter called OTUs) with less than 25 reads were removed. Taxonomy was assigned with Ribosomal Database Project Classifier (Wang et al., 2007), and a phylogenetic tree was built in QIIME using the fasttree algorithm (Price et al., 2010). Samples were subsequently rarefied at 4,000 reads per sample to normalize read counts across samples. Sequences have been archived in the SRA database (Bioproject accession number: PRJNA394790).

Data Analysis

Number of OTUs, Chao1, effective number of species [exp(Shannon index), Jost, 2006], and Faith's phylogenetic diversity were calculated for all samples as measures of alpha diversity, i.e., species richness and species diversity. General linear models (GLM) were used to test which factors were significant predictors of amphibian skin bacterial richness and diversity in SPSS v24 (IBM Corp, Armonk, NY, United States). The same factors described below for PERMANOVAs were included in the models to represent site parameters, host ecomorphology and host phylogeny.

Beta diversity was calculated as weighted and unweighted Unifrac distances in QIIME. The resulting matrices were used to explore patterns in beta diversity in two main ways: (1) Multiple Regression on distance Matrices (MRM), and (2) Permutational multivariate analysis of variance (PERMANOVA).

MRM was used to simultaneously explore the role of host phylogeny and host ecology on microbial community structure. We made distance matrices to represent (a) evolutionary divergences among host species (i.e., host phylogeny) and (b) ecomorphological differences (i.e., host ecology). Evolutionary divergences among host species (with 2 or more sampled individuals) were calculated as patristic phylogenetic distances from an ultrametric timetree. We first reconstructed a phylogenetic tree of all host species included in our study based on partial sequences of the mitochondrial 16S rRNA gene (Vieites et al., 2009), in MEGA7 (Kumar et al., 2016) under the Maximum Likelihood optimality criterion, with a general time-reversible (GTR + I) substitution model and using SPR branch swapping. We then manually corrected the tree topology for some wrongly reconstructed deep relationships, based on published multigene phylogenies of mantellids (Wollenberg et al., 2011), microhylids (Scherz et al., 2016), and intrafamilial relationships (Roelants et al., 2007). We then entered this topology as the usertree in MEGA7 along with the 16S alignment, and reconstructed an ultrametric tree using the Real-time method, without absolute calibration. Patristic pairwise distances among included amphibian species were calculated from this ultrametric tree in R using the ape and adephylo packages (Paradis et al., 2004; Jombart and Dray, 2008). Ecomorphological distances were calculated on the basis of major species traits that can be hypothesized to influence the cutaneous microbiota: (1) degree of arboreality, (2) degree of water dependence, (3) kind of breeding water body, (4) primary forest dependence, (5) reproductive mode, (6) aquatic or terrestrial egg deposition, and (7) body size (Glaw and Vences, 2007). The traits were coded as either ordered/ordinal (1,2,4,7) or unordered/categorical (3,5,6) characters. For a complete list of all character states see Supplementary Tables 3, 4. Distance matrices among species were calculated using PAUP v. 4b10 (Swofford, 2002). We considered *a priori* the first three traits as most likely to be important as they directly indicate distinct microhabitats that the frogs are exposed to, while we considered the remaining traits as possibly important, but probably less influential. MRM analyses were completed with the 'ecodist' package in R (Goslee and Urban, 2007; R Core Team, 2014), testing the correlation of the phylogenetic and ecomorphological matrices with microbial community structure for the full dataset (89 species) as well as for amphibians occurring at a single hyperdiverse site (38 species), Ranomafana. Microbial communities were represented by weighted Unifrac distance matrices derived from OTU tables averaged by frog species; that is, the rarified OTU table was first averaged by host species, and pair-wise weighted unifrac distances were subsequently calculated.

Topological congruency analysis was used to further explore the possible existence of phylosymbiosis. For this, we quantified congruence between the host phylogenetic tree and microbial dendrograms using the TreeCmp program. Microbial dendrograms were created in QIIME using both Weighted Unifrac and Bray-Curtis distances derived from OTU tables averaged by frog species. Using TreeCmp, we calculated the normalized Robinson–Foulds scores, where values of 0 indicates complete congruence and values of 1 indicate lack of congruence.

Because no topological congruence was found and the MRM analyses suggested a stronger correlation with host ecology than with phylogeny (see Results), we performed PERMANOVAs to further understand how frog ecomorphology as well as the other variables influence the skin bacterial communities. PERMANOVAs were completed in R (R Core Team, 2014)

[1]https://github.com/biocore/deblur

with the ADONIS2 function in the 'vegan' package (Oksanen et al., 2017) to test which factors significantly explained the observed variation in microbial community composition and structure. The "margin" option in ADONIS2 was used to assess marginal effects of each term in a model including all other variables. The following factors were included in the models: elevation, latitude, ecomorphological category [hereafter called host ecomorph, with three categories: (a) arboreal, (b) aquatic and semi-aquatic, or (c) terrestrial], as well as an approximate representation of host phylogeny. The phylogeny variable was chosen because the large number of frog species sampled made it infeasible to include host species as a categorical variable in any model, and because a categorical host species variable would not have captured the phylogenetic relationships among the amphibians. We, therefore, performed non-metric multidimensional scaling (nMDS) constrained to 1 dimension on the patristic distance matrix using SPSS v24 (IBM Corp, Armonk, NY, United States). The coordinates of the nMDS axis were subsequently extracted and used as a proxy variable in PERMANOVA models. PERMANOVA was also used to test whether frog skin microbiota differed from that of the environment in PRIMER7 (Clarke and Gorley, 2015).

Unweighted pair group method with arithmetic mean (UPGMA) was used to evaluate clustering patterns across host ecomorphs and Similarity Profile Analysis (SIMPROF) was used to statistically test for significant structure within the created UPGMA dendrogram. Both analyses were performed in PRIMER7 (Clarke and Gorley, 2015).

The Linear discriminant analysis Effect size (LEfSe) method (Segata et al., 2011) was used to identify which bacterial taxa were most likely explaining the observed differences between categories of interest. LEfSe was used to identify differentially abundant taxa between frogs and the environment, and also to identify differentially abundant taxa among host ecomorphs. Default parameters were used with the exception of increasing the LDA score; taxa with LDA scores greater than 3.0 were considered significant.

Bipartite networks were used to visualize the association of bacterial taxa with a given host ecomorph. These networks were calculated in R (Sedlar et al., 2016), and visualized with Gephi (Bastian et al., 2009).

RESULTS

Malagasy Frog Skin Microbiota Differs from Environmental Substrates

Frog cutaneous microbial communities were less species rich than those of the environment (# of OTUs – Frog: 219.9 3 ± 6.71(SE)/Env: 948.96 ± 72.98; Chao1 – Frog: 265.37 ± 8.57/Env: 1415.79 ± 130.76), and also less diverse than that of the environment (Effective # of species – Frog: 509.8 ± 40.0/Env: 8644.5 ± 1337.8; Faith's PD – Frog: 37.28 ± 0.81/Env: 106.86 ± 5.88).

Frog skin microbiotas were dominated by Proteobacteria (*Gamma* – 46.6%, *Beta* – 15.4%, *Alpha* – 9.4%, *Delta* – 1.6%), Bacteriodetes (8.1%), Actinobacteria (7.9%), Firmicutes (3.6%), and Acidobacteria (2.3%). Microbial community structure on frog skin strongly differed from that of the environment (PERMANOVA: Pseudo-F = 33.34, $p = 0.001$, **Figure 2A**). Soil environments were comprised predominantly of Acidobacteria (19.7%), Proteobacteria (*Alpha* – 17.3%, *Gamma* – 8.7%, *Beta* – 8.5%, *Delta* – 6.4%), Bacteriodetes (7.3%), Verrucomicrobia (5.3%), Chloroflexi (5.1%) Actinobacteria (4.8%), and Planctomycetes (3.3%). Water environments were comprised of Proteobacteria (*Beta* – 23.7%, *Alpha* – 21.4%, *Gamma* – 9.5%, *Delta* – 3.9%), Actinobacteria (11.8%), Bacteriodetes (7.7%), Planctomycetes (3.9%), Firmicutes (3.0%), Acidobacteria (2.7%), and Verrucomicrobia (2.5%). Leaf surfaces were comprised of Proteobacteria (*Alpha* – 30.2%, *Gamma* – 12.3%, *Beta* – 5.2%, *Delta* – 2.8 %), Bacteriodetes (24.7%), Actinobacteria (10.1%), Acidobacteria (3.7%), Cyanobacteria (2.8%), and Verrucomicrobia (2.7%).

Fifty-eight bacterial taxa were identified as differentially abundant between frog hosts and the environment using the LEfSe method (LDA > 3). More specifically, 47 taxa exhibited greater relative abundance in the environment and 11 taxa exhibited greater relative abundance on frogs (Supplementary Table 5). For example, Synthrophobacteraceae (Deltaproteobacteria) and Chthoniobacteraceae (Spartobacteria) were enriched in the environment, while Pseudomonadaceae (Gammaproteobacteria) and Enterobacteriaceae (Gammaproteobacteria) were enriched on frog skin (**Figure 2B**).

Drivers of Skin Microbial Composition, Structure and Diversity

To simultaneously explore the role of host phylogeny and host ecology we performed MRMs. When considering the entire dataset, both host phylogeny and host ecology were not significant (MRM -Phylo: $p = 0.400$, Eco: $p = 0.515$). However, when considering the single hyperdiverse site Ranomafana, host ecomorphology, derived from the 3-character matrix, was significant while host phylogeny was not (MRM – Eco: $p = 0.029$, Phylo: $p = 0.579$). Inclusion of additional ecological traits in the calculation of the ecomorphological distance matrix did not improve the correlation with weighted unifrac distances of the microbial communities (Supplementary Table 6), suggesting that arboreality, water dependence and breeding water body are the most important ecological traits influencing the cutaneous bacterial communities of Malagasy amphibians.

Topological congruency analysis also showed a lack of congruence between host phylogenetic trees and microbial dendrograms, suggesting a limited effect of host phylogeny (Full dataset – Weighted Unifrac: normalized RF score = 1, Bray-Curtis: normalized RF score = 0.98; Ranomafana-Weighted Unifrac: normalized RF score = 0.98, Bray-Curtis: normalized RF score = 1).

Using PERMANOVAs, host ecomorph was the strongest predictor of skin bacterial community structure [PERMANOVA – Weighted: Pseudo-$F_{(2,980)} = 30.308$, $p = 0.001$; Unweighted: Pseudo-$F_{(2,980)} = 7.142$, $p = 0.001$, **Table 1**]; however, host phylogeny (nMDS 1), latitude, and elevation also explained significant portions of the variation

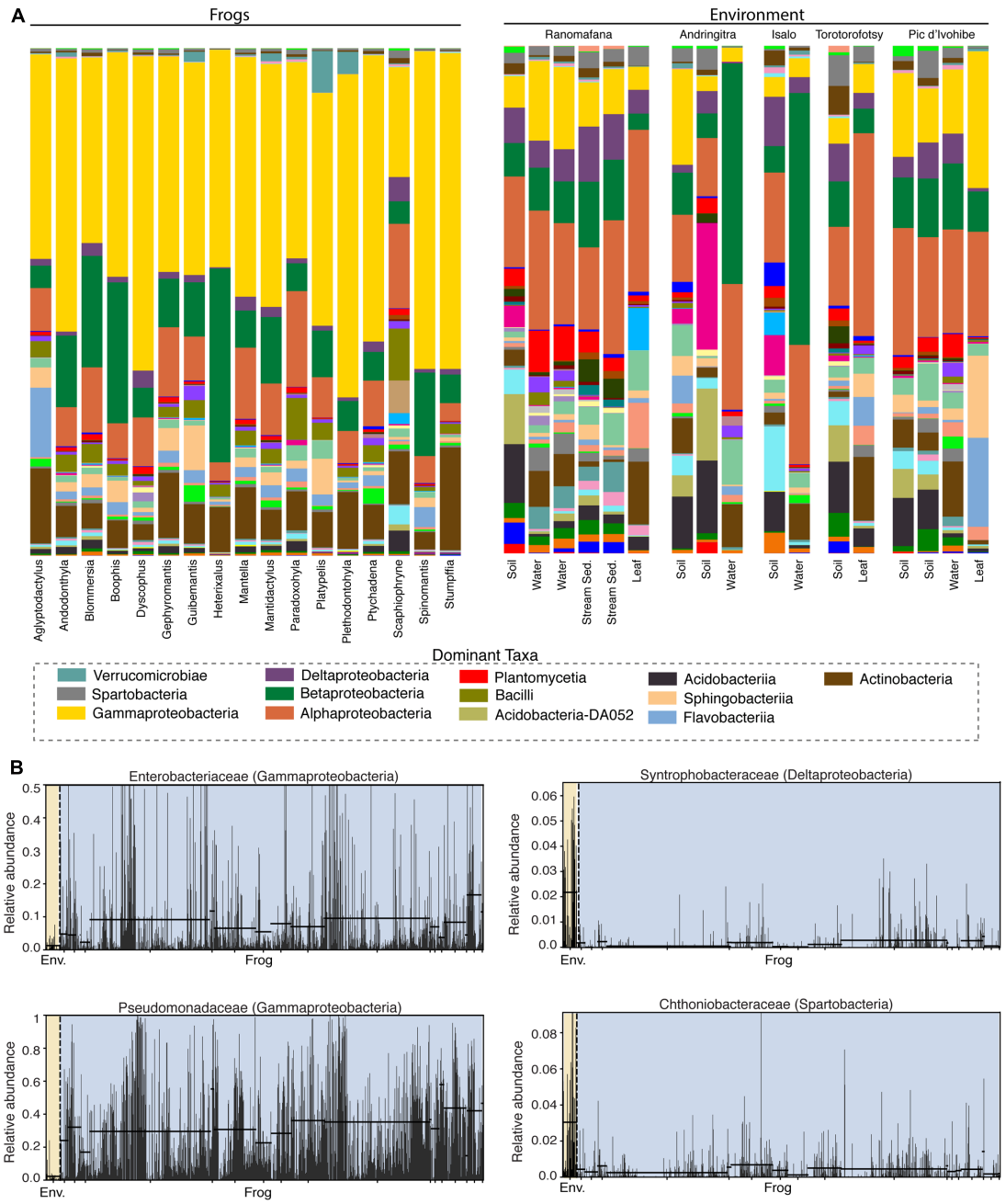

FIGURE 2 | Bacterial composition differs between the skin of Malagasy frog hosts and the environment. **(A)** Taxonomic bar plots for bacterial communities on frogs (by host genus) and in the environment (by substrate type) at the class level. Dominant taxa are identified in the legend. **(B)** Four representative differentially abundant taxa identified by LEfSe analysis. Two taxa that exhibited greater relative abundance on frogs (left) and two taxa that exhibited greater relative abundance in the environment (right) are presented. Yellow highlighting indicates the environment and blue highlighting indicates frogs. Each bar represents an individual sample. Supplementary Table 2 presents all LEfSe-identified taxa with LDA Scores.

(**Table 1**). Pairwise comparisons of individuals within ecomorphs were lower than the pairwise distances between ecomorphs (Supplementary Table 7), and pairwise comparisons of host ecomorph classes showed that each ecomorph was significantly different from the others (Arb-Ter: $t = 6.699$, $p = 0.001$; Arb-Aqu $t = 5.625$, $p = 0.001$; Ter-Aqu $t = 2.875$, $p = 0.001$; **Figure 3**). Multivariate dispersion also did not differ between host ecomorphs (PERMDISP- $F = 1.699$, $p = 0.223$). In addition, UPGMA clustering showed that host species within the same ecomorph class typically clustered together (**Figure 3**), and SIMPROF analysis revealed that there was significant structure within the dendrogram (SIMPROF – $p = 0.001$).

TABLE 1 | ADONIS results for main factors influencing beta diversity of cutaneous bacterial communities on Malagasy amphibians.

		Beta diversity metric	
Factor	**DF**	**Weighted Unifrac**	**Unweighted Unifrac**
Host ecomorph	2	**30.308**	7.142
	980	**0.001**	0.001
Latitude	1	5.930	**8.359**
	980	0.001	**0.001**
Elevation	1	4.282	3.052
	980	0.001	0.001
Host phylogeny (nMDS1)	1	5.034	4.057
	980	0.001	0.001

Model results for weighted and unweighted Unifrac matrices are provided. Pseudo-F and p-values are presented. The strongest predictor is bolded.

Fifty-eight bacterial taxa were identified to best explain the observed microbial community differences among host ecomorphs (LEfSe, LDA > 3); seven were differentially more abundant on arboreal frogs, 39 were more abundant on terrestrial frogs, and 12 were more abundant on aquatic frogs (Supplementary Table 8). For example, the genus *Pigmentiphaga* (Alcaligenaceae) was significantly enriched on arboreal frogs, *Agrobacterium* (Rhizobiaceae) was significantly enriched on terrestrial frogs, and *Methylotenera* (Methylophilaceae) was significantly enriched on aquatic frogs (**Figure 3**). In particular, the relationship between *Pigmentiphaga* and arboreal frogs was seen across host genera (e.g., *Boophis* and *Heterixalus*) and locations (**Figure 4**).

For selected frog hosts, variation through time was explored. While bacterial composition exhibited differences between sampling time points, key bacterial taxa [e.g., Alcaligenaceae in

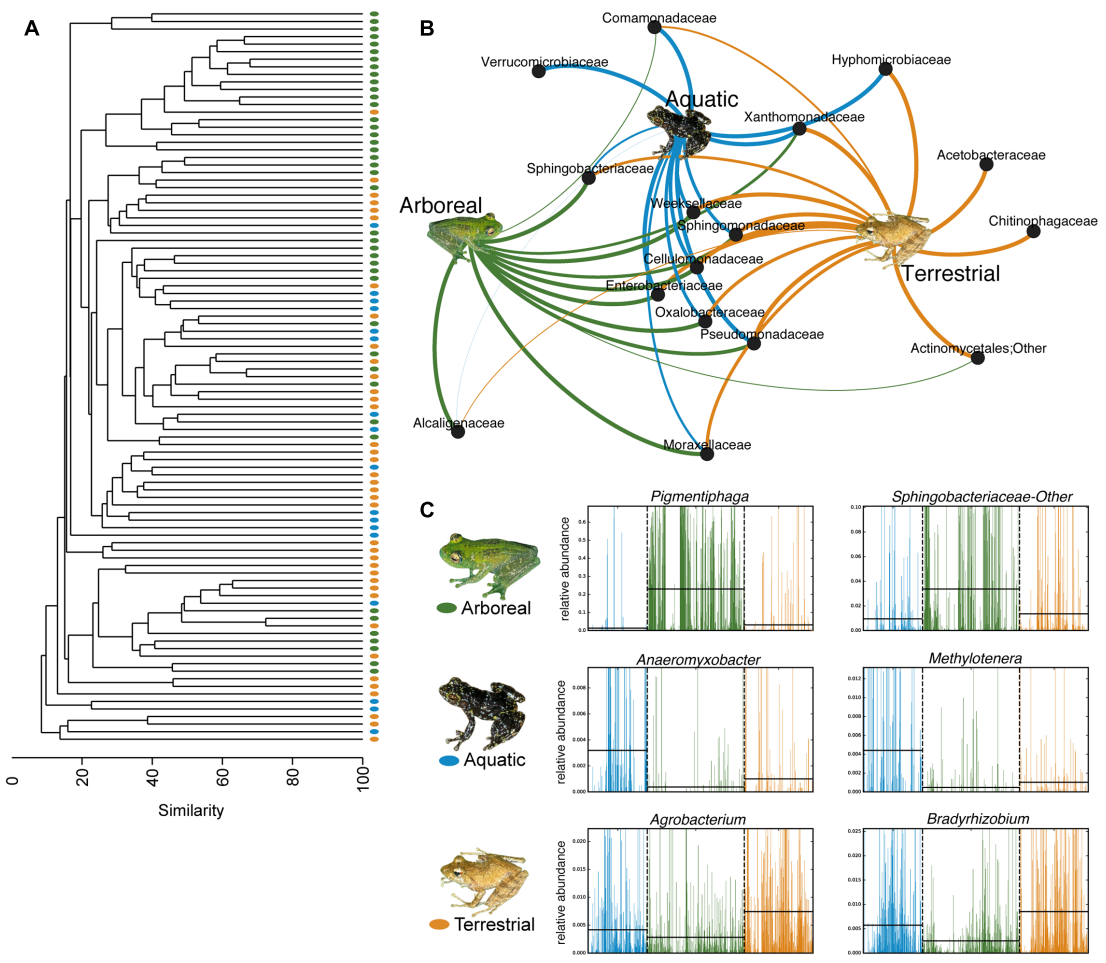

FIGURE 3 | Host ecology affects amphibian skin microbial community structure and composition. **(A)** UPGMA clustering of the microbial communities on the 88 sampled frog species. Each point represents a frog species. **(B)** Bipartite network showing the association of particular bacterial taxa with certain host ecomorphs (analysis based on a 0.01 relative abundance threshold for OTU inclusion). Lines connect family level OTUs to host ecomorph categories and are weighted by relative abundance. **(C)** Six representative differentially abundant taxa identified by LEfSe analysis. Two taxa that exhibited greater relative abundance on frogs from each host ecomorph are presented. Green represents arboreal frog species, blue represents aquatic frog species, and yellow represents terrestrial frog species. Supplementary Table 4 presents all LEfSe-identified bacterial families. Inset frog photos were taken by M. Vences.

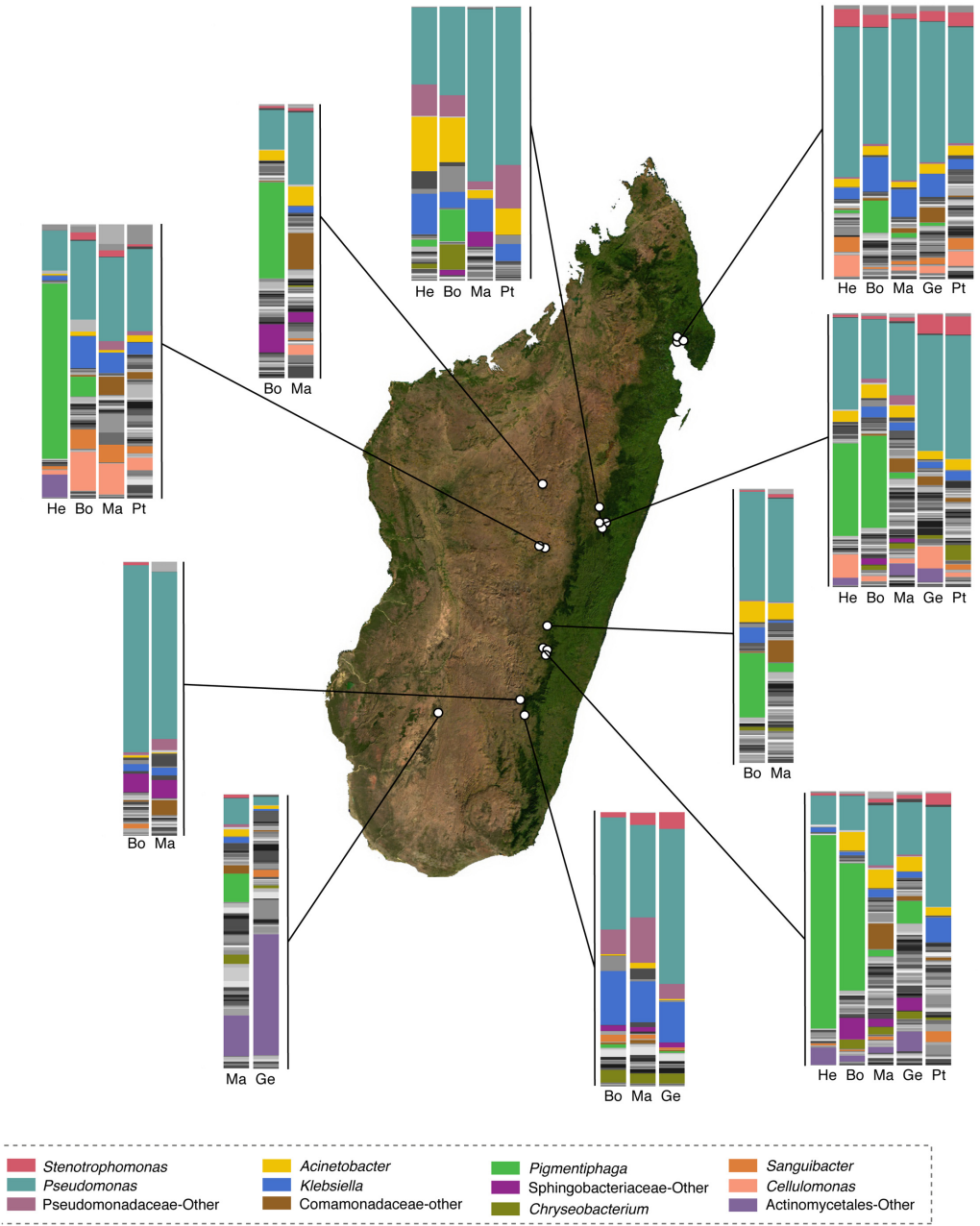

FIGURE 4 | Taxonomic composition of skin microbiota of selected genera of Malagasy frogs across locations. Within the bar plots, major taxa are colored, with each color representing a bacterial genus. Other taxa are presented in gray scale. Bar plots represent all OTUs with relative abundances greater than 0.1% across the dataset. Host genera are abbreviated as follows: He, *Heterixalus*; Bo, *Boophis*; Ma, *Mantidactylus*; Ge, *Gephyromantis*; and Pt, *Ptychadena*. The base map was obtained from www.worldofmaps.net. No permission is required from the copyright holders for the reproduction of this image. Points on the map were generated using Google Earth Pro and afterwords edited in Adobe® Illustrator® CS6 software.

arboreal frogs (*Boophis*)] were consistently present through time (**Figure 5**).

Richness and diversity of frog skin microbial communities were also affected by multiple factors, with host ecomorphology exerting the strongest influence in most cases (**Table 2**). Host phylogeny and latitude also influenced species richness and diversity (**Table 2**). Pair-wise comparisons between host ecomorphs revealed that the main effect of host ecomorph was driven by arboreal frogs having significantly less diverse bacterial communities than both aquatic and terrestrial frogs, and aquatic frog having slightly less diverse communities than terrestrial frogs (**Tables 3**, **4**).

From a presence-absence perspective, there were 990 (15%) OTUs, 1592 (24.1%) OTUs, and 1776 (26.9%) OTUs

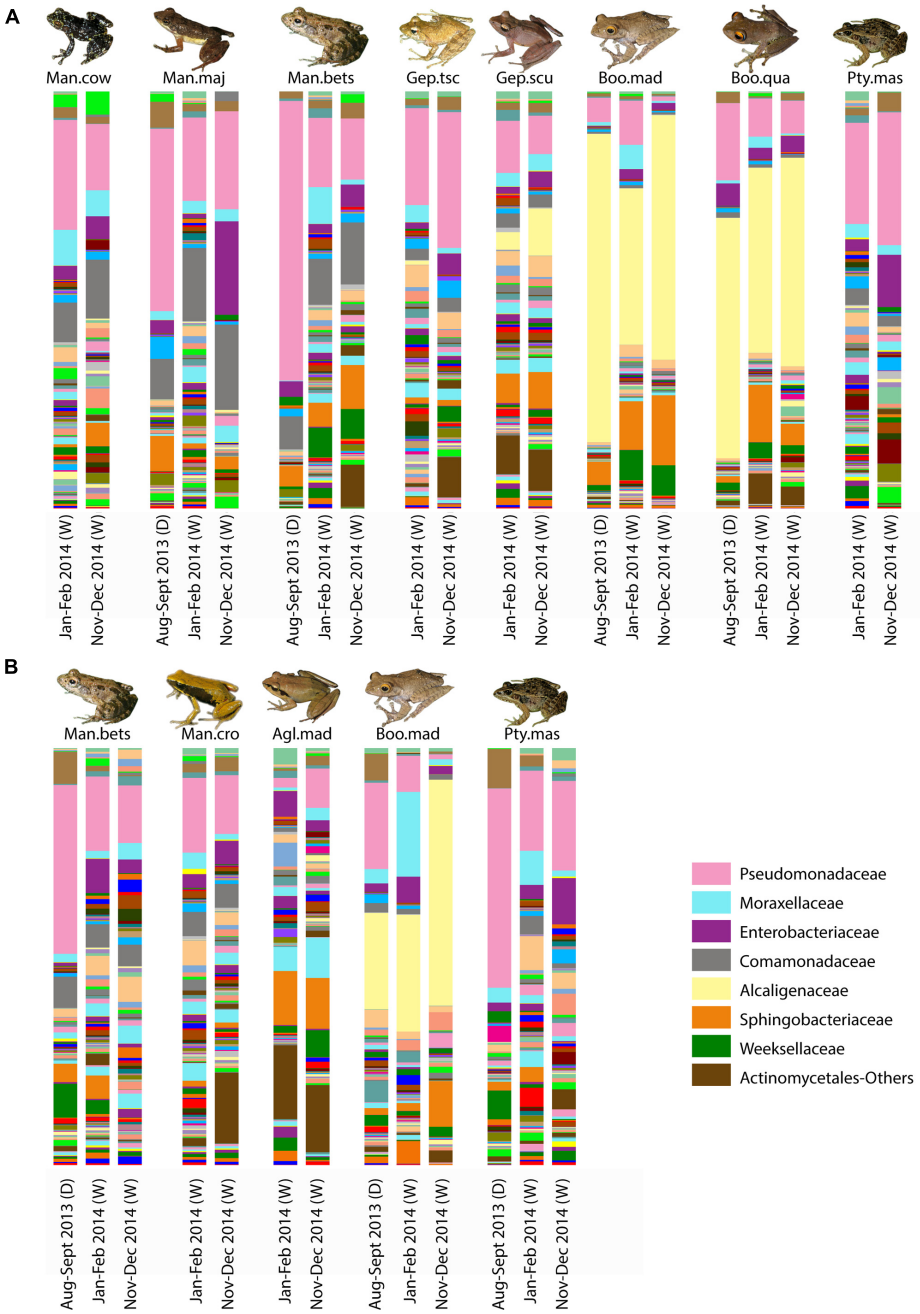

FIGURE 5 | Variation of skin bacterial composition for selected frog species in Madagascar across time. Taxonomic composition of skin bacterial communities at the family level for selected host species from Ranomafana **(A)** and Andasibe **(B)** across the three sampling time points. Each bar represents the average community composition for a given species at the given sampling time. Sampling time is given below each bar; parenthetical "D" indicates dry season, and "W" represents wet season. Dominant taxa are identified in the bottom right. Frog species are abbreviated as follows: Man.cow, *Mantidactylus cowani* "small"; Man.maj, *Mantidactylus majori*; Man.bet, *Mantidactylus betsileanus*; Gep.tscsch, *Gephyromantis tschenki*; Gep.scu, *Gephyromantis sculpturatus*; Boo.mad, *Boophis madagascariensis*; Boo.qua, *Boophis quasiboehmei*; Pty.mad, *Ptychadena mascareniensis*; Man.cro, *Mantella crocea*; and Agl.mad, *Aglyptodactylus madagascariensis*. Inset frog photos were taken by M. Vences.

(summed across all individuals of a given ecomorph) that were unique to arboreal, aquatic and terrestrial frogs, respectively. There were also OTUs shared between these groups; 15.3% of OTUs were shared across all ecomorphs, 7.8% of OTUs were shared between arboreal and terrestrial frogs, 7.3% were shared between terrestrial and aquatic frogs, and 3.6% were shared between aquatic and arboreal frogs (**Figure 6**).

TABLE 2 | Generalized linear model results for the main factors influencing species richness and diversity indices of cutaneous bacterial communities on Malagasy amphibians.

Factor	*DF*	Richness or Diversity Index			
		Number of OTUs	Chao1	Effective number of species [exp(Shannon Index)]	Faith's PD
Host ecomorph	2	**25.686**	**26.438**	**71.451**	21.339
	980	<0.01	<0.01	<0.01	<0.01
Latitude	21	21.781	19.168	6.293	**29.522**
	980	<0.01	<0.01	0.012	<0.01
Elevation	21	0.747	1.171	1.144	2.709
	980	0.388	0.279	0.285	0.100
Host phylogeny (nMDS1)	21	8.899	11.907	2.262	7.932
	980	0.003	0.001	0.133	0.005

Wald chi-square values and p-values are presented. The strongest predictor is bolded.

TABLE 3 | Richness and diversity of cutaneous bacterial communities on Malagasy amphibians across the three ecomorph categories given as mean ± standard error.

Richness/diversity metric	Amphibian ecomorph		
	Arboreal	Aquatic	Terrestrial
Number of OTUs	167.5 ± 7.3	239.4 ± 14.6	277.1 ± 13.9
Chao1	203 ± 9.3	284.3 ± 18.6	335.6 ± 18.1
Effective number of species [exp(Shannon Index)]	188.3 ± 98.5	707.9 ± 25.8	784.9 ± 87.2
Faith's phylogenetic diversity	31 ± 0.9	40.35 ± 1.8	43.5 ± 1.6

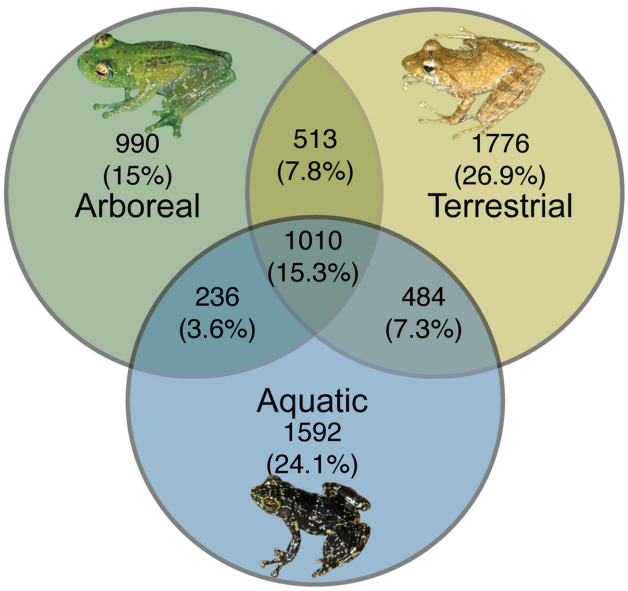

FIGURE 6 | Unique and shared bacterial OTUs across ecomorphs of Malagasy frogs. Bacterial OTUs were considered present if it had a minimum of 5 reads in the rarified dataset. Inset frog photos were taken by M. Vences.

DISCUSSION

The hyperdiverse amphibian communities of Madagascar offer a unique system for studying the factors that structure skin-associated microbial communities. This study is among the first to systematically explore amphibian skin microbiota on such a large geographical scale and to comparatively evaluate the roles of host phylogeny and host ecology.

Our results demonstrate that frogs skin microbiota differed from environmental substrates, suggesting that the skin is a unique niche in which only selected taxa can colonize and persist. In particular, Gammaproteobacteria were significantly enriched on frogs, while Alpha- and Betaproteobacteria were enriched in the environment. This distinct signature of the frog skin microbiota is in concordance with other studies that have found that skin communities are enriched for bacterial taxa that are in low relative abundance in the environment (Walke et al., 2014; Rebollar et al., 2016). The bacterial communities of Malagasy frog skin were predominantly composed of Proteobacteria, Bacteriodetes, Actinobacteria, Firmicutes, and

TABLE 4 | Sidak *post hoc* test results for pair-wise comparisons between host ecomorph categories for richness and diversity values of Malagasy frog cutaneous bacterial communities.

Comparison	Richness or Diversity Index			
	Number of OTUs	Chao1	Effective number of species [exp(Shannon Index)]	Faith's PD
Arboreal – Terrestrial	<0.01	<0.01	<0.01	<0.01
Arboreal – Aquatic	0.070	0.164	<0.01	0.017
Terrestrial – Aquatic	0.048	0.015	0.640	0.397

Acidobacteria. This is similar to that found in other studied amphibians around the world (e.g., Kueneman et al., 2014; Belden et al., 2015; Sabino-Pinto et al., 2016), further suggesting that amphibian skin may act as a selective niche favoring particular taxa. Moreover, the high relative abundance of *Pseudomonas* on frog hosts mirrors the findings in other tropical regions, such as Panama (Belden et al., 2015; Rebollar et al., 2016). The fact that a unique community inhabits amphibian skin suggests host filtering is occurring, which could arise via multiple mechanisms including, host-produced compounds secreted into the skin mucosal environment (i.e., antimicrobial peptides, mucosal polysaccharides and proteins, or other metabolite-like compounds). Such compounds could differentially affect potential colonizers, by exhibiting both antimicrobial (i.e., inhibiting growth) or promicrobial (i.e., facilitating growth) activity (Conlon, 2011; Rollins-Smith and Woodhams, 2012; Franzenburg et al., 2013; Colombo et al., 2015).

Host ecomorphology was the strongest predictor of diversity and structure of cutaneous bacterial communities; therefore, ecological characteristics, including arboreality and association with water appear to be important drivers of variation in these communities. One possible explanation is that these microhabitat preferences expose frogs to different environmental microbial pools. Thus, while the skin microbiotas remain distinct from the environmental community, highlighting that a filtering process occurs, the structure and diversity of the surrounding environmental pool could affect colonization and succession dynamics of the skin community. Indeed, in a study on the salamander, *Plethodon cinereus,* the structure of the environmental microbial community largely affected the skin-associated microbial community structure (Loudon et al., 2014). Given the role of environmental transmission in maintenance of amphibian skin microbiota, the fact that environmental substrates differ in microbial composition (Fierer and Jackson, 2006; Hullar et al., 2006), could, in part, explain the host ecology effect. Alternatively, hosts with similar ecologies may be exposed to similar abiotic and biotic stressors. For example, arboreal frogs are more likely to be exposed to ultraviolet radiation. UV radiation has been shown to affect soil and aquatic environmental microbial communities (Jacobs and Sundin, 2001; Piccini et al., 2009; Hunting et al., 2013), and therefore, may also influence the microbial communities on frog skin. Moreover, from a biotic perspective, similar pathogen stressors are likely to be experienced by frogs with similar ecologies. For example, the ecological preferences of amphibian hosts have been related to their susceptibility to infection by the cutaneous pathogen, *Batrachochytrium dendrobatidis* (Stuart et al., 2004; Lips et al., 2006). Therefore, it is plausible that, over time, microbes that can offer protection against such pathogens would be selected for via changes in the chemical properties of the skin environment or production of specific defensive peptides. If such selective pressures are exerted more strongly on particular ecomorphs, this could drive the observed host ecology effect.

One of the most striking patterns associated with the ecomorphology effect, is the apparent association of bacteria from the genus *Pigmentiphaga* (Alcaligenaceae) with arboreal frog species. This genus was observed on arboreal frogs from multiple genera including, *Boophis*, *Heterixalus*, and *Spinomantis*, and was found on these frog genera across numerous locations and seasons. While little is known about the specific niches of Malagasy frogs, individuals from different species and genera spatially overlap and individuals of different species are often seen close to each other on the same leafs or in the same section of a small stream. Thus, occasional physical contact among non-conspecific frogs is likely, and horizontal transmission of skin bacteria is possible. Interestingly, another genus of Alcaligenaceae, *Achromobacter*, was strongly associated with the treefrogs, *Agalychnis callidryas* and *Dendropsophus ebraccatus* in Panama (Belden et al., 2015). This concordant finding in two distinct regions of the world, suggests that taxa from this family may have a particular facility for establishing on arboreal frogs and perhaps provide some beneficial function to their arboreal hosts. Not much is known about *Pigmentiphaga*; however, *Achromobacter* has been isolated from pine needles (Favilli and Messini, 1990), and in some plants, *Achromobacter* sp. confer tolerance to drought (Mayak et al., 2004).

While host phylogeny was found to be a significant factor in shaping microbial community structure (albeit less than host ecology) in model-based analyses (i.e., PERMANOVAs), we found no further evidence for strong phylosymbiosis in frog skin microbial communities; that is, the similarity of amphibian skin microbial communities did not parallel host phylogeny (Brooks et al., 2016). Phylosymbiosis has been documented in gut microbiota across multiple host clades including humans, mice and various insects (Brooks et al., 2016), but has not been explored with respect to skin microbial communities. Based on current knowledge, gut microbiota are more intimately linked to host processes, such as metabolism and immune system development (Ley et al., 2008; Chung et al., 2012; Sommer et al., 2016), For example, amphibians and other hosts with specialized diets have unique microorganisms enabling proper digestion and metabolism of particular compounds (Kohl et al., 2013, 2014; Vences et al., 2016); Thus, co-evolution patterns could be expected to occur more easily, driving the existence of phylosymbiosis patterns (Amato, 2013; Shapira, 2016). Apart from bacterial symbionts, a recent study found no association between amphibian phylogeny and eukaryotic parasites in the diverse South American amphibian fauna, and attributed this to a pattern whereby the majority of amphibian parasites are generalists (Campião et al., 2015). Thus, it is possible that specialist symbionts may be more likely to demonstrate phylosymbiosis with host taxa.

Elevation and latitude were also secondary factors that influenced the skin bacterial communities. These factors both represent site parameters suggesting that geographic location plays at least a small role in assembly of amphibian skin microbiota, which has also been found in previous studies on amphibian skin microbiota (Kueneman et al., 2014; Rebollar et al., 2016). Amphibian microbial communities are thought, at least in part, to be assembled and maintained via environmental transmission (Loudon et al., 2014), and environmental microbial communities are also known to vary across geographic space

(Fierer and Jackson, 2006; Fierer, 2008). The sampled locations in Madagascar included rainforest sites at varying elevations, including, high-elevation montane sites, as well as semi-arid grasslands and canyon gallery forest sites; thus, differences in the environmental microbiota can be expected and may explain the observed effect of site-related parameters.

Overall, our findings illustrate that frog cutaneous microbiotas are distinct from the environment, and that while multiple factors influence the cutaneous microbial communities of amphibians, host ecomorphology is the main driver of cutaneous microbial diversity and structure in the biodiversity hotspot, Madagascar. For amphibians as well as other wildlife, microbes play an important role in mediating disease susceptibility. Gaining an understanding of the ecological forces structuring host-associated communities at different spatial scales provides a foundation for elucidating their role in host health and for understanding how these communities can be targeted with microbial therapies to promote positive health outcomes.

AUTHOR CONTRIBUTIONS

MCB, RNH, VM, and MV designed the project. MCB, RNH, FR, and AR collected field data. MCB and HA completed laboratory work. MCB and MV completed all data analysis. MCB wrote the paper. All authors contributed to revision of the manuscript and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01530/full#supplementary-material

REFERENCES

Amato, K. R. (2013). Co-evolution in context: the importance of studying gut microbiomes in wild animals. *Microbiome Sci. Med.* 1, 10–29. doi: 10.2478/micsm-2013-0002

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Xu. Z. Z., et al. (2017). Deblur rapidly resolves single-nucleotide community sequence patterns. *mSystems* 2:e00191-16. doi: 10.1128/msystems.00191-16

Austin, R. M. (2000). "Cutaneous microbial flora and antibiosis in *Plethodon ventralis*," in *The Biology of Plethodontid Salamanders*, eds R. C. Bruce, R. G. Jaeger, and L. D. Houck (Boston, MA: Springer), 451–462. doi: 10.1007/978-1-4615-4255-1_25

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science* 307, 1915–1920. doi: 10.1126/science.1104816

Bastian, M., Heymann, S., and Jacomy, M. (2009). "Gephi: an open source software for exploring and manipulating networks," in *Proceedings of the Third International AAAI Conference on Weblogs and Social Media*, San Jose, CA, 361–362. doi: 10.1136/qshc.2004.010033

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE* 5:e10957. doi: 10.1371/journal.pone.0010957

Belden, L. K., and Harris, R. N. (2007). Infectious diseases in wildlife: the community ecology context. *Front. Ecol. Environ.* 5:533–539. doi: 10.1890/060122

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Brooks, A. W., Kohl, K. D., Brucker, R. M., van Opstal, E. J., and Bordenstein, S. R. (2016). Phylosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. *PLoS Biol.* 14:e2000225. doi: 10.1371/journal.pbio.2000225

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008b). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Brune, A., and Dietrich, C. (2015). The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Annu. Rev. Microbiol.* 69, 145–166. doi: 10.1146/annurev-micro-092412-155715

Campião, K. M., De Aquino Ribas, A. C., Morais, D. H., Da Silva, R. J., and Tavares, L. E. R. (2015). How many parasites species a frog might have? Determinants of parasite diversity in South American anurans. *PLoS ONE* 10:e0140577. doi: 10.1371/journal.pone.0140577

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303.QIIME

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Cheng, T. L., Rovito, S. M., Wake, D. B., and Vredenburg, V. T. (2011). Coincident mass extirpation of Neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9502–9507. doi: 10.1073/pnas.1105538108

Chung, H., Pamp, J., Hill, J. A., Surana, N. K., Edelman, S. M., Troy, E. B., et al. (2012). Gut immune maturation depends on colonization with a host-specific microbiota. *Cell* 149, 1578–1593. doi: 10.1016/j.cell.2012.04.037

Clarke, K. R., and Gorley, R. N. (2015). *PRIMER v7: User Manual/Tutorial*. Plymouth: PRIMER-E.

Colombo, B. M., Scalvenzi, T., Benlamara, S., and Pollet, N. (2015). Microbiota and mucosal immunity in amphibians. *Front. Immunol.* 6:111. doi: 10.3389/fimmu.2015.00111

Conlon, J. M. (2011). The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. *Cell Tissue Res.* 343, 201–212. doi: 10.1007/s00441-010-1014-4

Daly, J. (1995). The chemistry of poisons in amphibian skin. *Proc. Natl. Acad. Sci. U.S.A.* 92, 9–13.

Engel, P., and Moran, N. A. (2013). The gut microbiota of insects-diversity in structure and function. *FEMS Microbiol. Rev.* 37, 699–735. doi: 10.1111/1574-6976.12025

Erspamer, V. (1994). "Bioactive secretions of the amphibian integument," in *Amphibian Biology*, eds G. Heatwole, T. Barthalmus, and A. Heatwole (Chipping Norton: Surrey Beatty & Sons), 178–350.

Favilli, F., and Messini, A. (1990). Nitrogen fixation at phyllospheric level in coniferous plants in Italy. *Plant Soil* 128, 91–95. doi: 10.1007/BF00009400

Fierer, N. (2008). "Microbial biogeography: patterns in microbial diversity across space and time," in *Accessing Uncultivated Microorganisms: From the Environment and Organisms and Genomes and Back*, ed. K. Zengler (Washington, DC: ASM Press), 95–115.

Fierer, N., Ferrenberg, S., Flores, G. E., González, A., Kueneman, J., Legg, T., et al. (2012). From animalicules to an ecosystem: application of ecological concepts to the human microbiome. *Annu. Rev. Ecol. Evol. Syst.* 43, 137–155. doi: 10.1093/femsec/fix044

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 103, 626–631. doi: 10.1073/pnas.0507535103

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Franzenburg, S., Walter, J., Künzel, S., Wang, J., Baines, J. F., Bosch, T. C. G., et al. (2013). Distinct antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3730–E3738. doi: 10.1073/pnas.1304960110

Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., et al. (2014). Bacteria-bacteria interactions within the microbiota of the ancestral metazoan *Hydra* contribute to fungal resistance. *ISME J.* 9, 1543–1556. doi: 10.1038/ismej.2014.239

Glaw, F., and Vences, M. (2007). *A Field Guide to the Amphibians and Reptiles of Madagascar*. Cologne: Vences & Glaw, Köln.

Goslee, S. C., and Urban, D. L. (2007). The ecodist package for dissimilarity-based analysis of ecological data. *J. Stat. Softw.* 22, 1–19.

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Hullar, M. A. J., Kaplan, L. A., and Stahl, D. A. (2006). Recurring seasonal dynamics of microbial communities in stream habitats. *Appl. Environ. Microbiol.* 72, 713–722. doi: 10.1128/AEM.72.1.713

Hunting, E. R., White, C. M., van Gemert, M., Mes, D., Stam, E., van, H. G., et al. (2013). UV radiation and organic matter composition shape bacterial functional diversity in sediments. *Front. Microbiol.* 4:317. doi: 10.3389/fmicb.2013.00317

Jacobs, J. L., and Sundin, G. W. (2001). Effect of solar UV-B radiation on a phyllosphere bacterial community. *Society* 67, 5488–5496. doi: 10.1128/AEM.67.12.5488

Jiménez, R. R., and Sommer, S. (2016). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Jombart, T., and Dray, S. (2008). *Adephylo: Exploratory Analyses for the Phylogenetic Comparative Method*. Available at: https://cran.rstudio.com/web/packages/adephylo/index.html

Jost, L. (2006). Entropy and diversity. *Oikos* 113, 363–375. doi: 10.1111/j.2006.0030-1299.14714.x

Kohl, K. D., Cary, T. L., Karasov, W. H., and Dearing, M. D. (2013). Restructuring of the amphibian gut microbiota through metamorphosis. *Environ. Microbiol. Rep.* 5, 899–903. doi: 10.1111/1758-2229.12092

Kohl, K. D., Weiss, R. B., Cox, J., Dale, C., and Dearing, M. D. (2014). Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecol. Lett.* 17, 1238–1246. doi: 10.1111/ele.12329

Krediet, C. J., Ritchie, K. B., Paul, V. J., and Teplitski, M. (2013). Coral-associated micro-organisms and their roles in promoting coral health and thwarting diseases. *Proc. Biol. Sci.* 280:20122328. doi: 10.1098/rspb.2012.2328

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33:msw054. doi: 10.1093/molbev/msw054

Kwon, S., Park, S., Lee, B., and Yoon, S. (2013). In-depth analysis of interrelation between quality scores and real errors in Illumina reads. *Conf. Proc. IEEE Eng. Med. Biol. Soc.* 2013, 635–638. doi: 10.1109/EMBC.2013.6609580

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H. M., Knight, R., McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Mayak, S., Tirosh, T., and Glick, B. R. (2004). Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Sci.* 166, 525–530. doi: 10.1016/j.plantsci.2003.10.025

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2017). *vegan: Community Ecology Package. R Package Version 2.4-2*. Available at: https://cran.r-project.org/package=vegan

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: analyses of phylogenetic and evolution in R language. *Bioinformatics* 20, 289–290.

Piccini, C., Conde, D., Pernthaler, J., and Sommaruga, R. (2009). Alteration of chromophoric dissolved organic matter by solar UV radiation causes rapid changes in bacterial community composition. *Photochem. Photobiol. Sci.* 8, 1321–1328. doi: 10.1039/b905040j

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

R Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org/

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Robinson, C. J., Bohannan, B. J. M., and Young, V. B. (2010). From structure to function: the ecology of host-associated microbial communities. *Microbiol. Mol. Biol. Rev.* 74, 453–476. doi: 10.1128/MMBR.00014-10

Roelants, K., Gower, D. J., Wilkinson, M., Loader, S. P., Biju, S. D., Guillaume, K., et al. (2007). Global patterns of diversification in the history of modern amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 104, 887–892. doi: 10.1073/pnas.0608378104

Rollins-Smith, L. A., and Woodhams, D. C. (2012). "Amphibian immunity: staying in tune with the environment," in *Ecoimmunology*, eds G. Demas and R. Nelson (New York, NY: Oxford University Press), 92–143.

Rosenthal, M., Goldberg, D., Aiello, A., Larson, E., and Foxman, B. (2011). Skin microbiota: microbial community structure and its potential association with health and disease. *Infect. Genet. Evol.* 11, 839–848. doi: 10.1016/j.meegid.2011.03.022.Skin

Sabino-Pinto, J., Bletz, M. C., Islam, M. M., Shimizu, N., Bhuju, S., Geffers, R., et al. (2016). Composition of the cutaneous bacterial bommunity in Japanese amphibians: effects of captivity, host species, and body region. *Microb. Ecol.* 72, 460–469. doi: 10.1007/s00248-016-0797-6

Sanford, J. A., and Gallo, R. L. (2013). Functions of the skin microbiota in health and disease. *Semin. Immunol.* 25, 370–377. doi: 10.1016/j.smim.2013.09.005

Scherz, M. D., Vences, M., Rakotoarison, A., Andreone, F., Köhler, J., Glaw, F., et al. (2016). Reconciling molecular phylogeny, morphological divergence and classification of Madagascan narrow-mouthed frogs (Amphibia: Microhylidae). *Mol. Phylogenet. Evol.* 100, 372–381. doi: 10.1016/j.ympev.2016.04.019

Sedlar, K., Videnska, P., Skutkova, H., Rychlik, I., and Provaznik, I. (2016). Bipartite graphs for visualization analysis of microbiome data. *Evol. Bioinform. Online* 12, 17–23. doi: 10.4137/EBO.S38546

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shapira, M. (2016). Gut microbiotas and host evolution: scaling up symbiosis. *Trends Ecol. Evol.* 31, 539–549. doi: 10.1016/j.tree.2016.03.006

Sommer, F., Ståhlman, M., Ilkayeva, O., Arnemo, J. M., Kindberg, J., Josefsson, J., et al. (2016). The gut microbiota modulates energy metabolism in the hibernating brown bear *Ursus arctos*. *Cell Rep.* 14, 1655–1661. doi: 10.1016/j.celrep.2016.01.026

Stecher, B., and Hardt, W. D. (2008). The role of microbiota in infectious disease. *Trends Microbiol.* 16, 107–114. doi: 10.1016/j.tim.2007.12.008

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Swofford, D. (2002). *PAUP* Phylogenetic Analysis using Parsimony/and other Methods*. Sunderland, MA: Sinauer Associates.

Vences, M., Lyra, M., Kueneman, J. G., Bletz, M. C., Archer, H. M., Canitz, J., et al. (2016). Gut bacterial communities across tadpole ecomorphs in two diverse tropical anuran faunas. *Sci. Nat.* 103, 25. doi: 10.1007/s00114-016-1348-1

Vences, M., Wollenberg, K. C., Vieites, D. R., and Lees, D. C. (2009). Madagascar as a model region of species diversification. *Trends Ecol. Evol.* 24, 456–465. doi: 10.1016/j.tree.2009.03.011

Vieites, D. R., Wollenberg, K. C., Andreone, F., Köhler, J., Glaw, F., and Vences, M. (2009). Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8267–8272. doi: 10.1073/pnas.0810821106

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog.* 12:e1005796. doi: 10.1371/journal.ppat.1005796

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wells, K. D. (2007). *Ecology and Behavior of Amphibians*. Chicago, IL: The University of Chicago Press.

Wollenberg, K. C., Vieites, D. R., Glaw, F., and Vences, M. (2011). Speciation in little: the role of range and body size in the diversification of Malagasy mantellid frogs. *BMC Evol. Biol.* 11:217. doi: 10.1186/1471-2148-11-217

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H. M., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96:595.

Woodhams, D. C., Bletz, M. C., Kueneman, J., and McKenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trends Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

Woodhams, D. C., Voyles, J., Lips, K. R., Carey, C., and Rollins-Smith, L. A. (2006). Predicted disease susceptibility in a Panamanian amphibian assemblage based on skin peptide defenses. *J. Wildl. Dis.* 42, 207–218. doi: 10.7589/0090-3558-42.2.207

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Composition of Micro-eukaryotes on the Skin of the Cascades Frog (*Rana cascadae*) and Patterns of Correlation between Skin Microbes and *Batrachochytrium dendrobatidis*

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Global amphibian decline linked to fungal pathogens has galvanized research on applied amphibian conservation. Skin-associated bacterial communities of amphibians have been shown to mediate fungal skin infections and the development of probiotic treatments with antifungal bacteria has become an emergent area of research. While exploring the role of protective bacteria has been a primary focus for amphibian conservation, we aim to expand and study the other microbes present in amphibian skin communities including fungi and other micro-eukaryotes. Here, we characterize skin-associated bacteria and micro-eukaryotic diversity found across life stages of Cascades frog (*Rana cascadae*) and their associated aquatic environments using culture independent 16S and 18S rRNA marker-gene sequencing. Individuals of various life stages of Cascades frogs were sampled from a population located in the Trinity Alps in Northern California during an epidemic of the chytrid fungus, *Batrachochytrium dendrobatidis*. We filtered the bacterial sequences against a published database of bacteria known to inhibit *B. dendrobatidis* in co-culture to estimate the proportion of the skin bacterial community that is likely to provide defense against *B. dendrobatidis*. Tadpoles had a significantly higher proportion of *B. dendrobatidis*-inhibitory bacterial sequence matches relative to subadult and adult Cascades frogs. We applied a network analysis to examine patterns of correlation between bacterial taxa and *B. dendrobatidis*, as well as micro-eukaryotic taxa and *B. dendrobatidis*. Combined with the published database of bacteria known to inhibit *B. dendrobatidis*, we used the network analysis to identify bacteria that negatively correlated with *B. dendrobatidis* and thus could be good probiotic candidates in the Cascades frog system.

Keywords: microbiome, micro-eukaryote, network, skin, *Rana cascadae*, *Batrachochytrium dendrobatidis*, Cascades frog

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INTRODUCTION

Microbial symbionts of vertebrate hosts facilitate many biological processes, such as nutrient acquisition, host behavior, training of the adaptive immune system and protection against environmental pathogens (Bravo et al., 2011; Ezenwa et al., 2012; Tuddenham and Sears, 2015). Thus, microbial associations can contribute to host phenotypes and may influence survival

(Fierer et al., 2012). The amphibian skin microbiome has become an active area of research due to the protective role that some bacteria provide against *Batrachochytrium dendrobatidis*, an emerging fungal skin pathogen (Harris et al., 2009; Kueneman et al., 2016). This aquatic pathogen, now the agent of a global pandemic, invades the keratinized epidermis of amphibians resulting in cell disruption and osmotic imbalances that can lead to death (Voyles et al., 2009). The use of 16S rRNA marker gene sequencing to characterize bacterial communities on amphibians has enabled many researchers to investigate the factors that shape those communities (McKenzie et al., 2012; Fitzpatrick and Allison, 2014; Jani and Briggs, 2014; Kueneman et al., 2014, 2015; Loudon et al., 2014; Walke et al., 2014). However, most studies have overlooked the fungal and micro-eukaryotic diversity that also occurs on amphibian skin and have yet to explore their function. Here, we use a parallel approach (18S rRNA marker gene sequencing) to explore micro-eukaryotic diversity alongside bacterial diversity on the skin of endangered Cascades frogs (*Rana cascadae*).

Bacterial-derived skin defenses have been experimentally shown to mitigate *B. dendrobatidis* infection (Harris et al., 2009; Jani and Briggs, 2014; Kueneman et al., 2016), however, interactions of other fungal taxa and micro-eukaryotes with *B. dendrobatidis* remain underexplored. Currently, it is unknown whether specific micro-eukaryotes may help mitigate infection of *B. dendrobatidis*. In plant systems, numerous experiments have found that some fungi are capable of excluding or reducing the effects of fungal pathogens. Examples include various leaf and root rots found in legumes, cacao, cotton, Dutch Elm, and fruit trees (Cotty and Bayman, 1993; Del Rio et al., 2002; Kabaluk et al., 2010). It is possible that symbiotic fungi on amphibians may be able to competitively exclude fungal pathogens through direct and indirect competition, however, this hypothesis remains to be tested on the amphibian skin.

In the absence of direct experimentation, network analyses provide one way to explore patterns of correlations within complex communities, identify microbes that negatively correlate with a pathogen, and infer possible antagonistic interactions (Rebollar et al., 2016a). While a negative correlation with a pathogen in a network does not confirm an antagonistic interaction, it is suggestive and should be tested independently. Microbes that are negatively correlated can help direct research to test specific interactions mechanistically and assess antibiosis that occurs with competing microbial taxa (Bletz et al., 2013). Previous field studies and lab experiments have demonstrated that individuals of species which have a higher proportion of their skin bacterial community composed of *B. dendrobatidis*-inhibitory taxa have lower abundance of *B. dendrobatidis* on their skin (Lam et al., 2010; Kueneman et al., 2016; Rebollar et al., 2016b). Thus, *B. dendrobatidis*-inhibitory bacteria negatively interacting with *B. dendrobatidis* within these networks are of special interest for conservation.

Cascades frogs are found in the Cascade and Olympic Mountains in the Pacific Northwest region of the United States. They have declined precipitously as a result of trout reintroduction and, more recently, *B. dendrobatidis* (Piovia-Scott et al., 2011). In Northern California, the Cascades frog population at Section Line Lake experienced a decline in juvenile frogs (>99%) attributed to *B. dendrobatidis* between 2009 and 2012 (Piovia-Scott et al., 2015). We had the opportunity to non-destructively sample the skin microbes from individuals in 2011, thus capturing a time point during an active *B. dendrobatidis* epidemic. Studying an epidemic in progress provides a unique perspective for examining the role of amphibian skin microbial communities in defense against pathogens, specifically by identifying microbes that have strong patterns of negative correlation with *B. dendrobatidis*. As Cascades frogs are imperiled, protective microbes can potentially offer new tools to augment the conservation of this species (Garcia et al., 2006; Piovia-Scott et al., 2015).

Here, we present a dataset that includes both the micro-eukaryotes and bacteria collected from the skin of wild *R. cascadae*. Our previous work contributed to a bacterial database of more than 1,200 isolates from amphibians around the globe that have demonstrated inhibitory action against *B. dendrobatidis* when tested in co-culture assays (Woodhams et al., 2015). No such database exists for the micro-eukaryotes, as of yet. We matched the bacterial sequence reads from the Cascades frogs against the *B. dendrobatidis*-inhibitory database to estimate the bacterial members of the skin community that are *B. dendrobatidis*-inhibitory. The aims of this study are threefold: (1) to characterize skin-associated micro-eukaryotes, including fungal skin communities among life-history stages of *R. cascadae*, (2) to examine correlational patterns among diverse microbes (bacteria and fungi), with a particular focus on interactions with the *B. dendrobatidis* pathogen, and (3) to identify candidate anti-*B. dendrobatidis* microbial taxa by leveraging the *B. dendrobatidis*-inhibitory sequence database.

MATERIALS AND METHODS

Amphibian and Environmental Sampling

Individuals of *Rana cascadae* were caught and sampled in the Trinity Alps of Northern California, August of 2011. All individuals were captured using a dip net, handled with new nitrile gloves and sampled on the same day. In order to remove any transient microbes from the environment, each individual was rinsed twice with 50 mL of sterile water. Each amphibian was then sampled using a sterile cotton-tipped swab. Swabbing consisted of brushing over the entire ventral surface and limbs of the amphibian for 30 s. Tadpoles were swabbed uniformly over entire body for 30 s (McKenzie et al., 2012). All sampling was done non-destructively and individuals were released back into the lake. Lake water samples were collected by moving a swab through the water for 30 s at a depth of 40 cm. Sediment samples were collected via embedding the swab into the sediment for 30 s. Swabbing amphibians and the environment for the same amount of time (30 s) was part of the effort to standardize the sampling across sample types. Each swab was stored in its original sterile container and stored on ice for transfer to a 20°C freezer for storage until DNA extraction. Micro-eukaryotic communities from *R. cascadae* at Section Line, an alpine lake in the Trinity Alps of California, included tadpoles ($N = 4$), subadults ($N = 6$),

adults ($N = 11$), sediment ($N = 3$), and lake water ($N = 2$). Permits and authorization were granted by California Fish and Game and the University of Colorado Institutional Animal Care and Use Committee.

DNA Extraction and Sample Processing

DNA extraction was completed utilizing the MoBio Power Soil extraction kit (MoBio Laboratories, Carlsbad, CA, United States). We used the standard MoBio protocol with minor adjustments including incubating samples in 65°C for 10 min after the addition of C1, vortexing the PowerBead tubes horizontally for 2 min, and allowing solution C6 to sit on the filter for 5 min before the final elution (Fierer et al., 2008; Lauber et al., 2008). Extraction controls were included. The PCR recipe was comprised of: 12 μL PCR water, 10 μL 5 Prime Master Mix, 1.0 μL of the forward and reverse primers at 10 uM concentrations, 1.0 μL $MgCl_2$, and 1.0 μL genomic DNA. For bacteria, PCR primers (515f/806r) were used to target the V4 region of the 16S rRNA gene and amplify region 533–786 in the *Escherichia coli* strain 83972 sequence (greengenes accession no. prokMSA_id:470367). The reverse PCR primer contained a 12-base error correcting Golay barcode developed in Caporaso et al. (2010). For micro-eukaryotes, primers 1391f/EukB were used (Amaral-Zettler et al., 2009). The PCR profiles included an initial denaturation step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and final extension at 72°C for 10 min. The PCR was performed in triplicate and combined after amplification. Extraction controls were processed and showed no amplification. Amplicons were quantified using the Quant-IT Picogreen dsDNA reagent and were pooled into one sample per plate by combining equal concentrations of each amplicon. These pools of DNA were cleaned using the MoBio UltraClean PCR clean-up DNA purification kit. Following cleanup, samples were again quantified using PicoGreen reagent with equal concentrations and pooled together one final time before sequencing. A NanoDrop spectrophotometer was used to determine the purity and DNA concentration of this pool. Finally, prepared DNAs and control samples were sequenced using an Illumina HiSeq 2000 instrument at the BioFrontiers Institute Next-Generation Genomics Facility at the University of Colorado Boulder, Boulder, CO, United States.

Sequence Filtering and Processing

18S amplicons were sequenced on one Illumina MiSeq run at the University of Colorado Boulder, Boulder, CO, United States, yielding 150 base pair reads. All analyses were performed using QIIME v1.9.0 (Caporaso et al., 2010), unless otherwise stated. Sequences were filtered and assigned to samples using default settings and Cutadapt v1.2 (MARTIN, M) was used to trim primers from the combined forward and reverse sequences. Low abundance OTUs (i.e., less than 0.00005 proportional abundance) were removed (Bokulich et al., 2013) according to the subsampling open reference protocol (Rideout et al., 2014) using eukaryotic reference library Silva111 (Yilmaz et al., 2013). The no-template sequencing control was clear of unintended DNA amplification. Sequences were assigned to amphibian and environmental samples collected from the research location of Section Line Lake. Total micro-eukaryotic sequences were less abundant on tadpoles limiting the utility of rarefaction. Thus, to visualize the full extent of diversity within each lifestage, we use a proportional taxonomic abundance table (**Figures 1A,B**). However, we acknowledge that comparison across lifestages in **Figures 1A,B** is invalid, due to differences in library size. To give the viewer an idea of original library size used to make the proportions, average library size is indicated in the **Figure 1** legend. To confirm our approach, and compare micro-eukaryotic communities (18S) combined across all lifestages, we used a rarefaction depth of 330 micro-eukaryotic sequences per sample (Supplementary Table 1 and Supplementary Figures A,C). Rarefaction reduced samples sizes in the micro-eukaryotic dataset to tadpoles ($N = 2$), subadults ($N = 6$), adults ($N = 9$), sediment ($N = 3$), and lake water ($N = 2$). Rarefied data was used in part due to the >10x library size difference between tadpoles and other life stages (Weiss et al., 2017). Data was not rarefied in the correlation (Network) or differential abundance analyses (ANCOM) to maximize statistical power. Bacterial community (16S) sequence filtering and processing was done on a rarefied dataset of 19,900 sequences per sample. Bacterial samples tadpoles ($N = 4$), subadults ($N = 8$), adults ($N = 12$), sediment ($N = 3$), and lake water ($N = 2$) (**Figure 3**).

Network Analysis

For the network analyses 16S and 18S datasets were merged and sediment and water samples were removed, resulting in 20 amphibian samples remaining with library sizes greater than 37,800. SparCC (Sparse Correlations for Compositional data) relies on sample proportions; therefore, analysis was done on the raw proportions without rarefying or other normalization. Then, OTUs not present in at least half the samples, and having total sum across samples less than 10, were removed prior to network analysis. This is because correlation detection performance degrades significantly with increased number of zero counts (Weiss et al., 2017). For SparCC in particular, performance degradation may be due to pseudo count addition. In this analysis 'edges' are positive and negative correlations with *B. dendrobatidis*. In the network, 'edges' with correlation values of 0.35 or higher were included. For SparCC (Friedman and Alm, 2012), correlation values are just as precise as *p*-value thresholds and computationally much faster (Weiss et al., 2017). In this study, we used default SparCC parameters. Since, we were mostly interested in *B. dendrobatidis*-inhibitory OTUs, only predicted 'edges' with *B. dendrobatidis* are shown in the network (**Figure 2**). A complete list of both positive and negative bacterial and micro-eukaryotic correlations with *B. dendrobatidis* can be found on the data repository DRYAD.

Batrachochytrium dendrobatidis-Inhibitory Bacterial Isolates Database

Culturing of bacterial isolates from diverse amphibians across the globe, including *R. cascadae,* have been collated into a *B. dendrobatidis*-inhibitory database including ∼1,200 isolates that have been tested and shown to inhibit *B. dendrobatidis* in co-culture (Roth et al., 2013; Woodhams et al., 2015). The

bacterial isolates within this database were Sanger sequenced (16S rRNA gene, 1,500 bp), then trimmed to 100 bp and used to pick OTUs with Greengenes reference database (August 2013 version). To explore the overlap between the *B. dendrobatidis*-inhibitory database and the naturally occurring microbial community of *R. cascadae*, we trimmed inhibitory OTUs based on sequences trimmed to the first 100 bp beyond primer 515f (GTGCCAGCMGCCGCGGTAA) to match the Illumina reads (819 isolates clustering to 304 OTUs). Then, we use expanded the dataset to include expected inhibitory OTUs within 0.1 Jukes-Cantor distances on the Greengenes phylogenetic tree (7,459 OTUs). This approach resulted in 7,459 unique expected inhibitory OTUs, forming the basis of the *B. dendrobatidis*-inhibitory database used in this study (available on Dryad). Using this strategy introduces some uncertainty, discussed below, however, it also provides a more comprehensive list of potential *B. dendrobatidis*-inhibitory taxa OTUs.

RESULTS

Total micro-eukaryotic sequences per sample ranged from 53 to 157,156, with a median observation level of 1,666. We observed 500 unique OTUs before rarefaction. Rarefication to a sequencing depth of 330 sequences per sample reduced the total number of unique OTUs to 255. Eukaryotic taxa found on amphibians included Fungi, Stramenopiles, and Metazoa. Analysis of Composition of Microbes (ANCOM) (Mandal et al., 2015) revealed six OTUs with significant differential abundance across life stages. Importantly, *B. dendrobatidis* was significantly lower on tadpoles and found highest on subadults. Both subadult and adult skin communities appeared to be dominated by pathogen *B. dendrobatidis* (**Figure 1B**). The five other taxa significantly differing across sample types were found to be more abundant in lake water (Supplementary Table 2). Qualitatively, fungi are the most abundant micro-eukaryotic taxa found on all life stages of *R. cascadae* and fungi shared between all amphibian life stages and sediment included Leotiomycetes and Eurotiomycetes. OTUs in the group Alveolata were found on both tadpoles and in the sediment (**Figure 1A**). Inter-sample variation for the proportional abundance of eukaryotic taxa across sample types was explored. Here, we see the proportional abundance of *B. dendrobatidis* is highest on all six subadults, compared with eight out of eleven adults revealing high proportional abundance of *B. dendrobatidis* (Supplementary Figure B). The combined patterns for proportion abundance using the full dataset are supported by the proportion abundance patterns using the rarefied dataset (Supplementary Figure C).

A list of the most abundant micro-eukaryotic OTUs found on *R. cascadae,* whether or not they were known to be pathogenic, and a brief description of where else they have been observed, is found in Supplementary Table 1. Surprisingly, we found nine OTUs of fungi, six OTUs of Stramenopiles, and five OTUs of Metazoa represented the majority of the micro-eukaryotic community. Of these 20 OTUs seven are believed to be pathogenic, and eight are believed to not have any negative consequences for the host. We did not find additional information for the remaining four OTUs.

The unequal and relatively small sample size of this dataset limits the strength of statistical measurements of diversity as well as identification of biologically relevant taxa. In particular, only a few tadpoles were found at this location and 18S sequencing yielded low library sizes for micro-eukaryotes. In contrast, 16S sequencing for bacteria yielded sufficient library sizes for comparison to other samples. The low library sizes for micro-eukaryotes warranted the multiple normalization methods described in the methods section. The normalization methods chosen were used to maximize information from the data while maintaining statistical integrity. We found Shannon diversity of micro-eukaryotes and fungi was higher in the sediment compared to amphibian samples. We also report no difference in Shannon diversity across the amphibian lifestages. These results are limited by the sample-sizes across groups and consequently the results and discussion of alpha diversity are otherwise excluded from the main text. A brief methods, results, and discussion of alpha diversity metrics are found in the Supplementary Figure A.

Using network analysis, we discovered that only two fungal OTUs, both Pezizomycota, were negatively correlated with *B. dendrobatidis* (**Figures 2A,B**). We observed that the majority of negative correlations occurred between bacterial OTUs that have not yet been challenged in co-culture (unknown status) and that the majority of both positively and negatively *B. dendrobatidis*-correlated bacterial taxa are in the class Burkholderiales and family Comomonadaceae (**Figure 2A**). Additional groups that negatively correlate with *B. dendrobatidis* are shown in a heat map (**Figure 2B**). Higher taxonomic resolution for both positive and negative correlation 'edges' can be found with supportive data materials on DRYAD. Specifically, taxa that negatively correlate with *B. dendrobatidis* and also match expected *B. dendrobatidis*-inhibitory database included Comamonadaceae (3) – OTU's 270402, 536916, and 823696; Enterobacteriaceae (2) – OTUs 537871 and 783638; Pseudomonadaceae (1) – OTU 279948, and *Bacillus* (1) – OTU 321618. *Janthinobacterium lividum* (OTU 351280), a common antifungal bacterial isolate, was also found to negatively correlate with *B. dendrobatidis* (**Figure 2A**). Taxa listed here warrant increased attention for future conservation applications.

The proportion of *B. dendrobatidis*-inhibitory bacteria was highest on tadpoles compared to subadults and adults (ANOVA, DF $= 2$, $F = 6.14$; $p = 0.0079$, **Figure 3**). The composition of expected *B. dendrobatidis*-inhibitory bacteria is shown in **Figure 3B**. Tadpole OTUs matching to *B. dendrobatidis*-inhibitory taxa were primarily in the family Pseudomonadaceae. Subadult OTUs primarily matched to members in the family Comamonadaceae, and more specifically an OTU in the genus *Ramlibacter*. Adult OTUs matched similarly to those of subadults, but also matched to low abundance inhibitory OTUs. Qualitatively, Enterobacteriaceae and *Bacillus* OTUs were comparatively very low in their sequence abundance, and Pseudomonadaceae, Comamonadaceae, and *Janthinobacterium lividum* were detected across all lifestages. A group significance (Kruskal-Wallis) test revealed that several OTUs matching to

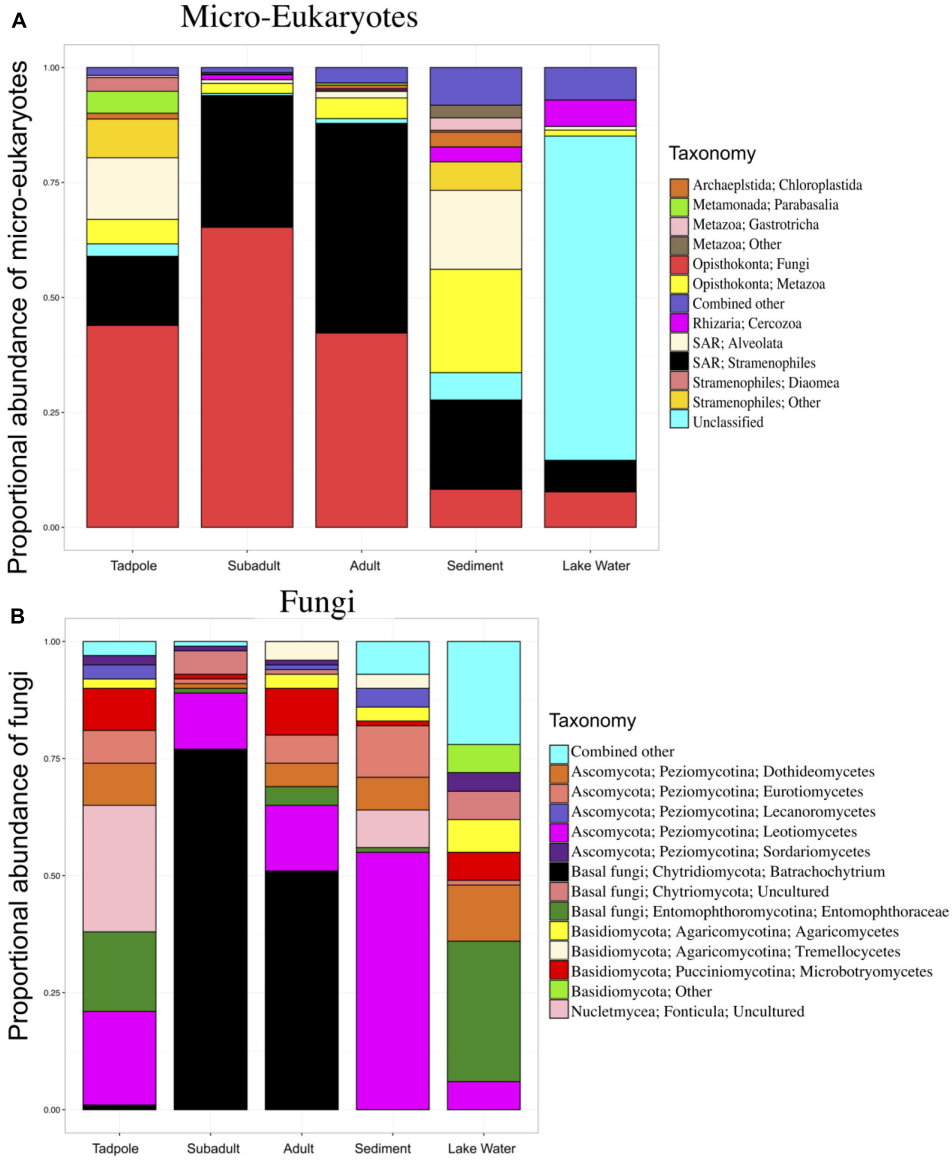

FIGURE 1 | Skin micro-eukaryotic taxa on *Rana cascadae* from Section Line at each life stage, collected on the same day. The proportional abundance of **(A)** micro-eukaryote and **(B)** Fungal OTU sequences per major taxon across lifestages: tadpoles (*N* = 4), subadults (*N* = 6), adults (*N* = 11), sediment (*N* = 3), and lake water (*N* = 2). Data for both figures is based on the proportional abundance of each microbial taxon per individual. OTUs with lower than 0.5% total abundance were grouped into the category combined other. Average number of sequences per sample type; tadpoles (*N* = 213), subadults (*N* = 2,661), adults (*N* = 3,490), sediment (*N* = 125,818) and lake water (*N* = 58,062).

Pseudomonas as well as *J. lividum* were more abundant on tadpoles.

DISCUSSION

One previous study has also used 18S rRNA marker gene sequencing to examine the micro-eukaryotic skin community of amphibian boreal toad (*Anaxyrus boreas*) (Kueneman et al., 2015), thus allowing for some comparison. In *R. cascadae* we detect a higher proportion of *B. dendrobatidis*-inhibitory taxa on tadpoles compared with post-metamorphic individuals (**Figure 3**). This same pattern was found for *Anaxyrus boreas* (Kueneman et al., 2015). The dominant taxonomy of *B. dendrobatidis*-inhibitory bacteria found on tadpoles of both *R. cascadae* and *A. boreas* are not equivalent. This suggests that each species maintains its own unique protective assemblages (Pseudomonadales for *R. Cascadae* and Burkholderiales in *A. boreas*), which may have differential ability to inhibit diverse fungal groups. Comparing other micro-eukaryotes of *R. cascadae* to *A. boreas* qualitatively, we find that the class Stramenopiles is shared across life stages. Taxa in class Alveolata are present

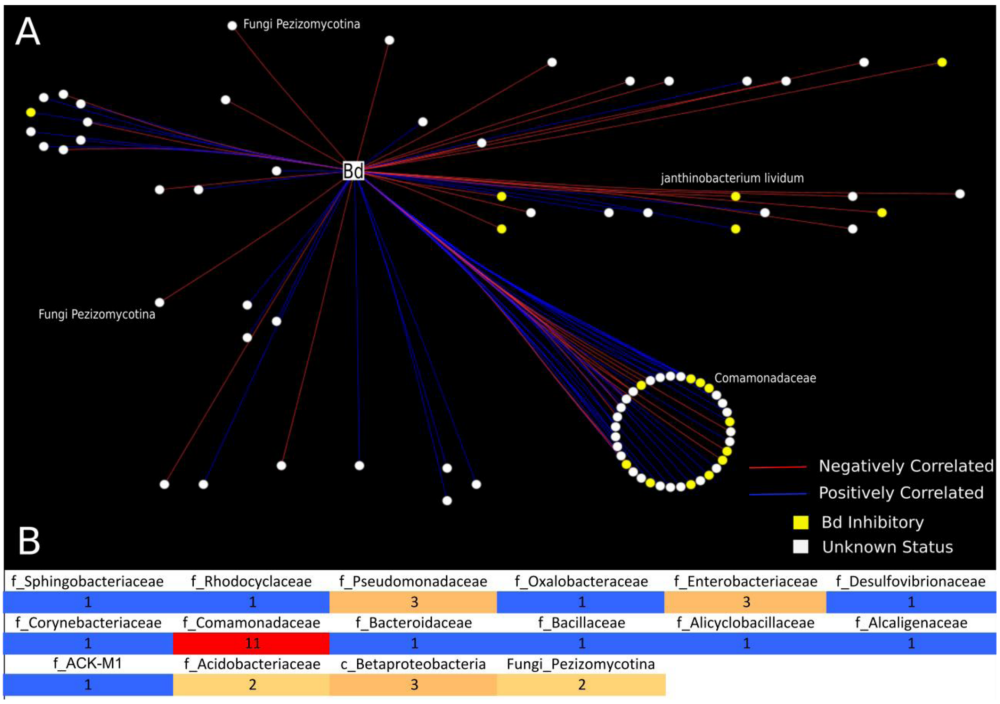

FIGURE 2 | Analysis of bacterial and fungal OTUs that correlate with *B. dendrobatidis* found on Cascades frogs at Section Line. This analysis included 21 frog individuals for which we had successful sequencing yields for both 16S and 18S: tadpoles (*N* = 4), subadults (*N* = 6), adults (*N* = 11). **(A)** Network analysis is depicting only significantly correlating bacterial and fungal OTUs with *B. dendrobatidis.* All circular nodes represent OTUs (either bacteria or fungi) with significant correlation with pathogen *B. dendrobatidis* in the network. Red lines indicate negative correlation between two OTUs. Blue lines indicate positive correlation between two OTUs. Yellow nodes = (*B. dendrobatidis*-inhibitory), White nodes (Unknown Status). The large circle represents bacterial OTUs of the family Comomonadaceae; the smaller circle shows taxa clustering at the order level (Burkholderiales). Two fungal OTUs (Pezizomycotina) were found to negatively correlate with pathogen *B. dendrobatidis*. **(B)** Heatmap is depicting the number of negatively correlating OTUs between bacterial and fungal OTUs and fungal taxon *B. dendrobatidis*. All positively correlated interactions are excluded from the heatmap. The numbers in the heatmap indicate the frequency of negative correlations between bacterial and fungal groups calculated as the sum of negative OTU correlations per bacterial taxon and *B. dendrobatidis*, which are also depicted as red lines in **A**. Taxonomic levels are described as f = Family, c = Class, and Fungi = subdivision.

among tadpoles in the Cascades frog and the boreal toad, but are uniquely absent in post-metamorphic stages of Cascades frogs. Thus, we observe important similarities and differences in the diversity of micro-eukaryotes across amphibian life stages of two different amphibian families (Ranidae and Bufonidae).

Interactions between naturally occurring communities of microbial taxa on amphibian skin can be difficult to infer. However, we show that network analysis can be a useful tool to predict interactions between diverse taxa on the skin of *R. cascadae*. To date, the sequence database for *B. dendrobatidis*-inhibitory bacterial taxa (Woodhams et al., 2015) includes 37 bacterial isolates from *R. cascadae* (Roth et al., 2013), but only five isolates were found from the individuals sampled at Section Line in this dataset. Additionally, OTUs that match these five inhibitory isolates are relatively rare in our dataset of negatively correlating bacterial taxa with *B. dendrobatidis*. Thus, we utilize a *B. dendrobatidis*-inhibitory dataset, described in the methods section, which enables us to consider taxa that are closely related to *B. dendrobatidis*-inhibitory taxa isolated from other amphibian species. We report possible negative interactions that may occur between bacterial and micro-eukaryote taxa and *B. dendrobatidis* (**Figure 2**). Specific taxa, identified as negatively correlating with *B. dendrobatidis,* should be tested experimentally in the lab to confirm activity against *B. dendrobatidis*. After additional confirmation, these bacterial taxa can be referenced by the *B. dendrobatidis*-inhibitory database and better inform risk assessment of individuals, life stages, or populations of *R cascadae,* as well as other amphibian species. Only one bacterial taxon, *Janthinobacterium lividum*, identified here as negatively correlating with *B. dendrobatidis,* and matching the *B. dendrobatidis*-inhibitory database, has been tested as a probiotic on amphibians (Harris et al., 2009; Becker et al., 2011; Walke et al., 2015; Kueneman et al., 2016). However, its activity against *B. dendrobatidis* on various amphibian species offered mixed results, and its efficacy as a probiotic for *R. cascadae* remains to be explored *in vivo*. Intriguingly, *J. lividum* was found most abundantly on tadpoles. Additionally, isolates of the fungal group Pezizomycota (shown to negatively correlate with *B. dendrobatidis*) and more specifically Pezizomycetidae, should also be tested for their bioactivity against *B. dendrobatidis*. Taken together, taxa that negatively correlate with *B. dendrobatidis* and also match the *B. dendrobatidis*-inhibitory database may serve to direct additional studies aimed at conservation of *R. cascadae* and should be tested *in vivo*.

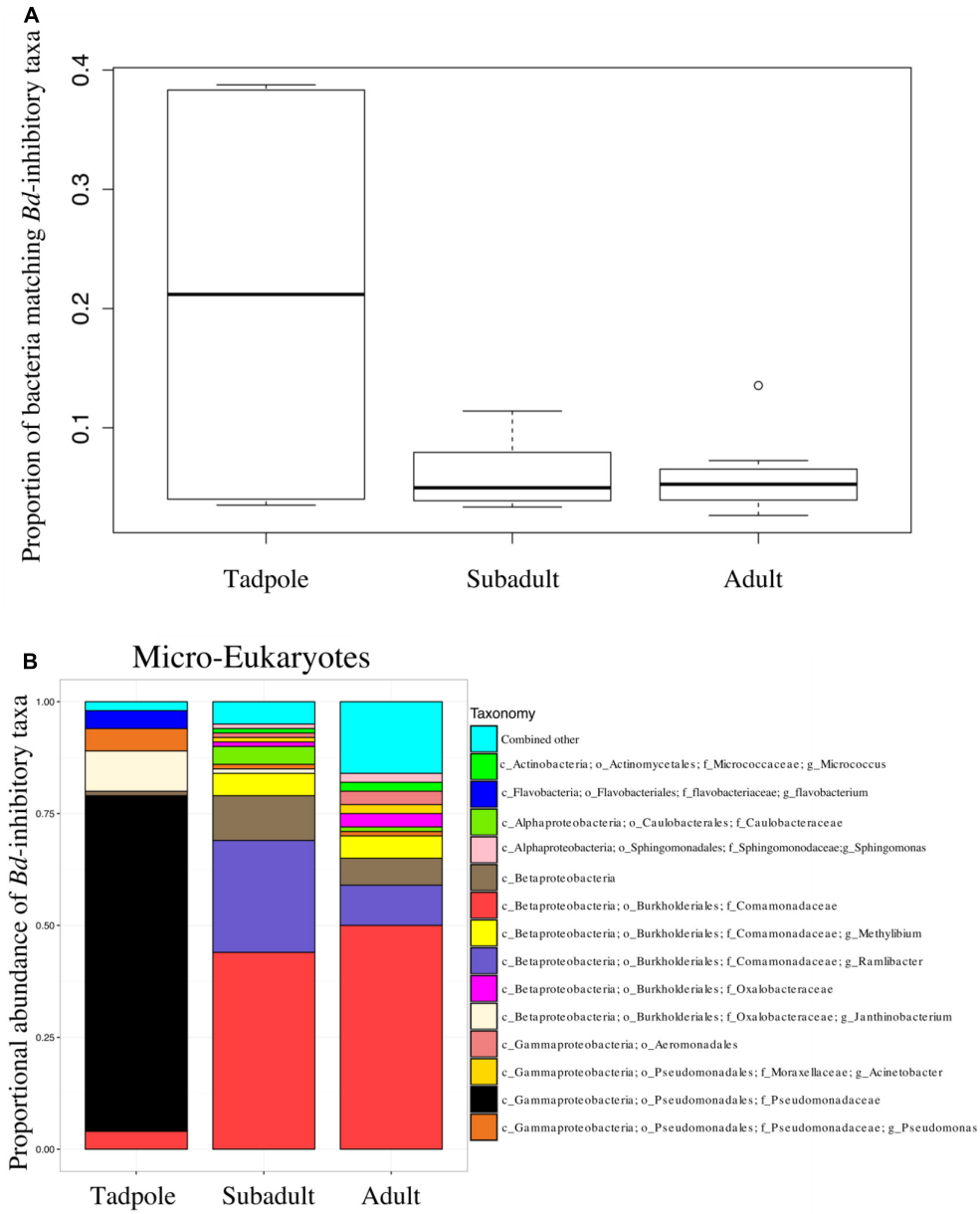

FIGURE 3 | (A) The proportion of sequences that match the *B. dendrobatidis*-inhibitory database found on individuals. There is a higher proportion of the skin community that is *B. dendrobatidis*-inhibitory on tadpoles compared with subadults and adults; ANOVA, DF = 2, $F = 6.143$; $p = 0.00793$. Calculation for A and B are conducted on a rarefied dataset of 19,900 sequences per sample; Lifestages: tadpoles ($N = 4$), subadults ($N = 6$), adults ($N = 11$), sediment ($N = 3$), and lake water ($N = 2$). **(B)** *B. dendrobatidis*-inhibitory skin bacteria on *Rana cascadae* from Section Line at each life stage, collected on the same day. OTUs with lower than 1% total abundance are grouped into the category combined other.

While the biology of many of the micro-eukaryotic taxa identified in this study remain unknown, several taxa identified, beyond *B. dendrobatidis,* have been previously shown to be antagonistic/pathogenic to free living organisms and could possibly influence the ecology of the frog skin microbiome (Supplementary Table 1). For example, *Entomophthora culicis* and *Leptolegnia caudata* are known parasites and pathogens of insects, respectively (Kerwin, 1982). Additionally, *Aphanomyces invadans* and *Karlodinium micrum* were found to be pathogens of fish and their eggs and *Saprolegnia* species are pathogens of both amphibians and fish, including their eggs (Bisht et al., 1996). Surprisingly, *Leptolegnia caudata* and *Aphanomyces invadans* were found to positively correlate with *B. dendrobatidis*. It is unknown at this point if the detection of these taxa signifies secondary infections or if they may have facilitated *B. dendrobatidis* infections. The ecology and consequence of the

aforementioned taxa should be considered in future studies of *R. cascadae* health. Additionally, network analysis tools, while having reasonable false positive rates on OTUs with <50% zeroes, need improvement. Many tools have poor detection of ecological relationships other than mutualism and commensalism. SparCC, while attempting to account for compositions, is also one of the strongest network analysis techniques for detecting competitive mutual exclusion (Weiss et al., 2017). Detection of multispecies relationships is also difficult. In this study, increased library size would have provided more insight. Despite these weaknesses, correlation techniques have proven to be useful here and in a variety of additional studies.

In this study, we acknowledge the limitations to the predictive power of the *B. dendrobatidis*-inhibitory database. This database lacks sufficient information on bacterial isolates that do not exhibit bioactivity against *B. dendrobatidis*. This is because isolates cultured from amphibians were tested in co-culture with *B. dendrobatidis*, and then Sanger sequenced. Isolates that did not show inhibition against *B. dendrobatidis* are often not sequenced and thus not included in the database. Consequently, it is not possible to fully consider microbial interactions not captured by the database. To address the limitation that many *B. dendrobatidis* inhibitory microbes are also not present in the database we utilize an expanded list of OTUs to capture additional bacterial taxa that may be biologically important. Additional limitations to the predictive power of the *B. dendrobatidis*-inhibitory dataset exist: For example, (1) most isolates are cultured from adults, and thus the database may be biased toward microbial taxa that colonize the skin of post-metamorphic individuals (Woodhams et al., 2015). (2) In captivity, bacterial taxa may interact differently with *B. dendrobatidis* than they do in their natural environments. (3) Bacterial taxa that are inhibitory to *B. dendrobatidis* growth under certain temperature regimes are known to behave differently at other temperature regimes (Woodhams et al., 2014). (4) *B. dendrobatidis*-inhibitory taxa can act differently in the context of other microbes in the environment, or at certain densities (Woodhams et al., 2014). (5) Bacterial strains of the same species also behave differently with respect to *B. dendrobatidis*. (6) There is intrinsic bias for amplification of taxa based on the primers used. Specifically, pairs of primers for both bacteria and micro-eukaryotes have limitations for equal sequencing across all groups (Tedersoo et al., 2015; Tedersoo and Lindahl, 2016). (7) Lastly, many of the naturally occurring microbial OTUs are absent from the *B. dendrobatidis*-inhibitory database which limit the strength of its predictive power. The limitations listed here are universal to the state of extracting meaningful microbial interactions from microbial marker-gene surveys, when there is limited system specific knowledge. Indeed, while metagenomic tools may introduce error, they can also help to direct future studies to address specific knowledge gaps.

The role of skin microbial communities in protecting individuals from *B. dendrobatidis* is an area open to discovery that may help inform the defenses of *R. cascadae* against *B. dendrobatidis* and provide tools for enhanced conservation efforts of *R. cascadae* in the future. Important to the inferences made from this study, a particularly lethal strain of pathogen *B. dendrobatidis* has been isolated from Section Line Lake and tested in an experimental setting against *R. cascadae* reared from eggs. Individuals reared from eggs collected from Section Line survived longer and were healthier than other local populations tested against the Section Line *B. dendrobatidis* isolate (Piovia-Scott et al., 2015). In addition, individuals from Section Line survived longer against an extraneous *B. dendrobatidis* isolate, compared to other local populations tested (Piovia-Scott et al., 2015). The evidence shows that *R. cascadae* at Section Line interact with an aggressive strain of *B. dendrobatidis* and thus it is particularly important to understand their defenses against the pathogen. Unfortunately, subadults retained very high infection intensities (**Figure 1B**), and high mortality for this age group is observed in population demographics (Piovia-Scott et al., 2015). This dwindling age group is responsible for the continued decline of this remnant population of the Cascades frog. The function of skin microbial associations on amphibians has yet to be fully explored. This contribution begins to address knowledge gaps regarding the diversity and role of micro-eukaryotes in the health of amphibians, in identifying naturally occurring microbial interactions that may protect *R. cascadae* from fungal disease epidemics, and in direct conservation research for the most promising protective bacterial groups.

AUTHOR CONTRIBUTIONS

JK and VM conceived the study, JK collected the data, JK and SW analyzed the data, JK, VM, and SW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02350/full#supplementary-material

REFERENCES

Amaral-Zettler, L. A., McCliment, E. A., Ducklow, H. W., and Huse, S. M. (2009). A method for studying protistan diversity using massively parallel sequencing of V9 hyper variable regions of small-subunit ribosomal RNA genes. *PLOS ONE* 4:e6372. doi: 10.1371/journal.pone.0006372

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., et al. (2011). Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16050–16055. doi: 10.1073/pnas.1102999108

Becker, M. H., Harris, R. N., Minbiole, K. P., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Bisht, G. S., Joshi, C.H., and Khulbe, R. D. (1996). Watermolds: potential biological control agents of malaria vector *Anopheles culicifacies*. *Curr. Sci.* 70, 393–395.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cotty, P. J., and Bayman, P. (1993). Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. *Phytopathology* 83, 1283–1287. doi: 10.1094/Phyto-83-1283

Del Rio, L. E., Martinson, C. A., and Yang, X. B. (2002). Biological control of Sclerotinia stem rot of soybean with *Sporidesmium sclerotivorum*. *Plant Dis.* 86, 999–1004. doi: 10.1094/PDIS.2002.86.9.999

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., and Xavier, J. B. (2012). Animal behavior and the microbiome. *Science* 338, 198–199. doi: 10.1126/science.1227412

Fierer, N., Ferrenberg, S., Flores, G. E., González, A., Kueneman, J., Legg, T., et al. (2012). From animalcules to an ecosystem: application of ecological concepts to the human microbiome. *Annu. Rev. Ecol. Evol. Syst.* 43, 137–155. doi: 10.1146/annurev-ecolsys-110411-160307

Fierer, N., Hamady, M., Lauber, C. L., and Knight, R. (2008). The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17994–17999. doi: 10.1073/pnas.0807920105

Fitzpatrick, B. M., and Allison, A. L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiol. Ecol.* 88, 482–494. doi: 10.1111/1574-6941.12314

Friedman, J., and Alm, E. J. (2012). Inferring correlation networks from genomic survey data. *PLOS Comput. Biol.* 8:e1002687. doi: 10.1371/journal.pcbi.1002687

Garcia, T. S., Romansic, J. M., and Blaustein, A. R. (2006). Survival of three species of anuran metamorphs exposed to UV-B radiation and the pathogenic fungus *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 72:163. doi: 10.3354/dao072163

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Kabaluk, J. T., Svircev, A. M., Goettel, M. S., and Woo, S. G. (2010). *The Use and Regulation of Microbial Pesticides in Representative Jurisdictions Worldwide*. St. Paul, MN: International Organization for Biological Control.

Kerwin, J. L. (1982). Chemical control of the germination of asexual spores of *Entomophthora culicis*, a fungus parasitic on dipterans. *Microbiology* 128, 2179–2186. doi: 10.1099/00221287-128-9-2179

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., McKenzie, V. J., et al. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., and McKenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc.* 283, 1839–1553. doi: 10.1098/rspb.2016.1553

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and McKenzie, V. J. (2015). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944 doi: 10.1038/ismej.2015.168

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40, 2407–2415. doi: 10.1016/j.soilbio.2008.05.021

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., and Peddada, S. D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26:27663.

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Piovia-Scott, J., Pope, K. L., Lawler, S. P., Cole, E. M., and Foley, J. E. (2011). Factors related to the distribution and prevalence of the fungal pathogen *Batrachochytrium dendrobatidis* in *Rana cascadae* and other amphibians in the Klamath Mountains. *Biol. Conserv.* 144, 2913–2921. doi: 10.1016/j.biocon.2011.08.008

Piovia-Scott, J., Pope, K., Worth, S. J., Rosenblum, E. B., Poorten, T., Refsnider, J., et al. (2015). Correlates of virulence in a frog-killing fungal pathogen: evidence from a California amphibian decline. *ISME J.* 9, 1570–1578. doi: 10.1038/ismej.2014.241

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L., K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., et al. (2014). Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* 2:545. doi: 10.7717/peerj.545

Roth, T., Foley, J., Worth, J., Piovia-Scott, J., Pope, K., and Lawler, S. (2013). Bacterial flora on Cascades frogs in the Klamath mountains of California. *Comp. Immunol. Microbiol. Infect. Dis.* 36, 591–598. doi: 10.1016/j.cimid.2013.07.002

Tedersoo, L., Anslan, S., Bahram, M., Põlme, S., Riit, T., Liiv, I., et al. (2015). Shotgun metagenomes and multiple primer pair-barcode combinations of amplicons reveal biases in metabarcoding analyses of fungi. *MycoKeys* 10, 1–43. doi: 10.3897/mycokeys.10.4852

Tedersoo, L., and Lindahl, B. (2016). Fungal identification biases in microbiome projects. *Environ. Microbiol. Rep.* 8, 774–779. doi: 10.1111/1758-2229.12438

Tuddenham, S., and Sears, C. L. (2015). The intestinal microbiome and health. *Curr. Opin. Infect. Dis.* 28, 464–470. doi: 10.1097/QCO.0000000000000196

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., and Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326, 582–585. doi: 10.1126/science.1176765

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a Chytrid fungus. *PLOS ONE* 10:e0139848. doi: org/10.1371/journal.pone.0139848

Weiss, S., Xu, Z. Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., et al. (2017). Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome* 5:27. doi: 10.1186/s40168-017-0237-y

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595–595. doi: 10.1890/14-1837.1

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLOS ONE* 9:e96375. doi: 10.1371/journal.pone.0096375

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2013). The SILVA and "all-species living tree project (LTP)" taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209

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Temporal Variation of the Skin Bacterial Community and *Batrachochytrium dendrobatidis* Infection in the Terrestrial Cryptic Frog *Philoria loveridgei*

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In animals and plants, symbiotic bacteria can play an important role in disease resistance of host and are the focus of much current research. Globally, amphibian population declines and extinctions have occurred due to chytridiomycosis, a skin disease caused by the pathogen *Batrachochytrium dendrobatidis* (Bd). Currently amphibian skin bacteria are increasingly recognized as important symbiont communities with a relevant role in the defense against pathogens, as some bacteria can inhibit the growth of *B. dendrobatidis*. This study aims to document the *B. dendrobatidis* infection status of wild populations of a terrestrial cryptic frog (*Philoria loveridgei*), and to determine whether infection status is correlated with changes in the skin microbial communities. Skin samples of *P. loveridgei* were collected along an altitudinal range within the species distribution in subtropical rainforests in southeast Australia. Sampling was conducted in two years during two breeding seasons with the first classified as a "La Niña" year. We used Taqman real-time PCR to determine *B. dendrobatidis* infection status and 16S amplicon sequencing techniques to describe the skin community structure. We found *B. dendrobatidis*-positive frogs only in the second sampling year with low infection intensities, and no correlation between *B. dendrobatidis* infection status and altitude, frog sex or size. Skin bacterial diversity was significantly higher in *P. loveridgei* frogs sampled in the 1st year than in the 2nd year. In addition, 7.4% of the total OTUs were significantly more abundant in the 1st year compared to the 2nd year. We identified 67 bacterial OTUs with a significant positive correlation between infection intensity and an OTU's relative abundance. Forty-five percent of these OTUs belonged to the family Enterobacteriaceae. Overall, temporal variation was strongly associated with changes in *B. dendrobatidis* infection status and bacterial community structure of wild populations of *P. loveridgei*.

Keywords: chytridiomycosis, skin bacteria, amphibians, *Philoria loveridge*, bacteria diversity

INTRODUCTION

Symbiotic relationships between bacteria and macro-organisms are ubiquitous in nature. The study of symbiotic interactions is a well-established field, yet recently has received increased attention due to the development of next generation sequencing technologies. Specifically, the role of beneficial bacterial communities has been the focus of research for wildlife and human hosts, as they can play important roles providing protection against pathogens (Harris et al., 2009; Jiménez and Sommer, 2016; Walke and Belden, 2016). In amphibians, some bacterial species on their skin inhibit the growth of pathogenic fungi (Harris et al., 2006; Becker et al., 2015; Woodhams et al., 2015) and have shown to play a protective role against chytridiomycosis, a deadly skin fungal disease (Harris et al., 2009; Becker and Harris, 2010; Holden et al., 2015).

Worldwide, many amphibian species are facing significant declines and extinctions attributable to chytridiomycosis (Collins and Storfer, 2003; Stuart et al., 2004; Wake and Vredenburg, 2008; Wake, 2012), the infectious diseases caused by *Batrachochytrium dendrobatidis* (Bd) and *B. salamandrivorans* (Bsal) (Berger et al., 1998; Longcore et al., 1999; Skerratt et al., 2007; Wake and Vredenburg, 2008; Martel et al., 2013). Interestingly, amphibian species exhibit differential susceptibility to Bd infections, which is reflected in the worldwide variation in mortality due to Bd and in Bd intensity and prevalence among species from different habitats, life histories and taxonomic affinities (reviewed in Skerratt et al., 2007; Kilpatrick et al., 2010). These variations are not completely understood, with research suggesting species-specific physiological and ecological traits, such as presence of symbiotic bacterial communities, differential immune response, production of antimicrobial peptides, behavioral patterns and environmental conditions (Kriger and Hero, 2008; Jiménez and Sommer, 2016; Rebollar et al., 2016a).

Given the beneficial role in disease resistance of symbiotic bacterial communities, recent attention has been given to the interaction occurring between amphibian skin microbiota and Bd. Recent evidence indicates that diversity and structure of skin bacterial communities is influenced by several factors such as species-specific traits (McKenzie et al., 2012; Rebollar et al., 2016b; Assis et al., 2017), developmental changes (Kueneman et al., 2014, 2016; Longo et al., 2015; Longo and Zamudio, 2017b), geographic location (Belden et al., 2015; Longo and Zamudio, 2017b; Muletz Wolz et al., 2017). Moreover, skin bacterial diversity is influenced by the microbial communities present in the environment (Fitzpatrick and Allison, 2014; Loudon et al., 2014, 2016; Walke et al., 2014; Rebollar et al., 2016b) as well as by pathogen infection (Jani and Briggs, 2014; Walke et al., 2015; Longo and Zamudio, 2017a).

It remains unclear how climatic conditions influence the composition of microbial communities on the amphibian skin (Kueneman et al., 2014; Jiménez and Sommer, 2016; Longo and Zamudio, 2017a; Medina et al., 2017; Muletz Wolz et al., 2017; Sabino-Pinto et al., 2017). However, in some amphibian species correlation between the variation in amphibian microbiota and abiotic factors (air temperature, water temperature, pH, conductivity, seasonality) has been reported (Kueneman et al., 2014; Krynak et al., 2015, 2016; Longo et al., 2015; Longo and Zamudio, 2017a; Muletz Wolz et al., 2017). For example, in *Lithobates yavapaiensis*, an increase in alpha diversity (Shannon index) of skin bacteria from summer to winter was found, as was a difference in community composition between seasons (Bray–Curtis dissimilarities) (Longo et al., 2015). Similarly, the bacterial composition of *Eleutherodactylus coqui* changed between seasons, potentially allowing frogs to limit Bd infection in the warm wet season (Longo and Zamudio, 2017a).

In addition to the potential role that climatic conditions and abiotic factors have on skin microbiota, Bd prevalence and infection has also been found to be influenced by these factors. Several environmental factors such as altitude, temperature and moisture have been associated with chytridiomycosis outbreaks (Berger et al., 2004; Drew et al., 2006; Lips et al., 2006; Kriger and Hero, 2007, 2008; Kriger et al., 2007; Longo et al., 2010). For example, several studies have shown a decrease in severity of infection at warmer temperatures and or dryer seasons (Retallick et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2007; Kriger et al., 2007; Rowley and Alford, 2013). Numerous studies suggest that chytridiomycosis presence, prevalence or intensity can be higher under cooler conditions (Berger et al., 2004; McDonald et al., 2005; Ouellet et al., 2005; Drew et al., 2006; Kriger and Hero, 2007; Kriger et al., 2007; Longo et al., 2010) and/or high elevation (Bosch et al., 2001; Burrowes et al., 2004; McDonald et al., 2005; Woodhams and Alford, 2005; Lips et al., 2006; Frías-Alvarez et al., 2008; Walker et al., 2010). Collectively, these studies support the hypothesis that amphibian populations declines associated with chytridiomycosis occur predominantly at high elevations and during cooler conditions. This pattern, along with the preference of Bd for relatively cool and humid conditions for growth in culture of approximately 23°C, suggests that environmental temperature and humidity are likely to be an important factor in disease dynamics (Muths et al., 2008).

Moreover, studies on Australian amphibian species have revealed that Bd infection is strongly associated with aquatic habitats (Kriger and Hero, 2007) and in frog species that hibernate in aquatic microhabitats rather than terrestrial (Skerratt et al., 2010). This pattern of aquatic amphibian species presenting higher infection and prevalence rates has also been reported among other amphibian species worldwide and has led researchers to hypothesize that terrestrial species may avoid Bd infection through microhabitat protection (Berger et al., 1998; Lips et al., 2006; Kriger and Hero, 2007; Brem and Lips, 2008; Catenazzi et al., 2011). Whilst Bd is more associated with aquatic habitats, terrestrial amphibians can also be infected with Bd, with some species experiencing population declines (Berger et al., 1998; Bell et al., 2004; Burrowes et al., 2004; Lips et al., 2006; Kriger and Hero, 2007; Brem and Lips, 2008; Longo and Burrowes, 2010; Catenazzi et al., 2011). To the best of our knowledge, only a few Australian terrestrial species have been reported with Bd infections. Negative Bd infection was reported on terrestrial Australian microhylids of the Wet Tropics (Hauselberger and Alford, 2012). However, Bd infection was reported for *Cophixalus ornatus* and *Assa darlingtoni* (Kriger and Hero, 2006, 2007). These limited and inconclusive results warrant further investigation of Bd suceptibility in Australian terrestrial

amphibians, and the role of skin microbiota on host-pathogen interactions.

Philoria loveridgei is a microendemic, diurnal terrestrial frog with terrestrial and nidicolous development to metamorphosis (embryos develop in the broken-down jelly capsules in a nest basin in mud, but they do not go to water) (Knowles et al., 2004; Anstis, 2013). This species is among the rarest vertebrates in eastern Australia, listed as vulnerable due to their habitat specialization and restricted mountaintop distribution (Hines et al., 1999; Knowles et al., 2004). Frogs from this fossorial species are small and highly cryptic making them difficult to find. Males construct shallow burrows under moist forest leaf litter or rocks, where they emit a soft mating call during breeding season to attract females. Inside this nest inguinal amplexus, egg laying and larval metamorphosis occur (Hines et al., 1999; Knowles et al., 2004; Anstis, 2013).

A few studies have addressed the role of climatic changes and temporal variation on host–pathogen interactions of amphibians including the role of skin microbiota and Bd (Jani and Briggs, 2014; Longo et al., 2015; Longo and Zamudio, 2017a; Medina et al., 2017; Muletz Wolz et al., 2017; Sabino-Pinto et al., 2017). However, temporal variation in these studies has been related to changes in seasons within the same year (Longo et al., 2015; Longo and Zamudio, 2017a; Muletz Wolz et al., 2017; Sabino-Pinto et al., 2017). Therefore, to better understand the influence skin microbiota has on host–pathogen interactions, we describe the relationship of the amphibian skin microbiota of wild populations and the climatic factors across two years. In this study, we hypothesized that climatic conditions would influence the extent of Bd infection as well as the structure of skin bacterial communities. To examine this hypothesis we documented Bd infection status and described skin microbial structure of wild populations of a terrestrial cryptic frog (*P. loveridgei*) during two years across their breeding season that showed clear differences in precipitation levels. We also determined whether infection status correlated with changes in the diversity and structure of skin microbial communities.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of a Scientific Purpose permit issued by the Department of Environment and Resource Management (WITK10308811) Queensland, Australia. Griffith University Animal Ethics Committee (ENV/21/12AEC) approved the protocol.

Field Sites

Field surveys were done in four national parks that make up part of the Tweed Caldera Rim within Gondwana Rainforests of Australia World Heritage Area, in mid-eastern Australia (**Figure 1**). This region is characterized by subtropical rainforest vegetation and is dominated by a moist subtropical climate. At altitudes above 800 m above sea level (asl) a cool high-altitude subtropical rainforest occurs as rainfall is significantly augmented by fog-drip. In these mountain ranges the monthly summer average temperatures range between a maximum of 24.1°C and a minimum of 19.7°C, while winter temperatures range from 17.8 to 12.3°C, correspondingly. Rainfall in this area is seasonal with 65–70% of the annual total (average annual rainfall >3000 mm) occurring in the summer months (McDonald, 2010).

Sample Collection

Surveys were conducted in two separate years during the frog species breeding season of each year (Australian spring and summer months) from November 2011 to January 2012 (here after year 1), and from November 2013 to January 2014 (here after year 2). Sample collection took place at nine sites covering the altitudinal and distribution ranges of *P. loveridgei*, including six sites in southeast Queensland (Springbrook and Lamington National Parks), and three sites in northeast New South Wales (Nightcap and Border Ranges National Parks). These sites were selected as they are highly occupied by the target species (MFL personal observation). The altitudes of the sites where frogs were encountered ranged from 680 to 1080 m asl (**Table 1**).

Sample collection consisted of microhabitat surveys and intensive burrow searches performed by carefully excavating under leaf litter or rocks at sites where calling males were heard, with a minimum target of 10 frogs per site. *P. loveridgei* frogs, particularly males, were found sitting inside burrows. Call mimicry was sometimes used to incite males to call, thereby aiding the discovery of their location. Frogs were captured with freezer plastic bags and kept individually, and two skin swabs samples were collected following standard published protocols for Bd first and skin bacteria second (Brem et al., 2007; Hyatt et al., 2007; Kriger and Hero, 2007; Vredenburg et al., 2010). Before swabbing, frogs were rinsed with sterile distill water to eliminate transient bacteria (Rebollar et al., 2014). Swabbing consisted of moving a swab across the frog's skin in a standardized way, using sterile rayon-tipped swabs (MW113; Lakewood Biochemical CO., United States), five strokes on each side of the abdominal midline, five strokes on the inner thighs of each hind leg, and five strokes on the foot webbing of each hind leg for a total of 30 strokes per frog (Kriger et al., 2006b; Vredenburg et al., 2010; Rebollar et al., 2014). Swabs were immediately placed in individually labeled microtubes and stored in a dry, cool place until transported back to the laboratory and stored at 4°C. To ensure consistency in swabbing technique all frogs were swabbed by MFL (Kriger et al., 2006b; Simpkins et al., 2014).

The morphological characteristics of all individuals, including body mass and size (snout-vent length, SVL), were recorded after skin samples were taken. All frogs were captured and handled at all times using individual, clean, unused freezer plastic bags. Additionally, all equipment used was washed with a solution of commercial bleach (1:9, bleach: water) and thoroughly cleaned between collection sites to prevent potential spread of the fungus pathogen (Phillott et al., 2010). Frog sampling was biased toward males as there sedentary calling behavior made them relatively more conspicuous than females. All burrows were individually marked to prevent resampling the same individual and to allow the release of individual frogs to their exact site of capture.

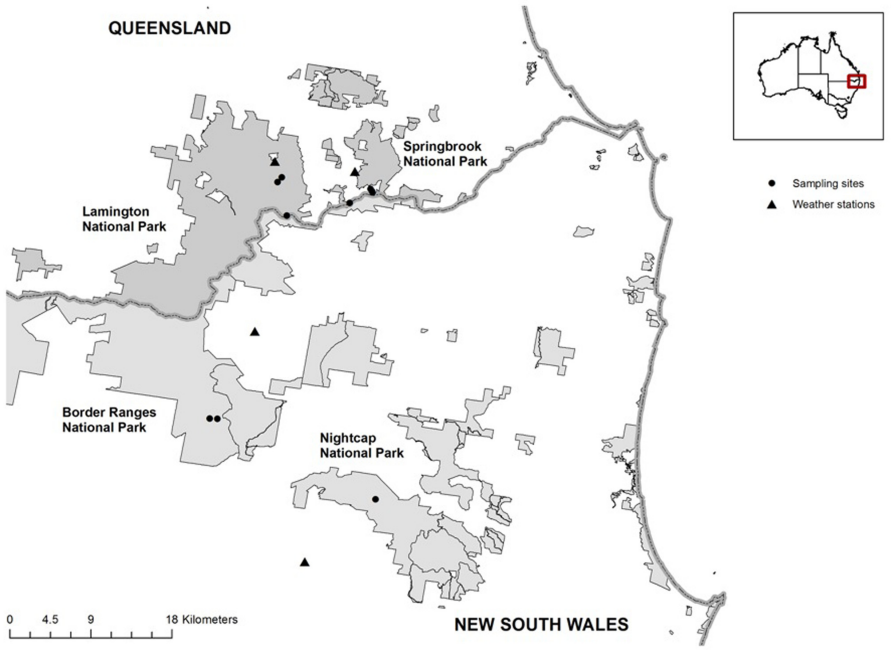

FIGURE 1 | Study area in south–east Queensland and north–east New South Wales National Parks, indicating *P. loveridgei* survey sites (dots) throughout the range of the species and the nearest Australian Bureau of Meteorology stations (triangles).

Molecular Methods and Data Analyses for Bd Analyses

Real-time quantitative PCR (qPCR) analyses of the frog swabs were used to test for Bd infection, prevalence and intensity. Swabs were analyzed at San Francisco State University (San Francisco, California, United States), using the protocol described by Boyle et al. (2004) with the following changes: swab extracts were analyzed singly instead of in triplicate since use of singletons and triplicates has been found to give similar results in previous studies (Kriger et al., 2006a; Vredenburg et al., 2010). In addition, BSA was added to the qPCR master mix (1 μL BSA per reaction) (Garland et al., 2010).

Prevalence of Bd was calculated as the number of frogs that were Bd positive (when zoospore equivalents were $\geq$0.1) relative to the total number of frogs sampled. Infection intensity was defined as the number of zoospore equivalents per swab. Zoospore equivalents were estimated by multiplying the qPCR genomic value by 80, as DNA extracts from swabs were diluted 80-fold during extraction and qPCR (Briggs et al., 2010; Vredenburg et al., 2010).

Spearman correlations were used to assess the relationship between altitude, body size, weight, and the intensity of Bd infection. Mann–Whitney *U* test was used to assess the relationship between sex of the frog and the intensity of Bd infection. Analyses were conducted using R statistical software (R Core Team, 2015) specifically using the function cor.test ("spearman") and wilcox.test().

Molecular Methods and Sequencing for Bacterial Analyses

Whole genomic DNA was extracted from 72 swab frog samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia,

TABLE 1 | Field surveys sites and dates of *P. loveridgei* across the species distribution range, across two years during the breeding seasons.

Site	National Park	Altitude (m)	Date sampled	Number of frogs
Bar Mt. picnic area	Border ranges	1083.1	November 2011; December 2013	13
Bar Mt.	Border ranges	977.1	December 2013; January 2014	13
Binna Burra 1	Lamington	892.8	November 2011	4
Binna Burra 2	Lamington	1020.6	November 2011; January 2012	4
Binna Burra 3	Lamington	859.9	November 2011	2
Mt. Nardi	Nightcap	683.4	December 2011; November and December 2013; January 2014	8
Best of all lookout	Springbrook	986.3	November and December 2011; November and December 2013	16
Bilborough 2	Springbrook	848.5	November 2011; December 2013; January 2014	7
Bilborough bridge	Springbrook	815.2	December 2013	5

CA, United States) following the manufacturer's instructions including a pretreatment with lysozyme included in the protocol titled: Pretreatment for Gram-Positive Bacteria (page 45). This pretreatment is recommended when trying to extract DNA from Gram-positive bacteria in addition to Gram-negative bacteria. DNA extracted from swabs was used to amplify the V4 region of the 16S rRNA gene using barcoded primers (F515/R806) and PCR conditions adapted from Caporaso et al. (2011). Amplicons were quantified using Quantifluor™ (Promega, Madison, WI, United States). Composite samples for sequencing were created by combining equimolar ratios of amplicons from the individual samples, followed by cleaning with the QIAquick PCR clean up kit (Qiagen, Valencia, CA, United States). Barcoded composite PCR products were sent to the Dana Farber Cancer Institute's Molecular Biology Core Facilities (Boston, MA, United States) for MiSeq Illumina sequencing using a 250 bp single read strategy.

16S Amplicon Data Processing

The 250 bp single reads were filtered and processed with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010b). Sequences were de-multiplexed and filtered to retain high quality reads using the following filtering parameters: no *N* characters were allowed in retained sequences, no errors in barcode sequence were allowed, a minimum of five high quality consecutive base pairs were needed to include a read, and a maximum of five consecutive low quality base pairs were allowed before truncating a read. After filtering, 5,991,855 sequences were retained for the 72 samples.

The de-multiplexed data set was deposited in the NCBI sequence read archive (SRA) with the accession number SRR5957156 which is linked to biosample SAMN07501977 and bioproject PRJNA398139.

De-multiplexed and filtered sequences were clustered into operational taxonomic units (OTUs) at a sequence similarity threshold of 97% with the UCLUST method (Edgar, 2010). Sequences were matched against the Greengenes database released in May 2013 (McDonald et al., 2012), and those that did not match were clustered as *de novo* OTUs at 97% sequence similarity. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) and the Greengenes database. Representative sequences were aligned to the Greengenes database with PyNAST (Caporaso et al., 2010a), and a ML phylogenetic tree was constructed with FastTree 2 (Price et al., 2010). The OTU table was filtered using a minimum cluster size of 0.001% of the total reads as suggested by Bokulich et al. (2013). Samples were rarefied according to the sample with lower number of reads (i.e., 25,605 reads). The final rarefied OTU table had 7,673 OTUs including a total of 1,843,560 reads.

Bacterial Skin Data Analysis and Statistics

To describe the diversity of host skin bacteria in *P. loveridgei* across two years in the breeding season Shannon diversity index was calculated for all 72 samples. ANOVA and *post hoc* Tukey's tests were carried out to determine differences between samples taken every month during the two years inbreeding seasons. Stacked bar charts of bacterial taxa at the class level were obtained to show the mean relative abundance of the 10 most abundant taxa on each month during the two years in the breeding seasons.

Weighted Unifrac and distance matrices were used to calculate the beta diversity and were visualized with a principal coordinates analysis. Differences in beta diversity between the two years samples and between Bd positive and negative samples were tested with non-parametric analysis of variance based on 999 permutations (PERMANOVA) using the software PRIMER-E (Clarke and Gorley, 2006). Alpha and beta diversity metrics and relative abundance comparisons at the class level were obtained using QIIME (Caporaso et al., 2010b). Analysis of multivariate homogeneity of group dispersions was calculated to determine whether skin communities from different breeding seasons had different dispersion values using the function betadisper in vegan package in R (Oksanen et al., 2015).

To determine the bacterial taxa that most likely explain differences between the two years, we used the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). Classes were defined as years 1 and 2, and subclasses corresponded to the months per breeding season. OTUs with LDA scores >2.0 were considered informative based on previous studies (Segata et al., 2011; Clemente et al., 2015; Rebollar et al., 2016a). Lefse was calculated using Galaxy (Afgan et al., 2016) implemented at the Huttenhower Lab[1].

To determine if Bd infection intensities were correlated with bacterial relative abundance across frog samples we calculated Spearman correlations with false discovery rate (Benjamini–Hochberg) to account for multiple comparisons using R (R Core Team, 2015) using the functions cor.test("spearman") and p.adjust("BH"). To perform this analysis we only included the data from year 2 since on year 1 Bd was not detected and the bacterial community was different than on year 2. Correlation values with $p < 0.05$ were considered significant. Wilcoxon Rank Sum Test in R (R Core Team, 2015), using the function wilcox.test(), was calculated to compare the relative abundance of bacterial taxa that had significant Spearman correlations between Bd-positive and Bd-negative samples.

RESULTS

Bd Infection Status of *Philoria loveridgei*

We detected Bd infection in seven individuals of 72 *P. loveridgei* tested (8 females and 64 males), with an overall prevalence of 9.7% ($n = 7$, 6 males and 1 female), across the two years during the breeding season (year 1: 2011/2012 and year 2: 2013/2014). The seven frogs infected were all sampled in year 2 ($n = 55$) while year 1 yielded none ($n = 17$). The pathogen was found only at two of the nine sites surveyed, both located in the southern limits of *P. loveridgei's* distribution range in New South Wales (**Figure 1**). The majority of infected frogs ($n = 6$) were from one site located in Border Ranges National Park (Bar Mt area), with a site infection prevalence of 35.3%. The only other Bd infected frog was from Nightcap National Park, with a site prevalence

[1]https://huttenhower.sph.harvard.edu/galaxy/

TABLE 2 | Disease prevalence and infection loads (Bd zoospore equivalents) of individual *P. loveridgei* per site and per year sampled (year 1 = 2011/2012 and year 2 = 2013/2014).

Site	Number of frogs (infected)	Year 1 Bd prevalence	Number of frogs (infected)	Year 2 Bd prevalence	Infection loads
Bar Mt. picnic area	1 (0)	0	12 (0)	0	0
Bar Mt.	0 (0)	0	**13 (6)**	**35.3%**	**19.1 ± 28.6**
Binna Burra 1	4 (0)	0	0 (0)	0	0
Binna Burra 2	4 (0)	0	0 (0)	0	0
Binna Burra 3	2 (0)	0	0 (0)	0	0
Mt. Nardi	1 (0)	0	**7 (1)**	**14.3%**	**124.8**
Best of all lookout	4 (0)	0	12 (0)	0	0
Bilborough 2	1 (0)	0	6 (0)	0	0
Bilborough bridge	0 (0)	0	5 (0)	0	0

Sites where Bd infection was detected are highlighted in bold.

of 12.5% (**Table 2**). These sites have a difference in altitude of 300 m asl; the Nightcap site was the lowest area surveyed in this study. Altitude, however, was not significantly correlated with Bd infection ($\rho = -0.126$, $P = 0.290$, $n = 72$).

We found that overall, infection intensity was relatively low with an average of 34.2 ± 47.7 zoospore equivalents (mean ± standard deviation), ranging from as low as 0.79 to 124.8 zoospore equivalents per individual. The frog presenting the highest infection intensity (124.8 zoospore equivalents) was from the Nightcap site (**Table 2**).

Male frogs weighed an average 2.2 ± 0.7 g and had a mean SVL of 25.6 ± 0.3 mm (mean ± standard deviation). As expected, female frogs were bigger than male frogs weighing an average 2.8 ± 0.6 g and had a mean SVL of 28.8 ± 0.2 mm. No association was found between sex of the frog and infection status (Mann–Whitney $U = 250$, $P = 0.848$, $n = 72$). Additionally, size and weight of frogs were not significantly correlated with Bd intensity (size; $\rho = 0.130$, $P = 0.278$, $n = 72$; weight; $\rho = -0.082$, $P = 0.496$, $n = 72$). No clinical signs of chytridiomycosis were observed in any of the frogs surveyed (Berger et al., 1998, 2005).

Temporal Variation of the Skin Bacterial Community Structure of ylum Proteobacteria was the most a

A total of 7,673 OTUs were identified from 72 frog samples collected during three months across two years in the breeding season. Overall, phylum Proteobacteria was the most abundant (49.9%) across all samples, followed by Actinobacteria (13.37%), Acidobacteria (7.71%), Verrucomicrobia (4.74%), and Planctomycetes (3.16%).

According to Shannon index estimates, skin bacterial alpha diversity was significantly higher in *P. loveridgei* skin samples from year 1 in comparison to year 2 [**Figure 2A**; ANOVA $F(5,66) = 8.215$, $p = 4.46 \times 10^{-6}$]. These diversity values indicate significant differences in bacterial community richness and relative abundance between months of different years but not between months within the same year.

Specifically, at the class level, Alphaproteobacteria were more abundant in year 1 while Gammaproteobacteria were more abundant in year 2 (**Figure 2B**). Beta diversity showed significant differences between these two years as seen with Bray–Curtis distances [Adonis test: $F(1,71) = 5.8841$, $p = 0.001$]. In addition, beta disperse analysis indicated a greater variance among samples from the 2nd year [**Figures 3A,B**; ANOVA $F(1,70) = 5.982$, $p = 0.016$].

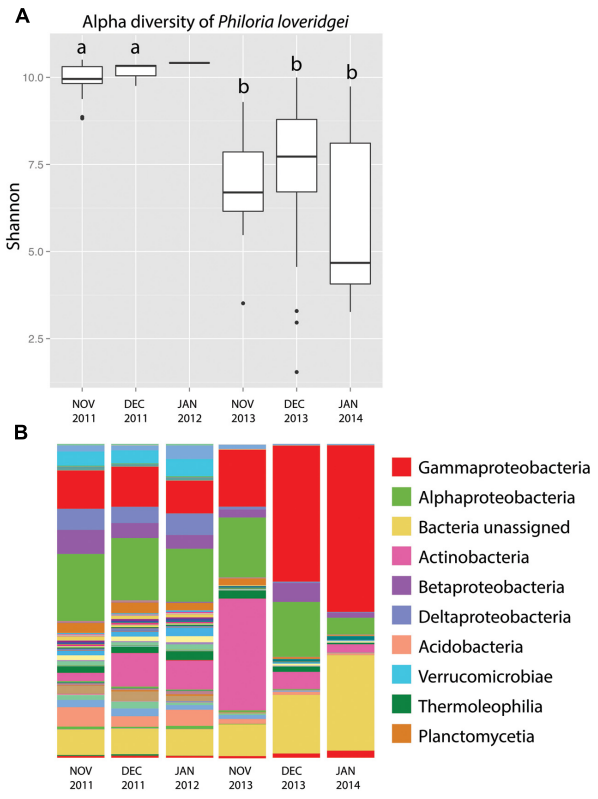

FIGURE 2 | Skin bacterial community structure of *P. loveridgei* across time in two breeding seasons. **(A)** Alpha diversity (Shannon) of *P. loveridgei* [ANOVA $F(5,66) = 8.215$, $P = 4.46 \times 10^{-6}$]. Different letters (a and b) signify statistically significant differences across months, as indicated by a Tukey's *post hoc* test. **(B)** Stacked bar chart of mean relative abundances of bacterial taxa (class level) of *P. loveridgei*. Colored bars (legend) indicate the 10 most abundant taxa.

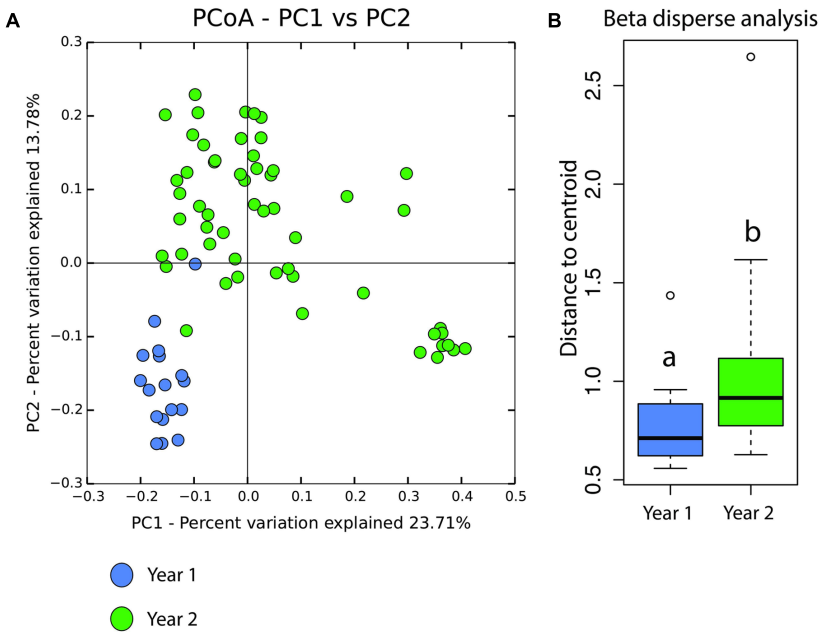

FIGURE 3 | (A) Beta diversity of *P. loveridgei* between year 1 (blue) and year 2 (green). Principal coordinate analysis is based on weighted Unifrac distances [ADONIS $F(1,71) = 5.8841$, $P = 0.001$]. **(B)** Analysis of multivariate homogeneity of group dispersions (variance) of *P. loveridgei* skin microbial communities. Box plot of distances to each centroid's group. Different letters (a and b) signify statistically significant differences between year 1 and year 2 [ANOVA $F(1,70) = 5.9827$, $P = 0.01696$].

With the aim to determine which OTUs significantly differed between years, a LEfse analysis was performed. This analysis determined that 569 out of 7673 OTUs (7.4% of the total OTUs) were significantly different and more abundant in year 1. In contrast, in year 2, only seven OTUs from the classes Alphaproteobacteria, Gammaproteobacteria, and Bacilli had a higher relative abundance (Supplementary Table 1).

Climatic Differences between Years

Differences between years 1 and 2 may be caused by several factors, but we found that rainfall patterns varied significantly between the two breeding seasons evaluated in this study (**Figure 4**). Specifically December and January, the rainy month of the year, showed significantly higher mean monthly rainfall in year 1 when compared to year 2 (Dec: $\rho = 0.024$; Jan: $\rho = 0.024$) (**Figure 4**). The 1st year being a "La Niña" year, which was characterized by heavy summer rainfalls, compared to a neutral phase year for the 2nd year. Furthermore, the "La Niña" year of the 1st year of the study (2011) followed a stronger "La Niña" event of 2010, bringing the highest two years of Australian rainfall on record, including heavy rainfall at the study area. Additionally, 2010 and 2011 were the coolest years recorded since 2001 due to the two consecutive "La Niña" events (CSIRO, 2012). Year 2 was a neutral year and Australia's warmest year on record, with widespread warmth throughout the year, and below average rainfall. Particularly, summer rainfall at the study area was in the lowest 10% of records, with some small areas recording their lowest summer rainfall on record. Additionally, year 2 (2013) was preceded by 2012 climatic patterns which reflected a shift from a "La Niña" year to a neutral year, but warmer than average (CSIRO, 2014).

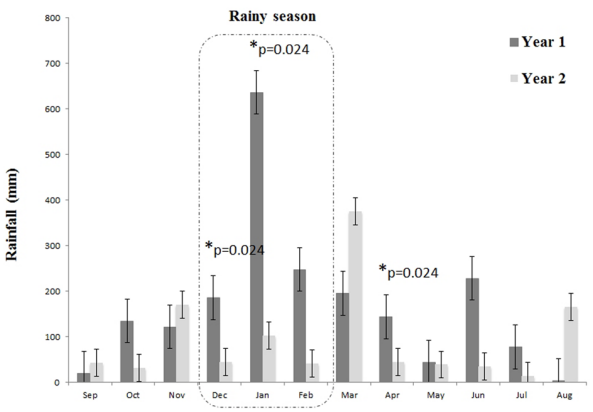

FIGURE 4 | Rainfall levels during the two study years including the breeding seasons were *P. loveridgei* frogs were surveyed. Asterisks indicate months were year 1 was significantly higher than year 2 according to *t*-test.

Correlation between Bd Infection Intensity and Skin Bacterial Diversity in Two Breeding Seasons

To determine the correlation between Bd infection status and the skin bacterial community structure we only analyzed the data from year 2, as Bd was not detected in year 1 and

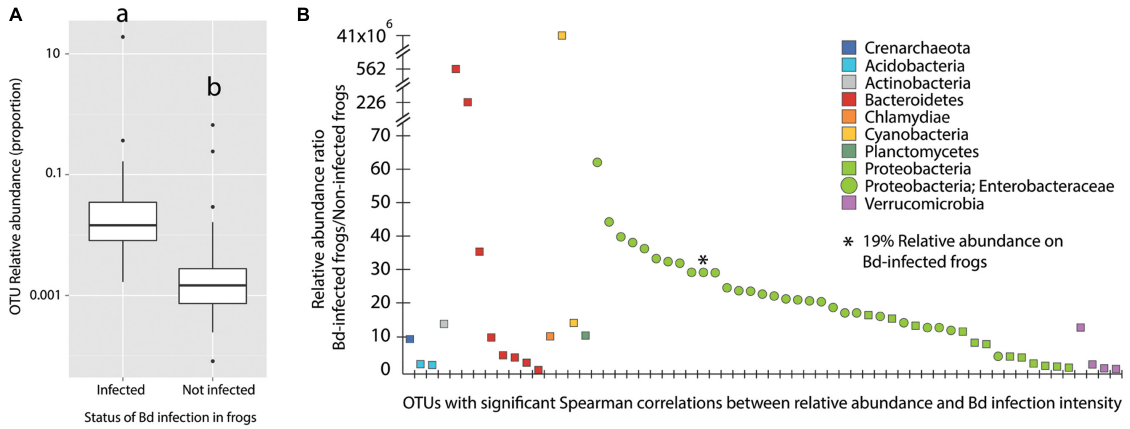

FIGURE 5 | (A) Relative abundance of 67 bacterial OTUs with a significant Spearman correlation (Bd loads vs. OTUs relative abundance) on Bd-infected individuals in comparison to non-infected individuals. Different letters (a and b) signify statistically significant differences, as indicated by a Wilcoxon rank sum test (W = 4102, p-value < $2.2e^{-16}$). **(B)** Relative abundance ratio (Bd-infected frogs/Non-infected frogs) of OTUs with a significant Spearman correlation. Y-axis was adapted to fit the highest values.

there were significant differences on the bacterial communities between years 1 and 2. We found no significant differences between Bd status (infected/not infected) based on bacterial alpha [Supplementary Figure 1A; ANOVA $F(1,70) = 2.579$, $p = 0.113$] and beta diversity estimates [Supplementary Figure 1B; Adonis test: $F(1,71) = 1.707$, $p = 0.07$]. However, when analyzing Bd infection loads we found positive significant Spearman correlations between Bd infection intensity and the relative abundance of 67 bacterial OTUs (Supplementary Table 2). These OTUs had a higher relative abundance in infected individuals than in non-infected individuals (**Figure 5A**) and Wilcoxon rank sum test indicated significant differences between these two groups ($W = 4102$, $p < 2.2 \times 10^{-16}$). OTUs with a higher relative abundance in Bd-infected individuals were classified in eight different bacterial phyla and one archaeal phylum (**Figure 5B**). Moreover, of the 67 OTUs, 41 (63%) were from the phylum Proteobacteria and 29 of these OTUs (45%) were from the family Enterobacteriaceae. Interestingly, one specific OTU from the family Enterobacteriaceae accounted for 19% of the relative abundance in Bd-infected frogs (**Figure 5B**).

DISCUSSION

In this study, we found differences on Bd status and skin microbial communities on *P. loveridgei* populations across the two years in the breeding season. Overall, even though sampling sites differed on altitude, temporal variation across the two breeding seasons was the factor that better explained the changes in Bd infection status and bacterial community structure of wild populations of *P. loveridgei*. Importantly, these two breeding seasons differed dramatically on their climatic conditions, with the 1st year being part of "la Niña year" characterized by high precipitation levels.

We found Bd in two wild populations of *P. loveridgei* at low (683 m) and mid-high (977 m) altitude subtropical rainforest sites in mid-eastern Australia in only one year of the two that were surveyed. Our results indicate low infection intensity and low disease prevalence in this frog species, however, we still lack knowledge on the impact that this disease may have on *P. loveridgei* wild populations. Our findings of low infection intensity are in agreement with those observed in two other Australian terrestrial species *C. ornatus* and *A. darlingtoni*, where Bd infection was also relatively low (31 and 189 zoospore equivalents, respectively) (Kriger and Hero, 2006, 2007). *B. dendrobatidis* presence and infection intensity on *P. loveridgei* had not been reported before, yet our study suggests that *P. loveridgei* populations are likely to be exposed to the pathogen throughout their altitudinal range. Moreover, some of the subtropical rainforest areas and lowlands that bordered our sites were previously known to have undergone disease outbreaks, with various stream breeding frog species found to be infected with Bd (Berger et al., 2004; Kriger and Hero, 2008; Narayan et al., 2014). Furthermore, disease dynamics in *P. loveridgei* may depend on additional variables associated to changes in climatic conditions, such as temperature and precipitation as reported for other species (Longo et al., 2010). For example, prevalence was related to annual environmental changes in *Litoria wilcoxii* (a stream breeding frog), with higher prevalence levels in winter and early spring months (Kriger and Hero, 2008). Similarly, a multi-species study of wild frogs from Queensland and New South Wales reported a higher incidence of chytridiomycosis during winter months compared to other seasons of the year, when mean temperatures are low and in the optimal growth range of Bd compared with summer temperatures which are higher and can restrict Bd (Berger et al., 2004). Interestingly, a study on wild populations of two direct-developing frogs, *E. coqui* and *E. portoricensis*, found an association between Bd prevalence and infection intensity and seasons (Longo et al., 2010). The authors proposed a lower infection intensity in the wet and warm season and a higher in the dry and cool, as a result of the climatic

differences but also of differential behavioral patterns (Longo et al., 2010).

The Bd infection intensity and prevalence patterns we observed in *P. loveridgei* suggest an enzootic state between the host and the pathogen. Despite finding populations of *P. loveridgei* infected with Bd, we found no clinical signs of chytridiomycosis or death related to this illness, nor have they been reported elsewhere. Additionally, no population declines related to Bd have been documented for any Australian direct-developing amphibian species (Hines et al., 1999; Hero et al., 2006; Hauselberger and Alford, 2012) in contrast with reports from other parts of the world (Bell et al., 2004; Burrowes et al., 2004; Lips et al., 2006; Longo and Burrowes, 2010; Longo et al., 2013). These results imply that Australian direct-developing amphibian species are surviving in an enzootic state with Bd perhaps through an innate immune response that could be providing them with protection against Bd, or by harboring beneficial skin microbiota. Brem and Lips (2008) suggest that terrestrial amphibians may share similar skin microbial fauna that provides them with protection to Bd and in turn make them less susceptible as a group. A study of four Australian frogs showed higher Bd resistance in species with powerful skin peptide defenses when experimentally infected with Bd (Woodhams et al., 2007b). Other studies have shown the inhibitory role of skin bacteria in the growth of Bd (Harris et al., 2006, 2009; Woodhams et al., 2007a; Walke et al., 2011). In wild *P. loveridgei* populations, higher Bd infection intensity may be found in seasons of the year we could not sample (e.g., cool and dry winter). Individuals of this fossorial cryptic frog species remain in their underground burrows during non-breeding months, making sampling extremely difficult. Because high Bd infection intensity implies susceptibility to chytridiomycosis (Vredenburg et al., 2010), the low level we discovered in this frog species could suggest resistance or tolerance to the infection, due to a more effective immune system, antimicrobial skin peptides or the presence of skin microbiota (Harris et al., 2009; Rollins-Smith, 2009).

We found differences in skin bacterial community structure across the two years in the breeding seasons. A high diversity of bacteria was found during the rainy and cool year (year 1: 2011), which was characterized by a higher abundance of Alphaproteobacteria. This was followed by an overall decrease on diversity during year 2 (2013) and the increase in abundance of Gammaproteobacteria and Actinobacteria. Interestingly, the 2nd year was the only time where Bd was detected on these frogs. The decrease of bacterial diversity has been correlated with the presence of Bd in previous field studies (Jani and Briggs, 2014; Rebollar et al., 2016b; Longo and Zamudio, 2017b). In *Rana sierrae* wild populations, Bd infection was strongly correlated with bacterial community composition with several taxa decreasing in abundance (Betaproteobacteria, Gammaproteobacteria, Actinobacteria and also a few members from the Acidobacteria and Alphaproteobacteria) (Jani and Briggs, 2014). Similarly, on a field survey of the terrestrial Panamanian frog *Craugastor fitzingeri*, Bd presence seemed to influence skin bacterial community structure with the Bd-naïve site dominated by OTUs from the genus *Acinetobacter* (Gammaproteobacteria) compared to the Bd-endemic sites dominated by *Pseudomonas* (Gammaproteobacteria), *Cellulomonas* and *Sanguibacter* (both Actinobacteria) (Rebollar et al., 2016b). Proteobacteria were also the most abundant phyla in skin samples of *R. sphenocephala* (Holden et al., 2015). Interestingly, this study found isolates of Proteobacteria and Actinobacteria that are able to inhibit the growth of Bd.

The changes in microbial diversity observed between breeding seasons may be due to several factors including climatic fluctuations (precipitation and temperature) as seen in previous studies (Longo and Zamudio, 2017a,b; Muletz Wolz et al., 2017; Sabino-Pinto et al., 2017). Climatic conditions may in turn influence host behavior, host susceptibility to pathogens, as well as Bd infection capacity (Longo et al., 2010; Longo and Zamudio, 2017a,b). For example, aggregation behavior was observed in two direct-developing frogs, *E. coqui* and *E. portoricensis*, during the dry season, while dispersion of the host occurred during the wet season (Longo et al., 2010). Heavily infected terrestrial frogs may remain inactive and hidden in fossorial retreats under dry and cool conditions, hindering their detection. In contrast, during warm and wet seasons frogs have lower infection levels and are more active in the forest, increasing the probability of sampling an infected frog and yielding higher prevalence estimates (Longo et al., 2010). Furthermore, climatic conditions can also lead to changes in microbial communities and in turn Bd infection and prevalence (Longo and Zamudio, 2017a). In another study of the two direct-developing frogs, Bd infection may have been limited during the warm and wet season by recruitment of putatively beneficial bacteria followed by a return to pre-infected levels of bacteria richness and diversity was observed (Longo and Zamudio, 2017a). Additionally, during the cool and dry season Bd infection increased through time and bacterial diversity did not change (Longo and Zamudio, 2017a). Overall, host-pathogen interactions may be strongly influenced by climatic fluctuations as seen in previous studies. Thus, we hypothesize that changes in climatic conditions between the two years in the breeding seasons that we surveyed could explain the presence of Bd and the decrease of skin microbial diversity. Thus, climatic changes may directly influence the skin bacteria community as well as the success of Bd to infect host, but also there might be an interaction occurring between the skin symbiotic community structure and the presence of the pathogen on the skin. However, it is important to consider that, besides climate, our results could be explained by other factors that we did not consider in this study.

Our study identified a group of 67 bacterial taxa that were enriched on the frogs from year 2, which were characterized by harboring higher Bd loads (significant positive Spearman correlations). A great proportion of these OTUs belonged specifically to the family Enterobacteriaceae. Interestingly, a data base for culturable Bd inhibitory bacteria published by Woodhams et al. (2015) showed that 94% of the culturable bacteria from this family inhibit Bd growth. Our field survey suggests that the increase in the abundance of Enterobacteriaceae OTUs might be related to Bd infection, e.g., these bacteria may be recruited to inhibit the pathogen or may be a consequence of Bd infection on the skin. To further test

this hypothesis additional experimental studies will need to be performed.

Overall, our study contributes on the understanding of the role of temporal variation on Bd infection and skin microbial community structure. However, in order to fully determine an effect of the climatic conditions on the skin microbial structure and pathogen infection, a long term study is essential. This study supports previous findings showing that Bd estimates and microbial community description are not static and therefore require multiple temporal and spatial samplings to fully understand the ecological interactions occurring between the host, the pathogen and bacterial symbiotic communities.

AUTHOR CONTRIBUTIONS

MFL contributed to the conception of the idea and design of the research, and carried out the fieldwork. MFL, ER, and VV contributed with laboratory analysis. MFL and ER analyzed the data with advise from RH and J-MH. All authors contributed with the interpretation of the data. MFL and ER wrote the manuscript and all authors provided critical feedback.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02535/full#supplementary-material

REFERENCES

Afgan, E., Baker, D., Van den Beek, M., Blankenberg, D., Bouvier, D., Èech, M., et al. (2016). The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. *Nucleic Acids Res* 44, W3–W10. doi: 10.1093/nar/gkw343

Anstis, M. (2013). *Tadpoles and Frogs of Australia*. Sydney, NSW: New Holland Publisher.

Assis, A. B., Barreto, C. C., and Navas, C. A. (2017). Skin microbiota in frogs from the Brazilian Atlantic forest: species, forest type, and potential against pathogens. *PLOS ONE* 12:e0179628. doi: 10.1371/journal.pone.0179628

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLOS ONE* 5:e10957. doi: 10.1371/journal.pone.0010957

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. Lond. B Biol. Sci.* 282:20142881. doi: 10.1098/rspb.2014.2881

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bell, B. D., Carver, S., Mitchell, N. J., and Pledger, S. (2004). The recent decline of a New Zealand endemic: how and why did populations of Archey's frog Leiopelma archeyi crash over 1996–2001? *Biol. Conserv.* 120, 189–199. doi: 10.1016/j.biocon.2004.02.011

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Berger, L., Speare, R., Hines, H., Marantelli, G., Hyatt, A., McDonald, K. R., et al. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.* 82, 434–439. doi: 10.1111/j.1751-0813.2004.tb11137.x

Berger, L., Spear, R., and Skerratt, L. F. (2005). Distribution of *Batrachochytrium dendrobatidis* and pathology in the skin of green tree frogs *Litoria caerulea* with severe chytridiomycosis. *Dis. Aquat. Organ.* 68, 65–70. doi: 10.3354/dao068065

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bosch, J., Martìnez-Solano, I., and Garcìa-Parìs, M. (2001). Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biol. Conserv.* 97, 331–337. doi: 10.1016/s0006-3207(00)00132-4

Boyle, D., Boyle, D., Olsen, V., Morgan, J., and Hyatt, A. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Brem, F., Mendelson, J. R. III., and Lips, K. R. (2007). *Field-Sampling Protocol for Batrachochytrium dendrobatidis from Living Amphibians, Using Alcohol Preserved Swabs. Version 1.0*. Arlington, VA: Conservation International.

Brem, F. M. R., and Lips, K. R. (2008). *Batrachochytrium dendrobatidis* infection patterns among Panamanian amphibian species, habitats and elevations during epizootic and enzootic stages. *Dis. Aquat. Organ.* 81, 189–202. doi: 10.3354/dao01960

Briggs, C. J., Knapp, R. A., and Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9695–9700. doi: 10.1073/pnas.0912886107

Burrowes, P. A., Joglar, R. L., and Green, D. E. (2004). Potential causes for amphibian declines in puerto rico. *Herpetologica* 60, 141–154. doi: 10.1655/03-50

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4516–4522. doi: 10.1073/pnas.1000080107

Catenazzi, A., Lehr, E., Rodriguez, L., and Vredenburg, V. (2011). *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. *Conserv. Biol.* 25, 382–391. doi: 10.1111/j.1523-1739.2010.01604.x

Clarke, K., and Gorley, R. (2006). *PRIMER v6: User Manual/Tutorial*. Plymouth: PRIMER-E.

Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., et al. (2015). The microbiome of uncontacted Amerindians. *Sci. Adv.* 1:e1500183. doi: 10.1126/sciadv.1500183

Collins, J. P., and Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. *Div. Distrib.* 9, 89–98. doi: 10.1046/j.1472-4642.2003.00012.x

CSIRO (2012). *State of the Climate 2012*. Melbourne, VIC: Australian Government Bureau of Meteorology.

CSIRO (2014). *State of the Climate 2014*. Melbourne, VIC: Australian Government Bureau of Meteorology.

Drew, A., Allen, E. J., and Allen, L. J. S. (2006). Analysis of climatic and geographic factors affecting the presence of chytridiomycosis in Australia. *Dis. Aquat. Organ.* 68, 245–250. doi: 10.3354/dao068245

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fitzpatrick, B. M., and Allison, A. L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiol. Ecol.* 88, 482–494. doi: 10.1111/1574-6941.12314

Frías-Alvarez, P., Vredenburg, V. T., Familiar-López, M., Longcore, J. E., González-Bernal, E., Santos-Barrera, G., et al. (2008). Chytridiomycosis survey in wild and captive Mexican amphibians. *EcoHealth* 5, 18–26. doi: 10.1007/s10393-008-0155-3

Garland, S., Baker, A., Phillott, A. D., and Skerratt, L. F. (2010). BSA reduces inhibition in a TaqMan assay for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 92, 113–116. doi: 10.3354/dao02053

Harris, R., James, T., Lauer, A., Simon, M., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Hauselberger, K. F., and Alford, R. A. (2012). Prevalence of *Batrachochytrium dendrobatidis* infection is extremely low in direct-developing Australian microhylids. *Dis. Aquat. Organ.* 100, 191–200. doi: 10.3354/dao02494

Hero, J.-M., Morrison, C., Gillespie, G., Roberts, J. D., Newell, D., Meyer, E., et al. (2006). Overview of the conservation status of Australian frogs. *Pac. Conserv. Biol.* 12:313. doi: 10.1071/PC060313

Hines, H., Mahony, M., and McDonald, K. (1999). "An assessment of frog declines in wet subtropical Australia," in *Declines and Disappearances of Australian Frogs*, ed. A. Campbell (Canberra, ACT: Environment Australia), 44–63.

Holden, W. M., Hanlon, S. M., Woodhams, D. C., Chappell, T. M., Wells, H. L., Glisson, S. M., et al. (2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (Rana sphenocephala) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 187, 91–102. doi: 10.1016/j.biocon.2015.04.007

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., et al. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 73, 175–192. doi: 10.3354/dao073175

Jani, A. J., and Briggs, C. J. (2014). The pathogen Batrachochytrium dendrobatidis disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jiménez, R. R., and Sommer, S. (2016). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodiver. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol. Evolut.* 25, 109–118. doi: 10.1016/j.tree.2009.07.011

Knowles, R., Mahony, M., Armstrong, J., and Donnellan, S. (2004). Systematics of sphagnum frogs of the Genus *Philoria* (Anura: Myobatrachidae) in Eastern Australia, with the description of two new species. *Rec. Aust. Mus.* 56, 57–74. doi: 10.3853/j.0067-1975.56.2004.1391

Kriger, K. M., and Hero, J.-M. (2006). *Cophixalus ornatus* (Ornate Nursery Frog) chytridiomycosis. *Herpetol. Rev.* 37:443.

Kriger, K. M., and Hero, J.-M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *J. Zool.* 271, 352–359. doi: 10.1111/j.1469-7998.2006.00220.x

Kriger, K. M., and Hero, J.-M. (2008). Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. *Austral. Ecol.* 33, 1022–1032. doi: 10.1111/j.1442-9993.2008.01872.x

Kriger, K. M., Hero, J.-M., and Ashton, K. J. (2006a). Cost efficiency in the detection of chytridiomycosis using PCR assay. *Dis. Aquat. Organ.* 71, 149–154.

Kriger, K. M., Hines, H., Hyatt, A. D., Boyle, D. G., and Hero, J.-M. (2006b). Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Dis. Aquat. Organ.* 71, 141–148.

Kriger, K. M., Pereoglou, F., and Hero, J.-M. (2007). Latitudinal variation in the prevalence and intensity of chytrid (*Batrachochytrium dendrobatidis*) infection in eastern Australia. *Conserv. Biol.* 21, 1280–1290. doi: 10.1111/j.1523-1739.2007.00777.x

Krynak, K. L., Burke, D. J., and Benard, M. F. (2015). Larval environment alters amphibian immune defenses differentially across life stages and populations. *PLOS ONE* 10:e0130383. doi: 10.1371/journal.pone.0130383

Krynak, K. L., Burke, D. J., and Benard, M. F. (2016). Landscape and water characteristics correlate with immune defense traits across Blanchard's cricket frog (*Acris blanchardi*) populations. *Biol. Conserv.* 193, 153–167. doi: 10.1016/j.biocon.2015.11.019

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and McKenzie, V. J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *Int. Soc. Microb. Ecol. J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Longcore, J. E., Pessier, A. P., and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91, 219–227. doi: 10.2307/3761366

Longo, A. V., and Burrowes, P. A. (2010). Persistence with chytridiomycosis does not assure survival of direct-developing frogs. *Ecohealth* 7, 185–195. doi: 10.1007/s10393-010-0327-9

Longo, A. V., Burrowes, P. A., and Joglar, R. L. (2010). Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests a mechanism for persistence. *Dis. Aquat. Organ.* 92, 253–260. doi: 10.3354/dao02054

Longo, A. V., Ossiboff, R. J., Zamudio, K. R., and Burrowes, P. A. (2013). Lability in host defenses: terrestrial frogs die from chytridiomycosis under enzootic conditions. *J. Wildl. Dis.* 49, 197–199. doi: 10.7589/2012-05-129

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Longo, A. V., and Zamudio, K. R. (2017a). Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. *Int. Soc. Microb. Ecol. J.* 11, 349–361. doi: 10.1038/ismej.2016.138

Longo, A. V., and Zamudio, K. R. (2017b). Temperature variation, bacterial diversity, and fungal infection dynamics in the amphibian skin. *Mol. Ecol.* 26, 4787–4797. doi: 10.1111/mec.14220

Loudon, A. H., Venkataraman, A., Van Treuren, W., Woodhams, D. C., Parfrey, L. W., McKenzie, V. J., et al. (2016). Vertebrate hosts as islands: dynamics of selection, immigration, loss, persistence, and potential function of bacteria on salamander skin. *Front. Microbiol.* 7:333. doi: 10.3389/fmicb.2016.00333

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *Int. Soc. Microb. Ecol. J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., et al. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc. Natl. Acad. Sci.* 110, 15325–15329. doi: 10.1073/pnas.1307356110

McDonald, B. (2010). "The tweed caldera group," in *Remnants of Gondwana: a Natural and Social History of the Gondwana Rainforests of Australia*, eds R.

Kitching, R. Braithwaite, and J. Cavanaugh (Chipping Norton, NSW: Surrey Beatty & Sons), 39–50.

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., et al. (2012). An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *Int. Soc. Microb. Ecol. J.* 6, 610–618. doi: 10.1038/ismej.2011.139

McDonald, K., Mendez, D., Müller, R., Freeman, A., and Speare, R. (2005). Decline in the prevalence of chytridiomycosis in frog populations in North Queensland, Australia. *Pac. Conserv. Biol.* 11, 114–120. doi: 10.1071/PC050114

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *Int. Soc. Microb. Ecol. J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Medina, D., Hughey, M. C., Becker, M. H., Walke, J. B., Umile, T. P., Burzynski, E. A., et al. (2017). Variation in metabolite profiles of amphibian skin bacterial communities across elevations in the Neotropics. *Microb. Ecol.* 74, 227–238. doi: 10.1007/s00248-017-0933-y

Muletz Wolz, C. R., Yarwood, S. A., Grant, E. H. C., Fleischer, R. C., and Lips, K. R. (2017). Effects of host species and environment on the skin microbiome of Plethodontid salamanders. *J. Anim. Ecol.* doi: 10.1111/1365-2656.12726 [Epub ahead of print].

Muths, E., Pilliod, D. S., and Livo, L. J. (2008). Distribution and environmental limitations of an amphibian pathogen in the Rocky Mountains, USA. *Biol. Conserv.* 141, 1484–1492. doi: 10.1016/j.biocon.2008.03.011

Narayan, E. J., Graham, C., McCallum, H., and Hero, J.-M. (2014). Over-wintering tadpoles of *Mixophyes fasciolatus* act as reservoir host for *Batrachochytrium dendrobatidis*. *PLOS ONE* 9:e92499. doi: 10.1371/journal.pone.0092499

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., et al. (2015). *vegan: Community Ecology Package, R Package Version 2.2*–1. Available at: https://github.com/vegandevs/vegan

Ouellet, M., Mikaelian, I., Pauli, B. D., Rodrigue, J., and Green, D. M. (2005). Historical evidence of widespread chytrid infection in North American amphibian populations. *Conserv. Biol.* 19, 1431–1440. doi: 10.1111/j.1523-1739.2005.00108.x

Phillott, A. D., Speare, R., Hines, H. B., Skerratt, L. F., Meyer, E., McDonald, K. R., et al. (2010). Minimising exposure of amphibians to pathogens during field studies. *Dis. Aquat. Organ.* 92, 175–185. doi: 10.3354/dao02162

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2–approximately maximum-likelihood trees for large alignments. *PLOS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Availabel at: http://www.R-project.org/

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "Omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Harris, R. N., Domangue, R. J., Medina, D., Ibáñez, R., et al. (2014). The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of far eastern Panamá. *PLOS ONE* 9:e95484. doi: 10.1371/journal.pone.0095484

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Retallick, R. W. R., McCallum, H., and Speare, R. (2004). Endemic infection of the amphibian chytrid fungus in a frog community post-decline. *PLOS Biol.* 2:e351 doi: 10.1371/journal.pbio.0020351

Rollins-Smith, L. A. (2009). The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochim. Biophys. Acta* 1788, 1593–1599. doi: 10.1016/j.bbamem.2009.03.008

Rowley, J. J. L., and Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Sci. Rep.* 3:1515. doi: 10.1038/srep01515

Sabino-Pinto, J., Galán, P., Rodríguez, S., Bletz, M. C., Bhuju, S., Geffers, R., et al. (2017). Temporal changes in cutaneous bacterial communities of terrestrial- and aquatic-phase newts (Amphibia). *Environ. Microbiol.* 19, 3025–3038. doi: 10.1111/1462-2920.13762

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Simpkins, C., Van Sluys, M., and Hero, J.-M. (2014). Swabber effect: swabbing technique affects the detectability of *Batrachochytrium dendrobatidis*. *Herpetol. Rev.* 45, 443–445.

Skerratt, L., Berger, L., Speare, R., Cashins, S., McDonald, K., Phillott, A., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* 4, 125–134. doi: 10.1007/s10393-007-0093-5

Skerratt, L., McDonald, K., Hines, H., Berger, L., Mendez, D., Phillott, A., et al. (2010). Application of the survey protocol for chytridiomycosis to Queensland, Australia. *Dis. Aquat. Organ.* 92, 117–129. doi: 10.3354/dao02272

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9689–9694. doi: 10.1073/pnas.0914111107

Wake, D. B. (2012). Facing extinction in real time. *Science* 335, 1052–1053. doi: 10.1126/science.1218364

Wake, D. B., and Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 105(Suppl. 1), 11466–11473. doi: 10.1073/pnas.0801921105

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *Int. Soc. Microb. Ecol. J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLOS ONE* 10:e0139848. doi: 10.1371/journal.pone.0139848

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLOS Pathog.* 12:e1005796. doi: 10.1371/journal.ppat.1005796

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A., and Woodhams, D. C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–400. doi: 10.1111/j.1744-7429.2011.00787.x

Walker, S. F., Bosch, J., Gomez, V., Garner, T. W., Cunningham, A. A., Schmeller, D. S., et al. (2010). Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecol. Lett.* 13, 372–382. doi: 10.1111/j.1461-0248.2009.01434.x

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Woodhams, D. C., and Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conserv. Biol.* 19, 1449–1459. doi: 10.1111/j.1523-1739.2005.004403.x

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595–595. doi: 10.1890/14-1837.1

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K., and Rollins-Smith, L. A. (2007a). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.* 10, 409–417. doi: 10.1111/j.1469-1795.2007.00130.x

Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007b). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, Rana muscosa. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

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Characterization of the Skin Microbiota of the Cane Toad *Rhinella cf. marina* in Puerto Rico and Costa Rica

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Rhinella marina is a toad native to South America that has been introduced in the Antilles, likely carrying high loads of microorganisms, potentially impacting local community diversity. The amphibian skin is involved in pathogen defense and its microbiota has been relatively well studied, however, research focusing on the cane toad microbiota is lacking. We hypothesize that the skin microbial communities will differ between toads inhabiting different geographical regions in Central America and the Caribbean. To test our hypothesis, we compared the microbiota of three populations of *R. cf. marina* toads, two from Costa Rican (native) and one Puerto Rican (exotic) locations. In Costa Rica, we collected 11 toads, 7 in Sarapiquí and 4 from Turrialba while in Puerto Rico, 10 animals were collected in Santa Ana. Separate swab samples were collected from the dorsal and ventral sites resulting in 42 samples. We found significant differences in the structure of the microbial communities between Puerto Rico and Costa Rica. We detected as much as 35 different phyla; however, communities were dominated by Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. Alpha diversity and richness were significantly higher in toads from Puerto Rico and betadiversity revealed significant differences between the microbiota samples from the two countries. At the genus level, we found in Santa Ana, Puerto Rico, a high dominance of *Kokuria*, *Niabella*, and Rhodobacteraceae, while in Costa Rica we found *Halomonas* and *Pseudomonas* in Sarapiquí, and *Acinetobacter* and *Citrobacter* in Turrialba. This is the first report of *Niabella* associated with the amphibian skin. The core microbiome represented 128 Operational Taxonomic Units (OTUs) mainly from five genera shared among all samples, which may represent the symbiotic *Rhinella*'s skin. These results provide insights into the habitat-induced microbial changes facing this amphibian species. The differences

in the microbial diversity in Puerto Rican toads compared to those in Costa Rica provide additional evidence of the geographically induced patterns in the amphibian skin microbiome, and highlight the importance of discussing the microbial tradeoffs in the colonization of new ecosystems.

Keywords: 16S rRNA gene sequencing, skin, toad, bacterial communities, bioinformatics

INTRODUCTION

In the last 30 years amphibians have undergone massive population declines (Whittaker et al., 2013). This phenomenon is attributed to climate change, habitat loss, pollution, and the presence of emerging infectious diseases, among other causes (Whitfield et al., 2016). It is suggested that the appearance of these emerging diseases is due to the introduction of exotic pathogens, such as *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999), Ranavirus (Price et al., 2014) or, more recently, *B. salamandrivorans* (*Bsal*), a fungus that affects salamanders (Martel et al., 2013). Pathogen spread has also been attributed to human trafficking of amphibian species (Bacigalupe et al., 2017). Due to the increase of infectious diseases, introduced species represent a constant threat to local fauna (Schloegel et al., 2009). Problems with introduced amphibians and reptiles have occurred worldwide, as in the case of the bullfrog (*Lithobates catesbeianus*) in the western areas of the United States, the Caribbean, and in South America (Young et al., 2004), the brown tree snake (*Boiga irregularis*) on Guam Island (Savidge et al., 2007), and also the giant toad or cane toad (*Rhinella cf. marina*) in Australia (Shine, 2010). In Puerto Rico, a decline of several native amphibian species has been documented, and among other possible factors is the introduction of the pathogen *Bd*, drought, and habitat loss (Burrowes et al., 2004). In addition, Puerto Rico has a great number of introduced species maintaining a constant threat to the native fauna, including six species of frogs (Joglar et al., 2007). The cane toad is one of such species, introduced in Puerto Rico in the early 20th century aiming at controlling a beetle infestation in sugarcane plantations, successfully halting the damage (Tyler, 1989; Thomas, 1999).

The cane toad has, in fact, a broad geographic distribution. It is native to the United States (South Texas), Central America (including Costa Rica), and South America, including Trinidad and Tobago. In these places the cane toad is not a threat and its populations appear to be stable (Solís et al., 2009). Its history of invasiveness dates back to the 1800s when it was introduced in Barbados and Jamaica, in 1920 in Florida and Puerto Rico, in 1930–1935 in Philippines and Australia, respectively, and from there to Japan in 1978 including other islands (Solís et al., 2009). Many of these introductions have been made with the aim of controlling agricultural pests, but have had little proven success. The cane toad has become a constant threat and the Invasive Species Specialist group of the Union for Conservation of Nature (IUCN) has declared it one of the 100 most damaging invasive species in the world (Lowe et al., 2000). Recent taxonomic changes subdivided this species into *R. horribilis* for Central America and *R. marina* for South America (Acevedo et al., 2016); however, the taxonomic status of the introduced populations is not clear and more genetic analyses are needed to verify these changes (Acevedo, personal communication).

When introducing an exotic species, either accidentally or intentionally, the potential pathogens that can be loaded are generally not analyzed, because molecular microbiological essays are never performed. It has been documented that the cane toad can carry *Salmonella* species that can affect other native species (Burrowes et al., 2004), and pathogen transmission between the cane toad and other species has even been documented in Panamá (Kelehear et al., 2015). These pathogens can be a severe problem to local fauna since invasive species are difficult to control and eliminate. Furthermore, some frog species are much less susceptible to death from particular pathogens and may act as carriers; for example, the cane toad is less susceptible to *Bd* but can still carry it as asymptomatic infections (Lips et al., 2006).

It is now possible to study the diversity of microbial communities in any habitat or species through next-generation sequencing, an approach that has allowed researchers to characterize the patterns of changes in the microbiota, revealing possible pathogens and symbionts associated with a given host (Rebollar et al., 2016a). One such example is the resistance of some frogs to pathogens, likely due to the presence of beneficial bacteria in their skin (Harris et al., 2009). Culture-independent techniques have shown differences in bacterial diversity depending on the degree of *Bd* infection among the same amphibian species (Rebollar et al., 2016a,b).

Variations in the skin microbiota of species across different geographies have been attributed to several factors, including: (1) the selective force excerpted by the chytrid fungus Bd (Walke et al., 2015; Rebollar et al., 2016b), (2) additive and non-additive mechanisms underlying the dilution effect (Becker et al., 2014), (3) environmental factors and host genetics and ecology (Kueneman et al., 2014; Bletz et al., 2017a), or (4) environmental connectivity (Walke et al., 2014).

Even though there have been several reports on the microbiota of amphibians, there are no studies on the Cane toad skin microbiota (Jiménez and Sommer, 2017), despite its wide distribution and propensity for acting as a vector of infectious diseases, and the capability of biotransformation of their chemical defenses in their parotid glands (Kamalakkannan et al., 2017). Similarly, amphibian bacterial communities have been compared between families in temperate and tropical regions (Belden et al., 2015) but to the best of our knowledge there are no studies comparing the same species in two geographically distant regions.

To bridge this knowledge gap, this work represents the first report comparing the microbial communities of *R. cf. marina* toads in its native (Costa Rica) and exotic (Puerto Rico) ranges,

a preliminary study on animals from two countries that share similar tropical ecosystems.

We hypothesize that there will be differences in the skin microbial communities between the dorsal and ventral sides of toads, and between the three sampling locations in its native (Costa Rica) and exotic (Puerto Rico) ranges. Here, we identify the differences between microbial communities of toads in Puerto Rico and Costa Rica, define the unique taxa for each locality, and define which bacterial groups compose the core microbiome of this species.

MATERIALS AND METHODS

Cane Toad Sampling

Field sampling was conducted between July and October 2016 in La Selva (LS) Biological Station Sarapiquí, Costa Rica (10, 25.816 N, 84, 0.550 W; elev. ∼60 m); Turrialba City (TC), Costa Rica (9, 53.897 N, 84, 40.330 W; elev. 600 m); and Centro Ambiental Santa Ana (SA) Bayamón, Puerto Rico (18, 24.480 N, 66, 8.651 W; elev. 20–60 m). Here we applied the Holdridge classification system (Holdridge, 1967) that considers tropical altitudinal height to be in a range of 0–700 m. A total of 21 Cane toads were collected using disposable nitrile gloves. Each toad was washed for 7 s using 50 ml of sterile distilled water to reduce transient surface bacteria (Madison et al., 2017). Sterile swabs were rubbed 10 times in the ventral and the dorsal area of toad, yielding two samples per individual. This study was exempt from IACUC protocol review since animals were collected without interfering with its environment. After the brief sterile skin swabbing *in situ*, toads were released immediately in their natural environment.

The swabs were placed in labeled Power Bead tubes (MoBio PowerSoil DNA Extraction Kit) into a cryobox in an ice-filled container and transported to the laboratory for −80°C storage. A total of 42 swab samples were obtained from the ventral and dorsal skin surfaces of toads, 20 from Puerto Rico and 22 from Costa Rica. For each individual toad, we measured the following parameters: skin surface pH in the dorsal area with a universal paper strip (Hydrion Paper); length and width employing a caliper, toads were placed inside the collection bag and weighed using a scale (Hanson). All sampled individuals were adults, although those from Sarapiquí Costa Rica were young adults. Environmental variables including temperature, humidity, and precipitation were obtained from nearby meteorological stations in both countries.

DNA Extraction

Genomic DNA was extracted from the swab material using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA, United States) following the manufacturer's instructions with the following modifications: (1) samples were incubated at 65°C after the addition of reagent C1; (2) the powerbead tubes were homogenized horizontally for 2 min at 2000 rpm, using a PowerLyzer^{TM} 24 Bench Top Bead-Based Homogenizer (MO BIO, Carlsbad, CA, United States); and (3) the elution buffer was allowed to sit on the filter for 5 min before the final centrifugation step.

To increase DNA yield, we used the pellet formed from the MO BIO powerbead for a second DNA extraction and pooled the two extractions per sample.

16S rRNA Gene PCR and Sequencing

The V4 hypervariable region of the 16S ribosomal RNA (∼291 bp length) was amplified by PCR using the universal bacterial and archaeal primers: 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) as described in the Earth Microbiome Project (EMP[1]) (Caporaso et al., 2012) using the following amplification conditions: 1 cycle of 94°C for 3 min, and 35 cycles of 94°C for 45 s and 50°C for 60 s and 72°C for 90 s, and a final extension of 72°C for 10 min.

16S amplicons were sent to the Sequencing and Genotyping Facility of the University of Puerto Rico for sequencing with the Illumina MiSeq System MSQ-M00883. The resulting post QC good-quality sequences for each sample were deposited in the NCBI BioProject ID PRJNA391810.

Sequence Processing and Data Analysis

A first quality control analyses using FastQC (Andrews, 2010) revealed that only forward reads were useful for downstream analyses. Sequences were de-multiplexed and processed using QIIME (Caporaso et al., 2010) with a Phred score of 20 and chimera filtering with the usearch61 hierarchical clustering method (Edgar et al., 2011). Sequences were clustered into Operational Taxonomic Units (OTUs) using *uclust* (Edgar, 2010) with a 97% identity threshold. Taxonomic assignment was performed using the RDP classifier with a minimum confidence threshold of 80%. Contaminant chloroplast and low abundance OTUs were removed from downstream analyses using the script filter_taxa_from_otu_table in QIIME (Kuczynski et al., 2012). Analyses were done in two ways: (1) considering both samples (dorsal and ventral) ($n = 42$) and (2) per individual, by collapsing ventral and dorsal samples in the BIOM table and mapping file into one data point using the -collapse_mode mean available in QIIME ($n = 21$). The resulting OTUs underwent rarefication to mitigate bias due to different sequence depth per sample. Values in the mapping file were also collapsed by grouping dorsal and ventral samples into one sample. The data analyses were done considering only those OTUs that were present in at least 50% of the samples; therefore, it eliminated much of the rare OTUs.

We used a QIIME diversity analyses workflow script core_diversity_analyses.py, for both alpha and beta diversity analyses for the main metadata categories of the mapping file country and location. The data analyses were performed using a rarefaction level of 3670 sequences per sample when considering all 42 samples (dorsal and ventral swabs), and of 32,900 sequences when collapsing dorsal and ventral samples in individuals, to avoid the bias caused by differences in sequence depth. This core diversity workflow does an extensive diversity analyses including alpha rarefaction diversity analyses such as the Chao 1 abundance-based richness estimator and the phylogenetic diversity (PD) metric of Faith, both computed in QIIME. Chao 1 values represent the estimated true species richness of a sample

[1]http://www.earthmicrobiome.org/emp-standard-protocols/16s/

and are calculated with the script for alpha rarefaction in QIIME that in turn implements the Chao 1 abundance-based estimator (Chao, 1987). It also calculates the PD metric of Faith, which does not take abundance into account but rather branch lengths of the phylogenies connecting all species to each community (Faith, 1992). The alpha rarefaction on the OTU table alpha_diversity.py, results in many files, that are then concatenated into a single file for generating rarefaction curves (collated file) to which statistical tests were applied. The rarefaction plots were recreated using the R package *Hmisc* (Harrell, 2006) using the output results of the rarefaction curves in QIIME.

Beta diversity analysis was performed as a non-metric multidimensional scaling plot (NMDS) using the Bray–Curtis distance metric and calculating *stress values* using the R packages Phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2008), and ggplot2 (Wickham, 2009) through the *ordinate* function. The Bray–Curtis matrix was calculated on the OTU table using script beta_diversity.py with the metrics option bray_curtis.

Taxonomic summaries at the Phyla and Genus levels were built by using QIIME's Taxa_Summary plot tables L2 and L6, respectively, using the *melt* function in the RESHAPE2 R package (Wickham, 2007). The significantly different phyla as determined by ANOVA, as well as the selected genus-level OTUs significantly associated with each location, were visualized as boxplots combining R packages ggplot2 (Wickham, 2009), RColorBrewer (Neuwirth, 2014), and scales (Wickham, 2017).

A heatmap of the significantly different taxa (FDR-adjusted *p*-values) for the two metadata categories (location and country) was built using heatmap.3 function in R (Zhao et al., 2015). Data normalization was done through DESeq2 negative binomial Wald normalization for visualization purposes due to differences in the numbers of individuals per sample. This normalization step was implemented in QIIME using the script normalize_table.py.

Additionally, the core microbiome was calculated for all samples using the compute_core_microbiome script in QIIME (Kuczynski et al., 2012) and the resulting OTU list was used to create a new OTU table used for plotting a Taxa Summary in QIIME (Kuczynski et al., 2012).

Statistical Analyses

Metadata categories were compared between each site using one-way ANOVA in R (v. 3.2.5) (R Development Core Team, 2008).

Significant differences of alpha diversity were calculated using a non-parametric two-sample *t*-test using 999 Monte-Carlo permutations using the QIIME (Caporaso et al., 2010) script compare_alpha_diversity.py using the collated alpha diversity file resulting from the alpha rarefaction analyses. The comparison was in fact done not between samples, but between groups of samples, created via the input category passed via "-c" on the mapping file. Significance tests were computed for each group comparison with the Chao1 abundance-based estimator, the alpha PD metric of Faith, and the Shannon index, for the 42-sample dataset. Same significance tests on alpha PD and Chao 1 were used on the 21-sample dataset.

Statistical tests on the beta diversity were done via nonparametric PERMANOVA significance in QIIME (Caporaso et al., 2010) through compare_categories.py script. This PERMANOVA test is determined through permutations and provides strength and statistical significance on sample groupings using a Bray–Curtis distance matrix as the primary input.

We performed Analyses of Variance tests using the *aov*( ) function in R (R Development Core Team, 2008) on the abundance values at each taxonomic Phyla, using the -biom-derived data matrices from QIIME (L2 table), comparing the relative abundance of each Phyla in the three sampling locations. Boxplots of the significant changes at the phyla level were plotted with ggplot2 (Wickham, 2009) and RColorBrewer (Neuwirth, 2014), using a normalized table of values, by running the R interface package of DESeq2 for table normalization, DESeq outputs negative values for lower abundant OTUs as a result of its log transformation.

Significantly different OTUs across countries and locations were detected through a log-likelihood ratio test, that detects what OTUs changed significantly in relative abundance between the two countries and the three habitats (locations) using the *G*-test with QIIME's group_significance script (Kuczynski et al., 2012), with the alternate hypothesis that the frequency of the OTUs would not be the same across all sample groups. Only FDR-adjusted *p*-values ($p < 0.05$) were taken in consideration.

RESULTS

A total of 5,296,165 good quality sequences were employed in the analyses. Among these, 1,967,761 sequences were obtained from Puerto Rican samples (Santa Ana) and they were binned into 3779 OTUs (**Table 1**). The Costa Rican samples included 1,296,254 sequences from Sarapiquí that were binned into 2253 OTUs and those from Turrialba in which 2,099,150 sequences were binned in 3516 OTUs (**Table 1** and **Supplementary Tables S2**, **S3**). **Supplementary Table S1** summarizes the number of sequences and OTUs for all 42 samples and **Supplementary Table S2** summarizes the number of sequences and OTUs for the 21 collapsed samples.

We compared differences in weight and pH among the two populations from which we had these values – Sarapiquí, Costa Rica, and Santa Ana, Puerto Rico, and found that animals in Sarapiquí weighed significantly less than those from Santa Ana (ANOVA, $df = 1$, F-value $= 117.1$, p-value $= 1.76e{-}08$), and their pH was also significantly higher ($df = 1$, F-value $= 13.97$, p-value $= 0.00198$). There were no significant differences in length between these animals although some of the individuals in Sarapiquí were smaller (**Supplementary Table S4**). Environmental measurements in the collection sites were very similar across the three locations, confirming that these sites have the same tropical environmental conditions in both countries.

We found no significant differences between the microbial community structure in dorsal and ventral samples in any of the three locations (**Figure 1**). We found a total of 35 assigned phyla, with 6 of these dominating across all the samples: Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria, and Verrucomicrobia; with the other 29 phyla

TABLE 1 | Number of sequences and OTUs across samples.

Country (site/habitat)	Number of animals	Number of samples	Number of sequences	Average number of OTUs ± Stdev
Puerto Rico (Santa Ana)	10	20	1,967,761	3779 ± 840
Costa Rica (Sarapiquí)	7	14	1,229,254	2253 ± 1013
Costa Rica (Turrialba)	4	8	2,099,150	3516 ± 798

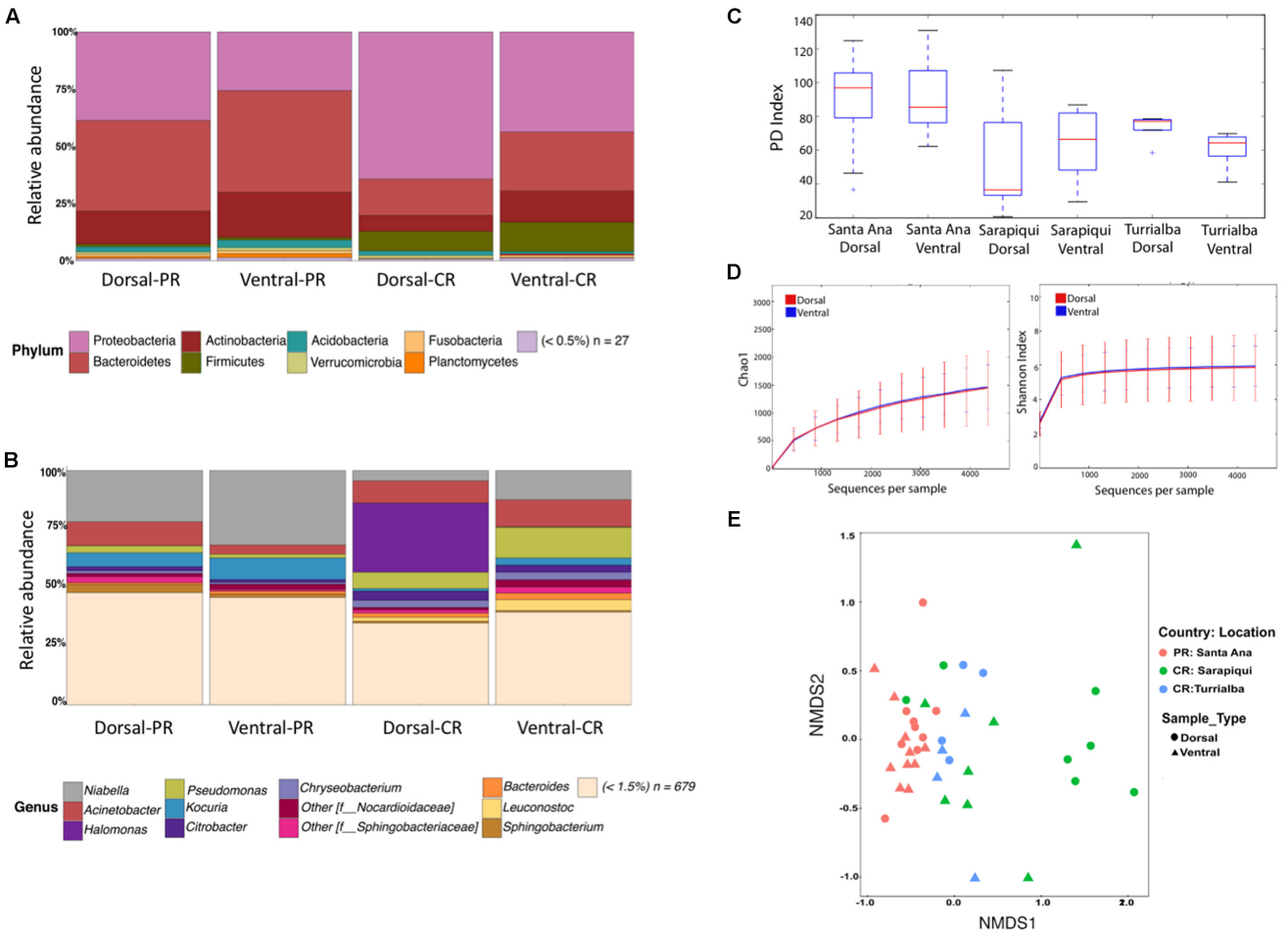

FIGURE 1 | Microbiota diversity in dorsal and ventral swab samples among toads in Puerto Rico and Costa Rica. **(A)** Taxonomic bar plots showing bacterial phyla among ventral and dorsal samples. **(B)** Taxonomic bar plots at the genus level. **(C)** Faith's phylogenetic diversity (PD) boxplots overall dorsal and ventral swabs per location. **(D)** Rarefaction plots of Chao1 (*t*-test, *t*-stat = –0.072, *p*-value = 0.94) and Shannon (*t*-test, *t*-stat = 0.164, *p*-value = 0.868) between dorsal and ventral skin sites. **(E)** Non-metric multidimensional scaling (NMDS) plots of samples according to location and sample type (stress = 0.15 and PERMANOVA Pseudo-*F*: 0.965, *p*-value = 0.461).

having a relative abundance lower than 1% (**Figure 1A**). Overall, at the genus level we found a dominance of *Niabella* and *Pseudomonas* across all samples (**Figure 1B**). The PD was nearly identical between ventral and dorsal swab samples at each of the three locations: Santa Ana dorsal vs. ventral (*t*-test, *t*-stat = 0.175, *p*-value = 1); Sarapiqui dorsal vs. ventral (*t*-test, *t*-stat = −0.477, *p*-value = 1), and Turrialba dorsal vs. ventral (*t*-test, *t*-stat = 1.591, *p*-value = 1) (**Figure 1C** and **Supplementary Table S4**). Rarefaction plots of Chao1 (*t*-test, *t*-stat = −0.072, *p*-value = 0.94) and Shannon (*t*-test, *t*-stat = 0.164, *p*-value = 0.868) confirm that there were no significant differences between dorsal and ventral skin sites (**Figure 1D**). Beta diversity comparisons between all 42 samples separated mostly samples from Turrialba (Costa Rica) from the rest, but did not separate ventral and dorsal samples (PERMANOVA, Pseudo-*F*: 0.9657, *p*-value = 0.461) (**Figure 1E** and **Supplementary Table S4**). As the analyses of the 42 samples did not show significant differences, we collapsed the dorsal

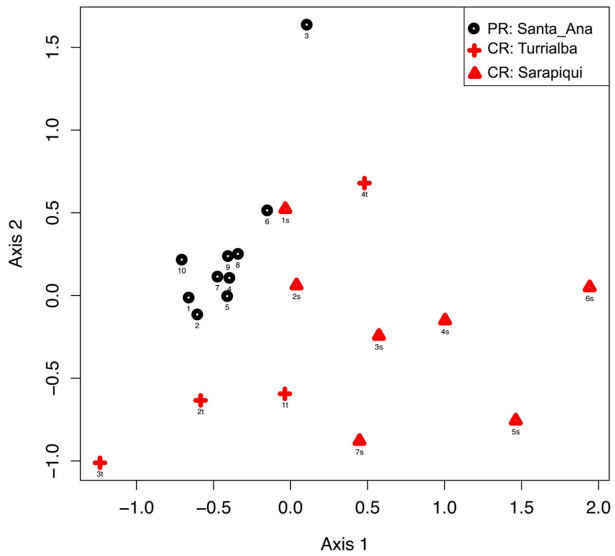

FIGURE 2 | Beta diversity comparisons by NMDS, stress = 0.156. Ordinations of Bray–Curtis dissimilarity between the bacterial communities inhabiting the three different locations in the two countries show a clear separation by country (PERMANOVA, Pseudo-*F*: 5.05, *p*-value = 0.001) and by location (PERMANOVA, Pseudo-*F*: 4.65, *p*-value = 0.001).

and ventral samples considering now 21 samples, one per individual.

Hence, considering the 21 individuals, microbial communities in the samples from Puerto Rico were clearly grouped together as shown by NMDS based on the relative dissimilarities of the samples (Bray–Curtis) with a stress value of 0.156. Costa Rican samples show a close aggregation with Puerto Rican samples, especially those from Turrialba (**Figure 2**). We found significant differences among microbial communities of the two countries (PERMANOVA, Pseudo-*F*: 5.05, *p*-value = 0.001); also, validated by an ANOSIM test (test statistic = 0.421 and *p*-value = 0.01). We also found the microbial communities in the three locations to be significantly different (PERMANOVA, Pseudo-*F*: 4.65, *p*-value = 0.001; **Supplementary Table S4**).

As discussed before, the dominating phyla were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes (**Figure 3**). Interestingly, we found that the Puerto Rican samples were significantly dominated by Bacteroidetes (ANOVA, *df* = 2, *F*-value = 19.25, *p*-value = 3.38*e*−05) while Costa Rican samples were dominated by Proteobacteria (ANOVA, *df* = 2, *F*-value = 8.99, *p*-value = 0.00196) (**Figure 4**). Regarding both Costa Rican sites, the most notorious difference at phylum level is that in Sarapiquí there is a higher abundance of Proteobacteria, Firmicutes, and Cyanobacteria compared to Turrialba (**Figure 3B** and **Supplementary Figure S1**).

At the genus-level, *Niabella* OTUs were highly dominant in Puerto Rico (∼25%) and the third most abundant in the two sites in Costa Rica. *Halomonas* OTUs had higher abundances in Sarapiquí (∼31%) compared to Santa Ana (<0.001%) and Turrialba (0.001%). *Bacteroides* OTUs were dominant in Turrialba samples (∼13%), as compared to Sarapiquí (0.006%) and Santa Ana (0.004%) (**Figures 3C,D**). Tables representing the relative abundance values for each sample at the phyla and genus levels can be found in the **Supplementary Tables S5**, **S6**.

The microbiota from Puerto Rican toads is significantly more diverse than the microbiota from Costa Rican toads (*t*-test, *t*-stat = 3.621, *p*-value = 0.004), as is its Chao 1 richness (*t*-test, *t*-stat = 3.723, *p*-value = 0.002) (**Figure 5** and **Supplementary Table S4**). As for the habitat/site, we found significant differences in diversity between the three locations (*p*-value = 0.01031). Nonetheless, pairwise comparisons showed that diversity was significantly different between Santa Ana and Sarapiquí (*t*-test, *t*-stat = −3.594, *p*-value = 0.021), as was richness (*t*-test, *t*-stat = −3.714, *p*-value = 0.009) (**Supplementary Table S4**).

Core diversity analyses between toads in the two countries interestingly revealed that 128 OTUs were shared across all 21 toads (100% samples) (**Figure 6**). At the genus level these 128 OTUs represent 24 different genera, these include a dominance of *Halomonas*, *Pseudomonas*, and *Acinetobacter* in Costa Rica, and the expected *Niabella* in the Puerto Rican samples (**Figure 6** and **Supplementary Table S7**).

We then proceeded to determine which taxa changed significantly (selected OTUs with FDR $p \leq 0.05$) between the two countries and the three locations/habitats, by employing a log-likelihood ratio test. Significantly different taxa between countries resulted in 20 OTUs, most remarkably an abundance in *Niabella* and Flavobacteriaceae in Puerto Rico, and a dominance of *Halomonas* in Costa Rica (**Figures 7**, **8**). In fact, *Halomonas* was significantly abundant in Sarapiquí as was *Pseudomonas* and *Leuconostoc*, while *Acinetobacter* and *Citrobacter* were highly abundant in Turrialba (**Figures 7**, **8**).

DISCUSSION

Capitalizing on advances in next-generation sequencing, several recent studies on amphibian skin microbiota have revealed the importance of cutaneous microbes for host disease resistance (Kueneman et al., 2014; Walke et al., 2015; Rebollar et al., 2016b; Bletz et al., 2017b). This is the first report of the microbiota of the successful toad colonist *R. marina* highlighting differences between habitats where animals are indigenous (two locations in Costa Rica) and those where it is invasive (Puerto Rico). Given that we had a small sample number at each location and only two countries were compared, we will limit the discussion to geographical differences and the possible effects of habitat and environment. Overall, many genera found in this study correspond to previous reports in other bufonids. In fact, *Pseudomonas*, *Sphingobacterium*, and *Bacteroides* were the most common genera found in the western toad, *Anaxyrus boreas* (Kueneman et al., 2014), while *Pseudomonas*, *Acinetobacter*, *Pantoea*, and *Chryseobacterium* were the most important genera in *Bufo japonicus* (Sabino-Pinto et al., 2016, 2017). All these genera, except *Pantoea*, were represented in the *Rhinella* microbiota.

Microbial symbioses have been considered a foundational principle for the invasive success of several different species. Microbiomes enhance the capability of species to adapt to

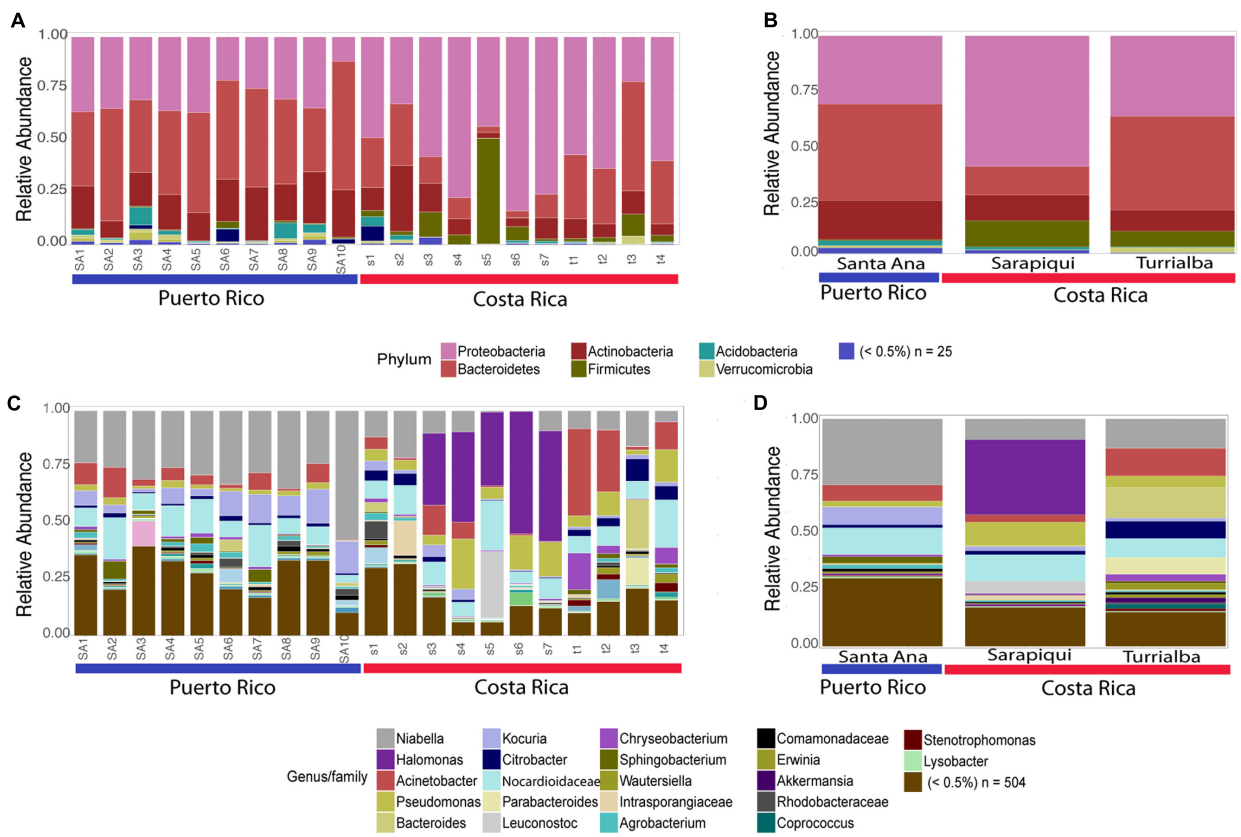

FIGURE 3 | Taxonomic profiles at the phyla-level **(A,B)** and genus-level **(C,D)**. **(A,C)** depict individual samples while **(B)** and **(D)** show the taxonomic profiles according to site/habitat.

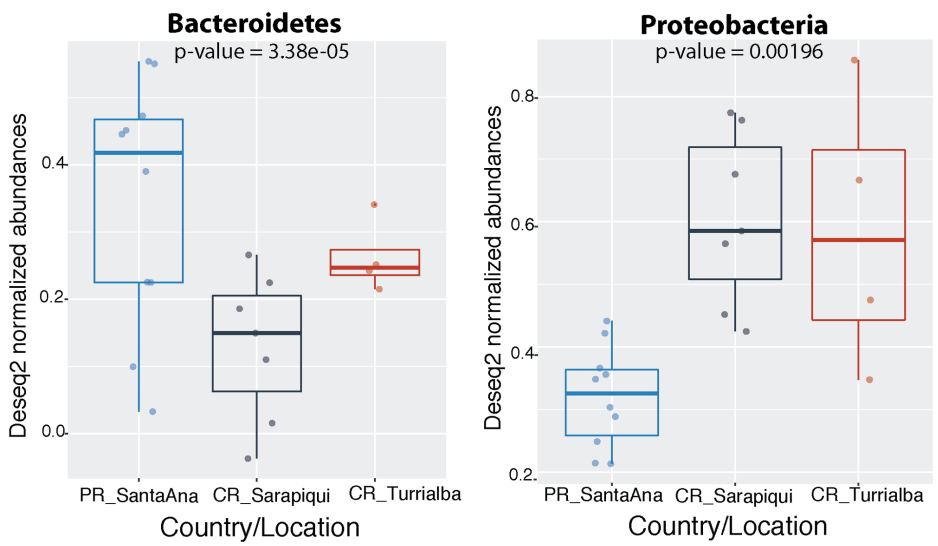

FIGURE 4 | Significantly different phyla among the two countries. Abundances were normalized through DESeq2 negative binomial Wald normalization.

new niches as was first reported by a large mammalian study (Ley et al., 2008), as well as in other non-mammalian cases including insects (Engel and Moran, 2013), fish (Ye et al., 2014), amphibians (Kohl et al., 2013), and even plants (Bulgarelli et al., 2013; Coats and Rumpho, 2014). We found that alpha diversity measures were significantly higher in Puerto

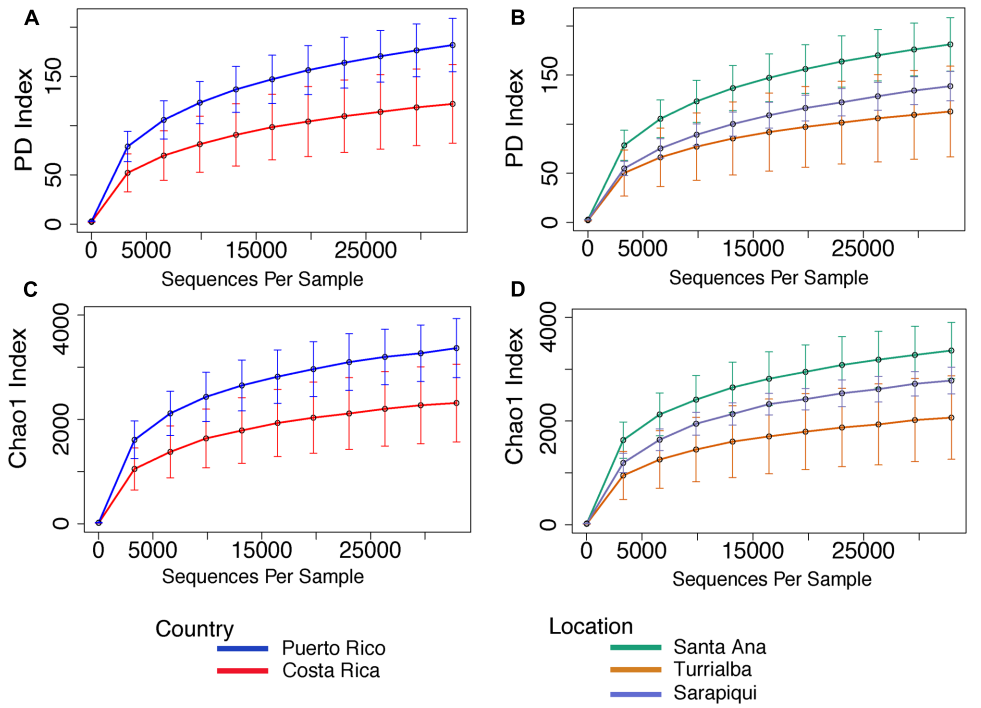

FIGURE 5 | Rarefaction curves for alpha diversity measures of Faith's PD index **(A,B)** and Chao 1 richness index **(C,D)** comparing country and location. Error bars in the figures correspond to one standard deviation out from the average (n = 10 biological replicates in Puerto Rico/Santa Ana; n = 7 biological replicates Sarapiquí, and n = 4 biological replicates Turrialba). PD measures per comparing countries indicate a significantly higher diversity in Puerto Rico (t-test, t-stat = 3.621, p-value = 0.004). Comparisons per location indicate that Santa Ana (PR) has significantly higher diversity compared to Sarapiquí (CR) (t-test, t-stat = –3.594, p-value = 0.021). Richness was significantly higher in Santa Ana compared to Sarapiquí (t-test, t-stat = –3.714, p-value = 0.009) but not compared to Turrialba (t-test, t-stat = –1.883, p-value = 0.291). Rarefaction analyses were based on 32,900 sequences per sample type.

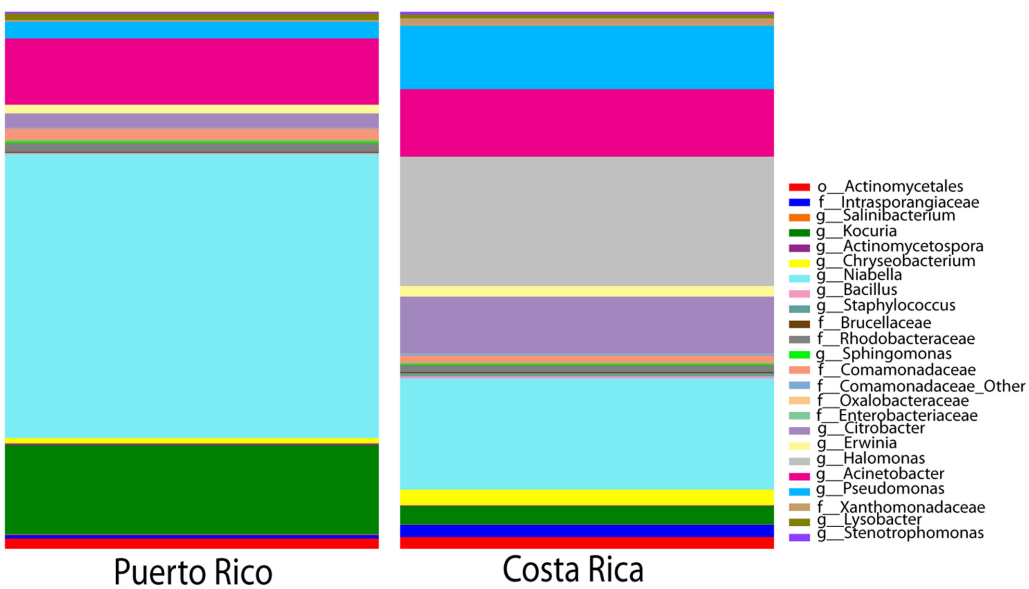

FIGURE 6 | Taxonomic profile of core OTUs. Includes only OTUs present in 100% of samples both in Costa Rica as well as in Puerto Rico. The number of OTUs shared across 100% of the samples in both countries is 128 OTUs out of the original 5,152 (~2.5% core species).

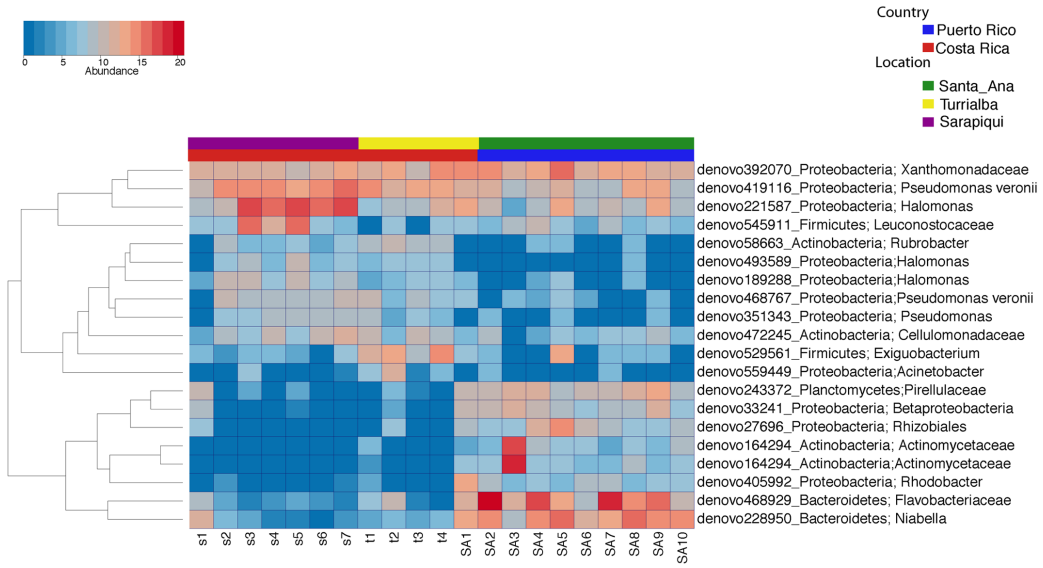

FIGURE 7 | Heatmap showing the significantly different taxa among country and location/habitat according to a parametric log-likelihood ratio test ($p < 0.05$).

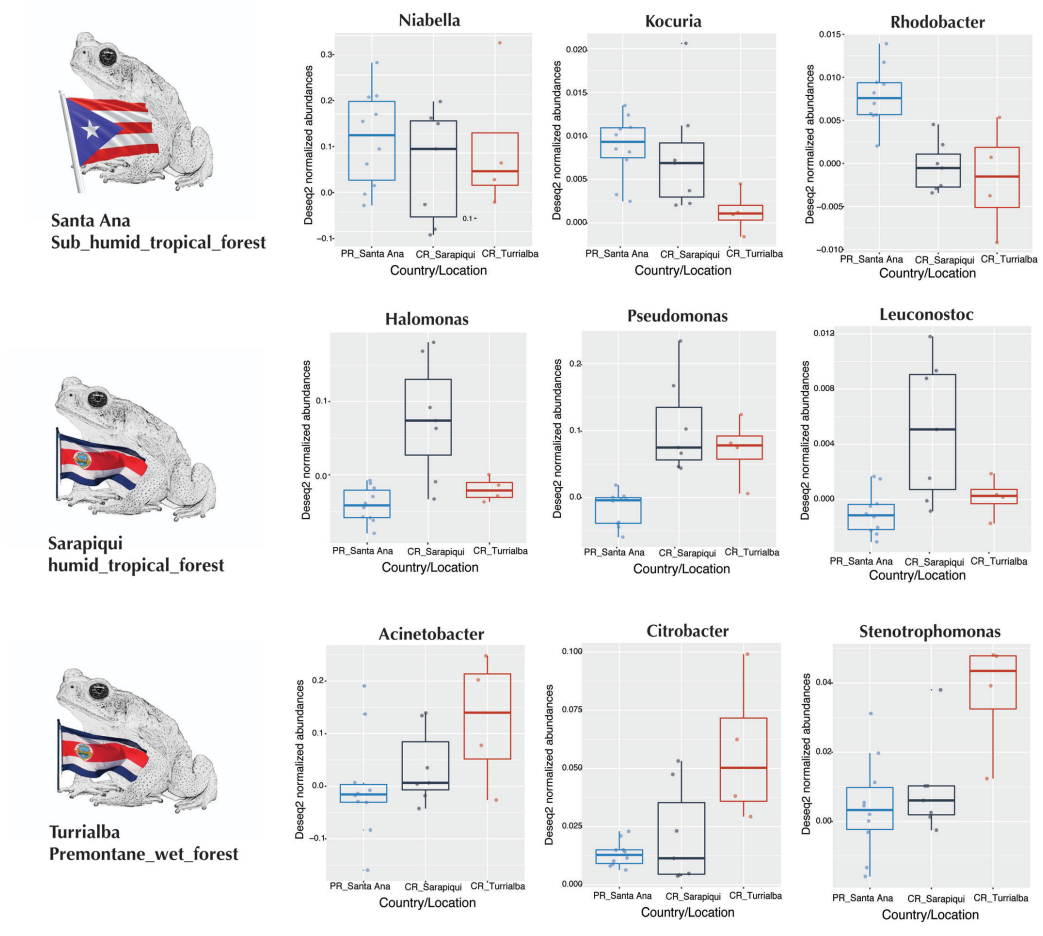

FIGURE 8 | Boxplots of taxa differentially abundant at each country/location.

Rico where *R. marina* toads were introduced, compared to the two locations in Costa Rica (native range), but these differences may be driven by the environmental differences of the habitats. Interestingly, a similar pattern was found in plant bacterial communities, where native plants shown to have lower microbial species diversity and increased abundance of pathogens compared to their invasive counterparts (Coats and Rumpho, 2014). The high diversity in the Puerto Rican samples may be related to a number of factors including environmental or genetic factors associated with different populations as seen in other amphibians (Kueneman et al., 2014; Rebollar et al., 2016b). A higher diversity in the Puerto Rican frogs (those in the native range) may provide the host with a plethora of antimicrobial peptides, and the capacity to use resources more efficiently than communities with low species richness in the native range.

Like plant roots, the toad skin surface is in close contact with the environment, mainly with soil and water; therefore, it would not be surprising to find microbial communities in frog skin to have similar patterns as those of plants in introduced environments. Interestingly, statistical tests on beta diversity confirm significant differences between toad microbes in the two geographies, similar to the separation between microbiota of frogs from tropical and temperate zones (Belden et al., 2015).

We also found a greater dispersion pattern in the microbiota of toads from Sarapiquí, a humid tropical forest. The complex conditions of the amphibian skin (pH and epithelial solutes) in the different locations may influence the structure of the microbiota, as animals from Sarapiquí have higher pH and communities are distant. Although the impact of host factors on the skin microbiota is acceptable, it is still poorly understood how environmental factors influence the biogeographic patterns of microbial communities in amphibians, which may be due to precipitation or even nitrogen deposition in these tropical ecosystems (Hietz et al., 2011).

Cane toads are very effective invaders and very resistant to adverse conditions (Solís et al., 2009) and infections (Lips et al., 2006). Resistance can occur, among other reasons, by the presence of beneficial bacteria in the skin of amphibians (Madison et al., 2017). Interestingly, some of the bacteria we found in these toads including genera like *Acinetobacter* and *Pseudomonas* in Turrialba and *Kocuria* or *Chryseobacterium* in Puerto Rico were reported to inhibit the pathogen *B. dendrobatidis* (Holden et al., 2015). The diversity of the microbial communities could be indicative of invasive success, however, because only three populations and two countries were compared, we recognize that more extensive sampling of individuals in different locations within both countries is needed to corroborate this trend.

Previous studies on amphibian microbes have shown that host species is a greater predictor of bacterial communities than habitat (McKenzie et al., 2012), however, it has also been shown that similar composition occurs at high taxonomic levels such as Phyla with only differences at the genus and species levels (Belden et al., 2015; Rebollar et al., 2016b).

The Cane Toad *R. cf. marina* besides having marked differences in structure between the two countries it also exhibits a core microbiome composed by 128 OTUS. Genera shared among all samples in both countries included *Niabella*, *Kokuria*, *Pseudomonas*, *Acinetobacter*, and *Chryseobacterium*, and this may be an indicator of a strong symbiotic relationship with this amphibian species, although more in-depth studies may be needed across several geographic regions to confirm this hypothesis. In fact, like the NMDS patterns of the current study, microbial communities in Panamanian frogs revealed different clusters according to sampling site (Belden et al., 2015). The Panamanian frog model has also showed that besides transient bacteria, there is a species-specific microbiota and the more distant bacterial communities correspond to samples infected with *Bd* (Rebollar et al., 2016b). Likewise, and regardless of its core microbiome, cane toads exhibit abundance-specific OTUs at each location such as *Niabella* and *Kocuria* in Puerto Rico, *Halomonas* in Sarapiquí, and *Acinetobacter* in Turrialba. Bacterial genera that have been associated with improved host defense against pathogens in other amphibian studies include *Pseudomonas*, *Acinetobacter*, *Stenotrophomonas*, and *Chryseobacterium* (Flechas et al., 2012), all of them are present in the core microbiome of cane toads from both countries. Some genera such as *Acinetobacter* are present at a similar relative abundance in both countries, while others, such as *Pseudomonas*, are more dominant in Costa Rica.

Niabella is the most dominant genus in the *Rhinella* population of Puerto Rico being shared by all Puerto Rican samples and the second most dominant taxa in Costa Rica, to our knowledge this is the first report of this bacteria symbiotically associated at high dominance with an amphibian. These are Gram-negative bacteria, aerobic, non-flagellated, and rod-shaped and they produce flexirubin-type pigments (Dai et al., 2011). There are seven species described (Glaeser et al., 2013) isolated from soils (Dai et al., 2011; Ngo et al., 2017), water (Siddiqi and Im, 2016) medicinal leeches (Kikuchi et al., 2009), as well as epiphytic communities in the green macroalgae *Cladophoraglomerata* (Zulkifly et al., 2012). This bacterium was indeed found associated with leeches and macroalgae, both highly humid environments, just like the toad skin. In fact, leeches are common in pathogenic or phoretic associations with amphibians (Stead and Pope, 2010; Maia-Carneiro et al., 2012). This is the first report of *Niabella* in association with a new world amphibian and its high dominance warrants further studies.

Halomonas is another bacterial genus worth discussing due to its high abundance in Costa Rica (mainly in Sarapiquí). Sarapiquí samples corresponded to young adults, compared to all the rest of the sampled toads both in Costa Rica and Puerto Rico and an ontogenic relationship of the frog skin microbiota has already been reported (Kueneman et al., 2014; Longo et al., 2015; Sabino-Pinto et al., 2017). Additionally, a comparison between adult and juvenile *Eleutherodactylus coqui* in Puerto Rico found that juveniles had a more diverse microbiota than adults, and certain OTUs present in juveniles were not found in adults (Longo et al., 2015). It is also possible that the habitat where these juveniles were captured could have influenced the microbiota of these young adults, such as debris and cellars. Cane toads have been identified as being capable of tolerating highly saline environments in the wild (De León and Castillo, 2015).

In fact, *Halomonas* have been isolated from saline environments (Sorokin and Tindall, 2006), rhizosphere (Borsodi et al., 2015), and have also been associated with rodents (Gavish et al., 2014). More studies comparing the skin microbiota of the cane toad at different stages of development should be done to further understand the type of association between *Halomonas* and this amphibian host.

The appearance of a new species in an ecosystem greatly impacts local diversity as already well described with the introduction of the pathogen *Bd* in frogs worldwide (Borzee et al., 2017) nonetheless, other animals such as geckos can bring different varieties of pathogenic bacteria (Gugnani et al., 1986) or parasites to the regions where they are introduced (Kelehear et al., 2015). Usually these risks are not well measured because the introductions are not controlled or monitored; therefore, next-generation sequencing tools take a special importance in the prevention of introduction of pathogens. In fact, amphibian microbiome studies have been increasing in recent years due to concerns about the disappearance of amphibians (Rebollar et al., 2016b; Jiménez and Sommer, 2017).

To our knowledge, this is the first study conducted to determine differences in skin microbiota between cane toads in two different geographical regions corresponding to exotic and native ranges. Our study confirms both the existence of bacterial OTUs composing a core microbiota in the *R. marina* sampled individuals, location-based patterns with significantly different taxa and reveals dominance of taxa such as *Niabella*, for the first time associated to the amphibian skin. We believe, therefore, that further sampling across global geographies in the native and exotic ranges are needed to further understand the microbial ecology of this species and to obtain a better understanding of the relationships between the microbiota in invasive species, likely leading to new insights into what microbes deem a successful invasion and allow the design of new microbiome-based control approaches.

AUTHOR CONTRIBUTIONS

JA conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared the figures and/or tables, and reviewed drafts of the paper. IZ and GO-M performed the experiments, analyzed the data, prepared the figures, and reviewed drafts of the paper. AL, MV-C, and NR-H performed the experiments, analyzed the data, and reviewed drafts of the paper. FV-S and CM-C performed the experiments and reviewed drafts of the paper. ET-R performed the experiments, contributed with materials, reviewed drafts of the paper, and funding. AP-T conceived and designed the experiments, performed the experiments, wrote the paper, reviewed drafts of the paper, and funding. FG-V conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared the figures and/or tables, reviewed drafts of the paper, and funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02624/full#supplementary-material

FIGURE S1 | Most abundant phyla across the three sampling locations. Abundances were normalized through DESeq2 negative binomial Wald normalization.

TABLE S1 | Total number of sequences and Operational Taxonomic Units (OTUs) for the 42 samples.

TABLE S2 | Total number of sequences and OTUs for the 21 collapsed samples.

TABLE S3 | Operational Taxonomic Unit table.

TABLE S4 | Statistical analyses comparing metadata, beta diversity and alpha rarefaction values for country, habitat and body site using Student's *t*-test.

TABLE S5 | Relative abundance for each Phyla-level OTU for each of the three habitats.

TABLE S6 | Relative abundance for each genus-level OTU for each of the three habitats.

TABLE S7 | Core OTUs shared by the 21 toad samples.

REFERENCES

Acevedo, A. A., Lampo, M., and Cipriani, R. (2016). The cane or marine toad, *Rhinella marina* (Anura, Bufonidae): two genetically and morphologically distinct species. *Zootaxa* 4103, 574–586. doi: 10.11646/zootaxa.4103.6.7

Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Bacigalupe, L. D., Soto-Azat, C., Garcia-Vera, C., Barria-Oyarzo, I., and Rezende, E. L. (2017). Effects of amphibian phylogeny, climate and human impact on the occurrence of the amphibian-killing chytrid fungus. *Glob. Chang Biol.* 23, 3543–3553. doi: 10.1111/gcb.13610

Becker, C. G., Rodriguez, D., Toledo, L. F., Longo, A. V., Lambertini, C., Correa, D. T., et al. (2014). Partitioning the net effect of host diversity on an emerging amphibian pathogen. *Proc. Biol. Sci.* 281:20141796. doi: 10.1098/rspb.2014.1796

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V. J., Rabemananjara, F. C. E., Rakotoarison, A., et al. (2017a). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.* 8:1530. doi: 10.3389/fmicb.2017.01530

Bletz, M. C., Perl, R. G. B., and Vences, M. (2017b). Skin microbiota differs drastically between co-occurring frogs and newts. *R. Soc. Open Sci.* 4:170107. doi: 10.1098/rsos.170107

Borsodi, A. K., Bárány, Á, Krett, G., Márialigeti, K., and Szili-Kovács, T. (2015). Diversity and ecological tolerance of bacteria isolated from the rhizosphere of halophyton plants living nearby Kiskunság soda ponds, Hungary. *Acta Microbiol. Immunol. Hung.* 62, 183–197. doi: 10.1556/030.62.2015.2.8

Borzee, A., Kosch, T. A., Kim, M., and Jang, Y. (2017). Introduced bullfrogs are associated with increased *Batrachochytrium dendrobatidis* prevalence and reduced occurrence of Korean treefrogs. *PLOS ONE* 12:e0177860. doi: 10.1371/journal.pone.0177860

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Burrowes, P. A., Joglar, R. J., and Green, D. E. (2004). Potential causes for amphibian declines in Puerto Rico. *Herpetologica* 60, 141–154. doi: 10.1655/03-50

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Chao, A. (1987). Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* 43, 783–791. doi: 10.2307/2531532

Coats, V. C., and Rumpho, M. E. (2014). The rhizosphere microbiota of plant invaders: an overview of recent advances in the microbiomics of invasive plants. *Front. Microbiol.* 5:368. doi: 10.3389/fmicb.2014.00368

Dai, J., Jiang, F., Wang, Y., Yu, B., Qi, H., Fang, C., et al. (2011). Niabella tibetensis sp. nov., isolated from soil, and emended description of the genus Niabella. *Int. J. Syst. Evol. Microbiol.* 61(Pt 5), 1201–1205. doi: 10.1099/ijs.0.022103-0

De León, L. F., and Castillo, A. (2015). *Rhinella marina* (Cane Toad). Salinity tolerance. *Herpetol. Rev.* 46, 237–238.

Edgar, R. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R., Haas, B., Clemente, J., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr1381

Engel, P., and Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol. Rev.* 37, 699–735. doi: 10.1111/1574-6976.12025

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* 61, 1–10. doi: 10.1016/0006-3207(92)91201-3

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus. *PLOS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Gavish, Y., Kedem, H., Messika, I., Cohen, C., Toh, E., Munro, D., et al. (2014). Association of host and microbial species diversity across spatial scales in desert rodent communities. *PLOS ONE* 9:e109677. doi: 10.1371/journal.pone.0109677

Glaeser, S. P., Galatis, H., Martin, K., and Kampfer, P. (2013). *Niabella hirudinis* and *Niabella drilacis* sp. nov., isolated from the medicinal leech *Hirudo verbena*. *Int. J. Syst. Evol. Microbiol.* 63, 3487–3493. doi: 10.1099/ijs.0.050823-0

Gugnani, H. C., Oguike, J. U., and Sakazaki, R. (1986). Salmonellae and other enteropathogenic bacteria in the intestines of wall geckos in Nigeria. *Antonie Van Leeuwenhoek* 52, 117–120. doi: 10.1007/BF00429314

Harrell, F. E. (2006). *The Hmisc Package*. Available at: https://cran.r-project.org/package=Hmisc

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Hietz, P., Turner, B. L., Wanek, W., Richter, A., Nock, C. A., and Wright, S. J. (2011). Long-term change in the nitrogen cycle of tropical forests. *Science* 334, 664–666. doi: 10.1126/science.1211979

Holden, W. M., Reinert, L. K., Hanlon, S. M., Parris, M. J., and Rollins-Smith, L. A. (2015). Development of antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*. *Dev. Comp. Immunol.* 48, 65–75. doi: 10.1016/j.dci.2014.09.003

Holdridge, L. R. (1967). *Life Zone Ecology*. San Jose, CA: Tropical Science Center.

Jiménez, R., and Sommer, S. (2017). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Joglar, R. L., Álvarez, A. O., Aide, T. M., Barber, D., Burrowes, P. A., García, M. A., et al. (2007). Conserving the Puerto Rican herpetofauna. *Appl. Herpetol.* 4, 327–345. doi: 10.1163/157075407782424566

Kamalakkannan, V., Salim, A. A., and Capon, R. J. (2017). Microbiome-mediated biotransformation of cane toad bufagenins. *J. Nat. Prod.* 80, 2012–2017. doi: 10.1021/acs.jnatprod.7b00134

Kelehear, C., Saltonstall, K., and Torchin, M. (2015). An introduced pentastomid parasite (*Raillietiella frenata*) infects native cane toads (*Rhinella marina*) in Panama. *Parasitology* 142, 675–679. doi: 10.1017/S0031182014001759

Kikuchi, Y., Bomar, L., and Graf, J. (2009). Stratified bacterial community in the bladder of the medicinal leech, *Hirudo verbana*. *Environ. Microbiol.* 11, 2758–2770. doi: 10.1111/j.1462-2920.2009.02004.x

Kohl, K. D., Cary, T. L., Karasov, W. H., and Dearing, M. D. (2013). Restructuring of the amphibian gut microbiota through metamorphosis. *Environ. Microbiol. Rep.* 5, 899–903. doi: 10.1111/1758-2229.12092

Kuczynski, J., Stombaugh, J., Walters, W. A., Gonzalez, A., Caporaso, J. G., and Knight, R. (2012). Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr. Protoc Bioinformatics* 36, 10.7.1–10.7.20. doi: 10.1002/9780471729259.mc01e05s27

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Ross, A. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Longcore, J. E., Pessier, A. P., and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91, 219–227. doi: 10.2307/3761366

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to

natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Lowe, S., Browne, M., and Boudjelas, S. (2000). *100 of the World's Worst Invasive Alien Species. A Selection from the Global Invasive Species Database*. Auckland: The Invasive Species Specialist Group.

Madison, J. D., Berg, E. A., Abarca, J. G., Whitfield, S. M., Gorbatenko, O., Pinto, A., et al. (2017). Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from amphibian populations in Costa Rica. *Front. Microbiol.* 8:290. doi: 10.3389/fmicb.2017.00290

Maia-Carneiro, T., Dorigo, T. A., Wachlevski, M., and Duarte Rocha, C. F. (2012). Evidence of phoresy by leeches (Hirudinoidea) on *Rhinella abei* (Anura: Bufonidae) in the Atlantic Rainforest in the state of Santa Catarina, southern Brazil. *Acta Herpetol.* 7, 163–169. doi: 10.13128/Acta_Herpetol-10807

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., et al. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15325–15329. doi: 10.1073/pnas.1307356110

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Neuwirth, E. (2014). *Package RColorBrewer: ColorBrewer Palettes. CRAN repository*. Available at: https://CRAN.R-project.org/package=RColorBrewer

Ngo, H. T. T., Trinh, H., Yan, Z. F., Moya, G., Kook, M., and Yi, T. H. (2017). *Niabella hibiscisoli* sp. nov., isolated from soil of a Rose of Sharon garden. *Int. J. Syst. Evol. Microbiol.* 67, 784–788. doi: 10.1099/ijsem.0.001595

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Simpson, G. L., Stevens, M. H. H., et al. (2008). *vegan: Community Ecology Package. R Package Version 1.17-2*. Available at: http://cran.r-project.org/package=vegan

Price, S. J., Garner, T. W. J., Nichols, R. A., Balloux, F., Ayres, C., Mora-Cabello de Alba, A., et al. (2014). Collapse of amphibian communities due to an introduced ranavirus. *Curr. Biol.* 24, 2586–2591. doi: 10.1016/j.cub.2014.09.028

R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "Omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibanez, R., and Belden, L. K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Sabino-Pinto, J., Bletz, M. C., Islam, M. M., Shimizu, N., Bhuju, S., Geffers, R., et al. (2016). Composition of the cutaneous bacterial community in Japanese Amphibians: effects of captivity, host species, and body region. *Microb. Ecol.* 72, 460–469. doi: 10.1007/s00248-016-0797-6

Sabino-Pinto, J., Galan, P., Rodriguez, S., Bletz, M. C., Bhuju, S., Geffers, R., et al. (2017). Temporal changes in cutaneous bacterial communities of terrestrial- and aquatic-phase newts (Amphibia). *Environ. Microbiol.* 19, 3025–3038. doi: 10.1111/1462-2920.13762

Savidge, J. A., Qualls, F. J., and Rodda, G. H. (2007). Reproductive biology of the brown tree snake, *Boiga irregularis* (Reptilia: Colubridae), during colonization of guam and comparison with that in their native range. *Pac. Sci.* 61, 191–199. doi: 10.2984/1534-6188(2007)61[191:RBOTBT]2.0.CO;2

Schloegel, L. M., Picco, A. M., Kilpatrick, A. M., Davies, A. J., Hyatt, A. D., and Daszak, P. (2009). Magnitude of the US trade in amphibians and presence of *Batrachochytrium dendrobatidis* and ranavirus infection in imported North American bullfrogs (*Rana catesbeiana*). *Biol. Conserv.* 142, 1420–1426. doi: 10.1016/j.biocon.2009.02.007

Shine, R. (2010). The ecological impact of invasive cane toads (Bufomarinus) in Australia. *Q. Rev. Biol.* 85, 253–291. doi: 10.1086/655116

Siddiqi, M. Z., and Im, W. T. (2016). *Niabella aquatica* sp. nov., isolated from lake water. *Int. J. Syst. Evol. Microbiol.* 66, 2774–2779. doi: 10.1099/ijsem.0.001053

Solís, F., Ibáñez, R., Hammerson, G., Hedges, B., Diesmos, A., Matsui, M., et al. (2009). *Rhinella marina. The IUCN Red List of Threatened Species*. Available at: http://www.iucnredlist.org/apps/redlist/details/full/41065/0

Sorokin, D. Y., and Tindall, B. J. (2006). The status of the genus name Halovibrio Fendrich 1989 and the identity of the strains *Pseudomonas halophila* DSM 3050 and Halomonas variabilis DSM 3051. Request for an opinion. *Int. J. Syst. Evol. Microbiol.* 56(Pt 2), 487–489. doi: 10.1099/ijs.0.63965-0

Stead, J. E., and Pope, K. L. (2010). Predatory leeches (Hirudinida) may contribute to amphibian declines in the Lassen Region, California. *Northwest. Nat.* 91, 30–39. doi: 10.1898/NWN08-56.1

Thomas, R. (1999). "The Puerto Rico area," in *Caribbean Amphibians and Reptiles*, ed. B. I. Crother (San Diego: Academic Press), 169–179. doi: 10.1016/B978-012197955-3/50017-3

Tyler, M. J. (1989). *Australian Frogs*. Ringwood: Penguin Books.

Walke, J., Becker, M., Loftus, S., House, L., Cormier, G., Jensen, R., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLOS ONE* 10:e0139848. doi: 10.1371/journal.pone.0139848

Whitfield, S. M., Lips, K. R., and Donnelly, M. A. (2016). Amphibian decline and conservation in central America. *Copeia* 104, 351–379. doi: 10.1643/CH-15-300

Whittaker, K., Koo, M. S., Wake, D. B., and Vredenburg, V. T. (2013). *Global Declines of Amphibians*. Waltham, MA: Academic Press. doi: 10.1016/B978-0-12-384719-5.00266-5

Wickham, H. (2007). Reshaping data with the reshape Package. *J. Stat. Softw.* 21, 1–20. doi: 10.3978/j.issn.2305-5839.2016.01.33

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verlag. doi: 10.1007/978-0-387-98141-3

Wickham, H. (2017). *Scale Functions for Visualization. CRAN Repository*. Available at: https://CRAN.R-project.org/package=scales

Ye, L., Amberg, J., Chapman, D., Gaikowski, M., and Liu, W. T. (2014). Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *ISME J.* 8, 541–551. doi: 10.1038/ismej.2013.181

Young, B. E., Stuart, S. N., Chanson, J. S., Cox, N. A., and Boucher, T. M. (2004). *Disappearing Jewels: The Status of New World Amphibians*. Arlington, VA: NatureServe.

Zhao, S., Guo, Y., Sheng, Q., and Shyr, Y. (2015). *heatmap3: An Improved Heatmap Package*. Available at: https://cran.r-project.org/web/packages/heatmap3/heatmap3.pdf

Zulkifly, S., Hanshew, A., Young, E. B., Lee, P., Graham, M. E., Graham, M. E., et al. (2012). The epiphytic microbiota of the globally widespread macroalga *Cladophora glomerata* (Chlorophyta, Cladophorales). *Am. J. Bot.* 99, 1541–1552. doi: 10.3732/ajb.1200161

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Composition and Functional Specialists of the Gut Microbiota of Frogs Reflect Habitat Differences and Agricultural Activity

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The physiological impact of agricultural pollution, habitat disturbance, and food source variability on amphibian remains poorly understood. By comparing the composition and predicted functions of gut microbiota of two frog species from forest and farmland, we quantified the effects of the exogenous environment and endogenous filters on gut microbiota and the corresponding functions. However, compositional differences of the gut microbiota between the frog species were not detected, even when removing roughly 80–88% of the confounding effect produced by common and shared bacteria (i.e., generalists) and those taxa deemed too rare. The habitat effect accounted for 14.1% of the compositional difference of gut microbial specialists, but host and host × habitat effects were not significant. Similar trends of a significant habitat effect, at an even higher level (26.0%), for the physiological and metabolic functions of gut microbiota was predicted. A very obvious skewing of the relative abundance of functional groups toward farmland habitats reflects the highly diverse bacterial functions of farmland frogs, in particular related to pathogenic disease and pesticide degradation, which may be indication of poor adaptation or strong selective pressure against disease. These patterns reflect the impacts of agricultural activities on frogs and how such stresses may be applied in an unequal manner for different frog species.

Keywords: 16S rRNA metagenome, adult Anura, functional predictions, gut microbiota, agricultural activity

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INTRODUCTION

Host habitat is the primary filter of the gut microbial community (Ley et al., 2006; Sullam et al., 2012). Through food intake, the gut becomes a reservoir of microbiota originating from the external habitat (Drake and Horn, 2007; Wiggins et al., 2011). For example, amphibians acquire soil microorganisms through the ingestion of prey and their own shed skin, both of which are covered with soil bacteria (Wiggins et al., 2011). The gastrointestinal environment also acts as a second-layer filter for selecting microbes that arrive from the external environment (Feld et al., 2008; Thomas et al., 2011). Epithelial cells and the fluids of the digestive tract maintain a homeostatic environment (Artis, 2008; Kohl et al., 2013) providing a constant adaptive pressure on intestinal microbes. These host effects which may potentially affect gut microbial composition are called endogenous factors. Gastrointestinal microbial assemblages also reflect the dispersal processes of hosts (via habitat shifts), environmental selection, and ecological drift (Costello et al., 2012). These effects are often called exogenous factors. These exogenous and endogenous factors

could synergistically shape the gut microbial community. For example, the tolerance, interaction, and adaptation to a specific niches, which is so called host adaptability, could also alter gut microbial composition (Hooper et al., 2002; Spor et al., 2011). Gut microorganisms reflect evolutionary selection pressure acting via the adaptation of the host to the external environment. The habitat-selected host genotypes may filter out immigration of unsuitable microorganisms, and may facilitate first colonization of mutualistic or pathogenic microbes from co-existence neighbors or parents (e.g., Lawley et al., 2008). The host adaptability to the habitat, the host internal (gut) environment, and dynamic of external and internal bacteria could together shape the gut microbial community (Ley et al., 2006).

The influence of habitat change on the homeostasis of gut microbiota is of particular importance for understanding the adaptability of hosts that undergo changes to their niche (Spor et al., 2011). Habitat-specific gut microbiota demonstrate how the external environment mediates the intestinal environment (Ley et al., 2006; Sullam et al., 2012; Wong and Rawls, 2012; Giatsis et al., 2015; Bletz et al., 2016; Chang et al., 2016). The functional convergence of differing gut microbial assemblages under shifting habitats indicates the taxonomic incoherence and metagenomic plasticity of gut microbiota (Bletz et al., 2016). Studies of primates have shown that artificial disturbance and habitat degradation decrease gut microbial diversity (Amato et al., 2013; Barelli et al., 2015), and can affect hosts' metabolism and health (Amato et al., 2013). Numerous medical studies demonstrate the association between gut microbiota and hosts' disease (Artis, 2008; Feld et al., 2008; Barbosa and Rescigno, 2010; Garrett et al., 2010; Manichanh et al., 2010; Schwabe and Jobin, 2013; Bultman, 2014; Hullar et al., 2014; Jasarevic et al., 2015; Johnson et al., 2016), highlighting the relationship between gut microbiota and the adaptability of a host. However, such relationships between gut microbiota and host physiology have focused primarily on experimental animals or humans. Few studies have included fauna in a natural setting.

Amphibians are highly sensitive to the environment and are very suitable for studying adaptability (Hopkins, 2007), although some have suggested caution (Kerby et al., 2010). Through the functional prediction of the gut microbiota, we may be able to more clearly understand the physiological status and niche divergence of species under differing environmental conditions (Stevenson et al., 2014; Bolanos et al., 2016). Furthermore, the functional change of the gut microbiota may be even more sensitive to impacts of environmental disturbance than the hosts themselves (Amato et al., 2013; Barelli et al., 2015).

Vertical transmission of maternal microbes through birth (and breastfeeding in mammals) also modifies the composition of gut microbiota assemblages (Funkhouser and Bordenstein, 2013), which also indirectly affects neurodevelopment (Jasarevic et al., 2015). However, it is hard to detangle the relative effect of external environment and vertical transmission on gut microbial community. Adult frogs offer this possibility. Significant differences between the gut microbiota of tadpoles and adult frogs have been demonstrated (Kohl et al., 2013), and change of the diet is the main contribution for the turnover of gut microbial composition during metamorphosis and distinct gut microbial composition among habitats (Vences et al., 2016). In tadpoles, as with fishes, there is a higher abundance of operational taxonomic units (OTUs) from the phylum Proteobacteria while Firmicutes and Bacteroidetes, common to terrestrial amniotes, dominate the gut environment in adult frogs (Kohl et al., 2013). The change of dietary strategy (from aquatic herbivore to a typically terrestrial insectivore) and gut pH, as well as the development of a gastric stomach and an epithelial immune function through metamorphosis of anuran amphibians (Hourdry et al., 1996; Du Pasquier et al., 2000) combine to reset the gut environment and succession of intestinal microflora (Vences et al., 2016), thereby eliminating influence from vertical transmission in mature frogs.

We selected two phylogenetically distinct frog species, *Fejervarya limnocharis* and *Babina adenopleura*, as hosts for the assessment of gut microbial composition. Both species are on the IUCN Red List Least Concern faunal list. *F. limnocharis* is usually found near the paddy fields, ponds, lakes, and ditches, while *B. adenopleura* is usually found in ponds or swamps (Supplementary Table S1). Both species have broad diets. Similar prey for both species includes Arachnida, Coleoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, and Stylommatophora. *F. limnocharis* has a more diverse diet than *B. adenopleura*, also feeding upon Blattodea, Collembola, Dermaptera, Diptera, and Psocoptera (Supplementary Table S2). Both species may be found together in similar habitats. We chose a forest habitat and a farmland habitat. These two sampling sites have an altitudinal difference of ∼340 m (F: ∼60 m a.s.l.; N: ∼400 m a.s.l.) and are situated ∼3 km away from each other, separated by the Beishi and Nanshi rivers, upstream of their confluence with the Xindian River. Traveling between these two sites seems to be infrequent because long distance migration is often maladaptive to juvenile frogs (Smith and Green, 2005; Semlitsch, 2008).

In this study, we compare 12 gut microbial communities, three samples for each of four different combinations (*F. limnocharis*–forest; *F. limnocharis*–farmland; *B. adenopleura*–forest; *B. adenopleura*–farmland). The four combinations were set and have passive interactions with each other (e.g., same species with different habitat or same habitat with different species). We determine whether the internal or external environment of a host, that is the host itself or its habitat, most strongly determines the compositional and functional diversity of the gut microbiota. Three main issues questions arise from this approach: (1) to what degree do external and internal environments filter the gut microbiome? (2) How different is the composition of gut microbial assemblages within sympatric hosts, and how similar is the gut microbial composition between allopatric host populations of the same species? (3) Do the predicted functional groups of gut microbiota reflect the characteristics of hosts and habitats? For testing the role of host and habitat effect on the composition of gut microbiota, we sampled two frog species from both paddy fields and ponds in a forest setting. We determined the relative abundance (RA) of gut microbiota using high-throughput 16S rRNA gene sequencing of each individual frog. We defined a gut microbial metacommunity as a gut microbial community that had either the same host

species or the same habitat. As such, we could compare the microbial community compositions between different hosts and between different habitats. Since the common microbes and those deemed as "too rare" could confound the results, we removed these microbes and retained only the host and habitat specialists for quantifying the host and habitat effects on gut microbiota composition. In addition, *in silico* profiling predicted and classified the metabolic and physiological functions of these gut microbiota. To elucidate the impact of the anthropogenic (agricultural activity) interference on the ecological functions of gut microbiota, we quantified the functional divergence between the hosts and habitats.

MATERIALS AND METHODS

Ethics

We sacrificed 12 frogs for obtaining the intestinal microbiota. To prevent contamination from bacteria outside the sample, the forceps and scissors for obtaining the intestinal tissue were sterilized by both autoclave and UV-light. The tissues were stored in −80°C before extraction. The Institutional Animal Care and Use Committee, National Taiwan Normal University (No. 104033) reviewed and approved the study protocols and the number of animals that could be used. All experiments involving animals followed the principles of the 3Rs (replace, reduce, and refine) to prevent excessive and unnecessary killing.

Sampling Sites

For this study, two sites near Taipei, Taiwan, having different environmental conditions, were sampled for *F. limnocharis* and *B. adenopleura*. The natural habitat (N) site is a secondary growth forest near a forest road and is adjacent to some small ponds (24°53′N, 121°33′E). The farmland (F) site lies in an agricultural field that produces rice and vegetables (24°55′N, 121°32′E).

Metagenomic Experiments

Intestinal microbial metagenomic DNA was extracted following the protocol of Sharma et al. (2003). Every extracted metagenomics DNA were adjusted to 50 ng/μL for subsequent analysis. We amplified the V4 hypervariable 16S rRNA region using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCGCGGCTGCTGGCAC-3′). Double distilled water was used as a control to ensure no contamination during amplification. The DNA library was assembled using a Roche GS FLX Titanium emPCR kit (Roche Applied Science, Indianapolis, IN, United States). We then sent the DNA libraries to Welgene Biotech Co., Ltd. (Taipei, Taiwan) for pyrosequencing. A Roche 454 FLX Titanium instrument and reagents were used for pyrosequencing and procedures followed the manufacturer's instructions. We removed V4 sequence fragments that were shorter than 200 bp, or that had barcodes, polyN, or polyA/T. We also eliminated readings having a quality score <Q25. The trimmed sequences were analyzed and aligned using the software mothur (Schloss et al., 2009). We normalized each OTU by its copy number using normalize.shared function implemented in mothur. We considered sequences that were >97% identical to be the same species and thus represented an OTU. Each OTU was classified using the SILVA rRNA database. We removed chimeras using the UCHIME algorithm (Edgar et al., 2011). The raw sequence data were deposited into the NCBI GenBank under Bioproject PRJNA279212 (accession number: SAMN04158746 for *B. adenopleura* and SAMN03434989 for *F. limnocharis*).

Microbial Community Diversity

We performed a rarefaction analysis to estimate the probable richness of each microbial community sample. To reduce the effect of sampling effort, we drew species accumulation curves using vegan packages of R (Dixon and Palmer, 2003) to correlate the number of microbial taxa and the number of sampled frogs and thereby assess the taxa-abundance distributions (van der Gast et al., 2011, 2013).

Functional Predictions of the Gut Microbiomes

For predicting physiological and metabolic functions of gut microbiota, we used PICRUSt v. 1.0.0, a functional prediction tool for estimating the shared gene content according to the corresponding microbiome phylogeny (Langille et al., 2013). PICRUSt can use an extended ancestral-state reconstruction algorithm to generate the composition of gene families for each metagenome. We used the online version of PICRUSt—Galaxy (https://huttenhower.sph.harvard.edu/galaxy/)—for assisting with our algorithms. In the PICRUSt prediction, the Greengenes v. 13.5 OTUs database (DeSantis et al., 2006) assigned the cleaned sequences to a closed reference OTU table using the 97% similarity implemented in QIIME v. 1.8.0 (Caporaso et al., 2010). We reconstructed and predicted the functional contribution of each OTU member by mapping 16S sequences with their nearest reference genome. A "virtual" metagenome with gene content abundance was then generated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog. The abundance of each KEGG Orthologs category was presented in a KEGG pathway at the third hierarchical level.

Comparing the Compositional Distribution of Gut Microbes from Different Hosts and Habitats

We used Venn diagrams to compare the intersections and unions of gut bacteria and functional groups among the different metacommunities. We used the ANOSIM function in the vegan package in R, which calculates difference of mean ranks between (r_B) and within (r_W) metacommunities (Clarke, 1993) to assess the dissimilarities of microbial composition between hosts and between habitats. We also used ggtern package in R to draw Ternary diagrams (Hamilton, 2016) to assesse the differences in the abundance of bacterial compositions or functional groups between hosts or habitats. For the ternary diagrams, we used the bacterial composition (or functional groups) of the other host as a baseline for comparing the compositional differences (or differences of functional groups) of the target host between habitats. For example, the bacterial composition

of *B. adenopleura* was used as the benchmark to compare the compositional differences of gut bacteria of *F. limnocharis* between forest and farmland. Similarly, we used the bacterial composition (or the functional groups) of one habitat as the benchmark for comparing the gut bacterial composition or functional groups between different hosts in the other habitat.

Data Filtering for Identifying the Specialists

Because "everything is everywhere" (Baas-Becking, 1934) and our purpose was to find the environment that selects and those taxa that are selected, we first wanted to remove those species not selected (i.e., the generalists) and identify those potentially being selected (i.e., the specialists). We used the supermajority rule (2/3 RA) to classify the generalist and specialist microbes of host habitats and host species (Chazdon et al., 2011) using the function CLAM in the R package vegan. We discarded those OTUs classified as "too rare." Similarly, we retained the functional group specialists of the host and habitats for the further analyses.

PCA and PERMANOVA

We performed principal component analysis (PCA) for two reasons: (1) to access the clustering pattern by hosts and habitats, and (2) the axis of PCA can provide quantitative weight on our variables, and can be used to transform the compositional matrix into vectors following the explanatory proportion for further analysis of multivariate logistic regression instead of principal coordinate analysis which used non-Euclidean distance matrix (Ramette, 2007). We performed PCA using the R package factoextra (Kassambara, 2015). As well, permutational multivariate analysis of variance (PERMANOVA) estimated the significance of the variance and covariance of independent factors "host habitat" and "host species" on the first three PCs for microbial composition and predicted functional groups (53.02 and 75.08% variation, respectively) using 999 permutations in the R package vegan (Dixon, 2003).

Redundancy Analysis to Assess the Explanatory Proportion of the Host and Habitat Effect

For understanding how host species and habitats affect the RA of gut microbiota, we applied distance-based redundancy analysis (dbRDA) to estimate the explanatory proportion of the RA of microbial compositions and functions. Analysis of variance (ANOVA) tested the significance of each independent factor through 999 permutations under a reduced model using the capscales function in the R package vegan.

RESULTS

High Beta-Diversity of Gut Microbial Communities and the Underestimation of Gut Bacterial Richness

We sequenced a total of 232,153 reads, retaining 197,260 reads (mean of 16438.33 reads per sample, range 7346–33,441 reads) for analyses after discarding (cleaning) the substandard sequences. Among these cleaned sequences, we obtained 562.33 ± 198.07 OTUs per sample (range 291–1011 OTUs) using the 97% similarity criterion for determining OTU (**Table 1**). The sequence depth obtained a mean richness of 76.87% (54.48–85.66%) or 77.06% (47.15–87.35%) as estimated through the Cha01 or ACE indices, respectively (**Table 1**). This suggested an underestimation of gut bacterial diversity in our sampling. This underestimation was also revealed by the linear increase of bacterial OTUs in the microbial assemblages of individual samples (Supplementary Figures S1–S3).

Major Bacterial Groups Dominate Gut Microbial Communities Influenced by Habitat

The five most dominant (top 5) phyla of bacteria were Firmicutes, Bacteroidetes, Proteobacteria, Tenericutes, and Verrucomicrobia. These phyla accounted for >90% of the gut microbial community composition for both frog species in both habitats (97.25 and 94.55%, for *F. limnocharis* and *B. adenopleura*, respectively, in the forest samples and 92.37 and 96.83% for *F. limnocharis* and *B. adenopleura*, respectively, in the farmland samples; **Figure 1** and **Table 2**). These values are roughly consistent with previous studies showing that these microbial phyla dominate amphibian gastrointestines (Kohl et al., 2013; Colombo et al., 2015; Vences et al., 2016; Weng et al., 2016; Zhang et al., 2016) However, RA differed slightly between sample habitats, in particular for the phyla Bacteroidetes and Proteobacteria (Mann–Whitney test and Kruskal–Wallis test, $P < 0.05$; **Table 2**). This significant difference for RA between habitats may reflect the frogs having different life habits, such as diet, for the different environments (Chang et al., 2016). However, as we know that both frog species have certain divergent feeding strategies (Supplementary Table S2), we wondered if there was an influence of the interaction between host and habitat on these dominant gut bacterial phyla. Hence, we performed a two-way ANOVA and revealed that only Bacteroidetes, a microbial phylum functionally involved in polysaccharide degradation (Thomas et al., 2011), had significant differences of variance between habitats ($P = 0.022$). Frog in two habitat types also revealed difference in food resources (Supplementary Tables S1, S2; Chang et al., 2016). We detected no significant effects for other phyla nor for the host effect nor for habitat × host (**Table 2**). This implied that only the habitat mattered and that not all bacterial groups were affected equally. This assessment supports the hypothesis of a constant gastric environment for maintaining an invariable core of microbiota. Only certain microbial indicators reflected the disturbance of the external environment (Barbosa and Rescigno, 2010; Garrett et al., 2010).

Compositional and Distributional Patterns of Bacteria across Hosts and Habitats

Venn diagrams comparing all four sampling groups of gut microbiota (i.e., the gut microbial communities in two species

TABLE 1 | Summary statistics of the 16S rRNA metagenome sequencing.

Host species	Habitat	Sample	Reads for cleaning			OTUs	ACE (95% CI)	Cha01 (95% CI)	Shannon (95% CI)	Simpson (95% CI)
			1st	2nd	Final					
F. limnocharis	Forest	AN1	17,442	13,700	12,380	540	640.45 (609.75 – 684.66)	653.55 (611.58 – 720.15)	4.878 (4.850 – 4.907)	0.024 (0.022 – 0.025)
F. limnocharis	Forest	AN2	17,343	15,656	14,737	291	361.32 (334.88 – 403.68)	370.88 (334.72 – 436.92)	3.552 (3.522 – 3.583)	0.088 (0.085 – 0.091)
F. limnocharis	Forest	AN3	37,167	34,860	33,441	458	524.32 (501.48 – 559.15)	541.72 (507.34 – 600.06)	3.420 (3.398 – 3.442)	0.102 (0.100 – 0.104)
F. limnocharis	Farmland	AF1	18,438	16,714	15,906	660	797.25 (759.73 – 848.88)	794.10 (749.07 – 861.90)	4.975 (4.950 – 5.001)	0.018 (0.018 – 0.019)
F. limnocharis	Farmland	AF2	23,334	21,971	20,716	1011	1358.82 (1287.07 – 1449.22)	1385.21 (1289.75 – 1513.36)	5.297 (5.275 – 5.320)	0.013 (0.013 – 0.014)
F. limnocharis	Farmland	AF3	20,095	18,963	18,403	590	863.05 (797.64 – 949.06)	839.50 (766.51 – 942.68)	2.960 (2.928 – 2.993)	0.152 (0.148 – 0.155)
B. adenopleura	Forest	BN1	15,562	13,495	12,137	473	577.71 (544.93 – 625.44)	573.44 (535.28 – 635.01)	4.760 (4.734 – 4.786)	0.017 (0.017 – 0.018)
B. adenopleura	Forest	BN2	17,135	15,531	14,281	523	611.29 (583.42 – 652.02)	610.55 (576.78 – 665.50)	4.881 (4.857 – 4.905)	0.015 (0.015 – 0.016)
B. adenopleura	Forest	BN3	11,948	8806	7346	427	511.07 (483.76 – 551.52)	512.85 (479.00 – 568.75)	4.700 (4.663 – 4.736)	0.024 (0.022 – 0.025)
B. adenopleura	Farmland	BF1	17,583	16,639	15,983	864	1104.41 (1051.07 – 1172.96)	1146.40 (1069.16 – 1252.73)	4.465 (4.430 – 4.499)	0.050 (0.048 – 0.052)
B. adenopleura	Farmland	BF2	17,350	16,103	15,564	583	825.47 (765.75 – 904.72)	842.37 (763.24 – 956.24)	3.568 (3.534 – 3.603)	0.097 (0.094 – 0.100)
B. adenopleura	Farmland	BF3	18,756	16,578	16,366	328	695.60 (619.92 – 790.92)	602.04 (506.14 – 749.57)	1.356 (1.322 – 1.390)	0.583 (0.573 – 0.592)

OTUs, operational taxonomic units defined by 97% identity threshold of the 16S rRNA gene; ACE, abundance-based coverage estimator.

in the farmland and in the forest sites) showed that 106 OTUs (14.54% of the total number) were found in all four groups (**Figure 2A**). There was a relatively high abundance of common gut microbes between the hosts in farmland sites (47.57%) as well as a higher RA than that found in forest sites (40.21%). On the other hand, *F. limnocharis* within different habitats shared more common gut microbes (35.86%) than *B. adenopleura* (25.93%, **Figure 2A**). Using ternary diagrams, we compared the bacterial distribution between hosts and between habitats (**Figures 3A–D**). In forest sites, we found most microbes common to both frog species were also common in farmland frogs (red points in **Figure 3A**). Forest-specific bacteria were also mostly host specific (i.e., clustering in the two corners of the triangle; **Figure 3A**). In contrast, when we compared the gut bacterial composition between hosts in farmland sites, we observed that the most dominant bacteria were in *F. limnocharis* although they were similar to forest frogs (red points in **Figure 3B**). Most farmland-specific bacteria were shared between the two hosts (i.e., located in the center of the bottom line; **Figure 3B**). This indicated that the gut microbes in forest sites were more divergent between frog species than those found in farmland sites. When we compared the bacterial distribution between habitats in both hosts separately, we saw similar patterns: very few of the common gut bacteria shared between habitats were found in both frog species. In the ternary diagram, the distribution was mainly concentrated around both sides and adjacent to the triangular vertex, in particular in the *B. adenopleura* (**Figures 3C,D**), which indicated the habitat divergence of gut bacteria. This inference was also confirmed by the significant difference for microbial RA between habitats based on random grouping (the Bray–Curtis similarity statistic $R = 0.246$, $P = 0.013$), although there was a non-significant difference between host species ($R = -0.048$, $P = 0.736$).

Identifying Host and Habitat Specialists

The top 3 common microbial phylum were used to verify the major composition between samples. No group can be clearly distinguished from other samples (Supplementary Figure S4). As the common microbes (i.e., the generalist or core microbiota) could confound estimates of habitat and/or host effects on gut microbial composition, we removed OTUs common to all habitats and hosts as well as those OTUs deemed too rare to be classified using the supermajority rule for assessing the host and habitat effects on gut microbial composition. By comparing different host species, we found 128 (17.6%) OTUs were generalists, 45 (6.2%) were specialists of *F. limnocharis*, 43 (5.9%) were specialists of *B. adenopleura*, and 513 (70.4%) were deemed "too rare" OTUs. When comparing habitats, 77 (10.6%) OTUs were generalists, 49 (6.7%) were specialists of *F. limnocharis*, 93 (12.8%) were specialists of *B. adenopleura*, and 510 (70.0%) were "too rare"-type OTUs (**Figure 4**). When comparing one group with the remaining samples, CLAM revealed similar results. There were 71.6–77.7% OTUs assigned to be "too rare," and 4.5–11.5% OTUs were generalists to specific habitat of different host (Supplementary Figure S5). We retained the specialists of these

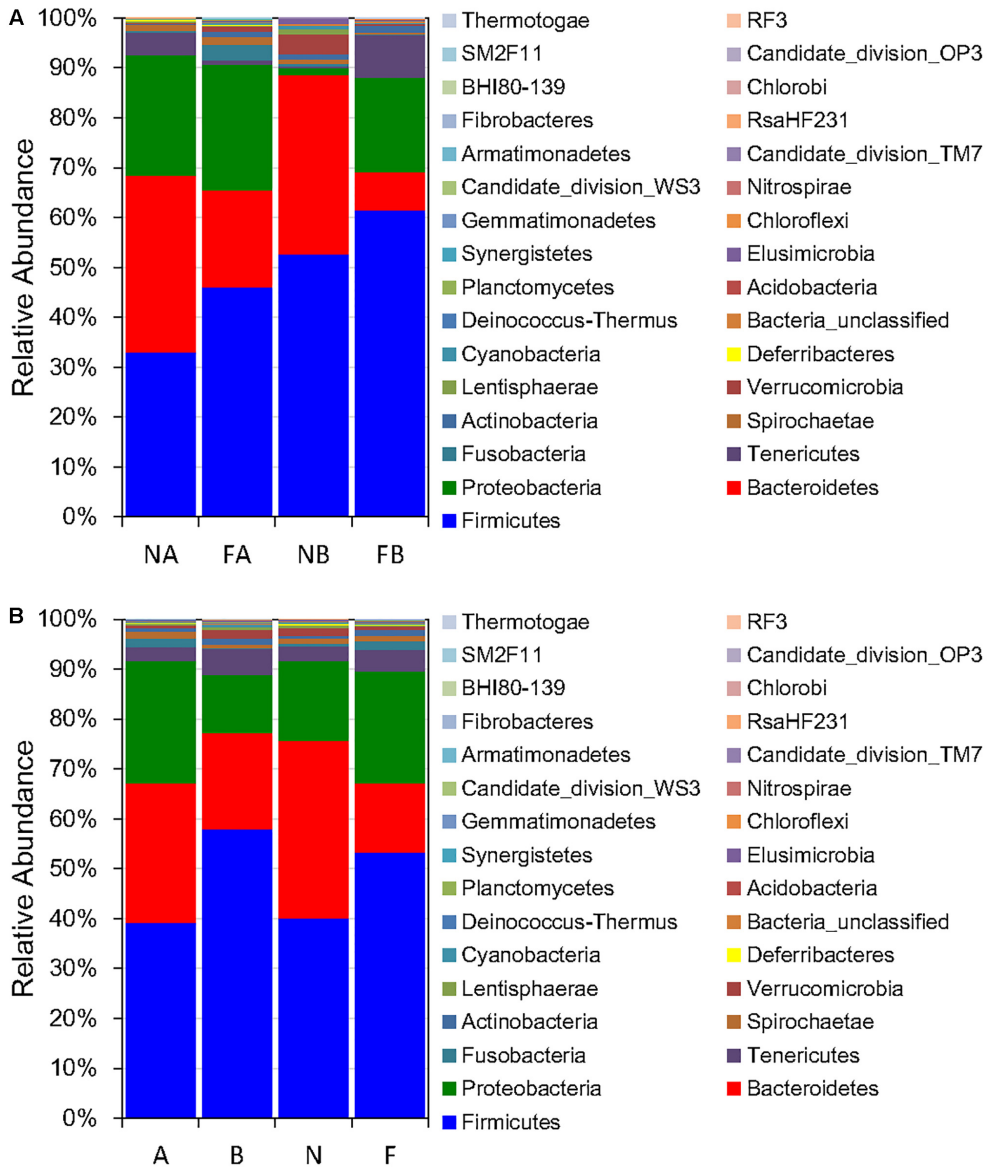

FIGURE 1 | Relative abundance of microbial phyla **(A)** classified by both habitat and host (F, farmland; N, forest; A, *Fejervarya limnocharis*; B, *Babina adenopleura*, $n = 3$ each), and **(B)** classified by either habitat or host ($n = 6$ each).

two host/habitat datasets (157 OTUs, 21.5%) for subsequent analyses. First, we used PCA to verify the compositional differences of these specialists. Gut microbial communities from different habitats were clearly separated along in the first two principal components (explaining 44.1% of total variation, **Figure 5A**). However, the microbial communities were not distinguished between host species (**Figure 5B**). Similar results were also revealed in PERMANOVA showing that the variance of microbial community composition could be significantly explained by habitat effect ($R^2 = 0.379$, $P = 0.001$) but not by host effect ($R^2 = 0.123$, $P = 0.109$) nor by the joint effect of habitat × host ($R^2 = 0.084$, $P = 0.210$, **Table 3**).

Quantifying the Explanatory Variance of Gut Microbial Beta-Diversity Due to Habitat

Given that most analyses showed that the habitat was most responsible for determining the beta-diversity of gut microbiota, we then wished to examine the proportion of variance explained by the habitats. As such, we applied a dbRDA based on the Euclidian distance of RA for OTUs. The dbRDA showed that the constrained variables (habitat, frog species, and host × habitat) explained 31.3% of the variance (14.1, 8.0, and 9.3%, respectively), while unconstrained factors (68.7%) explained a larger proportion of variation.

TABLE 2 | Relative abundance of the five most dominant" microbial phyla in the gut of sampled frogs and the significance tests for the differences of relative abundance between host species and between host habitats.

Phylum	Relative abundance				MW test[a]		KW test[b]		Two-way ANOVA[c]		
	AN	AF	BN	BF	Host	Habitat	Host	Habitat	Host	Habitat	Host × habitat
Firmicutes	0.377 ± 0.105	0.459 ± 0.016	0.566 ± 0.175	0.611 ± 0.215	0.180	0.699	0.150	0.631	0.143	0.562	0.869
Bacteroidetes	0.410 ± 0.138	0.195 ± 0.099	0.324 ± 0.146	0.076 ± 0.053	0.394	0.041*	0.337	0.037*	0.246	0.022*	0.847
Proteobacteria	0.156 ± 0.184	0.251 ± 0.127	0.015 ± 0.007	0.191 ± 0.107	0.310	0.041*	0.262	0.037*	0.285	0.161	0.660
Tenericutes	0.028 ± 0.038	0.009 ± 0.007	0.003 ± 0.002	0.087 ± 0.121	0.937	0.485	0.873	0.423	0.565	0.244	0.287
Verrucomicrobia	0.002 ± 0.002	0.010 ± 0.005	0.037 ± 0.035	0.003 ± 0.002	0.485	0.818	0.423	0.749	0.306	0.321	0.129

[a]*P-value of the two-group Mann–Whitney U-test.* [b]*P-value of the Kruskal–Wallis rank sum test.* [c]*P-value of the two-way ANOVA with a model including the interaction between host and habitat effects.* $^*P < 0.05$.

A further significance test via a type-II ANOVA on the effect of these two constraining factors showed that only habitat could significantly explain the beta-diversity of the gut microbiota ($P = 0.004$). Neither the host ($P = 0.626$) nor the host × habitat effects ($P = 0.246$; **Table 4**) explained gut beta-diversity. Thus, difference in habitat, despite representing only approximately a seventh of the total proportion of the variance (14.1%), governs the beta-diversity of frog gut microbial communities.

Prediction of Functional Content of Gut Bacteria Using the 16S rRNA Metagenome

As statistical analyses demonstrated that gut microbiota was differentiated by habitat, we investigated whether these gut bacteria function differentially on metabolism or physiology of host species. Bioinformatic functional profiling by PICRUSt (Langille et al., 2013) predicted 294 functional groups in the third-level KEGG pathways. Among them, we predicted 255 functional groups in all four sampling sets (**Figure 2B**) and 11 and 3 unique functional groups in *F. limnocharis* and *B. adenopleura*, respectively, in the farmland sites. Eleven functional groups were shared among frog species in the farmland sites (**Figure 2B**). In contrast, no unique or specific functional groups of gut bacteria were predicted for frogs from forest sites. The relatively high number of common functional groups (in contrast to the taxonomic composition of the gut microbiota) indicated highly conserved functions of gut microbiota among hosts and habitats (i.e., core functions). The farmland-specific functional groups of gut microbiota implied a higher niche differentiation between Anura species than within the highly disturbed habitats.

Such inference was supported by the ternary diagrams (**Figures 3E–H**). When we compared the two hosts in forest sites, most functional groups of gut bacteria were close to the vertex of the triangle (**Figure 3E**), indicating that there were no forest-specific functional groups. In contrast, the most abundant functional group, as well as most functions, were closer to the bottom line in **Figure 3F**, indicating a higher functional diversity in gut bacteria for the farmland frogs. This suggested that most metabolic or physiological functions were shared among the two frog species. When comparing functions between the different habitats in the two hosts, we observed a similar trend of more functional groups skewed toward the forest in both *F. limnocharis* (**Figure 3G**) and *B. adenopleura* (**Figure 3H**). This pattern argues for a higher functional diversity of frogs' gut bacteria in farmland sites relative to forest sites.

Identifying the Specific Functional Groups of the Gut Bacteria

By supermajority rule, we identified 17 (5.78%) forest-specific and 29 (9.86%) farmland-specific functional groups of gut bacteria (**Figure 4D**). In addition, we identified 18 (6.12%) and 8 (2.72%) *F. limnocharis*-specific and *B. adenopleura*-specific functional groups, respectively (**Figure 4C**). Bar-plots

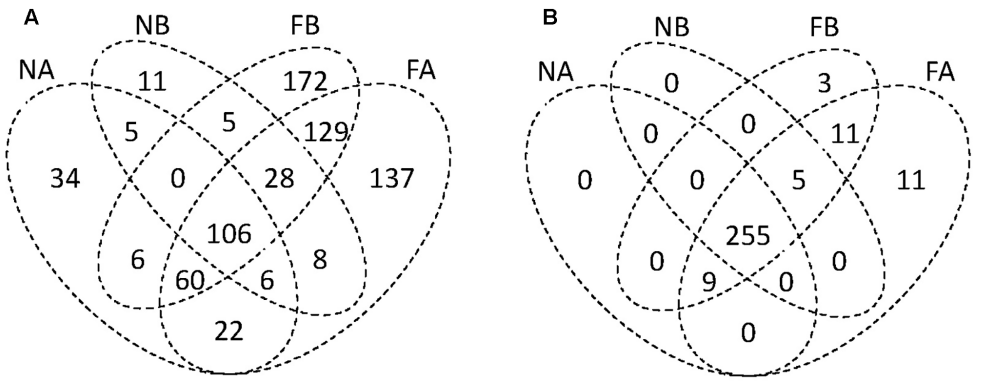

FIGURE 2 | Venn diagrams showing the compositional similarity and uniqueness of **(A)** bacterial OTUs and **(B)** functional groups among four metacommunities. A, *Fejervarya limnocharis*; B, *Babina adenopleura*; F, farmland; N, forest.

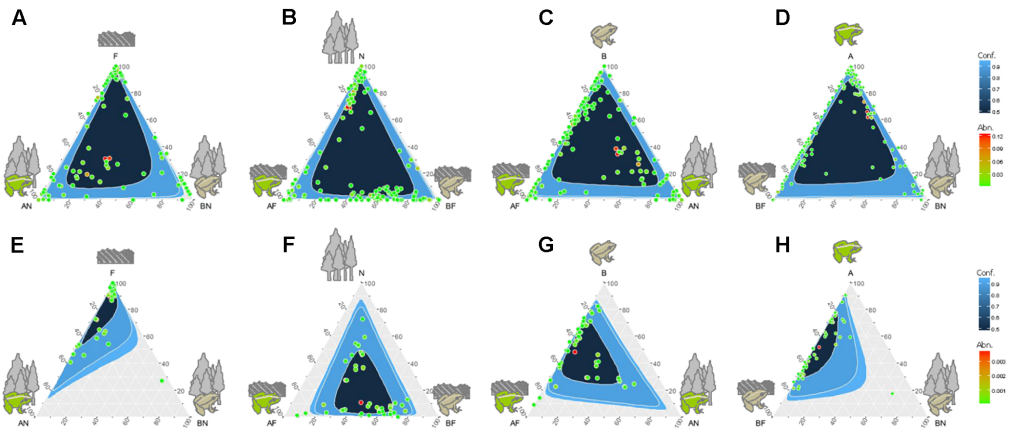

FIGURE 3 | Ternary diagrams comparing the abundance (Abn) of gut bacterial composition **(A)** between different host frogs in the forest; **(B)** between different host frogs species in the farmland; **(C)** between the different habitats of *Fejervarya limnocharis*; **(D)** between the different habitats of *Babina adenopleura*; comparisons of the abundance of functional groups **(E)** between different host frogs in the forest; **(F)** bacteria between different host frogs in the farmland; **(G)** between the different habitats of *Fejervarya limnocharis*; **(H)** between the different habitats of *Babina adenopleura*. A, *Fejervarya limnocharis*; B, *Babina adenopleura*; N, forest; F, farmland.

(**Figure 6**) clearly highlight the differences in RA of these predicted functions for gut microbiota. We then used these specific functional groups to perform PCA, PERMANOVA, and dbRDA (as performed for accessing gut bacterial composition). Our results showed that (1) in PCA, the functional groups were grouped by host habitats (**Figure 5C**), although undistinguished by host species (**Figure 5D**); (2) PERMANOVA demonstrated that the habitat significantly explained the variance of functional groups ($P = 0.004$) but that neither hosts ($P = 0.521$) nor host × habitat ($P = 0.927$; **Table 3**) could account for the variance; and (3) the constraining factors explained 45.3% of the variance of functional groups of gut bacteria, in which the habitat, host, and host × habitat explained 26.0, 14.6, and 4.7% of the total variance, respectively. Only the habitat was able to significantly explain the beta-diversity of gut bacterial functions ($P = 0.004$; **Table 4**). An observed difference for the estimates of microbial composition is that the hosts had a marginally significant explanation for the functional divergence of gut bacteria ($P = 0.062$), revealing different physiological adaptabilities between the two frog species. These results (1) indicate a higher proportion of generalists in functional groups than in the overall gut bacterial composition, regardless of hosts or habitats; and (2) suggest that habitat remains the main factor governing the beta-diversity of the metabolic and physiological functions of frog gut microbiota.

DISCUSSION

Composition of the gut microbiota is a consequence of competition between the foreign and the native (resident) microbiota, which prohibit the establishment of both probiotic and pathogenic microbes (Lozupone et al., 2012). Experiments involving antibiotic treatments with Lewis rates indicate that exogenous interference reduced, rather than facilitated, the colonization by exotic bacteria, while the resident bacteria may be more plastic than previously thought (Manichanh et al.,

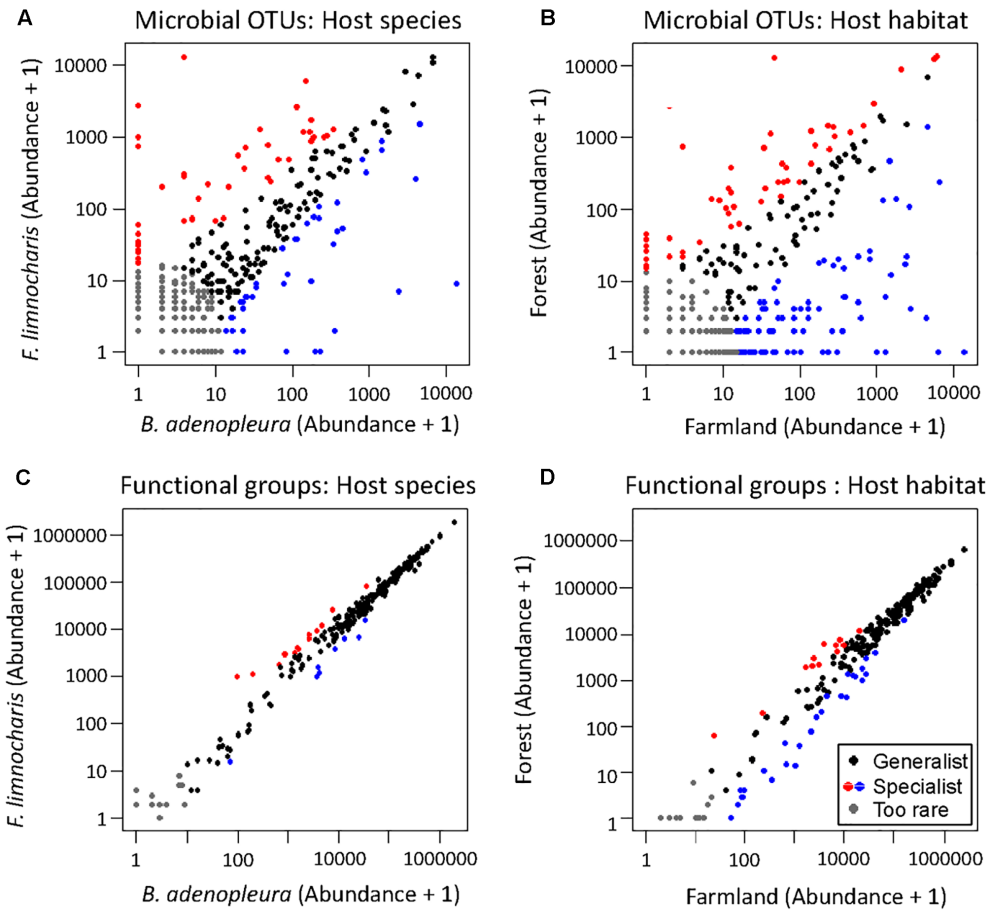

FIGURE 4 | CLAM plots showing the grouping of generalists, specialists, and "too rare" bacteria **(A,B)**, and the functional groups **(C,D)** within gut microbial communities for different host species **(A,C)** and different host habitats **(B,D)**. These plots show that the microbial composition and the functional groups of the gut microbiota are more sensitive to habitat than the host. These gut bacteria reveal a highly functional convergence of the species assemblage.

2010). In mice, the intestinal microflora is affected by host genotypes, emphasizing the dependence of gut microbiota on a particular host (Deloris Alexander et al., 2006). If such phenomena are prevalent in animals, we hypothesized that the composition of gut microbiota should be governed by the endogenous gut environment that is shaped by the physical, physiological, and immune properties of host species, and would be less influenced by the surrounding environment. Our hypothesis is supported given the high proportion of common gut microbes (i.e., generalists, **Figures 4A,B**), and the most abundant common phylum as indication in other vertebrates (Ley et al., 2008), Bacteroidetes, Firmicutes, and Proteobacteria, do not show obvious differences in composition among habitat or host species (Supplementary Figure S4). This implies that the endogenous environment selects microbes that are optimally fit for the gastrointestinal characteristics. Nevertheless, a significant effect of habitat was detected by multiple statistical assessments (**Tables 3**, **4**). This effect was noted even given the large proportion of generalists (Supplementary Tables S3, S4, estimated by total 16S rRNA sequences) meaning that the external environments still positively affect the composition of gut microbiota (see, for example, Ley et al., 2006; Sullam et al., 2012; Wong and Rawls, 2012; Giatsis et al., 2015; Bletz et al., 2016; Chang et al., 2016).

However, changes in the composition of the gut microbiota as the habitat is altered might not severely impact core physiological functions of gut microbiota. In metabolic functional predictions, a relatively higher proportion of functional generalists was inferred than generalists for microbial OTUs, implying that most of the replacing gut bacteria still harbored similar physiological functions (e.g., functional redundancy). This pattern could be beneficial for increasing the resilience and persistence of the functional stability of gut microbiota (Elmqvist et al., 2003; Lozupone et al., 2012). In other words, the stable physiological states in a changing gut environment are preserved (Bletz et al., 2016) by a tight interaction between microbial metabolic activities rather than the taxonomic composition of microbes. This is also shown by a more significant correlation, despite a greater dispersal, on a heat map of the microbial metabolic functional groups than of the microbial composition (**Figure 7**

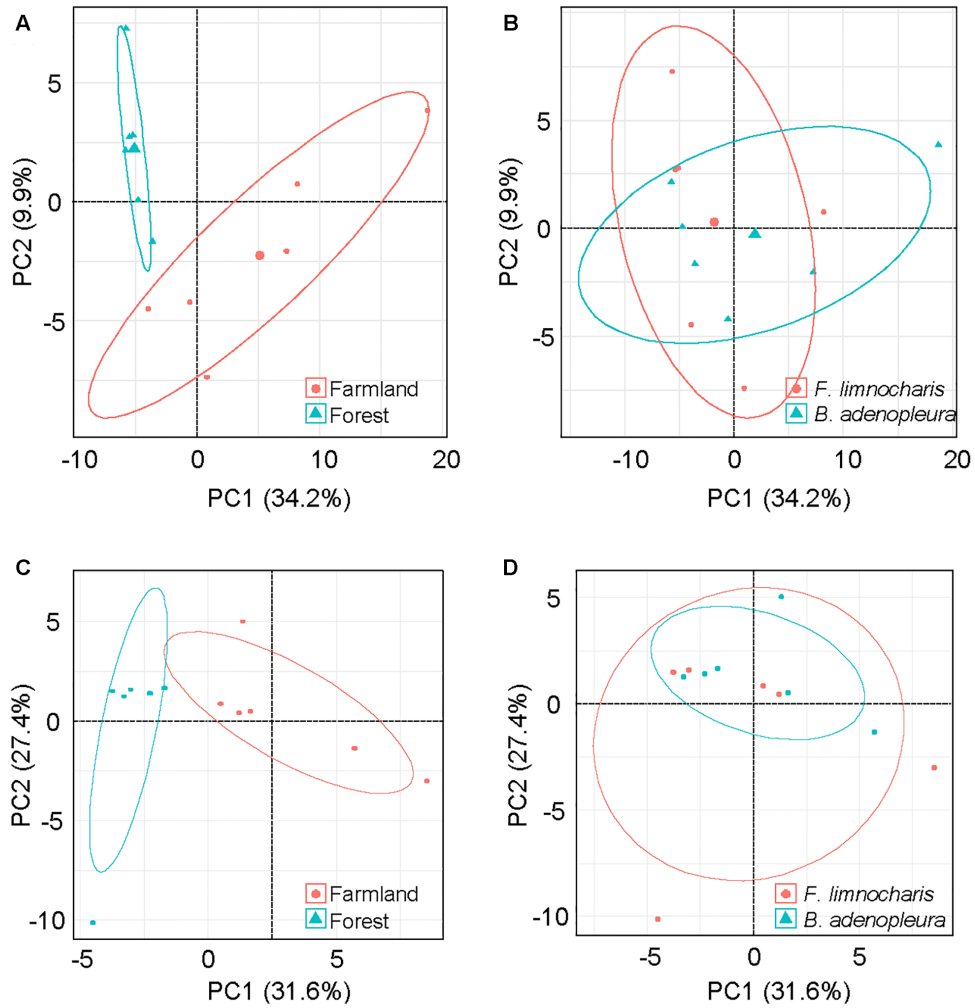

FIGURE 5 | Principal component analysis (PCA) of gut microbial communities based on the relative abundance of **(A,B)** microbial phyla and **(C,D)** functional groups of gut bacteria. Samples from the microbial community were grouped by **(A,C)** habitat, and **(B,D)** host.

and Supplementary Table S5). Such greater significant correlations in functional cohorts compared with taxonomic cohorts implies physiological links rather than phylogenetic associations between co-occurring microbes in frog gut microbiota.

The environment in which the host lives affects gut microbial diversity (Giatsis et al., 2015; Bletz et al., 2016; Chang et al., 2016). Such influence can be mediated by environment–diet–microbe–host interactions (Zhernakova et al., 2016). Natural habitats (e.g., forest) are believed to have

TABLE 3 | Permutational multivariate analysis of variance (PERMANOVA) for testing the effect of habitat and host classification on the variance of gut bacterial composition and the functional groups of gut bacteria as estimated by the specialists of host species and host habitats.

		Gut microbial composition					Functional groups of gut bacteria				
	df	Sums of Sqs	Mean Sqs	F. model	R^2	*P*	Sums of Sqs	Mean Sqs	F. Model	R^2	*P*
Habitat	1	347.27	347.27	7.331	0.379	0.001*	129.03	129.03	4.193	0.312	0.004*
Host	1	112.67	112.67	2.379	0.123	0.109	25.95	25.95	0.843	0.063	0.521
Habitat × host	1	76.69	76.69	1.619	0.084	0.21	11.77	11.77	0.383	0.029	0.927
Residuals	8	378.98	47.37		0.414		246.16	30.77		0.596	
Total	11	915.61			1		412.92			1	

*$P < 0.05$. *Sqs, sum of square.*

TABLE 4 | Distance-based redundancy analysis for quantifying the significance of habitat and host effects on the gut bacterial composition and the functional groups of gut bacteria as estimated by the specialists of host species and host habitats.

	Gut microbial composition				Functional groups of gut bacteria			
	Sum of Sqs	Proportion	*F*	*P*	Sum of Sqs	Proportion	*F*	*P*
Total	1.33	1			8.337E-5	1		
Constrained	0.417	0.313			3.777E-5	0.453		
Habitat	0.187	0.141	1.624	0.004*	2.165E-5	0.260	3.798	0.004*
Host	0.106	0.080	0.922	0.626	1.217E-5	0.146	2.135	0.062
Habitat × host	0.123	0.093	1.08	0.246	3.951E-6	0.047	0.693	0.671
Unconstrained	0.913	0.687			4.560E-5	0.547		

*$P < 0.05$. *Sqs, sum of square.*

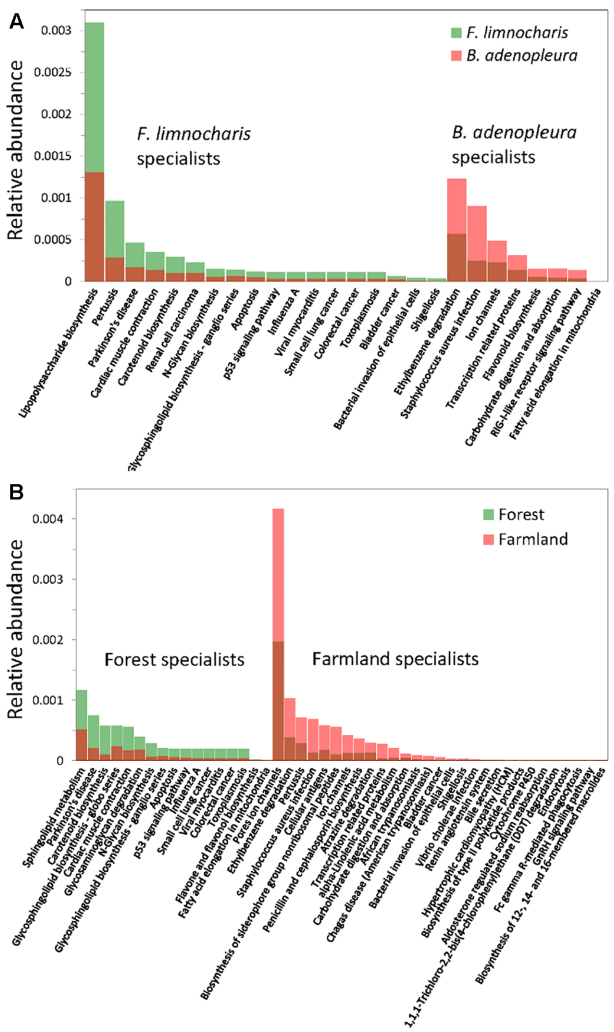

FIGURE 6 | Relative abundance of the functional group specialists of gut microbiota. **(A)** Specialists of hosts; **(B)** specialists of habitats.

diversified nutrient and food resources because of the presence of a healthier ecosystem (Polis et al., 1997) that harbors more diverse (gut) microbiota (Amato et al., 2013; Chang et al., 2016). A global study demonstrated a positive correlation between the plant community and environmental microbial community (Leff et al., 2015), confirming the positive impact of ecosystem diversity on microbial diversity. In our study, however, the farmland frogs, which are subject to frequent environmental disturbance due to agricultural activities, have a higher gut bacterial diversity and more habitat-specific gut bacteria than forest frogs (**Figures 2A**, **4B**). This result is contrary to previous studies suggesting a positive association between the ecosystem health and gut microbial diversity inferred in primates (Amato et al., 2013; Barelli et al., 2015). Surprisingly, farmland frogs harbored a more diversified gut bacterial flora than the forest frogs, in particular species-specific microbes (**Figure 2A**). The ternary diagram also showed an obvious skew toward the farmland frogs in terms of functional groups (**Figures 3G,H**). Under a disturbed environment, frog species should undergo some stress such as changes in food resources (Chang et al., 2016) that may alter the physiological conditions of frogs and further influence the gut environment and impact on the gut microbiota (Toft and Andersson, 2010). Our previous study inferred a high risk of invasive disease in frogs in farmlands due to a relatively high proportion of the phyla Proteobacteria, Actinobacteria, Acidobacteria, and Planctomycetes in frog guts (Chang et al., 2016). We suggest that such highly diversified gut microbiota of farmland frogs reflects their physiological, metabolic, and ecological responses to environmental disturbance. More specialized functional groups that skew toward gut bacteria in the farmland habitat (**Figures 3E–H**) also support a hypothesis of high "functional response diversity" of the gut microbiota in farmland frogs to compensate for the environmental disturbance (Elmqvist et al., 2003).

Although the physiological and metabolic functions of the gut microbiota are more stable than the microbial composition between different environments (**Figure 4**; Bletz et al., 2016), specialized functional groups seem more capable of responding to given habitat characteristics. Among them, the functional specialists of forest frog microbiota are mostly the physiological and cellular metabolites, while those of farmland frog microbiota are composed additionally of functions related to pesticide degradation (ethylbenzene, atrazine, and DDT degradation) and pathogenic diseases (e.g., pertussis, *Staphylococcus aureus* infection, penicillin and cephalosporin biosynthesis, trypanosomiasis, bacterial invasion of epithelial

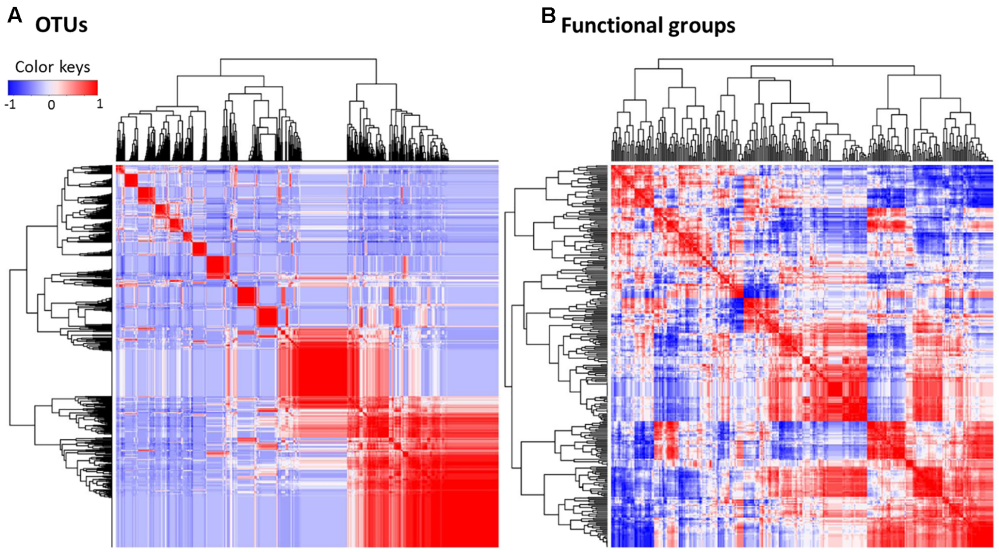

FIGURE 7 | Spearman's correlation of the relative abundance profiles, which were calculated between **(A)** one OTU and all other OTUs, or between **(B)** one predicted functional groups and other groups of the gut bacteria. The tighter and more distinguishable clusters in bacterial OTUs **(A)** in contrast to the more dispersed and indistinguishable clusters of the functional groups **(B)** suggest more stable physiological functions compared to the composition of the gut microbial assemblage in changing environments. Red and blue colors indicate the positive and negative correlations, respectively. Color intensity represents the strength of correlation.

cells, Shigellosis, and *Vibrio cholerae* infection) (**Figure 6B**). Since the microbial community is the unit of selection under specific conditions (Day et al., 2011), properties of microbial composition and the ecological functions of these microbial communities could be seen as the elements that reflect the hosts' survival risks (Weng et al., 2016). This inference of increased pathogenic bacteria and changes of physiological functions under artificial interference on amphibian gut microbiota is similar to the consequence of the increasing risk of bacterial infections under hibernation induced in laboratory (Weng et al., 2016). Given these results, gut microbial ecosystem not only mirrors the ecological condition of the habitat, but also reflects the fitness (e.g., health) of host species in that environment. Hence, our results also indicate that, even if an amphibian may not be sensitive to environmental pollution (Kerby et al., 2010), its gut microbes are sensitive.

Such functional specialists were also illustrated between hosts (**Figure 6A**). Functional groups related to the health status were highly abundant in *F. limnocharis* (e.g., pathogenic diseases: pertussis, influenza A, viral myocarditis, toxoplasmosis, Shigellosis, bacterial invasion of epithelial cells; physical health: cardiac muscle contraction, cancers; **Figure 6A**). We also found an interesting link to several cancer-related functions (renal cell carcinoma, small cell lung cancer, colorectal cancer, bladder cancer, N-glycosylation, glycosphingolipid biosynthesis, apoptosis, p53 signaling pathway) for the gut microbiota of *F. limnocharis* (**Figure 6A**). Several studies have also shown links between cancer and the gastrointestinal microbiome (Schwabe and Jobin, 2013; Bultman, 2014; Hullar et al., 2014; Johnson et al., 2016). The gut microbiota may reciprocally affect, and be affected by, the mucosal integrity, development, and activity of immune system of hosts (Schwabe and Jobin, 2013), reflecting the health status of hosts. Abundant disease-related functions in the gut microbiota of *F. limnocharis* imply the poor adaptation of this species within our sampling area, or under strong selective pressure of disease-associated inflammation (Börnigen et al., 2013; Kreisinger et al., 2014; Loudon et al., 2014). In contrast, *B. adenopleura* harbors a greater number of functions related to pesticide degradation (e.g., ethylbenzene degradation; **Figure 6A**). The uses of pesticide and agro-chemical were common in amphibian habitat, especially for frog inhabit near the farmland of Northern Taiwan, which is corresponding to our sampling sites (Lin et al., 2008), indicating that the selective pressure in farmland (e.g., pesticide and pathogens) is asymmetric for the two different frog species, despite there are still several factors which may be different among our sampling sites (e.g., Temperature or elevation) which may also contribute to the gut microbial composition difference between habitats. Therefore, we may not confidently conclude that differences in gut microbial compositions between samples were due to uses of pesticide. Our study suggests that compositional and functional prediction of gut microbiota reflects the specific environmental adaptability of adult frogs (Toft and Andersson, 2010).

AUTHOR CONTRIBUTIONS

P-CL and C-WC conceived and designed the experiments. C-WC contributed frog materials. B-HH performed the laboratory experiments. B-HH, C-WH, JG, and P-CL analyzed the data. P-CL wrote the paper. B-HH, C-WC, JG,

and C-WH critically reviewed the manuscript. All authors participated in the discussion and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02670/full#supplementary-material

REFERENCES

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., et al. (2013). Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J.* 7, 1344–1353. doi: 10.1038/ismej.2013.16

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. *Nat. Rev. Immunol.* 8, 411–420. doi: 10.1038/nri2316

Baas-Becking, L. G. M. (1934). *Geobiologie of Inleiding tot de Milieukunde*. The Hague: W.P. Van Stockum & Zoon N.V.

Barbosa, T., and Rescigno, M. (2010). Host-bacteria interactions in the intestine: homeostasis to chronic inflammation. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2, 80–97. doi: 10.1002/wsbm.48

Barelli, C., Albanese, D., Donati, C., Pindo, M., Dallago, C., Rovero, F., et al. (2015). Habitat fragmentation is associated to gut microbiota diversity of an endangered primate: implications for conservation. *Sci. Rep.* 5:14862. doi: 10.1038/srep14862

Bletz, M. C., Goedbloed, D. J., Sanchez, E., Reinhardt, T., Tebbe, C. C., Bhuju, S., et al. (2016). Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nat. Commun.* 7:13699. doi: 10.1038/ncomms13699

Bolanos, L. M., Rosenblueth, M., Castillo-Ramirez, S., Figuier-Huttin, G., and Martinez-Romero, E. (2016). Species-specific diversity of novel bacterial lineages and differential abundance of predicted pathways for toxic compound degradation in scorpion gut microbiota. *Environ. Microbiol.* 18, 1364–1378. doi: 10.1111/1462-2920.12939

Börnigen, D., Morgan, X. C., Franzosa, E. A., Ren, B., Xavier, R. J., Garrett, W. S., et al. (2013). Functional profiling of the gut microbiome in disease-associated inflammation. *Genome Med.* 5:65. doi: 10.1186/gm469

Bultman, S. J. (2014). Emerging roles of the microbiome in cancer. *Carcinogenesis* 35, 249–255. doi: 10.1093/carcin/bgt392

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Chang, C. W., Huang, B. H., Lin, S. M., Huang, C. L., and Liao, P. C. (2016). Changes of diet and dominant intestinal microbes in farmland frogs. *BMC Microbiol.* 16:33. doi: 10.1186/s12866-016-0660-4

Chazdon, R. L., Chao, A., Colwell, R. K., Lin, S. Y., Norden, N., Letcher, S. G., et al. (2011). A novel statistical method for classifying habitat generalists and specialists. *Ecology* 92, 1332–1343. doi: 10.1890/10-1345.1

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* 18, 117–143. doi: 10.1111/j.1442-9993.1993.tb00438.x

Colombo, B. M., Scalvenzi, T., Benlamara, S., and Pollet, N. (2015). Microbiota and mucosal immunity in amphibians. *Front. Immunol.* 6:111. doi: 10.3389/fimmu.2015.00111

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., and Relman, D. A. (2012). The application of ecological theory toward an understanding of the human microbiome. *Science* 336, 1255–1262. doi: 10.1126/science.1224203

Day, M. D., Beck, D., and Foster, J. A. (2011). Microbial communities as experimental units. *Bioscience* 61, 398–406. doi: 10.1525/bio.2011.61.5.9

Deloris Alexander, A., Orcutt, R. P., Henry, J. C., Baker, J. Jr., Bissahoyo, A. C., and Threadgill, D. W. (2006). Quantitative PCR assays for mouse enteric flora reveal strain-dependent differences in composition that are influenced by the microenvironment. *Mamm. Genome* 17, 1093–1104. doi: 10.1007/s00335-006-0063-1

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Dixon, P., and Palmer, M. W. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Drake, H. L., and Horn, M. A. (2007). As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. *Annu. Rev. Microbiol.* 61, 169–189. doi: 10.1146/annurev.micro.61.080706.093139

Du Pasquier, L., Robert, J., Courtet, M., and Mussmann, R. (2000). B-cell development in the amphibian *Xenopus*. *Immunol. Rev.* 175, 201–213. doi: 10.1034/j.1600-065X.2000.017501.x

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Elmqvist, T., Folke, C., Nyström, M., Peterson, G., Bengtsson, J., Walker, B., et al. (2003). Response diversity, ecosystem change, and resilience. *Front. Ecol. Environ.* 1, 488–494. doi: 10.1890/1540-9295(2003)001[0488:RDECAR]2.0.CO;2

Feld, L., Schjorring, S., Hammer, K., Licht, T. R., Danielsen, M., Krogfelt, K., et al. (2008). Selective pressure affects transfer and establishment of a *Lactobacillus plantarum* resistance plasmid in the gastrointestinal environment. *J. Antimicrob. Chemother.* 61, 845–852. doi: 10.1093/jac/dkn033

Funkhouser, L. J., and Bordenstein, S. R. (2013). Mom knows best: the universality of maternal microbial transmission. *PLOS Biol.* 11:e1001631. doi: 10.1371/journal.pbio.1001631

Garrett, W. S., Gordon, J. I., and Glimcher, L. H. (2010). Homeostasis and inflammation in the intestine. *Cell* 140, 859–870. doi: 10.1016/j.cell.2010.01.023

Giatsis, C., Sipkema, D., Smidt, H., Heilig, H., Benvenuti, G., Verreth, J., et al. (2015). The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Sci. Rep.* 5:18206. doi: 10.1038/srep18206

Hamilton, N. (2016). *ggtern: An Extension to 'ggplot2', for the Creation of Ternary Diagrams. R Package Version* 2.2.0. Available at: https://CRAN.R-project.org/package=ggtern [accessed November 23, 2016].

Hooper, L. V., Midtvedt, T., and Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22, 283–307. doi: 10.1146/annurev.nutr.22.011602.092259

Hopkins, W. A. (2007). Amphibians as models for studying environmental change. *ILAR J.* 48, 270–277. doi: 10.1093/ilar.48.3.270

Hourdry, J., LHermite, A., and Ferrand, R. (1996). Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. *Physiol. Zool.* 69, 219–251. doi: 10.1086/physzool.69.2.30164181

Hullar, M. A. J., Burnett-Hartman, A. N., and Lampe, J. W. (2014). Gut microbes, diet, and cancer. *Cancer Treat. Res.* 159, 377–399. doi: 10.1007/978-3-642-38007-5_22

Jasarevic, E., Rodgers, A. B., and Bale, T. L. (2015). A novel role for maternal stress and microbial transmission in early life programming and neurodevelopment. *Neurobiol. Stress* 1, 81–88. doi: 10.1016/j.ynstr.2014.10.005

Johnson, C. H., Spilker, M. E., Goetz, L., Peterson, S. N., and Siuzdak, G. (2016). Metabolite and microbiome interplay in cancer immunotherapy. *Cancer Res.* 76, 6146–6152. doi: 10.1158/0008-5472.CAN-16-0309

Kassambara, A. (2015). *Factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R Package Version 1.0.3*. Available at: http://www.sthda.com

Kerby, J. L., Richards-Hrdlicka, K. L., Storfer, A., and Skelly, D. K. (2010). An examination of amphibian sensitivity to environmental contaminants: are amphibians poor canaries? *Ecol. Lett.* 13, 60–67. doi: 10.1111/j.1461-0248.2009.01399.x

Kohl, K. D., Cary, T. L., Karasov, W. H., and Dearing, M. D. (2013). Restructuring of the amphibian gut microbiota through metamorphosis. *Environ. Microbiol. Rep.* 5, 899–903. doi: 10.1111/1758-2229.12092

Kreisinger, J., Èížková, D., Vohánka, J., and Piálek, J. (2014). Gastrointestinal microbiota of wild and inbred individuals of two house mouse subspecies assessed using high-throughput parallel pyrosequencing. *Mol. Ecol.* 23, 5048–5060. doi: 10.1111/mec.12909

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lawley, T. D., Bouley, D. M., Hoy, Y. E., Gerke, C., Relman, D. A., and Monack, D. M. (2008). Host transmission of *Salmonella enterica* Serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. *Infect. Immun.* 76, 403–416. doi: 10.1128/IAI.01189-07

Leff, J. W., Jones, S. E., Prober, S. M., Barberan, A., Borer, E. T., Firn, J. L., et al. (2015). Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. *Proc. Natl. Acad. Sci. U.S.A.* 112, 10967–10972. doi: 10.1073/pnas.1508382112

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788. doi: 10.1038/nrmicro1978

Ley, R. E., Peterson, D. A., and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell* 124, 837–848. doi: 10.1016/j.cell.2006.02.017

Lin, H. C., Cheng, L. Y., Chen, P. C., and Chang, M. H. (2008). Involving local communities in amphibian conservation: Taipei frog *Rana taipehensis* as an example. *Int. Zoo Yearb.* 42, 90–98. doi: 10.1111/j.1748-1090.2008.00049.x

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature* 489, 220–230. doi: 10.1038/nature11550

Manichanh, C., Reeder, J., Gibert, P., Varela, E., Llopis, M., Antolin, M., et al. (2010). Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome Res.* 20, 1411–1419. doi: 10.1101/gr.107987.110

Polis, G. A., Anderson, W. B., and Holt, R. D. (1997). Toward an integration of landscape and food web ecology: the dynamics of spatially subsidized food webs. *Annu. Rev. Ecol. Syst.* 28, 289–316. doi: 10.1146/annurev.ecolsys.28.1.289

Ramette, A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiol. Ecol.* 62, 142–160. doi: 10.1111/j.1574-6941.2007.00375.x

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schwabe, R. F., and Jobin, C. (2013). The microbiome and cancer. *Nat. Rev. Cancer* 13, 800–812. doi: 10.1038/nrc3610

Semlitsch, R. D. (2008). Differentiating migration and dispersal processes for pond-breeding amphibians. *J. Wildl. Manage.* 72, 260–267. doi: 10.2193/2007-082

Sharma, R., John, S. J., Damgaard, D. M., and McAllister, T. A. (2003). Extraction of PCR-quality plant and microbial DNA from total rumen contents. *Biotechniques* 34, 92–97.

Smith, A. M., and Green, D. M. (2005). Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? *Ecography* 28, 110–128. doi: 10.1111/j.0906-7590.2005.04042.x

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi: 10.1038/nrmicro2540

Stevenson, T. J., Duddleston, K. N., and Buck, C. L. (2014). Effects of season and host physiological state on the diversity, density, and activity of the arctic ground squirrel cecal microbiota. *Appl. Environ. Microbiol.* 80, 5611–5622. doi: 10.1128/AEM.01537-14

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., Knight, R., et al. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol. Ecol.* 21, 3363–3378. doi: 10.1111/j.1365-294X.2012.05552.x

Thomas, F., Hehemann, J. H., Rebuffet, E., Czjzek, M., and Michel, G. (2011). Environmental and gut *Bacteroidetes*: the food connection. *Front. Microbiol.* 2:93. doi: 10.3389/fmicb.2011.00093

Toft, C., and Andersson, S. G. E. (2010). Evolutionary microbial genomics: insights into bacterial host adaptation. *Nat. Rev. Genet.* 11, 465–475. doi: 10.1038/nrg2798

van der Gast, C., Cuthbertson, L., Rogers, G., Pope, C., Marsh, R., Redding, G., et al. (2013). Three clinically distinct chronic pediatric airway infections share a common core microbiota. *Ann. Am. Thorac. Soc.* 11, 1039–1048. doi: 10.1513/AnnalsATS.201312-456OC

van der Gast, C. J., Walker, A. W., Stressmann, F. A., Rogers, G. B., Scott, P., Daniels, T. W., et al. (2011). Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *ISME J.* 5, 780–791. doi: 10.1038/ismej.2010.175

Vences, M., Lyra, M. L., Kueneman, J. G., Bletz, M. C., Archer, H. M., Canitz, J., et al. (2016). Gut bacterial communities across tadpole ecomorphs in two diverse tropical anuran faunas. *Sci. Nat.* 103:25. doi: 10.1007/s00114-016-1348-1

Weng, F. C., Yang, Y. J., and Wang, D. (2016). Functional analysis for gut microbes of the brown tree frog (*Polypedates megacephalus*) in artificial hibernation. *BMC Genomics* 17(Suppl. 13):1024. doi: 10.1186/s12864-016-3318-6

Wiggins, P. J., Smith, J. M., Harris, R. N., and Minbiole, K. P. C. (2011). Gut of Red-backed Salamanders (*Plethodon cinereus*) may serve as a reservoir for an antifungal cutaneous bacterium. *J. Herpetol.* 45, 329–332. doi: 10.1670/10-231.1

Wong, S. D., and Rawls, J. F. (2012). Intestinal microbiota composition in fishes is influenced by host ecology and environment. *Mol. Ecol.* 21, 3100–3102. doi: 10.1111/j.1365-294X.2012.05646.x

Zhang, W., Guo, R., Yang, Y., Ding, J., and Zhang, Y. (2016). Long-term effect of heavy-metal pollution on diversity of gastrointestinal microbial community of *Bufo raddei*. *Toxicol. Lett.* 258, 192–197. doi: 10.1016/j.toxlet.2016.07.003

Zhernakova, A., Kurilshikov, A., Bonder, M. J., Tigchelaar, E. F., Schirmer, M., Vatanen, T., et al. (2016). Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* 352, 565–569. doi: 10.1126/science.aad3369

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Environmental and Host Effects on Skin Bacterial Community Composition in Panamanian Frogs

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Research on the amphibian skin microbiota has focused on identifying bacterial taxa that deter a pathogenic chytrid fungus, and on describing patterns of microbiota variation. However, it remains unclear how environmental variation affects amphibian skin bacterial communities, and whether the overall functional diversity of the amphibian skin microbiota is associated to such variation. We sampled skin microbial communities from one dendrobatoid frog species across an environmental gradient along the Panama Canal, and from three dendrobatoid frog species before and after the onset of the wet season in one site. We found frog skin microbial alpha diversity to be highest in frogs from sites with low soil pH, but no clear effect of the onset of the wet season. However, we found frog skin microbial community structure to be affected by soil pH and the onset of the wet season, which also resulted in a decrease in between-sample variation. Across the sampled frog species, bacterial functional groups changed with the onset of the wet season, with certain bacterial functional groups entirely disappearing and others differing in their relative abundances. In particular, we found the proportion of Bd-inhibitory bacteria to correlate with mean soil pH, and to increase in two of the frog species with the onset of the wet season. Taken together, our results suggest that structure and predicted function of amphibian bacterial skin communities may be influenced by environmental variables such as pH and precipitation, site effects, and host effects.

Keywords: frog skin microbiota, metabarcoding, abiotic factors, *Dendrobates auratus*, *Silverstoneia flotator*, *Allobates talamancae*, chytrid, *Batrachochytrium dendrobatidis*

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INTRODUCTION

The interactions between vertebrate hosts and their microbial communities have recently become a subject of accrued interest (Caporaso et al., 2010b, 2012; Escalona et al., 2016; Rebollar et al., 2016a), because microbiota may play an important role in animal ecology and evolution. For example, microbiota have been found to alter the behavior of their host (Bravo et al., 2011; Ezenwa et al., 2012; Marin et al., 2017), affect digestion (Schnorr et al., 2014) and development (McFall-Ngai et al., 2013), as well as contribute to immune system function (Kueneman et al., 2016a; Woodhams et al., 2016). However, how wildlife-associated microbiota vary according to abiotic factors remains poorly understood (Jiménez and Sommer, 2017; Medina et al., 2017; Pollock et al., 2017).

In amphibians, the skin microbiota are known to vary between host species (Kueneman et al., 2014; Belden et al., 2015; Pollock et al., 2017) and sampling sites (Kueneman et al., 2014; Krynak et al., 2016). It has also been suggested that ontogenetic and seasonal changes contribute to variation in the frog skin microbiota (Longo et al., 2015). Interestingly, microbes abundant on amphibians' skin are usually rare or present in low abundances in the environment, suggesting that the amphibian skin represents a unique and selective environment (Loudon et al., 2014; Walke et al., 2014; Rebollar et al., 2016b). However, it is also clear that amphibians depend on microbial environmental reservoirs, such as forest soil, to maintain diverse skin microbiota (Fitzpatrick and Allison, 2014; Loudon et al., 2014; Kueneman et al., 2016a).

Both moisture (Hartmann et al., 2014) and pH (Fierer and Jackson, 2006; Lauber et al., 2009; Zhalnina et al., 2015; Schappe et al., 2017) drive patterns of variation in soil microbial communities, but whether these abiotic factors indirectly shape the amphibian skin microbial communities remains unclear. Alternatively, soil pH and moisture could potentially have an effect on microbe–microbe and microbe–host interactions, for example, by affecting amphibian antimicrobial peptide secretions (Rinaldi, 2002; Tennessen et al., 2009). Even though many frog skin microbiota studies were focused on identifying sources of variation, whether changes in skin microbial communities correspond with changes in overall microbial functional groups remains poorly understood.

In amphibians, the skin microbiota appear to constitute a critical component of the immune system (Walke et al., 2015; Woodhams et al., 2015; Kueneman et al., 2016b), whereby certain bacterial taxa are capable of producing anti-fungal metabolites (Brucker et al., 2008; Lauer et al., 2008; Harris et al., 2009b). Some of these anti-fungal metabolites deter the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (Bd), which has been associated with certain amphibian declines (Lips et al., 2006; Pounds et al., 2006). One of these agents, the betaproteobacterium *Janthinobacterium lividum* has been isolated from a number of amphibians (e.g., *Plethodon cinereus*, Brucker et al., 2008; Becker and Harris, 2010; *Gastrotheca excubitor*, Burkart et al., 2017), and can reduce Bd-related mortality (Harris et al., 2009a; Becker and Harris, 2010).

To date, only a few studies have empirically assessed variation in the overall microbial functional groups in amphibian hosts (Loudon et al., 2014; Davis et al., 2017). However, ecologically important the anti-Bd function of the skin microbes may be, this constitutes only one of the many functions performed by the amphibian skin microbiota. To have a better understanding of the functional implications of amphibian skin microbiota disruption, for example due to chytridiomycosis or environmental variation, it is necessary to better characterize the composition of these microbiota as a first step in assessing the broad spectrum of functionality that they may represent for the amphibian host. Accordingly, we sought to investigate the possible effects of abiotic environmental variables (i.e., soil pH and precipitation levels) across four sites in one frog species, as well as seasonal and host effects in three species of dendrobatoid frogs within one site in shaping the diversity and predicted function of the skin microbiota. Sites along the Panama Canal inhabited by these frogs differ in soil pH and annual precipitation, with well-defined wet and dry seasons, within a relative small area. Only about 50 km separate the Pacific from the Atlantic termini of the Panama Canal, along which is a steep climatic gradient in precipitation, with average annual precipitation ranging from ca. 1,500 to over 4,000 mm per year (Santiago and Mulkey, 2005; Engelbrecht et al., 2007).

If amphibians require microbial environmental reservoirs, such as forest soil, to maintain diverse skin bacterial communities (Loudon et al., 2014), then environmental variables that drive variation in free-living microbial communities like (1) soil pH, (2) precipitation levels, and (3) the onset of the wet season should correlate with frog skin microbial community structure. Frog skin bacterial diversity should be expected to be highest in frogs sampled from sites with neutral soil pH and high humidity, where soil bacterial diversity is highest (Fierer and Jackson, 2006; Lauber et al., 2009; Hartmann et al., 2014; Zhalnina et al., 2015), in comparison to either pH extreme and dryer sites. We also explored (4) host-specific differences on the skin microbiota. Finally, (5) if soil pH, precipitation levels, the onset of the wet season, and host-related skin microbial community shifts are ecologically relevant to these three dendrobatoid frog species, then we should observe a relationship between them and predicted microbial functional group variation, including the presence or absence of bacteria that may either inhibit or facilitate the growth of Bd.

MATERIALS AND METHODS

Sampling Sites and Frog Species

In total, we sampled 70 dendrobatoid frogs (**Table 1**) from four sites along the Panama Canal in April 2016 (**Figure 1**). Cerro Ancon is a secondary growth urban forest, whereas the other three sites are characterized by old growth forests (Ibáñez et al., 2002). For each site, soil pH was recorded from soil samples (2–10 cm from surface) mixed in water (1:2, soil:water ratio; **Table 2**). We obtained historical annual mean and daily precipitation records from the Panama Canal Authority weather station closest to each site (20–136 years of records), and we used the daily values to calculate accumulated precipitation 5, 10, and 15 days before sampling. We used these accumulated precipitation values to confirm the beginning of the wet season. We believe that these time intervals could explain microbiota variation, as previous studies have recorded changes in amphibian skin microbiota in as little as 7 days in captivity (Loudon et al., 2014) and 3 days in field experiments (Longo and Zamudio, 2016).

To test the effects of soil pH and precipitation levels on skin microbiota, we focused our sampling on juvenile and adult *Dendrobates auratus* (N = 9–19 per site, four sites), a frog living in the leaf litter where soil pH is likely affecting its microbiota (dataset "Dend.aura"). We included *D. auratus* life stage as a fixed factor in our models to account for previously reported ontogenetic differences (Longo et al., 2015). To test seasonal effects on skin microbiota in one site (Pipeline Road), we sampled

TABLE 1 | Host species habitat and sample sizes of the three dendrobatoid frogs by life stage.

Host species (sample size)	Host's habitat	Site-specific sample sizes (before, after onset of wet season)			
		Fort Sherman	Cerro Ancon	Pipeline Road	Barro Colorado Island
Dendrobates auratus (58)	Leaf litter	8A, 1J	12A, 7J	9A, 2J (7A 1J, 2A 1J)	15A, 4J
Silverstoneia flotator (6)	Stream-associated			6A (2, 4)	
Allobates talamancae (6)	Stream-associated			6A (2, 4)	

Life stage: A, adult frogs or J, juvenile frogs.

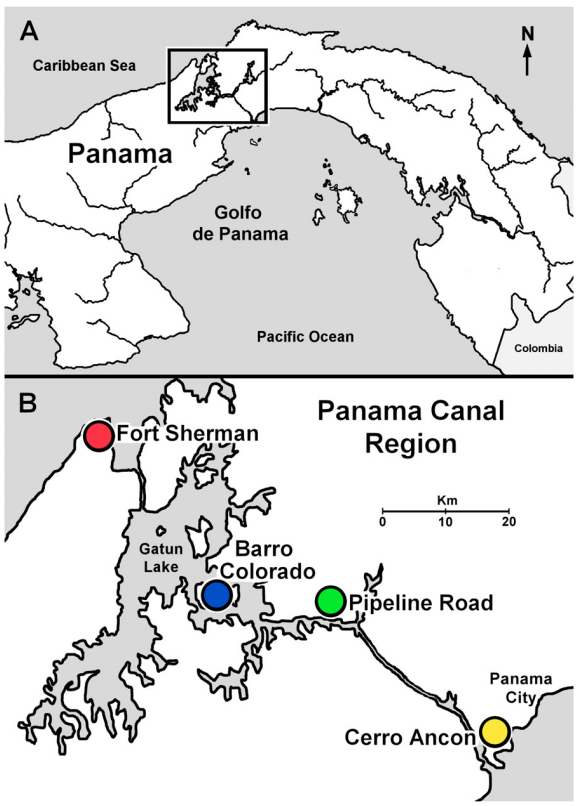

FIGURE 1 | Sample sites along the Panama Canal in Central Panama. **(A)** Central Panama showing location of the Panama Canal region (inset). **(B)** Panama Canal region indicating sample localities.

three dendrobatoid frog species before and after the onset of the wet season (*D. auratus* $N_{before} = 8$, $N_{after} = 3$; *Silverstoneia flotator* $N_{before} = 2$, $N_{after} = 4$; and *Allobates talamancae* $N_{before} = 2$, $N_{after} = 4$, dataset "Pipeline"). To test differences between frog species, we used the "Pipeline" dataset and controlled for sampling time (i.e., "before" or "after" the onset of the wet season).

To collect our samples, we caught frogs with gloved hands and rinsed them with 50 ml of distilled autoclaved water to wash off any transient microbes (Loudon et al., 2014; Rebollar et al., 2016b). After rinsing, we swabbed the frogs with two sterile cotton tipped swabs 10 times on the ventral side, 10 times on each leg and each toe once. We used new gloves for each frog to prevent cross-contaminating our samples. We stored the swabs in

TABLE 2 | Abiotic factors for the sites sampled along the Panama Canal: soil pH and precipitation levels.

Site (sampling date)	Mean soil pH ± SE (*n*)	Prec. 5/10/15	Mean annual precipitation
Fort Sherman (4 April 2016)	4.7 ± 0.3 (26)	0/0/2	255.5
Cerro Ancon (7 April 2016)	5.4 (1)	0/0/0	153.3
Pipeline road* (9 and 23 April 2016)	5.6 ± 0.3 (17)	0/0/0 7/7/8	178.9
Barro Colorado Island (11 and 12 April 2016)	6.2 ± 0.5 (10)	1/1/1	204.9

**We resampled Pipeline Road as soon as the rainy season started; thus, we provide two sets of cumulative precipitation values.*

sterile, autoclaved micro-centrifuge tubes on ice until they could be stored in a −20°C freezer prior to DNA extraction. We used one swab for DNA extraction to test Bd presence and to construct 16S rRNA libraries. We stored the second swab at −80°C as a reference.

Research permits allowing the collection of our samples were granted by the Panamanian authority Ministerio de Ambiente (Permit No. SE/A-38-16) and by the Authority of the Panama Canal (Permit No. 173). All procedures with animals were conducted under approved animal care protocols from the Smithsonian Tropical Research Institute's Animal Care Committee (2016-0301-2019-2) and by the McGill University's Animal Care Committee (2000-4569).

DNA Extraction, Bd Analyses, and 16S rRNA Library Preparation

We treated swabs with a lysozyme buffer to break up Gram-positive bacteria's cell walls, followed by a modified protocol of the QIAGEN DNeasy Blood & Tissue Kit.

To test for Bd presence, we performed the qPCR assay developed by Boyle et al. (2004) with the following modifications. We extracted DNA from swabs using the QIAGEN DNeasy Blood & Tissue Kit (Kosch and Summers, 2013; Bletz et al., 2015). We performed the qPCR reactions on a Roche LightCycler 96 System, and a volume of 20 µl per well (Kriger et al., 2006a). Instead of using Applied Biosystems 2× TaqMan Master Mix, we used Roche FastStart Essential DNA Probes Master and we added 0.25 µl of Roche LightCycler Uracil-DNA Glycosylase per reaction to eliminate potential PCR carryover contamination. We used Applied Biosystems TaqMan Exogenous

Internal Positive Control Reagents VIC Probe (Hyatt et al., 2007), and we included five negative and two Bd-positive controls on every plate (Kriger et al., 2006a,b). We performed a duplicate analysis by initially running a 1:10 dilution of the DNA extract from each sample in singlicate, followed by a second run of the undiluted DNA extract in singlicate to avoid false negatives due to a potentially low and undetectable quantity of DNA in the diluted samples. Samples in which Bd was not detected in both runs of the duplicate analysis were considered negative. Despite the fact that the sites we sampled have previously been reported as Bd endemic (Woodhams et al., 2008; Rebollar et al., 2014), and Bd might still be present in other amphibian species, all our samples tested negative for Bd; therefore, we did not include Bd as a factor in our statistical analyses.

To construct our 16S rRNA library, we amplified each sample following Caporaso et al. (2012), using primers F515 and R806. We pretreated the PCR pools with Just-a-Plate^{TM} PCR 96 Purification and Normalization Kit (Charm Biotech, San Diego, CA, United States). We used equimolar parts of each sample to construct the library and cleaned it with Serapure beads to remove any primer dimers. DNA concentration and quality of the library were assessed with NanoDrop^{TM} ND-1000 (Thermo Fisher Scientific Inc., 2008, Wilmington, DE, United States), Invitrogen Qubit® Fluorometer (Thermo Fisher Scientific Inc., 2007, Wilmington, DE, United States), and Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., 2000–2005, Santa Clara, CA, United States). The library was sequenced at the NAOS laboratories of the Smithsonian Tropical Research Institute in Panama using an Illumina MiSeq v3 600 cycles cartridge.

Sequence Reads Processing

We processed all samples in the same manner before separating them into the two data sets. Using Quantitative Insights into Microbial Ecology (QIIME, Caporaso et al., 2010b), we filtered sequences to retain high-quality reads. We clustered sequences according to 97% sequence similarity threshold using the UCLUST method (Edgar, 2010). The resulting operational taxonomic units (OTUs) were classified using the Greengenes database when possible (May 2013 release; McDonald et al., 2012); otherwise they were clustered *de novo* using a 97% sequence similarity threshold. We assigned taxonomy with the Greengenes database and the RDP classifier (Wang et al., 2007). We used PyNAST (Caporaso et al., 2010a) to align representative sequences to the Greengenes database and constructed a phylogenetic tree with FastTree2 (Price et al., 2010). Lastly, we filtered the resulting OTU table to only include clusters $\geq$0.001% of the total reads (Bokulich et al., 2013). To standardize sequencing effort, we rarefied the samples to a depth of 6,813 sequences. This initial dataset included 27,120 bacterial OTUs from 70 samples. As demonstrated by plateauing rarefaction curves, the samples had appropriate sequencing coverage, which indicates that our sampling captured most of the bacterial alpha diversity (Supplementary Figure S1). For downstream analyses, we divided the initial dataset into a subset of 22,846 OTUs from 58 samples of *D. auratus* from the four sites ("Dend.aura"), and a subset of 12,354 OTUs from 23 samples from all three species from Pipeline Road ("Pipeline"). Sequences for each sample were deposited in the NCBI SRA under BioProject ID PRJNA433445.

Comparing Alpha and Beta Diversity

Unless otherwise stated, all statistical and graphical analyses were performed using R 3.3.2 (R Core Team, 2017, Vienna, Austria) and the *ggplot2* package (Wickham, 2009). Full test statistics can be found in Supplementary Table S1.

To estimate alpha diversity of skin microbiota per frog species and site, we calculated Shannon's diversity index, which measures the number of species and their evenness within a sample. Using the *lme4* package (Bates et al., 2015) and the "Dend.aura" dataset, we tested for an effect of mean soil pH and precipitation levels on bacterial alpha diversity, including site as a random factor and life stage as a fixed factor. We tested for an effect of the onset of the wet season on alpha diversity using unpaired *t*-tests (two-tailed) for each of the three host species from Pipeline Road ("Pipeline") sampled before and after the onset of the wet season. We also tested for host effects on bacterial alpha diversity among the Pipeline Road sample using linear mixed effects model with sampling time as a random factor (i.e., "before" or "after" the onset of the wet season).

We used the data derived from *D. auratus* ("Dend.aura") samples to test the effect of mean soil pH and precipitation levels on bacterial beta diversity, and data from Pipeline Road ("Pipeline") samples of all three host species to test the effect of the onset of the wet season and host species effects on bacterial beta diversity. We calculated the UniFrac Weighted and UnWeighted (Lozupone and Knight, 2005) and Bray–Curtis distances matrices, and obtained NMDS ordinations. To determine differences between groups, we used the *adonis* function within the *vegan* package (Oksanen et al., 2016) and 999 permutations. We controlled for site effects by including site as *strata*, which does not correspond directly to a random factor, but in this case *strata* controlled for repeated measures within sites.

To determine which OTUs were driving the reported beta diversity differences, we performed linear discriminant analyses of effect sizes using LEfSe and normalizing the sum of values to 1M as recommended by Segata et al. (2011). We used 0.05 thresholds for both the Kruskal–Wallis (among class differences) and Wilcoxon (between classes differences) tests, and we ran a linear discriminant analysis (LDA) considering LDA scores >3 as ecologically relevant. LDA scores are an estimate of the effect size of features that are differentially abundant among the categories tested (Segata et al., 2011). For bacterial OTUs beta diversity and functional group richness analyses, we tested whether groups differed in their dispersion patterns by running the *betadisper* function from the *vegan* package (Oksanen et al., 2016), which is equivalent to a Levene's test comparing homogeneity of variances between groups. We only reported significant *betadisper* results.

Comparing Bacterial Diversity and Predicted Functional Group Variation

To assess whether the alpha and beta diversity differences associated with sites that differed in soil pH and precipitation levels, timing in relation to the onset of the wet season,

and host species corresponded with changes in predicted microbial functional groups, we compared our OTU table against the Functional Annotation of Prokaryotic Taxa (FAPROTAX) database (Louca et al., 2016). FAPROTAX extrapolates functions of cultured prokaryotes (identified at the genus or species level) to the rest of the prokaryotic genus to estimate putative function. There are two main limitations of applying this approach to our data: (1) the FAPROTAX database was constructed mainly to analyze biogeochemistry of water bodies and (2) the FAPROTAX database is non-exhaustive; thus, only a small percentage of our OTUs may be assigned to at least one functional group. Even considering these caveats, we believe that predicting microbial functional groups using FAPROTAX is an appropriate alternative, especially when a metatranscriptomics approach is not plausible. Additionally, because FAPROTAX only considers cultured bacterial species with characterized functions, it may be superior to genomic prediction approaches like PICRUSt (Langille et al., 2013) and PAPRICA (Bowman and Ducklow, 2015).

Estimating the Bd-Inhibitory and Bd-Enhancing Functions of the Skin Microbiota

Finally, to identify bacterial isolates that potentially inhibit or enhance Bd, we used the *pick_closed_reference_otus.py* script in QIIME (Caporaso et al., 2010b) and standard settings to compare our results to the database published by Woodhams et al. (2015). It should be noted that (a) the database only includes culturable bacteria and thus is not exhaustive, (b) the database is likely biased toward bacterial isolates that inhibit Bd, (c) some of the bacteria were isolated from non-Panamanian frogs (Woodhams et al., 2015), and (d) microbiota functions may be context dependent (Medina et al., 2017). To standardize the resulting OTU table, we calculated proportion of Bd-inhibitory and Bd-enhancing bacterial sequences per sampled frog. To do this, we considered each frog and computed the sum of the relative abundances of all Bd-inhibitory and Bd-enhancing bacteria separately. We compared these proportions between host species, sites, and in relation to the onset of the wet season using generalized linear models with the function *glm* (R Core Team, 2017). Because we were comparing proportions bound between 0 and 1 and to account for overdispersion, we used quasibinomial distributions in the case of Bd-inhibitory bacteria. Due to their low proportions, we used quasipoisson distributions to analyze Bd-enhancing bacteria.

RESULTS

Among-Site Skin Microbiota Variation in *D. auratus*

In *D. auratus*, Shannon diversity correlated negatively with mean soil pH and was highest in frogs sampled from the site with lowest soil pH (**Figure 2B**, $P < 0.05$). However, precipitation levels did not have an effect on Shannon diversity ($P > 0.05$; nor on observed OTUs or Phylogenetic Diversity, Supplementary Table S1). In terms of bacterial community structure, we found the sites with the lowest mean soil pH (Fort Sherman) and highest mean soil pH (Barro Colorado Island) to plot on opposite sides of the ordination, with both intermediate mean soil pH sites (Cerro Ancon and Pipeline Road) in the middle. The pattern is less clear with regards to precipitation levels. The two sites with highest precipitation (Fort Sherman and Barro Colorado Island) plotted on opposite sides of the ordination, whereas the two intermediate precipitation level sites (Cerro Ancon and Pipeline Road) plotted in the middle of the ordination. Nevertheless, we found soil pH and precipitation levels as statistically significant factors explaining variation in bacterial community structure (**Figure 2C**, PERMANOVA UniFrac Weighted, Bray–Curtis, and UniFrac UnWeighted, $P < 0.05$). Out of 2,084 OTUs, we found 33 most significant OTUs best explaining site differences in *D. auratus* skin bacterial beta diversity (LDA > 3, **Figure 2A**). In particular, we found clear overrepresentation of *Pseudomonadaceae*, *Oxalobacteraceae*, and *Xanthomonadaceae* in Fort Sherman; *Sphingomonadaceae* and *Weeksellaceae* in Cerro Ancon; *Brevibacteriaceae* in Pipeline Road; and *Moraxellaceae* and *Sphingobacteriaceae* in Barro Colorado.

At higher bacterial taxonomic levels, though, the bacterial skin communities among *D. auratus* populations appeared to be similar. To illustrate, OTUs belonging to the Gammaproteobacteria were abundant in all sites, whereas Betaproteobacteria were abundant in all sites except in Pipeline Road. Anaerolineae bacteria were abundant in Fort Sherman and Barro Colorado Island, the two sites with the lowest and highest mean soil pH, respectively, among the four sites. Bacilli were abundant in Cerro Ancon and in Barro Colorado Island, which are the sites with the most human traffic (i.e., tourists and residents in Cerro Ancon, and researchers and visitors in Barro Colorado Island). The rest of the overrepresentations at higher taxonomic levels were site-specific. In Fort Sherman, Actinobacteria and Alphaproteobacteria were abundant; whereas Deltaproteobacteria, Acidobacteria, Flavobacteriia, and Pseudanabaenales were abundant in Cerro Ancon. At this taxonomic resolution, we did not detect any site-specific overrepresentations in Pipeline Road. Gemmatimonadetes and Thaumarchaeota were abundant in Barro Colorado Island.

Within-Site Skin Microbiota Variation in Relation to the Wet Season and Host Species

We found no effect of the onset of the wet season on skin bacterial alpha diversity of Pipeline Road *D. auratus* or *A. talamancae* (both $P > 0.05$), but there was a barely significant decline in alpha diversity of *S. flotator* (**Figure 3B**; $t = 2.77$, $df = 4$, $P = 0.05$). Within Pipeline Road, we found frog skin bacterial beta diversity to be assorted into distinct clusters in relation to the onset of the wet season (**Figure 3C**; PERMANOVA UniFrac Weighted and UniFrac UnWeighted $P < 0.001$; Bray–Curtis $P < 0.01$). After the onset of the wet season, the frog skin microbiota became more homogeneous, which was illustrated by the wet season samples forming a tighter cluster than the dry season samples (**Figure 3C**; *betadisper* ANOVA on UniFrac UnWeighted

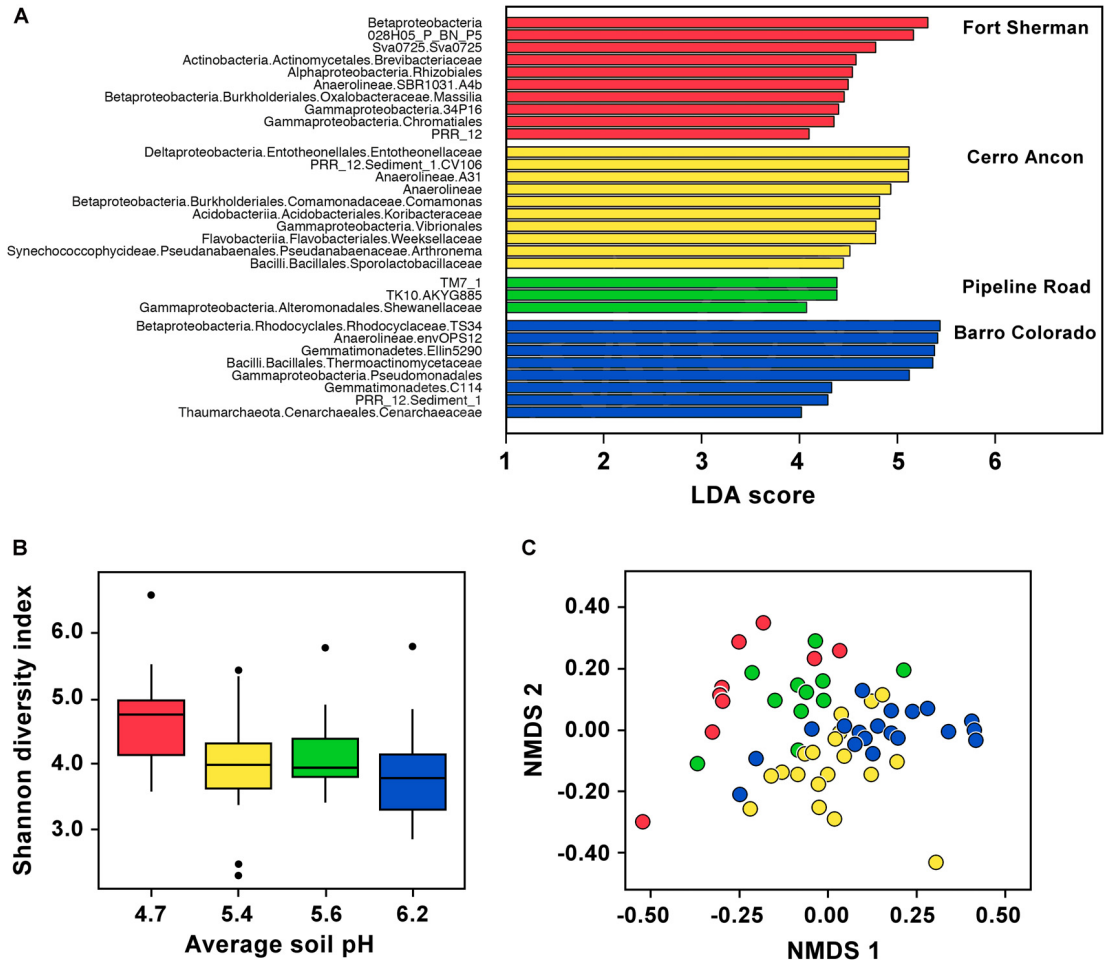

FIGURE 2 | Skin microbiota in *Dendrobates auratus* in relation to sample site and soil pH. **(A)** Bacterial operational taxonomic units (OTUs) that best define bacterial community structure in *D. auratus* across sites. Bars represent most significant linear discriminant analysis (LDA) scores computed using LEfSe, i.e., LDA > 3. **(B)** Box-and-whisker plot comparison of skin bacterial Shannon alpha diversity in *D. auratus* across sites in relation to average soil pH. Box indicates median, first, and third quartiles; points extending past 1.5 times the inter-quartile length were considered outliers and depicted as dots. **(C)** Non-metric multi-dimensional scaling (NMDS) plot of Bray–Curtis distances in skin bacterial beta diversity between individuals of *D. auratus* across sites. In all charts, sites are color-coded as follows: Fort Sherman is red, Cerro Ancon is yellow, Pipeline Road in green, and Barro Colorado is dark blue.

distances $F_{(1,21)} = 6.791$, $P < 0.05$). Out of 1,679 OTUs, we found 20 most significant OTUs explaining the differences in relation to the onset of the wet season (LDA > 3, **Figure 3A**). Actinobacteria OTUs were abundant both before and after the onset of the wet season. However, the abundant Actinobacteria before the onset of the wet season was a Micrococcaceae, whereas after the onset of the wet season the abundant OTU belonged to the Pseudonocardiaceae. The frogs sampled before the onset of the wet season were characterized by an abundance of OTUs belonging to Pedosphaerae, Synechococcophycidae, Clostridia, Alphaproteobacteria, Betaproteobacteria, and Brachyspirae. On the other hand, Gammaproteobacteria, Thermophilia, Flavobacteriia, and Cyanobacteria were abundant after the onset of the wet season. Overall, some bacterial classes appeared to be more abundant before the onset of the wet season (e.g., Actinobacteria and Bacilli), and decreased after the onset of the wet season when other classes became relatively more abundant (e.g., Flavobacteria and Sphingobacteria, Supplementary Figure S2).

We found Shannon diversity to differ between frog species in Pipeline Road ($P < 0.05$). However, these three species did not differ in observed OTUs or Phylogenetic Diversity (Supplementary Table S1). Out of the three frog species sampled, *D. auratus* had the lowest alpha diversity indexes, followed by *A. talamancae* and then *S. flotator*. Across frog species, microbial communities were dominated by Gammaproteobacteria, Alphaproteobacteria, and Betaproteobacteria (Supplementary Figure S2). The two stream-associated frogs, *A. talamancae* and *S. flotator*, also appeared to have higher relative abundances of Sphingobacteria than *D. auratus*. Flavobacteria and Actinobacteria seemed to be more abundant in *D. auratus*. After controlling for sampling time effects, we found no host effect on beta diversity (PERMANOVA Bray–Curtis, UniFrac Weighted and UnWeighted $P > 0.05$).

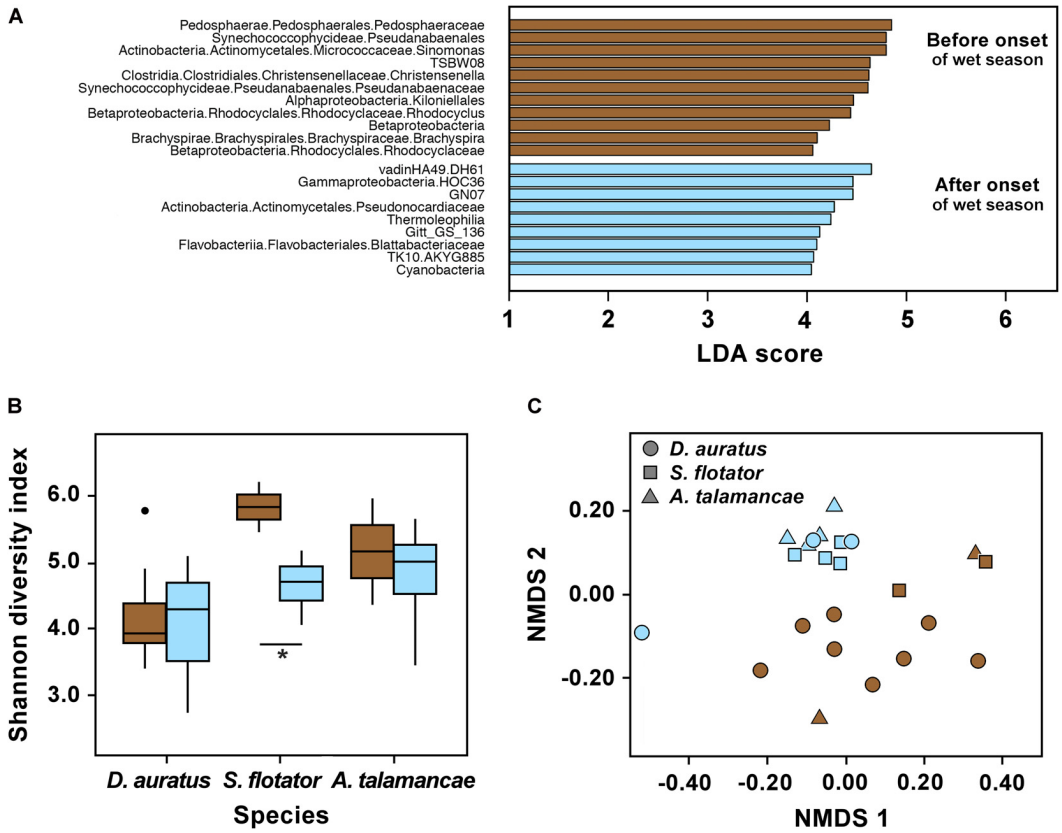

FIGURE 3 | Skin microbiota in three dendrobatoid frog species from Pipeline Road in relation to the onset of the wet season. **(A)** Bacterial OTUs that best define bacterial community structure among species before and after the onset of the wet season. Bars represent most significant linear discriminant analysis (LDA) scores computed using LEfSe, i.e., LDA > 3. **(B)** Box-and-whisker plot comparison of skin bacterial Shannon alpha diversity in dendrobatoid frog species before and after the onset of the wet season. Box indicates median, first, and third quartiles; points extending past 1.5 times the inter-quartile length were considered outliers and depicted as dots. * indicates a significant difference in alpha diversity before and after the onset of the wet season. **(C)** NMDS plot of Bray–Curtis distances in skin bacterial beta diversity between individual frogs of three dendrobatoid species at Pipeline Road. In all charts, brown indicates before the onset of the wet season, whereas light blue indicates after the onset of the set season.

Skin Microbiota Predicted Function Variation

We could assign 489 out of 2,184 bacterial OTUs (22.39%) to at least one microbial functional group using the FAPROTAX database, and most functional groups were represented by more than one OTU (**Table 3**). *D. auratus* skin microbiota had different microbial functional groups at sites differing in soil pH and precipitation levels (PERMANOVA Bray–Curtis $P < 0.001$). These site-to-site differences were best explained by 17 functional groups (**Table 3**), including fermentation in Fort Sherman, aerobic chemoheterotrophy in Cerro Ancon, anoxygenic photoautotrophy in Pipeline Road, and human pathogens in Barro Colorado Island.

At Pipeline Road and after controlling for frog species, skin microbial functional groups were different before and after the onset of the wet season (PERMANOVA Bray–Curtis $P < 0.01$), with 16 functional groups associated with this difference (**Figure 4**). These differences were explained by certain functional groups changing in their relative abundance or entirely disappearing after the onset of the wet season. To elaborate, functional groups involving nitrite, nitrate, and photoautotrophy were more abundant before the onset of the wet season, whereas chemoheterotrophy, cellulolysis, and nitrogenous compounds respiration were more abundant after the onset of the wet season.

Variation of Potential Bd-Interacting Microbiota

We could assign 185 out of 2,184 OTUs (8.47%) to one of the Bd-interacting categories using the Woodhams et al. (2015) database. The majority of these OTUs were isolated from Panamanian frogs (118/185 or 63.38%), and 14.05% (26/185) were isolated from the same species we report in this study (3 from *A. talamancae*, 6 from *D. auratus*, and 17 from *S. flotator*). *D. auratus* sampled from different sites differed in the proportion of Bd-inhibitory (**Figure 5A**, $\chi^2_{57,3} = 6.144$, $P < 0.010$) and Bd-enhancing microbiota (**Figure 5B**, $\chi^2_{57,3} = 0.196$, $P < 0.05$); yet, the two Bd-interacting microbial categories followed opposite trends. Bd-inhibitory bacterial OTUs were relatively more abundant in *D. auratus* from Pipeline Road, followed by Cerro Ancon, Fort Sherman, and Barro Colorado, whereas Bd-enhancing bacterial

TABLE 3 | Bacterial functional groups that best defined the site differences in the microbiota of *Dendrobates auratus* frogs between sampling sites, the number of bacterial operational taxonomic units (OTUs) identified for each functional group, and the relative abundance of bacteria in each functional group, presented in terms of linear discriminant analysis (LDA) scores computed using LEfSe.

Bacterial functional group	OTUs assigned	Relative abundance (LDA score)			
		Fort Sherman	Cerro Ancon	Pipeline Road	Barro Colorado Island
Chemoheterotrophy	294		4.500		
Aerobic chemoheterotrophy	199		4.569		
Phototrophy	90		3.954		
Photoautotrophy	84		4.219		
Fermentation	80	4.275			
Cyanobacteria	79		4.081		
Oxygenic photoautotrophy	79		4.081		
Intracellular parasites	39	3.793			
Animal parasites or symbionts	22				4.563
Chloroplasts	18	4.143			
Ureolysis	10			3.180	
Nitrogen fixation	8		3.158		
Human pathogens	5				4.567
Anoxygenic photoautotrophy sulfur oxidizing	5			3.819	
Anoxygenic photoautotrophy	5			3.819	
Aromatic compound degradation	3				4.555

OTUs were relatively more abundant in Fort Sherman, followed by Cerro Ancon, Pipeline Road, and Barro Colorado Island.

In Pipeline Road, the proportion of Bd-inhibitory bacteria differed in relation to the onset of the wet season ($\chi^2_{22,1} = 2.420$, $P < 0.05$) and between frog hosts (**Figure 5C**, $\chi^2_{20,2} = 2.420$, $P < 0.05$). Even though Bd-enhancing bacteria appeared to be relatively more abundant before the onset of the wet season across the three dendrobatoid species, this difference fell short from statistical significance (**Figure 5D**, $P > 0.05$), and we found no discernible difference in the proportion of Bd-enhancing bacteria among frog species ($P > 0.05$).

DISCUSSION

In this study, we assessed the role of two abiotic factors (i.e., soil pH and precipitation levels), seasonal, and host effects in shaping the amphibian skin microbiota diversity and predicted functional groups. To address our hypotheses, we sampled *D. auratus* across four sites differing in soil pH and precipitation levels, and three dendrobatoid frog species before and after the onset of the wet season within one site.

Our results demonstrate that variation in the skin microbiota of the Panamanian dendrobatoid frogs, *D. auratus*, *S. flotator*, and *A. talamancae*, from the Panama Canal region correlates with environmental variation in soil pH and changes in rainfall associated with the onset of the wet season. This is consistent with the hypothesis that these frogs depend on microbial environmental reservoirs to maintain their diverse skin microbiota (Fitzpatrick and Allison, 2014; Loudon et al., 2014; Kueneman et al., 2016a; Rebollar et al., 2016b). Thus, inhabiting an environment with conditions that promote higher microbial alpha diversity could translate into the frog's skin being colonized by more microbial species. Typically, soil bacterial alpha diversity is expected to correlate with soil pH following a quadratic model, with highest diversity in nearly neutral soils with pH ≈ 6.0 than soils with pH <4.5 or >8 (Fierer and Jackson, 2006; Lauber et al., 2009; Zhalnina et al., 2015). However, our findings that *D. auratus* from the lowest soil pH site, Fort Sherman, had the highest alpha diversity and that frogs from Fort Sherman and Barro Colorado, which had the most extremely acidic and basic soil pHs, respectively, among our sample sites had the most differentiated bacterial communities, suggest that the frog host may nevertheless influence the composition of its skin microbiota. This finding is in line with evidence that amphibian skin microbiota can be composed disproportionately of microbes that are otherwise present in relatively low abundances in the environment (Walke et al., 2014; Rebollar et al., 2016b).

Although precipitation levels have no apparent effect on skin microbiota alpha diversity in *D. auratus*, they do appear to correlate with skin bacterial community dispersion patterns, as frogs sampled from sites with wetter climates showed lower between sample variation than frogs sampled from sites with drier climates. This is in line with the thought that moisture promotes more diverse environmental microbial communities (Lauber et al., 2009; Hartmann et al., 2014). The site differences may be explained both in terms of the relative abundances and the presence (or absence) of particular OTUs. Three of the bacterial classes that we found to be overrepresented, Actinobacteria, Gammaproteobacteria, and Betaproteobacteria, were previously reported to be more abundant in frogs from Bd-positive sites, whereas Sphingobacteria were abundant in frogs from a Bd-negative site (Rebollar et al., 2016b).

A dependence on microbial environmental reservoirs to maintain frog skin bacterial communities is further indicated by the significant change in skin bacterial alpha diversity,

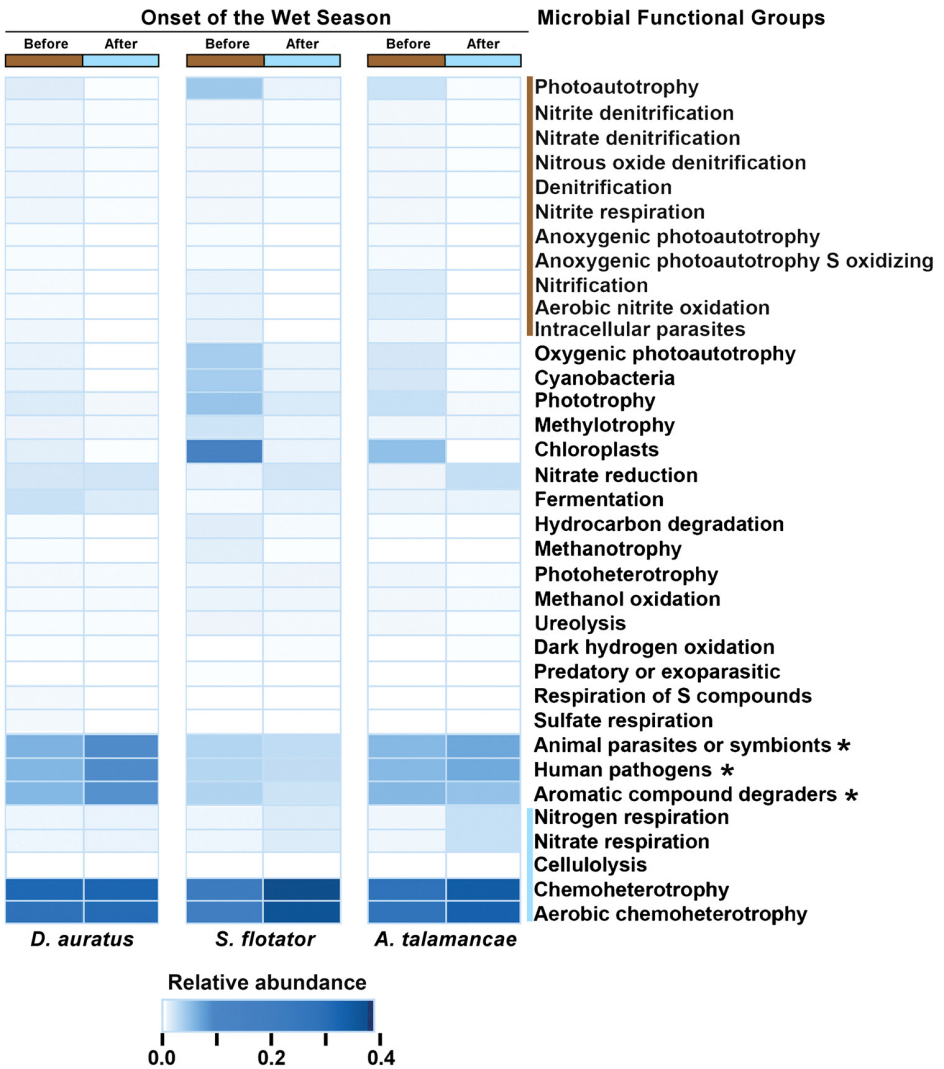

FIGURE 4 | Mean relative abundances of skin microbial functional groups before and after the onset of the wet season in three species of dendrobatoid frogs, *Dendrobates auratus*, *Silverstoneia flotator*, and *Allobates talamancae*, from Pipeline Road, Panama. Relative abundances are depicted in terms of color intensity from white (=0) to darkest blue (=0.433). Microbial functional groups more abundant before the onset of the wet season are preceded by a brown bar, whereas groups more abundant after the onset of the wet season are preceded by a blue bar. Asterisks (*) indicate microbial functional groups more abundant in *D. auratus*.

particularly in *S. flotator* at Pipeline Road, coincident with the onset of the wet season rains. The streamside habitat of these frogs tends to dry almost completely during the dry season but floods during the wet season, which may result in an increase in stress hormones, which has been related to a reduction in alpha diversity in the oral microbiota of certain species (Stothart et al., 2016). Our results also indicate that skin microbial communities of different species of frogs can become more similar with the onset of the wet season, which may be due to the more homogeneously damp environment after the start of the rains, allowing the frogs to forage more widely. Such seasonal changes in microbial community structure have also been reported in the skin microbiota of temperate ranid frogs (*Lithobates yavapaiensis*, Longo et al., 2015), and in the gut microbiota of other vertebrate groups (Keenan et al., 2013). Moreover, whether these seasonal patterns result in changes in the overall functional groups of the frog skin microbiota remained largely unexplored.

The redundancy that we found in microbial functional groups, whereby similar functions may be attributed to more than one bacterial OTU, may corroborate with evidence of an amphibian skin functional core (Hamady and Knight, 2009; Human Microbiome Project Consortium, 2012; Davis et al., 2017). From an evolutionary ecology perspective, such redundancy of microbial functional groups may be expected to be beneficial to the host in the face of potentially rapid microbial turnover (Loudon et al., 2014; Hughey et al., 2016), as an essential metabolic function, if carried out by only a single OTU could easily be lost, to the detriment of the host's fitness. Such redundancy of ecologically relevant microbial functional groups has also been observed in soil (Wellington et al., 2003).

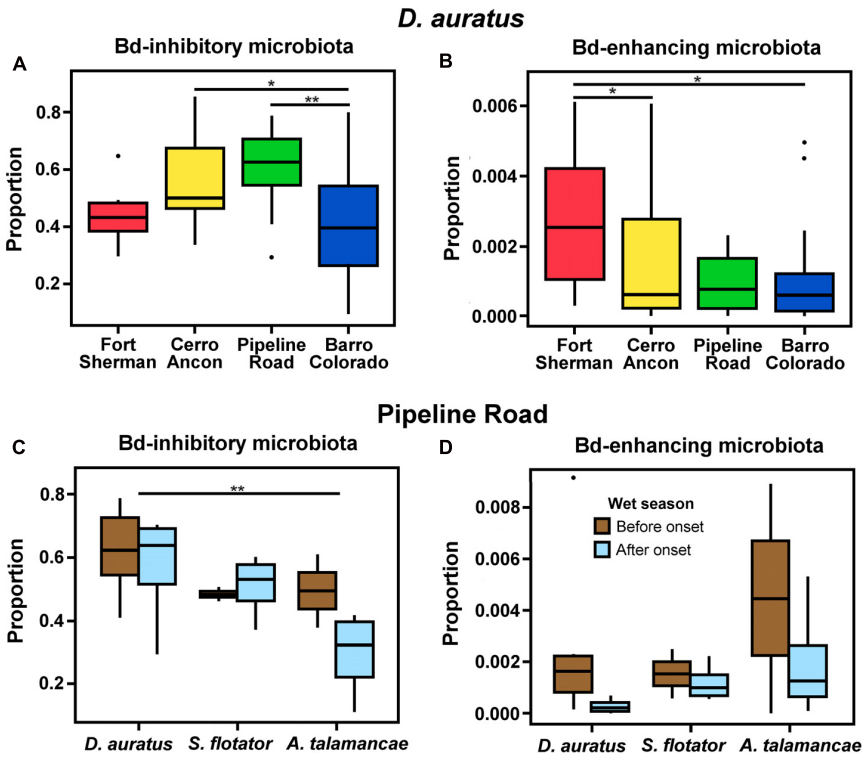

FIGURE 5 | Proportions of *Batrachochytrium dendrobatidis* (Bd) inhibitory **(A)** and enhancing **(B)** microbiota in *D. auratus* across sites in central Panama, arranged in order of increasing average soil pH values **(A,B)**, and in relation to the onset of the wet season in three Panamanian dendrobatoid frog species at Pipeline Road **(C,D)**.

In our study, the most abundant microbial functional groups were chloroplasts in Fort Sherman, aerobic chemoheterotrophs in Cerro Ancon, anoxygenic photoautotrophs in Pipeline Road, and human pathogens in Barro Colorado Island. The various bacteria representing these functional groups can exploit different carbon and energy sources (Balkwill et al., 1989; Johnston et al., 2009), which might indicate the position they occupy in the microbial biofilm and whether they interact predominantly with the frog host or with other microbes. The overabundance of human pathogens in frogs from Barro Colorado Island may also suggest that amphibians can play a role in the transmission of zoonotic diseases (Daszak et al., 2000) or, reciprocally, that human activities can affect the microbial communities of wildlife (Hacioglu and Tosunoglu, 2014). The shift in the most abundant functional groups from before to after the onset of the wet season from bacteria associated with photoautotrophy and nitrification to those associated to chemoheterotrophy and aerobic chemoheterotrophy suggests that when the environmental context of the host becomes more homogeneous, the skin microbiota follow a similar trend. That the microbial functional group differences we observe are ecologically relevant to the frog host remain, however, an assumption. How these functional groups actually interact with the host remains largely unknown.

The skin microbiota of these frogs may also be shaped by the properties of the frog's skin, as well as the environment. All amphibians produce and sequester a rich variety of nitrogenous compounds, including alkaloids, in skin granular glands that could represent a strong microbial selective force. Indeed, alkaloids purified from anuran skin extracts have been found to act as antimicrobials in *in vitro* trials (Mina et al., 2015). Like other species of dart-poison frogs, *D. auratus* sequesters dietary-derived alkaloids in its skin to a far greater extent than either *S. flotator* or *A. talamancae*. The relative high abundances of aromatic compound degrading bacteria, such as *Rhodococcus*, *Nocardioides* and *Acinetobacter*, that we found in the skin microbiota of *D. auratus* compared to those of *S. flotator* and *A. talamancae* suggest that certain microbes might interact with the frog's toxic alkaloids. This is similar to the case of the Midwife Toad, *Alytes obstetricans*, which secretes potent skin peptides that are also thought to play a role in shaping its microbiota (Davis et al., 2017). The potential for interactions between biogenically active amphibian skin compounds and amphibian skin microbiota regarding the frog's toxicity and its immunity from disease may have considerable ecological and evolutionary implications.

Even though all the frogs we sampled tested negative for Bd, this chytrid amphibian pathogen is known to be present on both sides of the Panama Canal (Woodhams et al., 2008; Rebollar et al., 2014) and our results suggest that environmental factors may mediate the abundance of Bd-inhibitory and Bd-enhancing microbiota. Variation in the proportion of Bd-inhibitory bacteria

in the skin of *D. auratus* correlated with mean soil pH, and decreasing proportions of Bd-enhancing bacteria in the skin of all three frog species in concert with the onset of the wet season, as we have found, may help explain seasonal variation observed in Bd prevalence (Lips et al., 2006; Longo et al., 2010; Whitfield et al., 2012). The lower proportions of Bd-inhibitory bacteria and higher proportions of Bd-enhancing bacteria we observed in the microbiota of *A. talamancae* compared to *D. auratus*, may signify the presence of differential effects on population persistence in these frogs (Lam et al., 2010). These findings may offer an explanation for previous suggestions that *A. talamancae* is more susceptible to Bd than *D. auratus* (Lips et al., 2006), and that susceptible frog species have distinct skin microbiota (Rebollar et al., 2016b).

Both the FAPROTAX database (Louca et al., 2016) and the Bd-interacting bacteria database (Woodhams et al., 2015) that we used to identify frog skin microbial functions have their limitations. As they are not exhaustive, they may be able to assign only a small percentage of discovered bacterial OTUs to one or more functional groups, enabling us to predict the function of less that 25% of the frog skin bacterial community. Furthermore, a particular bacterial OTU might carry out distinct functions depending on environmental context (Medina et al., 2017) and thus our predictions could be underestimating bacterial functional diversity. However, it is unlikely that we reported a bacterial functional group that was actually absent as most of these were largely redundant. Nevertheless, because both of these databases only consider cultured bacterial species with characterized functions, they may be considered superior to genomic approaches, like PICRUSt (Langille et al., 2013) or PAPRICA (Bowman and Ducklow, 2015), in minimizing Type I errors in predicting microbial functional groups.

We note that as both soil pH and precipitation levels are associated to the sampling sites, to be cautious we could interpret the differences discussed here as site differences. Similarly, given our reduced sample sizes and lack of seasonal replicates, we should be cautious when generalizing the results associated with the onset of the wet season. Despite these limitations, we present evidence that abiotic factors, site effects, and host effects shape bacterial skin community structure and predicted function in three tropical frog species.

The variability we observe in the skin microbiota of these three species of Panamanian frogs appears clearly to be correlated with abiotic factors, even though there may not yet be sufficient evidence to assign cause and effect. Frog skin microbial communities may plausibly be affected by interactions of constituent microbes with the environment, the host, and/or other co-occurring microbial species. Thus, there remains much more to be understood about such wildlife-associated microbiota and the complexities of their interactions with their amphibian hosts.

AUTHOR CONTRIBUTIONS

BV designed the study, collected the data, performed the analyses, and wrote the first version of the manuscript. DL, RI, and DG revised the manuscript. BV and DG prepared the figures. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00298/full#supplementary-material

REFERENCES

Balkwill, D. L., Fredrickson, J. K., and Thomas, J. M. (1989). Vertical and horizontal variations in the physiological diversity of the aerobic chemoheterotrophic bacterial microflora in deep southeast coastal plain subsurface sediments. *Appl. Environ. Microbiol.* 55, 1058–1065.

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Becker, M., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One* 5:e10957. doi: 10.1371/journal.pone.0010957

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bletz, M. C., Rebollar, E. A., and Harris, R. N. (2015). Differential efficiency among DNA extraction methods influences detection of the amphibian pathogen *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 113, 1–8. doi: 10.3354/dao02822

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Bowman, J. S., and Ducklow, H. W. (2015). Microbial communities can be described by metabolic structure: a general framework and application to a seasonally variable, depth-stratified microbial community from the coastal west Antarctic Peninsula. *PLoS One* 10:e0135868. doi: 10.1371/journal.pone.0135868

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in

amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., et al. (2011). Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16050–16055. doi: 10.1073/pnas.1102999108

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008). Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Burkart, D., Flechas, S. V., Vredenburg, V. T., and Catenazzi, A. (2017). Cutaneous bacteria, but not peptodes, are associated with chytridiomycosis resistance in Peruvian marsupial frogs. *Anim. Conserv.* 20, 483–491. doi: 10.1111/acv.12352

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinfomatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the illumina hiseq and miseq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Daszak, P., Cunningham, A. A., and Hyatt, A. D. (2000). Emerging infectious diseases of wildlife–threats to biodiversity and human health. *Science* 287, 443–449. doi: 10.1126/science.287.5452.443

Davis, L. R., Bigler, L., and Woodhams, D. C. (2017). Developmental trajectories of amphibian microbiota: response to bacterial therapy depends on initial community structure. *Environ. Microbiol.* 19, 1502–1517. doi: 10.1111/1462-2920.13707

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Engelbrecht, B. M. J., Comita, L. S., Condit, R., Kursar, T. A., Tyree, M. T., Turner, B. L., et al. (2007). Drought sensitivity shapes species distribution patterns in tropical forests. *Nature* 447, 80–82. doi: 10.1038/nature05747

Escalona, M., Rocha, S., and Posada, D. (2016). A comparison of tools for the simulation of genomic next-generation sequencing data. *Nat. Rev. Genet.* 17, 459–469. doi: 10.1038/nrg.2016.57

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., and Xavier, J. B. (2012). Microbiology. Animal behavior and the microbiome. *Science* 338, 198–199. doi: 10.1126/science.1227412

Fierer, N., and Jackson, R. B. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 103, 626–631. doi: 10.1073/pnas.0507535103

Fitzpatrick, B. M., and Allison, A. L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiol. Ecol.* 88, 482–494. doi: 10.1111/1574-6941.12314

Hacioglu, N., and Tosunoglu, M. (2014). Determination of antimicrobial and heavy metal resistance profiles of some bacteria isolated from aquatic amphibian and reptile species. *Environ. Monit. Assess.* 186, 407–413. doi: 10.1007/s10661-013-3385-y

Hamady, M., and Knight, R. (2009). Microbial community profiling for human microbiome projects: tools, techniques, and challenges. *Genome Res.* 19, 1141–1152. doi: 10.1101/gr.085464.108

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Hartmann, M., Niklaus, P. A., Zimmermann, S., Schmutz, S., Kremer, J., Abarenkov, K., et al. (2014). Resistance and resilience of the forest soil microbiome to logging-associated compaction. *ISME J.* 8, 226–244. doi: 10.1038/ismej.2013.141

Hughey, M. C., Walke, J. B., Becker, M. H., Umile, T. P., Burzynski, E. A., Minbiole, K. P., et al. (2016). Short-term exposure to coal combustion waste has little impact on the skin microbiome of adult spring peepers (*Pseudacris crucifer*). *Appl. Environ. Microbiol.* 82, 3493–3502. doi: 10.1128/AEM.00045-16

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Hyatt, A. D., Boyle, A. H. D., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., et al. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 73, 175–192. doi: 10.3354/dao073175

Ibáñez, R., Condit, R., Angehr, G., Aguilar, S., García, T., Martínez, R., et al. (2002). An ecosystem report on the panama canal: monitoring the status of the forest communities and the watershed. *Environ. Monit. Assess.* 80, 65–95. doi: 10.1023/A:1020378926399

Jiménez, R. R., and Sommer, S. (2017). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Johnston, D. T., Wolfe-Simon, F., Pearson, A., and Knoll, A. H. (2009). Anoxygenic photosynthesis modulated proterozoic oxygen and sustained earth's middle age. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16925–16929. doi: 10.1073/pnas.0909248106

Keenan, S. W., Engel, A. S., and Elsey, R. M. (2013). The alligator gut microbiome and implications for archosaur symbioses. *Sci. Rep.* 3:2877. doi: 10.1038/srep02877

Kosch, T. A., and Summers, K. (2013). Techniques for minimizing the effects of PCR inhibitors in the chytridiomycosis assay. *Mol. Ecol. Resour.* 13, 230–236. doi: 10.1111/1755-0998.12041

Kriger, K. M., Hero, J. M., and Ashton, K. J. (2006a). Cost efficiency in the detection of chytridiomycosis using PCR assay. *Dis. Aquat. Organ.* 71, 149–154.

Kriger, K. M., Hines, H. B., Hyatt, A. D., Boyle, D. G., and Hero, J. M. (2006b). Techniques for detecting chytridiomycosis in wild frogs: comparing histology with real-time Taqman PCR. *Dis. Aquat. Organ.* 71, 141–148.

Krynak, K. L., Burke, D. J., and Benard, M. F. (2016). Landscape and water characteristics correlate with immune defense traits across blanchard's cricket frog (*Acris blanchardi*) populations. *Biol. Conserv.* 193, 153–167. doi: 10.1016/j.biocon.2015.11.019

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and Mckenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016a). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. Biol. Sci.* 283:20161553. doi: 10.1098/rspb.2016.1553

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016b). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Langille, M. G., Zaneveld, J., Caporaso, J. G., Mcdonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lauber, C. L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil ph as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.* 75, 5111–5120. doi: 10.1128/AEM.00335-09

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A., and Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J.* 2, 145–157. doi: 10.1038/ismej.2007.110

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Longo, A. V., Burrowes, P. A., and Joglar, R. L. (2010). Seasonality of *Batrachochytrium dendrobatidis* infection in direct-developing frogs suggests

a mechanism for persistence. *Dis. Aquat. Organ.* 92, 253–260. doi: 10.3354/dao02054

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Longo, A. V., and Zamudio, K. R. (2016). Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. *ISME* 11, 349–361. doi: 10.1038/isme.2016.138

Louca, S., Parfrey, L. W., and Doebeli, M. (2016). Decoupling function and taxonomy in the global ocean microbiome. *Science* 353, 1272–1277. doi: 10.1126/science.aaf4507

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., Mckenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Lozupone, C., and Knight, R. (2005). Unifrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Marin, I. A., Goertz, J. E., Ren, T., Rich, S. S., Onengut-Gumuscu, S., Farber, E., et al. (2017). Microbiota alteration is associated with the development of stress-induced despair behavior. *Sci. Rep.* 7:43859. doi: 10.1038/srep43859

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., et al. (2012). An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Loso, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3229–3236. doi: 10.1073/pnas.1218525110

Medina, D., Hughey, M. C., Becker, M. H., Walke, J. B., Umile, T. P., Burzynski, E. A., et al. (2017). Variation in metabolite profiles of amphibian skin bacterial communities across elevations in the Neotropics. *Microb. Ecol.* 74, 227–238. doi: 10.1007/s00248-017-0933-y

Mina, A. E., Ponti, A. K., Woodcraft, N. L., Johnson, E. E., and Saporito, R. A. (2015). Variation in alkaloid-based microbial defenses of the dendrobatid poison frog *Oophaga pumilio*. *Chemoecology* 25, 169–178. doi: 10.1007/s10886-018-0930-8

Oksanen, J., Blancher, F. G., Friednly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2016). *Vegan: Community Ecology Package. R package Version* 2.4-1. Available at: https://CRAN.R-project.org.package=vegan

Pollock, F. J., Wada, N., Torda, G., Willis, B. L., and Bourne, D. G. (2017). White syndrome-affected corals have a distinct microbiome at disease lesion fronts. *Appl. Environ. Microbiol.* 83:e02799-16. doi: 10.1128/AEM.02799-16

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fogden, M. P., Foster, P. N., et al. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature* 439, 161–167. doi: 10.1038/nature04246

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree2 – approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

R Core Team (2017). *R: a Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Harris, R. N., Domangue, R. J., Medina, D., Ibáñez, R., et al. (2014). The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of far eastern Panamá. *PLoS One* 9:e95484. doi: 10.1371/journal.pone.0095484

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016b). Skin bacterial diversity of panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Rinaldi, A. C. (2002). Antimicrobial peptides from amphibian skin: an expanding scenario. *Curr. Opin. Chem. Biol.* 6, 799–804. doi: 10.1016/S1367-5931(02)00401-5

Santiago, L. S., and Mulkey, S. S. (2005). Leaf productivity along a precipitation gradient in lowland Panama: patterns from leaf to ecosystem. *Trees* 19, 349–356. doi: 10.1007/s00468-004-0389-9

Schappe, T., Albornoz, F. E., Tuner, B. L., Neat, A., Condit, R., and Jones, F. A. (2017). The role of soil chemistry and plant neighbourhoods in structuring fungal communities in three Panamanian forests. *J. Ecol.* 105, 569–579. doi: 10.1111/1365-2745.12752

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., et al. (2014). Gut microbiome of the hadza hunter-gatherers. *Nat. Commun.* 5:3654. doi: 10.1038/ncomms4654

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Stothart, M. R., Bobbie, C. B., Schulte-Hostedde, A. I., Boonstra, R., Palme, R., Mykytczuk, N. C., et al. (2016). Stress and the microbiome: linking glucocorticoids to bacterial community dynamics in wild red squirrels. *Biol. Lett.* 12:20150875. doi: 10.1098/rsbl.2015.0875

Tennessen, J. A., Woodhams, D. C., Chaurand, P., Reinert, L. K., Billheimer, D., Shyr, Y., et al. (2009). Variations in the expressed antimicrobial peptide repertoire of northern leopard frog (*Rana pipiens*) populations suggest intraspecies differences in resistance to pathogens. *Dev. Comp. Immunol.* 33, 1247–1257. doi: 10.1016/j.dci.2009.07.004

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLoS One* 10:e0139848. doi: 10.1371/journal.pone.0139848

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wellington, E. M., Berry, A., and Krsek, M. (2003). Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. *Curr. Opin. Microbiol.* 6, 295–301. doi: 10.1016/S1369-5274(03)00066-3

Whitfield, S. M., Kerby, J., Gentry, L. R., and Donnelly, M. A. (2012). Temporal variation in infection prevalence by the amphibian chytrid fungus in three species of frogs at La Selva, Costa Rica. *Biotropica* 44, 779–784. doi: 10.1111/j.1744-7429.2012.00872.x

Wickham, H. (2009). *Ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer-Verglag. doi: 10.1007/978-0-387-98141-3

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecol. Arch.* 96:595. doi: 10.1890/14-1837.1

Woodhams, D. C., Bletz, M., Kueneman, J., and Mckenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trends Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

Woodhams, D. C., Kilburn, V. L., Reinert, L. K., Voyles, J., Medina, D., Ibáñez, R., et al. (2008). Chytridiomycosis and amphibian population declines continue to spread eastward in Panama. *Ecohealth* 5, 268–274. doi: 10.1007/s10393-008-0190-0

Zhalnina, K., Dias, R., De Quadros, P. D., Davis-Richardson, A., Camargo, F. A., Clark, I. M., et al. (2015). Soil pH determines microbial diversity and composition in the park grass experiment. *Microb. Ecol.* 69, 395–406. doi: 10.1007/s00248-014-0530-2

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Skin Microbiomes of California Terrestrial Salamanders Are Influenced by Habitat More Than Host Phylogeny

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A multitude of microorganisms live on and within plant and animal hosts, yet the ecology and evolution of these microbial communities remains poorly understood in many taxa. This study examined the extent to which environmental factors and host taxonomic identity explain microbiome variation within two salamander genera, *Ensatina* and *Batrachoseps*, in the family Plethodontidae. In particular, we assessed whether microbiome differentiation paralleled host genetic distance at three levels of taxonomy: genus and high and low clade levels within *Ensatina eschscholtzii*. We predicted that more genetically related host populations would have more similar microbiomes than more distantly related host populations. We found that salamander microbiomes possess bacterial species that are most likely acquired from their surrounding soil environment, but the relative representation of those bacterial species is significantly different on the skin of salamanders compared to soil. We found differences in skin microbiome alpha diversity among *Ensatina* higher and lower clade groups, as well as differences between *Ensatina* and *Batrachoseps*. We also found that relative microbiome composition (beta diversity) did vary between *Ensatina* lower clades, but differences were driven by only a few clades and not correlated to clade genetic distances. We conclude this difference was likely a result of *Ensatina* lower clades being associated with geographic location and habitat type, as salamander identity at higher taxonomic levels (genus and *Ensatina* higher clades) was a weak predictor of microbiome composition. These results lead us to conclude that environmental factors are likely playing a more significant role in salamander cutaneous microbiome assemblages than host-specific traits.

Keywords: microbiome, amphibian, symbiosis, bacteria, *Ensatina eschscholtzii*, *Batrachoseps*

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INTRODUCTION

Just as plants and animals have evolved and adapted to particular habitats, forming complex interconnected communities, microbial species have also evolved to form communities that are particularly well-suited to specific environments. These unique microbial communities (here on referred to as microbiomes) can be found in a range of environments from hot springs

(Jackson et al., 2001) to vertebrate digestive tracts (Ley et al., 2008a,b). These microbiomes can play a crucial role in the health and well-being of their host (Moloney et al., 2014). Microbiomes contribute to host immunity, physiology, development, and behavior (McFall-Ngai et al., 2013). Despite a growing understanding of the function and complexity of microbiomes, we are just beginning to understand the factors that cause microbiomes to vary across host individuals, populations, and closely related species. Technological advancements in DNA sequencing have made it possible to get a more complete picture of microbiome community composition (Petrosino et al., 2009), and begin answering questions about variation in the host–microbiome relationship (Spor et al., 2011; Council et al., 2016; Moeller et al., 2016).

The amphibian skin microbiome has been a focus of recent research, due to the non-invasive method of sample collection as well as the relevance of skin microbiomes to amphibian health (McKenzie et al., 2012; Jani and Briggs, 2014; Kueneman et al., 2014). The skin of an amphibian is essential for the proper function of many biological processes, including moisture balance, gas exchange, and disease defense. Proper function of skin-related biological processes depends on host factors (physiology, metabolism, behavior, etc.) as well as the skin microbiome, which often provides essential benefits that affect host fitness. For example, in defense against the fungal pathogen, *Batrachochytrium dendrobatidis*, some amphibian species release antimicrobial peptides from glands that can inhibit *Batrachochytrium dendrobatidis* growth (Rollins-Smith and Conlon, 2005), while other hosts might also, or alternatively, harbor beneficial microbes, such as *Janthinobacterium lividum*, on their skin that protect the host from infection (Harris et al., 2009).

Despite evidence that microbiomes are often essential components of host fitness, the relative influence of environment versus host taxonomy in shaping amphibian microbiomes remains unclear. In aquatic amphibians, for example, evidence suggests host taxonomic identity more strongly predicts microbiome composition than environment (McKenzie et al., 2012; Walke et al., 2014; Kueneman et al., 2014). This result indicates there may be an evolutionary relationship between hosts and their microbiomes. For example certain taxonomic groups may harbor specific microbes that are passed vertically or horizontally among closely related hosts due to their unique life histories (Walke et al., 2014). Alternatively, unique taxonomic groups may have a genetic predisposition to acquire a biased subset of microbes from the environment. In terrestrial salamanders, habitat (rather than host taxonomy) seems to be the strongest predictor of microbiome composition (Fitzpatrick and Allison, 2014; Loudon et al., 2014; Muletz Wolz et al., 2017; Prado-Irwin et al., 2017), lending evidence to the theory that some amphibians may simply acquire skin associated microbes directly from their surrounding environment. Differences in the relative influence of host phylogeny is likely due to biological differences in aquatic versus terrestrial amphibians, but may also be due to a lack of studies with broad habitat sampling of terrestrial salamanders. However, one study on plethodontid salamanders did find good evidence that site rather than host species identity explained skin microbiome beta diversity patterns (Muletz Wolz et al., 2017). Further studies, such as the one we present here, focusing on groups with well-characterized phylogenies and geographic ranges encompassing several habitat types can help elucidate which factors influence the amphibian skin microbiome.

The terrestrial salamander *Ensatina eschscholtzii* (family Plethodontidae) is an ideal study system for exploring the relative influences of host taxonomy and environment on the amphibian skin microbiome. The evolutionary history of *Ensatina* as a ring species has been well established (Stebbins, 1949; Wake and Yanev, 1986; Moritz et al., 1992; Kuchta et al., 2009b): the species originated in Northern California and Southern Oregon and later dispersed (divergently) down both the coastal and inland regions of California, eventually meeting again in southern California (Stebbins, 1949; Moritz et al., 1992). Throughout its range *Ensatina* differentiates into 12 distinct genetic clades encompassing seven taxonomic subspecies (Kuchta et al., 2009a). This well-characterized genetic history provides a unique opportunity to assess the degree of similarity of microbiomes among clade groups with varying degrees of genetic distance. In addition, for much of its range, *Ensatina* also overlaps with other plethodontid species within the genus *Batrachoseps*, allowing for a within-family comparison. *Ensatina* and *Batrachoseps* co-occur across several different habitat types, providing an opportunity to disentangle host taxonomic versus environmental influences on the microbiome. If host taxonomy is a primary driving factor in determining the microbiome, we would expect that sympatric species would still harbor distinct microbiome communities. However, if environment plays a more important role, we would expect sympatric species to exhibit more similar microbiomes that more closely related allopatric individuals.

With *Ensatina* and *Batrachoseps* as our study system, we used 16S amplicon sequencing to evaluate to relative roles of host identity and habitat on the skin bacterial community (from here on referred to as the microbiome) of California terrestrial salamanders. The primary objective of our study was to assess whether varying degrees of host genetic distance could explain differences in the skin microbiome. We hypothesized that the microbiome would track the phylogeny of their hosts, with more closely related salamander groups harboring more similar microbiome communities. Correspondingly, we expected more distantly related salamander groups to exhibit more distinct microbiome communities from one another. To test this hypothesis, we assessed differences in the microbiome within the plethodontid family using different genera (*Ensatina* vs. *Batrachoseps*) and species (*Ensatina* and three species of *Batrachoseps*). We also looked at differences in the microbiome within the species *Ensatina* at two clade levels (referred to here as "higher clades" and "lower clades") as identified in Kuchta et al. (2009a). In addition to determining whether host phylogeny influenced skin microbiomes, we also considered the role of habitat in influencing microbiome variation. We predicted that habitat would explain some variation, but to a lesser degree than host taxonomic identity, as seen in previous studies (Kueneman et al., 2014). Lastly, we compared

salamander microbiomes to their surrounding soil microbiomes and hypothesized that the soil microbiome would house a greater diversity of microbial species, as soil and sediment have been shown to have enormous microbial diversity (Torsvik et al., 2002). We also expected the salamander skin microbiome to share some but not all microbial species with the soil, while also housing some unique microbial species not found in the soil as seen in previous work (Prado-Irwin et al., 2017). This would lend evidence to the theory that some degree of vertical or horizontal transmission may be occurring in these species.

MATERIALS AND METHODS

Field Sampling

We sampled 118 salamanders during the rainy season from March – May 2014 and December – February 2015 (**Figure 1** and Supplementary Table 1). Skin microbiome samples were collected from 10 of the 12 distinct genetic clades (here on referred to as "lower clades") of *Ensatina eschscholtzii* (Kuchta et al., 2009a) (**Figure 1**). These lower clades are nested within three higher clades [Coastal Clade (n = 22), Oregonensis [1] (n = 10), and Clade A (n = 54)], which was an additional level of genetic relatedness between hosts that we compared microbiomes across (Kuchta et al., 2009a). The molecular phylogeny and corresponding range map of these higher and lower genetically distinct clade groups can be found in Kuchta et al. (2009a). Clade identity was assumed based on morphology and known localities, and populations sampled for each clade were sufficiently geographically distant from populations of other clades to avoid hybrid individuals (Kuchta et al., 2009a). The lower clade *E. e. croceater* was not sampled because no individuals were found, likely due to extreme drought conditions within their range during the time of this study. The lower clade *E. e. xanthoptica* [2] was also not sampled because its range includes a high degree of geographic overlap with other clades, and we wanted to avoid sampling hybrid individuals. We also collected skin microbiome samples from *Batrachoseps* where they were found sympatrically with *Ensatina*. Sample size for each *Ensatina* lower clade and *Batrachoseps* species are summarized by location in **Table 1**.

Due to the difficulty of finding individuals in many of the clades, we could not standardize the total sampling area across clades (i.e., some individuals were found at closer distances to one another than others). For each lower clade group, we attempted to sample ten adult *Ensatina*, a comparable sample size relative to other amphibian microbiome studies (McKenzie et al., 2012; Bataille et al., 2016; Hughey et al., 2017; Walke et al., 2017). If 10 adults could not be found after extensive searching, we collected and included juveniles in our analysis for that *Ensatina* lower clade wherever possible. To ensure that including juvenile *Ensatina* in our analyses did not affect our results, we tested whether life stage explained variation in weighted and unweighted microbiome beta diversity within *Ensatina* and found that it did not explain significant differences (adonis R^2 = 0.03, $p > 0.05$). This is consistent with previous findings (Prado-Irwin et al., 2017). All of the *Batrachoseps* sampled were adults.

Individuals were located by turning over cover objects (logs, rocks, etc.). Salamanders were handled with new nitrile gloves and rinsed with approximately 50 mL (for *Ensatina*) or 25 mL (for *Batrachoseps*) of 18 MΩ/cm MilliQ water from a sterile syringe to remove any dirt and non-skin associated (i.e., transient) microbes. The salamander was then swabbed 30 times (10 times on dorsal surface, 10 times on ventral surface, and 5 times on each side) using a sterile fine tip rayon dryswab. The swab was then placed in a sterile Eppendorf tube that was immediately placed on dry ice for temporary storage until the sample could be transferred to a −80°C freezer. Every salamander was then swabbed a second time using the same method to test for the presence of the common amphibian fungal pathogen, *Batrachochytrium dendrobatidis*. We took *Batrachochytrium dendrobatidis* swabs to identify diseased individuals for exclusion from analyses, as *Batrachochytrium dendrobatidis* has been shown to cause significant shifts in the microbiome (Jani and Briggs, 2014; Walke et al., 2015; Bataille et al., 2016). We performed a standard real-time quantitative PCR assay to determine possible presence of fungal infection from *Batrachochytrium dendrobatidis* swabs (Boyle et al., 2004). We ultimately did not need to exclude any samples from analyses because all samples were negative for *Batrachochytrium dendrobatidis*.

For each salamander, we took GPS coordinates at the site of sample collection (Supplementary Table 1). GPS coordinates were later input into the Conservation Biology Institute's Data Basin platform[1] to determine habitat type (Scrub Oak Chaparral, Upland Redwood Forest, etc.) for each sample, using land-cover data provided by the "California landcover based on California Natural Diversity Data Base (CNDDB) system" layer (Supplementary Table 1).

For each *Ensatina* individual sampled, we also collected a soil sample in a sterile 2 mL Eppendorf tube from under the cover object where the salamander was found. The soil sample was immediately placed on dry ice for temporary storage until the sample could be transferred to a −80°C freezer. We randomly chose five soil samples per *Ensatina* lower clade to analyze for this study. For lower clade groups where less than five *Ensatina* individuals were found, we included all collected soil samples for that clade in our analyses (one associated with each cover object for each individual).

The protocol for the use of salamanders in this research was approved by the California Department of Fish and Wildlife (SC-12919) and the San Francisco State University Institutional Animal Care and Use Committee (Protocol #A12-07).

Microbiome DNA Extraction and Sample Processing

Bacterial DNA was extracted from each microbiome swab and soil sample using a PowerSoil Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States). Each swab or soil sample (0.25 g) was placed in a bead tube provided by the kit, and extraction

[1] www.databasin.org

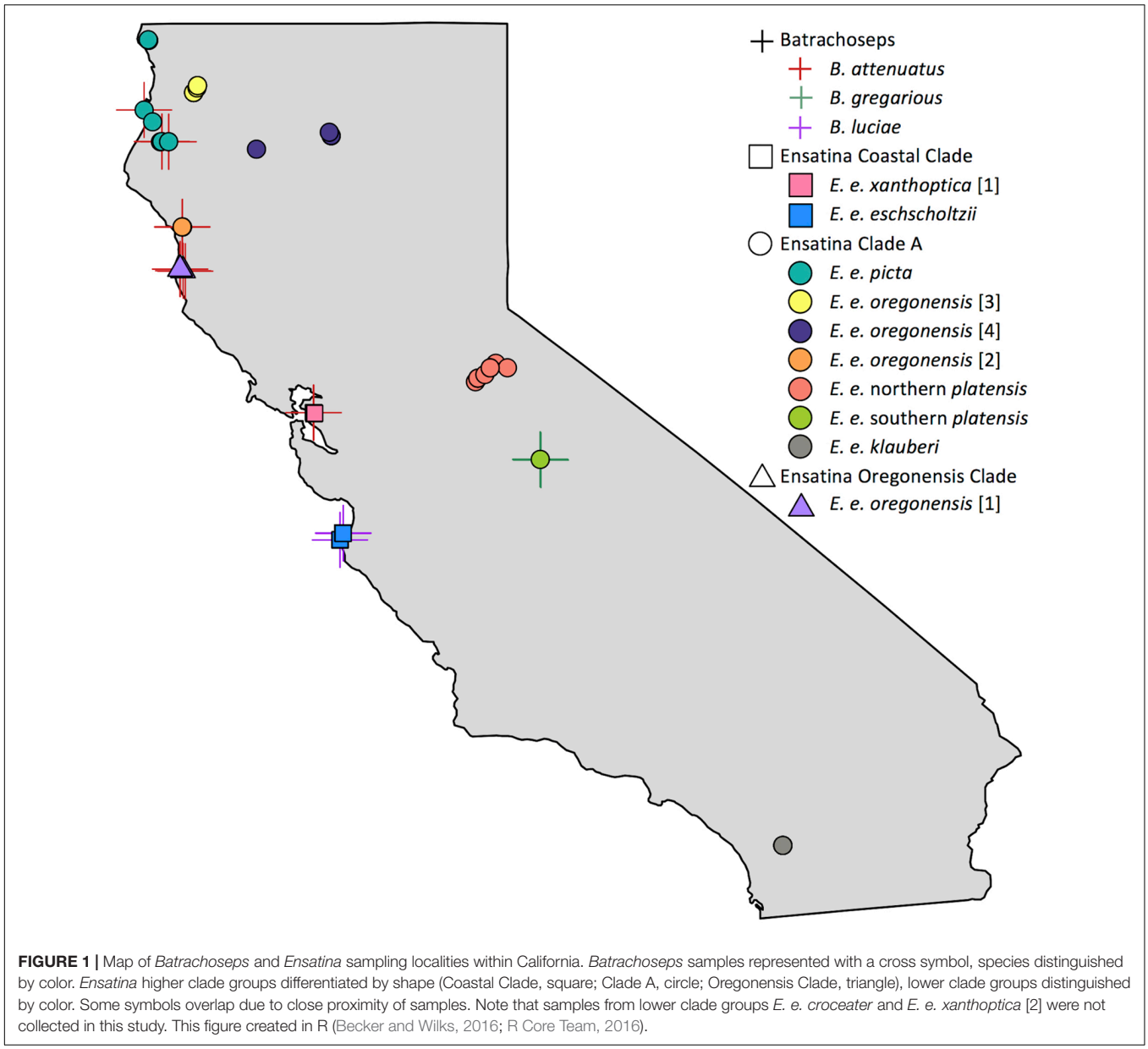

FIGURE 1 | Map of *Batrachoseps* and *Ensatina* sampling localities within California. *Batrachoseps* samples represented with a cross symbol, species distinguished by color. *Ensatina* higher clade groups differentiated by shape (Coastal Clade, square; Clade A, circle; Oregonensis Clade, triangle), lower clade groups distinguished by color. Some symbols overlap due to close proximity of samples. Note that samples from lower clade groups *E. e. croceater* and *E. e. xanthoptica* [2] were not collected in this study. This figure created in R (Becker and Wilks, 2016; R Core Team, 2016).

was completed using the manufacturer's protocol. The V3-V4 region of the bacterial 16S rRNA gene was amplified using Illumina primers (Supplementary Table 2) for each sample using a modified version of the Illumina protocol (we used 30 PCR cycles during the amplification step; Illumina Inc., San Diego, CA, United States). Each extract was amplified in triplicate, resulting in a total volume of 75 μl of amplified product per sample. After amplification, product was cleaned up using Agencourt AMPure XP beads to remove non-target DNA. Cleaned product was then re-amplified according to Illumina protocol using sample-specific Illumina Nextera Index primers (Supplementary Table 2). Indexed product was cleaned up with the same methods used for the amplicon product. Clean indexed product was then run on a gel to confirm the presence of product and to ensure no contamination had occurred. Samples were then quantified using qPCR. Each qPCR consisted of 6 μl KAPA SYBR FAST qPCR Master Mix and 4 μl sample. Samples were then pooled in equimolar concentrations and the pool was quantified using qPCR to confirm concentration, and further diluted if required. The pool was then sequenced at the Department of Biology's Genomics/Transcriptomics Analysis Core facility at San Francisco State University, on an Illumina MiSeq, using a v2 kit.

Sequence Analyses

Sequence analyses were conducted using QIIME v1.9.0 (Caporaso et al., 2010b). Default protocol was used unless otherwise indicated. Forward and reverse reads were joined and sequences were filtered using a quality score of Q20 (removes reads with <99% base call accuracy), resulting in approximately eight million sequences. Sequences were then

TABLE 1 | Sample sizes (*n*) for each *Batrachoseps* species and *Ensatina* subspecies by site.

Site	Species/subspecies	*n*
Alameda	*Ensatina eschscholtzii xanthoptica* [1]	12
	Batrachoseps attenuates	5
Calaveras	*Ensatina eschscholtzii* northern *platensis*	10
Humboldt	*Ensatina eschscholtzii picta*	10
	Batrachoseps attenuates	5
Jackson State Forest	*Ensatina eschscholtzii oregonensis* [1]	10
	Batrachoseps attenuates	5
Leggett	*Ensatina eschscholtzii oregonensis* [2]	10
	Batrachoseps attenuates	5
Monterey	*Ensatina eschscholtzii eschscholtzii*	10
	Batrachoseps luciae	8
Palomar	*Ensatina eschscholtzii klauberi*	9
Shasta	*Ensatina eschscholtzii oregonensis* [4]	4
Sierra National Forest	*Ensatina eschscholtzii* southern *platensis*	2
	Batrachoseps gregarious	4
Siskiyou	*Ensatina eschscholtzii oregonensis* [3]	9

clustered into operational taxonomic units (OTUs) at 97% similarity. We used the open-reference subsampling protocol in QIIME to assign OTU taxonomy using the Greengenes 13_8 reference database[2] (DeSantis et al., 2006; McDonald et al., 2012). Sequences were aligned using PyNAST (Caporaso et al., 2010a). Aligned sequences have been archived under BioProject accession number PRJNA434592. Any sequences that did not match the reference database were clustered into *de novo* OTUs using UCLUST (Edgar, 2010) and taxonomy was assigned using the RDP Classifier 2.2 (Wang et al., 2007). The final OTU table was then additionally filtered across all samples before analysis to remove rare OTUs with fewer than 100 reads and those represented in only one sample (soil or salamander), which removed OTUs representing less than ∼0.001% of all sequences (Bokulich et al., 2013; Kueneman et al., 2014; Walke et al., 2014; Longo et al., 2015), resulting in a total of 6,576 OTUs. Samples were rarefied by analysis to the number of sequences present in the sample with the lowest number of sequences (see below for rarefaction levels associated with each of the individual analyses).

Statistical Analyses

We assessed differences in the microbiome across five sampling categories, representing different levels of genetic relatedness: sample type (soil or salamander), genus, species, higher clade and lower clade. Higher clade groups and lower clade groups are identified from previous phylogenetic analyses of *Ensatina* (Kuchta et al., 2009a). Rarefaction levels of sequences per sample by analysis: soil vs. salamander = 7968, *Ensatina* vs. *Batrachoseps* = 9466, *Ensatina* vs. *Batrachoseps* (within overlapping range) = 15514, within *Ensatina* higher and lower clade comparisons = 9466. Refer to Supplementary Table 3 for rarefaction levels used for *Ensatina* pairwise comparisons.

Microbiome alpha diversity metrics calculated in QIIME for each sample included: OTU richness, phylogenetic diversity, Simpson diversity index, Shannon diversity index, and Shannon's equitability (evenness). We determined normality of alpha diversity data using Shapiro–Wilk tests. We compared alpha diversity between soil and salamanders and *Batrachoseps* and *Ensatina* using two-sample *t*-tests (parametric or non-parametric depending on normality). We used a one-way ANOVA to test for differences in Shannon's diversity and Kruskal–Wallis tests for differences in the other four diversity metrics across *Ensatina* higher and lower clade groups. All alpha diversity comparisons were done using R (R Core Team, 2016). For each of the five sampling categories defined above we identified dominant OTUs, which were defined as OTUs representing 3% or greater of the total sequences found within that respective sampling category. We also identified the number of OTUs that were unique to each sample type within each of the five sampling categories.

We used unweighted and weighted UniFrac distance metrics to calculate beta diversity in QIIME (Lozupone and Knight, 2005). Unweighted UniFrac distances account for the presence or absence of OTUs within each sample. Weighted UniFrac distances account for the presence or absence of OTUs, as well as relative OTU abundances within each sample. It is important to assess results from both UniFrac metrics (weighted and unweighted) as they each give distinct information about differences between microbial communities (Lozupone et al., 2007). The contribution of sample type, host taxonomy, habitat type, and site to beta diversity was analyzed using adonis in QIIME and plotted using a principle coordinates analysis (PCoA) in R (R Core Team, 2016). For a subset of *Ensatina* lower clade groups, we also tested for correlations between host genetic distance and Unifrac distances using a Pearson's correlation. We used previously published genetic distances between *Ensatina* populations for this analysis (Wake, 1997; Kuchta et al., 2009b).

For *Ensatina*, we also identified a core microbiome for each lower clade group, defined as all the OTUs found on 90% of samples within that group. We then compared relative core abundances across clades using a Kruskal–Wallis test. We also identified the OTUs that were found on 90% of *Batrachoseps attenuatus* samples. However, we did not assess a core microbiome for *B. luciae* or *B. gregarius* due to their low sample sizes.

RESULTS

Differences Between Soil and Salamanders

Among the 118 salamander samples and the 41 soil samples we analyzed, we found a large degree of variation in OTU richness among individual samples within each of the sample categories. For soil samples, OTU richness varied between 404 and 2072 OTUs per sample (mean = 776 OTUs per sample). For salamanders, OTU richness varied between 201 and 3087 OTUs per sample (mean = 622 OTUs per sample). Soil had significantly greater alpha diversity for all metrics when compared to salamander samples as a whole (non-parametric two-sample *t*-test: OTU richness, $p = 0.017$; phylogenetic diversity, $p = 0.009$; Shannon diversity index,

[2]http://greengenes.lbl.gov

p = 0.001; Simpson diversity index, p = 0.001). Soil samples were also significantly more even in relative OTU abundances than salamander samples (non-parametric two-sample t-test: Shannon's equitability, p = 0.001). Soil samples had no dominant OTUs that made up at least 3% the microbiome across all soil samples. However, 15% of total microbiome across all soil samples was made up of bacteria from three groups: the class Phycisphaerae, and the families Acidobacteriaceae, and Sphingobacteriaceae. By contrast, salamanders (as a whole) had four dominant OTUs, two belonging to the genus *Pseudomonas*, and one to the genera *Achromobacter,* and *Chlamydia*, which collectively made up 24.3% of the total salamander skin microbiome community (**Figure 2**).

The majority of OTUs were found in at least one soil sample and one salamander sample. However, when looking across all soil samples, we did find 94 unique OTUs that were not found in salamander samples. All of these unique soil OTUs were present in very low abundances, all together accounting for approximately 1.1% of the soil microbial community. Conversely, we found 756 OTUs that were unique to salamanders, and not found in any of our soil samples. These unique salamander OTUs were also present in very low abundances, together making up only 3.9% of the total salamander skin microbiome community. Note that while these unique OTU's might represent bacteria that are acquired from sources other than soil (including conspecifics), it is also possible that they are present in soil that we did not sample at our sites, given that soil is typically quite heterogeneous.

We assessed beta diversity across all samples (soil and salamander) using unweighted and weighted UniFrac metrics. Analysis of unweighted UniFrac distances across all samples showed that site was the greatest predictor of differences in bacterial communities among samples (adonis R^2 = 0.14, p = 0.001), with habitat type also explaining a significant amount of variation (adonis R^2 = 0.12, p = 0.001). Sample type ("Soil" versus "Salamander") explained a very small degree of variation between samples in an unweighted comparison (adonis R^2 = 0.058, p = 0.001; **Figure 3**). Conversely, when analyzing the weighted UniFrac distances, sample type ("Soil" versus "Salamander") was the greatest predictor of differentiation between bacterial communities (adonis R^2 = 0.19, p = 0.001; **Figure 3**). Site and habitat type also explained a significant amount of variation using the weighted comparison (adonis site R^2 = 0.15, p = 0.001, habitat type R^2 = 0.128, p = 0.001) across all samples.

Differences Within *Ensatina* Higher and Lower Clade Groups

Within *Ensatina* samples, Shannon's diversity was normally distributed (Shapiro–Wilk test, $p > 0.05$), and all other diversity metrics were non-normally distributed (Shapiro–Wilk test, $p < 0.001$). Simpson's diversity, Shannon's diversity and evenness did not differ between lower clade groups (all p-values > 0.05). Lower clade groups did differ in OTU richness (Kruskal–Wallis test, p = 0.003) and phylogenetic diversity (Kruskal–Wallis test, p = 0.001). *Post hoc* pairwise two-sample t-tests of lower clade groups showed no significant differences in any alpha diversity metric after Bonferroni correction. No alpha diversity metrics significantly differed between higher clade groups.

Ensatina lower clade groups harbored between three and seven dominant OTUs (≥3% of microbiome composition), comprising 11.5–57.8% of the microbiome for a given clade (**Figure 4**). Every lower clade group had at least one dominant OTU belonging to the genus *Pseudomonas*. Southern *platensis*, *oregonensis* [3] and *oregonensis* [4] all had dominant OTUs (OTUs making up >3% of the microbiome) that were not dominant in any other lower clade group. For the higher clade groups, there was a great amount of overlap in dominant OTUs (**Figure 2**). Clade A had three dominant OTUs and the Coastal Clade and Oregonensis [1] Clade each had five dominant OTUs. All higher clades shared two dominant OTUs belonging the

OTU ID	Taxon	Soil	Salamander	*Batrachoseps*	*B. attenuatus*	*B. luciae*	*B. gregarious*	*Ensatina*	Clade A	*Oregonensis* Clade	Coastal Clade
280459	*Pseudomonas sp.*	0.008	9.779	12.569	14.799	11.834	4.156	8.683	6.901	11.518	11.524
646549	*Pseudomonas sp.*	0.025	6.200	6.439	7.619	5.869	2.724	6.106	5.340	6.223	7.644
de novo	*Achromobacter sp.*	0.001	4.606	0.005	0.005	0.003	0.008	6.413	6.896	22.891	0.669
de novo	*Chlamydia* sp.	0.000	3.787	0.541	0.095	1.232	0.003	5.062	2.382	3.883	10.905
544847	f_Xanthomonadaceae	0.007	2.851	3.464	4.144	3.210	1.023	2.611	1.957	2.227	4.062
624310	*Sanguibacter* sp.	0.003	2.697	3.187	4.284	2.311	1.154	2.505	2.284	2.192	3.047
277094	*Pseudomonas sp.*	0.012	2.322	2.983	3.375	3.006	0.883	2.062	1.563	2.991	2.818
de novo	*Achromobacter sp.*	0.001	2.221	7.837	10.809	3.562	10.066	0.015	0.012	0.034	0.015
de novo	*Chlamydia* sp.	0.001	1.952	6.905	13.080	0.852	0.003	0.006	0.001	0.003	0.016

FIGURE 2 | Heat map of the relative abundances as percent values of dominant OTUs (≥3% of microbiome composition) for each sample type, genus, species and *Ensatina* higher clade groups.

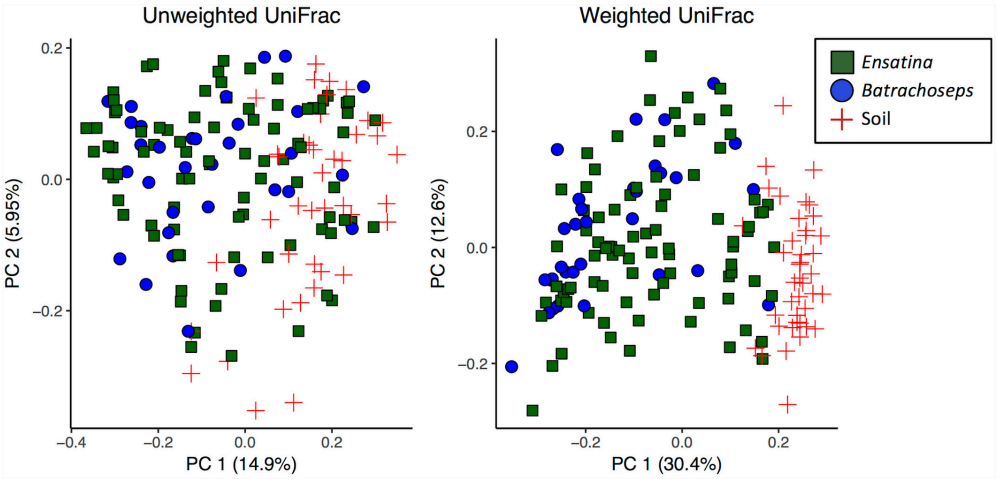

FIGURE 3 | Principle coordinates analysis plots of unweighted and weighted UniFrac distances of all salamander and soil samples.

		Coastal Clade		Clade A							
OTU ID	Taxon	*E. e. xanthoptica*	*E. e. eschscholtzii*	*E. e. picta*	*E. e. oregonensis* [2]	*E. e. oregonensis* [3]	*E. e. oregonensis* [4]	*E. e.* northern *platensis*	*E. e.* southern *platensis*	*E. e. klauberi*	*E. e. oregonensis* [1]
4323871	*Methylobacterium sp.*	0.001	0.092	1.068	0.946	0.085	7.756	1.551	0.000	2.991	2.104
573035	*Alicyclobacillus sp.*	0.002	0.001	0.845	1.160	0.002	5.629	2.446	0.008	2.172	0.056
186867	*Rickettsia sp.*	0.002	0.001	0.081	0.081	0.640	0.000	0.001	9.425	0.255	0.015
de novo	*Cellulomonas sp.*	0.006	0.018	0.024	0.034	5.396	1.175	0.007	0.025	0.186	0.004
de novo	f__Chitinophagaceae	0.019	0.075	0.015	0.009	0.004	0.001	4.390	0.000	0.013	0.023
106985	*Pseudomonas sp.*	0.024	0.066	0.454	0.028	9.449	4.348	0.069	0.015	3.404	0.186
295031	*Pseudomonas fragi*	0.049	0.426	0.584	0.180	4.272	2.726	1.084	0.148	0.765	0.131
350105	*Pseudomonas sp.*	0.055	0.032	0.527	0.032	0.152	0.507	3.366	0.001	1.747	0.772
589597	*Pseudomonas viridiflava*	0.108	0.431	0.267	0.219	3.611	0.670	0.031	0.010	0.083	0.087
de novo	*Achromobacter sp.*	0.191	1.532	30.336	4.037	2.153	0.886	0.100	0.022	0.027	22.891
250626	*Luteibacter rhizovicinus*	0.341	0.745	0.183	0.531	0.307	4.950	1.765	0.180	0.149	0.323
633252	*Pseudomonas sp.*	1.404	2.829	3.609	3.140	0.701	0.865	2.979	0.972	0.648	2.255
277094	*Pseudomonas sp.*	1.866	4.539	1.046	5.171	0.168	0.016	0.011	1.019	0.314	2.991
624310	*Sanguibacter sp.*	2.969	3.189	1.137	5.867	0.716	0.215	0.002	2.609	3.453	2.192
544847	f__Xanthomonadaceae	4.094	4.005	0.685	6.014	0.424	0.294	0.186	2.876	1.201	2.227
646549	*Pseudomonas sp.*	5.160	12.138	2.932	11.922	0.746	3.426	3.793	7.473	4.410	6.223
280459	*Pseudomonas sp.*	8.830	16.398	4.575	21.672	0.129	0.328	0.314	5.441	3.677	11.518
de novo	Chlamydia sp.	16.921	0.021	2.098	0.003	11.180	0.029	0.001	0.000	2.360	3.883

FIGURE 4 | Heat map of the relative abundances as percent values of dominant OTUs (>3% of microbiome composition within sample group) for each *Ensatina* clade.

genus *Pseudomonas*. Each lower clade group had unique OTUs not present in any other lower clade (Supplementary Table 4). The number of unique OTUs varied between four, as seen in *oregonensis* [4], and 151, as seen in *xanthoptica* [1]. Looking at *Ensatina* samples at the higher clade level, Clade A had 778 unique OTUs, the Coastal Clade had 278 unique OTUs, and *oregonensis* [1] had 26 unique OTUs.

Using unweighted UniFrac analyses comparing all *Ensatina* samples, we found that lower clade group and habitat type were the greatest predictors of similarity among samples (adonis, lower

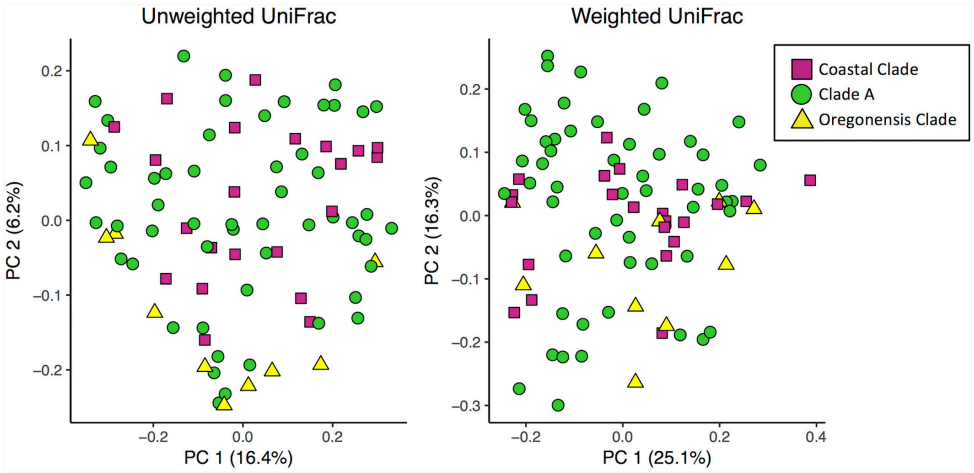

FIGURE 5 | Principle coordinates analysis plots of unweighted and weighted Unifrac distances of *Ensatina* samples by higher clade groups.

clade $R^2 = 0.228$, $p = 0.001$, habitat type $R^2 = 0.196$, $p = 0.001$). Higher clade group explained much less variation (adonis, $R^2 = 0.048$, $p = 0.001$) (**Figure 5**). Weighted UniFrac analysis of all *Ensatina* samples yielded similar results as unweighted results, but with habitat type explaining slightly more variation than lower clade group (adonis, lower clade $R^2 = 0.205$, $p = 0.001$, habitat type $R^2 = 0.221$, $p = 0.001$, higher clade $R^2 = 0.04$, $p = 0.001$; **Figure 5**). Therefore, when only examining bacterial species present, lower clade group identity appears to have a larger influence on the microbiome composition than habitat type. However, when we account for the relative abundances of those bacterial species among samples, habitat type appears to play a larger role than lower clade group identity. We also tested for a correlation between genetic distance and Unifrac distance for a subset of lower clade groups for which genetic distances have been established (Wake, 1997; Kuchta et al., 2009b). We found that there was no significant correlation between genetic distance and unweighted Unifrac distance, nor weighted Unifrac distance (all p-values > 0.05).

We also did pairwise comparisons of unweighted and weighted Unifrac distances of *Ensatina* lower clade groups (**Table 2** and Supplementary Table 3). After Bonferroni correction, three lower clade comparisons showed significant differences in both weighted and unweighted Unifrac distances. Two comparisons were significantly different for weighted, but not unweighted, Unifrac distances. Ten other lower clade comparisons were significantly different for unweighted Unifrac distances but no longer significantly different when looking at weighted data. There was no pattern, in terms of clades with higher genetic distance being more likely to show distinct microbiomes.

We determined that 29 OTUs made up the core microbiome of *Ensatina* (Supplementary Table 5). These OTUs were present in a minimum of 90% of *Ensatina* samples and made up 1.6–87.3% of the total microbiome for any given individual (mean = 38.6%). The average core abundance was non-normally distributed (Shapiro–Wilk test, $p < 0.001$) and varied between lower clade groups: *E. e. eschscholtzii* (53.9%), *E. e. klauberi* (26.4%), *E. e.* northern *platensis* (17.9%), *E. e. oregonensis* [1] (51.7%), *E. e. oregonensis* [2] (59.6%), *E. e. oregonensis* [3] (32.5%), *E. e. oregonensis* [4] (22.1%), *E. e. picta* (47.2%), *E. e.* southern *platensis* (26.6%), *E. e. xanthoptica* [1] (27.2%). Differences in core abundances were found to be significantly different across lower clade groups (Kruskal–Wallis test, $p < 0.001$).

Ensatina vs. *Batrachoseps*

Measures of alpha diversity were not significantly different, with the exception of evenness, between samples collected from *Ensatina* and *Batrachoseps*, even after excluding *Ensatina* samples from geographic ranges that did not overlap with *Batrachoseps*. *Ensatina*'s alpha diversity was significantly more even between samples than *Batrachoseps* (non-parametric two-sample t-test, Shannon's equitability, $p = 0.045$). When comparing all *Ensatina* samples to each species of *Batrachoseps* separately, *Ensatina* was significantly more even than *B. attenuatus* (non-parametric two-sample t-test, Shannon's equitability, $p = 0.03$) and did not differ from the other two *Batrachoseps* species. However, when only considering *Ensatina* samples that overlapped with the range of *Batrachoseps* samples (**Figure 1** and **Table 1**), *Ensatina* was not significantly more even than *Batrachoseps*, nor any one particular species of *Batrachoseps*. Additionally, when comparing *Batrachoseps* and *Ensatina* within each site, we found no significant differences in any alpha diversity metrics. Comparing *Batrachoseps* species to one another, the only difference we found was that *B. gregarious* was slightly more even than *B. attenuatus* (non-parametric two-sample t-test, Shannon's equitability, $p = 0.036$).

Batrachoseps had six dominant OTUs (≥3% of microbiome composition) and *Ensatina* had four, accounting for 40.5% and 26.3% of the overall microbiome community, respectively (**Figure 2**). *Batrachoseps* and *Ensatina* shared two dominant OTUs, both of which were from the family Pseudomonas. *Batrachoseps*' other dominant OTUs belonged to the

TABLE 2 | Summary of R^2 and *p*-values of adonis analyses from *Ensatina* lower clade group pairwise comparisons of weighted and unweighted Unifrac distances.

Lower clade 1	Lower clade 2	Unweighted R^2	Unweighted *p*-value	Weighted R^2	Weighted *p*-value
Eschscholtzii	Klauberi	0.15755	0.001*	0.19898	0.001*
Eschscholtzii	Oregonensis [2]	0.10078	0.001*	–	0.172
Northern Platensis	Oregonensis [2]	0.1683	0.001*	0.23664	0.001*
Northern Platensis	Oregonensis [1]	0.13087	0.009	0.16953	0.001*
Northern Platensis	Eschscholtzii	0.15971	0.001*	0.23874	0.001*
Oregonensis [1]	Klauberi	0.1706	0.001*	0.16023	0.01
Oregonensis [2]	Klauberi	0.18177	0.001*	0.17119	0.005
Oregonensis [4]	Klauberi	0.2117	0.001*	–	0.165
Picta	Eschscholtzii	0.10022	0.002	0.21106	0.001*
Picta	Klauberi	0.11739	0.001*	0.14155	0.014
Xanthoptica [1]	Oregonensis [2]	0.17621	0.001*	0.1362	0.012
Xanthoptica [1]	Oregonensis [3]	0.14161	0.001*	–	0.112
Xanthoptica [1]	Eschscholtzii	0.12407	0.001*	0.12109	0.021
Xanthoptica [1]	Oregonensis [1]	0.14648	0.001*	0.09914	0.05
Xanthoptica [1]	Klauberi	0.11135	0.001*	–	0.219

After Bonferroni correction, only p-values ≤ 0.001 (*) were considered significant. Only significant comparisons are included in this table. For full results from all comparisons, see Supplementary Table 3.

family Xanthomonadaceae, and the genera *Sanguibacter*, *Achromobacter*, and *Chlamydia*. *Ensatina*'s other dominant OTUs were in the genera *Achromobacter* and *Chlamydia*. When comparing salamander genera there were 865 OTUs unique to *Ensatina*, making up 2.0% of the community, and there were 108 OTUs unique to *Batrachoseps*, making up 0.65% of the community. Looking at individual species within *Batrachoseps*, we found that *B. attenuatus* had seven dominant OTUs, *B. luciae* had five, and *B. gregarious* had two (**Figure 2**). These dominant OTUs made up 58.1%, 27.5%, and 14.2% of the microbiome communities for each of these three species, respectively.

When analyzing beta diversity among samples, genus explained a very small amount of variation between all *Ensatina* and *Batrachoseps* samples in both unweighted (adonis $R^2 = 0.014$, $p = 0.017$) and weighted analyses (adonis $R^2 = 0.033$, $p = 0.001$) (**Figure 3**). However, when we excluded *Ensatina* samples from outside the geographic range where we found *Batrachoseps*, genus explained no significant variation in samples using an unweighted analysis (adonis $p > 0.05$), and explained a small amount of variation using a weighted analysis (adonis $R^2 = 0.027$, $p = 0.032$). We also compared *Ensatina* and *Batrachoseps* samples within each site. After Bonferroni correction, to account for the six pairwise comparisons, genus did not explain within site variation between salamander samples (all *p*-values > cut-off of 0.008).

Of the 29 core OTUs identified in *Ensatina* (Supplementary Table 5), 16 of them belonged to the family Pseudomonadaceae. The other core OTUs belonged to the families: Comamonadaceae, Methylobacteriaceae, Sanguibacteraceae, Microbacteriaceae, Propionibacteriaceae, Bradyrhizobiaceae, Sphingomonadaceae, Xanthomonadaceae, Enterobacteriaceae, and Alcaligenaceae. We identified 95 OTUs found on 90% of the *Batrachoseps* individuals sampled (Supplementary Table 5). It is important to note that these 95 OTUs should not be considered a true representation of the core microbiome for this genus, as we only sampled three of the 21 species of *Batrachoseps*. Within *Batrachoseps attenuatus* we found 67 core OTUs. The core microbiome for this salamander species made up an average of 70.5% of the total microbiome, varying between 14.3% and 93.2% of the microbiome per individual.

DISCUSSION

In this study, we characterized the skin microbiome of the plethodontid salamander *Ensatina eschscholtzii* throughout its range. Our analysis included 10 of the 12 lower clade groups, as well as the three higher clade groups, that make up the *Ensatina* species complex (Kuchta et al., 2009a). Additionally, we characterized the microbiome across three species in the genus *Batrachoseps*, which belongs to the same family (Plethodontidae) as *Ensatina*. We compared microbiome variation between groups of salamanders of different genetic distances to evaluate the potential for the skin microbiome to serve as a phylogenetic signal in these terrestrial salamanders. We also evaluated microbes present in the soil and various habitat variables to elucidate the respective roles of host and their surrounding environment in shaping the skin microbiome community.

Differences Between Soil and Salamanders

Soil had significantly higher alpha diversity than salamander samples. This is unsurprising given that the amount of microbes associated with organisms is predicted to be much less than the alpha diversity found within soil (Whitman et al., 1998; Curtis et al., 2002). It is important to note that while we did find a significant difference in alpha diversity, the magnitude of this difference was not as great as one might expect. The alpha diversity we observed in our soil samples is likely an underrepresentation of the full microbial diversity present in our terrestrial salamanders' environment. For each *Ensatina* lower clade group, we only analyzed up to 5 soil samples,

versus up to 15 salamander samples (10 *Ensatina* and 5 *Batrachoseps*). Additionally soil samples were collected from the very top surface level, rather than taking a deeper core sample, which would show greater diversity. This top layer of collected substrate was often predominated with decaying wood matter rather than silt, clay. or sand. Previous evaluation of bacterial communities on terrestrial salamanders versus free-living assemblages found on cover objects (i.e., decaying logs) identified similar magnitudes of difference in alpha diversity as reported here, with logs actually having lower richness than salamander skin (Fitzpatrick and Allison, 2014). Our soil samples were also localized from under cover objects, which may explain why we found many more unique OTUs on salamanders than within the soil, especially considering salamanders move through their environment, exposing them to a broad spectrum of substrates and surfaces.

We also found that soil samples exhibited more evenness in alpha diversity than salamander samples. High species diversity and uniform species distribution in soil can be explained by low competition between bacterial species due to spatial heterogeneity of resources (Zhou et al., 2002). The high diversity we observed in soil explains, in part, why we found no dominant OTUs ($\geq$3% relative abundance) within soil samples. Other studies have also found high alpha diversity to be associated with high evenness across bacterial species in the soil (Rubin et al., 2013).

It is notable that salamanders and soil shared no dominant OTUs from the same genera. Two dominant salamander OTUs belonged to the genus *Pseudomonas*. Many species in this genus have been identified as amphibian symbionts that protect against disease, including chytridiomycosis (Harris et al., 2006; Flechas et al., 2012; Woodhams et al., 2015), which may explain why none of the salamanders sampled in this study were positive for *Batrachochytrium dendrobatidis*. Several studies corroborate a lower susceptibility of salamanders to *Batrachochytrium dendrobatidis*, which could be explained in part by their symbiotic bacteria (Bancroft et al., 2011; Muletz et al., 2014; Sette et al., 2015). Alternatively, the salamanders evaluated in this study may also exhibit lower levels of susceptibility to *Batrachochytrium dendrobatidis* due to their terrestrial life history limiting their contact with this pathogen, or due to environmental conditions (i.e., drought) during this study, which was likely to inhibit fungal growth. It is unclear what potential symbiotic function dominant OTUs in the genera *Achromobacter* and *Chlamydia* found on *Ensatina* skin might play; however, species within *Chlamydia* have been identified as pathogenic to salamanders (Martel et al., 2012).

In our analyses of beta diversity using the unweighted UniFrac metric, sample type (soil or salamander) was not the strongest predictor of microbiome composition across all samples. Instead, site and habitat type explained the most variation. This indicates that the geographic location in which these salamanders live is playing a role in the microbes present in the environment and therefore affecting which microbes are available for acquisition by the host salamanders. However, interestingly, when relative abundances of the bacterial species present were considered (weighted UniFrac), sample type (soil or salamander) was the strongest predictor of microbiome composition. These results suggest that salamanders are likely procuring many bacterial species from their surroundings, but that salamander skin provides different conditions than the soil, allowing for skin and soil microbial assemblages to vary in relative species abundances, supporting previous findings (Fitzpatrick and Allison, 2014; Walke et al., 2014; Prado-Irwin et al., 2017). The skin's conditions for bacterial growth are likely different from the conditions provided in the environment. Indeed, soil microbial community richness and diversity have been strongly driven by pH conditions (Fierer and Jackson, 2006), whereas microbial communities on amphibian skin are influenced more by other factors such as diet (Antwis et al., 2014), immune defenses, and temperature (Woodhams et al., 2014).

Influences of Host Identity on the Cutaneous Microbiome

After establishing that salamander cutaneous microbial community composition is distinct from the surrounding soil, we sought to explore the extent to which host identity might be able to explain variation in the microbiome within salamanders. We first evaluated differences between the *Ensatina* lower clade groups. Overall, there was a general lack of differences in alpha diversity of the skin microbiome between the *Ensatina* clade groups. A previous study comparing subspecies on an aquatic salamander, *Cryptobranchus alleganiensis*, found a similar lack of differentiation in alpha diversity among conspecific hosts (Hernández-Gómez et al., 2017).

Lower clade group identity did indicate differences in the beta diversity of the microbial community. However, most pairwise comparisons of *Ensatina* lower clades showed no differences in unweighted or weighted analyses, and only three comparisons were significantly different for both metrics. Therefore, the role of lower clade group identity in explaining differences in beta diversity was likely driven by a few unique microbiomes of particular clades, rather than each clade being unique from all others (**Table 2**). Genetic distances between *Ensatina* subspecies have been previously described (Wake and Yanev, 1986; Kuchta et al., 2009b) and our comparisons of beta diversity did not show any pattern related to genetic distance between lower clade groups. For example, *E. e. eschscholtzii* and *E. e.* southern *platensis* are more distantly related than *E. e.* northern *platensis* and *E. e. oregonensis* [2], but only the latter comparison showed significant differences in beta diversity. Despite the fact that we did not see a correlation between differentiation in the microbiome and genetic distance of hosts, it is interesting to note that the two most distantly related clade groups, *E. e. klauberi* and *E. e. eschscholtzii*, did show significant differences in both the weighted and unweighted analyses of beta diversity. However, a previous study comparing populations within a single subspecies (*E. e. xanthoptica*) did not find a correlation between genetic distance and beta diversity (Prado-Irwin et al., 2017).

Habitat type also predicted the microbial community. Due to the fact that the clade identity is correlated with habitat type, it is possible that the differences we observed in the microbial communities between lower clade groups are driven by habitat differences rather than traits (or evolutionary history) of the

salamanders themselves. Further support for this conclusion comes from the analysis of higher-order clades (Coastal Clade, Clade A, Oregonensis Clade), which span larger geographic ranges and more habitat types. In our analysis of beta diversity at the higher clade level, host identity does not explain a large amount of variation between samples (**Figure 5**), lending credence to the conclusion that microbiome differences between these higher-order clades are likely more driven by habitat type. Previous studies have found that environmental variables such as land use type and elevation are important predictors of amphibian skin microbiome beta diversity (Hughey et al., 2017). Additionally, an experiment on salamander larvae showed that the skin microbiome shifts in response to transplantation into a different aquatic habitat (Bletz et al., 2016).

Due to the fact that different *Ensatina* clades are necessarily correlated to localities, we included *Batrachoseps* in our study, which are sympatric with *Ensatina* for much of their range, as a method of control. If the microbiomes of *Ensatina* clade groups were distinct, and *Ensatina* and *Batrachoseps* microbiomes were even more differentiated, this would provide strong evidence that host identity was contributing to differences in microbiome composition. However, as mentioned prior, we did not find *Ensatina* clade group (higher or lower) to be a strong predictor of microbiome variation. Instead, we were able to use our *Batrachoseps* data to evaluate if species identity within a family could serve as a predictor of microbiome composition for samples paired within a site. Studies on aquatic amphibians of different families have found species identity to be a significant predictor of the microbiome composition (McKenzie et al., 2012; Kueneman et al., 2014). We sought to determine if a similar pattern could be observed in California terrestrial salamanders within a family. This would help us determine which taxonomic level is most relevant for evaluating differences in the microbiome.

We found no differences in alpha diversity between *Ensatina* and *Batrachoseps*, and no differences in beta diversity when comparing sympatric *Ensatina* and *Batrachoseps* samples (**Figure 3**). From this, we conclude that taxonomic identity within these groups is not a reliable predictor of the microbial community and that any differences between these hosts due to size, diet, habitat use, etc. do not strongly influence the microbial communities that can reside on the skin. These results differ from what has been found in aquatic amphibians, where host species has been shown to be the strongest predictor of differences in skin microbiome samples, as opposed to site effects (McKenzie et al., 2012; Kueneman et al., 2014). This disparity may be explained by differences in aquatic and terrestrial amphibians, with major differences in host ecology driving patterns of microbiome composition and influencing the relative role of host phylogenetic effects (Bletz et al., 2017). Alternatively, it may be that *Batrachoseps* and *Ensatina* are not genetically differentiated enough to detect host effects, as found in aquatic amphibian communities. However, other work on plethodontids corroborates what we found (Muletz Wolz et al., 2017), while work on subspecies of aquatic salamanders show differences in beta diversity (Hernández-Gómez et al., 2017). We might expect plethodontids in particular to experience similar selective pressures on their skin microbiomes, and therefore exhibit more uniformity across species, due to the fact that they are lung-less and therefore rely heavily on their skin for biological processes.

Though we did not find that host phylogeny strongly influences microbiome variation in our system, more studies must be done to determine if this is true across other systems, including within amphibians at higher taxonomic levels. Studies done on primates have found evidence that differences in the gut and skin microbiome coincide with divergences between host species (Council et al., 2016; Moeller et al., 2016). A recent study on aquatic amphibians found that the skin microbiome beta diversity differed between host orders but not host species within the same order (Bletz et al., 2017). However, it can be difficult to determine if differences are due to host genetics or other factors such as diet (Ley et al., 2008b). Though studies have shown species identity to be connected to microbiome composition in amphibians, it is unclear which host factors drive those differences. Future studies should try and explore the respective roles of genetics, environment and the interaction between the two in shaping the microbiome.

While we did not find strong evidence that the microbial communities are significantly different between any taxonomic groups, we did identify unique OTUs to each genus, species, and clade group. Though collectively the unique OTUs only accounted for small proportions of the total microbiome, they could be playing an important role in host health (Podar et al., 2007; Hajishengallis et al., 2011; Human Microbiome Project Consortium, 2012; Davenport et al., 2014). The functionality of rare versus dominant antifungal symbionts differs depending on the host species (Walke et al., 2017). However, other studies have found that low abundance microorganisms do not play any important function in host physiology, and therefore the unique OTUs we found may be insignificant (Lee et al., 2016).

CONCLUSION

The primary aim of this study was to determine whether differences in amphibian skin microbial communities could be explained due to host divergence leading to deviating microbial acquisition. If this occurs, we would expect more distantly related hosts to have more distinct microbiomes, and more closely related hosts to have more similar microbiomes. Our study did not support this hypothesis for two plethodontid genera. Differences in beta diversity were low between genera and between intra-generic *Ensatina* clades, with taxonomic group explaining little to no variation among samples. While we did find that lower clade group was a predictor of microbiome composition, habitat type explained a similar amount of variation. Our results suggest that aspects of the environment that contribute to habitat type influence the skin microbiome of plethodontids, and host specific factors may also be playing a role. Further studies need to be done to fully detangle the relative

impacts of the host vs. the environment and explore how specific environmental factors influence the skin microbiome.

AUTHOR CONTRIBUTIONS

AB, AZ, and VV conceived and designed the study. AB and SP-I collected data and performed the lab work. AB analyzed the data and primarily wrote the manuscript with input regarding interpretation and drafts from AZ, VV, and SP-I.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00442/full#supplementary-material

REFERENCES

Antwis, R. E., Haworth, R. L., Engelmoer, D. J. P., Ogilvy, V., Fidgett, A. L., and Preziosi, R. F. (2014). *Ex situ* diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). *PLoS One* 9:e85563. doi: 10.1371/journal.pone.0085563

Bancroft, B. A., Han, B. A., Searle, C. L., Biga, L. M., Olson, D. H., Kats, L. B., et al. (2011). Species-level correlates of susceptibility to the pathogenic amphibian fungus *Batrachochytrium dendrobatidis* in the United States. *Biodivers. Conserv.* 20, 1911–1920. doi: 10.1007/s10531-011-0066-4

Bataille, A., Lee-Cruz, L., Tripathi, B., Kim, H., and Waldman, B. (2016). Microbiome variation across amphibian skin regions: implications for chytridiomycosis mitigation efforts. *Microb. Ecol.* 71, 221–232. doi: 10.1007/s00248-015-0653-0

Becker, R. A., and Wilks, A. R. (2016). *Maps: Draw Geographical Maps. [Online]*. Available at: https://cran.r-project.org/package=maps

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V. J., Rabemananjara, F. C. E., Rakotoarison, A., et al. (2017). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.* 8:1530. doi: 10.3389/fmicb.2017.01530

Bletz, M. C., Goedbloed, D. J., Sanchez, E., Reinhardt, T., Tebbe, C. C., Bhuju, S., et al. (2016). Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nat. Commun.* 7:13699. doi: 10.1038/ncomms13699

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Methods* 10, 57–59. doi: 10.1038/nmeth.2276

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2010b). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Council, S. E., Savage, A. M., Urban, J. M., Ehlers, M. E., Skene, J. H. P., Platt, M. L., et al. (2016). Diversity and evolution of the primate skin microbiome. *Proc. Biol. Sci.* 283:20152586. doi: 10.1098/rspb.2015.2586

Curtis, T. P., Sloan, W. T., and Scannell, J. W. (2002). Estimating prokaryotic diversity and its limits. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10494–10499. doi: 10.1073/pnas.142680199

Davenport, E. R., Mizrahi-Man, O., Michelini, K., Barreiro, L. B., Ober, C., and Gilad, Y. (2014). Seasonal variation in human gut microbiome composition. *PLoS One* 9:e90731. doi: 10.1371/journal.pone.0090731

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Fierer, N., and Jackson, R. (2006). The diversity and biogeography of soil bacterial communities. *Proc. Natl. Acad. Sci. U.S.A.* 103, 626–631. doi: 10.1073/pnas.0507535103

Fitzpatrick, B. M., and Allison, A. L. (2014). Similarity and differentiation between bacteria associated with skin of salamanders (*Plethodon jordani*) and free-living assemblages. *FEMS Microbiol. Ecol.* 88, 482–494. doi: 10.1111/1574-6941.12314

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS One* 7:e44832. doi: 10.1371/journal.pone.0044832

Hajishengallis, G., Liang, S., Payne, M. A., Hashim, A., Jotwani, R., Eskan, M. A., et al. (2011). A low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and the complement pathway. *Cell Host Microbe* 10, 497–506. doi: 10.1016/j.chom.2011.10.006

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Hernández-Gómez, O., Kimble, S. J. A., Briggler, J. T., and Williams, R. N. (2017). Characterization of the cutaneous bacterial communities of two giant salamander subspecies. *Microb. Ecol.* 73, 445–454. doi: 10.1007/s00248-016-0859-9

Hughey, M. C., Pena, J. A., Reyes, R., Medina, D., Belden, L. K., and Burrowes, P. A. (2017). Skin bacterial microbiome of a generalist Puerto Rican frog

varies along elevation and land use gradients. *PeerJ* 5:e3688. doi: 10.7717/peerj.3688

Human Microbiome Project Consortium (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Jackson, C. R., Langner, H. W., Donahoe-Christiansen, J., Inskeep, W. P., and McDermott, T. R. (2001). Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. *Environ. Microbiol.* 3, 532–542. doi: 10.1046/j.1462-2920.2001.00221.x

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, e5049–e5058. doi: 10.1073/pnas.1412752111

Kuchta, S. R., Parks, D. S., Mueller, R. L., and Wake, D. B. (2009a). Closing the ring: historical biogeography of the salamander ring species *Ensatina eschscholtzii*. *J. Biogeogr.* 36, 982–995. doi: 10.1111/j.1365-2699.2008.02052.x

Kuchta, S. R., Parks, D. S., and Wake, D. B. (2009b). Pronounced phylogeographic structure on a small spatial scale: geomorphological evolution and lineage history in the salamander ring species *Ensatina eschscholtzii* in central coastal California. *Mol. Phylogenet. Evol.* 50, 240–255. doi: 10.1016/j.ympev.2008.10.019

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Lee, M. J., Jeong, H. J., Jang, S. H., Lee, S. Y., Kang, N. S., Lee, K. H., et al. (2016). Most low-abundance "background" *Symbiodinium* spp. are transitory and have minimal functional significance for symbiotic corals. *Microb. Ecol.* 71, 771–783. doi: 10.1007/s00248-015-0724-2

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008a). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725

Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. I. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 776–788. doi: 10.1038/nrmicro1978

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P. C., and Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* 5:441. doi: 10.3389/fmicb.2014.00441

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228

Lozupone, C. A., Hamady, M., Kelley, S. T., and Knight, R. (2007). Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl. Environ. Microbiol.* 73, 1576–1585. doi: 10.1128/AEM.01996-06

Martel, A., Adriaensen, C., Bogaerts, S., Ducatelle, R., Favoreel, H., Crameri, S., et al. (2012). Novel *Chlamydiaceae* disease in captive salamanders. *Emerg. Infect. Dis.* 18, 1020–1022. doi: 10.3201/eid1806.111137

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., et al. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 6, 610–618. doi: 10.1038/ismej.2011.139

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3229–3236. doi: 10.1073/pnas.1218525110

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. V., Lonsdorf, E. V., Muller, M. N., et al. (2016). Cospeciation of gut microbiota with hominids. *Science* 353, 380–382. doi: 10.1126/science.aaf3951

Moloney, R. D., Desbonnet, L., Clarke, G., Dinan, T. G., and Cryan, J. F. (2014). The microbiome: stress, health and disease. *Mamm. Genome* 25, 49–74. doi: 10.1007/s00335-013-9488-5

Moritz, C., Schneider, C. J., and Wake, D. B. (1992). Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. *Syst. Biol.* 41, 273–291. doi: 10.1093/sysbio/41.3.273

Muletz, C., Caruso, N. M., Fleischer, R. C., McDiarmid, R. W., and Lips, K. R. (2014). Unexpected rarity of the pathogen *Batrachochytrium dendrobatidis* in appalachian *Plethodon* salamanders: 1957-2011. *PLoS One* 9:e103728. doi: 10.1371/journal.pone.0103728

Muletz Wolz, C. R., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., and Lips, K. R. (2017). Effects of host species and environment on the skin microbiome of plethodontid salamanders. *J. Anim. Ecol.* 87, 341–353. doi: 10.1111/1365-2656.12726

Petrosino, J. F., Highlander, S., Luna, R. A., Gibbs, R. A., and Versalovic, J. (2009). Metagenomic pyrosequencing and microbial identification. *Clin. Chem.* 55, 856–866. doi: 10.1373/clinchem.2008.107565.Metagenomic

Podar, M., Abulencia, C. B., Walcher, M., Hutchison, D., Zengler, K., Garcia, J. A., et al. (2007). Targeted access to the genomes of low-abundance organisms in complex microbial communities. *Appl. Environ. Microbiol.* 73, 3205–3214. doi: 10.1128/AEM.02985-06

Prado-Irwin, S. R., Bird, A. K., Zink, A. G., and Vredenburg, V. T. (2017). Intraspecific variation in the skin-associated microbiome of a terrestrial salamander. *Microb. Ecol.* 74, 745–756. doi: 10.1007/s00248-017-0986-y

R Core Team (2016). *R: A Language and Environment for Statistical Computing. [Online]*. Available at: http://www.r-project.org/

Rollins-Smith, L. A., and Conlon, J. M. (2005). Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Dev. Comp. Immunol.* 29, 589–598. doi: 10.1016/j.dci.2004.11.004

Rubin, B. E. R., Gibbons, S. M., Kennedy, S., Hampton-Marcell, J., Owens, S., and Gilbert, J. A. (2013). Investigating the impact of storage conditions on microbial community composition in soil samples. *PLoS One* 8:e70460. doi: 10.1371/journal.pone.0070460

Sette, C. M., Vredenburg, V. T., and Zink, A. G. (2015). Reconstructing historical and contemporary disease dynamics: a case study using the California slender salamander. *Biol. Conserv.* 192, 20–29. doi: 10.1016/j.biocon.2015.08.039

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi: 10.1038/nrmicro2540

Stebbins, R. C. (1949). Speciation in salamanders of the plethodontid genus Ensatina. *Univ. Calif. Publ. Zool.* 48, 377–526.

Torsvik, V., Øvreås, L., and Thingstad, T. F. (2002). Prokaryotic diversity: magnitude, dynamics, and controlling factors. *Science* 296, 1064–1066. doi: 10.1126/science.1071698

Wake, D. B. (1997). Incipient species formation in salamanders of the Ensatina complex. *Proc. Natl. Acad. Sci. U.S.A.* 94, 7761–7767.

Wake, D. B., and Yanev, K. P. (1986). Geographic variation in allozymes in a "ring species," the plethodontid salamander *Ensatina eschscholtzii* of Western North America. *Evolution* 40, 702–715. doi: 10.1111/j.1558-5646.1986.tb00532.x

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2017). Dominance-function relationships in the amphibian skin microbiome. *Environ. Microbiol.* 19, 3387–3397. doi: 10.1111/1462-2920.13850

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, V. R., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLoS One* 10:e0139848. doi: 10.1371/journal.pone.0139848

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6578–6583. doi: 10.1073/pnas.95.12.6578

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated

bacteria and function against emerging fungal pathogens. *Ecology* 96:595. doi: 10.1890/14-1837.1

Woodhams, D. C., Brandt, H., Baumgartner, S., and Kielgast, J. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* 9:e96375. doi: 10.1371/journal.pone.0096375

Zhou, J., Xia, B., Treves, D. S., Wu, L., Marsh, T. L., Neill, R. V. O., et al. (2002). Spatial and resource factors influencing high microbial diversity in soil spatial and resource factors influencing high microbial diversity in soil. *Appl. Environ. Microbiol.* 68, 326–334. doi: 10.1128/AEM.68.1.326

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The Skin Microbiome of the Neotropical Frog *Craugastor fitzingeri*: Inferring Potential Bacterial-Host-Pathogen Interactions From Metagenomic Data

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Skin symbiotic bacteria on amphibians can play a role in protecting their host against pathogens. Chytridiomycosis, the disease caused by *Batrachochytrium dendrobatidis*, Bd, has caused dramatic population declines and extinctions of amphibians worldwide. Anti-Bd bacteria from amphibian skin have been cultured, and skin bacterial communities have been described through 16S rRNA gene amplicon sequencing. Here, we present a shotgun metagenomic analysis of skin bacterial communities from a Neotropical frog, *Craugastor fitzingeri*. We sequenced the metagenome of six frogs from two different sites in Panamá: three frogs from Soberanía (Sob), a Bd-endemic site, and three frogs from Serranía del Sapo (Sapo), a Bd-naïve site. We described the taxonomic composition of skin microbiomes and found that *Pseudomonas* was a major component of these communities. We also identified that Sob communities were enriched in Actinobacteria while Sapo communities were enriched in Gammaproteobacteria. We described gene abundances within the main functional classes and found genes enriched either in Sapo or Sob. We then focused our study on five functional classes of genes: biosynthesis of secondary metabolites, metabolism of terpenoids and polyketides, membrane transport, cellular communication and antimicrobial drug resistance. These gene classes are potentially involved in bacterial communication, bacterial-host and bacterial-pathogen interactions among other functions. We found that *C. fitzingeri* metagenomes have a wide array of genes that code for secondary metabolites, including antibiotics and bacterial toxins, which may be involved in bacterial communication, but could also have a defensive role against pathogens. Several genes involved in bacterial communication and bacterial-host interactions, such as biofilm formation and bacterial secretion systems were found. We identified specific genes and pathways enriched at the different sites and determined that gene co-occurrence networks differed between sites. Our results

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suggest that skin microbiomes are composed of distinct bacterial taxa with a wide range of metabolic capabilities involved in bacterial defense and communication. Differences in taxonomic composition and pathway enrichments suggest that skin microbiomes from different sites have unique functional properties. This study strongly supports the need for shotgun metagenomic analyses to describe the functional capacities of skin microbiomes and to tease apart their role in host defense against pathogens.

Keywords: skin microbiome, shotgun metagenomics, host-bacteria interactions, amphibians, *Batrachochytrium dendrobatidis*

INTRODUCTION

Microbial symbiotic communities are ubiquitous in animals and plants. For decades, most of the studies in animals have focused on insect endosymbionts, which generally involve only a few bacterial species (Dale and Moran, 2006), whereas more recent studies, mainly in humans, have revealed a complex community of microbes coexisting with their host (Turnbaugh et al., 2007; Grice, 2014; Jorth et al., 2014). Many of these symbionts complete a variety of functions for their hosts, such as nutrient acquisition (Dale and Moran, 2006) and pathogen protection (Fraune et al., 2014; Walke and Belden, 2016). Recent studies have determined that the skin of amphibians harbors bacterial communities that are unique to different species (McKenzie et al., 2012; Kueneman et al., 2014; Walke et al., 2014; Belden et al., 2015; Costa et al., 2016; Rebollar et al., 2016; Bletz et al., 2017a) and might play an important role in protecting the host against *Batrachochytrium dendrobatidis*, Bd (Harris et al., 2009; Becker et al., 2011; Kueneman et al., 2016). This pathogenic fungus causes a skin disease called chytridiomycosis, and it has been considered one of the greatest global threats to amphibian populations (Fisher et al., 2009; Kilpatrick et al., 2010). Field studies have shown that not all species are susceptible to Bd, as some species persist in Bd-endemic regions with no apparent population declines (Crawford et al., 2010; Rebollar et al., 2014; Rodríguez-Brenes et al., 2016). One important component of defense against pathogens that could explain the presence of tolerant and resistant frog species is skin symbiotic bacteria. Hundreds of Bd-inhibitory bacteria have been isolated from the skin of multiple amphibian species from many sites around the world (Antifungal Isolates Database: Woodhams et al., 2015). Moreover, the addition of some of these bacteria (e.g., *Janthinobacterium lividum*) to captive amphibians protected the hosts from Bd infection and reduced morbidity and mortality (Harris et al., 2009; Becker et al., 2011; Kueneman et al., 2016). In addition, skin bacterial community structure before infection can predict mortality and morbidity after infection (Becker et al., 2015a; Walke et al., 2015).

Even though our knowledge of amphibian skin microbial communities has grown considerably in the past decade, we still lack knowledge of the full range of functional capabilities of these communities and their interactions with their hosts. Most of the studies have focused on describing the skin community via 16S rRNA gene amplicon sequencing (Kueneman et al., 2014; Rebollar et al., 2016; Bletz et al., 2017a) and/or culturing bacteria to characterize their inhibitory capacities against Bd using *in vitro* challenge assays (Harris et al., 2006; Flechas et al., 2012; Bell et al., 2013; Becker et al., 2015b; Medina et al., 2017). In other cases, functions have been predicted using bacterial data bases (Kueneman et al., 2015; Bletz et al., 2017b) or predictive tools such as PICRUSt (Loudon et al., 2014a). Moreover, changes in skin bacterial community structure associated with Bd infection have been observed in experimental settings (Jani and Briggs, 2014; Becker et al., 2015a; Walke et al., 2015; Longo and Zamudio, 2017); however, we still lack knowledge of the functional changes that may occur in the bacterial community once Bd is present.

Recent studies on several symbiotic systems have identified genes that were originally described in pathogens, which are also important in mutualistic bacterial species, such as bacterial secretion systems (Dale and Moran, 2006; Preston, 2007; Medina and Sachs, 2010) and biofilm formation (Frese et al., 2013; Schmid et al., 2015). In addition, the host can also affect the symbiotic community through the production of molecules, such as antimicrobial peptides (AMPs) (Gallo and Hooper, 2012; Franzenburg et al., 2013). Furthermore, the study of bacterial symbionts on other animal hosts, like amphibians, has revealed symbiotic bacterial interactions that are important in protecting the hosts against pathogens (Walke and Belden, 2016). One mechanism through which bacteria protect their host is through the production of secondary metabolites that have antimicrobial properties (Flórez et al., 2015). In amphibians, some members of the skin microbiota can produce secondary metabolites that inhibit the growth of the fungal pathogen Bd. Examples of these antifungal metabolites are violacein (Brucker et al., 2008b), prodigiosin (Woodhams et al., 2017), tryptophol (Loudon et al., 2014b), indole-3-carboxaldehyde (I3C) (Brucker et al., 2008b) and 2,4-diacetylphloroglucinol (2,4 DAPG) (Brucker et al., 2008a).

Here, we describe the genes involved in bacterial defense and communication that are present in skin microbiomes of the terrestrial Neotropical frog *Craugastor fitzingeri* using shotgun metagenomics. Our *a priori* goals were to expand our knowledge of the metabolic capacities that symbiotic bacteria have and to explore the presence of genes that are potentially involved in bacteria-bacteria, bacteria-host and bacteria-pathogen interactions. Previous studies have described the skin bacterial structure of *C. fitzingeri* across several regions in Panamá (Belden et al., 2015; Rebollar et al., 2016). These studies showed that the skin bacterial community is dominated by the phyla Proteobacteria and Actinobacteria. Specifically, Rebollar et al. (2016) described differences in bacterial OTU relative abundance in frog skin communities between a Bd-endemic site and a Bd-naïve site. The bacterial

community structure in the Bd-endemic site was enriched for taxa known to have antifungal properties (e.g., *Pseudomonas* and members of the Actinobacteria class). These differences may be related to natural selection caused by the presence or absence of Bd in these lowland regions, although other explanations are possible.

In this study, we analyzed samples from three individuals of *C. fitzingeri* from a Bd-endemic site and three from a Bd-naïve site. These samples were previously analyzed by Rebollar et al. (2016) with 16S rRNA gene amplicon sequencing for bacterial community profiling. We described the taxonomic composition and analyzed the genetic metabolic pathways present in skin microbiomes. Lastly, we compared the bacterial composition and function among frogs from a Bd-endemic site and a Bd-naïve site. We hypothesized that frog skin microbiomes will include genes associated with bacterial communication and bacterial-host interactions, as well as pathways involved in the production of antifungal metabolites and resistance to bacterial toxins, as a result of potential cooperative or competitive interactions within the community. We also hypothesized that taxonomic and functional composition of skin microbiomes will differ between sites with contrasting Bd incidence. A finding of genes associated with bacterial-host interactions would increase our confidence that amphibian skin bacteria are resident species and not transient. Moreover, unraveling the potential functions present in these bacterial communities will advance our knowledge of the interactions occurring among the host, the bacterial symbiotic community and the pathogen, Bd.

METHODS

Sample Selection and Molecular Procedures

C. fitzingeri frogs were collected and swabbed from two lowland forests sites in Panamá: Soberanía National Park (Sob), a Bd endemic site, and Serranía del Sapo (Sapo), a Bd naïve site. DNA from these swab samples was extracted and used in a previous study for 16S amplicon sequencing and qPCR detection of Bd (Rebollar et al., 2016), which contains the details of the sample collection and the molecular procedures used to extract the DNA from the swabs. Six DNA samples extracted from this previous study were used to sequence the metagenome as explained in section Shotgun metagenome sequencing. We chose these samples considering their Bd infection status (infected vs. not infected) and collection site (Bd endemic and Bd naïve) (Table S1). Two other samples were sequenced but were not included in further analyses because they could not be properly annotated.

Shotgun Metagenome Sequencing

Six DNA samples (three from Sob and three from Sapo) were used to obtain frog skin shotgun metagenomes (Table S1). The six barcoded samples were randomly distributed on two lanes (three samples on each lane) and were sequenced on HiSeq 2500 (Genomics Sequencing Center, Bioinformatics Institute, Virginia Tech), generating over 500 million, 100 bp, paired end reads (with ∼200 bp insert size). The numbers of reads for each of the six samples ranged from 84 million to 124 million reads (Table S1). Metagenomic reads were deposited in the SRA Database (NCBI) with the accession number SRP130893 as part of Bioproject PRJNA429199.

Metagenome Binning for Filtering Out Host Reads

With the purpose of filtering out the host (*C. fitzingeri*) reads, we assembled the reads for each of the six samples into metagenome contigs using Ray Meta (Boisvert et al., 2012) *de novo* assembler. Four million reads for each frog sample were then mapped to all the contigs greater than 1,000 bp from all six frogs to determine the relative abundance of each contig. The covariance of these abundances across samples was then used to cluster all contigs with >100 mapped reads into metagenome bins using the PAM k-medoids algorithm in R with $k = 10$. Contigs in three of these bins or clusters (Clusters 1–3) exhibited similar abundance profiles across the six samples and appeared to be predominantly frog (with consistently low GC content ∼42% and best Blastn hits to eukaryotic sequences). We also mapped the reads for each sample to the frog 18S rRNA sequence (assembled from the data) and a database of bacterial 16S rRNA sequences (from www.patricbrc.org) to estimate the relative proportion of eukaryotic (mainly frog) and prokaryotic (mainly bacteria) DNA from each sample. The 18S/16S ratios for the six samples showed good correspondence with the variation in the abundance of the frog DNA clusters. The remaining seven clusters (Cluster 4–10) were composed of contigs belonging to different bacterial groups.

Taxonomic Assignment and Procrustes Analysis

We determined the bacterial species composition of the frog skin samples using Metaphlan (Segata et al., 2012), a method used to profile bacterial communities based on clade-specific marker genes. We determined the relative abundance of bacterial taxa at different taxonomic levels based on the annotated reads that had been previously filtered to eliminate host-derived reads (Table S1).

We used a Procrustes analysis to evaluate how similar the 16S rRNA gene amplicon sequencing data (obtained with QIIME in Rebollar et al., 2016) was to the shotgun metagenome data (obtained with Metaphlan) using the vegan R package (R Core Team, 2015; Oksanen et al., 2016). Procrustes analysis evaluates the congruency between two data sets by the superimposition of principal component analyses (McHardy et al., 2013; Luo et al., 2016). We performed Procrustes analysis on Bray-Curtis distance matrices calculated from the bacterial relative abundance at the genus level in both data sets. We used the PROTEST test function from the vegan R package (R Core Team, 2015; Oksanen et al., 2016), which performs repeated symmetric Procrustes analyses to estimate if the degree of concordance between matrices is greater than expected by random association (Jackson, 1995). Significant *p*-values below 0.05 indicate that the matrices are more similar than expected by random association.

Read Level Functional Annotation

To annotate the metagenome reads (read level analysis), we assigned reads to the bacterial clusters obtained in section Metagenome binning for filtering out host reads by re-mapping reads from each sample to contigs assembled from the same sample using blastn (Altschul et al., 1990) with an e-value cutoff of 1. This resulted in 8,323,746 reads (1.57% out of the total number of reads) that mapped to contigs with frog-associated cluster assignments, which were discarded.

We assigned KEGG orthology (KO) group (Kanehisa and Goto, 2000) annotations to non-frog-associated reads from the six frog samples using blastx with an e-value cutoff of 1 to map reads to the KEGG gene database downloaded on July 15th, 2013. This produced 192,761,569 (36.36%) reads with best hits to genes with KO annotations (Table S1). We calculated KO abundances using the number of reads assigned to each KO. In the case where a read had best hits to multiple KOs, the read count was evenly distributed among the corresponding KOs. For example, if a read mapped equally well to 3 KOs, each KO received 1/3 of the count. We normalized the abundances of KOs using MUSiCC (Manor and Borenstein, 2015) with the inter-sample correction option, which corrects for biases in quantification using the abundance of universal single-copy KOs.

Descriptive and Comparative Analyses Using Read Level Annotations

To evaluate differences in the functional repertoire among frog skin communities at multiple levels of detail, we aggregated the annotated KO assignments into modules, pathways, and classes based on a custom-curated version of the BRITE hierarchy. We summed the relative abundances of all KOs associated with each pathway, module, or class, following Manor et al. (2016). Pathways and modules were further filtered to verify that downstream analysis considers only bacterial pathways or modules. Specifically, a pathway or module was included in our analysis only if at least 1% of bacterial genomes in KEGG contained at least 1 KO from that pathway or module, and if these bacterial genomes contained at least 5% (20%) of the KOs in the pathway or module, on average.

For each pathway, module or class, normalized abundances from samples from the Sapo region were compared to those from Sob using LefSe Analysis (Linear Discriminant Analysis Effect Size) which incorporates the use of two non-parametric tests (Kruskal-Wallis and Wilcoxon) and a linear discriminant analysis to detect biomarkers or genes that are differentially abundant among the groups tested (Segata et al., 2011).

Contig Level Functional Annotation and Assembly

To determine the taxonomic identity of the genes present on frog skin metagenomes we assembled reads into contigs (contig level analysis), annotated them and determined their taxonomy (**Table 1**). Reads were assembled into contigs using stringent criteria to facilitate gene prediction. Forward and reverse reads were assembled using MEGAHIT version 1.3.0 (Li et al., 2016) with default parameters, except for a minimum length of 200 bp for the assembled contigs and a starting kmer size of 23 in increasing steps of 10 until reaching a kmer size of 93. Gene prediction was performed on the newly assembled contigs using Prokka (Seemann, 2014). For metabolic and taxonomic classifications of the predicted genes, we used GhostKOALA from KEGG (Kanehisa et al., 2016).

Descriptive and Comparative Analyses Using Contig Level Annotations

Metabolic and taxonomic data inferred from GhostKOALA were visualized in stacked bar charts using *ad hoc* scripts in R (R Core Team, 2015). We plotted gene counts from five broad functional classes based on KEGG classification: Membrane Transport (MT), Cellular Communication (CC), Metabolism of Terpenoids and Polyketides (MTP), Biosynthesis of Secondary Metabolites (BSM) and Antimicrobial Drug Resistance (ADR). Functional classes or pathways with abundance values < 3 were not plotted. We used LefSe analysis to test for differentially enriched genes (KOs) between sites for all five broad classes and for all the pathways contained within each class (55 pathways in total). Methods on co-occurrence network construction can be found on Supplementary Methods.

RESULTS

Bacterial Composition of *C. fitzingeri* Skin Microbiomes

We determined the taxonomic composition and relative abundance of the bacteria present in the frog metagenomes from *C. fitzingeri*. Based on a Metaphlan analysis of the

TABLE 1 | Assembly and annotation data of the six skin metagenomes of *C. fitzingeri* from sites Sapo ($N = 3$) and Sob ($N = 3$).

Sample ID	Assembly				Annotation			
	Contigs	Max(bp)	avg (bp)	N50	CDS	miscRNAs	tRNAs	rRNAs
Sapo01	311185	154616	501	458	79219	759	704	205
Sapo02	187876	404914	603	568	75067	885	877	134
Sapo03	400815	278576	655	702	231429	2431	3324	195
Sob01	407419	232836	561	516	139736	1196	1548	218
Sob02	148664	42792	703	874	93277	868	1025	66
Sob03	99132	250402	921	1532	82916	858	1379	80

skin metagenome reads, the dominant genera in the Bd-endemic site (Sob) were *Pseudomonas* (74.43%), *Variovorax* (4.72%), *Sanguibacter* (4.65%) and *Stenotrophomonas* (3.23%), while the Bd-naïve site (Sapo) was dominated by *Pseudomonas* (40.46%), *Acinetobacter* (30.6%), *Staphylococcus* (6.38%) and *Delftia* (5.15%). A hierarchical clustering analysis based on the relative abundance of bacterial taxa suggested that the community structure was different between the three samples from Sob and the three samples from Sapo (**Figure 1A**). To determine whether the taxonomic composition obtained with 16S rRNA gene amplicon sequencing (Rebollar et al., 2016) was congruent with the shotgun metagenome approach presented in this study, we performed a Procrustes analysis (**Figure 1B**). Our results indicate that both methods gave similar results when trying to define the dominant groups present on amphibian skin bacterial communities (PROTEST nperm = 999 $p = 0.0069$).

Presence of Main Functional Classes and Gene Relative Abundance in *C. fitzingeri* Skin Microbiomes

We annotated between 25 and 47% of the reads from the six samples obtained from *C. fitzingeri* skin (Table S1) and classified them according to main functional classes from KEGG. Skin microbiomes contained most of the functional classes identified in KEGG and had a similar proportion of classes among samples (**Figure 2A**). The most abundant gene classes were amino acid metabolism (mean = 13.87%, SD = 0.11), carbohydrate metabolism (mean = 12.59%, SD = 0.35), energy metabolism (mean = 9.89%, SD = 0.27), membrane transport (mean = 8.76%, SD = 0.60) and metabolism of cofactors and vitamins (mean = 8.24%, SD = 0.16). When we analyzed the KO relative abundances across samples, these were clustered by site based on Bray Curtis distances (**Figure 2B**). We found significant differences (p-value < 0.05 and LDA score > 2) between sites at the KO level (Table S2). Specifically, we identified 36 KOs that discriminated between Sob and Sapo sites. Of the 36 KOs, 27 and 9 were enriched in Sob and Sapo, respectively. In Sob, most of the enriched genes were part of functional classes involved in membrane transport (13), cellular communication (5), biosynthesis of secondary metabolites (2) and antimicrobial drug resistance (1). In Sapo, enriched genes were associated with the cell cycle (1), lipid metabolism (1) and xenobiotic degradation metabolism (2). In addition to differences found between sites, there is also individual variation among samples within each site as shown in **Figure 2B**.

Genes Involved in Bacterial Communication, Transport and Defense: Unique and Shared Functional Traits Between Sites

Based on assembled contigs, we specifically decided to explore genes from five functional classes associated with bacterial communication, molecular transport and defense mechanisms: biosynthesis of secondary metabolites (BSM), metabolism of terpenoids and polyketides (MTP), membrane transport (MT), cellular communication (CC) and antimicrobial drug resistance (ADR).

Skin metagenomes from both sites had genes for the five functional classes tested, but these genes were associated with different taxonomic groups depending on the site. As seen in **Figure 3**, most of the genes within each category belonged to *Pseudomonas*, *Delftia, Azotobacter,* and *Acinetobacter* in Sapo and *Pseudomonas*, *Variovorax*, *Sanguibacter*, *Stenotrophomonas,* and *Microbacterium* in Sob. However, no significant differences in the number of genes were found between Sapo and Sob sites for these functional categories (LefSe p-values > 0.05).

We further analyzed the gene abundance for all pathways within each functional category (55 in total). In the case of the BSM class, 16 out of 25 pathways described in KEGG were present in the skin metagenomes. The most abundant

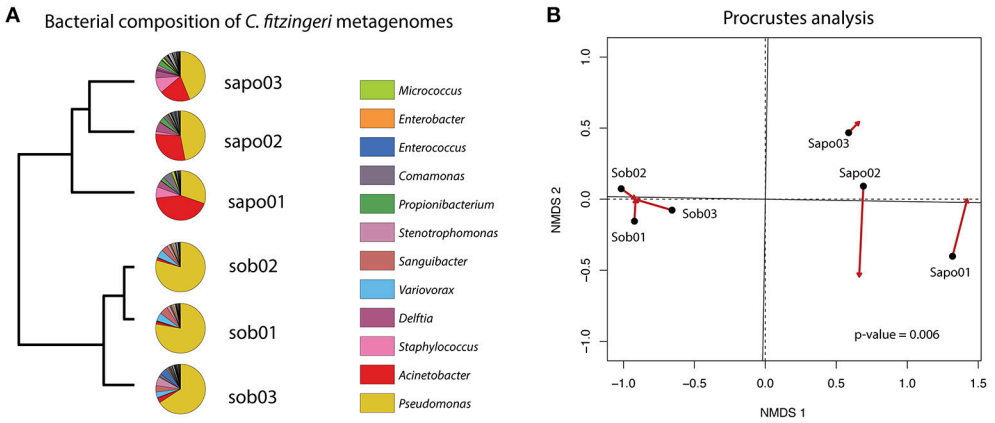

FIGURE 1 | (A) Pie charts of the most abundant genera obtained with metaphlan for each frog microbiome sample. An UPGMA on the left shows grouping of similar samples based on the relative abundance of bacterial taxa. **(B)** Procrustes analysis comparing the relative abundance of bacterial taxa (Bray-Curtis dissimilarity matrices) of the 16S rRNA gene amplicon data (arrowheads) and the shotgun metagenome data (black circles). A p-value of 0.006 (PROTEST test) indicates that the matrices are more similar than expected by random association.

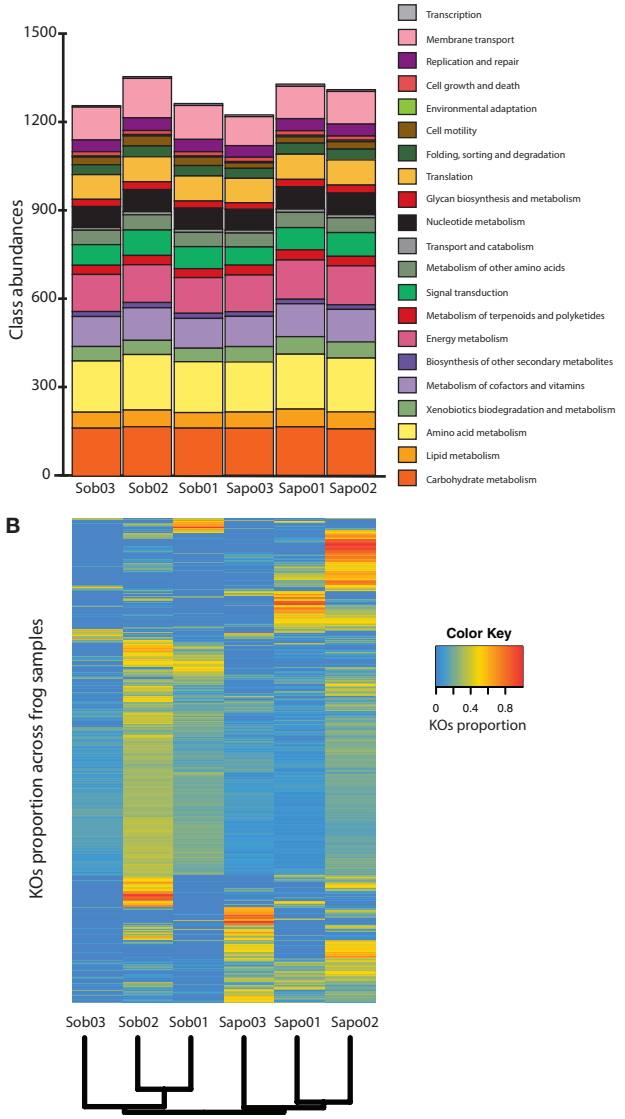

FIGURE 2 | Read level analysis **(A)** Class abundances normalized with MUSiCC. **(B)** Heatmap showing the relative abundance of KOs across samples. Rows are individual KOs and columns are frog samples. Dendogram at the bottom indicates clustering of samples based on Bray-Curtis distances. Colors indicate the relative abundance (proportions) of KOs across samples (see color legend on the right hand side of the figure).

were the biosynthesis of monobactam, prodigiosin, streptomycin and novobiocin (Figure S1). In the case of the MTP class, 16 out of 21 pathways described in KEGG were present in the skin metagenomes. The most abundant were terpenoid backbone biosynthesis, geraniol degradation, polyketide sugar unit biosynthesis and biosynthesis of siderophore group nonribosomal peptides (Figure S2). In the case of classes MT, CC and ADR all pathways were present (Figure S3). In the case of the MT class, and as part of the bacteria secretion system pathway, we found almost the complete Type II and Type VI secretion systems, which are involved in secreting molecules (including toxins) to the external environment (Green and Mecsas, 2016). Also, we identified several complete ABC transporters from all the different types of prokaryotic transporters according to KEGG. Within the CC pathways, several genes involved in biofilm formation were identified, as well as quorum sensing genes that had previously been found in Gammaproteobacteria and Bacilli classes. In the case of ADR pathways, we identified most of the genes involved in beta-lactam and vancomycin resistance. In addition, several genes involved in antimicrobial peptide (AMP) resistance were found including the two-component system *PhoQ-PhoP* which has been identified as an important component in pathogenic and symbiotic bacteria to adapt to host environments (Clayton et al., 2017).

Of all pathways from the five classes, seven were significantly different between sites (LefSe analysis: LDA score > 2 and p-value < 0.05) (**Figure 4**). We found a significant enrichment of phenylpropanoid biosynthesis genes (BSM class) in Sob in comparison to Sapo. Genes from this biosynthetic pathway were enriched in most of the abundant genera in Sob (*Cellulomonas, Cellulosimicrobium, Sanguibacter,* and *Microbacterium*), which were not as abundant in Sapo, and all are from the Actinobacteria class. We found a significant enrichment of prodigiosin biosynthesis genes in Sapo. Genes from this biosynthetic pathway were enriched in most of the abundant genera in Sapo (*Pseudomonas, Agromyces,* and *Acinetobacter*), which are all from the Gammaproteobacteria class. In addition, genes from biofilm formation, AMP resistance, bacterial secretion systems and carotenoid biosynthesis were significantly enriched in Sapo (**Figure 4**).

We obtained gene co-occurrence networks for each site based on the relative abundance of genes from the same five functional classes (BSM, MTP, MT, CC, and ADR) (Figure S4). Both networks had a similar number of nodes (KOs): 298 nodes in Sapo and 280 nodes in Sob (Table S3). Interestingly, the number of significant Spearman correlations in the Sob network was twofold greater than in the Sapo network (Figure S4) and included an equivalent amount of negative (mutual exclusions) and positive ones (co-occurrences). Accordingly, the clustering coefficient and network centralization were higher in Sob (Table S3). Moreover, the 229 nodes (KOs) shared between the Sob and Sapo networks had strikingly distinct degree values (number of connections per node) (Figure S5). Thus, we did not find a significant correlation between the degree values of the genes shared in the two networks ($\tau = 0.0239$, p-value $= 0.5987$), indicating that connections between nodes are not maintained across sites.

Anti-Bd Secondary Metabolite Pathways Are Present in *C. fitzingeri* Skin Metagenomes

We looked for genes involved in the production of metabolites that have anti-Bd properties such as violacein (Brucker et al., 2008b), indole-3-carboxaldehyde (I3C) (Brucker et al., 2008b), 2,4-diacetylphloroglucinol (2,4-DAPG) (Brucker et al., 2008a), indole-3-ethanol (tryptophol) (Loudon et al., 2014b) and

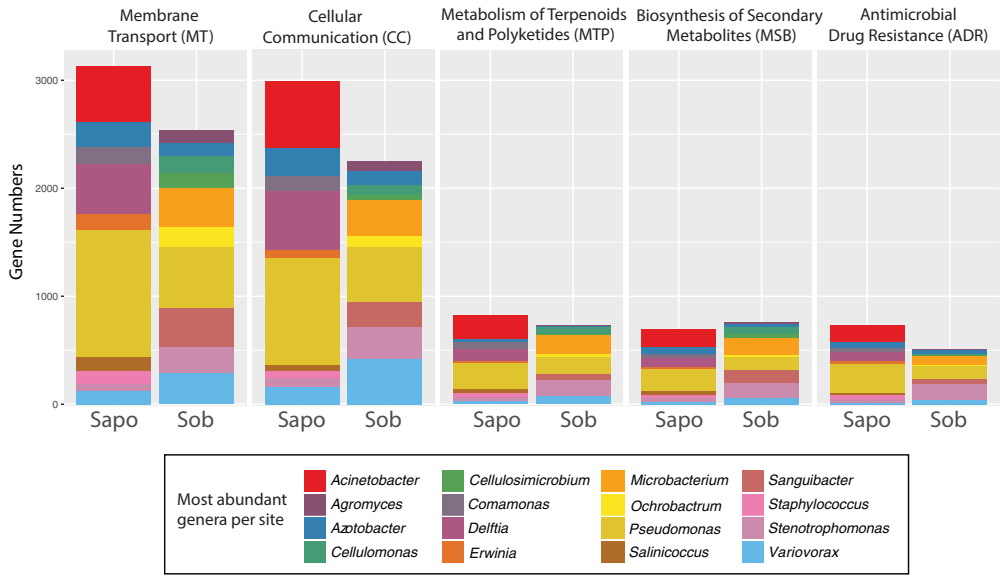

FIGURE 3 | Contig level analysis. Gene abundance stacked graphs of five functional categories from KEGG that are involved in bacterial communication and bacterial-host-pathogen interactions. Each category represents the gene abundance for Sapo ($N = 3$) and Sob ($N = 3$) sites.

prodigiosin (Woodhams et al., 2017). We identified 16 genes from pathways involved in the production of anti-Bd metabolites (**Table 2**).

Specifically, we identified three genes from the prodigiosin biosynthesis pathway (BSM) that were present in both sites and significantly enriched in Sapo (**Figure 4**). One or more genes from this pathway were present in all the abundant bacterial taxa from both sites except for *Erwinia*, which was only abundant in Sapo. We also identified the coding gene of the enzyme responsible for producing 2,4 DAPG from the terpenoid backbone biosynthesis pathway (MTP) in both sites (K01641). This gene was found only in *Staphyloccocus* (abundant in Sapo) and *Microbacterium* (abundant in Sob). In addition, we identified 12 genes that are part of the tryptophan metabolism from which I3C and tryptophol are produced. Specifically, we detected the enzyme that produces indole-3-acetic acid (K00128), from which I3C can be produced (Stutz, 1958). Most of these genes, including K00128, were present in all the abundant bacterial taxa from both sites with a few exceptions (see Table S4).

DISCUSSION

In this study, we described the taxonomic and functional diversity of *C. fitzingeri* skin metagenomes, and we compared these microbiomes between two populations, one being from a Bd-endemic site (Sob) and the other from a Bd-naïve site (Sapo). To our knowledge, this is the first study describing the functional diversity of skin microbiomes on adult amphibians using shotgun metagenomics. First, we discuss genes and pathways found in both populations that may be required for bacterial interactions and bacterial-host-pathogen interactions. Then, we discuss differences between populations in genes and pathways. We used two approaches (read-level and contig-level) that showed consistent results, although contig-level analyses allowed us to better describe taxonomic and functional features present on *C. fitzingeri* skin metagenomes.

We focused on the analysis of five main functional classes that could inform us on the capacity of these bacterial communities to communicate with each other, to interact with the host, and to interact with pathogens like Bd: membrane transport (MT), cell communication (CC), biosynthesis of secondary metabolites (BSM), metabolism of terpenoid and polyketides (MTP) and antimicrobial drug resistance class (ADR). Many of the pathways present in these five functional classes are considered essential for bacteria to communicate with each other and to respond to stimuli from the environment. Examples of these pathways are ABC transporters (MT) (Davidson et al., 2008), quorum sensing (CC) (Rutherford and Bassler, 2012), biofilm formation (CC) (Moons et al., 2009), and secondary metabolite production (BSM and MTP) (Flórez et al., 2015). Here, we found that these essential pathways were prominent in *C. fitzingeri* skin metagenomes independent of bacterial community structure. Based on these results, it appears that many different bacterial taxa can provide the same functions within these communities, suggesting that functional redundancy may be one of the properties present in these symbiotic communities (Foster et al., 2017). Thus, differences in community structure that have been identified among populations and sites in previous studies, may not always mean these communities differ in function (Belden et al., 2015).

In the case of symbiotic bacteria in other hosts, some genes and pathways within these functional classes are known to be important for bacteria to adapt to the host environment, such as the mammalian gut (Frese et al., 2013). One example is the bacterial secretion system pathways (MT), which were initially described in pathogenic bacteria, but have been recently

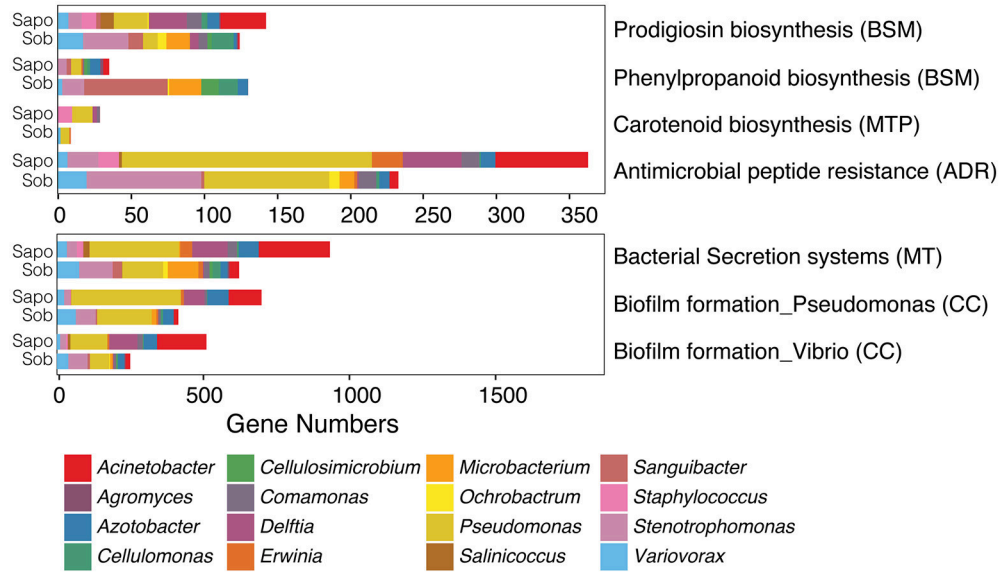

FIGURE 4 | Contig level analysis. Gene abundance plots of significant pathways enriched in Sob or Sapo sites within five functional classes from KEGG involved in bacterial communication and bacterial-host-pathogen interactions. Each bar represents the gene abundances for Sapo ($N = 3$) and Sob ($N = 3$) sites. Colors indicate the taxonomic assignment.

described in symbiotic bacteria (Green and Mecsas, 2016). The presence of almost the complete set of genes for secretion systems II and VI in *C. fitzingeri* metagenomes suggest that skin bacteria export molecules (perhaps toxins) through these mechanisms and may in turn exert an effect on their host. In addition to the secretion system, other pathways can be important for bacteria to survive on the host; such is the case of the antimicrobial peptide (AMP) production pathways within the ADR class. In *C. fitzingeri* metagenomes, we found genes from the AMP production pathway. Symbiotic skin bacteria would be expected to evolve mechanisms to avoid the effects of AMPs to colonize the skin. Specifically, the presence of genes within the AMP resistance pathway suggests that skin microbiomes can adapt to the host environment in part through this mechanism. Many amphibian species produce a vast array of AMPs (Conlon, 2011) but so far, no information on these defense molecules has been published for *C. fitzingeri*.

Skin metagenomes of *C. fitzingeri* also have a broad range of secondary metabolite pathways (BSM, MTP and ADR classes). These pathways include the biosynthesis of antibiotics, toxins and aromatic compounds, as well as antibiotic resistance pathways, which may be involved in bacterial competition within the community but could also play a role in protecting the host against pathogens. Indeed, it has been proposed that defense of the host arises as a byproduct of microbial competition (Scheuring and Yu, 2012). One example is the microbial symbionts from sponges and corals which produce terpenoids and polyketides (MTP) and play a protective role (Flórez et al., 2015). In *C. fitzingeri* metagenomes, we identified genes from the MTP class involved in terpenoid and polyketide biosynthetic pathways, antibiotic biosynthesis pathways like ansamycins and vancomycin from the MTP class and antibiotic resistance pathways within the ADR class.

The MTP class also includes pathways involved in the degradation of several compounds produced mainly by plants such as geraniol, pinene and limonene. These pathways have been previously described in other bacterial genera like *Pseudomonas* and *Rhodococcus* (Marmulla and Harder, 2014), which can use these toxic compounds as carbon sources. We found these pathways present in *C. fitzingeri* metagenomes, which is expected considering that frogs are exposed to many of the same or similar carbon sources in their leaf-litter habitat. Also, terpenoid degradation pathways have been found in other bacterial symbionts (Marmulla and Harder, 2014).

We expected to find genes for the production of anti-Bd metabolites, since *C. fitzingeri* is apparently resistant or tolerant to Bd infection in the wild. We identified some of the genes involved in the production of prodigiosin, I3C, tryptophol and 2,4 DAPG in both sites (Sapo and Sob). However, proving whether these metabolites are produced on *C. fitzingeri* skin communities would require additional studies that test the presence of their respective transcripts or the metabolites themselves *in vivo*.

In this study, we identified clear differences in bacterial community structure between sites that were consistent with previous studies using 16S rRNA gene amplicon sequencing (Rebollar et al., 2016). Some members of these skin communities were shared between sites, but some genera were clearly enriched in either Sapo or Sob. Based on the results obtained here, we consider that the genetic/functional differences between sites may be explained by the unique bacterial genera found in each of the populations that we studied. We expect bacterial communities

TABLE 2 | Genes involved in the production of anti-Bd metabolites that are present on frog skin metagenomes.

KEGG class	KEGG pathway	KO	Gene description	Anti-Bd metabolite
BSM	Prodigiosin biosynthesis	K00059	fabG; 3-oxoacyl-[acyl-carrier protein] reductase	Prodigiosin
BSM	Prodigiosin biosynthesis	K00208	fabI; enoyl-[acyl-carrier protein] reductase I	Prodigiosin
BSM	Prodigiosin biosynthesis	K00645	fabD; [acyl-carrier-protein] S-malonyltransferase	Prodigiosin
MTP	Terpenoid backbone biosynthesis	K01641	hydroxymethylglutaryl-CoA synthase	2,4 DAPG
MTP	Geraniol degradation	K00022	HADH; 3-hydroxyacyl-CoA dehydrogenase	I3C and tryptophol
MTP	Insect hormone biosynthesis	K00128	ALDH; aldehyde dehydrogenase (NAD+)	I3C and tryptophol
AAM	Tryptophan metabolism	K00164	2-oxoglutarate dehydrogenase E1 component	I3C and tryptophol
BSM	Isoquinoline alkaloid biosynthesis	K00274	MAO, aofH; monoamine oxidase	I3C and tryptophol
AAM	Tryptophan metabolism	K00452	3-hydroxyanthranilate 3,4-dioxygenase	I3C and tryptophol
AAM	Tryptophan metabolism	K00453	tryptophan 2,3-dioxygenase	I3C and tryptophol
AAM	Tryptophan metabolism	K00466	tryptophan 2-monooxygenase	I3C and tryptophol
AAM	Tryptophan metabolism	K01432	arylformamidase	I3C and tryptophol
AAM	Tryptophan metabolism	K01556	kynureninase	I3C and tryptophol
MTP	Limonene and pinene degradation	K01692	paaF, echA; enoyl-CoA hydratase	I3C and tryptophol
AAM	Tryptophan metabolism	K10217	aminomuconate-semialdehyde/2-hydroxymuconate-6-semialdehyde dehydrogenase	I3C and tryptophol
AAM	Tryptophan metabolism	K14338	cypD_E, CYP102A2_3	I3C and tryptophol

The first column indicates broad functional classes: BSM, Biosynthesis of secondary metabolites; MTP, Metabolism of terpenoids and polyketides; AAM, Amino acid metabolism.

in both sites to provide a defensive function since potential pathogens are ubiquitous in nature; however, we also expected increased selection for a defensive function in Sob since this is considered a Bd-endemic site (Rebollar et al., 2014).

One clear example of the differences between sites in terms of function and taxonomic composition is the enrichment of genes from the phenylpropanoid biosynthesis pathway at the Sob site (**Figure 4**). Phenylpropanoids are a diverse family of metabolites that are common plant natural products and play several roles, including resistance to pests in plants (reviewed in Vogt, 2010). In this study, most of the bacteria harboring genes from this biosynthetic pathway in Sob were *Sanguibacter, Microbacterium, Cellulomonas,* and *Cellulosimicrobium,* which are all Actinobacteria. The Phenylpropanoid biosynthesis pathway has been previously found in Actinobacteria (Moore et al., 2002), and several bioactive molecules from this family have been identified using metabolomics (Wu et al., 2016). In addition, members of the Actinobacteria class also produce a wide range of antibiotics, and have been identified as symbionts that play a crucial role for the protection of several animal hosts (mainly insects) against pathogens (Flórez et al., 2015). Considering that Sob is a Bd-endemic site, we hypothesize that the enrichment of Actinobacteria in Sob provides *C. fitzingeri* with protection against the pathogen Bd. Further analyses would be necessary to determine if the presence of Actinobacteria in *C. fitzingeri* skin indeed plays a role in protecting the host against Bd and which molecular mechanisms are involved. We also identified six metabolic pathways that were enriched in Sapo from the CC, ADR, MT and BSM functional classes (**Figure 4**) that were mainly explained by genes associated to *Acinetobacter, Pseudomonas,* and *Delftia*.

The functional traits enriched in either Sapo or Sob likely reflect distinct interactions within the members of the skin microbiome and potentially different ways to interact with the host. Moreover, the differences found in co-occurrence networks between sites (mainly on the number of connection that nodes have) may be caused by the presence of distinct taxonomic groups that harbor unique genetic repertoires. In particular, the functional repertoire of Actinobacteria in Sob and Gammaproteobacteria in Sapo may cause distinct degree values of the KOs within each network.

We suggest that shotgun metagenomics is a promising tool that could allow a deeper understanding of the functions present in amphibian skin microbiomes. This approach could be used not only in the field but also in experimental settings since it could unveil functional changes in time-series or bacterial manipulation experiments (Davis et al., 2017). In the case of the *C. fitzingeri* skin microbiome, we have been able to describe important pathways involved in bacterial communication, as well as genes involved in potential bacterial-host-pathogen interactions. However, an important caveat of this study is the sample size (3 microbiomes per site) which may influence our results and may not allow us to detect other significant differences between sites. Thus, in the future, we strongly suggest increasing the sample size to fully describe the functional diversity present in amphibian skin microbiomes.

ETHICS STATEMENT

Scientific collection permits were provided by the Panamanian authorities (Autoridad Nacional del Ambiente): permits SE/A-47-12, SEX/A-65- 12, SEX/A-77-12 and SEX/A-89-12. Animal care protocols were approved by the Smithsonian Tropical Research Institute's Animal Care Committee: protocol 2011-1110- 2014 and by Virginia Tech's Animal Care Committee: protocol 11-105- BIOL.

AUTHOR CONTRIBUTIONS

ER, RH, and LB: contributed to the original idea; ER, RH, LB, RJ, JW, and MH: contributed to the design of the research; MH and DM: carried out the fieldwork; ER, JW, MH, and DM: contributed with laboratory analyses; ER, RJ, AG-P, CN, and AE: performed all data analyses; ER, AG-P, CN, AE, EB, and RH: contributed to analyses interpretation; ER: wrote the manuscript and all authors provided critical feedback.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00466/full#supplementary-material

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L., K. et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015a). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B* 282:20142881. doi: 10.1098/rspb.2014.2881

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015b). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.* 103, 77–85. doi: 10.3354/dao02560

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V. J., Rabemananjara, F. C. E., Rakotoarison, A., et al. (2017a). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.* 8:1530. doi: 10.3389/fmicb.2017.01530

Bletz, M. C., Perl, R. G. B., Bobowski, B. T., Japke, L. M., Tebbe, C. C., Dohrmann, A. B., Bhuju, S., et al. (2017b). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME J.* 11, 1521–1534. doi: 10.1038/ismej.2017.41

Boisvert, S., Raymond, F., Godzaridis, E., Laviolette, F., and Corbeil, J. (2012). Ray Meta: scalable de novo metagenome assembly and profiling. *Genome Biol.* 13:R122. doi: 10.1186/gb-2012-13-12-r122

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander plethodon cinereus. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B., et al. (2008b). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Clayton, A. L., Enomoto, S., Su, Y., and Dale, C. (2017). The regulation of antimicrobial peptide resistance in the transition to insect symbiosis. *Mol. Microbiol.* 103, 958–972. doi: 10.1111/mmi.13598

Conlon, J. M. (2011). The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. *Cell Tissue Res.* 343, 201–212. doi: 10.1007/s00441-010-1014-4

Costa, S., Lopes, I., Proença, D. N., Ribeiro, R., and Morais, P. V. (2016). Diversity of cutaneous microbiome of *Pelophylax perezi* populations inhabiting different environments. *Sci. Total Environ.* 572, 995–1004. doi: 10.1016/j.scitotenv.2016.07.230

Crawford, A. J., Lips, K. R., and Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13777–13782. doi: 10.1073/pnas.0914115107

Dale, C., and Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell* 126, 453–465. doi: 10.1016/j.cell.2006.07.014

Davidson, A. L., Dassa, E., Orelle, C., and Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* 72, 317–364. doi: 10.1128/MMBR.00031-07

Davis, L. R., Bigler, L., and Woodhams, D. C. (2017). Developmental trajectories of amphibian microbiota: response to bacterial therapy depends on initial community structure. *Environ. Microbiol.* 19, 1502–1517. doi: 10.1111/1462-2920.13707

Fisher, M. C., Garner, T. W. J., and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annu. Rev. Microbiol.* 63, 291–310. doi: 10.1146/annurev.micro.091208.073435

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving Chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Flórez, L. V., Biedermann, P. H. W., Engl, T., and Kaltenpoth, M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Nat. Prod. Rep.* 32, 904–936. doi: 10.1039/C5NP00010F

Foster, K. R., Schluter, J., Coyte, K. Z., and Rakoff-nahoum, S. (2017). The evolution of the host microbiome as an ecosystem on a leash. *Nat. Publ. Gr.* 548, 43–51. doi: 10.1038/nature23292

Franzenburg, S., Walter, J., Künzel, S., Wang, J., Baines, J. F., Bosch, T. C. G., et al. (2013). Distinct antimicrobial peptide expression determines host species-specific bacterial associations. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3730–E3738. doi: 10.1073/pnas.1304960110

Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., et al. (2014). Bacteria–bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance. *ISME J.* 9, 1543–1556. doi: 10.1038/ismej.2014.239.

Frese, S. A., MacKenzie, D. A., Peterson, D. A., Schmaltz, R., Fangman, T., Zhou, Y., et al. (2013). Molecular characterization of host-specific biofilm formation in a vertebrate gut symbiont. *PLoS Genet.* 9:e1004057. doi: 10.1371/journal.pgen.1004057

Gallo, R. L., and Hooper, L. V (2012). Epithelial antimicrobial defence of the skin and intestine. *Nat. Rev. Immunol.* 12, 503–516. doi: 10.1038/nri3228

Green, E. R., and Mecsas, J. (2016). Bacterial secretion systems – an overview. *Am. Soc. Microbiol.* 4, 1–32. doi: 10.1128/microbiolspec.VMBF-0012-2015

Grice, E. A. (2014). The skin microbiome: potential for novel diagnostic and therapeutic approaches to cutaneous disease. *Semin. Cutan. Med. Surg.* 33, 98–103. doi: 10.12788/j.sder.0087

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Jackson, D. A. (1995). Protest - a procrustean randomization test of community environment concordance. *Ecoscience* 2, 297–303. doi: 10.1080/11956860.1995.11682297

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jorth, P., Turner, K. H., and Gumus, P. (2014). Metatranscriptomics of the human oral microbiome during *Health* 5, 1–10. doi: 10.1128/mBio.01012-14

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* 28, 27–30.

Kanehisa, M., Sato, Y., and Morishima, K. (2016). BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. *J. Mol. Biol.* 428, 726–731. doi: 10.1016/j.jmb.2015.11.006

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol. Evol.* 25, 109–118. doi: 10.1016/j.tree.2009.07.011

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc. B Biol. Sci.* 283:20161553. doi: 10.1098/rspb.2016.1553

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and McKenzie, V. J. (2015). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (Anaxyrus *boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168.

Li, D., Luo, R., Liu, C. M., Leung, C. M., Ting, H. F., Sadakane, K., et al. (2016). MEGAHIT v1.0: a fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods* 102, 3–11. doi: 10.1016/j.ymeth.2016.02.020

Longo, A. V., and Zamudio, K. R. (2017). Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. *ISME J.* 11, 349–361. doi: 10.1038/ismej.2016.138

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P., and Harris, R. N. (2014b). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* 5:441. doi: 10.3389/fmicb.2014.00441

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2014a). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Luo, G., Fotidis, I. A., and Angelidaki, I. (2016). Comparative analysis of taxonomic, functional, and metabolic patterns of microbiomes from 14 full-scale biogas reactors by metagenomic sequencing and radioisotopic analysis. *Biotechnol. Biofuels* 9:51. doi: 10.1186/s13068-016-0465-6

Manor, O., and Borenstein, E. (2015). MUSiCC: a marker genes based framework for metagenomic normalization and accurate profiling of gene abundances in the microbiome. *Genome Biol.* 16:53. doi: 10.1186/s13059-015-0610-8

Manor, O., Levy, R., Pope, C. E., Hayden, H. S., Brittnacher, M. J., Carr, R., et al. (2016). Metagenomic evidence for taxonomic dysbiosis and functional imbalance in the gastrointestinal tracts of children with cystic fibrosis. *Sci. Rep.* 6:22493. doi: 10.1038/srep22493

Marmulla, R., and Harder, J. (2014). Microbial monoterpene transformations-a review. *Front. Microbiol.* 5:346. doi: 10.3389/fmicb.2014.00346.

McHardy, I. H., Goudarzi, M., Tong, M., Ruegger, P. M., Schwager, E., Weger, J. R., et al. (2013). Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. *Microbiome* 1:17. doi: 10.1186/2049-2618-1-17

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Medina, D., Walke, J. B., Gajewski, Z., Becker, M. H., Swartwout, M. C., and Belden, L. K. (2017). Culture media and individual hosts affect the recovery of culturable bacterial diversity from Amphibian skin. *Front. Microbiol.* 8:1574. doi: 10.3389/fmicb.2017.01574

Medina, M., and Sachs, J. L. (2010). Symbiont genomics, our new tangled bank. *Genomics* 95, 129–137. doi: 10.1016/j.ygeno.2009.12.004

Moons, P., Michiels, C. W., and Aertsen, A. (2009). Bacterial interactions in biofilms bacterial interactions in biofilms. *Crit. Rev. Microbiol.* 35, 157–168. doi: 10.1080/10408410902809431

Moore, B. S., Hertweck, C., Hopke, J. N., Izumikawa, M., Kalaitzis, J. A., Nilsen, G., et al. (2002). Plant-like biosynthetic pathways in bacteria: from benzoic acid to chalcone. *J. Nat. Prod.* 65, 1956–1962. doi: 10.1021/np020230m

Oksanen, J., Guillaume, F. B., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2016). *vegan: Community Ecology Package. R package Version 2.4-0*. Available online at: https://CRAN.R-project.org/package=vegan

Preston, G. M. (2007). Metropolitan microbes: type III secretion in multihost symbionts. *Cell Host Microbe* 2, 291–294. doi: 10.1016/j.chom.2007.10.004

R Core Team (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: http://www.R-project.org/

Rebollar, E. A., Hughey, M. C., Harris, R. N., Domangue, R. J., Medina, D., Ibáñez, R., et al. (2014). The lethal fungus *Batrachochytrium dendrobatidis* is present in lowland tropical forests of Far Eastern Panamá. *PLoS ONE* 9:e95484. doi: 10.1371/journal.pone.0095484

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. 10, 1682–1695. doi: 10.1038/ismej.2015.234

Rodríguez-Brenes, S., Rodriguez, D., Ibañez, R., and Ryan, M. J. (2016). Spread of amphibian chytrid fungus across lowland populations of Túngara frogs in panamá. *PLoS ONE* 11:e0155745. doi: 10.1371/journal.pone.0155745

Rutherford, S. T., and Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb. Perspect. Med.* 2, 1–25. doi: 10.1101/cshperspect.a012427

Scheuring, I., and Yu, D. W. (2012). How to assemble a beneficial microbiome in three easy steps. *Ecol. Lett.* 15, 1300–1307. doi: 10.1111/j.1461-0248.2012.01853.x

Schmid, J., Sieber, V., and Rehm, B. (2015). Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Front. Microbiol.* 6:496. doi: 10.3389/fmicb.2015.00496

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., and Huttenhower, C. (2012). Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat. Methods* 9, 811–814. doi: 10.1038/nmeth.2066

Stutz, R. E. (1958). Enzymatic formation of indole-3-carboxaldehyde from indole-3-acetic acid. *Plant Physiol.* 33, 207–212. doi: 10.1104/pp.33.3.207

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-liggett, C., Knight, R., and Gordon, J. I. (2007). The human microbiome project: exploring the microbial part of ourselves in a changing world. *Nature* 449, 804–810. doi: 10.1038/nature06244

Vogt, T. (2010). Phenylpropanoid biosynthesis. *Mol. Plant* 3, 2–20. doi: 10.1093/mp/ssp106

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 1–11. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with Bullfrogs exposed to a Chytrid fungus. *PLoS ONE* 10:e0139848. doi: 10.1371/journal.pone.0139848

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog.* 12, 6–11. doi: 10.1371/journal.ppat.1005796

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595–595. doi: 10.1890/14-1837.1

Woodhams, D. C., Labumbard, B. C., Barnhart, K. L., Becker, M. H., Bletz, M. C., Escobar, L. A., et al. (2017). Prodigiosin, violacein, and volatile organic compounds produced by widespread cutaneous bacteria of amphibians can inhibit two Batrachochytrium fungal pathogens. *Microb. Ecol.* doi: 10.1007/s00248-017-1095-7. [Epub ahead of print].

Wu, C., Zhu, H., van Wezel, G. P., and Choi, Y. H. (2016). Metabolomics-guided analysis of isocoumarin production by Streptomyces species MBT76 and biotransformation of flavonoids and phenylpropanoids. *Metabolomics* 12, 1–11. doi: 10.1007/s11306-016-1025-6

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Pesticides Could Alter Amphibian Skin Microbiomes and the Effects of *Batrachochytrium dendrobatidis*

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At least 32% of amphibian species are threatened or extinct (Stuart et al., 2004; IUNC, 2017). Amphibians are thought to be especially sensitive to a milieu of stressors because they rely on their skin to regulate fluid balance, ion transport, and respiration. The important role that amphibian skin plays in these critical physiological processes makes them vulnerable to desiccation and environmental pollutants (McCoy and Guillette, 2009). Amphibian skin, also plays a critical role in regulating health by producing antioxidants (Liu et al., 2010), antimicrobial peptides (reviewed in Rollins-Smith et al., 2005, 2011), and by harboring diverse microbial communities that protect against pathogens (Harris et al., 2006). The symbiotic skin bacteria that persist in the presence of antimicrobial mucosal peptides can inhibit pathogen colonization and infection of the skin (Woodhams et al., 2007; Piovia-Scott et al., 2017). Thus, the skin microbiome is an essential part of the amphibian's innate immune system, and changes to the skin microbiome can lead to higher mortality (Harris et al., 2009a).

It is thought that host-mediated microbiome selection can result in disease resistant phenotypes (reviewed in Mueller and Sachs, 2015). The infectious skin disease, chytridiomycosis, caused by the fungal species *Batrachochytrium dendrobatidis* (Bd) is responsible for more than 200 amphibian population declines and extinctions (Skerratt et al., 2007). Importantly, those amphibian populations that successfully persist in the presence of this fungal pathogen include more individuals with (culturable) skin bacterial isolates that produce antifungal compounds compared to amphibian populations that experience major BD-induced declines (Woodhams et al., 2007; Harris et al., 2009a; Rebollare et al., 2016).

Colonization of skin-associated microbes varies over the amphibian life stage, especially before and after metamorphosis (Kueneman et al., 2014, 2016). Changes in microbiome composition over amphibian life stages influences disease suppression. Resident skin bacteria are known to compete for available space and nutrients leading to Bd inhibition and play a critical role in limiting the colonization and establishment of Bd zoospores of various amphibian species (reviewed in Bletz et al., 2013). For example, in Colorado's boreal toads *Anaxyrus boreas* early life stages depended on the skin microbiome to enhance immune function (Kueneman et al., 2016). Specifically, during the tadpole life stage, microbiomes were enriched in Bd-inhibitory bacteria and reduced in fungal taxa (Kueneman et al., 2016). How early microbiome communities influence the structure of later (metamorph and adult) microbiomes and resistance to Bd is unknown, but data presented below suggests that priority effects might control susceptibility. Determining the environmental factors that alter amphibian microbiomes will inform strategies for mitigating the devastating effects of infectious skin diseases such as Bd (Jiménez and Sommer, 2016).

Pollution influences microbial communities across many contexts, and could be influencing amphibian skin microbiomes leaving species more vulnerable to infectious diseases. In fact, microbial communities are typically the first taxa to respond to synthetic chemicals (Lew et al., 2009). For example, polychlorinated biphenyls (PCBs) and heavy metals are known to alter

amphibian gut microbiomes (Kohl et al., 2015; Zhang et al., 2016). In fact, pesticides cause significant shifts in the composition of the GI microbiota across diverse taxa from honey bees to humans (Kakumanu et al., 2016; Velmurugan et al., 2017). Pesticides are also known to decrease soil microbial activity, alter microbial metabolic potential, and alter soil bacteria diversity (Lupwayi et al., 2009; Muñoz-Leoz et al., 2013; Jiao et al., 2017). Repeated annual application of the herbicide glyphosate over 4 years reduced beneficial soil organisms (i.e., free-living diazotrophs, arbuscular mycorrhizal fungi, and dark septate endophytes) in a warm-season grassland community (Druille et al., 2016). Pesticides also reduce microbial diversity and alter microbial community structure in aquatic systems (Muturi et al., 2017). In addition to pollutants affecting bacteria, microbes are also capable of metabolizing pollutants which can lead to variation in host responses (reviewed in Claus et al., 2016).

Here, we argue that if pollutants can directly alter gut, soil, and aquatic microbial communities, and microbial communities can alter toxicity, then environmental contaminants could play an important role in altering the amphibian skin microbiome and disease susceptibility (briefly reviewed in Rollins-Smith et al., 2011). Pollutants have contributed to amphibian declines, and agrochemicals are thought to be especially problematic in a number of contexts (Davidson et al., 2002; Hayes et al., 2006; Davidson and Knapp, 2007; McCoy et al., 2008). Pesticides are globally distributed, transported atmospherically, and are deposited and accumulate in areas where amphibian populations have suffered massive declines or extinctions (Daly et al., 2007a,b; Wania et al., 2007). For example, population declines, and extinctions of several California (USA) amphibian species are associated with wind-borne agricultural chemicals (Davidson et al., 2001, 2002; Sparling et al., 2001; Davidson, 2004; Davidson and Knapp, 2007).

Initially, the idea that pesticides were playing a role in amphibian declines seemed unlikely. Many amphibian declines occurred in natural ecosystems that had not experienced obvious human modification and were considered "pristine" environments. However, we now know that many remote ecosystems, such as the artic and relatively isolated montane forests, are contaminated with synthetic pollutants from distant origins (Sonne et al., 2004; Daly et al., 2007a,b; Wania et al., 2007). Soils in some neotropical montane forests in Costa Rica have much higher concentrations of pesticides than what is found elsewhere in the country (Daly et al., 2007a). Some of the pollutants accumulating in remote montane regions of Costa Rica are known to disrupt the endocrine system and can lead to reproductive feminization [e.g., organochlorines (reviewed in Hayes and Hansen, 2017)]. The unusual female-biased sex ratios observed before devastating chytridiomycosis-induced declines that occurred in Costa Rica suggest that endocrine disrupting pesticides in conjunction with skin infectious disease, could have played an important role in the species declines and extinctions occurring in the region (Lips, 1998).

Many pesticides are known immunotoxins and increase host susceptibility to disease (Hayes et al., 2006; Coors et al., 2008), and this link has been known for more than two decades (e.g., reviewed in Banerjee et al., 1996). For example, exposure to the organochlorine DDT suppresses the humoral immune response, and atrazine exposure suppresses thioglycolate-stimulated recruitment of white blood cells and decreases phagocytic activity (Koner et al., 1998; Brodkin et al., 2007). Pollutants can also contribute to host stress and alter host microbiomes resulting in more disease susceptible hosts (reviewed in Alverdy and Luo, 2017).

Although the mechanisms are rarely determined, an increasing number of studies show interactions between pesticides and disease susceptibility. Although, some studies do not find this connection (Gaietto et al., 2014; Wise et al., 2014; Buck et al., 2015; Rumschlag and Boone, 2015), others have argued that these chemicals can facilitate emergence of infectious disease (e.g., Ross, 2002). For example, sublethal exposure of *Rana clamitans* to pesticides increased their susceptibility to trematode infection (Rohr et al., 2013). Some anti-fungal agents (e.g., itraconazole) have been used as therapeutics in hopes of clearing Bd infections (Garner et al., 2009; Berger et al., 2010; Cashins et al., 2013). The herbicide glyphosate and insecticide carbaryl reduce Bd growth in culture, but host-associated Bd growth was not tested (Hanlon and Parris, 2012). The herbicide atrazine and fungicide chlorothalonil were found to inhibit Bd growth in culture, and when associated with tadpoles (McMahon et al., 2013). Although, Bd infections were reduced they were not completely cleared, and atrazine is a reproductive toxicant that feminizes male frogs, and thus will not aid amphibian conservation efforts (McCoy and Guillette, 2009 reviewed in Hayes et al., 2011).

Pesticide exposure can have long lasting effects and influence vulnerability to disease later in life. Frogs that were exposed to atrazine as tadpoles experienced higher mortality when exposed to chytrid fungus post-metamorphosis relative to non-atrazine exposed animals with the same pathogen loads (Rohr et al., 2013), showing that early pesticide exposure influences later disease susceptibility. In another study, tadpoles that were exposed to one of three fungicides along with Bd showed similar Bd loads relative to the no-fungicide control. In contrast, individuals exposed to pesticides as tadpoles and then exposed to Bd as metamorphs (∼2 months after fungicide exposure) had significantly greater Bd abundance and Bd-induced mortality than frogs similarly exposed to Bd but with no previous pesticide exposure (Rohr et al., 2017). Importantly, the fungicides used in these studies are all directly toxic to Bd, but paradoxically increased future Bd infections. One hypothesis that could explain the enhanced mortality induced by early pesticide exposure is that toxicants might alter the community shift that occurs during metamorphosis that establishes a healthy skin microbiome making exposed individuals less well protected against future infections. Indeed, Blanchard's Cricket Frog (*Acris blanchardi*) larvae exposed to 2.5 mg/L of the glyphosate containing pesticide Rodeo correlated with distinct skin bacterial communities compared to control Cricket Frogs (Krynak et al., 2017). Additional studies that investigate the effects of pesticide exposure on amphibian skin microbiome form and function are, in our opinion, desperately needed.

Here we argue that pesticides might exacerbate disease progression, transmission, and mortality by altering host-associated microbiomes in ways that enhance successful colonization of pathogenic microorganisms and increase virulence of colonizers. Environmental pollutants can also directly impact soil and aquatic environmental microbial communities (Lupwayi et al., 2009; Muñoz-Leoz et al., 2013; Karimi et al., 2017), which changes the microbial species pool available to colonize amphibian skin microbiomes. For example, microbial community richness and phylogenetic diversity were lowest at a coal ash contaminated site compared to reference sites (Hughey et al., 2016). Although, the skin microbiomes of the frogs from these sites were not compared, it is known that the microbial species pool in the environment are important for maintaining a diverse skin bacterial community (Loudon et al., 2014). It is possible that the coal ash-induced changes in the environmental microbial pool could alter the resident amphibian skin microbiome leaving them more susceptible to pathogens. However, a brief 12 h exposure of adult spring peepers (*Pseudacris crucifer*) to coal ash, which mimics a single night's breeding event, did not induce noticeable changes in skin microbiota (Hughey et al., 2016). The effects of chronic exposure to coal ash, or exposure at earlier life stages on the structure and function of the adult microbiome are still unknown.

Few studies have directly tested how pesticides, or other pollutants, affect the microbiome of amphibian skin or have determined how those alterations scale up to affect colonization by and virulence of pathogens. However, adult frogs that have reduced bacterial diversity as tadpoles have three times more parasitic worms than adults with unmanipulated microbiota as tadpoles. (Knutie et al., 2017). The identity of the pollutant or mixture, dose, and the life history stage in which the animal is exposed will determine the how the chemicals interact with the microbiome, specific disease organisms and host immune system (Jones et al., 2017). For example, skin peptide defenses were significantly reduced in newly metamorphosed foothill yellow-legged frogs (*Rana boylii*) after exposure to carbaryl. However, these changes did not result in altered survival, growth, or antimicrobial defenses in froglets that were also exposed to chytrid (Davidson et al., 2007). Not all pesticides, will induce immunotoxicity or interact with disease organisms (Buck et al., 2012), nor will we always identify effects if we focus on single concentrations of contaminants. Our challenge is to determine the contexts under which environmental contaminants are interacting with disease organisms.

We focus on pesticides as they are globally distributed and are known to induce amphibian population declines, but other types of pollutants affect microbial and amphibian communities. Before we can fully understand the interaction between toxicant exposure, disease, and their combined role in driving amphibian declines, we must understand how pollutants directly affect the amphibian skin (and gut) microbiome, the disease-causing microorganisms, and how those effects scale up to play a critical role in amphibian disease dynamics (Harris et al., 2006, 2009a,b). Pollutant-disease-microbiome interactions are critically understudied aspects of amphibian disease ecology.

AUTHOR CONTRIBUTIONS

KM and AP contributed to the development of the ideas, writing, and final approval of this manuscript. KM handled incorporating reviewers comments.

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REFERENCES

Alverdy, J. C., and Luo, J. N. (2017). The influence of host stress on the mechanism of infection: lost microbiomes, emergent pathobiomes, and the role of interkingdom signaling. *Front. Microbiol.* 8:322. doi: 10.3389/fmicb.2017.00322

Banerjee, B. D., Koner, B. C., and Ray, A. (1996). Immunotoxicity of pesticides: perspectives and trends. *Indian J. Exp. Biol.* 34, 723–733.

Berger, L., Speare, R., Pessier, A., Voyles, J., and Skerratt, L. F. (2010). Treatment of chytridiomycosis requires urgent clinical trials. *Dis. Aquat. Org.* 92, 165–174. doi: 10.3354/dao02238

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Brodkin, M. A., Madhoun, H., Rameswaran, M., and Vatnick, I. (2007). Atrazine is an immune disruptor in adult Northern Leaopard Frogs (*Rana pipiens*). *Environ. Toxicol. Chem.* 26, 80–84. doi: 10.1897/05-469.1

Buck, J. C., Hua, J., Brogan Iii, W. R., Dang, T. D., Urbina, J., Bendis, R. J., et al. (2015). Effects of pesticide mixtures on host-pathogen dynamics of the amphibian chytrid fungus. *PLoS ONE* 10:e0132832. doi: 10.1371/journal.pone.0132832

Buck, J. C., Scheessele, E. A., Relyea, R. A., and Blaustein, A. R. (2012) The effects of multiple stressors on wetland communities:pesticides, pathogens and competing amphibians. *J. Freshw. Biol.* 57, 61–73. doi: 10.1111/j.1365-2427.2011.02695.x

Cashins, S. D., Grogan, L. F., Mcfadden, M., Hunter, D., Harlow, P. S., Berger, L., et al. (2013). Prior infection does not improve survival against the amphibian disease chytridiomycosis. *PLoS ONE* 8:e56747. doi: 10.1371/journal.pone.0056747

Claus, S. P., Guillou, H., and Simatos-Ellero, S. (2016). The gut microbiota: a major player in the toxicity of environmental pollutants? *Biofilms Microbiomes* 2:16003. doi: 10.1038/npjbiofilms.2016.3

Coors, A., Decaestecker, E., Jansen, M., and De Meester, L. (2008). Pesticide exposure strongly enhances parasite virulence in an invertebrate host model. *Oikos* 117, 1840–1846. doi: 10.1111/j.1600-0706.2008.17028.x

Daly, G. L., Lei, Y. D., Teixeira, C., Muir, D. C. G., Castillo, L. E., Jantunen, L. M. M., et al. (2007a). Organochlorine pesticides in the soils and atmosphere of costa rica. *Environ. Toxicol. Chem.* 41, 1124–1130. doi: 10.1021/es062349d

Daly, G. L., Lei, Y. D., Teixeira, C., Muir, D. C. G., and Wania, F. (2007b). Pesticides in western Canadian mountain air and soil. *Environ. Sci. Technol* 41, 6020–6025. doi: 10.1021/es070848o

Davidson, C. (2004). Declining downwind: amphibian population declines in California and historical pesticide use. *Ecol. Appl.* 17, 1892–1902. doi: 10.1890/03-5224

Davidson, C., Benard, M. F., Shaffer, H. B., Parker, J. M., O'Leary, C., Conlon, M. J., et al. (2007) Effects of chytrid and carbaryl exposure on survival, growth and skin peptide defenses in foothill yellow-legged frogs. *Environ. Sci. Technol.* 41, 1771–1776. doi: 10.1021/es0611947

Davidson, C., and Knapp, R. A. (2007). Multiple stressors and amphibian declines-Dual impacts of pesticides and fish on yellow-legged frogs. *Ecol. Appl.* 17, 587–597. doi: 10.1890/06-0181

Davidson, C., Shafer, H. B., and Jennings, M. R. (2002). Spatial tests of the pesticide drift, habitat destruction, UV-B, and climate-change hypotheses for California amphibian declines. *Conserv. Biol.* 16, 1588–1601. doi: 10.1046/j.1523-1739.2002.01030.x

Davidson, C., Shaffer, H. B., and Jennings, M. R. (2001). Declines of the California red-legged frog- Climate, UV-B, habitat, and pesticides hypotheses. *Ecol. Appl.* 11, 464–479. doi: 10.1890/1051-0761(2001)011[0464:DOTCRL]2.0.CO;2

Druille, M., García-Parisi, P., Golluscio, R., Cavagnaro, F., and Omacini, M. (2016). Repeated annual glyphosate applications may impair beneficial soil microorganisms in temperate grassland. *Agric. Ecosyst. Environ.* 230, 184–190. doi: 10.1016/j.agee.2016.06.011

Gaietto, K. M., Rumschlag, S. L., and Boone, M. D. (2014). Effects of pesticide exposure and the amphibian chytrid fungus on gray treefrog (Hyla chrysoscelis) metamorphosis. *Environ. Toxicol. Chem.* 33, 2358–2362. doi: 10.1002/etc.2689

Garner, T., Garcia, G., Carroll, B., and Fisher, M. (2009). Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of Alytes muletensis tadpoles. *Dis. Aquat. Org.* 83, 257–260. doi: 10.3354/dao02008

Hanlon, S. M., and Parris, M. J. (2012). The impact of pesticides on the pathogen *Batrachochytrium dendrobatidis* independent of potential hosts. *Arch. Environ. Contam. Toxicol.* 63, 137–143. doi: 10.1007/s00244-011-9744-1

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Hayes, T. B., Anderson, L. L., Beasley, V. R., de Solla, S. R., Iguchi, T., Ingraham, H., et al. Willingham, E. (2011). Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. *J. Steroid Biochem. Mol. Biol.* 127, 64–73. doi: 10.1016/j.jsbmb.2011.03.015

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., et al. (2006). Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? *Environ. Health Perspect.* 114, 40–50. doi: 10.1289/ehp.8051

Hayes, T. B., and Hansen, M. (2017). From silent spring to silent night: agrochemicals and the anthropocene. *Elementa Sci. Anthropocene* 5, 1–24. doi: 10.1525/elementa.246

Hughey, M. C., Walke, J. B., Becker, M. H., Umile, T. P., Burzynski, E. A., Minbiole, K. P. C., et al. (2016). Short-term exposure to coal combustion waste has little impact on the skin microbiome of adult sprin. *Appl. Mol. Microbiol.* 82, 3493–3502. doi: 10.1128/AEM.00045-16

IUNC (2017). *The IUCN Red List of Threatened Species [Online]*. Available online at: http://www.iucnredlist.org/initiatives/amphibians/analysis (Accessed Ocober 30, 2017).

Jiao, S., Luo, Y., Lu, M., Xiao, X., Lin, Y., Chen, W., et al. (2017). Distinct succession patterns of abundant and rare bacteria in temporal microcosms with pollutants. *Environ. Pollut.* 225, 497–505. doi: 10.1016/j.envpol.2017.03.015

Jiménez, R. R., and Sommer, S. (2016). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Jones, D. K., Dang, D. T., Urbina, J., Bend, R. J. (2017) Effect of Simultaneous amphibian exposure to pesticides and an emerging fungal pathogen, *Batrachochytrium dendrobatidis*. *Environ. Sci. Technol.* 51, 671–679. doi: 10.1021/acs.est.6b06055

Kakumanu, M. L., Reeves, A. M., Anderson, T. D., Rodrigues, R. R., and Williams, M. A. (2016). Honey Bee gut microbiome is altered by in-hive pesticide exposures. *Front. Microbiol.* 7:1255. doi: 10.3389/fmicb.2016.01255

Karimi, B., Maron, P. A., Chemidlin-Prevost Boure, N., Bernard, N., Gilbert, D., and Ranjard, L. (2017). Microbial diversity and ecological networks as indicators of environmental quality. *Environ. Chem. Lett.* 15, 265–281. doi: 10.1007/s10311-017-0614-6

Knutie, S. A., Gabor, C., Kohl, K. D., and Rohr, J. R. (2017). Do host-associated gut microbiota mediate the effect of an herbicide on disease risk in frogs? *J. Anim. Ecol.* 87, 489–499. doi: 10.1111/1365-2656.12769

Kohl, K. D., Cary, T. L., Karasov, W. H., and Dearing, M. D. (2015). Larval exposure to polychlorinated biphenyl 126 (PCB-126) causes persistent alteration of the amphibian gut microbiota. *Environ. Toxicol. Chem.* 34, 1113–1118. doi: 10.1002/etc.2905

Koner, B. C., Banerjee, B. D., and Ray, A. (1998). Organochlorine pesticide-induced oxidative stress and immune suppression in rats. *Indian J. Exp. Biol.* 36, 395–398.

Krynak, K. L., Burke, D. J., and Benard, M. F. (2017). Rodeo^{TM} Herbicide negatively affects blanchard's cricket frogs (Acris blanchardi) survival and alters the skin-associated bacterial community. *J. Herpetol.* 51, 402–410. doi: 10.1670/16-092

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and Mckenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Lew, S., Lew, M., Szarek, J., and Mieszczynski, T. (2009). Effect of pesticides on soil and aquatic environmental microorganisms—a short review. *Fresenius Environ. Bull.* 18, 1390–1395.

Lips, K. (1998). Decline of a tropical montane amphibian fauna. *Conserv. Biol.* 12, 106–117. doi: 10.1046/j.1523-1739.1998.96359.x

Liu, C., Hong, J., Yang, H., Wu, J., Ma, D., Li, D., et al. (2010). Frog skins keep redox homeostasis by antioxidant peptides with rapid radical scavenging ability. *Free Radic. Biol. Med.* 48, 1173–1181. doi: 10.1016/j.freeradbiomed.2010.01.036

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., Mckenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Lupwayi, N. Z., Harker, K. N., Dosdall, L. M., Turkington, T. K., Blackshaw, R. E., O'donovan, J. T., et al. (2009). Changes in functional structure of soil bacterial communities due to fungicide and insecticide applications in canola. *Agric. Ecosyst. Environ.* 130, 109–114. doi: 10.1016/j.agee.2008.12.002

McCoy, K. A., Bortnick, L. J., Campbell, C. M., Hamlin, H. J., Guillette, L. J., and St Mary, C. M. (2008). Agriculture alters gonadal form and function in the toad Bufo marinus. *Environ. Health Perspect.* 116, 1526–1532. doi: 10.1289/ehp.11536

McCoy, K. A., and Guillette, L. J. (2009). "Endocrine disruptors," in *Amphibian Decline: Diseases, Parasites, Maladies, and Pollution*, eds H. F. Heatwole and J. W. Wilkinson (Baulkham Hills, NSW: Surrey Beatty and Sons), 3208–3238.

McMahon, T. A., Romansic, J. M., and Rohr, J. R. (2013). Nonmonotonic and monotonic effects of pesticides on the pathogenic fungus *Batrachochytrium dendrobatidis* in culture and on tadpoles. *Environ. Sci. Technol.* 47, 7958–7964. doi: 10.1021/es401725s

Mueller, U. G., and Sachs, J. L. (2015). Engineering microbiomes to improve plant and animal health. *Trends Microbiol.* 23, 606–617. doi: 10.1016/j.tim.2015.07.009

Muñoz-Leoz, B., Garbisu, C., Charcosset, J.-Y., Sánchez-Pérez, J. M., Antigüedad, I., and Ruiz-Romera, E. (2013). Non-target effects of three formulated pesticides on microbially-mediated processes in a clay-loam soil. *Sci. Total Environ.* 449, 345–354. doi: 10.1016/j.scitotenv.2013.01.079

Muturi, E. J., Donthu, R. K., Fields, C. J., Moise, I. K., and Kim, C.-H. (2017). Effect of pesticides on microbial communities in container aquatic habitats. *Sci. Rep.* 7:44565. doi: 10.1038/srep44565

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., Mckenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

Rebollare, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Rohr, J. R., Brown, J., Battaglin, W. A., Mcmahon, T. A., and Relyea, R. A. (2017). A pesticide paradox: fungicides indirectly increase fungal infections. *Ecol. Appl.* 27, 2290–2302. doi: 10.1002/eap.1607

Rohr, J. R., Raffel, T. R., Halstead, N. T., Mcmahon, T. A., Johnson, S. A., Boughton, R. K., et al. (2013). Early-life exposure to a herbicide has enduring effects on pathogen-induced mortality. *Proc. R. Soc. B Biol. Sci.* 280, 20131502–20131502. doi: 10.1098/rspb.2013.1502

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K., and Woodhams, D. C. (2011). Amphibian immune defenses against chytridiomycosis: impacts of changing environments. *Integr. Comp. Biol.* 51, 552–562. doi: 10.1093/icb/icr095

Rollins-Smith, L. A., Reinert, L. K., O'leary, C. J., Houston, L. E., and Woodhams, D. C. (2005). Antimicrobial peptide defenses in amphibian skin. *Integr. Comp. Biol.* 45, 137–142. doi: 10.1093/icb/45.1.137

Ross, P. S. (2002). The role of immunotoxic environmental contaminants in facilitating the emergence of infectious diseases in marine mammals. *Hum. Ecol. Risk Assess.* 8, 277–292. doi: 10.1080/20028091056917

Rumschlag, S. L., and Boone, M. D. (2015). How time of exposure to the amphibian chytrid fungus affects *Hyla chrysoscelis* in the presence of an insecticide. *Herpetologica* 71, 169–176. doi: 10.1655/HERPETOLOGICA-D-13-00070

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4:125. doi: 10.1007/s10393-007-0093-5

Sonne, C., Dietz, R., Born, E. W., Riget, F. F., Kirkegaard, M., Hyldstrup, L., et al. (2004). Is bone mineral composition disrupted by organochlorines in east greenland polar bears (*Ursus maritimus*)? *Environ. Health Perspect.* 112, 1711–1716. doi: 10.1289/ehp.7293

Sparling, D. W., Fellers, G. M., and McConnell, L. L. (2001). Pesticides and amphibian population declines in California, USA. *Environ. Toxicol. Chem.* 20, 1591–1595. doi: 10.1002/etc.5620200725

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Velmurugan, G., Ramprasath, T., Gilles, M., Swaminathan, K., and Ramasamy, S. (2017). Gut microbiota, endocrine-disrupting chemicals, and the diabetes epidemic. *Trends Endocrinol. Metabol.* doi: 10.1016/j.tem.2017.05.001

Wania, F., Shen, L., D., L. Y., Teixeira, C., and Muir, D.C.G. (2007). Accumulation of current-use pesticides in neotropical montane forests. *Environ. Sci. Technol.* 41, 1118–1123. doi: 10.1021/es0622709

Wise, R. S., Rumschlag, S. L., and Boone, M. D. (2014). Effects of amphibian chytrid fungus exposure on American toads in the presence of an insecticide. *Environ. Toxicol. Chem.* 33, 2541–2544. doi: 10.1002/etc.2709

Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Zhang, W., Guo, R., Yang, Y., Ding, J., and Zhang, Y. (2016). Long-term effect of heavy-metal pollution on diversity of gastrointestinal microbial community of Bufo raddei. *Toxicol. Lett.* 258, 192–197. doi: 10.1016/j.toxlet.2016.07.003

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Assessment of Bacterial Communities Associated With the Skin of Costa Rican Amphibians at La Selva Biological Station

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Amphibian skin is a suitable environment for rich communities of microorganisms, both beneficial and detrimental to the host. The amphibian cutaneous microbiota has been hypothesized to play an important role as symbionts, protecting their hosts against disease. Costa Rica has one of the most diverse assemblages of amphibians in the world and we know very little about the microbiota of these tropical animals. For comparison with other studies, we explore the diversity of the skin bacterial communities employing16S rRNA amplicon sequencing of swab samples from twelve species of frogs at La Selva Biological Station in Sarapiquí, Heredia province. The predominant phylum detected in our studies was Proteobacteria, followed by Bacteroidetes and Actinobacteria, with these three phyla representing 89.9% of the total bacterial taxa. At the family level, Sphingobacteriaceae and Comamonadaceae were highly represented among samples. Our results suggest that host species and host family are significant predictors of the variation in microbiota composition. This study helps set the foundation for future research about microbiota composition and resilience to unfavorable conditions, leading to improvement in managing strategies for endangered amphibian species.

Keywords: amphibian, microbiota, La Selva Biological Station, bacterial communities, *Batrachochytrium dendrobatidis*

INTRODUCTION

Dramatic declines in amphibian populations around the world have been a subject of research in recent years. Since the 1980s, amphibian populations have declined to the point where 42% of the 7854 species described worldwide (AmphibiaWeb, 2018) have experienced some form of population declines (Stuart et al., 2004; Whittaker et al., 2013). Population declines in amphibians have been attributed to several factors, mainly related to human activities such as habitat

destruction, pesticide abuse and climate change, among others (Whitfield et al., 2007; Kerby et al., 2011). However, population declines in protected areas, so called "enigmatic declines," have drawn the attention to the emerging disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Lips et al., 2006; Whitfield et al., 2007, 2012; Lips, 2016).

Costa Rica has become a focus for amphibian research. In the late 1990s, a study revealed declines of up to 40% of amphibian populations in the Monteverde Cloud Forest Reserve (Pounds et al., 1997). Also, Lips (1998) documented population declines in Costa Rican amphibians at Las Tablas. Another hotspot for amphibian studies in Costa Rica is La Selva Biological Station in Heredia Province. This is a 16 km^2 private protected area, managed by the Organization of Tropical Studies, where amphibians have been studied for almost 40 years. Whitfield and collaborators analyzed data collected for over 35 years and they found that all species of terrestrial amphibians declined approximately 75% in this site. Interestingly, that study showed a correlation between population declines and a climate driven reduction of the quantity of leaf litter, but no correlation to *Bd* emergence was detected (Whitfield et al., 2007).

Over the last years, next generation sequencing technologies allowed scientists to explore oceans, soils, humans and a variety of host related microbiota (Jani and Briggs, 2014). These technologies can be implemented to study amphibians, specifically their skin microbiota. Skin is especially important for amphibian physiology; it is responsible for the exchange of respiratory gasses and osmoregulation (Voyles et al., 2009). Furthermore, the skin is in continuous exposure to microorganisms in the environment, hence these potentially symbiotic microbes may be involved in different physiological processes and interacting with pathogens such as *Bd* (Madison et al., 2017). Knowledge of the ecology of bacterial communities associated with amphibians has only a few years of development (Bletz et al., 2017a). Previous research showed that bacterial communities of amphibian skin were significantly different to their environments and one of the most important factors driving the structure of the microbiota was host species (McKenzie et al., 2012; Kueneman et al., 2013; Sabino-Pinto et al., 2016). More recently, it has been suggested that environmental factors may also play an important role in the variation of the amphibian bacterial microbiota (Muletz et al., 2017; Bird et al., 2018; Varela et al., 2018). It has also been reported that bacterial communities can vary according to geography (Abarca et al., 2018; Rebollar et al., 2018), ecomorphs (Bletz et al., 2017a) and ontogeny (Longo et al., 2015).

The discovery of bacteria with antagonistic activity to *Bd* (Harris et al., 2006, 2009) opens the possibility of employing new strategies to control the pathogen; however, due to the rapid loss of species and habitat, it is necessary to speed up the search for beneficial bacteria to be employed in captivity or in wildlife (Flechas et al., 2017). To accelerate this search, the use of culture independent techniques can be a very efficient tool to describe local bacterial communities (Rebollar et al., 2016a). Due to the variation in the inhibitory response of bacteria to *Bd* (Antwis and Harrison, 2018), and because skin bacterial communities differed strongly from those of the surrounding environment (Bletz et al., 2017a), it has been recommended to implement mitigation strategies at the local level, using bacteria to counteract the circulating pathogen in the same region (Flechas et al., 2017). The aim of this study is to explore and compare the diversity of the skin bacterial communities of twelve tropical amphibian species at La Selva Biological Station in Costa Rica. Further, these communities are compared to previous studies with other amphibian species and were examined for potential anti-*Bd* function. Our goal is to describe this host-associated microbiota and set the foundation to understand microbiota composition and potential interaction with *Bd*, as well as to describe host specific relationships between amphibians and microbes in Costa Rica.

MATERIALS AND METHODS

Study Site, Species and Sampling

All sampling was performed at La Selva Biological Station in Sarapiquí, Heredia Province, northeastern Costa Rica (10°26′ N, 83°59′ W), between January 18th–19th, 2013. La Selva is a private biological reserve with elevation ranging from 35 m to 137 m.a.s.l., managed by the Organization for Tropical Studies for over 45 years and has been described as lowland wet forest. About 73% of the station territory is primary forest, the rest of the reserve includes forests regenerating from pasture and other agricultural uses (Whitfield et al., 2012). We sampled 66 specimens within twelve species of frogs from nine different families. These included 8 terrestrial species, 3 arboreal and one arboreal-aquatic species (**Table 1** and **Supplementary Table 1**). We sampled by visual encounter on the trails, every specimen was kept in individual clean plastic bags. Once in the lab, each amphibian individual was handled with new sterile nitrile gloves. Each frog was washed for 7 s using 50 ml of sterile distilled water. Sterile swabs were rubbed 10 times on the ventral and dorsal areas and 5 more times on each side (Boyle et al., 2004; Bird et al., 2018). After skin swabbing, frogs were released to their natural environment in less than 3 h. The swabs were placed directly in dry labeled 1.5 ml tubes, transported to the laboratory the same day in an ice-filled container and stored at −80°C until DNA was extracted 2 days later.

DNA Extraction and Sequencing

DNA extractions were performed using the MOBIO PowerSoil Extraction kit (MO BIO, Carlsbad, CA, United States) according to manufacturer's protocols, with the following modifications: samples were incubated at 65°C after the addition of reagent C1, the powerbead tubes were vortexed horizontally for 2 min (Lauber et al., 2008), and the reagent C6 was left on the filter for 5 min before the final elution. Eighteen negative controls were extracted from sterile swabs. DNA extractions were transported in ice packs to the University of Colorado Boulder and stored at −80°C for 15 days until barcoding addition. DNA was sequenced 3 weeks later. PCR reactions contained 11 μL PCR water, 10 μL

TABLE 1 | Amphibian samples processed for skin bacteria community analysis at La Selva Biological Station.

Family	Species	Common name	Individuals	Habitat
Craugastoridae	*Craugastor bransfordii*	Bransford's Litter Frog	5	Terrestrial
	Craugastor fitzingeri	Common Rain Frog	10	Terrestrial
	Craugastor megacephalus	Veragua Robber Frog	2	Terrestrial
Bufonidae	*Rhinella horribilis*	Cane toad	1	Terrestrial
	Rhaebo haematiticus	Truando Toad	10	Terrestrial
Phyllomedusidae	*Agalychnis callidryas*	Red Eyed Tree Frog	4	Arboreal
Hylidae	*Smilisca manisorum*	Mexican Tree Frog	1	Aquatic-arboreal
Strabomantidae	*Pristimantis ridens*	Pygmy Robber Frog	2	Arboreal
Dendrobatidae	*Oophaga pumilio*	Strawberry Poison Frog	25	Terrestrial
Eleutherodactylidae	*Diasporus diastema*	Dink Frog	2	Arboreal
Leptodactylidae	*Leptodactylus savagei*	Jungle Smoking Frog	1	Terrestrial
Microhylidae	*Hypopachus pictiventris*	Southern Narrow-mouthed Toad	2	Terrestrial

of 5 Prime Master Mix (5 PRIME GmbH), 1.0 μL each of the forward and reverse primers (at 10 μM), 1.0 μL $MgCl_2$, and 1.0 μL genomic DNA. PCR primers (F515/R806) were used to target the V4 region of 16S rRNA. This primer pair amplifies the 533–786 region in *Escherichia coli* strain 83972 genome sequence (Greengenes accession no. prokMSA_id:470367). The reverse PCR primer contained a 12-base Golay error-correcting barcode originally developed by Caporaso et al. (2011). PCR conditions consisted of an initial denaturation step of 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s; and final extension of 10 min at 72°C. From 18 negative controls, five yielded PCR bands, these samples were sequenced as described below and filtered out from the sequence analysis. Each sample was amplified in triplicate and combined. Amplicons were quantified using the Quant-IT Picogreen dsDNA reagent in 1X TE buffer. A composite sample for sequencing was created by combining equimolar ratios of amplicons from the individual samples and cleaned using the MO BIO UltraClean PCR clean up DNA purification kit. Purity and DNA concentration were determined using a NanoDrop spectrophotometer (Thermo Scientific). Samples were processed on an Illumina MiSeq Sequencer (Illumina Inc., San Diego, CA, United States) at the Next Generation Sequencing Facility located at the University of Colorado Boulder. The resulting raw sequences for each sample were deposited as Sequence Read Archive (SRA) in the NCBI BioProject ID PRJNA470570.

Sequence Analyses

Quantitative Insights Into Microbial Ecology (QIIME v1.9.0) (Caporaso et al., 2010b) was employed for all sequence analyses. Sequences were filtered for quality and assigned to their respective sample using default settings. The resulting 3,551,436 reads were clustered into 89,504 operational taxonomic units (OTUs) according to the subsampling open reference protocol using the Greengenes May 2013 97% reference sequences[1]. The reads that did not match the reference dataset were clustered into *de novo* OTUs. Chimeric reads, reads matching archaea and mitochondria, as well as low abundance OTUs (lower than 0.00005 of the total read count) were removed from downstream analyses. We detected 116 different OTUs in the negative controls, of which 10 had abundance percentages greater than 1.0%, these OTUs were filtered and removed from further analyses. Samples were subsequently rarefied at 7,800 reads per sample to normalize read counts across samples. Sequences were aligned to the Greengenes (v. gg_13_8) reference alignment using PyNast (Caporaso et al., 2010a). We then constructed a phylogenetic tree using FastTree according to standard procedures within QIIME. Taxonomic summaries at the phylum and genus levels were built by using QIIME's plot tables L2 and L6, respectively, using the melt function in the RESHAPE2 R package (Wickham, 2007). Then we calculated the core OTUs (OTUs present in 90% of the samples) and the more abundant OTUs, a heatmap of these OTUs was built using heatmap.3 function in R. We determined Shannon diversity index and Faith's phylogenetic diversity (PD_whole_tree) and employed a parametric ANOVA test to compare the means of the estimators by species and habitat.

Beta diversity analysis was performed with a multidimensional scaling plot (PCoA) using the weighted Unifrac distances and plotted with the ordinate function in the PHYLOSEQ R packages (McMurdie and Holmes, 2013). We also performed analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) to examine the contribution of host species, host family and habitat in the variation of bacterial community composition. Additionally, we employed an ADONIS test to examine the simultaneous effects of host species and habitat. We compared the diversity of tropical versus temperate amphibian skin microbiota using our Costa Rica data set and similar datasets of amphibians from California and Virginia (Kueneman et al., 2013; Loudon et al., 2014). Finally, the OTU table was filtered to retain only reads matching sequences of bacteria from the Antifungal Isolates Database (Woodhams et al., 2015) that were previously shown to inhibit the growth of *Bd*. We calculated the proportion of these putatively anti-*Bd* bacteria for each sample and tested for differences among species and between habitat types. Non-parametric tests were employed in IBM SPSS Statistics v21 because data did not meet the assumptions of equality of variance.

[1] greengenes.secondgenome.com

RESULTS

After rarefaction, we obtained a total of 2,621,143 good quality sequences and 6330 OTUs distributed in 38 bacterial phyla (**Figure 1A**). Eight out of the twelve species under study were dominated by the phylum Proteobacteria (43.95% of the total OTUs). Other abundant phyla were Bacteroidetes (35.59%) and Actinobacteria (8.79%), while Acidobacteria (3.91%), Firmicutes (2.61%), Verrucomicrobia (1.79%) and Planctomycetes (1.04%) were other phyla represented in at least 1% of the OTUs. Among the four species that were not dominated by Proteobacteria, *Hypopachus pictiventris*, *Leptodactylus savagei,* and *Rhaebo haematiticus* presented a majority of Bacteroidetes OTUs, with 89.02, 69.47, and 39.40%, respectively. The bacterial community associated with *Rhinella horribilis* showed a dominance of Bacteroidetes (43.23%) and Actinobacteria (42.08%), besides this species, only *Smilisca manisorum* presented a relative abundance of actinomycete OTUs above 10% (23.72%).

We identified 463 bacterial families in our dataset. In terms of relative abundance of OTUs, the Sphingobacteriaceae family presented the highest percentage (13.82%), followed by Comamonadaceae (9.08%), Weeksellaceae (8.14%), Chitinophagaceae (7.95%), and Alcaligenaceae (7.46%) (**Supplementary Table 2**). The phylogenetic distribution at family and genus level was noteworthy (**Figure 1B**), for example, showing the dominance of *Pedobacter* in the skin microbiota of *H. pictiventris* (84.34%). In *C. bransfordii* the bacterial community was weakly dominated by OTUs of the genus *Chryseobacterium* (7.00%) and *Agrobacterium* (4.51%). The bacterial community of *C. fitzingeri* was dominated by OTUs of the genus *Sphingobacterium* (11.34%) and one OTUs in Chitinophagaceae family (6.28%); *C. megacephalus* had a bacterial community dominated weakly by *Agrobacterium* (5.10%). The bacterial community of *O. pumilio* had a dominance of OTUs related to Enterobacteriaceae (16.54%) and the bacterial community of *R. haematiticus* was dominated by the genus *Sphingobacterium* (11.98%). The bacterial community of

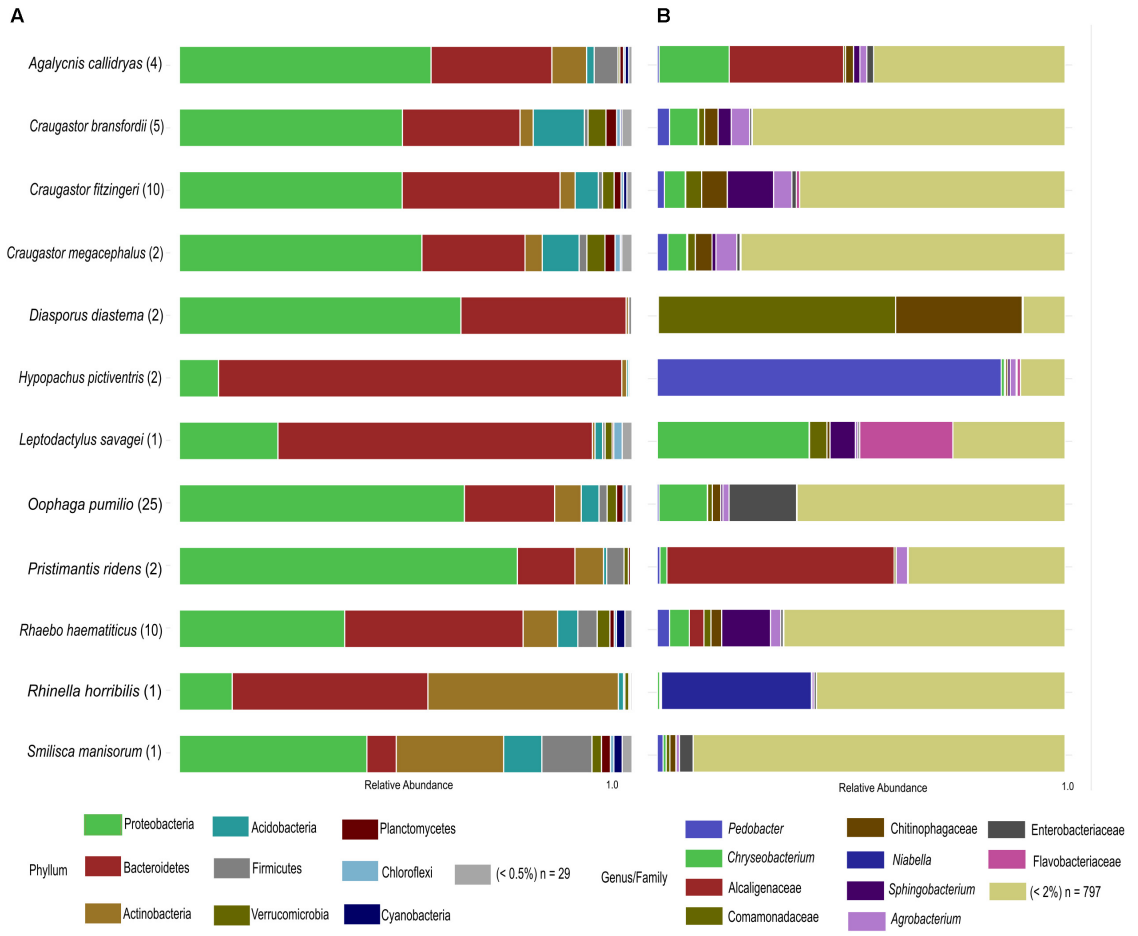

FIGURE 1 | Taxonomic profile of skin bacterial communities of Costa Rican frogs from La Selva Biological Station. **(A)** Phylum-level relative abundance profiles of the host-associated skin bacterial communities. OTUs with abundances lower than 0.5% within each Phylum were clustered together. **(B)** Genus/family level relative abundance profiles of the frog-associated skin bacterial communities. OTUs with percentages lower than 2% of the microbiota composition within species were clustered together. The number of individuals sampled for each species is shown in parenthesis.

A. callidryas was dominated by Alcaligenaceae OTUs (28.04%). The Cane toad, *R. horribilis,* showed an abundance of OTUs in the genus *Niabella*, family Chitinophagaceae (36.75%). *P. ridens* was dominated by OTUs in the family Alcaligenaceae (55.7%), while the *L. savagei* community was dominated by *Chryseobacterium* (37.21%) followed by another OTU belonging to the family Flavobacteriaceae (22.82%).

Figure 2 shows a heatmap of the core microbiota (OTUs that are present in 90% of the samples), as well as the OTUs that have relative abundances significantly different between the bacterial communities. All amphibian species shared OTUs belonging to the genera *Chryseobacterium* and *Pedobacter*, as well as several OTUs assigned to the families Alcaligenaceae, Comamonadaceae and Rhizobiaceae. The three most abundant OTUs detected in this study were assigned to the family Enterobacteriacea and to the genera *Methylibium* and *Pedobacter*.

The relative abundance of OTUs varied among individuals within each species, as follows: In *O. pumilio* (n = 25), 12 individuals were dominated by Enterobacteriaceae OTUs, 7 individuals were dominated by *Methylibium* OTUs, while the other 6 had higher abundance of Chitinophagaceae and *Stenotrophomonas*. In *C. fitzingeri* (n = 10), 3 individuals were dominated by *Sphingobacterium* OTUs, 2 by *Chryseobacterium* OTUs and the 5 remaining individuals each had a different most abundant OTU. Regarding *R. haematiticus* (n = 10), 4 individuals were dominated by *Sphingobacterium* OTUs, 2 by *Chryseobacterium* OTUs and the 4 remaining individuals each had a different most abundant OTU. In two species, *C. bransfordii* (n = 5) and *A. callidryas* (n = 4), all individuals were dominated by a different OTU. Finally, all species with 2 individuals sampled had the same dominant OTU (*G. pictiventris, P. ridens* and *D. diastema*), except for *C. megacephalus*, in which one

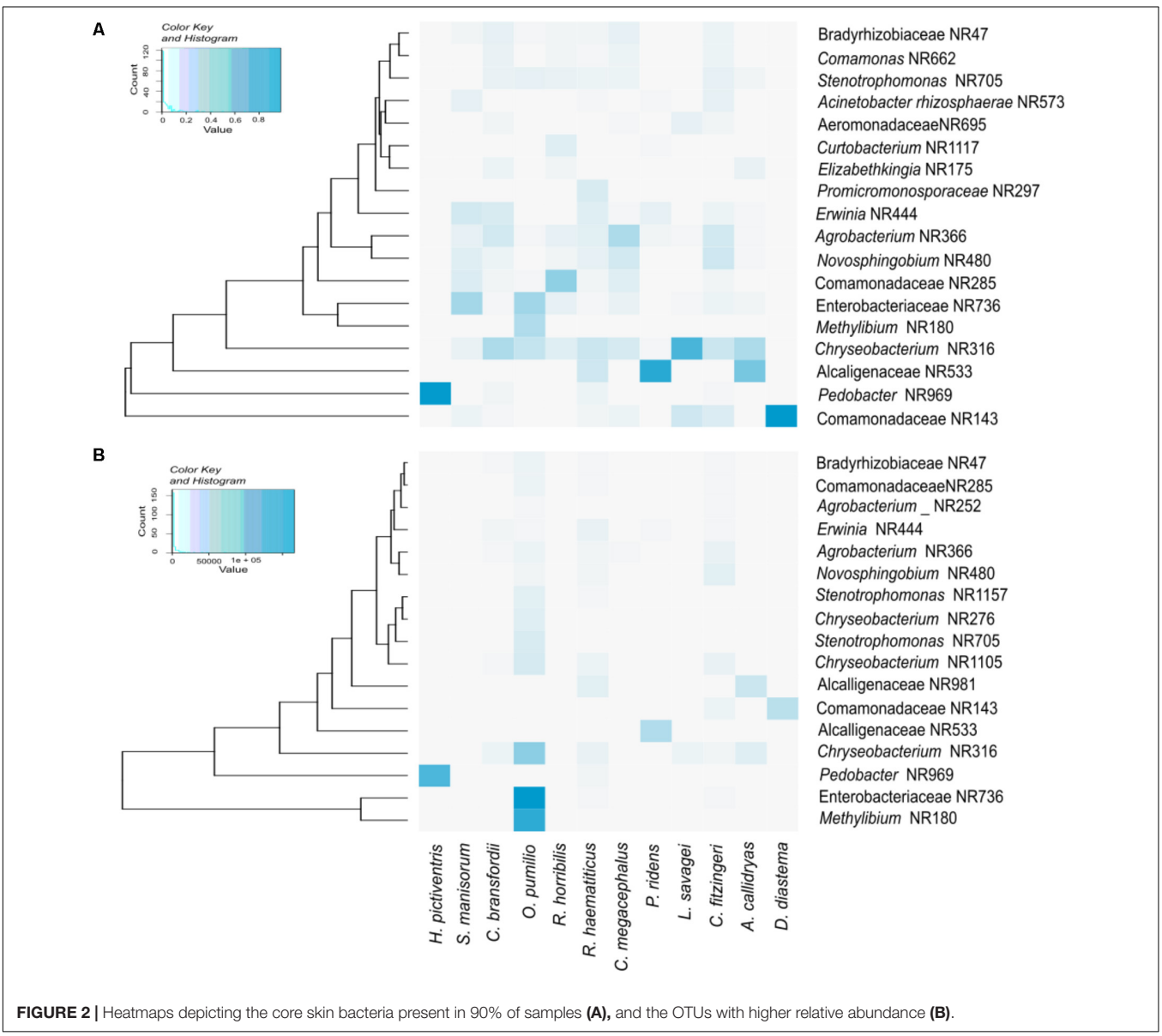

FIGURE 2 | Heatmaps depicting the core skin bacteria present in 90% of samples **(A),** and the OTUs with higher relative abundance **(B)**.

individual was dominated by *Chryseobacterium* and the other by *Agrobacterium* OTUs.

The beta diversity analysis did not show clear clustering among the different bacterial communities; however, some trends are worth noticing. **Figure 3** shows *H. pictiventris* communities separately from the rest of the frogs. Other species did cluster together such as *D. diastema*, while some samples of *O. pumilio* and one of *A. callidryas* grouped close to them. Most *O. pumilio* samples are widely distributed around the plot, indicating great variation within this species. Beta-diversity analysis showed that host species (ANOSIM $R = 0.416$, $p = 0.01$; PERMANOVA PseudoF = 3.388, $p = 0.001$) and host family (ANOSIM $R = 0.392$, $p = 0.01$; PERMANOVA PseudoF = 3.751, $p = 0.001$) are significant predictors of the variation in bacterial community composition. Contrary, host habitat did not show significant results (ANOSIM $R = 0.171$, $p = 0.11$; PERMANOVA PseudoF = 1.866, $p = 0.0599$). The combined effect of host species and host habitat also influenced significantly the variation in bacterial community composition (ADONIS $F = 3.3884$, $p = 0.001$). Regarding alpha-diversity, both Shannon and phylogenetic diversity indexes presented similar results (**Figure 4**): they did not vary significantly between amphibian species ($P > 0.005$ in both indexes). Frogs of the genus *Craugastor*, *O. pumilio*, *R. haematiticus* and *S. baudinii* have the most diverse skin bacterial communities. On the other hand, *D. diastema, P. ridens* and *H. pictiventris* showed lower diversity (**Supplementary Table 3**). Regarding habitat, there was a significant variation between indexes according to habitat ($p < 0.005$ in both), showing a higher diversity associated to terrestrial species when compared to arboreal species. On the other hand, there were no differences between diversity indexes when we compared terrestrial species versus aquatic species or arboreal species versus aquatic ones ($p > 0.005$ in all indexes).

Finally, we compared tropical and temperate species, but no significant latitudinal differences were found in alpha diversity; furthermore, two overlapping families, Bufonidae and Hylidae, also presented similar alpha diversity values (**Figure 5**). The proportion of putatively anti-*Bd* bacteria showed no significant differences among species (Kruskal–Wallis test, $p = 0.149$) or between arboreal and terrestrial habitat types (Mann–Whitney *U*-test, 0.714). However, all 12 species and all samples contained putatively anti-*Bd* bacterial taxa with an average of 13.3% of the 16S rRNA sequencing reads (**Figure 5**).

DISCUSSION

Amphibian skin is an important organ for electrolyte and gas exchange with the environment, and many diseases and

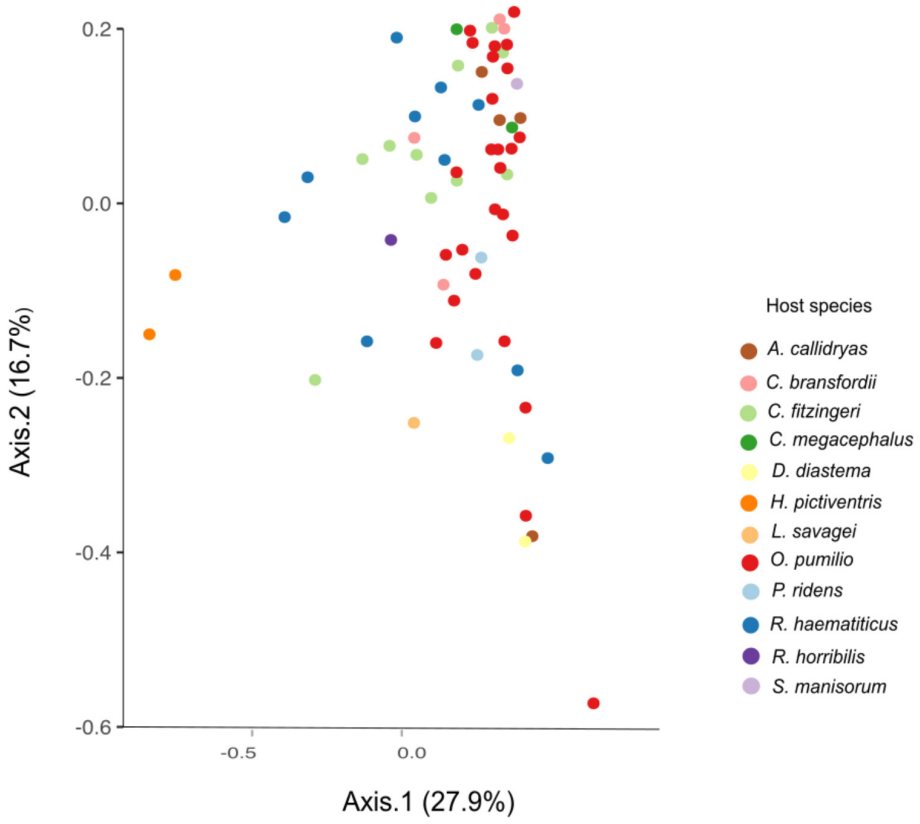

FIGURE 3 | Beta diversity PCoA by weighted UNIFRAC of amphibian skin bacterial communities from La Selva Biological Station. Axes show the major components that explain 44.6% of the differences between samples.

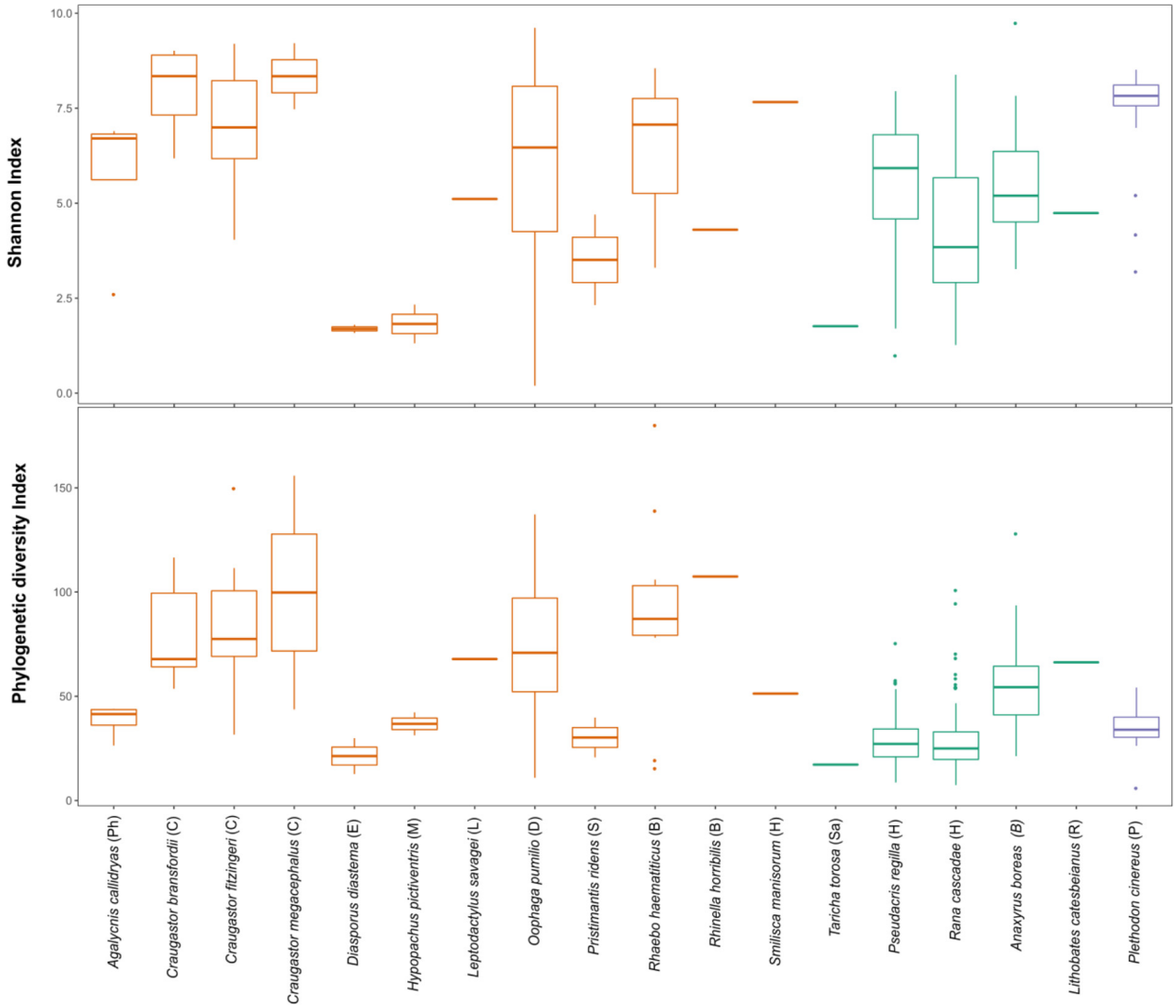

FIGURE 4 | Shannon index and Faith Phylogenetic diversity of amphibian skin bacterial communities from Costa Rica (orange), California (green) and Virginia (purple). Letters in parenthesis represent family: **Ph**, Phyllomedusidae; **C**, Craugastoridae; **E**, Eleutherodactylidae; **M**, Microhylidae; **L**, Leptodactylidae; **D**, Dendrobatidae; **S**, Strabomantidae; **B**, Bufonidae; **H**, Hylidae; **Sa**, Salamandridae; **R**, Ranidae; **P**, Plethodontidae.

contaminants can affect amphibians through it (Campbell et al., 2012). Unfortunately, very little is known about amphibian-associated microbiota in a biodiversity hotspot that has suffered from severe amphibian decline such as Costa Rica. To start bridging this knowledge gap, we studied the skin microbiota of several frog species at La Selva Biological Station. We found that skin bacterial communities differ among amphibian species. Species-level differences in the amphibian skin microbiota have been previously documented in temperate (McKenzie et al., 2012; Kueneman et al., 2013; Walke et al., 2014) and tropical systems (Flechas et al., 2012; Belden et al., 2015; Rebollar et al., 2016b). Furthermore, we found that host family is also a good predictor of bacterial community variation. In tropical regions there are more frog families occupying different microhabitats, this is not the case for temperate zones, where host family was not a strong driver of microbiota when compared to host habitat (Kueneman et al., 2013).

It has been reported that host ecology and habitat are more important factors of variation than host taxonomy (Bletz et al., 2017a; Muletz et al., 2017; Bird et al., 2018), and that most amphibian microbiota comes from the environment (Rebollar et al., 2016b). Despite this, we did not find that habitat was a predictor of variation in the bacterial community of the population under study. This result may be caused by the limited sampling range, or it could reflect amphibian ecology at La Selva, where species transition between different habitats. For example, *O. pumilio* is principally terrestrial but they climb trees to bring tadpoles to bromeliads, *R. haematiticus* can be seen in the forest floor as well as in rivers or even

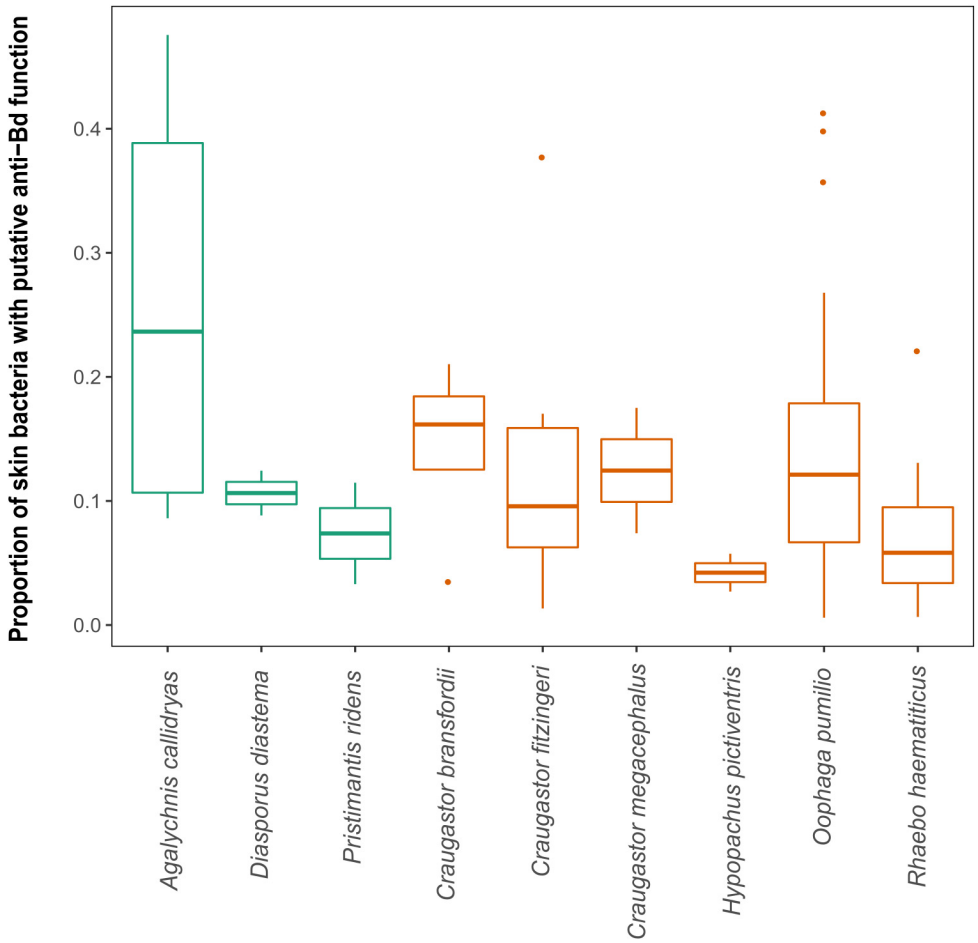

FIGURE 5 | Proportion of putatively anti-*Bd* bacteria from the Antifungal Isolates Database. Note that 9 of the 12 species are shown after removing species with only one sample. No significant differences were found in proportion of putatively anti-*Bd* bacteria among species or between arboreal (orange) and terrestrial (green) habitat types.

in low vegetation and *C. fitzingeri* can be observed in forest leaf litter, vegetation, near rivers and open areas with pastures (Savage, 2002). However, further research is needed to confirm these results, including analyzing more species from aquatic habitats.

Proteobacteria was the most commonly detected phylum and was more abundant in leaf litter species. This is the main phylum recovered from soils (Kim et al., 2014), tree leaves (Kembel et al., 2014) and amphibians (Belden et al., 2015; Bletz et al., 2017a; Varela et al., 2018) in the tropics. The other three dominant phyla agree with the main four phyla of bacteria associated with amphibians in temperate or tropical areas (Lauer et al., 2008; McKenzie et al., 2012; Walke et al., 2014; Becker et al., 2014; Belden et al., 2015). However, we found that Comamonadaceae and Alcaligenaceae were the most common Proteobacteria families in the bacterial communities of the amphibians sampled, in contrast with other studies in both tropical and temperate zones that identified Pseudomonadaceae as the most abundant family (Lam et al., 2010; Rebollar et al., 2016b, 2018).

Bacteroidetes was the second most abundant phylum in skin of the frogs at La Selva. This phylum is also widely distributed in the environment, including soils, sediments and sea water (Thomas et al., 2011). Bacteroidetes is common in four aquatic and terrestrial frog species. Important genera within this phylum present in the microbiota of amphibians at La Selva were *Pedobacter*, *Chryseobacterium* and *Niabella*. High dominance of *Pedobacter* OTUs was found in *H. pictiventris*. The abundance of this genus is important because it has been shown to have inhibitory activity against *Bd* (Harris et al., 2006; Lauer et al., 2008). *Pedobacter* has been isolated from multiple habitats and it can dominate aerobic communities in different aquatic and terrestrial environments, due to its high metabolic diversity (Gordon et al., 2009). *H. pictiventris* spends most of its life underground but in the breeding season they congregate in puddles in the forest (Savage, 2002). This sudden change of microclimate could affect the skin bacterial community allowing *Pedobacter* to thrive over other genera. However, further research is needed to establish whether these two organisms maintain a symbiotic relationship.

Craugastor spp., *R. haematiticus, R. horribilis,* and *O. pumilio* had higher overall bacterial diversity compared with the rest of species; *P. ridens* and *H. pictiventris* have the least bacterial diversity. At La Selva, alpha diversity metrics as Shannon and Phylogenetic diversity indexes show that typical leaf-litter dwelling families (Craugastoridae, Bufonidae and Dendrobatidae) have greater bacterial diversity than arboreal or aquatic families such as Hylidae, Phyllomedusidae, Leptodactylidae, Microhylidae or Eleutherodactylidae. It is possible that more soil bacteria can colonize amphibians in the leaf litter than in arboreal or aquatic habitats, this pattern agrees with previous studies (Belden et al., 2015; Rebollar et al., 2016b; Bletz et al., 2017a).

Our results show differences with similar studies performed in nearby Panama. In those studies, different frog species were analyzed (except for *C. fitzingeri* and *A. callidryas*), suggesting that differences in bacterial composition between countries may be due to different host species tested. One important difference is that Actinobacteria was the second most abundant phylum in frogs of Panama (Belden et al., 2015; Rebollar et al., 2016b), while in Costa Rica this group was in third place. For shared species, however, the alpha diversity indexes appear to be similar, with higher values for *C. fitzingeri*. The microbiota of *C. fitzingeri* from La Selva is enriched with OTUs of the Sphingobacteriaceae family, while those of Panama are enriched with other families such as Comamonadaceae and Moraxellaceae (Rebollar et al., 2018). The Shannon index of *C. fitzingeri* in Costa Rica is similar to that of *C. fitzingeri* in "*Bd-naive*" sites in Panamá (Rebollar et al., 2016b), although La Selva is a "*Bd*-endemic" site where it has been reported that *C. fitzingeri* along with other species of amphibians carry *Bd* (Whitfield et al., 2012). Additionally, in our study, in every species with more than 4 amphibians sampled, the most abundant OTU was not the same in all of them. Both observations suggest that there are intraspecific variations in amphibian bacterial communities, these variations are possibly associated with environmental factors, presence of *Bd* or internal population variations of amphibian species, as has been reported by other studies (Muletz et al., 2017; Abarca et al., 2018; Rebollar et al., 2018).

We found similarities in Shannon diversity index and phylogenetic diversity between temperate zones and tropical zones. At bacterial composition level, temperate zones frogs appear to have more Firmicutes than tropical ones (Kueneman et al., 2013; Loudon et al., 2014; Belden et al., 2015). It is important to point out that the temperate studies included mostly aquatic species, and only one terrestrial family: Bufonidae, while at La Selva we studied mostly terrestrial and arboreal species; therefore, we can only compare two families between Costa Rica and temperate zones: Bufonidae and Hylidae. Those families showed similar richness and phylogenetic diversity. In Bufonidae, Kueneman et al. (2013) found similar abundance of three phyla, the same found in *R. horribilis* at La Selva. Both in Kueneman et al. (2013) and in Belden et al. (2015) the aquatic temperate species was *Lithobates catesbeianus*, and they found different microbiota composition between tropical and temperate populations. To establish a better comparison, it is necessary to characterize the bacterial community of a fully aquatic tropical frog, ideally in the Family Ranidae. Even though the tropics present very different conditions than temperate zones, it is possible that the skin texture and habits of certain species may represent a more homogeneous microhabitat for some bacterial genera regardless of geographical location.

We recovered several sequences related to putatively anti-*Bd* bacterial strains in the Antifungal Isolates Database (Woodhams et al., 2015). This database has been used to determine proportions of OTUs of anti-*Bd* bacteria among other species of amphibians (Kueneman et al., 2016), as well as to study associations between the degrees of infection and the presence of OTUs related to inhibitory bacteria (Jani et al., 2017). *Chryseobacterium* and *Pseudomonas* comprised the majority of putatively anti-*Bd* bacteria from all species examined. These two genera have been reported as the most abundant among inhibitory *Bd* bacteria within their phylum (Park et al., 2014; Becker et al., 2015). In this study over 13% of sequences were related to putatively anti-*Bd* strains. This percentage of putatively anti-*Bd* bacteria is similar to that found using culture-based techniques in Costa Rican amphibians (11.1% in cell-free supernatant assays) (Madison et al., 2017). The presence of OTUs associated with putatively anti-*Bd* bacteria suggests the existence of bacteria that can help fight the disease, but this is still a hypothetical value. For this reason, inhibition tests with cultivable bacteria from each amphibian species (Antwis et al., 2015; Bletz et al., 2017b; Burkart et al., 2017) are necessary to confirm if some amphibians indeed carry higher proportions of anti *Bd* bacteria.

Understanding the microbial community in each region is one of the first steps to implement mechanisms to mitigate chytridiomycosis, including employing probiotic bacteria (Woodhams et al., 2016). Here we find that skin bacterial community of amphibians at La Selva varies according to species and family. Within the bacterial community there are potential symbiotic bacteria that should be the focus of future studies. It is important to know and describe local bacterial communities associated with amphibian skin to understand the mechanisms of interaction between these microorganisms and thus implement efficient bioaugmentation strategies (Flechas et al., 2012). This work represents an effort to characterize the bacterial communities of amphibians in Costa Rica. La Selva Biological Station is a tropical forest research center of worldwide recognition, and this research can serve as a foundation for further studies aimed at designing mitigation strategies to control the threat of *Bd* and human-associated factors as drivers of amphibian extinction.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Commission on Biodiversity at the University of Costa Rica. Our work

with amphibians has been reviewed and approved by the Animal Care and Ethics Committee from the University of Costa Rica (Resolution CICUA-029-15).

AUTHOR CONTRIBUTIONS

SW, JK, VM and AP-T conceived and designed the experiments. JA, GV, IZ, and CM-C performed the experiments. JA, GV, IZ, SW, DW, JK, and VM analyzed the data. JK, VM, and CM-C contributed reagents, materials, and analysis tools. JA, GV, IZ, DW, VM, and AP-T wrote the paper. SW, DW, JK, and CM-C reviewed draft on the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02001/full#supplementary-material

REFERENCES

Abarca, J. G., Zuniga, I., Ortiz-Morales, G., Lugo, A., Viquez-Cervilla, M., Rodriguez-Hernandez, N., et al. (2018). Characterization of the skin microbiota of the cane toad rhinella cf. marina in puerto rico and costa rica. *Front. Microbiol.* 8:2624. doi: 10.3389/fmicb.2017.02624

AmphibiaWeb (2018). *University of California, Berkeley*. Available at: http://amphibiaweb.org [accessed May 2, 2018].

Antwis, R. E., and Harrison, X. (2018). Probiotic consortia are not uniformly effective against different amphibian chytrid pathogen isolates. *Mol. Ecol.* 27, 577–589. doi: 10.1111/mec.14456

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/AEM.00010-15

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B., and Belden, L. K. (2014). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biol. Cons.* 176, 199–206. doi: 10.1016/j.biocon.2014.05.029

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B.* 282, 28–81. doi: 10.1098/rspb.2014.2881

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bird, A. K., Prado-Irwin, S. R., Vredenburg, V. T., and Zink, A. G. (2018). Skin microbiomes of california terrestrial salamanders are influenced by habitat more than host phylogeny. *Front. Microbiol.* 9:442. doi: 10.3389/fmicb.2018.00442

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V. J., Rabemananjara, F. C. E., Rakotoarison, A., et al. (2017a). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of madagascar. *Front. Microbiol.* 8:1530. doi: 10.3389/fmicb.2017.01530

Bletz, M. C., Perl, R. G., Bobowski, B. T., Japke, L. M., Tebbe, C. C., Dohrmann, B., et al. (2017b). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME J.* 11, 1521–1534. doi: 10.1038/ismej.2017.41

Boyle, D. G., Boyle, D. B., Olsen, V., Skerratt, L. F., Meyer, E., McDonald, K. R., et al. (2004). Rapid quantitative detection of amphibians to pathogens during field studies. *Dis. Aquat. Org.* 92, 175–185. doi: 10.3354/dao02162

Burkart, D., Flechas, S. V., Vredenburg, V. T., and Catenazzi, A. (2017). Cutaneous bacteria, but not peptides, are associated with chytridiomycosis resistance in Peruvian marsupial frogs. *Anim. Conserv.* 20, 483–491. doi: 10.1111/acv.12352

Campbell, C. R., Voyles, J., Cook, D. I., and Dinudom, A. (2012). Frog skin epithelium: electrolyte transport and chytridiomycosis. *Int. J. Biochem. Cell Biol.* 44, 431–434. doi: 10.1016/j.biocel.2011.12.002

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods.* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Flechas, S. V., Blasco-Zúñiga, A., Merino-Viteri, A., Ramírez-Castañeda, V., Rivera, M., and Amézquita, A. (2017). The effect of captivity on the skin microbial symbionts in three Atelopus species from the lowlands of Colombia and Ecuador. *PeerJ* 5:e3594. doi: 10.7717/peerj.3594

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving Chytridiomycosis: differential anti-*batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus. *PLoS One* 7:e44832. doi: 10.1371/journal.pone.0044832

Gordon, N. S., Valenzuela, A., Adams, S. M., Ramsey, P. W., Pollock, J. L., Holben, W. E., et al. (2009). Pedobacter nyackensis sp. nov., *Pedobacter alluvionis* sp. nov.and *Pedobacter borealis* sp. nov., isolated from montana flood-plain

sediment and forest soil. *Int. J. Syst. Evol. Microbiol.* 59, 1720–1726. doi: 10.1099/ijs.0.000158-0

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). The amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *PNAS Plus* 111, 5049–5058. doi: 10.1073/pnas.1412752111

Jani, A. J., Knapp, R. A., and Briggs, C. J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proc. R. Soc. B* 284:20170944. doi: 10.1098/rspb.2017.0944

Kembel, S. W., O' Connor, T. K., Arnold, H. K., Hubbell, S. P., Wright, S. J., and Green, J. L. (2014). Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *PNAS* 111, 13715–13720. doi: 10.1073/pnas.1216057111

Kerby, J. L., Hart, J. A., and Storfer, A. (2011). Combined effects of virus, pesticides, and predator cue on larval tiger salamander (*Ambystoma trigrinum*). *EcoHealth* 8, 46–54. doi: 10.1007/s10393-011-0682-1

Kim, M., Kim, W.-S., Tripathi, B. M., and Adams, J. (2014). Distinct bacterial communities dominate tropical and temperate zone leaf litter. *Microb. Ecol.* 67, 837–848. doi: 10.1007/s00248-014-0380-y

Kueneman, J. G., Wegener, L., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2013). The amphibian skin associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and McKenzie, V. J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Cons.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Lauber, C. L., Strickland, M. S., Bradford, M. A., and Fierer, N. (2008). The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biol. Biochem.* 40:2407e2415. doi: 10.1016/j.soilbio.2008.05.021

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A., and Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J.* 2, 145–157. doi: 10.1038/ismej.2007.110

Lips, K. R. (1998). Decline of a tropical montane amphibian fauna. *Conserv. Biol.* 12, 106–107. doi: 10.1046/j.1523-1739.1998.96359.x

Lips, K. R. (2016). Overview of chytrid emergence and impacts on amphibians. *Phil. Trans. R. Soc. B* 371:20150465. doi: 10.1098/rstb.2015.0465

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., Valerie McKenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Madison, J. D., Berg, E. A., Abarca, J. G., Whitfield, S. M., Gorbatenko, O., Pinto, A., et al. (2017). Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from amphibian populations in costa rica. *Front. Microbiol.* 8:290. doi: 10.3389/fmicb.2017.00290

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8:e61217. doi: 10.1371/journal.pone.0061217

Muletz, C. R., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., and Lips, K. R. (2017). Effects of host species and environment on the skin microbiome of plethodontid salamanders. *J. Anim. Ecol.* 87, 341–353. doi: 10.1111/1365-2656.12726

Park, S., Collingwood, A., St-Hilaire, S., and Sheridan, P. P. (2014). Inhibition of *Batrachochytrium dendrobatidis* caused by bacteria isolated from the skin of boreal toads, Anaxyrus (Bufo) boreas, from grand teton national park, Wyoming, USA. *Microbiol. Insights* 7, 1–8. doi: 10.4137/MBI.S13639

Pounds, J. A., Fogden, M. P. L., Savage, J. M., and Gorman, G. C. (1997). Tests of null models for amphibian declines on a tropical mountain. *Cons. Biol.* 11, 1307–1322. doi: 10.1046/j.1523-1739.1997.95485.x

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "Omics" and integrated Multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging Infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Gutiérrez-Preciado, A., Noecker, C., Eng, A., Hughey, M. C., Medina, D., et al. (2018). The skin microbiome of the neotropical frog *Craugastor fitzingeri*: inferring potential bacterial-host-pathogen interactions from metagenomic data. *Front. Microbiol.* 9:466. doi: 10.3389/fmicb.2018.00466

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. H., Ibáñez, R., and Belden, L. K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1–14. doi: 10.1038/ismej.2015.234

Sabino-Pinto, J., Bletz, M. C., Islam, M. M., Shimizu, N., Bhuju, S., Geffers, R., et al. (2016). Composition of the Cutaneous bacterial community in japanese amphibians: effects of captivity. Host Species, and Body Region. *Microb. Ecol.* 72, 460–469. doi: 10.1007/s00248-016-0797-6

Savage, J. M. (2002). *The Amphibians and Reptiles of Costa Rica: A Herpetofauna between two Continents, Between Two Seas*. Chicago: The University of Chicago Press.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodriguez, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Thomas, F., Hehemann, J. H., Rebuffet, E., Cjzek, M., and Michel, G. (2011). Enviromental and gut Bacteroidetes: the food connection. *Front. Microbiol.* 2:93. doi: 10.3389/fmicb.2011.00093

Varela, B. J., Lesbarrères, D., Ibáñez, R., and Green, D. M. (2018). Environmental and host effects on skin bacterial community composition in panamanian frogs. *Front. Microbiol.* 9:298. doi: 10.3389/fmicb.2018.00298

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326:582e585. doi: 10.1126/science.1176765

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Whitfield, S., Kerby, J., Gentry, L. R., and Donnelly, M. A. (2012). Temporal variation in infection prevalence by the amphibian chytrid fungus in three species of frogs at la selva. *Costa Rica. Biotropica* 44, 779–784. doi: 10.1111/j.1744-7429.2012.00872.x

Whitfield, S. M., Bell, K. E., Philippi, T., Sassa, M., Bolaños, F., Chaves, G., et al. (2007). Amphibian and reptile declines over 35 years at La Selva. Costa Rica. *PNAS* 104, 8352–8356.

Whittaker, K., Koo, M. S., Wake, D. B., and Vredenburg, V. T. (2013). "Global Declines of Amphibians," in *Encyclopedia of Biodiversity*, 2nd edition, Vol. 3, ed. S. A. Levin (Waltham, MA: Academic Press), 691–699. doi: 10.1016/B978-0-12-384719-5.00266-5

Wickham, H. (2007). Reshaping data with the reshape package. *J. Stat. Softw.* 21, 1–20. doi: 10.3978/j.issn.2305-5839.2016.01.33

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595. doi: 10.1890/14-1837.1

Woodhams, D. C., Bletz, M., Kueneman, J., and McKenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trends Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

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Characterization of *Batrachochytrium dendrobatidis* Inhibiting Bacteria from Amphibian Populations in Costa Rica

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Global amphibian declines and extinction events are occurring at an unprecedented rate. While several factors are responsible for declines and extinction, the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been cited as a major constituent in these events. While the effects of this chytrid fungus have been shown to cause broad scale population declines and extinctions, certain individuals and relict populations have shown resistance. This resistance has been attributed in part to the cutaneous bacterial microbiome. Here, we present the first study characterizing anti-*Bd* bacterial isolates from amphibian populations in Costa Rica, including the characterization of two strains of *Serratia marcescens* presenting strong anti-*Bd* activity. Transcriptome sequencing was utilized for delineation of shifts in gene expression of the two previously uncharacterized strains of *S. marcescens* grown in three different treatments comprising *Bd*, heat-killed *Bd*, and a no *Bd* control. These results revealed up- and down-regulation of key genes associated with different metabolic and regulatory pathways. This information will be valuable in continued efforts to develop a bacterial-based approach for amphibian protection as well as providing direction for continued mechanistic inquiries of the bacterial anti-*Bd* response.

Keywords: amphibian, microbiome, *Serratia marcescens*, RNA-sequencing, *Batrachochytrium dendrobatidis*

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INTRODUCTION

The role of the bacterial microbiome in conferring disease resistance has been the subject of intensive study in a number of disease systems. This area has critical importance in elucidating the mechanisms of disease dynamics in the context of important host relationships with bacteria. Inquiries into such systems is an important frontier in many taxa undergoing biodiversity loss. Examples include diseases such as white-nose syndrome caused by *Pseudogymnoascus destructans* in bats (Hoyt et al., 2015) and chytridiomycosis caused by *Batrachochytrium dendrobatidis* (hereafter *Bd*) and *Batrachochytrium salamandrivorans* (hereafter *Bsal*) in amphibians (Longcore et al., 1999; Martel et al., 2013). There are also several anthropocentric reasons to decipher these

relationships. Indeed, the role of the bacterial microbiome in agriculturally important plant diseases (Berendsen et al., 2012) and medical applications in human disease (Schwabe and Jobin, 2013; Kostic et al., 2014) necessitates the need for basic research in this field of study. Specifically, the underlying genetic mechanisms that bacteria utilize in mitigating disease risk is an understudied area in disease dynamics. While previous work has examined bacterial gene expression in response to disease in humans (Duran-Pinedo et al., 2014), this is generally lacking in many of the other important disease systems of animals where conservation priorities are a concern.

This study seeks to characterize amphibian cutaneous bacteria and their role in ameliorating amphibian population declines. These declines have been attributed to a variety of factors including habitat loss, global climate change, disease, and environmental contaminants (Collins and Storfer, 2003). In conjunction with other factors, disease has been directly attributed to extinction of various amphibian species (Crawford et al., 2010). Specifically, the *Ranavirus* of the Iridoviridae and the fungal pathogens *Bd* and the now emerging *Bsal* are of concern vis-à-vis the health of at-risk amphibian populations (Martel et al., 2013).

In the wake of devastation that often follows these epizootic events, there are sometimes small relict populations that seem to have an innate resistance to avoid disease (Chaves-Cordero et al., 2014). This survival has been attributed to a variety of factors including the cutaneous bacterial microbiome and host-produced skin peptides (Woodhams et al., 2007a,b,c). An adaptive immune response has also been investigated with results that vary by amphibian species and stage of development (Rollins-Smith, 1998; Ramsey et al., 2010; Poorten et al., 2015). While all of these aspects are important, the possibility of developing probiotic based protection through exploitation and manipulation of the amphibian cutaneous bacterial microbiome has gained recognition as a major research objective. It has been shown that while whole bacterial community composition is important (Rebollar et al., 2016a), there are often specific bacterial species with strong anti-*Bd* properties (Harris et al., 2009). While previous work has examined the transcriptomic response of Anurans to *Bd* (Rosenblum et al., 2012a; Ellison et al., 2014; Price et al., 2015) and also the transcriptome of *Bd* (Rosenblum et al., 2008, 2012b), there have been no studies delineating the transcriptomic response of bacteria to *Bd*. However, there have been studies examining the bacterial metabolites produced in known anti-*Bd* bacteria (Brucker et al., 2008a,b; Belden et al., 2014; Loudon et al., 2014). One important bacteria that has been the subject of various papers is the anti-*Bd* bacteria *Janthinobacterium lividum* (Brucker et al., 2008a). This bacteria produces a secondary metabolite, violacein, which has important similarities to the secondary metabolite prodigiosin produced in another known anti-*Bd* bacteria, *Serratia marcescens*. *J lividum* is also known to produce extracellular chitinases similar to those of *S. marcescens* (Gleave et al., 1995). These similarities could present an important pathway for general anti-*Bd* inhbition. These studies also provide an important overall context for interpreting results presented herein. The need for work addressing these issues has been suggested in recent communications (Woodhams et al., 2016).

To explore the mechanisms by which individual bacteria may be deterring *Bd* growth and thus allowing amphibian persistence in a disease outbreak, our group has sampled various relict, recovering, and unaffected amphibian populations in Costa Rica. Bacterial species were isolated in pure culture and assayed for *Bd* inhibition. Of those bacteria that were determined to be strong anti-*Bd* candidates, two strains of *S. marcescens* were selected for further characterization. Previous studies have examined other *Serratia* spp. that have known anti-*Bd* activity *in vitro* (Woodhams et al., 2014; Becker et al., 2015). It was hypothesized that there would be significant gene up- and down-regulation in *S. marcescens* gene expression as a response to *Bd* which could highlight transcriptomic shifts associated with specific bacterial response mechanisms. Also examined was the expression of genes that are involved in canonical *S. marcescens* antifungal pathways. Known antifungal pathways include the production of broadly antifungal enzymes such as extracellular chitinases and glucanases, as well as the production of secondary metabolites such as prodigiosin (Duzhak et al., 2012; Gutiérrez-Román et al., 2015; Tan et al., 2015). Elucidations from this data will allow for more in-depth studies to occur on the mechanisms by which *S. marcescens* inhibits *Bd* growth. Such mechanistic determinations could be utilized in work developing probiotic bioaugmentation tools for use on both captive and wild amphibian populations.

MATERIALS AND METHODS

Bacterial Isolation

Field sampling for cutaneous bacteria was conducted between March and November 2012 by searching for amphibians in relict populations at sites where they had been previously reported (Puschendorf-Fahrenkrug et al., 2005; Hoffmann, 2006; Abarca et al., 2010; Chaves-Cordero et al., 2014). Bacterial isolates were obtained by swabbing 191 frogs belonging to 12 amphibian species in neotropical montane regions of Costa Rica (**Figure 1**; species collected given in Supplementary Table 4). Frogs were captured with plastic bags and handled using fresh disposable nitrile gloves. The capture of transient surface bacteria was reduced by washing the entire body of individuals for seven seconds with sterile distilled water. Individuals were swabbed over the entire body using a sterile cotton swab. First, swabs were streaked onto the surface of Reasoner's 2A (R2A) agar in a petri dish to obtain bacteria; subsequently the washing was continued with sterile distilled water, and a second swab from the frog was streaked onto chitin agar media for obtaining actinomycetes. Plates were cultured at 25–28°C and colony formation was observed daily. In the case of R2A media, the formation of bacterial colonies was observed at 2 or 3 days of isolation, and one to 2 weeks for actinomycetes. The different colonies were then purified on a new medium: Luria-Bertani (LB) for bacteria from R2A media and yeast malt extract agar (YMEA) for actinomycetes. Each bacterial isolate was cryopreserved with liquid LB media with 40% glycerol and YMEA liquid media with 20% glycerol, respectively. All work with amphibians was carried out in accordance with the recommendations of the Institutional

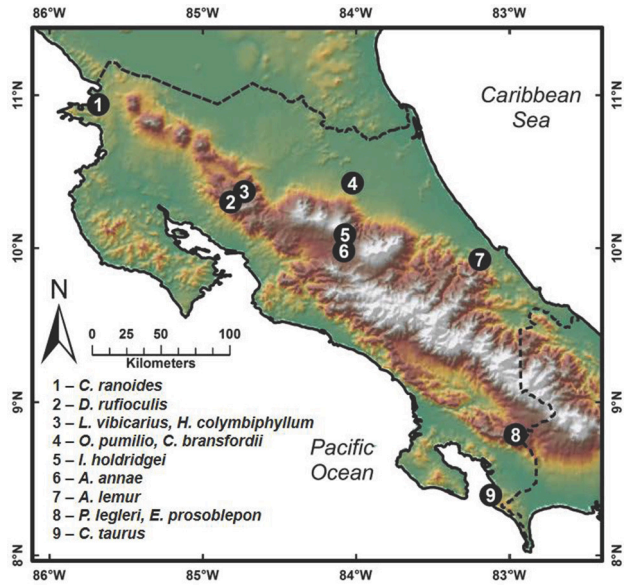

FIGURE 1 | Map showing amphibian collection sites in Costa Rica where amphibians were swabbed for bacterial isolates. Species collected at each site are given.

Commission on Biodiversity at the University of Costa Rica. Amphibian sampling resulting in the bacteria used in this study was done in conjunction with Adrian Pinto at the University of Costa Rica, with research permits approved by the Institutional Commission on Biodiversity at the University of Costa Rica (Resolutions 014).

Cell-Free Supernatant Challenge Assay

A cell-free supernatant assay was initially used to examine anti-*Bd* potential of bacterial isolate extracts. This assay followed closely to the protocol outlined by Bell et al. (2013). Briefly, bacterial isolates were first inoculated into 2 mL of sterile tryptone gelatin hydrolysate lactose (TGhL) medium and incubated at 25°C for 48 h. They were then centrifuged at 5,796 g for 10 min to pellet cells. The supernatant was transferred to 1.5 mL filter microtubes and centrifuged again at 5,796 g for 10 min until only peptides remained suspended in the broth. Experimental and control wells were divided on 96-well microplates. The experimental wells contained 50 μL of *Bd*-inoculated TGhL broth (3.15 × 106 zoospores per mL), 45 μL TGhL medium, and 5 μL of the bacterial isolate. Three controls were used. The positive control wells contained 50 μL of *Bd*, 45 μL TGhL medium, and 5 μL deionized water. The negative control wells contained 50 μL of heat killed *Bd* (raised to 60°C for 30–60 min), 45 μL of TGhL medium, and 5 μL deionized water. The final wells were blanks and contained 95 μL TGhL medium and 5 μL deionized water. Complete plates contained four replicates of each bacterial isolate and eight replicates of the positive, negative, and medium-only controls. Absorbance values were recorded after plate setup was completed (day 0) and then every 24 h on a spectrophotometer (BioTek Take 3) at 492 nm until maximum *Bd* growth (day 14). Plates were kept at room temperature in the dark between absorbance value recordings. Ninety bacterial isolates were examined using this method. The mean daily absorbance values of all replicate and control wells were calculated daily using a spectrophotometer. A generalized linear model (GLM) was used to compare each isolate with replicated controls. Increasing absorbance indicates zoospore reproduction and growth, while decreasing absorbance indicates zoospore death.

Agar Plate Challenge Assay

For the agar plate challenge assay, plates with TGhL agar were inoculated with a lawn of *Bd* (strain JEL 731, isolated from *Craugastor bransfordii* at La Selva Biological Station, Costa Rica). This lawn was then streaked with one of the bacterial isolates with each isolate being examined in triplicate. Each bacteria was incubated for 48–72 h at 25°C and then flooded with 3 mL of *Bd*-inoculated broth (3.15×10^6 zoospores per mL) and left for 1–2 h until agar had absorbed most of the *Bd*-inoculated broth. Each plate had two bacterial strains, with pairs randomized across four replicates. Pictures were taken of the bacteria every 24 h for 3 days. ImageJ software was used to standardize measurements. Zone of inhibition (ZOI) was measured by calculating the area of each zone [Area = (ZOI length–bacteria length) × (ZOI width–bacteria width)]. A GLM was used to compare each isolate to a theoretical control where the ZOI = 0. All of the isolates but one exhibited a ZOI, but only 38 of the 86 had a significant *p*-value ($p < 0.05$). After 2 days of growth at 20°C, the resulting zone of inhibition was quantified. Of those bacteria that were determined to be strong anti-*Bd* candidates and identified using 16S rDNA sequencing, two strains of *S. marcescens* were selected for further characterization. The two strains had 91% 16S rDNA partial sequence homology (BLAST Needleman-Wunsch alignment). Both strains of S. marcescens used in this study were isolated from a single captured and released specimen of *Agalychnis annae* from a successfully translocated population established from a relict population in Costa Rica.

16S rDNA Sequencing

DNA was extracted from 36 bacterial isolates with the greatest *Bd*-inhibitory properties. DNA extraction utilized a Qiagen DNeasy Blood and Tissue kit (Qiagen-Hilden, Germany). DNA preparation for sequencing was done according to manufacturer directions. Briefly, extracted DNA was amplified using the eubacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-ACCTTGTTACGACTT-3′). This amplified product was then sequenced with the original primers as well as the internal primers 907r (5′-CCGTCAATTCMTTTRAGTTT-3′) and 704f (5′-GTAGCGGTGAAATGCGTAGA-3′). The BigDye Terminator v3.1 cycle sequencing kit was utilized in sequencing. Sequencing was carried out using an Applied Biosystems 3500 Genetic Analyzer (Life Technologies-Carlsbad, California, USA). The output sequencing data was assembled with Geneious (version 7.0) and subjected to taxonomic identification using BLAST in the NCBI database.

Bacterial and Fungal Growth Analysis

All fungal growth was done in lactose TGhL broth as per previous studies (Bell et al., 2013). Growth curves of *S. marcescens* were also acquired under the varying treatments (control, Bd, heat-killed Bd). These growth curves were acquired using serial dilutions followed by plating and counting of colony forming units to avoid the non-distinguishing reading of zoospores from bacterial cells by the spectrophotometer which would have resulted in skewed bacterial cell counts between treatments.

RNA-Sequencing

Bd strain JEL731 was used in this experiment. *S. marcescens* (described above) were isolated from the cutaneous layer of *A. annae*. Both *S. marcescens* strains were subsequently shipped from Costa Rica to the University of South Dakota and grown in culture on LB agar. TGhL broth was inoculated with *Bd*, mixed, and spiked with *S. marcescens* according to our experimental conditions. Each experimental condition was done in triplicate with bacterial populations of both strains (at 20°C). Experimental conditions included a no-*Bd* zoospore control, heat-killed *Bd* zoospore control (killed by exposure to 20 min at 50°C; Johnson et al., 2003), and live-*Bd* zoospores. The concentration of *Bd* zoospores used for both heat-killed and live-*Bd* zoospore experimental conditions were 25×10^4 zoospores/mL (measured with a hemocytometer). Cultures were incubated at 20°C with RNA being extracted in bacterial exponential growth phase at 12 h (**Figure 2**). RNA extraction was done by initially stabilizing bacterial RNA with RNAprotect bacteria reagent (Qiagen-Hilden, Germany). Enzymatic lysis was subsequently used using an EDTA (ethylenediaminetetraacetic acid)/lysozyme solution buffered with Tris. RNA purification was then carried out using an RNeasy kit (Qiagen-Hilden, Germany) following standard protocols. Purified RNA was then transported from the University of South Dakota to the WestCore DNA core facility for sequencing preparation and sequencing. Subsequent rRNA depletion of total-RNA for both bacteria and fungi was accomplished using a modified protocol for a Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA). The modified protocol included probes from both the yeast and bacterial rRNA Removal Kits which were combined in a 1:1 ratio to the recommended concentration for a one probe kit. Clean-up of the rRNA reduced RNA was done using a RNA-Clean and Concentrator kit (Zymo Research-Irvine, CA) following manufacturer instructions. RNA quality (RIN score) was obtained on a LabChip GX (Caliper, a PerkinElmer company, Hopkin, MA) using the PicoRNA assay. RNA quantity was measured on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequent library preparation for sequencing utilized the Illumina ScriptSeq RNA-Seq Library Kit. Library quantification was done with a LabChip GX using the DNA High Sensitivity assay and Qubit 2.0 Fluorometer. Indices were added for a six sample multiplex on a one lane flowcell. The Illumina MiSeq platform was used for the sequencing run using the version 3 reagents kit to obtain 2×76 bp reads (paired-end).

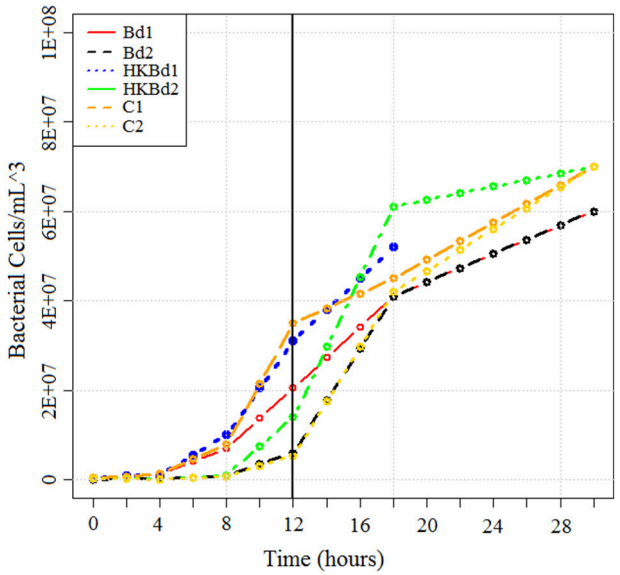

FIGURE 2 | Growth curve of bacteria in all treatments obtained using serial dilution plating. RNA was extracted at $T = 12$ h (indicated by vertical line). Bd1 and Bd2 indicate *S. marcescens* strains one and two grown with *Bd*, respectively. Heat-killed Bd1 and heat-killed Bd2 indicates *S. marcescens* strains one and two grown with heat killed *Bd*, respectively. No Bd1 and no Bd2 indicates *S. marcescens* strains one and two grown without any *Bd*, respectively.

qPCR Verification

A subset of differentially expressed genes (DEGs) identified through RNA-sequencing were verified using RT-qPCR. RNA was from the same samples utilized in the RNA sequencing and were reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen-Hilden, Germany). Real-time PCR was done on the StepOnePlus Real-Time qPCR-system (Applied Biosystems-Foster City, California) using SYBR Green chemistry as provided by the SYBR Green PCR Kit (Qiagen-Hilden, Germany). All qPCR was carried out with no-RT and no-template negative controls. The *narG* (nitrate reductase) gene and *fadE* (acyl-conezyme A dehydrogenase) gene were examined due to their significant upregulation observed in RNA-seq analysis. Other genes examined included *chiA* (chitinase A) and *pigM* (key-regulatory enzyme in the prodigiosin production pathway) to confirm lack of differential expression as seen in RNA-seq analysis. The reference genes *dnaE* and *rplU* were utilized for statistical comparison. Every gene analyzed had expression determined under every experimental condition (*Bd* treatment, HK-*Bd* treatment, and no-*Bd* control). All primer sequences used were either from the literature or designed in NCBI using the Primer BLAST tool followed by verification in our laboratory (primer sequences, Supplementary Table 1).

Statistical Analyses

De-multiplexing of raw data was done on Illumina BaseSpace as part of the BaseSpace MiSeq Reporter workflow. Analysis of raw RNA-seq data was done using the Rockhopper 2 software platform (McClure et al., 2013; Tjaden, 2015). Raw

g-zipped fastq files were uploaded directly to the Rockhopper platform with paired-end reads being combined. Assembly and mapping of the raw data was done using the default settings. Reads were mapped to the *S. marcescens* WW4 reference genome (NCBI Reference Sequence NC_020211.1). Significant differential gene expression analysis was also done using Rockhopper 2. Briefly, assembled/mapped files were subjected to upper-quartile normalization. Expression data was then subjected to the Rockhopper algorithm assuming a negative binomial distribution for estimation of *p*-values. *P*-values were then used to obtain q-values in which false discovery rate is taken into account using the Benjamini-Hochberg procedure. Analysis of qPCR data for differential gene expression was completed using the ΔΔCt method (Livak and Schmittgen, 2001).

RESULTS

Hosts and Isolates

Amphibian species *A. annae*, *Agalychnis lemur*, *C. bransfordii*, and *Oophaga pumilio* had the greatest number of anti-*Bd* bacterial isolates (Supplementary Table 3). *A. lemur* hosted two bacterial species that were shown to enhance *Bd* growth. None of the tested isolates from species *Craugastor ranoides*, *Craugastor taurus*, or *Hyalinobatrachium colymbiphyllum* exhibited anti-*Bd* properties. However, we note that not all of the strains isolated were tested, and bacterial strains are known to exist from *H. colymbiphyllum* that are not culturable or have culturing bias (Walke et al., 2011). *A. annae* exhibited the highest diversity of anti-*Bd* bacteria, with 7 different genera represented in anti-*Bd* isolates.

Identified anti-*Bd* bacteria were in the phyla Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Proteobacteria was the most common phyla being found on 5 of the 12 amphibian species. The following genera were also identified: *Alcaligenes*, *Bacillus*, *Chyrseobacterium*, *Enterobacteriaceae*, *Lysinibacillus*, *Microbacterium*, *Pseudomonas*, *Sphingobacterium*, *Staphylococcus*, and *Stenotrophomonas*. The *Serratia* genus was the most common identified within the selected anti-*Bd* bacteria isolates; *S. marcescens* was the most common species (Supplementary Table 5) and was found on three of the amphibian species.

Bacterial Challenge Assays

Of the 90 bacterial isolates examined in the cell-free supernatant challenge assay, 10 (11.1%) exhibited *Bd* inhibition where $p < 0.05$ and 2 (2.2%) enhanced *Bd* growth (**Figure 3**; Supplementary Table 2). Of the 86 isolates examined in the agar-based challenge assay (**Figure 4**; Supplementary Table 3), 38 (44.2%) of isolates were characterized as anti-*Bd* ($p < 0.05$). All but one isolate produced a zone-of-inhibition during the experiment (Supplementary Table 3).

We also compared the 68 isolates which were examined in both assays; overall 30 exhibited anti-*Bd* properties (44.1%; Supplementary Table 4). Of these 30 isolates, 28 isolates (93.3%) were shown to inhibit *Bd* using the agar-based method but not the cell-free supernatant method, and four isolates (13.3%) were shown to inhibit *Bd* using both methods. There were 2 isolates that exhibited anti-*Bd* properties ($p < 0.05$) in the cell-free supernatant assay but not in the agar-based assay.

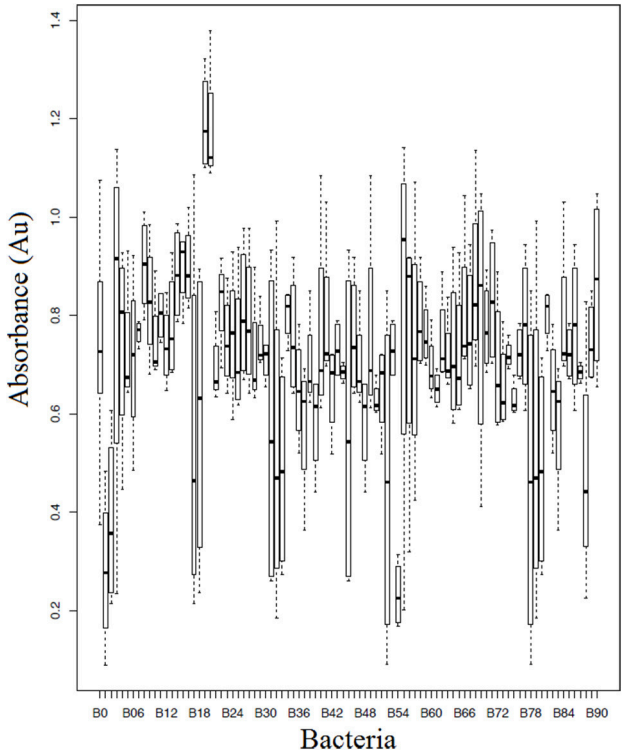

FIGURE 3 | Box plot estimates of the cell-free supernatant challenge assay for each of the bacteria strains. The box represents the interquartile range (IQR) and the top and bottom whiskers represent Q3−(1.5 IQR) and Q1−(1.5 IQR), respectively. Bacteria IDs on the x-axis are referenced in Supplementary Table 3. Lower values signify greater inhibition of *Bd* growth. B0 corresponds to a *Bd*-only control.

Transcriptomic Analysis

All extracted RNA had RNA integrity numbers (RINs) >9.0 indicating that all extracted RNA samples were within tolerance for use in sequencing. Raw sequencing data of two *S. marcescens* strains from *A. annae* were referenced against the *S. marcescens* WW4 genome with >80% mapping (**Table 1**). Additionally, 47–74% of reads were mapped to protein coding genes (**Table 1**). All DEGs identified in each treatment were compared to a no-*Bd* control. In the live *Bd* treatment this experiment found 111 differentially expressed genes (DEGs) in *S. marcescens* strain one (Supplementary Table 6) and 100 DEGs in *S. marcescens* strain two (Supplementary Table 7). For the heat-killed *Bd* treatment, *S. marcescens* strain one had 96 DEGs (Supplementary Table 8) and *S. marcescens* strain two had 73 DEGs (Supplementary Table 9). Pooling results from both strains of *S. marcescens* used in this study, only three significant DEGs were identified in the *Bd* treatment while five significant DEGs were identified in the heat-killed *Bd* treatment. We were able to identify the top 15 up- and down-regulated genes in each treatment in each strain as well as the significant DEGs that were shared with both strains pooled (**Figure 5**). Genes that were significantly

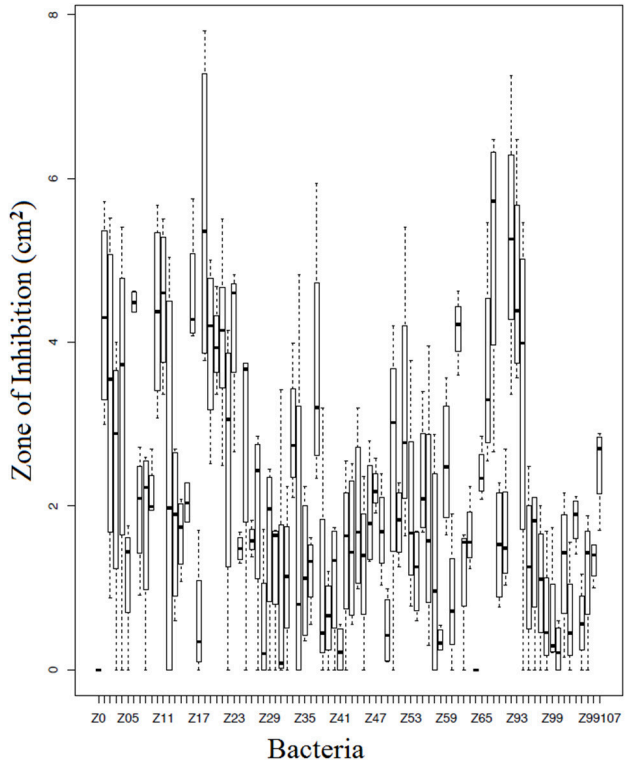

FIGURE 4 | Box plot estimates of the agar based challenge assay for each of the bacteria strains. The box represents the interquartile range (IQR) and the top and bottom whiskers represent Q3—(1.5 IQR) and Q1—(1.5 IQR), respectively. Bacteria IDs on the x-axis are referenced in Supplementary Table 4. Larger values signify greater inhibition of *Bd* growth.

upregulated in both strains included those associated with the nitrate reductases and the associated transport machinery (*narG*, *narH*, and *nirC*). Also of interest and shared between both strains was a putatative oxalate-formate antiporter coding gene (*yhjX*) and the acyl-conezyme A dehydrogenase gene (*fadE*). We also used a Venn diagram to visualize the differentially expressed genes of *S. marcescens* in strains one and two as a response to live-*Bd* and heat-killed *Bd* (**Figure 6**).

RNA-sequencing expression results were validated by examination of six genes in the presence of the two experimental treatments and control (**Figure 7**). The *narG* gene showed upregulation in *Bd* and heat-killed *Bd* treatments with much stronger upregulation in response to the live *Bd* treatment. The *fadE* gene was also significantly upregulated but only in the *Bd* treatment. Other genes examined included *chiA* (chitinase A) and *pigM* (key-regulatory enzyme in the prodigiosin production pathway) to confirm the lack of differential expression as seen in RNA-sequencing. Reference genes *dnaE* and *rplU* showed no significant differential expression.

DISCUSSION

This work is the first to highlight gene expression in anti-*Bd* bacterial strains. More importantly, many of these bacteria

TABLE 1 | Sequencing results including total reads for each multiplexed sample, reads mapped to reference genome, and mapped genes aligning to protein coding genes.

*Sequenced sample	Total reads	Mapped reads	Aligning to protein coding genes
SMWW4 Control (1)	3239079	2664279 (82%)	1491996 (56%)
SMWW4 Control (2)	3814486	3258485 (85%)	2378694 (73%)
SMWW4 *Bd* (1)	3938104	3201797 (81%)	2145204 (67%)
SMWW4 *Bd* (2)	3912551	3348231 (86%)	2477691 (74%)
SMWW4 HK*Bd* (1)	4097349	3361055 (82%)	1579696 (47%)
SMWW4 HK*Bd* (2)	3139545	2623075 (84%)	1914845 (73%)

**Both strains were mapped to the S.marcescens strain WW4 (SMWW4) with correlating experimental growth condition (Control, B. dendrobatidis present (Bd), heat-killed B. dendrobatidis present (HKBd) and biological replicate number.*

were isolated from amphibian species in recovering and relict populations from a major epizootic event. These strains represent important data for understanding the role the bacterial microbiome has in conferring *Bd* resistance. Understanding which bacterial species are anti-*Bd* and the underlying gene expression is meaningful for proposing mechanisms of anti-*Bd* action and subsequent verification studies using gene knockout and complementation. Such work will be invaluable to understanding disease resistance and the role of the bacterial microbiome. Additionally, this work is critical for the future development of novel *in situ* microbiome engineering tools for conservation.

In general, the bacterial genera that were identified in this study as having anti-*Bd* activity were similar to those found in other studies both in the tropics and in temperate zones (Lam et al., 2010; Flechas et al., 2012). The bacterial isolates most common in this study were members of the genus *Serratia*, part of the phylum Proteobacteria. *Serratia* were the strongest candidates for bioaugmentation efforts identified in this study owing to their rapid growth *in vitro*, apparent anti-*Bd* properties, and common distribution across various Costa Rican amphibian species. They have also been shown to exhibit anti-*Bd* properties in previous work examining amphibian microbiomes (Woodhams et al., 2007b; Antwis et al., 2015). While the *S. marcescens* used in this study have excellent potential for bioaugmentation efforts and mechanistic inquiries, certain strains of *S. marcescens* are known opportunistic human pathogens (Hejazi and Falkiner, 1997). Caution should be taken in working with uncharacterized wild-type strains of this bacteria including safety education of personnel and BSL-2 standards in all laboratory work.

In understanding *S. marcescens* and their anti-*Bd* mechanisms in more detail as it pertains to the cutaneous microbiome, we found that the two *S. marcescens* strains examined in this study had various transcriptomic shifts in response to *Bd*. The responses seen were varied and implicated various metabolic and regulatory processes. The genes validated using RT-qPCR had complete congruency with the RNA-sequencing data. This should be seen as a small but important validation of the significantly expressed genes identified in the RNA-sequencing portion of the study as well as validation of lack of expression in genes coding for enzymes known to have antifungal activity.

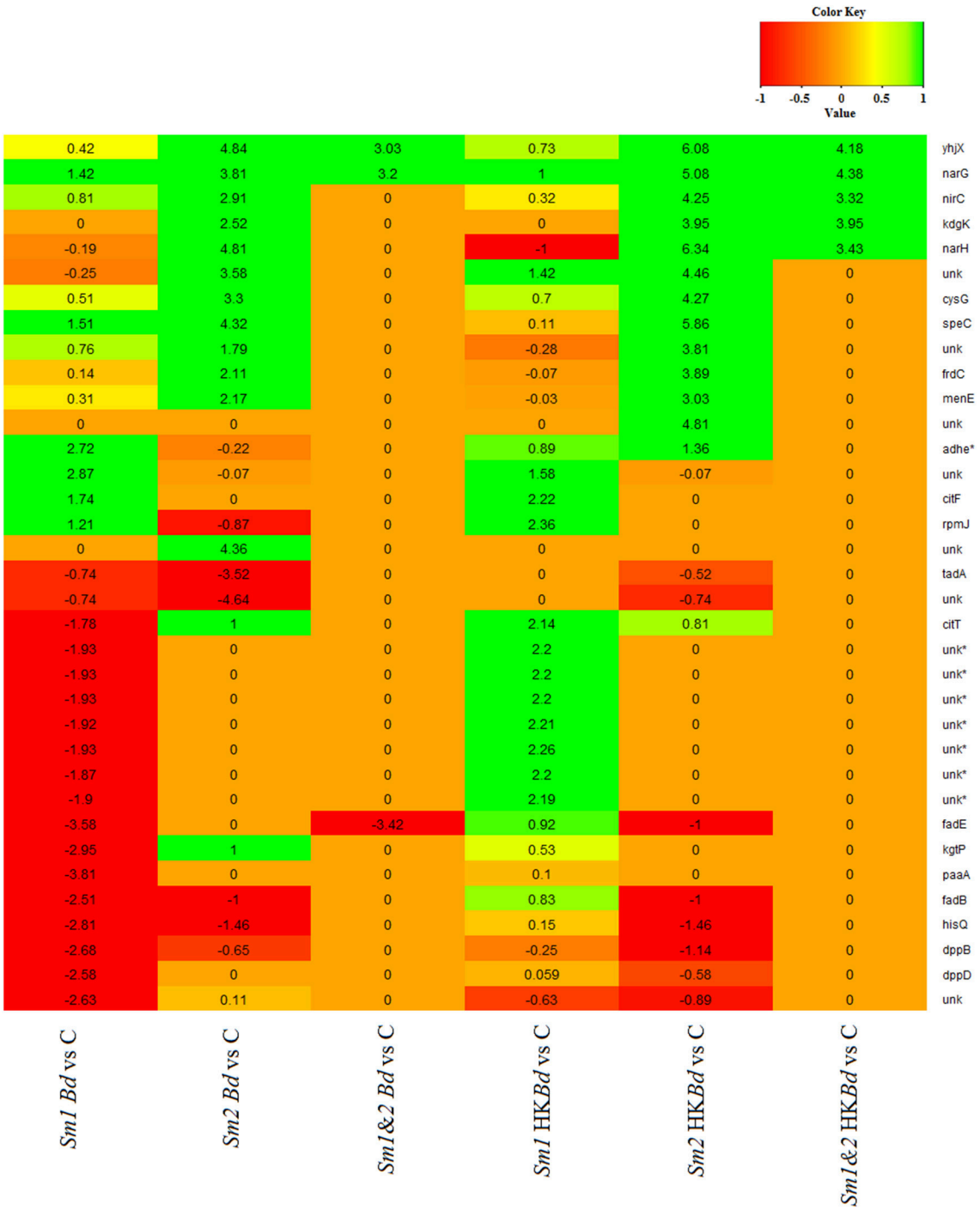

FIGURE 5 | Gene expression data presented as a heat map for the 15 most up- and down-regulated genes for *Bd* and heat-killed *Bd* treatments relative to a no-*Bd* control. Corresponding gene expression values are given for those only in the top 15 in one treatment. Corresponding genes that were not significantly up or down-regulated relative to a no-*Bd* control but were in the top 15 in a different treatment are denoted by a value of 0. Gene names are given on the vertical axis. Treatments are given on the bottom horizontal axis. Sm1 and Sm2 denote *S. marcescens* strain one and two. Bd indicates live *B. dendrobatidis* and HKBd indicates heat-killed *B. dendrobatidis*. 1.2 indicates pooled expression data for both strains. A gene name of *unk* indicates an unnamed gene. An asterisk (*) by a gene name indicates an antisense gene.

The upregulation of nitrate reductases and nitrite transporters was one of the few gene sets shown to be upregulated in both strains and in both treatments. Specifically, the significant upregulation of genes encoding the catalytic nitrate reductase α-subunit and transitory β-subunit which are both part of the membrane bound reductase (NAR) complex is of interest. This

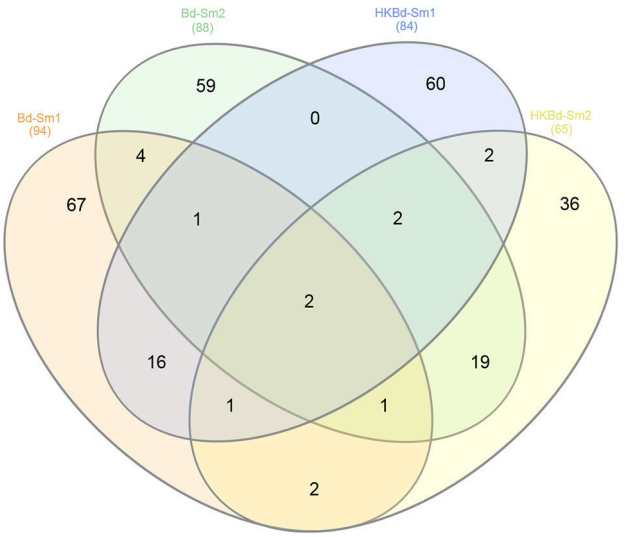

FIGURE 6 | Venn diagram depicting shared significantly expressed DEGs from each of the treatment conditions (*Bd* and heat killed-*Bd*; both vs. no-*Bd* control) in both *S. marcescens* isolates examined.

data coupled with upregulation of the inner membrane nitrite transporter in both strains seems to indicate a response to anaerobic conditions in which nitrate reduction is utilized for energy conservation (Richardson et al., 2001). The reason for the utilization of the membrane-bound nitrate reductase machinery other than a response to anaerobic conditions and available nitrate is unclear but certainly occurring in both *Bd* and heat-killed *Bd* treatments.

Also upregulated in both strains under both conditions was a putative oxalate-formate antiporter coding gene. The need to upregulate the antiport machinery for oxalate decarboxylation to formate in response to *Bd* is at this point not fully understood. However, the production of oxalate by other fungi and the necessary genes in the *Bd* genome for production of oxalate suggest such production by *Bd* is theoretically possible and potentially necessary (Benny, 1995; Bindschedler et al., 2016). This coupled with recent work showing amphibian mortality due to oxalate nephropathy from an unknown oxalate source may suggest a specific mechanism by which *Bd* could kill amphibians (Tokiwa et al., 2015). This possible mechanism deserves further consideration, especially owing to the fact that specific mechanisms for *Bd* induced mortality are not fully understood. Evidence for the epidermal dysfunction hypothesis presented by Voyles et al. (2009) still has no clear biochemical mechanism delineated for the disruption of epidermal channels involved in electrolyte transport. Whether this response and the formation of nitrite into the periplasmic space has any direct inhibitory effects on *Bd* growth is also unclear but worth further consideration.

Differential expression of the commonly associated antimicrobial genes of *S. marcescens* was not seen. These include the upregulation of extracellular chitinases and glucanases as well as the key regulatory enzymes involved in prodigiosin biosynthesis. Chitinases have been previously shown to target the β-1,4 n-acetylglucosamine linkages of the fungal chitin polymer which constitutes a substantial component of the fungal cell wall (Chet and Inbar, 1994). Also, bacterial glucanases have been shown to have a similar effect in the targeting of glucan linkages critical for fungal cell wall structure (Hong and Meng, 2003). The importance of prodigiosin, a secondary metabolite shown to have antifungal characteristics, was also examined in the context of the key regulatory enzymes of the prodigiosin biosynthesis pathway. These enzymes, which are encoded by the prodigiosin biosysnthesis gene cluster (*pig* cluster), were not shown to be upregulated in this study. The downstream effects of this lack of expression was also confirmed visually as *S. marcescens* in culture exhibited only a faint red color (color consistent with the production of the red pigment prodigiosin) under the experimental conditions used in this study. This can most likely be attributed to the known temperature dependence of prodigiosin production in *S.* spp. (Williams, 1973; Woodhams et al., 2014). The temperatures used for co-culturing in this study (20°C) are not generally known to be the most advantageous for prodigiosin production. The lack of upregulation seen in the chitinase (*chi*) genes as well as those involved in glucanase production was also of interest. While not being upregulated directly, there was still a consistent amount of basal expression which may indicate constitutive production of these products which is unaffected by the presence of *Bd* or heat-killed *Bd*. While differential expression is of interest and often informative of an organism's response (e.g., bacteria to *Bd*) there may be products that are basally expressed and indifferent to the presence of a stimulatory organism such as *Bd*. The only way in which to empirically evaluate the importance of such products being produced in an anti-*Bd* response would be the generation of knockouts or knockdowns of the genes of interest in line with molecular Koch's postulates (Falkow, 1988).

While not showing differential expression, the observed expression of the proteins associated with the type six secretion system (T6SS) was also of interest. The expression of a majority of the 13 core genes including *VgrG* (*TssI*) and *Hcp* (*TssD*) associated with the T6SS could have implications in direct inhibition of *Bd* by *S. marcescens*. While the use of such a system has been shown to inhibit competing bacteria in other *S. marcescens* strains (Murdoch et al., 2011), the existence of effector proteins associated with the T6SS that are antifungal in nature are at this point unclear. However, direct competition with other bacteria could allow *S. marcescens* to propagate to higher levels at the bacterial community level allowing other factors discussed above to play a larger role in the system. This preliminary evidence on the role of a T6SS in *S. marcescens* and other gram negative bacteria in the amphibian-*Bd* system therefore merits further examination.

The upregulation of 2-dehydro, 3-deoxy-phosphogluconate aldolase gene, coding for a key regulatory enzyme in the Entner-Duodoroff (ED) pathway, was seen in both *S. marcescens* strains in response to heat-killed *Bd*. The reason behind this upregulation in response to both strains of heat-killed *Bd* treatments is unclear but could also merit further consideration.

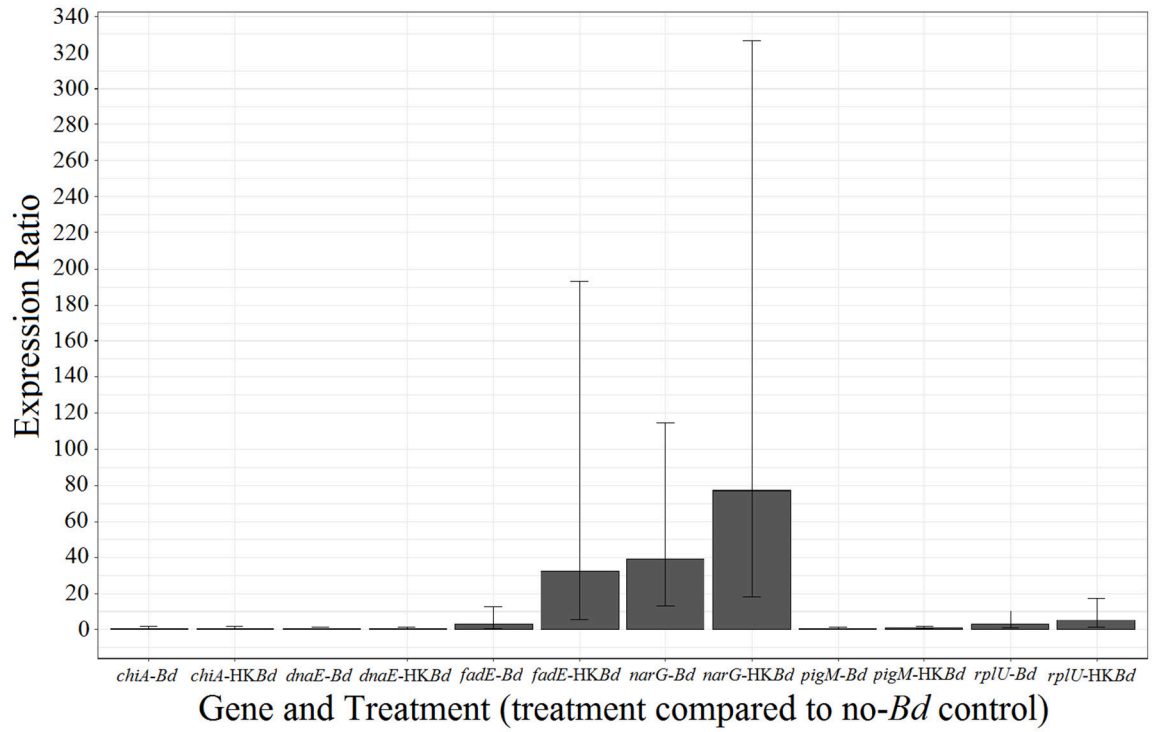

FIGURE 7 | qPCR verification of a select group of genes identified in RNA-sequencing. Error bars represent 95% confidence intervals. All gene expression was compared to *dnaE* and *rplU* reference genes.

The upregulation of various antisense-RNAs was also intriguing and could represent a point of regulation which is part of a response to *Bd*. All asRNAs were associated with ribosomal RNAs representing possible regulation of translation. Curiously, all differentially expressed asRNAs were only observed in strain one with down-regulation as a response to *Bd* and upregulation as a response to heat-killed *Bd*. These results seem to indicate clear differences of a bacterial reaction to *Bd* and heat-killed *Bd* in strain one. The generation of asRNAs are known to have significant regulatory effects on gene expression as well as functional roles (Sesto et al., 2013). Specifically, asRNAs have been shown to induce (i.e., *agr* locus of *Staphylococcus aureus*) as well as attenuate (i.e., asRNA dependent *rep*-mRNA conformations for attenuation of replication control) various bacterial functions (Brantl, 2002). However, the exact role of such regulation and function in this system remains unclear.

The two strains of *S. marcescens* used in this study both had high homology to sequenced strain WW4. However, there are obvious physiological differences in these strains as indicated by the few (eight) genes that were differentially expressed in both. The strains used were collected at the same time but remained in culture for different amounts of time before being cryoarchived representing a possible source of the observed differences. The unstable genomes of wild-type bacteria and potential for lateral gene transfer could account for differences in the aforementioned timeframe and highlights the difficulty in working with such bacteria. While the inclusion of more biological replicates could have been beneficial, both strains came from the same individual and were the only two strains collected in suburban Heredia, Costa Rica. It should also be noted that each sample of assumedly clonal bacterial cells should have variation following an approximate normal distribution due to the high population (n > 10,000,000) of bacterial cells in each tube. Setting up multiple growth flasks would be a better indicator of population sensitivity (and resulting expression profile) to small and potentially uncontrollable differences in growth conditions. We have included results from both the comparisons between individual bacterial populations as well as comparisons with both populations pooled. The introduction of other *S. marcescens* from other amphibian species and individuals would nullify the comparison of bacteria growing in the same environment although would still be interesting but ultimately outside the scope of this study.

The results from the transcriptomic analysis portion of this study provide important information of a bacterial species with strongly inhibitory *Bd* properties. The use of meta-omics approaches as suggested by Rebollar et al. (2016b) could also be utilized in further studies delineating whole bacterial microbiome response to *Bd*. However, we suggest that conclusions delineating species-specific bacterial mechanisms of interest for probiotic development from such methods should be done with caution. Many bacteria identified through such techniques have no reference genome or at the least have large repeat regions creating challenging areas for *de*

novo assembly in species-specific determinations (NP-hard). Interpreting data and trusting available workflows is also problematic and fails to recognize these considerations in such analyses. Determination of error in workflows can also be challenging and would entail complex error propagation (e.g., Taylor series expansion) strategies that are currently limited in implementation. Any meta-omic interpretations should be reserved for the community-level in the interest of excluding erroneous conclusions. Such data would still be of great benefit in continued research initiatives.

Several of the amphibian species examined had few to no anti-*Bd* bacterial isolates. The number of isolates examined from each amphibian species is small compared to the breadth of the entire microbial community on the cutaneous layer. Additionally, not all isolates from field sampling were used in this study. Microbial communities on amphibians are extremely variable between populations and individuals within the same population across temporal and spatial distributions (Vredenburg et al., 2010). Continued sampling and bioassays of the bacteria of these relict populations may uncover additional bacteria that exhibit anti-*Bd* properties. Additionally, the number of anti-*Bd* isolates does not necessarily correlate with the success of an amphibian population; few isolates may still provide protection against chytridiomycosis.

It is also possible that these relict populations have not yet acquired anti-*Bd* bacteria, but instead persist in the presence of *Bd* because of other immune defenses. While symbiotic bacteria are an effective defense against *Bd*, other innate defenses such as antimicrobial peptides (AMPs) secreted by dermal glands are important in conferring anti-*Bd* properties (Rollins-Smith and Conlon, 2005; Conlon, 2011). AMP secretion has been shown to be variable between amphibian species and families creating many possible dynamics between AMPs and the amphibian bacterial microbiome (Conlon, 2011). Three of the amphibian species examined in this study belong to families known to secrete AMPs with anti-fungal properties and also host isolates identified as anti-*Bd* by this study (Apponyi et al., 2004; Amiche et al., 2008; Conlon, 2011). However, *Incilius holdridgei* of the Bufonidae family are known to not secrete any distinguishable AMPs with antifungal properties. Interestingly, this species also lives in areas with the ideal environmental conditions for *Bd* growth (Abarca et al., 2010) thus making it possible that symbiotic bacteria play a strong role in resistance to chytridiomycosis. In addition to innate immune responses, the adaptive immune system can also provide significant benefit in anti-*Bd* activity. The relationship between innate and immune responses may also be important in continuing amelioration efforts.

Several control efforts have been suggested to mitigate amphibian decline due to chytridiomycosis, such as inoculating ponds with sodium chloride or the use of anti-fungal drugs (Stockwell et al., 2012). Also proposed is probiotic bioaugmentation with protective bacteria known to inhibit *Bd*. Bioaugmentation targets amphibian species that are being released into areas where *Bd* has colonized and where populations are experiencing decline due to chytridiomycosis. All of these approaches though, have seen limitations and lack broad applicability. Therefore, we recommend *in situ* microbiome engineering tools as a novel direction for continued amelioration efforts. Specifically, addition of known antifungal genes and the conferring of protective mechanisms to specific members of a resident bacterial population with plasmid or phage-based genetic modifications may be possible. This is an area our group is currently exploring with great interest for use in the amphibian-disease system and beyond. Recent interest in the literature (Sheth et al., 2016) seems to address this potential use of the bacterial microbiome.

The results and insights provided here will serve as a critical foundation for future studies interested in bacterial responses to *Bd* and continued work on bioaugmentation efforts with *S. marcescens*. Specifically, those genes shown upregulated in both strains of *S. marcescens* would be of interest for further work including the construction of bacterial knockouts as well as complementary studies in *Bd*. Lastly, this data will aid in future and ongoing work addressing manipulation of the amphibian microbiome as well as other disease systems through microbiome engineering efforts.

AUTHOR CONTRIBUTIONS

JM was involved in the following: anti-Bd assays, RNA sequencing, RT-qPCR, data analysis, and writing the manuscript. EB was involved in the following: anti-Bd assays and related data analysis. JA was involved in the following: isolation and initial characterization of the bacterial isolates and writing the manuscript. OG was involved in the following: RNA sequencing laboratory work. SW was involved in the following: isolation and initial characterization of the bacterial isolates and writing the manuscript. AP was involved in the following: isolation and initial characterization of the bacterial isolates and writing the manuscript. JK was involved in the following: project oversight of all aspects of the study. All authors partook in commenting/editing of the manuscript and have approved the given manuscript for submission. Additionally, all authors agree to be accountable for all aspects of the presented work.

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SUPPLEMENTARY MATERIAL

Additional Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00290/full#supplementary-material

All transcriptome sequencing files are accessible through the NCBI database with accession number GSE84057 and ID 200084057. Included are raw data files, processed data files, and metadata files giving the experimental parameters and conditions. All 16S rDNA sequencing and metadata files are available through the NCBI database with accession numbers KX928035-KX928072. All assembled sequences were checked for chimeras using DECIPHER (Wright et al., 2012). All R scripts used for the generation of graphs and statistical analyses are available through GitHub, (user: kvasir7, repository: dissertation_research).

REFERENCES

Abarca, J., Chaves, G., García-Rodríguez, A., and Vargas, R. (2010). Reconsidering extinction: rediscovery of *Incilius holdridgei* (Anura: Bufonidae) in Costa Rica after 25 years. *Herpetol. Rev.* 41, 150–152.

Amiche, M., Ladram, A., and Nicolas, P. (2008). A consistent nomenclature of antimicrobial peptides isolated from frogs of the subfamily Phyllomedusinae. *Peptides* 29, 2074–2082. doi: 10.1016/j.peptides.2008.06.017

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/AEM.00010-15

Apponyi, M. A., Pukala, T. L., Brinkworth, C. S., Maselli, V. M., Bowie, J. H., Tyler, M. J., et al. (2004). Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. *Peptides* 25, 1035–1054. doi: 10.1016/j.peptides.2004.03.006

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2014). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Org.* 103, 77–85. doi: 10.3354/dao02560

Benny, G. L. (1995). Classical morphology in zygomycete taxonomy. *Can. J. Bot.* 73, 725–730. doi: 10.1139/b95-315

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Bindschedler, S., Cailleau, G., and Verrecchia, E. (2016). Role of fungi in the biomineralization of calcite. *Minerals* 6:41. doi: 10.3390/min6020041

Brantl, S. (2002). Antisense-RNA regulation and RNA interference. *Biochim. Biophys. Acta* 1575, 15–25. doi: 10.1016/S0167-4781(02)00280-4

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. (2008b). The identification of 2, 4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008a). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Chaves-Cordero, G. A., Zumbado-Ulate, H., García-Rodríguez, A., Gómez, E., Vredenburg, V. T., and Ryan, M. J. (2014). Rediscovery of the critically endangered streamside frog, *Craugastor taurus* (Craugastoridae), in Costa Rica. *Trop. Conserv. Sci.* 7, 628–638. doi: 10.1177/194008291400700404

Chet, I., and Inbar, J. (1994). Biological control of fungal pathogens. *Appl. Biochem. Biotechnol.* 48, 37–43. doi: 10.1007/BF02825358

Collins, J. P., and Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. *Divers. Distrib.* 9, 89–98. doi: 10.1046/j.1472-4642.2003.00012.x

Conlon, J. M. (2011). The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. *Cell Tissue Res.* 343, 201–212. doi: 10.1007/s00441-010-1014-4

Crawford, A. J., Lips, K. R., and Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13777–13782. doi: 10.1073/pnas.0914115107

Duran-Pinedo, A. E., Chen, T., Teles, R., Starr, J. R., Wang, X., Krishnan, K., et al. (2014). Community-wide transcriptome of the oral microbiome in subjects with and without periodontitis. *ISME J.* 8, 1659–1672. doi: 10.1038/ismej.2014.23

Duzhak, A. B., Panfilova, Z. I., Duzhak, T. G., Vasyunina, E. A., and Shternshis, M. V. (2012). Role of prodigiosin and chitinases in antagonistic activity of the bacterium *Serratia marcescens* against the fungus *Didymella applanata*. *Biochemistry* 77, 910–916. doi: 10.1134/S0006297912080123

Ellison, A. R., Savage, A. E., DiRenzo, G. V., Langhammer, P., Lips, K. R., and Zamudio, K. R. (2014). Fighting a losing battle: vigorous immune response countered by pathogen suppression of host defenses in the chytridiomycosis-susceptible frog *Atelopus zeteki*. *G3 (Bethesda)*. 4, 1275–1289. doi: 10.1534/g3.114.010744

Falkow, S. (1988). Molecular Koch's postulates applied to microbial pathogenicity. *Rev. Infect. Dis.* 10(Suppl. 2), S274–S276. doi: 10.1093/cid/10.supplement_2.s274

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Gleave, A. P., Taylor, R. K., Morris, B. A., and Greenwood, D. R. (1995). Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum*. *FEMS Microbiol. Lett.* 131, 279–288. doi: 10.1111/j.1574-6968.1995.tb07788.x

Gutiérrez-Román, M. I., Holguín-Meléndez, F., Dunn, M. F., Guillén-Navarro, K., and Huerta-Palacios, G. (2015). Antifungal activity of *Serratia marcescens* CFFSUR-B2 purified chitinolytic enzymes and prodigiosin against *Mycosphaerella fijiensis*, causal agent of black Sigatoka in banana (*Musa* spp.). *BioControl* 60, 565–572. doi: 10.1007/s10526-015-9655-6

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Hejazi, A., and Falkiner, F. R. (1997). *Serratia marcescens*. *J. Med. Microbiol.* 46, 903–912. doi: 10.1099/00222615-46-11-903

Hoffmann, H. (2006). Some ecological notes on *Agalychnis annae* (Annura: Hylidae). *Brenesia* 65, 73–77.

Hong, T. Y., and Meng, M. (2003). Biochemical characterization and antifungal activity of an endo-1, 3-β-glucanase of *Paenibacillus* sp. isolated from garden soil. *Appl. Microbiol. Biotechnol.* 61, 472–478. doi: 10.1007/s00253-003-1249-z

Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., Frick, W. F., and Kilpatrick, A. M. (2015). Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. *PLoS ONE* 10:e0121329. doi: 10.1371/journal.pone.0121329

Johnson, M. L., Berger, L., Phillips, L., and Speare, R. (2003). Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid, *Batrachochytrium dendrobatidis*. *Dis. Aquat. Org.* 57, 255–260. doi: 10.3354/dao057255

Kostic, A. D., Xavier, R. J., and Gevers, D. (2014). The microbiome in inflammatory bowel disease: current status and the future ahead. *Gastroenterology* 146, 1489–1499. doi: 10.1053/j.gastro.2014.02.009

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Longcore, J. E., Pessier, A. P., and Nichols, D. K. (1999). *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. *Mycologia* 91, 219–227. doi: 10.2307/3761366

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P., and Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* 5:441. doi: 10.3389/fmicb.2014.00441

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., et al. (2013). *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proc. Natl. Acad. Sci.* 110, 15325–15329. doi: 10.1073/pnas.1307356110

McClure, R., Balasubramanian, D., Sun, Y., Bobrovskyy, M., Sumby, P., Genco, C. A., et al. (2013). Computational analysis of bacterial RNA-Seq data. *Nucleic Acids Res.* 41, e140. doi: 10.1093/nar/gkt444

Murdoch, S. L., Trunk, K., English, G., Fritsch, M. J., Pourkarimi, E., and Coulthurst, S. J. (2011). The opportunistic pathogen *Serratia marcescens* utilizes type VI secretion to target bacterial competitors. *J. Bacteriol.* 193, 6057–6069. doi: 10.1128/JB.05671-11

Poorten, T. J., Stice-Kishiyama, M. J., Briggs, C. J., and Rosenblum, E. B. (2015). Mountain yellow-legged frogs (*Rana muscosa*) did not produce detectable antibodies in immunization experiments with *Batrachochytrium dendrobatidis*. *J. Wildl. Dis.* 52, 154–158. doi: 10.7589/2015-06-156

Price, S. J., Garner, T. W., Balloux, F., Ruis, C., Paszkiewicz, K. H., Moore, K., et al. (2015). A de novo assembly of the common frog (*Rana temporaria*) transcriptome and comparison of transcription following exposure to *Ranavirus* and *Batrachochytrium dendrobatidis*. *PLoS ONE* 10:e0130500. doi: 10.1371/journal.pone.0130500

Puschendorf-Fahrenkrug, R., Chaves-Cordero, G. A., Crawford, A. J., and Brooks, D. R. (2005). Eleutherodactylus ranoides (NCN). Dry forest population, refuge from decline? *Herpetol. Rev.* 36, 53.

Ramsey, J. P., Reinert, L. K., Harper, L. K., Woodhams, D. C., and Rollins-Smith, L. A. (2010). Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infect. Immun.* 78, 3981–3992. doi: 10.1128/IAI.00402-10

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016b). Using "Omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016a). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell. Mol. Life Sci.* 58, 165–178. doi: 10.1007/PL00000845

Rollins-Smith, L. A. (1998). Metamorphosis and the amphibian immune system. *Immunol. Rev.* 166, 221–230. doi: 10.1111/j.1600-065X.1998.tb01265.x

Rollins-Smith, L. A., and Conlon, J. M. (2005). Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. *Dev. Comp. Immunol.* 29, 589–598. doi: 10.1016/j.dci.2004.11.004

Rosenblum, E. B., Poorten, T. J., Joneson, S., and Settles, M. (2012b). Substrate-specific gene expression in *Batrachochytrium dendrobatidis*, the chytrid pathogen of amphibians. *PLoS ONE* 7:e49924. doi: 10.1371/journal.pone.0049924

Rosenblum, E. B., Poorten, T. J., Settles, M., and Murdoch, G. K. (2012a). Only skin deep: shared genetic response to the deadly chytrid fungus in susceptible frog species. *Mol. Ecol.* 21, 3110–3120. doi: 10.1111/j.1365-294X.2012.05481.x

Rosenblum, E. B., Stajich, J. E., Maddox, N., and Eisen, M. B. (2008). Global gene expression profiles for life stages of the deadly amphibian pathogen *Batrachochytrium dendrobatidis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 17034–17039. doi: 10.1073/pnas.0804173105

Schwabe, R. F., and Jobin, C. (2013). The microbiome and cancer. *Nat. Rev. Cancer* 13, 800–812. doi: 10.1038/nrc3610

Sesto, N., Wurtzel, O., Archambaud, C., Sorek, R., and Cossart, P. (2013). The excludon: a new concept in bacterial antisense RNA-mediated gene regulation. *Nat. Rev. Microbiol.* 11, 75–82. doi: 10.1038/nrmicro2934

Sheth, R. U., Cabral, V., Chen, S. P., and Wang, H. H. (2016). Manipulating bacterial communities by *in situ* microbiome engineering. *Trends Genet.* 32, 189–200. doi: 10.1016/j.tig.2016.01.005

Stockwell, M. P., Clulow, J., and Mahony, M. J. (2012). Sodium chloride inhibits the growth and infective capacity of the amphibian chytrid fungus and increases host survival rates. *PLoS ONE* 7:e36942. doi: 10.1371/journal.pone.0036942

Tan, D., Fu, L., Han, B., Sun, X., Zheng, P., and Zhang, J. (2015). Identification of an endophytic antifungal bacterial strain isolated from the rubber tree and its application in the biological control of banana *Fusarium* wilt. *PLoS ONE* 10:e0131974. doi: 10.1371/journal.pone.0131974

Tjaden, B. (2015). *De novo* assembly of bacterial transcriptomes from RNA-seq data. *Genome Biol.* 16, 1. doi: 10.1186/s13059-014-0572-2

Tokiwa, T., Kadekaru, S., Ito, M., Yoshida, M., and Une, Y. (2015). Oxalate nephropathy in free-living American bullfrog tadpoles. *Dis. Aquat. Org.* 116, 199–203. doi: 10.3354/dao02936

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326, 582–585. doi: 10.1126/science.1176765

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9689–9694. doi: 10.1073/pnas.0914011107

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A., and Woodhams, D. C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–400. doi: 10.1111/j.1744-7429.2011.00787.x

Williams, R. P. (1973). Biosynthesis of prodigiosin, a secondary metabolite of *Serratia marcescens*. *Appl. Microbiol.* 25, 396–402.

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K., and Rollins-Smith, L. A. (2007a). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.* 10, 409–417. doi: 10.1111/j.1469-1795.2007.00130.x

Woodhams, D. C., Bletz, M., Kueneman, J., and McKenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trends Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE*, 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Rollins-Smith, L. A., Alford, R. A., Simon, M. A., and Harris, R. N. (2007b). Innate immune defenses of amphibian skin: antimicrobial peptides and more. *Anim. Conserv.* 10, 425–428. doi: 10.1111/j.1469-1795.2007.00150.x

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007c). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Wright, E. S., Yilmaz, L. S., and Noguera, D. R. (2012). DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl. Environ. Microbiol.* 78, 717–725. doi: 10.1128/AEM.06516-11

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Inhibition of Fungal Pathogens across Genotypes and Temperatures by Amphibian Skin Bacteria

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Symbiotic bacteria may dampen the impacts of infectious diseases on hosts by inhibiting pathogen growth. However, our understanding of the generality of pathogen inhibition by different bacterial taxa across pathogen genotypes and environmental conditions is limited. Bacterial inhibitory properties are of particular interest for the amphibian-killing fungal pathogens (*Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*), for which probiotic applications as conservation strategies have been proposed. We quantified the inhibition strength of five putatively *B. dendrobatidis*-inhibitory bacteria isolated from woodland salamander skin against six *Batrachochytrium* genotypes at two temperatures (12 and 18°C). We selected six genotypes from across the *Batrachochytrium* phylogeny: *B. salamandrivorans*, *B. dendrobatidis*-Brazil and four genotypes of the *B. dendrobatidis* Global Panzootic Lineage (GPL1: JEL647, JEL404; GPL2: SRS810, JEL423). We performed 96-well plate challenge assays in a full factorial design. We detected a *Batrachochytrium* genotype by temperature interaction on bacterial inhibition score for all bacteria, indicating that bacteria vary in ability to inhibit *Batrachochytrium* depending on pathogen genotype and temperature. *Acinetobacter rhizosphaerae* moderately inhibited *B. salamandrivorans* at both temperatures ($\mu = 46$–53%), but not any *B. dendrobatidis* genotypes. *Chryseobacterium* sp. inhibited three *Batrachochytrium* genotypes at both temperatures ($\mu = 5$–71%). *Pseudomonas* sp. strain 1 inhibited all *Batrachochytrium* genotypes at 12°C and four *Batrachochytrium* genotypes at 18°C ($\mu = 5$–100%). *Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp. moderately to strongly inhibited all six *Batrachochytrium* genotypes at both temperatures ($\mu = 57$–100%). All bacteria consistently inhibited *B. salamandrivorans*. Using cluster analysis of inhibition scores, we found that more closely related *Batrachochytrium* genotypes grouped together, suggesting that bacterial inhibition strength may be predictable based on *Batrachochytrium* relatedness. We conclude that bacterial inhibition capabilities change among bacterial strains, *Batrachochytrium* genotypes and temperatures. A comprehensive understanding of bacterial inhibitory function, across pathogen genotypes and temperatures, is needed to better predict

the role of bacterial symbionts in amphibian disease ecology. For targeted conservation applications, we recommend using bacterial strains identified as strongly inhibitory as they are most likely to produce broad-spectrum antimicrobial agents at a range of temperatures.

Keywords: symbiont, salamander, *Batrachochytrium*, antifungal, disease ecology

INTRODUCTION

Interactions between host microbiomes and pathogens influence the severity of host disease. The outcome of microbiome-pathogen interactions can depend on microbiome composition (Chang et al., 2008; Jani and Briggs, 2014; Rovenich et al., 2014), pathogen genotype (Antwis et al., 2015), and environmental context (Duffy and Defago, 1999; Lokmer and Wegner, 2015). In amphibians, the skin microbiome has been implicated in variable host susceptibility to the disease chytridiomycosis (Harris et al., 2009a; Becker and Harris, 2010; Muletz et al., 2012). Chytridiomycosis has been linked to extreme loss of global amphibian biodiversity (Berger et al., 1998; Lips et al., 2006; Martel et al., 2014; Spitzen-van der Sluijs et al., 2016; Carvalho et al., 2017; Stegen et al., 2017), and is caused by skin infection by either of two congeneric chytrid fungi, *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, hereafter *B. dendrobatidis* and *B. salamandrivorans*, respectively. One mechanism by which bacterial symbionts offer protection from *Batrachochytrium* is through production of inhibitory metabolites that can kill zoospores (Brucker et al., 2008a,b) or cause zoospores to move away from the metabolites (Lam et al., 2011). However, the impacts of amphibian skin microbiomes on fungal disease are difficult to predict because little is known about the ecological and evolutionary factors shaping microbiome functions, such as antifungal properties (e.g., Madison et al., 2017). To better predict the role of bacterial symbionts in amphibian disease ecology it is necessary to quantify antifungal properties across bacterial strains, *Batrachochytrium* genotypes and temperatures.

Application of antifungal bacteria has been proposed as a preventative strategy and a treatment option for chytridiomycosis in the wild (Muletz et al., 2012; Bletz et al., 2013). Of the approximately 250 bacterial operational taxonomic units (OTUs) identified as *B. dendrobatidis*-inhibitory (Woodhams et al., 2015; Muletz-Wolz et al., 2017a), nine have been used in bioaugmentation trials (e.g., Harris et al., 2009a,b; Woodhams et al., 2012; Nebergall, 2013; Becker et al., 2015a). These trials have had mixed success in mitigating chytridiomycosis. For instance, three studies found no effect of augmenting *Janthinobacterium lividum* on amphibian skin in reducing *B. dendrobatidis*-associated disease symptoms (Becker et al., 2011; Bletz, 2013; Nebergall, 2013), even though *J. lividum* inhibits *B. dendrobatidis* growth *in vitro* (Harris et al., 2006) and can be effective against *B. dendrobatidis* on amphibian skin (Harris et al., 2009a; Muletz et al., 2012). The variation in impact of bacterial augmentation *in vivo* suggests that environment and/or pathogen specific factors influence antifungal activity of bacterial symbionts through regulation of metabolite production or pathogen genotype specificity.

Batrachochytrium is a globally distributed genus with a complex evolutionary history within the two described species, *B. dendrobatidis* and *B. salamandrivorans* (Farrer et al., 2013, 2017; Rosenblum et al., 2013; Jenkinson et al., 2016). *B. dendrobatidis* originated at least 30 mya and is comprised of multiple, deeply diverged lineages including a Global Panzootic Lineage (GPL) and four enzootic lineages that are generally confined to their respective regions, *B. dendrobatidis*-Brazil, *B. dendrobatidis*-Cape, *B. dendrobatidis*-CH, and *B. dendrobatidis*-Korea (Schloegel et al., 2012; Farrer et al., 2013; Rosenblum et al., 2013; Martel et al., 2014; James et al., 2015; Jenkinson et al., 2016). Recent mass mortality events and population declines linked to *B. dendrobatidis* have been primarily associated with *B. dendrobatidis*-GPL (Fisher et al., 2009; Farrer et al., 2011; James et al., 2015). *B. dendrobatidis*-GPL is rapidly evolving with genetic differentiation that generally form two clades, *B. dendrobatidis*-GPL1, which is found primarily in North America, and *B. dendrobatidis*-GPL2, which is a geographically dispersed group (Schloegel et al., 2012; Rosenblum et al., 2013; James et al., 2015). *B. salamandrivorans* originated at least 30 mya where it coexisted with an Asian salamander clade (Martel et al., 2014) until its emergence in Western Europe resulted in rapid population declines of European fire salamanders (Martel et al., 2013; Stegen et al., 2017). To date, only *B. dendrobatidis*-GPL isolates have been tested to identify amphibian skin bacteria with *Batrachochytrium*-inhibitory traits (Antwis et al., 2015; Woodhams et al., 2015; Madison et al., 2017; Muletz-Wolz et al., 2017a).

Amphibian population declines and mass mortality events linked to *B. dendrobatidis* often have been more devastating in cooler seasons and higher elevations (Berger et al., 2004; Lips et al., 2006, 2008; Kriger and Hero, 2008; Carvalho et al., 2017). Host and pathogen responses may explain increased virulence at lower temperatures, including changes in (i) *B. dendrobatidis* fecundity as a life-history tradeoff (Woodhams et al., 2008), (ii) host immune response to infection (Ribas et al., 2009; Longo and Zamudio, 2017), and (iii) antifungal activity by bacterial symbionts (Daskin et al., 2014; Woodhams et al., 2014; Bresciano et al., 2015). For amphibian skin symbionts, temperature influences bacterial growth rate and population size, and high cell density is often needed to produce inhibitory metabolites (Yasumiba et al., 2016). Compared to *B. dendrobatidis* (Piotrowski et al., 2004; Stevenson et al., 2013), *B. salamandrivorans* generally has a lower optimal growth temperature (Martel et al., 2013) and *B. dendrobatidis*-inhibitory bacteria may not be effective against *B. salamandrivorans* due to temperature-dependent growth

constraints of pathogen and bacteria, among other reasons [e.g., genetic and phenotypic variation between *B. dendrobatidis* and *B. salamandrivorans* (Farrer et al., 2017; Stegen et al., 2017)].

We quantified the inhibition strength of five amphibian skin bacteria cultured from North American woodland salamanders (*Plethodon cinereus* and *P. cylindraceus*), previously shown to inhibit *B. dendrobatidis* (GPL1-JEL404: Muletz-Wolz et al., 2017a) across *Batrachochytrium* genotypes and temperatures. We had three main objectives, (i) quantify the inhibitory proprieties of putatively anti-*B. dendrobatidis* bacterial strains against *B. salamandrivorans*, (ii) quantify the effect of temperature (12 and 18°C), *Batrachochytrium* genotype and their interaction on bacterial inhibition strength, and (iii) determine if *Batrachochytrium* relatedness predicts bacterial inhibition strength. Quantifying interactions between pathogen and bacterial symbionts in an environmental and genetic framework strengthens our understanding of disease dynamics and guides conservation measures.

MATERIALS AND METHODS

We performed *in vitro* challenge assays with five bacterial strains and six *Batrachochytrium* genotypes at two temperatures (12 and 18°C) using 96-well plates in a full factorial experimental design. We selected five bacterial strains to represent a range of inhibition based on their previously quantified inhibition strength against *B. dendrobatidis*-GPL1-JEL404 at 20°C (**Table 1**; Muletz-Wolz et al., 2017a). All bacterial strains were isolated from either *Plethodon cinereus* or *P. cylindraceus* at Shenandoah National Park, Virginia, in May 2012, and were widespread in these populations (Muletz-Wolz et al., 2017b). We selected six *Batrachochytrium* isolates from across the *Batrachochytrium* phylogeny, two GPL1 isolates (JEL647 and JEL404), two GPL2 isolates (SRS810 and JEL423), a Brazilian *B. dendrobatidis* isolate (JEL649), and *B. salamandrivorans* (**Table 2**). Hereafter, we refer to these *Batrachochytrium* isolates as *Batrachochytrium* genotypes because genetic analyses have shown that each of these isolates represent distinct genotypes (Schloegel et al., 2012; Martel et al., 2013; James et al., 2015).

Experimental Set-up

We performed the experiment using a total of 16 96-well plates. Each plate was assigned to one of four randomly generated configurations of bacterial by *Batrachochytrium* combinations (Supplementary Figure S1), and housed in one of four incubators (Percival model DR-36VL; two chambers per temperature, Supplementary Figure S2). Each incubator contained a total of four plates, with one plate per configuration.

We set up challenge assays following a protocol based on Muletz-Wolz et al. (2017a), with the following modifications to accommodate the design of the experiment. We passaged cryopreserved bacteria on 1% tryptone plates three times, then inoculated each bacterial strain in 25 mL of 1% tryptone broth and grew for 3 days at room temperature (approximately 21°C) on a shaker at 100 rpm. By 3 days of incubation, the bacterial cultures reached high densities where inhibitory metabolites are produced (Bérdy, 2005). We obtained cell-free supernatants (CFSs) from bacterial monocultures, following the centrifuging and filtering methods outlined in Muletz-Wolz et al. (2017a). By using bacterial CFS, we determined the inhibitory properties of bacterially produced extracellular factors against live *Batrachochytrium* zoospores, and eliminated the possibility of direct competition or priority effects between bacterial and fungal cultures. As the bacterial strains were

TABLE 1 | Five bacteria strains used in the study, including their phylogenetic designation and previously quantified inhibition strength against *B. dendrobatidis*-GPL1-JEL404 (Muletz-Wolz et al., 2017a).

Bacteria strain	Strain ID	GenBank accession no. of 16S rRNA gene	Inhibition score for *B. dendrobatidis* -GPL1-JEL404 at 20°C (%)
Acinetobacter rhizosphaerae	THA6-B68	KU739019	32
Pseudomonas sp. strain 1	RSB5-4	KU738948	99
Pseudomonas sp. strain 2	SFB8-6	KU738987	82
Chryseobacterium sp.	SFA2-10	KU738960	54
Stenotrophomonas sp.	LSB7-4	KU738931	100

TABLE 2 | Six *Batrachochytrium* genotypes used in the study.

Isolate ID	Phylogenetic lineage	Genotype	Location of isolation	Approximately # passages since isolation
JEL649	*B. dendrobatidis*-Brazil	Brazil-JEL649	São Paulo, Brazil	8
JEL647	*B. dendrobatidis*-GPL1	GPL1-JEL647	California, United States	3
SRS810	*B. dendrobatidis*-GPL2	GPL2-SRS810	Georgia, United States	6
AMFP13/1	*B. salamandrivorans*	*B. salamandrivorans*	Zuid-Limburg, Netherlands	9
JEL423	*B. dendrobatidis*-GPL2	GPL2-JEL423	El Cope, Panama	6
JEL404	*B. dendrobatidis*-GPL1	GPL1-JEL404	Maine, United States	8

Each isolate represented a distinct genotype based on genetic analyses by Schloegel et al. (2012), Martel et al. (2013), and James et al. (2015). We used the combination of Batrachochytrium lineage and isolate to specify the Batrachochytrium genotype.

grown at one temperature prior to the experiment all effects of temperature on inhibition relate to temperature-dependent activity of CFS extracellular factors and/or fungal physiology. For *Batrachochytrium* genotypes, we passaged cryopreserved isolates (prior passage history ranged between 3 and 9 times; **Table 2**) on 1% tryptone plates twice, and then grew them for 1 week on multiple 1% tryptone plates at 15°C. We harvested zoospores by flooding the plates with 1% tryptone broth, filtered out the zoosporangia using a sterile coffee filter, and homogenized the zoospore mixture.

To set up the assays, we added 50 μl of approximately 1×10^6 zoospores/ml of each *Batrachochytrium* genotype (counted with a hemocytometer; approximately 50,000 zoospores in each well) to their designated wells in a 96-well plate (Supplementary Figure S1). In sample wells, we added 50 μl of the CFS from each bacterial strain to four wells for each bacterial-*Batrachochytrium* combination. In total, each bacterial-*Batrachochytrium* combination was represented in 16 wells distributed over four plates per temperature. In each 96-well assay, we included two positive controls (PCs) and one negative control for each *Batrachochytrium* genotype using four wells per control. The positive controls were: 50 μl of *Batrachochytrium* zoospores + 50 μl 1% tryptone broth PC and 50 μl of *Batrachochytrium* zoospores + 50 μl of water [nutrient-depleted positive control (NDPC)]. The negative control was 50 μl of *Batrachochytrium* zoospores heat-killed at 60°C for 60 min + 50 μl of 1% tryptone broth (heat-killed *B. dendrobatidis*: HK). We measured optical density ($OD_{492\ nm}$) of each well for 16 96-well plates using a microplate reader every other day starting on day 1, for 27 days.

B. dendrobatidis Inhibition Score Calculations

We used R version 3.2.5 for all calculations and statistical analyses (R Core Team, 2016). We visually inspected the optical density (OD) readings for each plate, and excluded data points for 32 wells (2% of wells) with unusually high densities (+0.1 or greater well OD compared to replicate wells on same plate), indicating contamination or error. We corrected for baseline zoospore OD by subtracting the average heat-killed OD of each *Batrachochytrium* genotype from the corresponding experimental wells in each plate. To achieve a normal distribution, we transformed the corrected OD readings using the following equation, $\log(OD_{corrected}(1-OD_{corrected})+1)$. Next, we fit linear regressions to the transformed OD readings over time for each well with the intercept set at zero. We extracted the slope of the linear regression, and interpreted this as *Batrachochytrium* growth (i.e., Δ optical density/time). We excluded wells in which the linear model had an r^2 less than 0.20, given the poor fit of the data. After quality filtering, we had slopes for 867 of the 960 sample wells (Supplementary Table S1). Then, we calculated *Batrachochytrium* growth inhibition, hereafter referred to as inhibition score, by dividing the slope of each sample well by the slope of the average NDPC wells of the corresponding *Batrachochytrium* genotype on the same plate, and subtracting the subsequent fraction from one, [Inhibition score = 1 – (slope sample well/average slope NDPC)]. We compared the slopes of sample wells to the NDPC wells because this accounts for the issue of nutrient depletion in PC wells and is a more conservative approach in identifying anti-*Batrachochytrium* bacteria (Bell et al., 2013; Muletz-Wolz et al., 2017a). We created this standardized inhibition scoring system to be able to compare inhibition scores between the two experimental temperatures. We interpreted inhibition scores greater than zero as inhibitory, indicating that the bacterial-*Batrachochytrium* sample well had less growth than the NDPC wells. Values less than zero we interpreted as non-inhibitory, and to determine if any bacterial symbionts promoted *Batrachochytrium* growth, we compared the inhibition scores to those of the PC wells (see Statistical Analyses below; Supplementary Figure S3).

Statistical Analyses

We quantified the effects of *Batrachochytrium* genotype, temperature, and their interaction (explanatory variables) on inhibition scores (response variable) using a linear mixed-effects model for each bacterial strain separately, using the *lmer* function in the 'lme4' package (Bates et al., 2015). We included plate nested within incubator as a random effect in each model. Next, we used the *Anova* function in the 'car' package with type II sum of squares to determine the significance of each of the fixed-effects (Fox and Weisberg, 2011). Using the 'lsmeans' package (Lenth, 2016), we used the *lsmeans* function to perform *post hoc* analyses to determine significant difference among *Batrachochytrium* genotypes and between temperatures. We used the *lsmip* function to generate *Batrachochytrium* genotypes by temperature interaction plots for each bacterial strain.

We determined if any bacterial symbiont promoted *Batrachochytrium* growth by comparing the inhibition score of each bacterial *Batrachochytrium* combination at each temperature to the inhibition score of the PC well for the corresponding *Batrachochytrium* genotype (Supplementary Figure S3). We used a linear mixed-effects model for each *Batrachochytrium* genotype examining the effects of well type (i.e., bacteria or PC), temperature, and their interaction (explanatory variables) on inhibition scores (response variable). We included the same random effects, determined significance and conducted *post hoc* analyses as described above.

We determined if patterns of bacterial inhibition score reflected *Batrachochytrium* phylogenetic relatedness by conducting a cluster analysis using the mean inhibition score for each bacteria-*Batrachochytrium* combination at each temperature. We used two clustering methods to confirm similar clustering patterns: (i) Ward's hierarchical clustering with Euclidean distances using the *pvclust* function in the 'pvclust' package (Suzuki and Shimodaira, 2006), and (ii) k-means clustering using a plot of within groups sum of squares by number of clusters to determine the appropriate number of clusters (Everitt and Hothorn, 2009). We were unable to perform a full phylogenetic analysis because we could not calculate branch length due to missing genotype data for *B. salamandrivorans* and GPL1-JEL404 (Schloegel et al., 2012, T. James, pers. comm.).

RESULTS

Batrachochytrium salamandrivorans was the only *Batrachochytrium* genotype that was inhibited by all bacterial strains, with moderate to strong inhibition ($\mu = 43$–92%) at both temperatures. *Stenotrophomonas* sp. and *Pseudomonas* sp. strain 2 were the most inhibitory against *B. salamandrivorans* at both temperatures ($\mu = 91$–92%).

We detected a *Batrachochytrium* genotype by temperature interaction effect on inhibition score for all bacterial strains (*post hoc* analyses: Supplementary Tables S2, S3), indicating that bacterial inhibition strength is affected by both *Batrachochytrium* genotype and temperature. No bacterial strain promoted the growth of any *Batrachochytrium* genotype (Supplementary Figure S3).

Acinetobacter rhizosphaerae only inhibited *B. salamandrivorans* (**Figure 1A**), and was moderately inhibitory of *B. salamandrivorans* at both temperatures ($\mu = 46$–53%). Inhibition scores for *A. rhizosphaerae* differed among pathogen genotypes ($X^2 = 202.7$, df $= 5$, $p < 0.001$) and this depended on temperature (interaction term: $X^2 = 25.3$, df $= 5$, $p < 0.001$), but the significant interaction was for scores that were non-inhibitory (**Figure 1A**).

Two bacterial strains, *Chryseobacterium* sp. and *Pseudomonas* sp. strain 1, were weakly to strongly inhibitory of most *Batrachochytrium* genotypes. Inhibition scores for *Chryseobacterium* sp. differed among *Batrachochytrium* genotypes ($X^2 = 932.9$, df $= 5$, $p < 0.001$) and this depended on temperature (interaction term: $X^2 = 122.0$, df $= 5$, $p < 0.001$). *Chryseobacterium* sp. was moderately inhibitory of *B. salamandrivorans* and Brazil-JEL649 at both temperatures ($\mu = 38$–71%), and was weakly inhibitory of the GPL1 genotypes with GPL1-JEL647 only inhibited at 18°C and GPL1-JEL404 only inhibited at 12°C (**Figure 1B**). Inhibition scores for *Pseudomonas* sp. strain 1 differed among genotypes ($X^2 = 479.1$, df $= 5$, $p < 0.001$), temperatures ($X^2 = 22.8$, df $= 1$, $p < 0.001$) and showed a significant interaction ($X^2 = 57.4$, df $= 5$, $p < 0.001$). *Pseudomonas* sp. strain 1 inhibited all *Batrachochytrium* genotypes ($\mu = 5$–100%), except for GPL1-JEL404 and GPL2-SRS810 at 18°C (**Figure 1C**). *Pseudomonas* sp. strain 1 was significantly more inhibitory of GPL1-JEL404, GPL1-JEL647, GPL2-SRS810 at 12°C compared to 18°C, and was differentially inhibitory among *Batrachochytrium* genotypes (Supplementary Table S3). For instance, *Pseudomonas* sp. strain 1 was more inhibitory of Brazil-JEL649 at both temperatures compared to all other *Batrachochytrium* genotypes.

Two bacterial strains, *Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp., were inhibitory of all *Batrachochytrium* genotypes at both temperatures. Inhibition scores for *Pseudomonas* sp. strain 2 differed among genotypes ($X^2 = 219.2$, df $= 5$, $p < 0.001$), temperatures ($X^2 = 10.4$, df $= 1$, $p = 0.001$) and showed a significant interaction ($X^2 = 101.1$, df $= 5$, $p < 0.001$). *Pseudomonas* sp. strain 2 moderately to strongly inhibited all *Batrachochytrium* genotypes at both temperatures ($\mu = 57$–98%). Inhibition scores for *Stenotrophomonas* sp. differed among *Batrachochytrium* genotypes ($X^2 = 40.6$, df $= 5$, $p < 0.001$), temperatures ($X^2 = 5.5$, df $= 1$, $p = 0.02$) and showed a significant interaction ($X^2 = 31.9$, df $= 5$, $p < 0.001$). *Stenotrophomonas* sp. strongly inhibited all *Batrachochytrium* genotypes at both temperatures ($\mu = 70$–100%), but was less inhibitory of GPL1-JEL647 and Brazil-JEL649 at 12°C than all other genotypes, except for GPL2-JEL423 (**Figure 1D** and Supplementary Table S3). Similar to *Pseudomonas* sp. strain 2, *Stenotrophomonas* sp. was less inhibitory of GPL1-JEL647 and Brazil-JEL649 at 12°C than all other *Batrachochytrium* genotypes (**Figure 1E** and Supplementary Table S3).

Bacterial inhibition strength may be predictable based on *Batrachochytrium* phylogenetic relatedness. Both clustering methods supported the same two clusters within the data, with *B. dendrobatidis*-Brazil and *B. salamandrivorans* clustering together and the *B. dendrobatidis*-GPL genotypes forming a separate cluster (**Figure 2**).

DISCUSSION

Our findings suggest that many bacteria identified as *Batrachochytrium*-inhibitory *in vitro* are unlikely to be consistently effective in mitigation of chytridiomycosis *in vivo*. We found that all bacterial strains consistently inhibited *B. salamandrivorans* growth across temperatures (12 and 18°C), but that three of the five bacterial strains did not consistently inhibit the five *B. dendrobatidis* genotypes. Similarly, Antwis et al. (2015) found that 3 of 11 previously identified *B. dendrobatidis*-inhibitory bacterial strains did not consistently inhibit three *B. dendrobatidis* isolates at 18°C. These results highlight the importance of considering *Batrachochytrium* genotype and bacterial strain in host–pathogen interactions, and may explain the variation in effectiveness of probiotics in experimental trials (e.g., Harris et al., 2009a; Woodhams et al., 2012; Becker et al., 2015a).

Our results also highlight the importance of considering environmental context in host–pathogen interactions. The effect of temperature on inhibition was not unidirectional, but depended on the context of the interacting *Batrachochytrium* genotype and bacterial strain. For example, *Pseudomonas* sp. strain 1 was less inhibitory of GPL1-JEL647 at 12°C compared to 18°C, whereas *Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp. were more inhibitory of GPL1-JEL647 at 12°C compared to 18°C. The differences in inhibition between temperatures of the same *Batrachochytrium* genotype likely related to temperature-dependent changes in bacterial extracellular factor activity (Daskin et al., 2014) and/or *Batrachochytrium* physiology (Woodhams et al., 2008). Bacterially produced extracellular factors include bacteriocins, siderophores, organic acids, lysozymes, proteases, and secondary metabolites. However, the most likely factors contributing to inhibition are secondary metabolites (reviewed by Verschuere et al., 2000; Raaijmakers et al., 2002), and antimicrobial activity of metabolites can vary among temperatures (Humair et al., 2009; Aguilar and Klotz, 2010). In addition, *Batrachochytrium* isolates vary in phenotypic traits (e.g., growth rate and zoosporangium size) depending on temperature (Piotrowski et al., 2004; Martel et al., 2013;

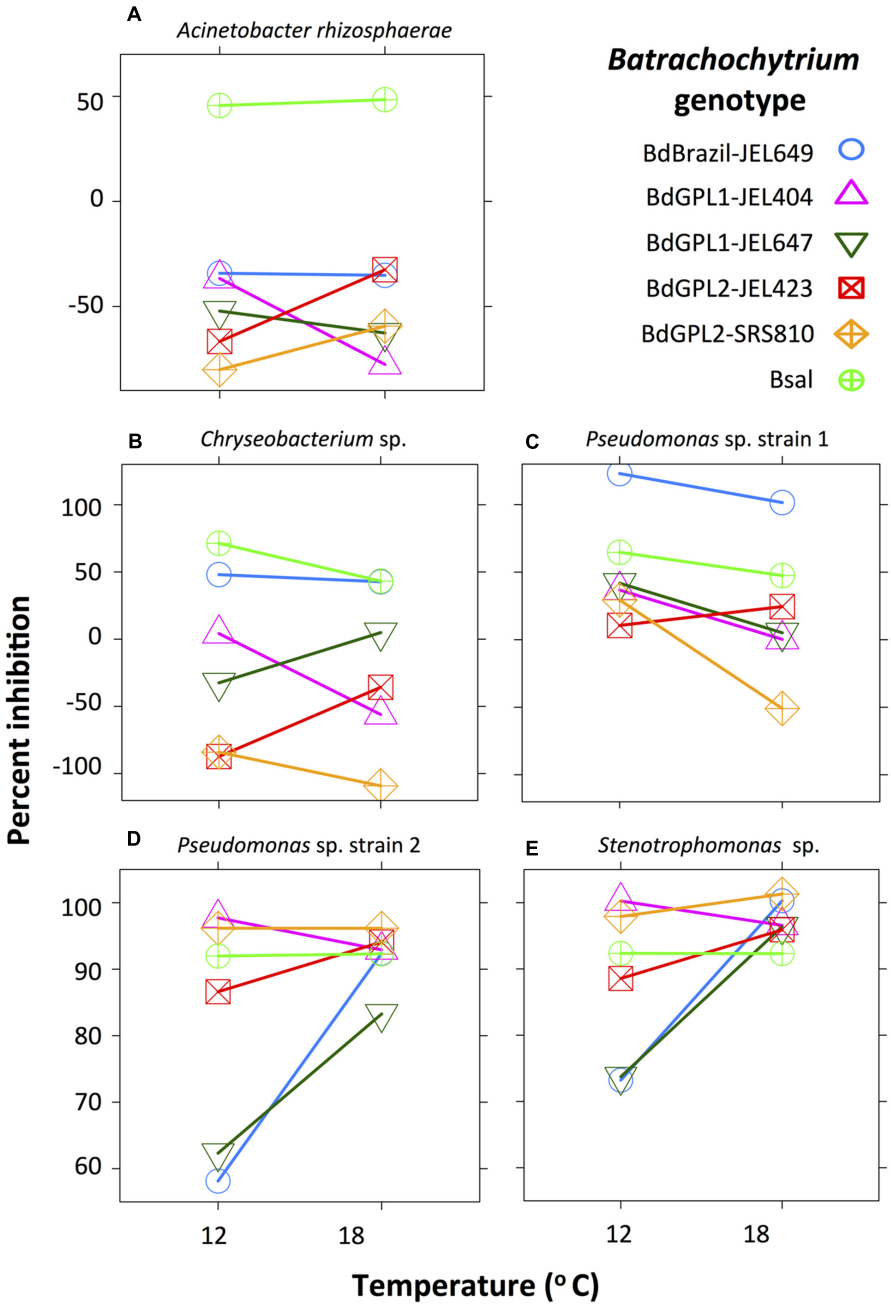

FIGURE 1 | Interaction plot showing the effect of *Batrachochytrium* genotype and temperature on bacterial inhibition score (percent inhibition: note different scale on y-axis). *Acinetobacter rhizosphaerae* **(A)** was moderately inhibitory of *B. salamandrivorans* at both temperatures, but did not inhibit any *B. dendrobatidis* genotypes. *Chryseobacterium* sp. **(B)** and *Pseudomonas* sp. strain 1 **(C)** were weakly to strongly inhibitory of most *Batrachochytrium* genotypes. *Pseudomonas* sp. strain 2 **(D)** and *Stenotrophomonas* sp. **(E)** were moderately to strongly inhibitory of all *Batrachochytrium* genotypes at both temperatures, and showed similar inhibition patterns. Percent inhibition is based on the parameter estimates from the linear-mixed effects models.

Stevenson et al., 2013) and this may impact their susceptibility to inhibitory compounds. Investigation into the inhibition strength of *Batrachochytrium*-inhibitory bacterial metabolites (e.g., violacein) at different temperatures would provide insight into whether bacterial factors or *Batrachochytrium* physiology underpin the temperature-dependent differences in inhibition.

Our study supports the hypothesis that similarity of fungal genomes can predict strength of bacterial inhibition. Our results showed separation between two clusters: the two highly

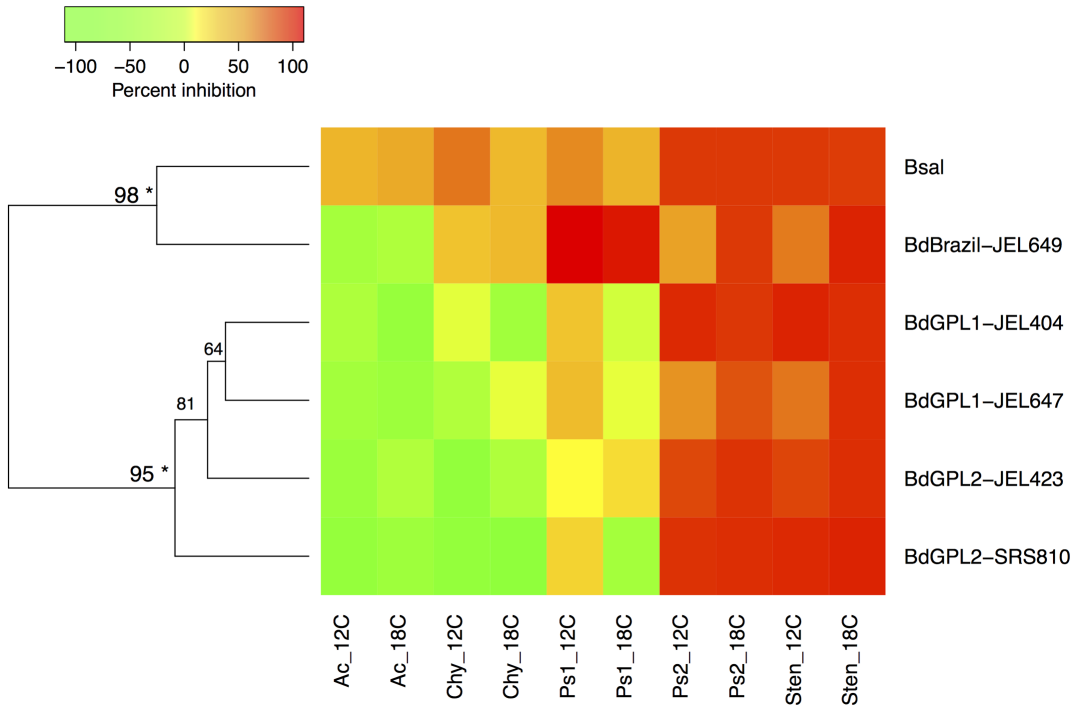

FIGURE 2 | Heatmap displaying Ward's hierarchical clustering of *Batrachochytrium* genotypes based on mean bacterial inhibition scores at each temperature. Approximately unbiased *p*-values are indicated for each cluster, with values larger than 95% being strongly supported by the data (*). Two clusters were supported by the data, one including *B. dendrobatidis*-Brazil and *B. salamandrivorans*, and one clustering all *B. dendrobatidis*-GPL genotypes. Ac = *Acinetobacter rhizosphaerae,* Chy = *Chryseobacterium* sp., Ps1 = *Pseudomonas* sp. strain 1, Ps2 = *Pseudomonas* sp. strain 2, Sten = *Stenotrophomonas* sp.

divergent lineages (*B. salamandrivorans* and *B. dendrobatidis*-Brazil) and the GPL genotypes. Generally, *B. salamandrivorans* and *B. dendrobatidis*-Brazil were more likely to be inhibited than the GPL genotypes. The GPL genotypes have increased chromosomal copy numbers (CCNs) and loss of heterozygosity compared to the endemic *B. dendrobatidis* lineages, and these measures are associated with increased virulence (Schloegel et al., 2012; Farrer et al., 2013; Rosenblum et al., 2013; James et al., 2015; Becker et al., 2017). This association may relate to resistance to inhibitory compounds released by host or symbionts. For instance, Farrer et al. (2013) found that *B. dendrobatidis* CCN increased following exposure to a host-produced antimicrobial peptide, which then resulted in reduced inhibition. A greater sampling across *Batrachochytrium* genotypes would be a useful next step to confirm this lineage-based similarity in pathogen response to bacterial inhibition.

While there was a relationship between *Batrachochytrium* relatedness and inhibition patterns, we did not detect a relationship between bacterial relatedness and inhibition patterns. For instance, we found variation within the two *Pseudomonas* sp. strains tested, which were defined as the same OTU (16S rRNA sequence similarity = 99%). *Pseudomonas* sp. strain 1 was generally less inhibitory compared to *Pseudomonas* sp. strain 2, and also non-inhibitory of two pathogen genotypes at 18°C. These findings are similar to other studies that profiled larger numbers of bacterial strains in a phylogenetic framework and found that inhibition strength was not correlated to bacterial phylogeny (Becker et al., 2015b; Muletz-Wolz et al., 2017a). While the majority of genomic information can be highly similar within a bacterial OTU, gene clusters associated with inhibitory metabolite production can differ among strains (Chen et al., 2015), potentially explaining the discrepancy in inhibition strength within an OTU. On the other hand, different OTUs can show similar inhibition patterns as homologous recombination and horizontal gene transfer of genes encoding antifungal compounds can occur between bacterial lineages with levels of DNA divergence as high as 25% (Kinashi et al., 1987; Ravel et al., 2000; Cohan, 2001). For example, we found a strong similarity in the inhibition patterns of *Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp. (16S rRNA sequence similarity = 75%). Documented modes of antibiosis by *Pseudomonas* differ from those of *Stenotrophomonas* spp. (Raaijmakers et al., 2002; Compant et al., 2005); whole genome sequencing or chemical analyses of these bacterial strains may identify the specific agent(s) underlying this similarity in *Batrachochytrium* inhibition.

For targeted conservation applications, we suggest testing bacterial strains *in vitro* against multiple *Batrachochytrium* genotypes across a range of temperatures to identify probiotics that would be most effective at mitigating

Batrachochytrium infection *in situ*. If probiotic-based conservation strategies are needed rapidly, previously identified *Batrachochytrium*-inhibitory bacterial strains that are strongly inhibitory are the most likely to be effective. For instance, we found that two bacterial strains (*Pseudomonas* sp. strain 2 and *Stenotrophomonas* sp.) were strongly inhibitory of all *Batrachochytrium* genotype tested across temperatures. These bacterial strains are good probiotic candidates as they likely produce antifungal compounds at a range of temperatures that inhibit a range of pathogen genotypes (Bletz et al., 2013).

CONCLUSION

We quantified the inhibition of diverse *Batrachochytrium* genotypes by five bacterial strains that were isolated from woodland salamanders in the eastern United States and previously shown to inhibit an eastern US *B. dendrobatidis* genotype, GPL1-JEL404 (Muletz-Wolz et al., 2017a). Inhibition capabilities of the symbiotic bacteria changed as a function of bacterial strain, *Batrachochytrium* genotype and temperature. This has practical implications for understanding host–pathogen dynamics and developing conservation measures. Amphibians and their microbiomes will continue to be exposed to rapidly evolving *Batrachochytrium* genotypes, and hosts harboring higher numbers of microbial taxa and hence more potentially inhibitory species may provide greater resistance to pathogen invasion than microbiome communities with fewer taxa (Chang et al., 2008; Ling et al., 2015; Lokmer and Wegner, 2015; Longo et al., 2015). Our findings should also be considered in other systems, such as white-nose syndrome in bats and pathogens in agriculture, where probiotic application is used as a disease management strategy (Verschuere et al., 2000; Raaijmakers et al., 2002; Teplitski and Ritchie, 2009; Hoyt et al., 2015; Xue et al., 2015).

DATA ACCESSIBILITY

All experimental data and statistical analyses (R code) will be deposited upon acceptance in figshare (doi: 10.6084/m9.figshare.5297416).

AUTHOR CONTRIBUTIONS

CM-W, GD, KZ, LT, and KL designed the research. AM, FP, KZ, and LT provided the *Batrachochytrium* isolates. CM-W, JA, and SB conducted the lab work. CM-W and JA analyzed the data with advice from SB and GD. All authors contributed to the interpretation of the data. CM-W wrote the manuscript and all authors provided critical feedback. All authors approved the manuscript's content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01551/full#supplementary-material

REFERENCES

Aguilar, C., and Klotz, B. (2010). Effect of the temperature on the antagonistic activity of lactic acid bacteria against *Escherichia coli* and *Listeria monocytogenes*. *J. Food Saf.* 30, 996–1015. doi: 10.1111/j.1745-4565.2010.00257.x

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/Aem.00010-15

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–47. doi: 10.18637/jss.v067.i01

Becker, C. G., Greenspan, S. E., Tracy, K. E., Dash, J. A., Lambertini, C., Jenkinson, T. S., et al. (2017). Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. *Fungal Ecol.* 26, 45–50.

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE* 5:e10957. doi: 10.1371/journal.pone.0010957

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015a). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. Biol. Sci.* 282:20142881. doi: 10.1098/rspb.2014.2881

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015b). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.* 103, 77–85. doi: 10.3354/dao02560

Bérdy J. (2005). Bioactive microbial metabolites - a personal view. *J. Antibiot.* 58, 1–26. doi: 10.1038/ja.2005.1

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.903

Berger, L., Speare, R., Hines, H. B., Marantelli, G., Hyatt, A. D., McDonald, K. R., et al. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.* 82, 434–439. doi: 10.1111/j.1751-0813.2004.tb11137.x

Bletz, M. (2013). *Probiotic Bioaugmentation of an Anti-Bd Bacteria, Janthinobacterium lividum, on the Amphibian, Notophthalmus viridescens, Transmission Efficacy and Persistence of the Probiotic on the Host and Non-Target Effects of Probiotic Addition on Ecosystem Components.* Master's thesis, James Madison University, Harrisonburg, VA.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation, characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bresciano, J. C., Salvador, C. A., Paz-y-Mino, C., Parody-Merino, A. M., Bosch, J., and Woodhams, D. C. (2015). Variation in the presence of anti-*Batrachochytrium dendrobatidis* bacteria of amphibians across life stages and elevations in Ecuador. *Ecohealth* 12, 310–319. doi: 10.1007/s10393-015-1010-y

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008b). Amphibian chemical defense, Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Carvalho, T., Becker, C. G., and Toledo, L. F. (2017). Historical amphibian declines and extinctions in Brazil linked to chytridiomycosis. *Proc. Biol. Sci.* 284:20162254. doi: 10.1098/rspb.2016.2254

Chang, J. Y., Antonopoulos, D. A., Kalra, A., Tonelli, A., Khalife, W. T., Schmidt, T. M., et al. (2008). Decreased diversity of the fecal microbiome in recurrent Clostridium difficile-associated diarrhea. *J. Infect. Dis.* 197, 435–438. doi: 10.1086/525047

Chen, Y. W., Shen, X. M., Peng, H. S., Hu, H. B., Wang, W., and Zhang, X. H. (2015). Comparative genomic analysis and phenazine production of *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium. *Genomics Data* 4, 33–42. doi: 10.1016/j.gdata.2015.01.006

Cohan F. M. (2001). Bacterial species and speciation. *Syst. Biol.* 50, 513–524. doi: 10.1080/10635150118398

Compant, S., Duffy, B., Nowak, J., Clement, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases, Principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/aem.71.9.4951-4959.2005

Daskin, J. H., Bell, S. C., Schwarzkopf, L., and Alford, R. A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians - implications for disease management and patterns of decline. *PLoS ONE* 9:e100378. doi: 10.1371/journal.pone.0100378

Duffy, B. K., and Defago, G. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.* 65, 2429–2438.

Everitt B.S. and Hothorn T. (2009). *A Handbook of Statistical Analyses Using R*, 2nd Edn. Boca Raton, Fl: CRC Press.

Farrer, R. A., Henk, D. A., Garner, T. W. J., Balloux, F., Woodhams, D. C., and Fisher, M. C. (2013). Chromosomal copy number variation, selection and uneven rates of recombination reveal cryptic genome diversity linked to pathogenicity. *PLoS Genet.* 9:e1003703. doi: 10.1371/journal.pgen.1003703

Farrer, R. A., Martel, A., Verbrugghe, E., Abouelleil, A., Ducatelle, R., Longcore, J. E., et al. (2017). Genomic innovations linked to infection strategies across emerging pathogenic chytrid fungi. *Nat. Commun.* 8:14742. doi: 10.1038/ncomms14742

Farrer, R. A., Weinert, L. A., Bielby, J., Garner, T. W. J., Balloux, F., Clare, F., et al. (2011). Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18732–18736. doi: 10.1073/pnas.1111915108

Fisher, M. C., Garner, T. W. J., and Walker, S. F. (2009). Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annu. Rev. Microbiol.* 63, 291–310. doi: 10.1146/annurev.micro.091208.073435

Fox J., and Weisberg S. (2011). *An R Companion to Applied Regression*, Second Edn. Thousand Oaks, CA: Sage.

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *Isme J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., Frick, W. F., and Kilpatrick, A. M. (2015). Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of White-Nose Syndrome. *PLoS ONE* 10:e0121329. doi: 10.1371/journal.pone.0121329

Humair, B., Gonzalez, N., Mossialos, D., Reimmann, C., and Haas, D. (2009). Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* Cha0. *Isme J.* 3, 955–965. doi: 10.1038/ismej.2009.42

James, T. Y., Toledo, L. F., Rodder, D., Leite, D. D., Belasen, A. M., Betancourt-Roman, C. M., et al. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease, lessons from the first 15years of amphibian chytridiomycosis research. *Ecol. Evol.* 5, 4079–4097. doi: 10.1002/ece3.1672

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jenkinson, T. S., Betancourt-Román, C. M., Lambertini, C., Valencia-Aguilar, A., Rodriguez, D., Nunes-de-Almeida, C. H. L., et al. (2016). Amphibian-killing chytrid in Brazil comprises both locally endemic and globally expanding populations. *Mol. Ecol.* 25, 2978–2996. doi: 10.1111/mec.13599

Kinashi H., Shimaji M., and Sakai A. (1987). Giant linear plasmids in Streptomyces which code for antibiotic biosysthesis genes. *Nature* 328, 454–456. doi: 10.1038/328454a0

Kriger, K. M., and Hero, J. -M. (2008). Altitudinal distribution of chytrid (*Batrachochytrium dendrobatidis*) infection in subtropical Australian frogs. *Austral Ecol.* 33, 1022–1032. doi: 10.1111/j.1442-9993.2008.01872.x

Lam, B. A., Walton, D. B., and Harris, R. N. (2011). Motile zoospores of *Batrachochytrium dendrobatidis* move away from antifungal metabolites produced by amphibian skin bacteria. *Ecohealth* 8, 36–45. doi: 10.1007/s10393-011-0689-7

Lenth, R. (2016). *lsmeans, Least-Squares Means. R Package Version* 2.20–23. Available at: http://CRAN.R-project.org/package=lsmeans

Ling, Z., Liu, X., Cheng, Y., Jiang, X., Jiang, H., Wang, Y., et al. (2015). Decreased diversity of the oral microbiota of patients with Hepatitis B virus-induced chronic liver disease, a pilot project. *Sci. Rep.* 5:17098. doi: 10.1038/srep17098

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Lips, K. R., Diffendorfer, J., Mendelson, J. R. III, and Sears, M. W. (2008). Riding the wave: reconciling the roles of disease and climate change in amphibian declines. *PLoS Biol.* 6:e72. doi: 10.1371/journal.pbio.0060072

Lokmer, A., and Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J.* 9, 670–682. doi: 10.1038/ismej.2014.160

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Longo, A. V., and Zamudio, K. R. (2017). Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. *ISME J.* 11, 349–361. doi: 10.1038/ismej.2016.138

Madison, J. D., Berg, E. A., Abarca, J. G., Whitfield, S. M., Gorbatenko, O., Pinto, A., et al. (2017). Characterization of *Batrachochytrium dendrobatidis* inhibiting

bacteria from amphibian populations in Costa Rica. *Front. Microbiol.* 8:290. doi: 10.3389/fmicb.2017.00290

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., et al. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* 346, 630–631. doi: 10.1073/pnas.1307356110

Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M. C., et al. (2013). *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15325–15329. doi: 10.1126/science.1258268

Muletz, C. R., Myers, J. M., Domangue, R. J., Herrick, J. B., and Harris, R. N. (2012). Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 152, 119–126. doi: 10.1016/j.biocon.2012.03.002

Muletz-Wolz, C. R., Direnzo, G. V., Yarwood S. A., Campbell Grant E. H., Fleischer R. C., and Lips, K. R. (2017a). Antifungal bacteria on woodland salamander skin exhibit high taxonomic diversity and geographic variability. *Appl. Environ. Microbiol.* 83:e00186–17. doi: 10.1128/AEM.00186-17

Muletz-Wolz, C. R., Yarwood S. A., Campbell Grant E. H., Fleischer R. C., and Lips, K. R., (2017b). Effects of host species and environment on the skin microbiome of Plethodontid salamanders. *J. Anim. Ecol.* doi: 10.1111/1365-2656.12726 [Epub ahead of print].

Nebergall, E. E. (2013). *Ecology and Applications of Cutaneous Mechanisms of Resistance to Amphibian Chytridiomycosis.* Master's thesis, Oregon State University, Corvallis, OR.

Piotrowski, J. S., Annis, S. L., and Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96, 9–15. doi: 10.2307/3761981

R Core Team (2016). *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing. Available at: https://www.R-project.org/

Raaijmakers, J. M., Vlami, M., and de Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* 81, 537–547. doi: 10.1023/a:1020501420831

Ravel J., Wellington E. M. H., and Hill R. T. (2000). Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. *Appl. Environ. Microbiol.* 66, 529–534. doi: 10.1128/AEM.66.2.529-534.2000

Ribas, L., Li, M. -S., Doddington, B. J., Robert, J., Seidel, J. A., Kroll, J. S., et al. (2009). Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE* 4:e8408. doi: 10.1371/journal.pone.0008408

Rosenblum, E. B., James, T. Y., Zamudio, K. R., Poorten, T. J., Ilut, D., Rodriguez, D., et al. (2013). Complex history of the amphibian-killing chytrid fungus revealed with genome resequencing data. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9385–9390. doi: 10.1073/pnas.1300130110

Rovenich, H., Boshoven, J. C., and Thomma, B. (2014). Filamentous pathogen effector functions, of pathogens, hosts and microbiomes. *Curr. Opin. Plant Biol.* 20, 96–103. doi: 10.1016/j.pbi.2014.05.001

Schloegel, L. M., Toledo, L. F., Longcore, J. E., Greenspan, S. E., Vieira, C. A., Lee, M., et al. (2012). Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. *Mol. Ecol.* 21, 5162–5177. doi: 10.1111/j.1365-294X.2012.05710.x

Spitzen-van der Sluijs, A., Martel, A., Asselberghs, J., Bales, E. K., Beukema, W., Bletz, M. C., et al. (2016). Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerg. Infect. Dis.* 22, 1286–1288. doi: 10.3201/eid2207.160109

Stegen, G., Pasmas, F., Schmidt, B. R., Rouffaer, L. O., Van Praet, S., and Schaub, M. et al. (2017). Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans*. *Nature* 544, 353–356. doi: 10.1038/nature22059

Stevenson, L. A., Alford, R. A., Bell, S. C., Roznik, E. A., Berger, L., and Pike, D. A. (2013). Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS ONE* 8:e73830. doi: 10.1371/journal.pone.0073830

Suzuki, R., and Shimodaira, H. (2006). Pvclust, an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 22, 1540–1542. doi: 10.1093/bioinformatics/btl117

Teplitski, M., and Ritchie, K. (2009). How feasible is the biological control of coral diseases? *Trends Ecol. Evol.* 24, 378–385. doi: 10.1016/j.tree.2009.02.008

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671. doi: 10.1128/mmbr.64.4.655-671.2000

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595–595. doi: 10.1890/14-1837.1

Woodhams, D. C., Alford, R. A., Briggs, C. J., Johnson, M., and Rollins-Smith, L. A. (2008). Life-history trade-offs influence disease in changing climates, Strategies of an amphibian pathogen. *Ecology* 89, 1627–1639. doi: 10.1890/06-1842.1

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Kuepfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Geiger, C. C., Reinert, L. K., Rollins-Smith, L. A., Lam, B., Harris, R. N., et al. (2012). Treatment of amphibians infected with chytrid fungus, learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Dis. Aquat. Organ.* 98, 11–25. doi: 10.3354/dao02429

Xue, C., Penton, C. R., Shen, Z. Z., Zhang, R. F., Huang, Q. W., Li, R., et al. (2015). Manipulating the banana rhizosphere microbiome for biological control of Panama disease. *Sci. Rep.* 5 14596. doi: 10.1038/srep11124

Yasumiba, K., Bell, S., and Alford, R. (2016). Cell density effects of frog skin bacteria on their capacity to inhibit growth of the chytrid fungus, *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 71, 124–130. doi: 10.1007/s00248-015-0701-9

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Culture Media and Individual Hosts Affect the Recovery of Culturable Bacterial Diversity from Amphibian Skin

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One current challenge in microbial ecology is elucidating the functional roles of the large diversity of free-living and host-associated bacteria identified by culture-independent molecular methods. Importantly, the characterization of this immense bacterial diversity will likely require merging data from culture-independent approaches with work on bacterial isolates in culture. Amphibian skin bacterial communities have become a recent focus of work in host-associated microbial systems due to the potential role of these skin bacteria in host defense against the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd), which is associated with global amphibian population declines and extinctions. As there is evidence that some skin bacteria may inhibit growth of Bd and prevent infection in some cases, there is interest in using these bacteria as probiotic therapy for conservation of at-risk amphibians. In this study, we used skin swabs from American toads (*Anaxyrus americanus*) to: (1) assess the diversity and community structure of culturable amphibian skin bacteria grown on high and low nutrient culture media, (2) determine which culture media recover the highest proportion of the total skin bacterial community of individual toads relative to culture-independent data, and (3) assess whether the plated communities from the distinct media types vary in their ability to inhibit Bd growth in *in-vitro* assays. Overall, we found that culture media with low nutrient concentrations facilitated the growth of more diverse bacterial taxa and grew distinct communities relative to media with higher nutrient concentrations. Use of low nutrient media also resulted in culturing proportionally more of the bacterial diversity on individual toads relative to the overall community defined using culture-independent methods. However, while there were differences in diversity among media types, the variation among individual hosts was greater than variation among media types, suggesting that swabbing more individuals in a population is the best way to maximize culture collections, regardless of media type. Lastly, the function of the plated communities against Bd did not vary across culture media type or between high and low nutrient media. These results inform current efforts for developing a probiotic-based approach for amphibian conservation and help to ensure that culture collections are capturing the majority of the important diversity in these systems.

Keywords: amphibian, amphibian skin bacteria, *Batrachochytrium dendrobatidis*, low nutrient culture media, microbiome, R2A

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INTRODUCTION

Microbial ecologists currently face the challenge of characterizing the ecological role of the vast bacterial diversity associated with different environments and hosts, identified by culture-independent methods, such as 16S rRNA gene amplicon sequencing (Zengler et al., 2002; Vartoukian et al., 2010). A complete understanding of the metabolic, pathogenic and functional features of this diversity will be enhanced by the use of "-omics" methods, such as shotgun metagenomics, the cultivation and isolation of the bacterial species (Zengler et al., 2002) and by linking culture-independent and culture-dependent approaches (Rebollar et al., 2016a). Importantly, culturable bacteria have provided a good system for assessing ecological processes (Jessup et al., 2005). For instance, bacterial systems have been used to assess the generality of some key factors known to influence and determine diversity patterns in plants and animals, such as habitat type, habitat heterogeneity, disturbance and primary productivity (reviewed in Horner-Devine et al., 2004). In addition, expanding our understanding about the natural history of bacteria, which are essential in critical natural processes, such as nutrient cycling, requires at some level the use of cultured isolates (Jessup et al., 2004). Lastly, the cultivation of bacteria can play a key role in the development of probiotics to mitigate pathogen infection in an array of species (Bletz et al., 2013; Walke and Belden, 2016). While "-omics" approaches can assist with identification of potential probiotics, implementation will still require the cultivation of bacteria for biological assays and probiotic application (e.g., Rebollar et al., 2016a).

Bacterial communities associated with amphibian skin have received attention due to the potential defensive role they play against the amphibian fungal skin pathogen *Batrachochytrium dendrobatidis* (Bd) (Bletz et al., 2013; Walke and Belden, 2016). The potential defensive role of the amphibian skin bacteria is important from an ecological and conservation standpoint given that chytridiomycosis, the disease caused by Bd, has been associated with amphibian extinctions and population declines around the world (Berger et al., 1998; Bosch and Martínez-Solano, 2006; Lips et al., 2006; Crawford et al., 2010; Vredenburg et al., 2010), and has caused a substantial disease-related loss of biodiversity (Fisher et al., 2012; Lips, 2016). Amphibian skin bacterial communities might also serve as a microbial system to address fundamental ecological questions, in particular those focused on the processes influencing diversity, community assembly, and function. For example, amphibian skin bacterial communities have been used to determine the roles of neutral (e.g., dispersal and ecological drift) and deterministic processes (e.g., habitat filtering and competition) in shaping bacterial communities (Loudon et al., 2016), to assess the influence and feedbacks caused by an invasive species, such as a host skin pathogen (Jani and Briggs, 2014), to examine the relationship between community structure and function (Becker et al., 2015; Belden et al., 2015; Walke et al., 2015b), and to elucidate the factors potentially driving context-dependent function (Daskin et al., 2014; Loudon et al., 2014; Woodhams et al., 2014).

The use of culture-dependent techniques has played an important role in the study of bacteria associated with amphibian skin, mainly within a conservation context to assist in the development of a probiotic-based conservation method. Culture-dependent techniques have facilitated the development of protocols to identify skin bacterial isolates that inhibit Bd growth *in vitro* (e.g., Harris et al., 2006; Lauer et al., 2007, 2008; Flechas et al., 2012; Bell et al., 2013; Becker et al., 2015), and also to identify some of the ecological factors (e.g., interspecific competition and temperature) affecting the production of bacterially-produced secondary metabolites (Daskin et al., 2014; Loudon et al., 2014).

In ecology, studies manipulating nutrient concentration and composition have advanced understanding of the relationship between primary productivity and diversity in terrestrial plant communities (Goldberg and Miller, 1990; Wilson and Tilman, 1991), aquatic communities (Schindler, 1990) and microbial systems (Kassen et al., 2000). Within this context, when cultivating bacteria, the composition and diversity of the cultured community is limited by factors, such as incubation time and culture media nutrient concentration and composition (Stevenson et al., 2004). Thus, nutrient composition and availability can influence bacterial communities and enhance the cultivation of previously uncultured bacteria (Vartoukian et al., 2010). For instance, nutrient-rich culture media (i.e., complex media) favors the growth of fast-growing bacteria over slower-growing bacteria (Connon and Giovannoni, 2002; Vartoukian et al., 2010), and the dilution of nutrients in culture media has been used to cultivate previously uncultured bacteria (Connon and Giovannoni, 2002; Zengler et al., 2002; Vartoukian et al., 2010).

In this study, we aimed, from an ecological perspective, to assess how high and low nutrient culture media influence the diversity of cultured amphibian skin bacteria that are recovered. In addition, and within an applied context, we also assessed the ability of different culture media types to grow a high portion (i.e., number of OTUs) of amphibian skin bacteria, and determined what culture media can recover the most representative fraction of the bacterial community relative to a culture-independent method. Lastly, given the ability of some bacterial isolates to inhibit Bd growth *in vitro*, particularly when grown with other bacteria (Loudon et al., 2014), and their potential as a conservation approach to mitigate Bd infection, we aimed to determine whether the bacterial communities growing on the different media types differed in their ability to inhibit Bd growth.

MATERIALS AND METHODS

Sample Collection

We analyzed bacterial communities from skin swabs from 12 American toads (*Anaxyrus americanus)* collected in Jefferson National Forest near Blacksburg, VA (USA). In the field, toad skin bacterial communities were sampled as described by Walke et al. (2015a). Briefly, each individual toad was handled with a new pair of nitrile gloves, rinsed twice with sterile water to remove transient microbes (Walke et al., 2014; Belden et al., 2015), and swabbed sequentially with two sterile rayon swabs (MW113; Medical Wire & Equipment). The swabbing technique

was standardized and consisted of 20 strokes on the ventral side of the toad and 5 strokes along each thigh and foot. The first swab was placed in a 1.5 ml sterile microcentrifuge tube and was used to characterize the skin bacterial communities via culture-independent 16S rRNA gene amplicon sequencing. The second swab was stored in another 1.5 ml sterile microcentrifuge tube containing 100 µl of TSYE-glycerol medium (2% Trypticase soy broth, 1% yeast extract, 20% glycerol) and was used for the characterization of bacterial isolate communities grown on four different types of culture media. These cultured communities were also subsequently characterized via 16S rRNA gene amplicon sequencing and were tested for whole community *in vitro* inhibition of Bd growth. Both field-collected swabs were stored at −80C until processing. All animal use was approved by the Institutional Animal Care and Use Committees of Virginia Tech.

Culture-Independent Assessment of the Skin Bacterial Communities

DNA was extracted from the first swab using the Qiagen DNeasy blood and tissue kit (Valencia, CA) protocol for Gram positive bacteria, with an initial incubation step of 1 h at 37°C. For the community characterization, the V4 region of the 16S rRNA gene was amplified using the primers 515F and the barcoded 806R and sequenced using a 250 bp paired-end strategy on the Illumina MiSeq platform as described by Walke et al. (2015a). The culture-independent data produced and used in the present study was part of Walke et al. (2015a), and is available in NCBI's Sequence Read Archive (SRA) under the accession number SRP062395.

Bacterial Community Comparison among Culture Media

We plated the second swab onto four different media types to compare the bacterial isolate richness (alpha-diversity) and community structure (beta-diversity). The culture media types comprised two with a high concentration of nutrients, LB (Luria-Bertani, Fisher Scientific) and TSA (Tryptic soy agar, Remel), and two with a low concentration of nutrients, R2A (Reasoner's 2A, Difco, Becton, Dickinson and Company) and dR2A (1/10 dilution of R2A with an addition of granulated agar so the amount of agar was the same as in the undiluted R2A). We chose commonly used media for our study. For example, LB is often used to culture and maintain *Escherichia coli*, TSA is often used as a non-selective media for general purposes, and R2A, which was initially developed to culture bacteria from potable water (Reasoner and Geldreich, 1985), is the most commonly used media for culturing bacteria from amphibian skin (Harris et al., 2006; Flechas et al., 2012; Antwis et al., 2015; Walke et al., 2015a). For this study, we tested each of the four media types with an aliquot of the TSYE-glycerol solution from each of the 12 toads, resulting in 48 culture plates. We inoculated 30 µl of a briefly vortexed 1:10 dilution of the TSYE-glycerol solutions from the freezer stock of the culture swab onto each media type, followed by spreading the solution across the plate. In addition, a non-inoculated plate for each media type was used as a control to assess the potential for contamination of the media. Following inoculation, all plates were incubated at room temperature in the lab (∼24°C).

Collection of Bacterial Cultures from Plates

After 6 days of incubation, when bacterial colonies started to cover the plates in the high nutrient concentration media, we sampled the entire bacterial community on each plate by applying a slightly modified version of the plate wash PCR procedure (PWPCR) developed by Stevenson et al. (2004). Culture plates were flooded with 3 ml of 1% tryptone broth, and a sterile spreader was used to suspend all visible bacterial colonies. Then the tryptone broth with the suspended bacteria was collected and transferred to a 2 ml sterile collecting tube and centrifuged at 10,000 rpm for 5 min. The resulting supernatant potentially containing bacterially-produced secondary metabolites was filtered through a 0.22 µm filter, and the cell-free supernatant (CFS) was used to conduct *in vitro* challenge assays to determine whether there was community-level variation in the ability to inhibit Bd growth among media types. After removing the supernatant for the challenge assays, we added 2 ml of MicroBead solution (MoBio Laboratories, from Ultraclean Microbial Isolate kit) to each tube of pelleted bacteria and then vortexed to homogenize the solution. These were stored at −20°C prior to DNA extraction. DNA from these cultured bacterial communities was extracted by adding 50 µl of lysozyme solution (20 mg/ml) to each tube followed by an incubation at 56°C for 45 min (Stevenson et al., 2004). After incubation, DNA extractions were performed following the manufacturer's protocol of the UltraClean microbial DNA isolation kit (MoBio Laboratories), which yielded a volume of 100 µl of template DNA.

Characterization of the Cultured Bacterial Communities

Following DNA extraction, assessment of the cultured communities was done as for the initial culture-independent swab, following methods of Walke et al. (2015a), with the exception that we used 2 µl of DNA template in the PCR. Out of the 48 experimental samples, five (two from the TSA culture media and one from each of the other culture media) were removed from the dataset because they would not amplify. In addition, out of the four control plates, only two could be included because most contained too little DNA for sequencing (as was expected), and even the two that were included had very little DNA. From each of the 45 remaining samples, 200 ng of PCR product was pooled to make a composite sample, which was then cleaned with the QIAquick PCR Purification Kit (Qiagen, Valencia, California). The final pooled sample was sent for sequencing on an Illumina Mi-Seq instrument at the Dana-Farber Cancer Institute of Harvard University following Caporaso et al. (2012) using a 250 bp single-end strategy.

Raw forward 16S rRNA amplicon sequences were demultiplexed and quality-filtered using the default parameters of the Quantitative Insight into Microbial Ecology (QIIME)

pipeline (Caporaso et al., 2010b), with a few exceptions: we allowed for no errors in the barcodes, increased the number of minimum consecutive low-quality base calls allowed before truncating a read (r) to 10, and decreased the fraction of the minimum number of consecutive high-quality base calls to include a read (p) to 0.5. Sequences matching PhiX, added to increase base diversity in Illumina sequencing runs, were removed from the dataset using Geneious (Biomatters, Ltd, version 8.1.8). For remaining sequences, a 97% similarity threshold was used to cluster sequences into operational taxonomic units (OTUs, ∼bacterial species) using the UCLUST method (Edgar, 2010). Each OTU was represented by the most abundant sequence clustered within it, which was aligned to the Greengenes 13_8 reference database (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a) and assigned taxonomy using the RDP classifier (Wang et al., 2007). OTUs assigned to chloroplast or mitochondria were removed, and then OTUs with fewer than 0.01% (524 sequences) of the total reads were removed (Bokulich et al., 2012; Hughey et al., 2016). The sequencing depth per sample ranged from 3,991 to 164,519, including the two controls, which, as expected, had the lowest read counts at 3,991 and 8,299 reads. We removed the controls from the set of samples, given that their removal did not change the total number of OTUs, but would have reduced substantially the cut-off for the standardization of the sampling effort across samples. Thus, we rarefied the sample set to a depth of 59,000 sequences/sample. This final dataset for the culture plates consisted of 347 OTUs across the 43 samples, with 93–277 OTUs per sample (mean ± SD = 174 ± 44). 16S rRNA amplicon sequences are deposited in the NCBI's SRA under the accession number SRP112779.

Comparison of Culture-Dependent and Culture-Independent Communities

To determine whether there was variation among media types in the proportion of OTUs obtained from culture plate washes relative to the culture-independent swab from the same individual, we produced a second dataset that incorporated both the amplicon data from the plate washes ($N = 43$) and the culture-independent swabs ($N = 12$). The resulting file was processed in QIIME, as described above, to produce a final OTU table containing all 55 samples. The OTU table was rarefied to 18,000 sequences/sample, which resulted in 459 total OTUs, with 54-254 OTUs per sample (mean ± SD = 148 ± 47). To avoid overestimation in analyses, 139 OTUs associated only with the culture plate samples were removed from the OTU table. These 139 OTUs occurred at very low relative abundances in the culture-independent dataset, and thus were eliminated when filtering out the OTUs below a relative abundance threshold of 0.01 and/or when rarifying the OTU table. By eliminating these OTUs, the dataset contained OTUs present only in the culture-independent swabs or shared between the culture-independent swabs and the culture plates. The final OTU table had a total of 320 OTUs, with 37–220 OTUs per sample (mean ± SD = 106 ± 43).

Community-Level Inhibition of Bd Growth across Individuals and Media Types

We also assessed whether there was variation among the cultured communities in their ability to inhibit Bd growth. We conducted *in vitro* challenge assays following the method of Bell et al. (2013) and Becker et al. (2015), with the exception that we did not initially co-culture the bacterial isolates with Bd prior to the assay because we were testing whole bacterial communities collected via plate washes. Instead, we used the cell-free supernatant (CFS) that was collected during the plate washes, as described above.

Prior to the challenge assay, zoospores from a Bd culture (JEL404, Maine-USA) were inoculated onto a 1% tryptone agar plate and grown for 3 days at 23°C. The plate was then flooded with 3 ml 1% tryptone broth and the zoospore suspension collected and filtered through a 20 μm filter. The challenge assay was prepared by adding 100 μl of the CFS containing the metabolites from the bacterial communities ($N = 48$ samples; 12 samples/media type) and 100 μl of the Bd zoospore suspension (2×10^6 zoospores per ml) in each well of a sterile 96-well plate. In addition, positive and negative controls were included in each of the 96-well plates. Positive controls were prepared by adding 100 μl of the zoospore suspension and 100 μl of 1% tryptone broth. Negative controls were prepared by adding 100 μl of heat-killed zoospore suspension and 100 μl of 1% tryptone broth. Samples and controls were run in triplicate, and plates were incubated at 23°C. Challenge assay plates were loaded the same day and with the same Bd stock solution. The optical density of each well was measured with a spectrophotometer at 492 nm immediately after plate set up was completed, and then at days 4, 7, and 11. The measurements of optical density were transformed using the formula Ln[OD/(1-OD)]. For each culture plate, the growth rate of Bd in the presence of CFS was calculated by performing a linear regression of the transformed measurements of optical density through time (day 0, 4, 7, and 11). Bd inhibition was calculated by dividing the slope of the triplicates by the average growth rate of the positive control from the respective 96-well plate, and subtracting from 1. Lastly, inhibition values of each triplicate were averaged to calculate the mean inhibition of each culture plate. Negative inhibition values suggest facilitation of Bd growth, while positive values suggest inhibition, with estimates = 1 representing a complete inhibition of Bd growth (Becker et al., 2015).

Data Analysis

Our specific goals were to: (1) compare the diversity of the cultured bacterial communities associated with the different media types representing high and low nutrient concentrations, and identify the cultured bacterial taxa that differed among the media types; (2) determine which media type cultured the highest proportion of OTUs from the full community based on the culture-independent samples; and (3) determine whether the communities growing on the different media types, or from different individual toads, differed in their ability to inhibit Bd growth. Unless noted, all statistical analyses were completed in R version 3.2.4 (R Core Team, 2016). For all generalized linear models (GLMs) and generalized linear mixed models (GLMMs),

we performed visual assessments of residual plots with model predictions to confirm that the error distributions used were appropriate.

Alpha and Beta Diversity Analysis

Alpha diversity estimates were calculated with QIIME for the metrics: richness (OTUs/culture plate), Faith's phylogenetic diversity (measure of diversity based on the branch length of the phylogenetic tree) and the Shannon Index (H', which assesses community evenness). We fitted the diversity metrics to GLMMs. We considered the predictor variable "Media" as a fixed factor and "Individual toad" as a random factor given the nestedness of the media types within individuals. The GLMMs were performed using appropriate error distributions for the diversity metrics to account for heteroscedasticity. For richness, a negative binomial error distribution was applied to the model using the log link function. For phylogenetic diversity and the Shannon index, which was transformed to Hill number (effective number of species; MacArthur, 1965), we used a Gamma error distribution with the inverse link function. The models were run using the R functions glmer.nb for richness, and glmer for phylogenetic diversity and the Shannon Index, from the package *lmer4* (Bates et al., 2014). Multiple comparisons were conducted with Tukey tests using the function glht in the package *multcomp* (Hothorn et al., 2008), which includes multiple comparisons for GLMs. Although not an explicit goal of this study, we also compared the alpha diversity estimates among individual toads since variation in total bacterial diversity across individuals can influence the culturable diversity. For instance, individual toads with high bacterial diversity had higher alpha diversity estimates in their plated communities relative to that from other individuals (Walke et al., 2015a). We used GLMs for this purpose. GLMs were performed as described above, with a negative binomial error distribution for richness estimates and a Gamma error distribution for Shannon and phylogenetic diversity.

Changes in the structure of the bacterial communities across media types were determined by the calculation of dissimilarity distances based on the Bray-Curtis distance measure, which takes into account OTU relative abundance in each community. We are only including the results based on Bray-Curtis since the Jaccard distance measure, which takes into account only the presence/absence composition of the communities, produced consistent results. A statistical comparison of the communities across media types based on the Bray-Curtis dissimilarities was done with a permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001). In addition, we compared the bacterial communities of the media types based on nutrient level, where LB and TSA were grouped as high nutrient concentration media types, and R2A and dR2A as low. When performing the PERMANOVAs, the argument "strata" was used to define the group within which to limit the permutations (i.e., individual toad) due to the nestedness of the replicates of the media types and the potential variation in the skin bacterial communities among toads. Importantly, an analysis excluding those toads that did not have replicates of all media types (toads 4, 11, and 12) was consistent with results from the inclusive analysis that is presented. Lastly, due to potential influence of the individual toads on the plated bacterial communities, as shown in the ordination (**Figure 2**), we also compared the communities across toads where the argument "strata" was not used when performing the PERMANOVAs. Bray-Curtis dissimilarity distances were calculated with the function vegdist, and PERMANOVAs were performed with the function adonis, both functions from the *vegan* package (Okansen et al., 2016). To visualize the results, we used principal coordinate analysis (PCoA).

Reduced variation in community structure across samples could occur on high nutrient media due to fast-growing bacteria out-competing slow-growing bacteria, and among samples from the same individual toad. As a way to test this, we compared the multivariate homogeneity of dispersion (i.e., distance from objects to cluster centroid) across media types based on Bray-Curtis dissimilarity distances. We used the function betadisper from the *vegan* package and conducted a hypothesis test to determine whether there are statistical differences in dispersion among groups using the function anova from the default R package *stats*.

Species Indicator Analysis

To identify OTUs associated with particular media types (e.g., TSA vs. LB or low nutrient concentration media vs. high nutrient concentration media), we performed an indicator species analysis using the function multipatt from the *indicspecies* package (De Cáceres and Legendre, 2009). Overall, the function multipatt quantifies, via the estimation of an Indicator Value (IndVal), the association between species (e.g., OTUs) and a group of samples (e.g., replicates within media types) based on the relative abundance and relative frequency of each species, and calculates the statistical significance of the relationships using a permutational approach (De Cáceres and Legendre, 2009).

Estimating the Proportion of OTUs Cultured Relative to the Culture-Independent Swabs

To account for the individual-level variation in OTU richness, the proportion of OTUs recovered by each media type was calculated at the individual toad level. We then used a GLMM to determine whether media types differed in the number of OTUs cultured relative to the culture-independent swabs. Similar to the alpha diversity analyses, we considered the predictor variable "Media type" as a fixed factor and "Individual toad" as a random factor given the nestedness of the media types within individuals. We used the Gamma error distribution with the inverse link function using the function glmer from the package *lmer4*.

Comparing Community Level Ability to Inhibit Bd Growth among Culture Media Types and Individual Toads

We compared the functional ability to inhibit Bd growth of each bacterial community growing on the culture media plates. We fitted the mean inhibition values to a linear mixed effects model given that the data were normally distributed, as determined by the Lilliefors normality test (function lillie. test from the package *nortest*; Gross and Ligges, 2015). The model included

the predictor variable "Media" as a fixed factor and "Individual toad" as a random factor. In addition, to assess the potential effect of individual toads in the inhibition values, we fitted the mean inhibition values to a linear model using the variable "toad" as the predictor variable. The linear mixed effect model was performed using the function lmer from the package *lmer4* (Bates et al., 2014), and the linear model was performed using the function lm from the default R package *stats*.

RESULTS

Alpha diversity metrics differed significantly among media types for richness and Faith's phylogenetic diversity (richness: Chisq = 15.68, $P = 0.0013$; phylogenetic diversity: Chisq = 25.67, $P < 0.0001$), with the low nutrient concentration media, R2A and dR2A, having a significantly higher diversity compared to the high nutrient concentration media, LB and TSA (**Figures 1A,B**, Richness: LB-dR2A: $z = -3.23$, $P = 0.007$; TSA-dR2A: $z = -3.52$, $P = 0.002$; R2A-LB: $z = 2.91$, $P = 0.019$; R2A-TSA: $z = -3.233$, $P = 0.007$; Faith's phylogenetic diversity: LB-dR2A: $z = 4.82$, $P < 0.001$; TSA-dR2A: $z = 3.84$, $P < 0.001$; R2A-LB: $z = -4.58$, $P < 0.001$; R2A-TSA: $z = 3.61$, $P = 0.002$). There were no significant differences in the pairwise comparisons between media types within nutrient concentration levels. In contrast to richness and Faith's phylogenetic diversity, we did not find significant differences when comparing the diversity among media types based on Shannon index Hill numbers (Shannon Hill number: Chisq = 7.17, $P = 0.067$), although there was a trend for dR2A and R2A to be higher (**Figure 1C**). There were also significant differences in the alpha diversity estimates of the plated communities among toads (richness: Deviance = 42.59, $P < 0.001$; phylogenetic diversity: F *statistic* = 3.96, $P = 0.001$; Shannon Hill number: F *statistic* = 6.68, $P < 0.001$).

Bacterial community structure differed among the four media types and also between high and low nutrient concentration media (**Figure 2**, media types: pseudo-F = 0.68, $R^2 = 0.05$, $P = 0.002$; nutrient concentration: pseudo-F = 1.14, $R^2 = 0.03$, $P < 0.001$). However, differences among individual toads explained substantially more variation (pseudo-F = 5.32, $R^2 = 0.65$, $P < 0.001$). Lastly, the average distances to centroids (∼multivariate variance) did not differ significantly among the media types ($F = 0.83$, $P = 0.48$; dR2A 0.57; R2A 0.60; LB 0.61; TSA 0.61). In contrast, distances to centroids did differ among individual toads (**Figure 3**, $F = 14.57$, $P = 0.001$), which supports the substantial variation explained by individual toads mentioned above, and suggests that individual toads are the main drivers of the observed clustering in **Figure 2**.

We identified a total of 50 indicator OTUs that were significantly ($p < 0.05$) associated with either a specific culture media or high/low nutrient media types. The distribution of these indicator OTUs across media types was: six associated

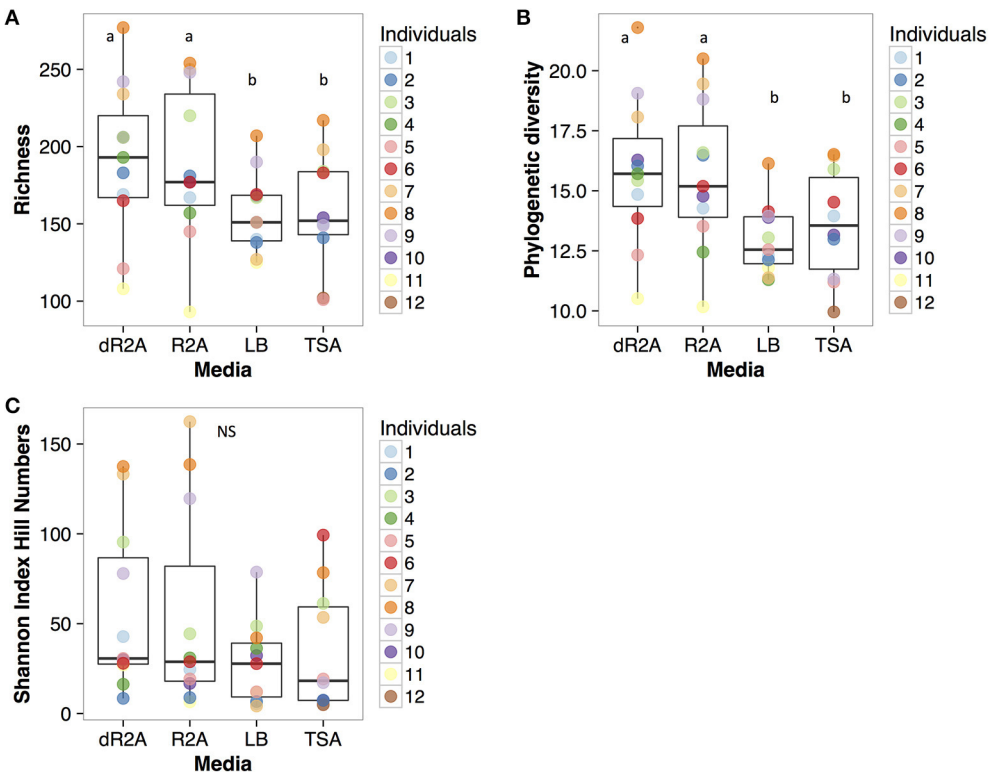

FIGURE 1 | Alpha diversity estimates for the different metrics (**(A)**: OTU richness; **(B)**: Faith's phylogenetic diversity; **(C)**: Shannon Index) by culture media type. Alpha diversity estimates are color-coded at the toad (individual #). Boxplots represent the median, upper and lower quartile, and maximum and minimum values.

with dR2A, one with TSA, and 41 with the low nutrient media group of R2A and dR2A, suggesting that the low nutrient agars did pick up a unique set of bacteria (**Table 1**, **Figures 4**, **5**). In addition, there were two indicator OTUs absent in only one of the 4 media types (**Table 1**): one absent only in LB and another one in dR2A. The relative abundance distribution of the indicator OTUs suggested that some taxa seemed to favor a particular type of culture media. For example, the family *Pseudomonadaceae* had higher relative abundance on LB media, the family *Phyllobacteriaceae* on TSA media, and the families *Sphingomonadaceae* and *Xanthomonadaceae* on the low nutrient media group, R2A and dR2A (**Figure 5**). Lastly, we also found that a higher proportion of OTUs from the culture-independent swabs were cultured on R2A and dR2A, relative to LB and TSA media plates (Chisq = 26.19, $P < 0.0001$; mean ± sd: R2A 59.65% ± 13.88; dR2A 60.71% ± 14.29; LB 48.47% ± 9.5; TSA 45.38% ± 14.31). This result is further supported by the lower Bray-Curtis dissimilarity distances between the cultured communities and those from the culture-independent swabs (**Figure 6**).

Despite differences across media types in community structure and proportion of recovered OTUs from the culture-independent swabs, we did not find differences among media types in the ability of metabolites from the plated communities to inhibit Bd growth (**Figure 7**, Chisq = 4.14, $P = 0.25$; mean ± sd: R2A 0.15 ± 0.53; dR2A 0.15 ± 0.25; LB 0.36 ± 0.52; TSA 0.44 ± 0.53). Likewise, despite differences among toads in community structure, we also did not find significant differences in the inhibition values among them ($F = 1.55$, $P = 0.16$). Overall, inhibition estimates across samples ranged from −1.16 (facilitating Bd growth) to 1.01 (completely inhibiting Bd growth), which suggests a substantial amount of variation across samples.

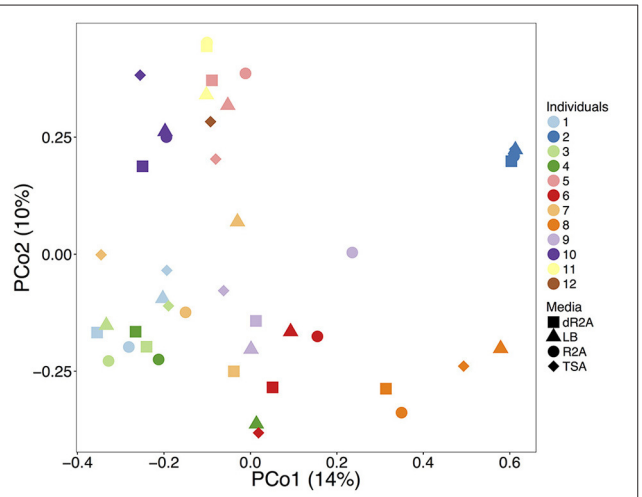

FIGURE 2 | Beta diversity comparisons of the cultured bacterial communities using a principal coordinate analysis (PCoA) ordination based on Bray-Curtis dissimilarity distances. Points represent each plated community. Colors and shapes represent individual toads and culture media types, respectively.

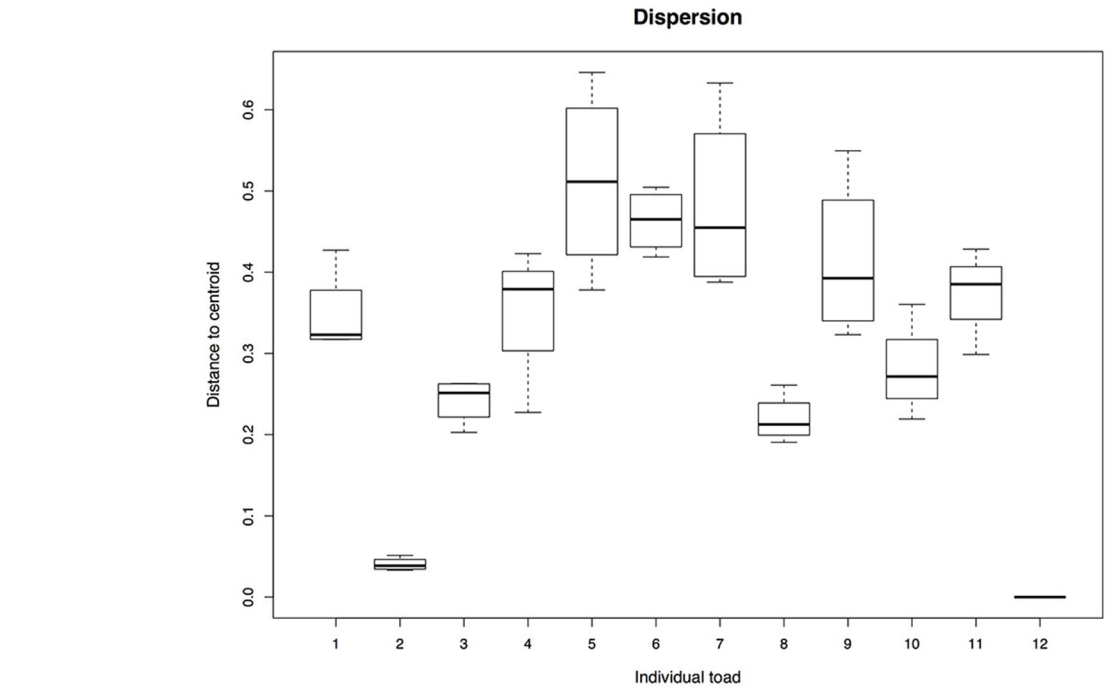

FIGURE 3 | Dispersion among the plated bacterial communities from each individual toad. The analysis was conducted based on Bray-Curtis dissimilarity distances. The figure shows significant differences in dispersion among the individual toads. Boxplots represent the median, upper and lower quartile, and maximum and minimum values.

TABLE 1 | List of indicators OTUs associated with the different culture media (LB, TSA, R2A, and dR2A) or group of culture media types.

Culture media and groups of media	Indicator OTU ID	Phyla	Family	Genus	Indicator value index	*P*-value
dR2A	156722	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.78	0.001
	817982	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.03
	540793	Proteobacteria	Enterobacteriaceae	*Serratia*	0.45	0.047
	4315079	Firmicutes	Paenibacillaceae	*Paenibacillus*	0.43	0.017
	348478	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.42	0.022
	533999	Proteobacteria	Xanthomonadaceae	–	0.37	0.039
dR2A + R2A + TSA	654003	Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	0.90	0.01
LB + R2A + TSA	141365	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.86	0.035
R2A + dR2A	4449609	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.95	0.01
	241289	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.91	0.001
	5364	Proteobacteria	Rhizobiaceae	–	0.91	0.002
	denovo77494	Proteobacteria	Enterobacteriaceae	*Erwinia*	0.86	0.001
	denovo73459	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.82	0.001
	denovo188538	Proteobacteria	Enterobacteriaceae	–	0.81	0.003
	denovo143862	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.77	0.001
	4421805	Actinobacteria	Microbacteriaceae	*Salinibacterium*	0.76	0.002
	denovo55330	Proteobacteria	Enterobacteriaceae	–	0.73	0.004
	denovo78257	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.73	0.001
	denovo22220	Proteobacteria	Enterobacteriaceae	–	0.73	0.001
	denovo196304	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.72	0.002
	denovo54352	Proteobacteria	Enterobacteriaceae	–	0.72	0.001
	135993	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.72	0.013
	denovo202891	Proteobacteria	Enterobacteriaceae	–	0.72	0.001
	denovo92894	Proteobacteria	Enterobacteriaceae	–	0.68	0.001
	4304056	Proteobacteria	Rhizobiaceae	–	0.68	0.003
	102915	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.68	0.012
	103410	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.68	0.031
	denovo166093	Proteobacteria	Burkholderiaceae	–	0.63	0.002
	4311005	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.59	0.007
	denovo67862	Proteobacteria	Xanthomonadaceae	*Luteibacter*	0.59	0.002
	denovo116903	Proteobacteria	Rhodobacteraceae	–	0.58	0.001
	4423410	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.58	0.015
	denovo167134	Proteobacteria	Enterobacteriaceae	–	0.57	0.027
	3180137	Proteobacteria	Rhodobacteraceae	–	0.56	0.004
	denovo145069	Actinobacteria	Nocardiaceae	*Rhodococcus*	0.55	0.019
	denovo191161	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	0.55	0.001
	denovo1424	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.54	0.029
	218154	Proteobacteria	Rhizobiaceae	*Agrobacterium*	0.54	0.006
	denovo150449	Proteobacteria	Phyllobacteriaceae	*Phyllobacterium*	0.54	0.003
	1104627	Proteobacteria	Rhizobiaceae	*Rhizobium*	0.54	0.008
	673343	Proteobacteria	Alcaligenaceae	–	0.52	0.027
	662915	Proteobacteria	Aurantimonadaceae	–	0.50	0.009
	denovo83855	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.005
	543890	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.50	0.01
	125947	Proteobacteria	Burkholderiaceae	*Burkholderia*	0.50	0.022
	5162	Proteobacteria	Rhizobiaceae	–	0.50	0.036

(Continued)

TABLE 1 | Continued

Culture media and groups of media	Indicator OTU ID	Phyla	Family	Genus	Indicator value index	*P*-value
	4479484	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.45	0.006
	4337890	Proteobacteria	Xanthomonadaceae	*Lysobacter*	0.45	0.045
	denovo164114	Proteobacteria	Sphingomonadaceae	*Sphingomonas*	0.41	0.03
TSA	denovo1626	Proteobacteria	Rhizobiaceae	–	0.30	0.032

Indicator OTUs are listed in a decreasing order based on their indicator value index for a culture media or group of media. The indicator value index represents a measure of the association between an OTU and a culture media or group of media, and range from 0 to 1, where values close to 1 imply a relative stronger association.

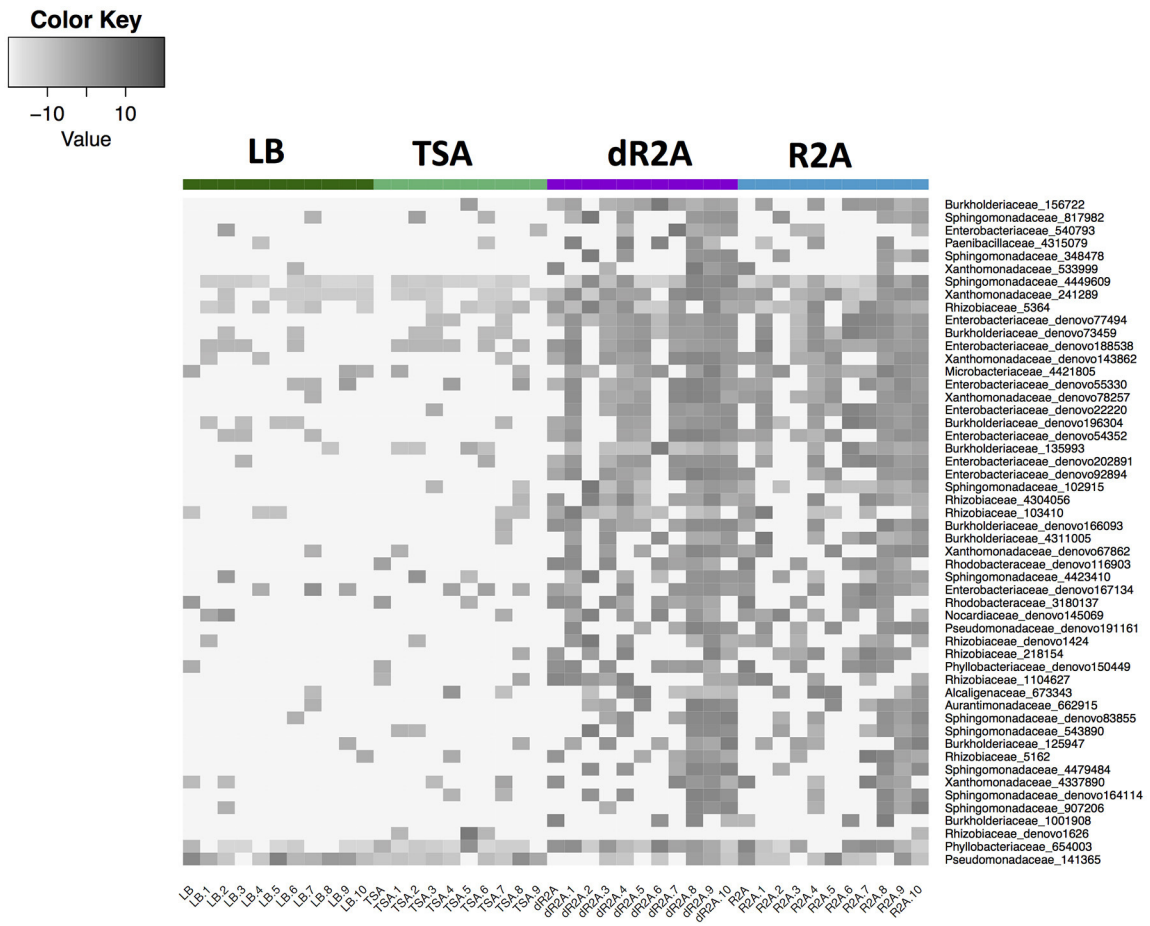

FIGURE 4 | Heatmap of log-transformed relative abundances of the indicator OTUs associated with the different media types (LB, TSA, R2A, and dR2A). Rows indicate each indicator OTU family and ID, and columns indicate the individual toads/replicates within each culture media type.

DISCUSSION

We used amphibian skin bacterial samples to conduct a comparative analysis of the diversity and community structure among plated communities growing on media that varied in nutrient concentration. We addressed the present study from two perspectives: first, from an ecological perspective that aimed to assess how variation in productivity (i.e., distinct nutrient concentrations in the culture media) influences the diversity of the cultured bacterial communities. Second, from an applied perspective that aimed to determine what culture media recovers the highest fraction of the amphibian skin bacterial communities relative to data derived from a culture-independent approach, and to assess whether the plated communities differ in their ability to inhibit the growth of an amphibian pathogenic fungus (Bd strain JEL404). Overall, we found that culture media with low nutrient concentration, R2A and dR2A, facilitated the growth of more diverse and distinct communities relative to the culture

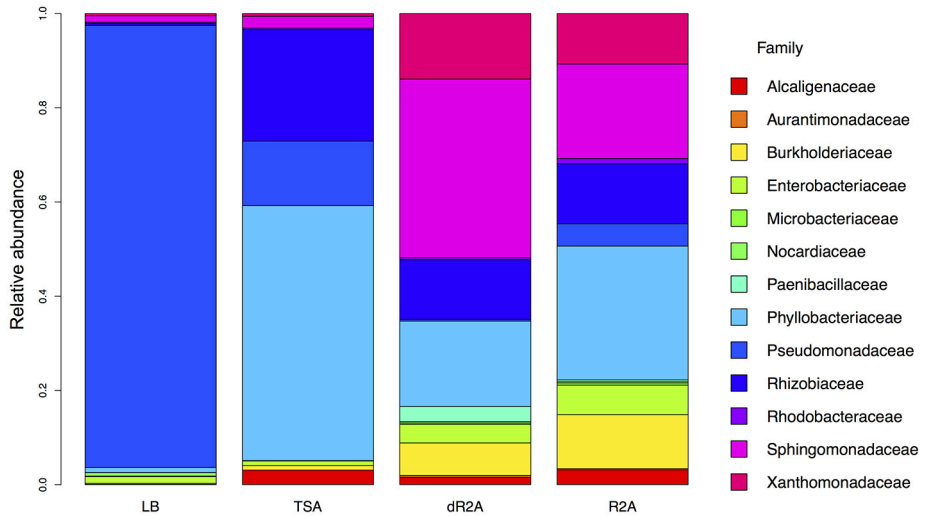

FIGURE 5 | Stacked barplot showing the relative abundance, across toads/replicates within each culture media, of the taxonomic families of the indicator OTUs associated with the distinct culture media types.

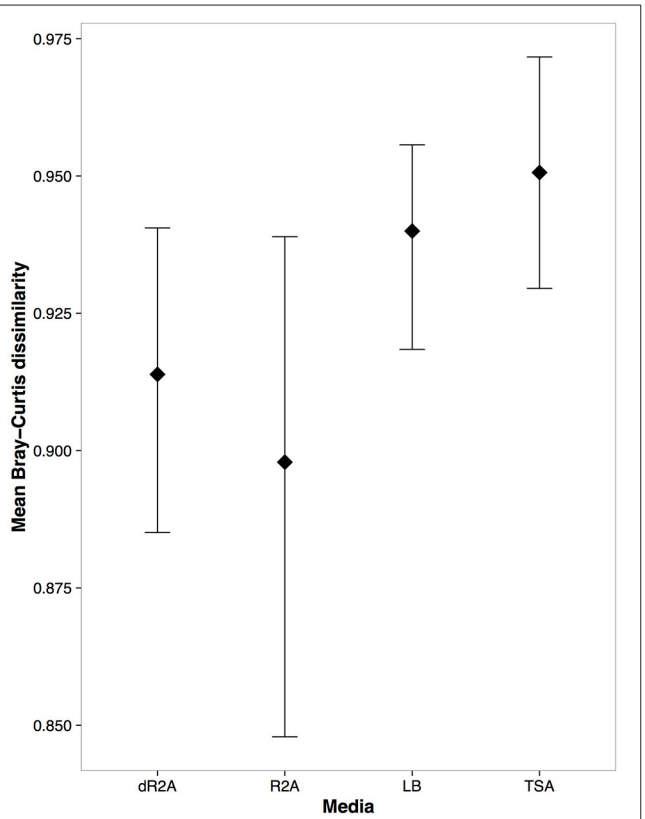

FIGURE 6 | Mean and 95% CI of Bray-Curtis dissimilarity estimates between the cultured communities from the culture media types and the culture-independent bacterial communities from the skin swabs.

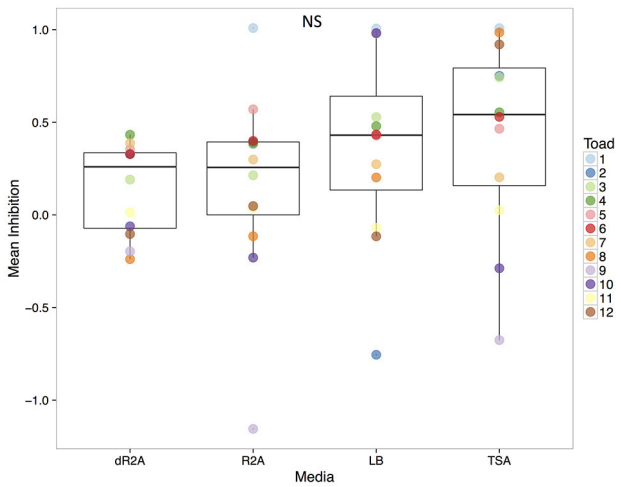

FIGURE 7 | Mean inhibition estimates for each plated community by culture media. Estimates are color-coded at the toad level (individual #). Boxplots represent the median, upper and lower quartile, and maximum and minimum values. Inhibition estimates with negatives values suggest facilitation of Bd growth, while positive values suggest inhibition, with estimates = 1 representing a complete inhibition of Bd growth.

media with higher nutrient concentration, LB and TSA. In addition, the high bacterial diversity observed and exclusively associated with R2A and dR2A reflects a higher proportion of the culturable diversity that can be recovered from amphibian skin. However, the estimated function of the plated communities against Bd, based on the whole-plate wash method, did not vary across the culture media or productivity levels. Interestingly, we found that variation among individual hosts influenced the structure of the plated bacterial communities more than the media type. Thus, when attempting to maximize diversity of cultured isolates, swabbing more individuals in the population may be more valuable than using diverse media (Walke et al., 2015a).

The relationship between primary productivity and diversity is considered a key factor determining spatial and temporal diversity patterns in ecological systems (Jessup et al., 2004). Interestingly, in contrast to the general positive quadratic pattern observed in plants and animals (Rosenzweig, 1995), diversity patterns across productivity gradients in microbial systems have been inconsistent across studies (Horner-Devine et al., 2004). The relationship between productivity and diversity in microbial systems has been examined via correlational studies focusing on the variation of bacterial diversity across gradients of nutrient concentration in both laboratory and field studies (Benlloch et al., 1995; Bohannan and Lenski, 2000; Kassen et al., 2000; Horner-Devine et al., 2003). Though our study did not examine the effects of a concentration gradient of a specific nutrient, it did represent microcosms with distinct arrays of nutrients at different concentrations. Moreover, the observed results are consistent with a study showing that diversity patterns in microbial systems can be mediated by the role of ecological factors, such as changes in the relative importance of competition across a gradient of nutrient concentrations (Bohannan and Lenski, 2000). For instance, the observed higher alpha diversity estimates in culture media with low concentrations of nutrients, relative to nutrient-rich ones, highlight the role of competition in plated communities, where faster-growing bacteria tend to outcompete slow-growing bacteria. In addition, culture media with high concentrations of nutrients favoring fast growing bacteria can limit the occurrence of positive relationships between bacterial species, such as cross-feeding and co-aggregation (Vartoukian et al., 2010; Faust and Raes, 2012), which might enhance the growth of slow-growing bacterial taxa.

We observed variation in the structure of the plated communities across media types even though most of the observed variation was driven by differences among individual toads. The observed variation across media types can be explained, in part, due to the fact that culture media types are commonly developed for different purposes and with different degrees of selectivity. For instance, LB and TSA are considered non-selective complex media (i.e., nutrient-rich), whose composition is poorly defined because they usually include complex ingredients, such as yeast extract (Slonczewski and Foster, 2017). In contrast, R2A is considered a defined medium whose chemical components are known (Slonczewski and Foster, 2017) and which selects for slow-growing bacteria, in particular those from aquatic environments (Reasoner and Geldreich, 1985). In the present study, the number and taxonomic classification of the indicator OTUs associated with R2A and dR2A suggests that R2A is the most appropriate media to recover a high diversity of the culturable members of the amphibian skin community. In addition, R2A also recovers some of the most abundant and prevalent taxa occurring on amphibian skin, based on studies of tropical and temperate species (Kueneman et al., 2013; Walke et al., 2014; Becker et al., 2015; Belden et al., 2015; Rebollar et al., 2016b; Medina et al., 2017). For example, we identified several indicator OTUs from the families *Nocardiaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, *Sphingomonadaceae* and *Xanthomonadaceae* associated with R2A and dR2A that were the most abundant and prevalent bacterial taxa on these individual toads according to 16S rRNA amplicon data (Walke et al., 2015a). Thus, the selection for slow-growing bacteria that characterizes R2A could facilitate the recovery of a higher diversity of culturable bacteria from these communities relative to LB and TSA, which are non-selective complex media and allow faster-growing bacteria to outcompete slow-growing bacteria.

The study by Walke et al. (2015a) that used the same toads aimed to examine the cultured portion of the amphibian skin bacterial communities identified with a culture-independent method, and determined that for each individual toad an average of 0.95% of the community was recovered using R2A. The recovery value estimated by Walke et al. (2015a) is substantially lower compared to that estimated in our study using R2A (i.e., 60%). There are a number of differences in the methods between our study and Walke et al. (2015a) that likely contribute to the observed differences in the number of OTUs recovered. For example, the sampling technique (plate wash in our study vs. pure culture isolation in Walke et al., 2015a) likely influenced the final set of cultured isolates. Pure culture isolation requires manually selecting morphologically-distinct isolates in serial plating, which could result in over-looking related taxa that are morphologically similar, or missing those that require longer incubation periods to have visible colony formation. This could result in an under-estimation of what is cultured. In addition, our present study used a filtering cutoff of 0.01% (maintained only those OTUs with $>$ 0.01% relative abundance), whereas Walke et al. (2015a) used a cutoff of 0.001%. The higher cutoff value 0.01% reduces the number of OTUs in the dataset, which could result in a higher overlap among cultured and culture-independent samples in terms of the OTU composition. We think a cutoff of 0.01%, which was determined in the present study as the cutoff level at which OTU richness leveled off in the dataset (per Bokulich et al., 2012), potentially represents a more realistic view of the actual bacterial species present in the community (Bokulich et al., 2012; Hughey et al., 2016).

Bacterial secondary metabolites produced in response to interspecific interactions have been suggested as a mechanism by which skin bacterial communities can protect their amphibian hosts against Bd infection (Bletz et al., 2013; Walke and Belden, 2016). Furthermore, recent evidence suggests that co-culturing bacterial isolates from amphibian skin can enhance the production of emergent antifungal metabolites, which then have a stronger inhibitory ability against Bd growth relative to monocultures (Loudon et al., 2014; Piovia-Scott et al., 2017). Within the context of our study, even though we observed variation in the diversity of the plated communities across culture media and individual toads, we found no significant differences across these factors in the ability to inhibit Bd growth in challenge assays. In fact, we found a substantial amount of variation, ranging from plated communities that completely inhibited Bd growth to others that enhanced growth. This result is somewhat surprising considering that the cell-free supernatant collected from each of the plated communities should have comprised bacterially-produced secondary metabolites resulting from interspecific interactions. However, given the high diversity of taxa that grew on the plates, variation in density of the

distinct bacterial taxa across plated communities, combined with stochastic variation, could have influenced our results (Loudon et al., 2014). Moreover, it is possible that we could have induced additional variation with the method used for collecting the bacterial community and the supernatant from each culture plate (i.e., plate wash; Stevenson et al., 2004). To our knowledge, no previous studies have used the plate wash approach to collect bacterially-produced metabolites. We based this approach on the plate wash method of Stevenson et al. (2004) that was used to survey bacterial communities, as we were interested in trying to get a whole community estimate of potential Bd inhibition. This method was applied under the assumption that anti-fungal metabolites might be produced, at least in part, on the surface of the solid media because the zone of Bd inhibition is commonly observed on the surface of culture plates during Bd challenge assays (Harris et al., 2006; Woodhams et al., 2007; Flechas et al., 2012). Further research will likely be required to fine tune and verify this method as a viable option for assessing whole community Bd inhibition. Our method also did not include the step of co-culturing the plated bacterial communities with Bd, as has been done in previous studies (Becker et al., 2015). However, Becker et al. (2015) found that co-culturing bacterial isolates with Bd had no effect on the ability of bacterial isolates to inhibit Bd growth.

Using culture media with low nutrient concentrations and/or diluted culture media is known to improve the probability of capturing a better representation of bacterial communities living in nature (Vartoukian et al., 2010). The present study provides evidence that low nutrient R2A, relative to other common culture media, allows the growth of a higher diversity of bacterial taxa and recovers a higher proportion of the overall diversity occurring on the amphibian skin. These findings are relevant given that R2A has been widely used in the isolation of amphibian skin bacteria (e.g., Harris et al., 2006; Flechas et al., 2012; Daskin et al., 2014; Shaw et al., 2014; Antwis et al., 2015; Becker et al., 2015; Madison et al., 2017), despite no evidence of its proficiency. We suggest that an understanding of the ecological interactions influencing the plated communities and the nature of the culture media will likely improve our ability to culture rare or previously uncultured microbes. Lastly, we would like to emphasize that, at least in our study species (and see Walke et al., 2015a), swabbing more individuals in a population is the best way to maximize culture collections, regardless of media type. These results can inform current efforts for developing a probiotic-based approach for amphibian conservation, and help to ensure that culture collections are capturing the majority of the important diversity in host-associated microbial systems.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptual framework and design of the experiment. DM, ZG, and MS conducted the experiment. DM processed samples in the laboratory. DM, JW, and LB completed data processing and analyses. DM and LB produced the first draft of the manuscript, and all authors edited the manuscript.

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REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/AEM.00010-15

Bates, D., Maechler, M., Bolker, B. M., and Walker, S. C. (2014). Fitting linear mixed-effects models using lme4. *J. of Stat. Soft.* 67, 1–48. doi: 10.18637/jss.v067.i01

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015). Phylogenetic distribution of symbiotic bacteria from *Panamanian amphibians* that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Org.* 103, 77–85. doi: 10.3354/dao02560

Benlloch, S., RodriguezValera, F., and MartinezMurcia, A. J. (1995). Bacterial diversity in two coastal lagoons deduced from 16S rDNA PCR amplification and partial sequencing. *FEMS Microbiol. Ecol.* 18, 267–279. doi: 10.1111/j.1574-6941.1995.tb00183.x

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bohannan, B., and Lenski, R. E. (2000). The relative importance of competition and predation varies with productivity in a model community. *Amer. Nat.* 156, 329–340. doi: 10.1086/303393

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2012). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Meth.* 10, 57–59. doi: 10.1038/nmeth.2276

Bosch, J., and Martínez-Solano, I. (2006). Chytrid fungus infection related to unusual mortalities of *Salamandra salamandra* and Bufo bufo in the Pe-alara Natural Park, Spain. *Oryx* 40, 84–86. doi: 10.1017/S0030605306000093

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Meth.* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Connon, S. A., and Giovannoni, S. J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl. Environ. Microbiol.* 68, 3878–3885. doi: 10.1128/AEM.68.8.3878-3885.2002

Crawford, A. J., Lips, K. R., Bermingham, E., and Wake, D. B. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13777–13782. doi: 10.1073/pnas.0914115107

Daskin, J. H., Bell, S. C., Schwarzkopf, L., and Alford, R. A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians–implications for disease management and patterns of decline. *PLoS ONE* 9:e100378. doi: 10.1371/journal.pone.0100378

De Cáceres, M., and Legendre, P. (2009). Associations between species and groups of sites: indices and statistical inference. *Ecology* 90, 3566–3574. doi: 10.1890/08-1823.1

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/AEM.03006-05

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Faust, K., and Raes, J. (2012). Microbial interactions: from networks to models. *Nat. Rev. Microbiol.* 10, 538–550. doi: 10.1038/nrmicro2832

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–194. doi: 10.1038/nature10947

Flechas, S. V., Sarmiento, C., Cárdenas, M. E., Medina, E. M., Restrepo, S., and Amézquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Goldberg, D. E., and Miller, T. E. (1990). Effects of different resource additions of species diversity in an annual plant community. *Ecology* 71, 213–225. doi: 10.2307/1940261

Gross, J., and Ligges, L. (2015). *nortest: Tests for Normality*. R package version 1.0-4. Available online at: https://CRAN.R-project.org/package=nortest

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Horner-Devine, M. C., Carney, K. M., and Bohannan, B. J. (2004). An ecological perspective on bacterial biodiversity. *Proc. R. Soc. B* 271, 113–122. doi: 10.1098/rspb.2003.2549

Horner-Devine, M. C., Leibold, M. A., Smith, V. H., and Bohannan, B. (2003). Bacterial diversity patterns along a gradient of primary productivity. *Ecol. Lett.* 6, 613–622. doi: 10.1046/j.1461-0248.2003.00472.x

Hothorn, T., Bretz, F., and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biom. J.* 50, 346–363. doi: 10.1002/bimj.200810425

Hughey, M. C., Walke, J. B., Becker, M. H., Umile, T. P., Burzynski, E. A., Minbiole, K. P., et al. (2016). Short-term exposure to coal combustion waste has little impact on the skin microbiome of adult spring Peepers (*Pseudacris crucifer*). *Appl. Environ. Microbiol.* 82, 3493–3502. doi: 10.1128/AEM.00045-16

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jessup, C. M., Forde, S. E., and Bohannan, B. J. M. (2005). Microbial experimental systems in ecology. *Adv. Ecol. Res.* 273–307. doi: 10.1016/S0065-2504(04)37009-1

Jessup, C. M., Kassen, R., Forde, S. E., Kerr, B., Buckling, A., Rainey, P. B., et al. (2004). Big questions, small worlds: microbial model systems in ecology. *Trends Ecol. Evol.* 19, 189–197. doi: 10.1016/j.tree.2004.01.008

Kassen, R., Buckling, A., Bell, G., and Rainey, P. B. (2000). Diversity peaks at intermediate productivity in a laboratory microcosm. *Nature* 406, 508–512. doi: 10.1038/35020060

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2013). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Lauer, A., Simon, M. A., Banning, J. L., Andre, E., Duncan, K., and Harris, R. N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 630–640. doi: 10.1643/0045-8511(2007)2007[630:CCBFTE]2.0.CO;2

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A., and Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J.* 2, 145–157. doi: 10.1038/ismej.2007.110

Lips, K. R. (2016). Overview of chytrid emergence and impacts on amphibians. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371, 20150465–20150469. doi: 10.1098/rstb.2015.0465

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P., and Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* 5:441. doi: 10.3389/fmicb.2014.00441

Loudon, A. H., Venkataraman, A., Van Treuren, W., Woodhams, D. C., Parfrey, L. W., McKenzie, V. J., et al. (2016). Vertebrate hosts as islands: dynamics of selection, immigration, loss, persistence, and potential function of bacteria on salamander skin. *Front. Microbiol.* 7:333. doi: 10.3389/fmicb.2016.00333

MacArthur, R. H. (1965). Patterns of species diversity. *Biol. Rev. Camb. Philos. Soc.* 40, 510–533. doi: 10.1111/j.1469-185X.1965.tb00815.x

Madison, J. D., Berg, E. A., Abarca, J. G., Whitfield, S. M., Gorbatenko, O., Pinto, A., et al. (2017). Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from amphibian populations in Costa Rica. *Front. Microbiol.* 8:290. doi: 10.3389/fmicb.2017.00290

Medina, D., Hughey, M. C., Becker, M. H., Walke, J. B., Umile, T. P., Burzynski, E. A., et al. (2017). Variation in metabolite profiles of amphibian skin bacterial communities across elevations in the Neotropics. *Microb. Ecol.* 74, 227–238. doi: 10.1007/s00248-017-0933-y

Okansen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., OHara, R. B., et al. (2016). *vegan: Community Ecology Package*. R package version 2.3-5. Available online at: https://CRAN.R-project.org/package=vegan

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., McKenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

R Core Team (2016). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Reasoner, D. J., and Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.* 49, 1–7.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016a). Using "Omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibá-ez, R., and Belden, L. K. (2016b). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Rosenzweig, M. L. (1995). *Species Diversity in Space and Time*. Cambridge, NY: Cambridge University Press.

Schindler, D. W. (1990). Experimental perturbations of whole lakes as tests of hypotheses concerning ecosystem structure and function. *Oikos* 57, 25–41. doi: 10.2307/3565733

Shaw, S. D., Berger, L., Bell, S., Dodd, S., James, T. Y., Skerratt, L. F., et al. (2014). Baseline cutaneous bacteria of free-living New Zealand native frogs (*Leiopelma archeyi* and *Leiopelma hochstetteri*) and implications for their role in defense against the amphibian chytrid (*Batrachochytrium dendrobatidis*). *J. Wild. Dis.* 50, 723–732. doi: 10.7589/2013-07-186

Slonczewski, J., and Foster, J. W. (2017). *Microbiology: an Evolving Science*. New York, NY: W.W. Norton & Company, Inc.

Stevenson, B. S., Eichorst, S. A., Wertz, J. T., Schmidt, T. M., and Breznak, J. A. (2004). New strategies for cultivation and detection of previously uncultured microbes. *Appl. Environ. Microbiol.* 70, 4748–4755. doi: 10.1128/AEM.70.8.4748-4755.2004

Vartoukian, S. R., Palmer, R. M., and Wade, W. G. (2010). Strategies for culture of "unculturable" bacteria. *FEMS Micro. Lett.* 309, 1–7. doi: 10.1111/j.1574-6968.2010.02000.x

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9689–9694. doi: 10.1073/pnas.0914111107

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2015a). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *Appl. Environ. Microbiol.* 81, 6589–6600. doi: 10.1128/AEM.01486-15

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P., et al. (2015b). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLoS ONE* 10:e0139848. doi: 10.1371/journal.pone.0139848

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog.* 12:e1005796. doi: 10.1371/journal.ppat.1005796

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Wilson, S. D., and Tilman, D. (1991). Component of plant competition along an experimental gradient of nitrogen availability. *Ecology* 72, 1050–1065. doi: 10.2307/1940605

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conser* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M., et al. (2002). Cultivating the uncultured. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15681–15686. doi: 10.1073/pnas.252630999

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Estimating Herd Immunity to Amphibian Chytridiomycosis in Madagascar Based on the Defensive Function of Amphibian Skin Bacteria

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For decades, Amphibians have been globally threatened by the still expanding infectious disease, chytridiomycosis. Madagascar is an amphibian biodiversity hotspot where *Batrachochytrium dendrobatidis* (*Bd*) has only recently been detected. While no *Bd*-associated population declines have been reported, the risk of declines is high when invasive virulent lineages become involved. Cutaneous bacteria contribute to host innate immunity by providing defense against pathogens for numerous animals, including amphibians. Little is known, however, about the cutaneous bacterial residents of Malagasy amphibians and the functional capacity they have against *Bd*. We cultured 3179 skin bacterial isolates from over 90 frog species across Madagascar, identified them via Sanger sequencing of approximately 700 bp of the 16S rRNA gene, and characterized their functional capacity against *Bd*. A subset of isolates was also tested against multiple *Bd* genotypes. In addition, we applied the concept of herd immunity to estimate *Bd*-associated risk for amphibian communities across Madagascar based on bacterial antifungal activity. We found that multiple bacterial isolates (39% of all isolates) cultured from the skin of Malagasy frogs were able to inhibit *Bd*. Mean inhibition was weakly correlated with bacterial phylogeny, and certain taxonomic groups appear to have a high proportion of inhibitory isolates, such as the Enterobacteriaceae, Pseudomonadaceae, and Xanthamonadaceae (84, 80, and 75% respectively). Functional capacity of bacteria against *Bd* varied among *Bd* genotypes; however, there were some bacteria that showed broad spectrum inhibition against all tested *Bd* genotypes, suggesting that these bacteria would be good candidates for probiotic therapies. We estimated *Bd*-associated risk for sampled amphibian communities based on the concept of herd immunity. Multiple amphibian communities, including those in the amphibian diversity hotspots, Andasibe and Ranomafana, were estimated to be below the 80% herd immunity threshold, suggesting they may be at higher risk to chytridiomycosis if a lethal *Bd* genotype

emerges in Madagascar. While this predictive approach rests on multiple assumptions, and incorporates only one component of hosts' defense against *Bd,* their culturable cutaneous bacterial defense, it can serve as a foundation for continued research on *Bd*-associated risk for the endemic frogs of Madagascar.

Keywords: anti-Bd bacteria, chytridiomycosis, amphibians, skin bacteria, *Batrachochytrium dendrobatidis*

INTRODUCTION

Host-associated symbiotic bacterial communities mediate protection against pathogens in multiple hosts, including plants (Haas and Défago, 2005), corals (Krediet et al., 2013), insects (Cafaro et al., 2011), bats (Hoyt et al., 2015), humans (Sanchez et al., 2016), and amphibians (Bletz et al., 2013; Walke and Belden, 2016). Next generation sequencing technologies have rapidly advanced our understanding of community composition and structure of host microbiotas; however, understanding the function of these communities requires alternative technologies and can be complicated. Culture-based studies can be of great value for determining microbial function. Understanding the functional capacity of culture isolates may help identify phylogenetic patterns of function and thus help to further elucidate how community composition is linked to function.

Bacteria can provide protection against the cutaneous chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*), which can cause the lethal disease, chytridiomycosis (Berger et al., 1998; Stuart et al., 2004; Lips et al., 2006; Cheng et al., 2011). Resident cutaneous microbes work together with the host's innate immune system to provide a first line of defense against invading pathogens, such as *Bd* (Becker and Harris, 2010). Bacterial symbionts isolated from amphibian skin can inhibit *Bd* growth through the production of anti-fungal compounds (Harris et al., 2006; Brucker et al., 2008a,b; Flechas et al., 2012; Woodhams et al., 2015); however, inhibitory strength of bacterial metabolites can differ among *Bd* genotypes (Antwis et al., 2015). Furthermore, the addition of particular bacterial species, such as *Janthinobacterium lividum,* to the skin of amphibians can increase host survival by reducing the burden of chytridiomycosis (Harris et al., 2009a,b; Vredenburg et al., 2011; Kueneman et al., 2016).

In a study of amphibians from the western USA, population persistence through the emergence of *Bd* has been linked to the proportion of amphibians with *Bd*-inhibitory bacteria residing on their skin (Lam et al., 2010). Lam et al. (2010) propose that a mechanism analogous to herd immunity may, in part, explain variation in population persistence when *Bd* emerges. This concept states that when a given percentage of the population is immunized or protected against a communicable disease, the disease will die out and the population will persist. This critical threshold is a function of an intrinsic property of the pathogen—its reproductive rate (R0). For several amphibian populations and communities, a herd immunity threshold of 80% appears to be a consistent cut-off, below which populations crash when the pathogen emerges, and above which populations persist in coexistence with *Bd* (Woodhams et al., 2007; Lam et al., 2010; Figure S1). Interestingly, for many human diseases, the herd immunity threshold percentage is also around 80% (Anderson and May, 1985; Fine, 1993; Gonçalves, 2008; Fine et al., 2011). From this, a hypothetical model can be derived for further testing: if 80% of amphibian individuals maintain at least one strongly *Bd*-inhibitory bacterium on their skin, the population may persist and coexist with *Bd*.

Madagascar is a hotspot for biodiversity conservation, home to more than 400 frog species, most of which are found nowhere else in the world (Vieites et al., 2009; Perl et al., 2014). Ecological niche modeling suggests that the eastern rainforest of Madagascar is highly suitable for *Bd* (Lötters et al., 2011) and it has a higher amphibian species richness compared to the more arid west (Brown et al., 2016). Until recently, Madagascar was considered naïve to *Bd*, but this pathogen has recently been detected in samples from multiple locations across Madagascar (Vredenburg et al., 2012; Weldon et al., 2013; Bletz et al., 2015). The lineage of *Bd* that is present in Madagascar has not yet been characterized, nor is its virulence known. The potential risk of *Bd*-associated declines is presumed high; however, there is essentially nothing known about the resident bacteria on Madagascar frogs and the role they may have in the hosts' defense against *Bd*. Probiotic therapies have been proposed as a possible disease mitigation strategy for combating chytridiomycosis (Bletz et al., 2013; Walke and Belden, 2016; Woodhams et al., 2016); therefore, culturing and characterizing the function of microbes from amphibian skin works toward a possible mitigation strategy against *Bd*.

We collected samples from over 500 Malagasy frogs from 14 locations, and cultured, sequenced, and characterized the *Bd*-inhibiting functional capacity of over 3,000 bacterial isolates in order to address the following questions: (1) Are bacteria residing on Madagascar frogs able to inhibit *Bd*?, (2) What is the phylogenetic distribution of cultured isolates and does *Bd*-inhibitory function correlate with bacterial phylogeny?, and (3) How does functional capacity of bacteria against *Bd* vary among different *Bd* genotypes? In addition, we use these functional data to estimate *Bd*-associated risk of amphibian communities based on their bacterial defense following the model proposed in Lam et al. (2010). Thus, we ask the questions, (4) Are certain amphibian communities in Madagascar likely to be at risk of developing chytridiomycosis based on their bacterial defense? and (5) Are particular host genera likely to be at risk of developing chytridiomycosis based on their bacterial defense? While bacterial defense makes up only one component of a host's defense against *Bd*, this approach can provide a step toward understanding *Bd*-associated risk for the endemic frogs of Madagascar.

METHODS

Field Sampling

Field sampling took place during three field visits: 14 August–12 September 2013, 4 January–9 February 2014, and 5 November–15 December 2014. In total, 540 culturable skin microbe samples were collected from 14 localities and 93 different host species (**Figure 1**).

Amphibians were captured during day and night surveys with clean nitrile gloves and were placed in sterile Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA). For skin microbe sampling, individuals were removed from the bag with a clean pair of nitrile gloves and were rinsed with 50 ml of filter- or UV-sterilized water. After rinsing, individuals were swabbed with 10 strokes on the ventral abdomen, 5 strokes on each ventral thigh, and 5 strokes on each foot using sterile rayon swabs (MW113, Medical Wire Equipment & Co. Ltd., Corsham, UK). Swabs were stored in microcentrifuge tubes containing 100–200 ul of Tryptic-Soy-Yeast-Extract + 20% Glycerol (TSYE+G) and were transported on ice (∼4–10°C) until transfer to a −20°C freezer. Frogs were immediately released at the location of capture after sampling. This study was approved by the Institutional Animal Care and Use Committee of James Madison University (protocol #A01-15), and necessary research and access permits were obtained from the Malagasy Direction Générale des Forêts (DGF) and Madagascar National Parks for all sampling.

Bacterial Culturing

Samples were thawed, gently vortexed, and 25 µl of the TSYE+G storage solution was plated on 1% tryptone agar. While using only one culture medium may limit the diversity of bacteria cultured, the one used represents a general low nutrient medium that supports a wide variety of microorganisms, and was used, in part, because it is also a standard one used for culturing *Bd*. Plates were incubated at 21°C for 2 weeks, and were checked every 3 days for morphologically distinct bacterial colonies. For each sample, each morphologically distinct colony was isolated into pure culture, and subsequently was cryopreserved in TSYE+G solution for later *Bd*-growth inhibition testing and 16S rRNA sequencing.

Bd-Growth Inhibition Assays

A *Bd* isolate from the Global Pandemic Lineage (GPL), JEL 423, was used to characterize function of all bacterial isolates. In addition, a subset of 77 isolates (all from Isalo, Madagascar) were tested against a panel of *Bd* isolates, including four GPL isolates from different regions of the world (USA, Panama, Africa, and Australia), as well as one isolate endemic to Brazil, one isolate endemic to Switzerland, and one isolate endemic to Korea (**Table 1**). Bacterial cell-free supernatant (CFS) obtained from a single liquid culture of each isolate was used for testing against all *Bd* genotypes. Each isolate was tested for its functional capacity against *Bd* using the 96-well assay method described in Bell et al. (2013) and Becker et al. (2015). Briefly, *Bd* zoospores were collected by flooding 3–5 day-old plate cultures with 1% tryptone, allowing zoospores to be released from mature sporangia into the tryptone media. *Bd* zoospores (2×10^6) were grown in the presence of the CFS of each bacterial isolate in triplicate. Bacterial CFS was obtained by filtering a liquid culture grown in co-culture with *Bd* for 3 days on a shaker (250 rpm), through a 0.22 um filter. The following controls were included with each assay in triplicate: (1) positive control–1% tryptone + *Bd* zoospores; (2) nutrient-depleted control–sterile water + *Bd* zoospores; (3) heat-killed control–heat-killed *Bd* zoospores + 1% tryptone; and (4) negative control–1% tryptone only. Assay plates were incubated at 21°C, and growth was measured as optical density (OD) at 492 nm on a spectrophotometer on days 0, 3, and 7.

Bacterial Sequencing and Identification

DNA was extracted from bacterial isolates using one of three methods: (1) PrepMan Ultra (ThermoFisher Scientific, Waltham,

	Year	# of individuals	# of species	# of bacterial isolates
Farankaraina	2013	53	18	233
Maroantsetra	2013	10	1	84
Nosy Mangabe	2013	25	11	122
Ambohitantely	2014(JF)	17	12	45
Fierenana	2014(JF)	10	5	39
Andasibe	2013, 2014(JF)	125	28	722
Mitsinjo Breeding center	2014(JF)	9	1	45
Torotorofotsy–Prolemur Camp	2013, 2014(JF)	53	17	364
Torotorofotsy –*Mantella* Site	2014(JF)	10	1	104
Vohimana	2014(JF)	43	16	250
Ambatolampy	2013	14	2	115
Ankaratra	2013	36	5	235
Antoetra	2014(JF)	21	8	73
Ranomafana–Vatoharanana	2013, 2014(JF)	74	28	363
Ranomafana–Vohiparara	2014(JF)	30	12	290
Isalo	2014(ND)	15	15	2

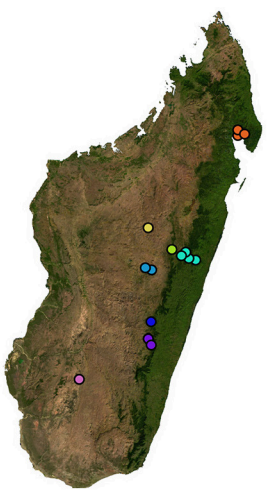

FIGURE 1 | Sampling locations and sample sizes across Madagascar throughout the sampling period. Mitsinjo Breeding center is located in Andasibe (no additional point has been added for this location on the map). Parenthetical "JF" indicates sampling occurred in January–February 2014, and "ND" indicates sampling occurring in November–December 2014. The base map was obtained from www.worldofmaps.net. No permission is required from the copyright holders for the reproduction of this image. Points on the map were generated using Google Earth Pro and afterwards edited on Adobe Illustrator CS6 (Adobe, 2012).

TABLE 1 | Genotypes of *Bd* used for growth-inhibition assays.

Bd Genotype	Lineage	Region	Isolated from	Isolated by
JEL 423	GPL	Panama	*Phyllomedusa lemur*	Joyce Longcore
JEL 242	GPL	Africa	*Xenopus*	Joyce Longcore
VMV 813	GPL	Georgia (USA)	*Lithobates catesbeianus*	Victoria Vasquez
Aus-*L. leseuri*	GPL	Australia	*Litoria lesueri*	Lee Berger
Bd-Swiss	CH	Switzerland	*Alytes obstetricans*	Trent Garner
KR *Bombina* -323	Korea	South Korea	*Bombina orientalis*	Arnaud Bataille
Brazil-LFT001/10	Brazil	Brazil	*Hylodes ornatus*	Felipe Toledo

MA, USA), (2) Chelex (Bio-rad, Hercules, CA, USA), or (3) MoBio UltraClean Microbial DNA isolation kit (MoBio, Carlsbad, CA, USA). The PrepMan protocol was as follows: suspend bacterial cells in 100 µl of PrepMan Ultra solution; vortex and incubate for 10 min at 100°C; centrifuge for 3 min at max speed; transfer supernatant to clean tube. The Chelex protocol was as follows: suspend bacterial cells in 100 µl of 5% Chelex solution; vortex and incubate for 20 min at 99°C; centrifuge for 2 min at max speed; transfer supernatant to clean tube. For MoBio extractions, the manufacturer's protocol was followed. Different methods were used to maximize cost efficiency and to extract troublesome bacterial cells.

Polymerase Chain Reactions (PCR) were used with the bacterial primers 27F and 907R to amplify part of the bacterial 16S rRNA gene from the extracted DNA of each bacterial isolate. Amplification was verified using gel electrophoresis, and each isolate was sequenced either using an in-house capillary sequencer (ABI 3130xl) or was sent for sequencing to LGC Genomics in Berlin, Germany. Sequencing produced approximately 500–800 bp for each bacterial isolate. Sequences were cleaned and a preliminary alignment was completed in order to trim to approximately equal lengths (∼500–600 bp) in CodonCode Aligner. Trimmed sequences were then aligned with PyNAST in QIIME, and a phylogenetic tree was built using fasttree (Price et al., 2010). The resulting tree was visualized using the Interactive Tree of Life tool (Letunic and Bork, 2007). Taxonomy was assigned to each bacterial isolate with the Ribosomal Database Project Classifier using QIIME (Wang et al., 2007; Caporaso et al., 2010). Sequences were deposited in GenBank (accession numbers GenBank MF523799–MF526895).

Data Analysis

For each tested bacterial CFS, the proportional *Bd* growth was determined by dividing the slope (OD/Time) of *Bd* growth in the presence of a given bacterial CFS by the slope of *Bd* growth in the nutrient-depleted control. Using the nutrient depleted control represents the effect of bacterially-secreted metabolites on *Bd* growth while accounting for the potential effect on growth due to additional nutrients in the culture medium added into the positive control (Bell et al., 2013). This value was subtracted from 1 to obtain a proportional inhibition score for each isolate. Triplicates of each tested bacterial isolate were averaged to obtain a mean inhibition score.

Mantel correlations were used to test the phylogenetic independence of mean inhibition scores. More specifically, distance matrices of the patristic distances between bacterial isolates were compared to distances derived from mean inhibition scores of each isolate. Using the inferred phylogenetic tree, patristic distances between bacterial isolates were calculated with the ape and adephylo packages in R (Paradis et al., 2004; Jombart et al., 2010; R Core Team, 2016). Euclidean distances between mean inhibition scores were calculated in QIIME with the distance_matrix_from_mapping.py script. Mean inhibition was compared across bacterial orders using Kruskal-Wallis tests because the data could not be normalized. Two-way analysis of variance (ANOVA) was used to compare bacterial inhibition of *Bd* across *Bd* genotypes. *Bd* genotype and bacterial isolate ID were the main factors.

To apply the herd immunity model proposed in Lam et al. (2010), the following steps were taken. First, bacterial isolates exhibiting inhibition scores greater than 0.8 (i.e., reduced *Bd* growth by 80%) were considered "inhibitory." This threshold was chosen because it represents a strong reduction in *Bd* growth, and similar thresholds have been used in other studies (e.g., Becker et al., 2015); note that this value is not related to the threshold in the herd immunity concept associated with the R0, which coincidentally is also 80% (see below). Second, each individual amphibian was classified as protected or not-protected based on the existence of at least one *Bd*-inhibitory isolate cultured from its skin. Next, the proportion of "protected" individuals was determined (1) for each sampled amphibian community (i.e., location) with more than 10 individuals sampled, and (2) for each host genera at two high diversity sites, Andasibe and Ranomafana (that is, the proportion of protected individuals was calculated considering all individuals within a given genus at the particular site). We estimated herd immunity from both the "community" and "host genera" perspective to address it from two scales: (1) a larger scale community framework, and (2) a finer scale examining specific host taxonomic groups within locations. Both frameworks were implemented because they carry different inherent assumptions. For example, variation in protection at the genus or species level could affect community level protection dynamics. For our estimates, we applied the hypothesis of an 80% herd immunity threshold, i.e., we considered a group of amphibians as protected by herd immunity if 80% of the individuals had at least one strongly *Bd*-inhibitory bacterium on their skin. It

is important to note that community level investigations make assumptions about potential pathogen transmission dynamics in that they assume contact would be equally likely within or across species (i.e., spatial and contact homogeneity). While contact rates within a species are undoubtedly higher than across species, interspecies or inter-genera contact can be expected to occur frequently given the high spatial overlap and sympatry of amphibians in hyper-diverse locations (i.e. multiple species inhabit small microhabitat areas at relatively high densities). During breeding season when amphibian species congregate at water bodies inter-species contact could be even more probable. In this context, a community level framework can be seen as valid and informative.

RESULTS

From the 540 sampled individuals, 3179 bacterial isolates were cultured and were successfully tested in *Bd*-growth inhibition assays. On average, 7.5 bacterial morphotypes were collected per frog. The cultured isolates were predominantly from the phylum Actinobacteria (57.3%) followed by Proteobacteria (27.1%, [Alpha-59.3%, Gamma-31.2%, Beta-9.4%]), Firmicutes (9.6%), and Bacteriodetes (5.2%) (**Figure 2**). Inhibitory isolates (reducing *Bd* growth by 80% or more) were identified in all four bacterial phyla represented in the data as well as in all the represented bacterial families (Figure S2).

Mean inhibition was weakly correlated with bacterial phylogeny (Mantel test- $R = 0.04$ $p = 0.01$). Furthermore, mean inhibition varied significantly across bacterial orders (KW chi-squared = 438.2, $df = 16$, p-value < 0.001, **Figure 3**). The orders Caulobacterales, Burkholderiales, Enterobacteriales, Pseudomonadales, Xanthamonadales, and Flavobacteriales consistently exhibited stronger inhibition than the other represented bacterial orders (**Figure 3**, **Table 1**). Mean inhibition also varied significantly at the family level (KW chi-squared = 675.2, $df = 56$, p-value < 0.001). In some bacterial families, the majority of the isolates were classified as inhibitory, including Caulobacteraceae (102/140), Weeksellaceae (60/83), Enterobacteriaceae (47/57), Pseudomonadaceae (18/24), and Xanthamondaceae (80/100), while other families had only a few inhibitory isolates, including Rhizobiaceae (7/39), Methylobacteriaceae, (4/70) and Staphylococcaceae (6/75) (Figure S2).

Testing of a subset of 77 bacterial isolates cultured from two frog species in Isalo (Mantella expectata: $n = 57$, Scaphiophyrne gottlebei: $n = 20$) showed that inhibition across *Bd* genotypes was not consistent (**Table 2**). Bacterial inhibition of *Bd* varied significantly among *Bd* genotypes [ANOVA–$F_{(6, 76)} = 15.46$, $p <$ 0.001, Table S1).

We determined the proportion of protected individuals within each amphibian community (i.e., each sampled locations) and within each host genus at Andasibe and Ranomafana by defining an individual as "protected" if at least one of its cultured isolates was classified as inhibitory. Proportion of protected individuals differed across locations, ranging from 57 to 100%. Locations predicted to be unprotected included Farankaraina, Ambohitantely, Andasibe, Torotorofotsy, Antoetra, and Ranomafana-Vatoharanana, while Nosy Mangabe, Maroantsetra, Ankaratra, Ambatolampy, Ranomafana-Vohiparara, and Isalo were predicted to be protected (**Figure 4**). Proportion of protected individuals also varied among host genera at both selected sites. In Ranomafana, our predictions suggest that *Boophis*, *Mantidactylus*, and *Platypelis* fall below the herd immunity threshold, while *Gephyromantis* meets this threshold. In Andasibe, *Boophis*, *Mantidactylus*, *Spinomantis*, and *Heterixalus* fell below the herd immunity threshold, while the genera *Aglyptodactylus, Blommersia*, and *Ptychadena* surpassed this threshold (**Figure 5**).

DISCUSSION

Phylogenetic and Taxonomic Distribution of *Bd*-Inhibitory Function

Bd inhibition by bacterial isolates derived from the skin of Malagasy amphibians was widespread across the bacterial phylogenetic tree, but mean inhibition was weakly correlated with bacterial phylogeny, suggesting that anti-*Bd* function may be at least in part phylogenetically conserved. This finding differs from that of a Panamanian frog skin bacteria study where inhibition was not correlated with bacterial phylogeny (Becker et al., 2015). The correlation herein was rather weak (Mantel R statistic = 0.04, but p-value of 0.01); therefore, bacterial phylogeny is likely not the main driver of inhibitory function against *Bd*, which could be associated with the highly flexible genomes of bacteria (Fuhrman, 2009). Bacterial genes can be readily transferred via horizontal gene transfer (Smillie et al., 2011). In fact, different bacterial species have been observed to transfer genes encoding for antifungal compounds (Ravel et al., 2000). Different bacterial taxa are also known to produce the same anti-fungal compounds, and this is even the case for known *Bd*-inhibitory compounds. For example, 2,4 DAPG is produced by both *Pseudomonas* (Pseudomonadaceae) and *Lysobacter* (Xanthamonadaceae), and violacein is produced by multiple taxa spanning across different bacterial genera including *Janthinobacterium*, *Collimonas*, *Duganella*, *Pseudoalteromonas*, and *Microbulbifer* (Brucker et al., 2008b; Choi et al., 2015).

Inhibitory taxa were found within all major bacterial orders and families which mirrors the findings of other studies (Harris et al., 2006; Woodhams et al., 2007; Flechas et al., 2012; Becker et al., 2015). Despite the fact that *Bd* inhibition was documented across nearly all taxonomic groups, certain bacterial groups appear to be composed of mainly inhibitory isolates [e.g., Caulobacteraceae (72%), Weeksellaceae (73%), Enterobacteriaceae (82%), Pseudomonadaceae (80%), and Xanthamondaceae (80%)] which might be responsible for the weak phylogenetic effect found in our overall data set. Many of these groups have been identified to have high proportions of inhibitory isolates in other amphibian studies as well (Becker et al., 2015). Pseudomonads, in particular, are well-documented in other systems including plants, crustaceans, fish, and bats as having pathogen-inhibiting effects (Spanggaard et al., 2001; Ramette et al., 2003, 2011; Balcázar et al., 2007; Kim et al., 2007; Cheng et al., 2016). Additionally, it is important to note that *in vitro* assays do not directly indicate *in vivo* function; other biotic and abiotic factors can influence the functional behavior of bacteria on amphibian skin.

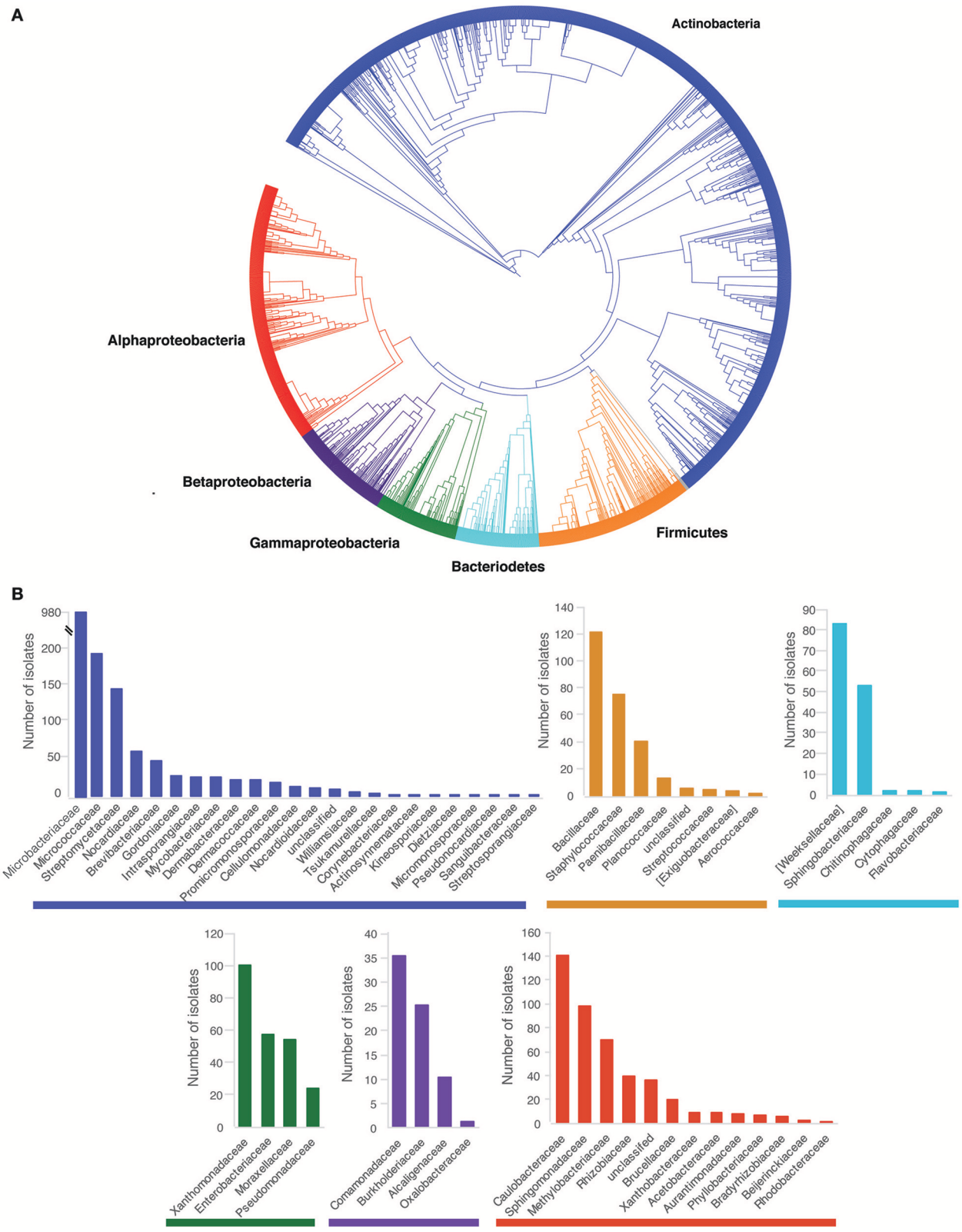

FIGURE 2 | Phylogenetic and taxonomic distribution of cultured isolates. **(A)** Phylogenetic tree of cultured isolates based on DNA sequences of the 16S rRNA gene (~500–600 bp in length). **(B)** Number of cultured isolates within each family of each phylum. Color coding corresponds to bacterial phyla.

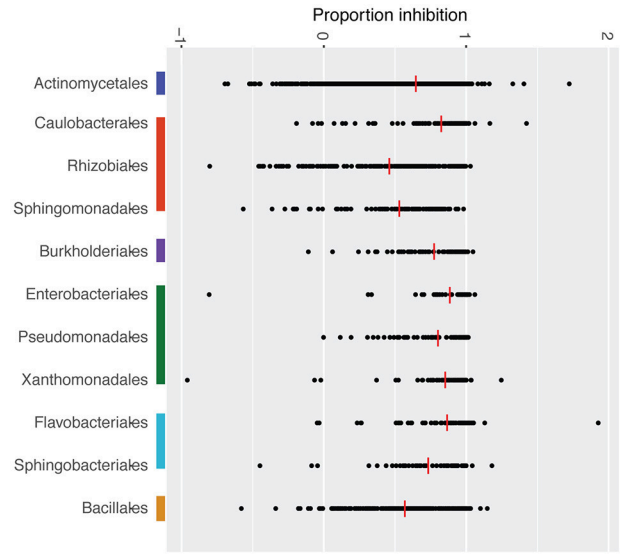

FIGURE 3 | Mean *Bd* inhibition for bacterial isolates within each dominant order. Each black point represents the mean inhibition of a given bacterial isolate, and the red bars represent the mean inhibition score for each order. On the horizontal axis: 1 equals complete inhibition of *Bd* growth; 0 equals no inhibition; values less than 0 indicate facilitation of *Bd* growth. Color bars beside each order name correspond to bacterial phyla in **Figure 2**.

Estimating *Bd*-Associated Risk based on Bacterial Defense

The functional characterization of the resident bacteria's ability to inhibit *Bd* was used to estimate the potential risk or susceptibility to *Bd*-associated declines in the context of the herd-immunity model proposed by Lam et al. (2010). This model is based on results from the *Rana muscosa*/*sierrae* system in the USA and from consideration of the concept of herd immunity in other systems. A population found co-existing with *Bd* had 80% of individuals with at least one *Bd*-inhibitory isolate, and a population below this 80% threshold was declining once *Bd* emerged in the population (Woodhams et al., 2007). In addition, a naïve population that met the 80% threshold did not go extinct while other naïve populations in this region went extinct (Lam et al., 2010). Several additional studies support this model (Figure S1).

While predictions within this framework provide an integrative look at how protection provided by bacteria varies across amphibian taxa and locations in Madagascar, it is important to note the following limitations of the model: (i) only the culturable community is considered, (i) bacterial interactions (antagonistic or synergistic) are not considered, (iii) the bacterial function assessment is based on high density *in vitro* testing (i.e., the hypothesis of antifungal activity does not take the *in vivo* density of a specific bacterium into account), and (iv) predictions are based on one GPL *Bd* isolate only. In general, dominant bacteria associated to the amphibian skin can be cultured with common techniques (Walke et al., 2015). To further support this within our study system, a comparison with data from Illumina-based sequencing of bacterial 16S amplicons from the skin of Malagasy amphibians (Bletz et al., 2017) suggests that a relatively high proportion of the dominant community members are represented among the cultured isolates [75% of top 80 illumina OTUs were present in the cultured isolates (Bletz, personal observation)]. Nevertheless, because additional (uncultured) members of the community might also inhibit *Bd*, our assessment of the number of protected individuals is conservative, and more individuals than estimated may have at least one *Bd*-inhibiting bacterium on their skin.

Our predictions based on the herd immunity model suggest that risk of developing chytridiomycosis varies across the landscape in Madagascar. Amphibian communities at some locations appear protected (above 80%), while others fall below this herd-immunity threshold. Locations predicted to be unprotected included Farankaraina, Ambohitantely, Andasibe, Torotorofotsy, Antoetra, and Ranomafana-Vatoharanana, while Nosy Mangabe, Maroantsetra, Ankaratra, Ambatolampy, Ranomafana-Vohiparara, and Isalo appear to be protected. There is no clear biogeographical or ecological pattern in the protected vs. unprotected categories; both contain localities from low-, mid-, and high-elevations, from eastern humid regions, and from drier regions of the central plateau (Brown et al., 2016). However, the fact that locations like Andasibe, Torotorofotsy, and Ranomafana-Vatoharanana are predicted to be at risk is particularly concerning considering these are all hyperdiverse mid-high elevation rainforest sites also predicted by ecological niche modeling to be highly suitable for *Bd* (Lötters et al., 2011). These sites are ecologically similar to places in Central America where drastic populations declines have occurred (La Marca et al., 2005; Lips et al., 2006). In addition, locations, such as Ambohitantely and Antoetra, are home to critically endangered species (*Anodonthyla vallani* and *Mantella cowani*, respectively) that have restricted distributions. It is important to note that our community predictions are based of variable numbers of species within the sampled locations and protection may vary non-randomly across host species or genera (see below); thus, these results should be taken as a preliminary look and continued research is needed to investigation complex community–infection dynamics that may occur in diverse amphibian assemblages.

Proportions of protected individuals also differed across host genera in Andasibe and Ranomafana, suggesting that Bd-associated risk would not be equal across amphibian hosts. Our predictions suggest that *Boophis*, *Mantidactylus*, *Platypelis*, *Spinomantis*, and *Heterixalus*, may be more at risk, at least at the sampled locations, while the genera *Aglyptodactylus, Blommersia*, *Guibemantis, Gephyromantis*, and *Ptychadena* are predicted to be protected. Interestingly, all of the potentially protected species, except *Gephyromantis,* are pond-breeding species, whereas the majority of *Boophis* and *Mantidactylus*, as well as all *Spinomantis*, are stream breeders. In general, stream-breeding amphibians are considered more susceptible to chytridiomycosis (Stuart et al., 2004). We hypothesize that genera such as *Aglyptodactylus, Blommersia, Guibemantis,* and *Ptychadena* (e.g., the pond breeders) might be protected against this disease by two mechanisms: (1) due to a high-proportion of individuals possessing *Bd*-inhibiting cutaneous

TABLE 2 | Mean *Bd* inhibition (1 = 100% inhibition of *Bd* growth) across multiple genotypes of *Bd* for the 57 bacterial isolates cultured from *Mantella expectata*. Isolates from *Scaphiophryne gottlebei* are not shown.

Isolate ID	JEL 242 (GPL)	JEL423 (GPL)	UMV813 (GPL)	Aus- *L. leseuri* (GPL)	BdBrazil (Brazil)	KR *Bombina* -323 (Korea)	BdSwiss (Swiss)
4260	0.153	0.438	0.448	-0.594	0.706	0.897	0.687
4261	0.547	0.848	0.697	0.723	1.253	0.837	0.415
4264	0.586	0.869	0.846	0.946	0.977	0.812	0.482
4265	0.44	0.813	0.292	0.906	1.027	0.627	0.634
4268	1.002	0.903	0.748	0.858	1.013	0.878	0.597
4271	0.983	1.01	0.798	0.966	0.914	0.828	0.945
4272	0.999	0.979	0.899	1.031	1.061	0.526	0.643
4273	0.078	0.463	0.098	-0.258	0.848	0.727	0.152
4274	1.008	0.943	0.843	0.884	1.053	0.768	0.556
4275	0.606	0.418	0.191	1.015	0.98	1.038	0.937
4276	0.983	0.858	0.765	0.718	1.021	0.838	0.507
4277	0.98	0.927	0.774	0.857	1.049	0.834	0.499
4278	0.951	0.971	0.692	0.746	0.921	0.832	0.542
4279	0.942	1.027	0.748	0.937	0.934	0.826	0.596
4280	0.893	1.024	0.834	0.324	0.993	0.955	0.947
4281	0.995	0.913	0.942	0.903	1.027	0.896	0.594
4283	0.988	1.018	1.002	0.951	1.047	0.806	0.492
4285	0.895	0.863	0.901	0.619	0.997	0.832	0.441
4286	0.955	0.933	0.908	0.902	0.982	0.84	0.511
4288	0.994	1.022	0.875	0.645	0.931	0.845	0.53
4289	0.86	1.007	0.901	1.026	0.956	0.875	0.59
4290	1.004	1.024	0.871	0.953	0.772	0.959	0.937
4291	-0.641	0.42	0.536	0.1	0.547	0.922	0.796
4292	-0.029	0.255	0.154	-0.965	-1.075	0.321	-0.772
4293	0.142	0.352	0.102	-1.964	-0.052	0.661	0.492
4294	-0.047	0.015	0.292	-1.44	-0.557	0.365	-0.12
4295	0.702	0.342	0.075	0.667	0.312	0.808	0.879
4298	0.978	0.925	0.658	0.999	1.032	1.006	0.949
4301	0.889	1.025	0.653	0.929	1.007	0.992	0.915
4304	0.031	0.528	-0.092	0.891	0.291	0.53	0.688
4305	0.931	0.913	0.685	0.985	1.01	1.019	0.918
4306	1.017	1	0.703	1.015	1.023	1.098	1.001
4307	1.008	1	0.761	1.022	1.004	1.008	0.949
4308	0.996	1.012	0.71	1.012	0.978	1.042	0.948
4309	0.989	1.024	0.898	0.986	0.902	0.978	0.972
4310	0.928	1.03	0.459	1.002	1.084	0.958	1.004
4311	0.977	1.036	0.892	1.008	1.01	1.008	0.977
4312	1.018	0.954	0.869	1.007	1.046	0.757	0.988
4313	1.021	0.976	0.866	1.016	1.049	1.01	0.986
4314	0.951	0.867	0.742	1.011	1.027	1.004	0.932
4316	-0.779	0.966	0.898	0.99	1.015	0.997	0.961
4318	0.546	0.452	0.119	0.843	0.62	0.748	0.622
4320	-0.016	0.353	-0.126	0.394	0.12	0.617	0.071
4322	0.951	0.919	0.887	1.014	0.944	1.016	0.976
4323	0.94	0.92	0.905	1.045	0.988	0.962	0.953
4325	1.019	0.762	0.881	-0.124	0.854	0.454	0.925
4326	1.025	0.705	0.933	-0.187	0.754	0.857	0.616
4328	0.947	0.394	0.897	-0.097	0.714	0.989	0.892
4329	0.986	0.691	0.927	0.058	0.884	0.797	0.908
4330	1.049	0.81	0.902	0.328	0.916	1.012	0.998
4331	1.013	0.855	1.022	0.517	1.112	1.043	0.875
4332	0.927	0.751	0.891	0.916	1.128	1.08	0.977
4333	1.033	0.659	0.92	0.202	0.812	1.091	1.003
4334	0.973	0.44	0.887	0.132	0.654	0.957	0.955
4335	0.766	0.541	0.384	0.027	0.694	0.93	0.942
4336	0.989	0.597	0.919	-0.155	0.739	1.005	0.918
4339	0.677	0.514	0.824	-0.496	0.859	0.225	0.753

Gradient of blue-red corresponds to increasing Bd inhibition, with red indicating strong inhibition.

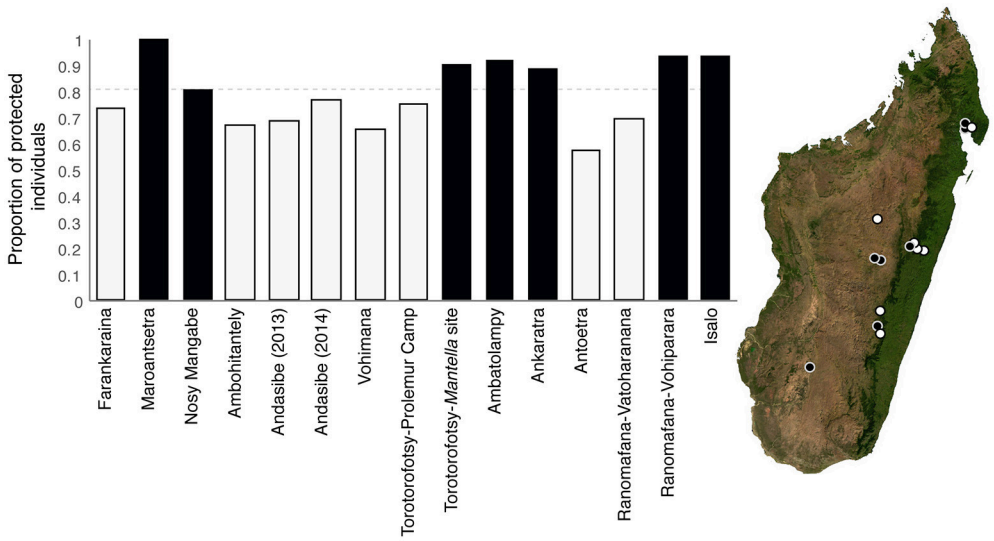

FIGURE 4 | Proportion of "protected" individuals across amphibian communities in Madagascar. Black coloring denotes location that meet or surpass the herd immunity threshold of 80% (i.e., predicted to be protected), and white coloring denotes locations that are below this threshold (i.e. predicted to be at risk). Dotted line represents the herd immunity threshold (80%). Map on the right shows the distribution of "protected" and "unprotected" locations across Madagascar. The base map was obtained from www.worldofmaps.net. No permission is required from the copyright holders for the reproduction of this image. Points on the map were generated using Google Earth Pro and afterwards edited on Adobe Illustrator CS6 (Adobe, 2012).

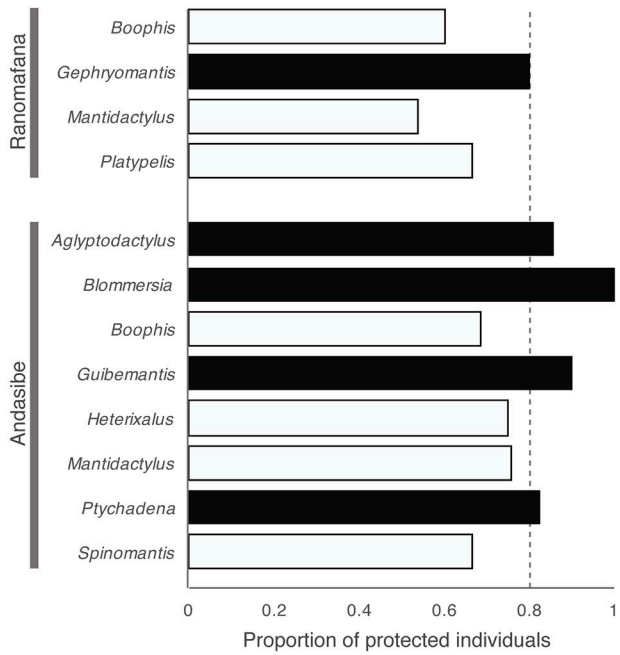

FIGURE 5 | Proportion of "protected" individuals across host genera at two hyperdiverse sites in Madagascar. Black coloring denotes genera that meet or surpass the herd immunity threshold of 80% (i.e., predicted to be protected), and white coloring denotes genera that are below this threshold (i.e., predicted to be at risk). Dotted line represents the herd immunity threshold (80%).

bacteria, and (2) due to their microhabitat preferences, which include periodic stays in or near warm, stagnant water bodies that do not provide suitable conditions for survival of *Bd* (Kriger and Hero, 2007; Forrest and Schlaepfer, 2011). In addition, *Aglyptodactylus* and *Ptychadena*, and partly *Blommersia* and *Gephyromantis*, are ground-dwelling frogs, and perhaps their association with terrestrial habitats increases the abundance of transient and established fungal-inhibiting bacteria on their skin, as soil is known to be a species-rich and functionally diverse environment (Torsvik and Øvreås, 2002).

Toward Probiotics for Malagasy Amphibians

Probiotic therapies have been proposed as a possible disease mitigation strategy for combating chytridiomycosis (Bletz et al., 2013; Walke and Belden, 2016; Woodhams et al., 2016). To establish such therapies, culturing and characterizing function of microbes from amphibian skin is an important first step. One of the main objectives of this research was to evaluate the functional capacity of bacteria isolated from Madagascar frogs against *Bd*, and to determine whether *Bd*-inhibitory taxa are present. Indeed, inhibitory bacterial taxa were cultured: 39% (1241 isolates) of the cultured isolates inhibited *Bd* by at least 80%, and 26% (829 isolates) inhibited *Bd* by at least 90%. These strongly inhibitory taxa can all serve as potential probiotic candidates for Madagascar's frogs if a lethal *Bd* genotype arrives in Madagascar. *Bd*-inhibition was found herein and in other studies (Antwis et al., 2015) to vary across *Bd* genotypes; that is, not all bacteria could consistently inhibit a panel of *Bd* genotypes, which has important implications for development of probiotic disease mitigation strategies. Ideal probiotics will be bacterial isolates that do demonstrate broad spectrum *Bd*-inhibitory function; that is, they have high

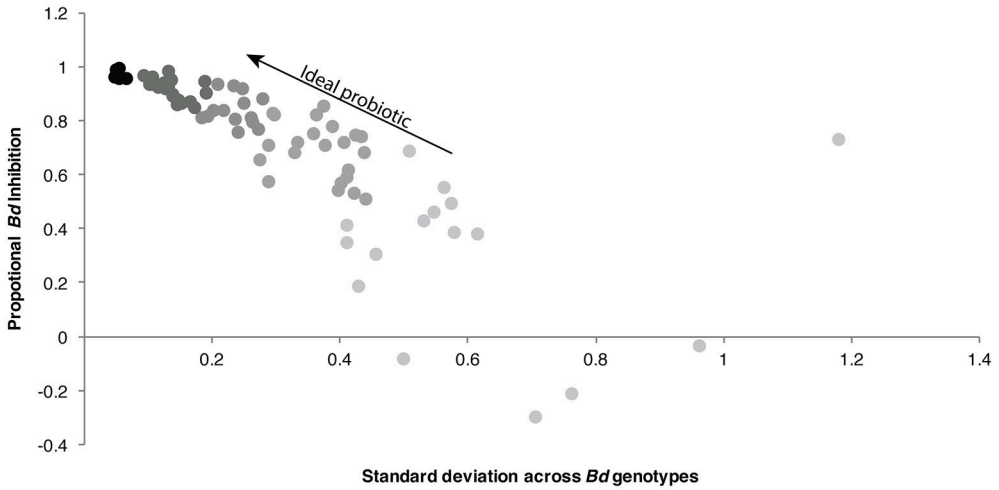

FIGURE 6 | Selecting bacterial isolates with functional consistency across *Bd* genotypes. Scatterplot displays mean inhibition vs. standard deviation of inhibition across *Bd* genotypes. The ideal probiotic candidates will be those with strong inhibitory function and low standard deviation. Points are colored from gray to black to illustrate increasing potential effectiveness as a probiotic.

inhibition scores (>80%) across *Bd* variants, and have a low standard deviation across replicates (**Figure 6**). While function across *Bd* genotypes varied for the bacterial isolates tested, there were some bacterial isolates with broad-spectrum *Bd*-inhibitory function, such as a *Chryseobacterium trutae*, a *Elizabethkingia miricola*, a *Pedobacter nutrimenti*, and a *Delftia acidovorans* (Table S2).

These results serve as a basis for continued development of probiotic disease mitigation strategies for the frogs of Madagascar by providing a bank of potential probiotics. In addition, they provide an initial estimate of *Bd*-associated risk across Madagascar, which can facilitate prioritization of locations and host genera that appear to be more at risk. It is important to acknowledge that bacterial defense is only one component of a host's defense against *Bd*; therefore, our predictive approach must be taken as preliminary hypothesis, and as a stimulus for future research on *Bd*-associated risk for the frogs of Madagascar. Continued research on host protection (bacterial and host-produced defenses) against disease is needed to improve our understanding of disease risk across the landscape in Madagascar and help inform integrative conservation management planning. Future research should continue along the probiotic selection steps outlined in Bletz et al. (2013) by working toward identifying which *Bd*-inhibitory taxa can colonize and persist on frog hosts. Bioaugmentation as a mitigation tool requires a deeper understanding of bacterial community assembly and stability in the context of the amphibian host community and their skin secretions (Garner et al., 2016), which will be an important part of future research. Before probiotics can be widely implemented as a long-term management strategy for wild amphibian populations broader aspects including the potential risk probiotics pose to ecosystems and public health must been assessed (Woodhams et al., 2016). However, provided that candidate bacteria meet the necessary criteria, bioaugmentation could be far more cost-effective, ethical and less controversial than the current alternative treatment, namely chemicals (Garner et al., 2016). Continued probiotic research will bring us one step closer to an integrative probiotic approach for mitigating possible *Bd*-associated declines in Madagascar.

AUTHOR CONTRIBUTIONS

MB and RH designed project with significant input from FR, CW, DE, and MV. MB, FR, AR, CW, DE and RH conducted sampling. MB, JM, and AR performed laboratory work. MB completed data analysis and wrote the paper. All authors contributed to revision of the manuscript and have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01751/full#supplementary-material

REFERENCES

Anderson, R. M., and May, R. M. (1985). Vaccination and herd immunity to infectious diseases. *Nature* 318, 323–329. doi: 10.1038/318323a0

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. (2015). Amphibian symbiotic bacteria do not show universal ability to inhibit growth of the global pandemic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/AEM.00010-15

Balcázar, J. L., Rojas-Luna, T., and Cunningham, D. P. (2007). Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*. *J. Invertebr. Pathol.* 96, 147–150. doi: 10.1016/j.jip.2007.04.008

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS ONE* 5:e10957. doi: 10.1371/journal.pone.0010957

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.* 103, 77–85. doi: 10.3354/dao02560

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Bletz, M. C., Archer, H., Harris, R. N., McKenzie, V., Rabemananjara, F. C. E., Rakotoarison, A., et al. (2017). Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Front. Microbiol.* 8:1530. doi: 10.3389/fmicb.2017.01530

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bletz, M. C., Rosa, G. M., Crottini, E. A., Andreone, F., Schmeller, D. S., Rabibisoa, N. H. C., et al. (2015). Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. *Sci. Rep.* 5:8633. doi: 10.1038/srep08633

Brown, J. L., Sillero, N., Glaw, F., Bora, P., Vieites, D. R., and Vences, M. (2016). Spatial biodiversity patterns of Madagascar's amphibians and reptiles. *PLoS ONE* 11:e144076. doi: 10.1371/journal.pone.0144076

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43. doi: 10.1007/s10886-007-9352-8

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008b). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Cafaro, M. J., Poulsen, M., Little, A. E. F., Price, S. L., Gerardo, N. M., Wong, B., et al. (2011). Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. *Proc. Biol. Sci.* 278, 1814–1822. doi: 10.1098/rspb.2010.2118

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., and Costello, E. K. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cheng, T. L., Mayberry, H., McGuire, L. P., Hoyt, J. R., Langwig, K. E., Nguyen, H., et al. (2016). Efficacy of a probiotic bacterium to treat bats affected by the disease white-nose syndrome. *J. Appl. Ecol.* 54, 701–708. doi: 10.1111/1365-2664.12757

Cheng, T. L., Rovito, S. M., Wake, D. B., and Vredenburg, V. T. (2011). Coincident mass extirpation of Neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9502–9507. doi: 10.1073/pnas.1105538108

Choi, S. Y., Yoon, K., Lee, J. I., and Mitchell, R. J. (2015). Violacein: properties and production of a versatile bacterial pigment. *Biomed. Res. Int.* 2015, 1–8. doi: 10.1155/2015/465056

Fine, P. E. (1993). Herd immunity: history, theory, practice. *Epidemiol. Rev.* 15, 265–302. doi: 10.1093/oxfordjournals.epirev.a036121

Fine, P., Eames, K., and Heymann, D. L. (2011). "Herd immunity": a rough guide. *Clin. Infect. Dis.* 52, 911–916. doi: 10.1093/cid/cir007

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus. *PLoS ONE* 7:e44832. doi: 10.1371/journal.pone.0044832

Forrest, M. J., and Schlaepfer, M. A. (2011). Nothing a hot bath won't cure: Infection rates of amphibian chytrid fungus correlate negatively with water temperature under natural field settings. *PLoS ONE* 6:e28444. doi: 10.1371/journal.pone.0028444

Fuhrman, J. A. (2009). Microbial community structure and its functional implications. *Nature* 459, 193–199. doi: 10.1038/nature08058

Garner, T. W. J., Schmidt, B. R., Martel, A., Pasmans, F., Muths, E., Cunningham, A. A., et al. (2016). Mitigating amphibian chytridiomycoses in nature. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 371:1709. doi: 10.1098/rstb.2016.0207

Gonçalves, G. (2008). Herd immunity: recent uses in vaccine assessment. *Expert Rev. Vaccines* 7, 1493–1506. doi: 10.1586/14760584.7.10.1493

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319. doi: 10.1038/nrmicro1129

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., and Frick, W. F. (2015). Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. *PLoS ONE* 10:e0121329. doi: 10.1371/journal.pone.0121329

Jombart, T., Balloux, F., and Dray, S. (2010). Adephylo: new tools for investigating the phylogenetic signal in biological traits. *Bioinformatics* 26, 1907–1909. doi: 10.1093/bioinformatics/btq292

Kim, J., Kim, B., and Lee, C. (2007). Alga-lytic activity of *Pseudomonas fluorescens* against the red tide causing marine alga *Heterosigma akashiwo* (Raphidophyceae). *Biol. Control* 41, 296–303. doi: 10.1016/j.biocontrol.2007.02.010

Krediet, C. J., Ritchie, K. B., Paul, V. J., and Teplitski, M. (2013). Coral-associated micro-organisms and their roles in promoting coral health and thwarting diseases. *Proc. Biol. Sci.* 280:20122328. doi: 10.1098/rspb.2012.2328

Kriger, K. M., and Hero, J. M. (2007). The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. *Divers. Distrib.* 13, 781–788. doi: 10.1111/j.1472-4642.2007.00394.x

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., McKenzie, V. J., et al. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc.* 283.1839:20161553. doi: 10.1098/rspb.2016.1553

La Marca, E., Lips, K. R., Lötters, S., Puschendorf, R., Ibáñez, R., Rueda-Almonacid, J. V., et al. (2005). Catastrophic population declines and extinctions in Neotropical harlequin frogs (Bufonidae: *Atelopus*). *Biotropica* 37, 190–201. doi: 10.1111/j.1744-7429.2005.00026.x

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Letunic, I., and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127–128. doi: 10.1093/bioinformatics/btl529

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in

a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Lötters, S., Rödder, D., Kielgast, J., and Glaw, F. (2011). "Hotspots, conservation, and diseases: Madagascar's megadiverse amphibians and the potential impact of chytridiomycosis," in *Biodiversity Hotspots*, eds F. E. Zachos and J. C. Habel (Berlin; Heidelberg: Springer), 255–274.

Paradis, E., Claude, J., and Strimmer, K. (2004). APE: analyses of phylogenetic and evolution in R language. *Bioinformatics* 20, 289–290. doi: 10.1093/bioinformatics/btg412

Perl, R. G. B., Nagy, Z. T., Sonet, G., Glaw, F., Wollenberg, K. C., and Vences, M. (2014). DNA barcoding Madagascar's amphibian fauna. *Amphibia Reptilia* 35, 197–206. doi: 10.1163/15685381-00002942

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone.0009490

R Core Team (2016). *R: A Language and Environment for Statistical Computing*. Vienna. Available online at: http://www.r-project.org/

Ramette, A., Frapolli, M., Saux, M. F. L., Gruffaz, C., Meyer, J. M., Défago, G., et al. (2011). *Pseudomonas protegens* sp. nov., widespread plant-protecting bacteria producing the biocontrol compounds 2,4-diacetylphloroglucinol and pyoluteorin. *Syst. Appl. Microbiol.* 34, 180–188. doi: 10.1016/j.syapm.2010.10.005

Ramette, A., Moënne-Loccoz, Y., and Défago, G. (2003). Prevalence of fluorescent pseudomonads producing antifungal phloroglucinols and/or hydrogen cyanide in soils naturally suppressive or conducive to tobacco black root rot. *FEMS Microbiol. Ecol.* 44, 35–43. doi: 10.1111/j.1574-6941.2003.tb01088.x

Ravel, J., Wellington, E. M. H., and Hill, R. T. (2000). Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. *Appl. Environ. Microbiol.* 66, 529–534. doi: 10.1128/AEM.66.2.529-534.2000

Sanchez, B., Delgado, S., Blanco-Miguez, A., Lourenco, A., Gueimonde, M., and Margolles, A. (2016). Probiotics, gut microbiota, and their influence on host health and disease. *Mol. Nutr. Food Res.* 201600240, 1–15. doi: 10.1002/mnfr.201600240

Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A., and Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480, 241–244. doi: 10.1038/nature10571

Spanggaard, B., Huber, I., Nielsen, J., Sick, E. B., Pipper, C. B., Martinussen, T., et al. (2001). The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environ. Microbiol.* 3, 755–765. doi: 10.1046/j.1462-2920.2001.00240.x

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Torsvik, V., and Øvreås, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5, 240–245. doi: 10.1016/S1369-5274(02)00324-7

Vieites, D. R., Wollenberg, K. C., Andreone, F., Köhler, J., Glaw, F., and Vences, M. (2009). Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8267–8272. doi: 10.1073/pnas.0810821106

Vredenburg, V. T., Briggs, C. J., and Harris, R. N. (2011). "Host-pathogen dynamics of amphibian chytridiomycosis: the role of the skin microbiome in health and disease," in *Fungal Diseases: an Emerging Threat to Human, Animal, and Plant Health*, eds L. Olson, E. Choffnes, D. Relman, and L. Pray (Washington, DC: National Academy Press), 342–355.

Vredenburg, V. V. T., Preez, L., Raharivololoniaina, L., Vieites, D. R., Vences, M., and Weldon, C. (2012). A molecular survey across Madagascar does not yield positive records of the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *Herpetol. Notes* 5, 507–517. Available online at: http://www.herpetologynotes.seh-herpetology.org/Volume5_PDFs/Vredenburg_Herpetology_Notes_Volume5_pages507-517.pdf (Accessed March 22, 2014).

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog.* 12:e1005796. doi: 10.1371/journal.ppat.1005796

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2015). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *App. Environ. Microbiol.* 81, 6589–6600. doi: 10.1128/AEM.01486-15

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Weldon, C., Crottini, A., Bollen, A., Rabemananjara, F. C. E., Copsey, J., Garcia, G., et al. (2013). Pre-emptive national monitoring plan for detecting the amphibian chytrid fungus in Madagascar. *Ecohealth* 10, 234–240. doi: 10.1007/s10393-013-0869-8

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H. M., Becker, M. H., Belden, L. K., et al. (2015). Antifungual isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595. doi: 10.1890/14-1837.1

Woodhams, D. C., Bletz, M. C., Kueneman, J., and McKenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trends Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

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Fight Fungi with Fungi: Antifungal Properties of the Amphibian Mycobiome

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Emerging infectious diseases caused by fungal taxa are increasing and are placing a substantial burden on economies and ecosystems worldwide. Of the emerging fungal diseases, chytridomycosis caused by the fungus *Batrachochytrium dendrobatidis* (hereafter *Bd*) is linked to global amphibian declines. Amphibians have innate immunity, as well as additional resistance through cutaneous microbial communities. Despite the targeting of bacteria as potential probiotics, the role of fungi in the protection against *Bd* infection in unknown. We used a four-part approach, including high-throughput sequencing of bacterial and fungal communities, cultivation of fungi, *Bd* challenge assays, and experimental additions of probiotic to Midwife Toads (*Altyes obstetricans*), to examine the overlapping roles of bacterial and fungal microbiota in pathogen defense in captive bred poison arrow frogs (*Dendrobates* sp.). Our results revealed that cutaneous fungal taxa differed from environmental microbiota across three species and a subspecies of *Dendrobates* spp. frogs. Cultivation of host-associated and environmental fungi realved numerous taxa with the ability to inhibit or facilitate the growth of *Bd*. The abundance of cutaneous fungi contributed more to *Bd* defense (∼45% of the fungal community), than did bacteria (∼10%) and frog species harbored distinct inhibitory communities that were distinct from the environment. Further, we demonstrated that a fungal probiotic therapy did not induce an endocrine-immune reaction, in contrast to bacterial probiotics that stressed amphibian hosts and suppressed antimicrobial peptide responses, limiting their long-term colonization potential. Our results suggest that probiotic strategies against amphibian fungal pathogens should, in addition to bacterial probiotics, focus on host-associated and environmental fungi such as *Penicillium* and members of the families Chaetomiaceae and Lasiosphaeriaceae.

Keywords: disease ecology, mycobiome, microbiome, chytrid, ITS, 16S rRNA, amphibian, fungal disease

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INTRODUCTION

Amphibians are globally at risk from a range of factors, not least of which are emerging fungal pathogens (Wake and Vrendenburg, 2008; Fisher et al., 2012). The chytridiomycete fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), induces a skin infection in amphibians that disrupts osmotic balance, leading to mortality (Voyles et al., 2009), and has been linked to the extinction or population decline of numerous amphibian species. While chytridiomycosis often has a high mortality rate, species and populations can exhibit variable resistance to *Bd* infection and this differential susceptibility is due in part to skin microbiota (Woodhams et al., 2014).

The microbiome, or the micro-organisms that live on and in organisms, can affect host health by influencing development, behavior, metabolism, and inflammation response (Cho and Blaser, 2012). In amphibians, the bacterial populations associated with the skin can provide protection against *Bd* infection through the production of anti-fungal metabolites and can be considered part of the amphibian's immune system (Bletz et al., 2013). While fungi have been known to produce anti-microbial compounds for some time (e.g., *Penicillin*, Strobel and Daisy, 2003), their effects on amphibian health and interactions with host immune defense are not well known. In particular, studies of cutaneous non-*Bd* fungal communities on amphibians infected with *Bd* are lacking, despite a growing literature on bacteria that are often suggested for probiotic applications (Rebollar et al., 2016; Woodhams et al., 2016).

Fungi are diverse, and play prominent roles in ecosystems as decomposers, pathogens, and parasites (Kirk et al., 2008; Fierer, 2017). In addition, fungi form intricate symbioses with plants, aiding them in host defense and nutrient acquisition (Philippot et al., 2013). The role of bacteria in non-plant host-associated systems has received substantial attention, however the role fungi play in animal hosts is unclear (Huffnagle and Noverr, 2013). Non-*Bd* fungi have been detected on amphibians (Gugnani et al., 1980; Czeczuga et al., 1998) and other non-*Bd* fungi have been shown to cause disease in amphibians (e.g., Frank, 1976; Wright and Whitaker, 2001). Despite the abundance of information about *Bd*-host interactions, we lack an understanding of the diversity and metabolic capabilities of non-pathogenic fungi associated with amphibians.

Because of the efficacy of fungi at inhibiting other infectious diseases (Fox, 2015) and the potential role microbes can play in the host immune response (Cho and Blaser, 2012), we tested the hypothesis that that cutaneous fungal taxa supplement host defense against chytridiomycosis. Further we hypothesized that *Bd* inhibitory fungal taxa would comprise a significantly greater proportion (as percentage of reads) of the cutaneous microbiome than bacteria on three species and one subspecies of poison arrow frogs (*Dendrobates* spp.). To test these hypotheses we partnered with the New England Aquarium to examine the microbiota of captive poison arrow frogs including *Dendrobates auratus*, *D. leucomelas,* and two subspecies of *D. tinctorius* as well as their tank environment using high-throughput sequencing of bacterial and fungal communities and cultivation of fungal taxa. In 2008, the Aquarium's *Dendrobates* collection experienced a *Bd* infection that eliminated populations of *D. auratus* and *D. tinctorius* while *D. leucomelas* individuals survived this exposure (Hirokawa et al., 2008). The controlled rearing conditions of these frogs on exhibit at the New England Aquarium, coupled with their divergent history of response to *Bd* infection, provides an excellent system to examine the relative contributions of bacterial and fungal skin communities on differential host defense. In addition, we tested whether fungal or bacterial probiotics can elicit a corticosterone stress response and examined whether skin peptide defense capacity was altered by the treatment.

MATERIALS AND METHODS

Sample Collection

Frogs were housed on exhibit at the New England Aquarium (Boston, MA, USA) and in its related holding facility 12 km south in Quincy, MA (**Table 1**). All frog species on exhibit at the New England Aquarium were housed in a single tank. This 3 m^3 display had a glass public facing front and a solid fiberglass housing with an 80 L sump for water reserve that was pumped up to the exhibit. Water and biofilm samples were collected from the sump. The frogs at the Quincy holding facility were housed in one of four species-specific plexiglass holding cages. In 2015, holding cages had a paper-towel on the floor and the cage was tilted so there was a 3-cm deep water pool along one edge. In 2016, the holding cages were enhanced with soil, moss, and other plants, and had a 4-cm deep pool integrated into the enclosure. Each cage held 5 to 8 frogs and the closest five frogs to the door were sampled to avoid inducing stress from "chasing." In these holding cages, biofilms were collected from the edge of the water pool. Approximately 75% of the frogs of each species in collection were bred at the Quincy holding facility, with the remainder originating in other Association of Zoo and Aquarium accredited institutions.

Frogs were individually selected, rinsed with 15-mL of sterile water to remove transitory microbes, and swabbed on the ventral surface (**Table 1**) with *fine-tipped rayon swabs (Molecular Wire and Equipment MW113)*. Swabs were placed in cryovials and stored on dry ice for transportation to the lab. All samples were kept at −80°C until DNA extraction. Water was collected in 15 mL sterile tubes, stored on ice, and filtered at University of Massachusetts, Boston through 0.2 μm sterivex filters to capture bacteria and fungi. Biofilms were also swabbed from the tank surface and stored frozen in sterile cryovials.

DNA Extraction, PCR, and Sequencing

DNA was extracted from frog and tank biofilm swabs using the MoBio PowerSoil Total DNA Isolation kit (Carlsbad, CA, USA) following manufacturer's instructions. Water samples were extracted using the MoBio PowerWater Total DNA Isolation kit following manufacturer's instructions. DNA extractions were verified by gel electrophoresis and when bands weren't visible, samples were checked with PCR using general bacterial primers (Caporaso et al., 2012). Samples for bacterial community analysis were amplified in triplicate using the primer pair 515F and 806R (Caporaso et al., 2010) following previously published conditions

TABLE 1 | Table of the number of amphibians and environmental samples collected in 2015 and 2016 from the two locations (Boston and Quincy, MA, USA) sampled.

	2015- Quincy	2016- Quincy	2016- Boston
D. leucomelas	5	5	1
D. auratus	5	5	3
D. tinctorious "azureus"	4	4	3
D. tinctorious	Not sampled	5	6
Water	5	5	2
Tank biofilm	5	5	4

(Caporaso et al., 2012). Primer constructs had Illumina adaptors and 12-bp GoLay barcodes. Proper product formation was verified with gel electrophoresis and samples were purified with the Qiagen QiaQuick Gel Purification Kit (Qiagen, Valencia, CA). DNA from each sample was quantified with a Qubit fluorometer (ThermoFisher, Waltham, MA, USA) and pooled in equal masses for paired-end 151-bp sequencing on an Ilumina MiSeq using V2 chemistry at the University of Massachusetts Boston.

Fungal communities were amplified in triplicate with primers ITS1F and ITS2R (Walters et al., 2016) targeting the fungal internal transcribed spacer region (ITS). We used primer constructs that had overhang sequences to allow downstream addition of dual Illumina indices and adapters. Proper product formation was verified with gel electrophoresis and samples were purified with the Qiagen QiaQuick Gel Purification kit. A second 8-cycle PCR was performed with the Illumina Nextera XT2 kit following the manufacturer's instructions to ligate dual indices and Illumina adaptors to each sample. Amplified DNA from each sample were purified using the Qiagen PCR Purification kit, quantified with a Qubit, and pooled in equal mass for paired-end 151-bp sequencing on an Illumina MiSeq using V2 chemistry (Caporaso et al., 2011).

Sequence and Statistical Analyses

Paired-end reads from 16S rRNA gene or ITS gene sequences were first joined with fastq-join (Aronesty, 2011) and then quality filtered and demultiplexed in QIIME (version 1.91; Caporaso et al., 2010) following previously published guidelines (Bokulich et al., 2013). Fungal reads were further quality filtered using ITSx to remove 5S and 18S fragments, which improves fungal analyses (Bengtsson-Palme et al., 2013). Both bacterial and fungal sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using uClust (Edgar, 2010) against the GreenGenes (version 13.5) and UNITE (version 7.0) databases respectively. Following clustering, OTUs appearing only once (singeltons) and OTUs matching archaea, chloroplasts, and protists were removed from both datasets. Beta diversity was calculated using Bray-Curtis similarity on OTU tables normalized to the lowest sampling depth (9,563 for bacteria and 6,753 for fungi). Beta diversity was visualized with a principal coordinates analysis. Due to the concerns with rarefaction (e.g., McMurdie and Holmes, 2014), we calculated beta diversity on unrarefied OTU tables and compared the distance matrix to a rarefied distance matrix using a mantel test. The mantel test revealed no significant difference between rarefied and unrarefied distance matricies, thus we used rarefied data for subsequent analyses. Significant differences in community composition were assessed with a permutational multivariate analysis of variance with 10,000 permutations in QIIME (Anderson, 2001). We included habitat (frog species, water, biofilm) as variables in the overall PERMANOVA, and used additional PERMANOVAs to assess all pair-wise comparisons.

Quantitative PCR

Quantitative PCR (qPCR) was performed to assess the copy number of the 16S rRNA gene and fungal ITS region. DNA and standards were first quantified with a Qubit fluorometer (ThermoFisher, Waltham, MA, USA). All samples were normalized to 3 ng μL^{-1} and serial dilutions of standards were prepared from purified PCR product of each gene. DNA from each sample was amplified in triplicate, along with standards and internal controls on a Strategene MX-3005P quantitative thermal cycler (Stratagene, La Jolla, CA, USA). 16S rRNA and ITS genes were amplified in 25 μL reactions using 0.25 μL of each primer, 12.5 μL of Qiagen QuantiTect SYBR Green PCR Master Mix, 1 μL of DNA template, and 11 μL PCR grade water. Bacterial qPCR was performed with primers 357F and 515R (Biddle et al., 2008) following conditions described by Bowen et al. (2011). Fungal qPCR was performed with primers ITS1F and ITS2R with the same cycling conditions as the 16S rRNA gene. Proper product formation was verified with melt curves and gel electrophoresis. All standard curves possessed a high degree of linearity (>0.99 R^2) and PCR efficiency ranged from 95 to 101% for both bacterial and fungal qPCR. To assess differences in the abundance of 16S rRNA gene and ITS copy number among frog species and their environment we used an ANOVA in R (R Core Team, 2012) with a Tukey HSD test for multiple comparisons. We ensured data met the assumptions of an ANOVA including assessing variance with a Bartlett test.

Fungal Isolation and *Bd* Assays

To isolate fungi we swabbed the tank biofilm and the ventral surface of frogs in 2015 and plated the swabs on Potato Dextrose Agar and Sabouraud Dextrose Agar. One milliliter of water was spread on Potato Dextrose Agar and Sabouraud Dextrose Agar as well. Plates were incubated at 25°C in the dark for 3 days and all visually distinct (shape and color) isolates were picked and isolated on Potato Dextrose Agar. Isolates were identified with sequencing at the Massachusetts General Hospital DNA Core Facility using the primer pair ITS1F and ITS4R (White et al., 1990). We clustered our isolates into OTUs at 97% identity as described above and assigned taxonomy in QIIME with BLASTn and the UNITE database. We defined a taxonomic hit with a *e*-value $< 1e^{-5}$ and a percent identity of 97% or greater.

To test the efficacy of isolates for inhibition of *Bd* zoospore growth we followed a protocol outlined previously (Woodhams et al., 2014). Briefly, all isolates were grown in 1% tryptone broth overnight in sterile centrifuge tubes at room temperature and growth was verified by checking for turbidity. Following confirmation of growth, samples were centrifuged at 2,225 × g

for 5 min to pellet the cells and the liquid was filtered through 0.22 μm filters. Isolate filtrates were kept at −20°C until needed for growth inhibition assays. Two strains of *Bd* from the Global Panzootic Hypervirulent lineage (JEL 197 and 423) were grown on 1% tryptone agar for 4–7 days to allow for the production of zoospores. Plates were flooded with 1% tryptone and the liquid was filtered through 0.45 μm filters. *Bd* zoospores were counted on a haemocytometer and diluted to 5×10^6 zoospores mL^{-1}. To assay the inhibition of *Bd* we inoculated 96-well plates with 50 μL *Bd* zoospores and 50 μL isolate filtrates. For negative controls we used heat killed *Bd* and wells containing no zoospores and for positive controls 50 μL of tryptone was added to zoospores. Growth was measured as changes in optical density at 480 nm at days 0, 3, 5, and 7. Percent growth of *Bd* in the presence of metabolites was calculated by taking the slope of the optical density over the 7-day incubation, subtracting the optical density of the negative controls, and dividing by the average slope of the growth in the positive control wells. Differences in growth between isolates and controls was measured with a *t*-test in R (R Core Team, 2012) using a Benjamini-Hochberg correction for multiple comparisons. To determine the phylogenetic relationship among fungal taxa we aligned ITS sequences with clustalW (Thompson et al., 2003) and constructed a phylogenetic tree based on maximum likelihood using RAxML (Stamatakis, 2014) and visualized the tree with the Interactive Tree of Life (Letunic and Bork, 2007). We tested confidence in tree topology using bootstrapping with 1,000 restarts. To test for a phylogentic signal of *Bd*-growth inhibition or facilitation within our isolates, we used a UniFrac significance test (Lozupone et al., 2006) and a phylogenetic signal analysis in the R package picante (Kembel et al., 2010).

The Abundance of *Bd* Inhibitory/Facilitating Microbes

To assess the proportion of reads in the bacterial communities that were inhibitory toward *Bd* we filtered our data set against a database of known *Bd*-inhibiting bacteria (Woodhams et al., 2015) in QIIME and filtered our fungal dataset against our *Bd* inhibiting/facilitating isolates. To do this, we performed a closed reference OTU pick against the GreenGenes and UNITE databases and filtered OTUs matching inhibitory/facilitating taxa from our dataset and compared the number of reads before and after filtering. We assessed significant differences among fungi and bacteria as well as among frog species with an ANOVA followed by a Tukey HSD test for pair-wise comparisons. We visualized the results using a heatmap and dendrograms to assess similarity of *Bd*-inhibitory communities. To test for differential abundance of OTUs between frog species and the environment, we used a Kruskal-Wallis test in R. Dendrograms were calculated using the weighted pair group method and arithmetic mean (WPGMA) clustering and significant differences among communities (between frog species and between frog species and their environment) were tested with a PERMANOVA. We tested for differences in percentages of inhibitory taxa between frogs and the environment using an ANOVA with a Tukey HSD test for multiple comparisons. We used a bipartite network analysis to determine the interaction of inhibitory and facilitating bacterial and fungal taxa using a previously described method (Bowen et al., 2013). This network determines positive association (presence of OTUs) of OTUs to a given environment. We included sample types as their own nodes using the Fruchterman and Reingold (1991) algorithm for ease of visualization. Networks were visualized using the R package network (Butts et al., 2012).

Testing Amphibian Immune and Stress Response to Bacteria and Fungi

Midwife toads, *Alytes obstetricans* ($n = 29$), were raised in captivity from larvae at the Breeding Centre of Endangered Amphibians of the Guadarrama Mountains in Spain. Toad research conformed to the legal requirements of Consejerias de Medio Ambiente of Madrid. The toads were maintained on a 12:12 h light cycle and fed *Acheta domesticus ad libitum*. After experimental treatments described below, toads were released into an outdoor mesocosm containing natural vegetation, a small pond, and pile of rocks for shelter. Each toad was photographed for individual identification upon recapture based on unique markings.

Midwife toads were randomly assigned to one of four treatments. Control toads ($n = 12$) were bathed in 20 ml sterile water for 1 h. Toads treated with probiotics were bathed in 20 ml water containing 1 ml of either *Penicillium expansum* ($n = 9$), *Janthinobacterium lividum* (isolate 77.5b1, 56×10^7 CFU, $n = 4$), or *Flavobacterium johnsoniae* (isolate 70c, 19×10^7 CFU, $n = 4$) for 1 h. The *P. expansum* was grown on Sabouraud Dextrose agar, while freshly growing bacteria were rinsed directly from 15 mm Petri plates with R2A agar media supplemented with 1% tryptone. Probiotic isolates were originally collected from wild *A. obstetricans* near Basel, Switzerland and chosen for this experiment based on their ability to inhibit *B. dendrobatidis* growth (Woodhams et al., 2014). Isolates used in this study were deposited in the Culture Collection of Switzerland (CCOS 423 & 433, http://www.ccos.ch/).

We assessed the stress response of frogs to probiotics using water-borne corticosterone release rates. Corticosterone is the primary amphibian stress hormone and water-borne corticosterone release rates are highly correlated with circulating corticosterone levels measured from plasma (Gabor et al., 2013). An hour after removing the toads from probiotic treatments, they were placed in 40 ml of sterile water within a 100 ml beaker for 1 h to collect water-borne hormones. Frogs were carefully lifted out of the beaker and the remaining water sample was saved to assay corticosterone release rates. Water samples were immediately frozen at −20°C and the hormones were extracted from the thawed water using C18 solid phase extraction columns (SepPak Vac 3 cc / 500 mg; Waters, Inc., Milford, MA, USA) with Tygon tubing (Saint Bobain formulation 2475) under vacuum pressure. After extraction the columns were immediately frozen at −20°C and sent to Texas State University where they were eluted with methanol and then evaporated with nitrogen gas following Gabor et al. (2013). The residue was then resuspended in 5% ethanol and 95% EIA buffer (provided by Cayman Chemicals Inc. Ann Arbor, MI, USA) for a final re-suspension volume of 250 μL.

Corticosterone release rates were measured in duplicate for all samples with an enzyme-immunoassay (EIA) kit (Cayman Chemicals Inc.) on a fluorescent plate reader (BioTek Powerwave XS). We examined the differences among treatment groups in the initial corticosterone stress (natural log transformed) using ANOVA with Tukey HSD pairwise comparisons (R Core Team, 2012), ensuring data met the assumptions of an ANOVA, including assessing variance with a Bartlett test.

Following the hormone assay we released toads into the same outdoor mesocosm for 4 weeks at which point toads were recaptured and sampled to measure their skin peptide defense capacity according to established methods (Woodhams et al., 2014). Peptide quantities recovered were compared among treatment groups. Peptides at a concentration of 500 $\mu g\ ml^{-1}$ were tested for ability to inhibit the growth of *B. dendrobatidis*, *J. lividum*, and *F. johnsoniae*. The differences among treatment groups were compared using ANOVA with Tukey HSD pairwise comparisons (R Core Team, 2012), ensuring all assumptions of an ANOVA were met.

Ethics Statement

Experiments at the New England Aquarium were conducted under the supervision of trained veterinary staff and were conducted in accordance with the New England Aquarium Animal Care and Use Committee Proposal 2015-01. Experiments with midwife toads were conducted under permit number 10/032921.9/12 from Conseneria de Medio Ambiente of Comunidad de Madrid.

RESULTS

Community Composition and Abundance

In the winter of 2015 and 2016 we sampled skin microbiomes from poison arrow frogs both from a mixed-species exhibit at the New England Aquarium (Boston, MA, USA), as well as from single species holding (Animal care facility, Quincy MA, USA). High-throughput sequencing of the 16S rRNA gene and fungal intergenic transcribed spacer (ITS) region revealed distinct communities of microbes associated with host skin compared to the microbial communities found in the frog's environment [**Figure 1**; PERMANOVA, Bacteria: $p < 0.001$, $F_{(5, 62)} = 7.89$, all pair-wise $p < 0.01$; Fungi: $p < 0.01$, $F_{(5, 45)} = 29.43$, all-pairwise $p < 0.001$]. Furthermore, we identified species-specific bacterial and fungal communities within the *Dendrobates* genus [**Figure 1**; PERMANOVA, Bacteria: $p < 0.01$, $F_{(5, 45)} = 6.98$, all pair-wise $p < 0.001$; Fungi: $p < 0.01$, $F_{(5, 45)} = 41.43$, all pair-wise $p < 0.001$] and this species specificity was maintained whether the frogs were reared in separate tanks at the Animal Care Facility, or in a shared tank on exhibit. We found lower copies of the 16S rRNA gene [Figure S1; ANOVA, $p < 0.01$, $F_{(5, 45)} = 29.01$, all pair-wise $p < 0.001$] and higher ITS copies [Figure S1B, $p < 0.01$, $F_{(5, 53)} = 63.21$, all pair-wise $p < 0.01$) on frog skin compared to the environment. There was no significant difference in the number of copies of the16S rRNA gene and ITS region among the species of frogs we examined.

Fungal Isolation and *Bd* Challenge Assays

We isolated 135 visually distinct fungal strains, of which 90 were unique at 97% sequence identity, from the skin and environment of *Dendrobates* frogs (Table S1). The cultured isolates, when screened against the ITS amplicon sequences, accounted for 71% of the sequences present. Secondary metabolites were collected from each isolate and tested for activity against zoospores from two strains of *Bd* (JEL 197 and 423). For fungi isolated from the environment ($N = 25$), most enhanced ($N = 12$) or had no effect on ($N = 5$) *Bd* growth. Those that were inhibitory toward *Bd,* 3 could inhibit both strains of *Bd*, 3 could only inhibit JEL 423, and 3 could only inhibit JEL 197. For fungi isolated from frogs ($N = 65$), 6 had no effect on *Bd* growth. For isolates tested against *Bd* JEL 197, 19 facilitated the growth of JEL 197, while 19 inhibited the growth of JEL 197. For isolates tested against *Bd* JEL 423, 28 facilitated growth of JEL 423 and 20 inhibited the growth of JEL 423. For OTUs with more than one visually distinct isolate associated with it, they displayed similar levels of inhibition or facilitation. Phylogenetic analysis of the fungal taxa (Table S1, Figure S2) revealed diverse isolates primarily associated with phylum Ascomycota. A UniFrac significance test (Lozupone et al., 2006) indicated a significant phylogenetic signal ($p < 0.001$) for inhibition of *Bd* growth. Further, a phylogenetic signal test also indicated a significant ($p = 0.003$, $K = 2.68$) phylogenetic signal of *Bd* inhibition among fungal taxa. Together, these suggest a phylogenetic conservation of *Bd* inhibition in fungi.

Distribution of *Bd* Inhibiting/Facilitating Taxa

We assessed the community composition of *Bd*-inhibitory and *Bd*-facilitating taxa by screening our high throughput sequencing data against databases of known *Bd* inhibitory/facilitating bacteria (Woodhams et al., 2015) and fungi (Table S1). Our result revealed that a significantly higher percentage of the cutaneous bacterial and fungal communities, assessed by the proportion of reads, were inhibitory than were found in the frog's environment (**Figures 2A,B**). However, there were significantly more *Bd* inhibitory fungi than *Bd* inhibitory bacteria [$F_{(11, 96)} = 21.44$, $p < 0.0001$]. In fact, approximately 45% of the fungal community on a given frog was capable of inhibiting *Bd*. Further, the percent of *Bd*-facilitating fungal taxa (**Figure 2C**) was significantly lower on frog skin than the environment [$F_{(5, 45)} = p < 0.01$, all pair-wise $p < 0.01$] and no *Bd*-facilitating bacteria were found on the frog skin.

We next examined patterns of antifungal community structure among different *Dendrobates* species by comparing abundance and community composition of bacterial taxa that demonstrated antifungal properties. Our results indicate species specific antifungal bacterial communities and distinct anti-microbial fungal communities on the frogs relative to their environment [PERMANOVA, Bacteria- $p < p < 0.001$, $F_{(5, 45)} = 12.34$, all pairwise $p < 0.01$, Fungi- $p < 0.001$, $F_{(5, 45)} = 29.43$, all pairwise $p < 0.05$; **Figure 3A**]. Because the communities associated with *D. leucomelas* are considerably dissimilar to the other frogs, this potentially links the differential

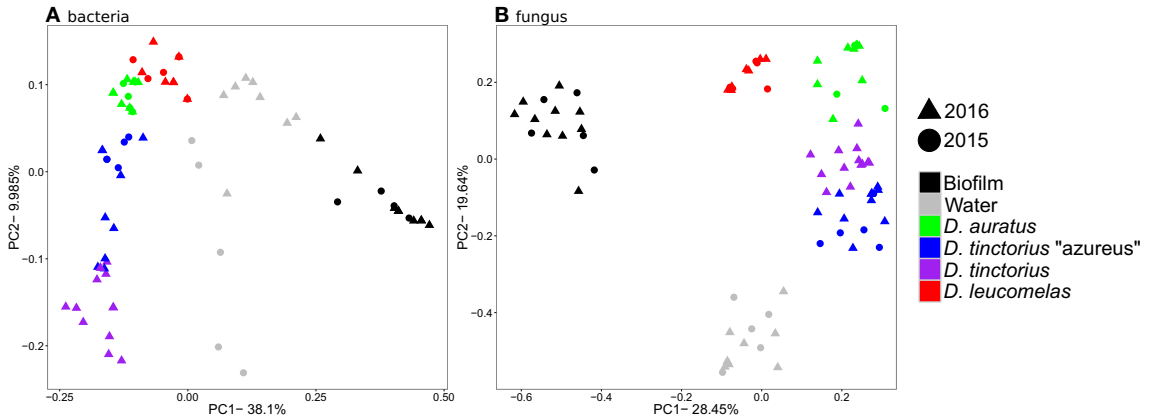

FIGURE 1 | Principal coordinates analysis based on Bray-Curtis similarity for the bacterial **(A)** and fungal **(B)** communities from *Dendrobates* spp. frogs and their environment. The data for 2016 include frogs from both single species and mixed species enclosures.

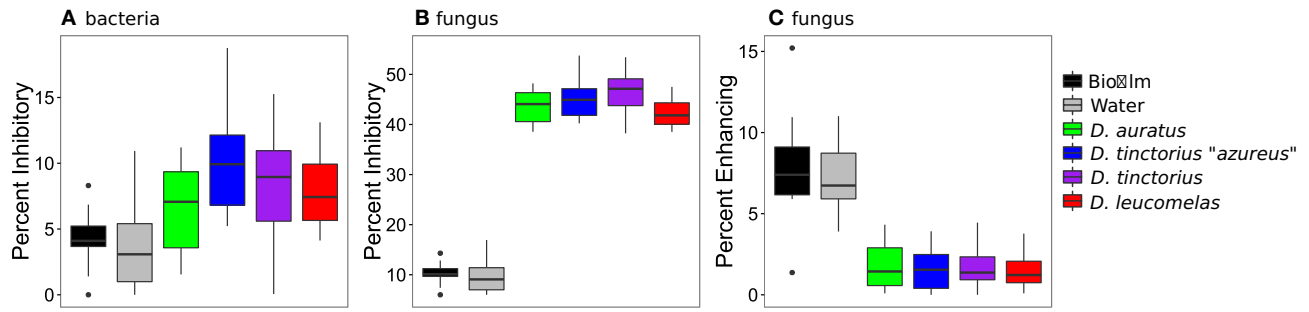

FIGURE 2 | Percentage of bacterial **(A)** and fungal **(B)** communities that inhibit the growth of *Bd* and the percentage of fungal communities that facilitate the growth of *Bd* **(C)** from *Dendrobates* spp. frogs and their enclosure. Boxes represent 25–75% quartiles, and the solid black line is the median value. Note, no *Bd* facilitating bacteria were found on the frogs sampled. *Bd*-inhibitory bacteria were determined by Woodhams et al. (2015) and fungi were identified in this study.

susceptibility among species of *Dendrobates* (Hirokawa et al., 2008) to their specific microbial communities (**Figure 1**). While many *Bd* inhibitory bacterial taxa were in similar abundance between frogs and their environment, all frogs possessed a higher proportion of bacteria from the families Aeromonadaceae, Enterobacteriaceae, Pseudomonadaceae, and Xanthomonadaceae and from the genera *Cryseobacterium*, *Flavobacterium*, and *Comamonas* (**Figure 3A**; Kruskal-Wallis test, Benjamini-Hochberg corrected $p < 0.001$). *Dendrobates leucomelas*, the species that withstood a previous *Bd* infection (Hirokawa et al., 2008), was enriched ($p < 0.001$) in bacteria from the genus *Pseudomonas*, which was in very low abundance on other frogs and in the environment. Like the bacterial communities, the *Bd* inhibitory fungal communities displayed species-specific inhibitory communities (**Figure 3B**). All frog species had skin containing a large number of a highly divergent fungal taxa from the phylum Ascomycota, including a taxon that was not closely related to known fungi even at the kingdom level, and from the genus *Cladosporium* (**Figure 3**). Compared to the other frog species, *D. leucomelas* was enriched in *Bd* inhibitory taxa from the phylum Ascomycota, in particular taxa from the families Chaetomiaceae, Lasiosphaeriaceae, and the genus *Pestalotiopsis* ($p < 0.001$), suggesting that these taxa may play a role in *Bd* defense and are potential candidates for probiotics.

To determine the interactions between *Bd*-facilitating and -inhibiting taxa within the bacterial and fungal datasets we performed a bipartite network analysis (**Figure 4**), which assessed the presence/absence of taxa in different environments or frogs. In this analysis, dots represent inhibitory/facilitating OTUs and lines represent positive associations (i.e., presence) to the sample categories. Bacterial and fungal networks had similar topology, where there are several nodes (OTUs) unique to the frogs and environment. Further, there's a distinct group of OTUs shared between all sample types and OTUs shared only between frog species. Both bacterial and fungal networks indicated that frogs had a higher degree of network connectivity (associations; mean = 65.25 for bacteria, 38.75 for fungus) to *Bd*-inhibitory taxa than the environment (mean = 32 for bacteria, 28 for fungus). Further, the number of *Bd*-inhibitory taxa unique to frog skin ($n = 47$ for bacteria, $n = 25$ for fungi) was higher than those unique to the environment ($n = 0$ for bacteria, $n = 1$ for fungi) and *D. leucomelas* had the highest number

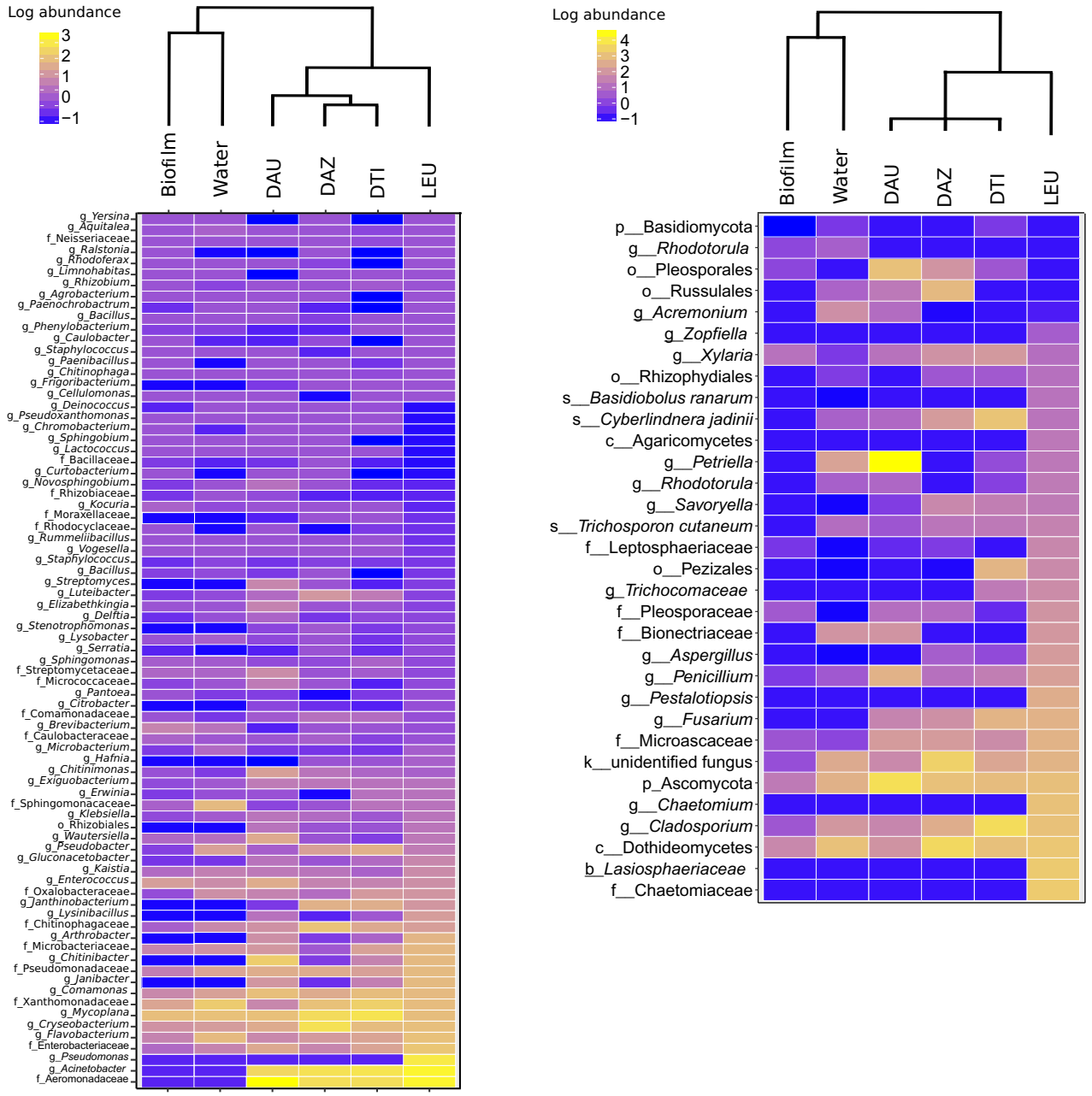

FIGURE 3 | Heat maps of $\log_{10}$ abundance of *Bd*-inhibitory bacterial **(A)** and fungal **(B)** OTUs. Dendrograms are based on weighted pair group method and arithmetic mean (WPGMA) clustering. The highest level of taxonomic identification of each taxon is denoted by k, kingdom; p, phylum; o, order; f, family; and g, genus; DAU, *Dendrobates auratus;* DAZ, *D. tinctorius* "azureus"; DTI, *D. tinctorius;* LEU, *D. leucomelas.*

of unique inhibitory taxa ($n = 4$ for bacteria, $n = 6$ for fungi). The presence of *Bd*-inhibitory bacteria and fungi on frog skin and not in the environment suggests that host factors may facilitate these microbes, while *Bd* inhibitory microbes present on both frog skin and environment may be viable candidates for use as probiotics in the treatment or prevention of chytridiomycosis.

Probiotic Stress and Immune Tests on Midwife Toads (*A. obstetricans*)

To determine potential endocrine-immune interactions produced by applications of fungal or bacterial probiotics, we exposed a non-*Bd* resistant species, Midwife toads (*A. obstetricans*), to two bacterial strains (*J. lividum* and *F. johnsoniae*) and a fungus closely related to an isolate from

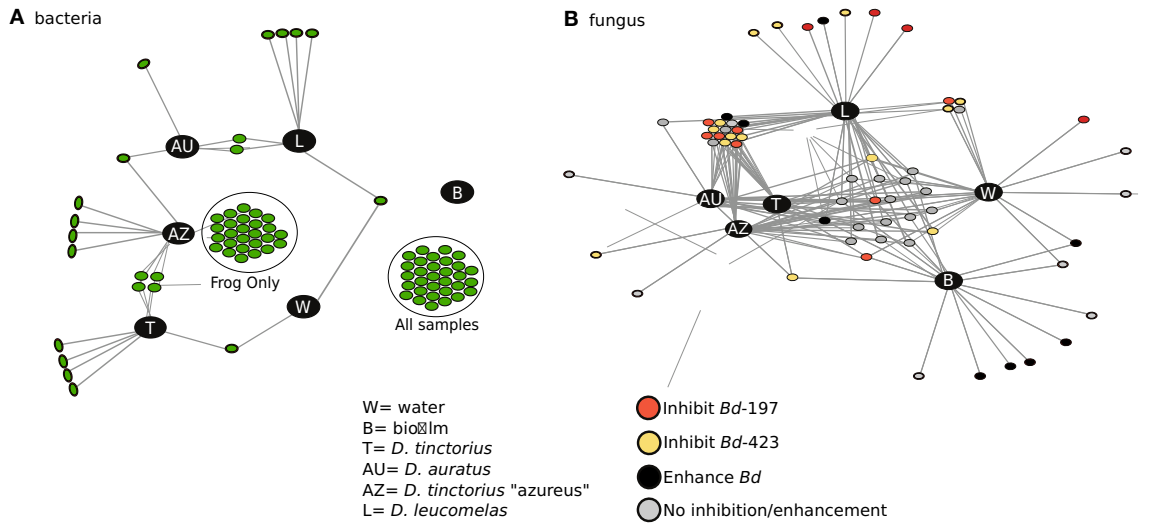

FIGURE 4 | Network analysis depicting the connectivity among sample types for *Bd* inhibitory bacterial **(A)** and *Bd*-inhibitory/facilitating fungal **(B)** taxa. Each dot represents an OTU and the lines represent associations (presence) to a sample category. Different colors in **(B)** indicate the taxa's ability to either inhibit, facilitate, or have no effect on *Bd* growth. In **(A)** "Frog only" taxa are found only in frogs while "all samples" are found in all sample types, the interactions have been removed for ease of viewing. No *Bd*-facilitating bacteria were found in this study. AU, *Dendrobates auratus*; AZ, *D. tinctorius* "azureus"; T, *D. tinctorius*; L, *D. leucomelas;* W, water; B, Biofilm.

our data set, *P. expansum* (Table S1). These probiotics were previously isolated from the target host species. Exposure to bacterial strains significantly increased corticosterone (stress hormone) release rates relative to *P. expansum* and control frogs (**Figure 5A**, Figure S3; ANOVA, $F = 21.83$, $p < 0.001$). Exposure to each bacterial strains decreased host antimicrobial peptide capacity against *Bd* (**Figure 5B**, Figure S3; ANOVA, $F = 4.26$, $p = 0.015$), while exposure to *P. expansum* did not significantly alter peptide activity relative to control frogs.

DISCUSSION

Host-associated microbial communities can have profound effects on host health and immune response (Cho and Blaser, 2012). While microbes can directly modulate the host immune function through direct interactions with the host (i.e., inflammation), host associated taxa can provide additional immune defense against pathogens through the production of secondary metabolites. Our study examined the role of host-associated bacterial and fungal communities in immune defense against chytridiomycosis in three species of poison arrow frogs (*Dendrobates* spp.). We observed species-specific bacterial and fungal communities associated with each frog that was distinct from their enclosure. Species-specific microbial communities have been observed at broad (Givens et al., 2015), narrow (Lee et al., 2011), and sub-species levels (Micallef et al., 2009). Previous work on fungi in other host-associated systems has documented similar findings to ours (e.g., Porras-Alfaro and Bayman, 2011; Bálint et al., 2015), however, our work builds upon previous work by linking host-environmental differences to the presence of beneficial, anti-fungal taxa. Further, the differences in microbiota observed among different frog species held within the same exhibit, as well as the consistency in community structure sampled in two different years, suggest that these frogs may recruit and maintain specific taxa, including those that may provide protection against *Bd*. Our study did not include field-caught frogs and future studies of wild frogs will help elucidate the strength of the pattern.

Much effort has been spent identifying bacteria capable of inhibiting *Bd* to be used as probiotics (Bletz et al., 2015; Woodhams et al., 2015; Walke and Belden, 2016) and some bacteria have been shown to facilitate the growth of *Bd* zoospores (Woodhams et al., 2015). The role of fungal taxa in inhibiting *Bd*, in contrast, has been understudied. Our study isolated 135 previously uncultivated fungi from amphibians, 44 of which inhibited the growth of *Bd* zoospores. Further, the proportion of the fungal community inhibitory toward *Bd* was significantly higher than the proportion of bacteria on frog skin, supporting our hypothesis that fungi contribute more to host defense than bacteria. Further, it suggests fungi have the potential to be an important source of host defense against pathogens on amphibians and perhaps for other organisms (Dean et al., 2012). The higher proportion of *Bd*-inhibitory fungi, while not an explicit test of the efficacy of fungi to supplement host immunity, suggests that there is the potential of fungi to aid in host defense. These data support our second hypothesis, however, an explicit test of fungal probiotics in *Bd* infected amphibians is needed to fully elucidate the role of fungi in the amphibian immune response.

In addition to fungal taxa with the ability to inhibit the growth of *Bd* zoospores, we identified several ($n = 20$ from environment, 19 from frog) taxa capable of facilitating the growth of *Bd* zoospores. The taxa that could facilitate the growth of *Bd* were predominately from the phylum Basidiomycota, a phylum distantly related to the Chytridiomycota phylum (Choi and Kim, 2017).While cooperation between pathogens and microbes is more common

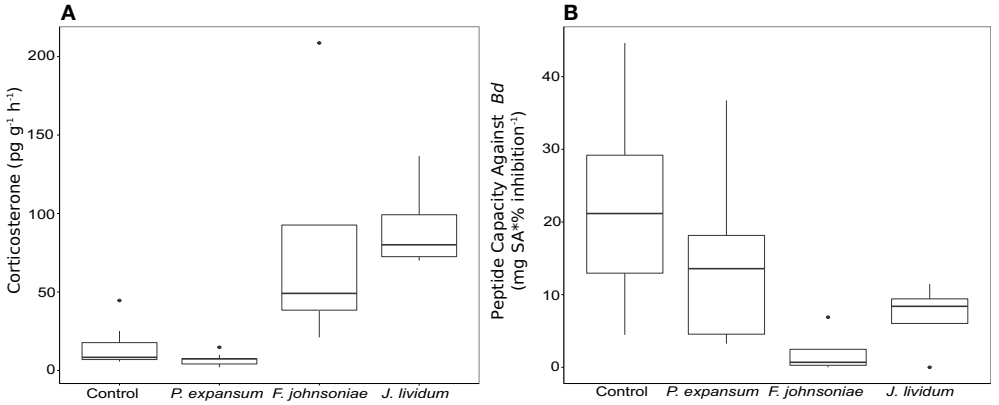

FIGURE 5 | Box plot of corticosterone release rates **(A)** and toad peptide capacity against *Bd* **(B)** in Midwife Toads, *Alytes obstetricans*, measured after exposure to one fungal and two bacterial probiotic strains.

between closely related taxa (West and Buckling, 2003; Griffin et al., 2004), our results suggest that cooperation between fungal taxa may occur across greater phylogenetic distance, indicating a lack of specificity in these interactions. Further, the presence of *Bd*-inhibiting and *Bd*-facilitating fungal taxa suggests that the interactions between skin-associated taxa and potential probiotics is important for not only the establishment of potential probiotics (Becker et al., 2011; Küng et al., 2014; Kueneman et al., 2016) but also for the immunological function of host-associated microbial communities.

Frogs from the *Dendrobates* population at the New England Aquarium have demonstrated differential susceptibility to *Bd* infection, with *D. leucomelas* having previously demonstrated the ability to clear *Bd* infection (Hirokawa et al., 2008). Analysis of bacterial and fungal communities revealed species-specific fungal and bacterial communities, suggesting the microbial communities may play an important role in host defense, in particular for *D. leucomelas*. Further, network analysis revealed groups of *Bd*-inhibitory bacteria and fungi found only on the skin of *D. leucomelas*, suggesting these taxa may play a role in the ability of *D. leucomelas* to clear *Bd* infection. *Bd* inhibiting taxa from the genus *Pseudomonas* were enriched on the microbiome of *D. leucomelas* relative to other frogs and taxa from this genus was absent in other species and the environment. The genus *Pseudomonas*, a common bacterial genus across many biomes, is known to produce numerous extracellular and often antimicrobial metabolites (Holmström and Kjelleberg, 1990) and its use as a probiotic has proven effective in plants (Picard and Bosco, 2008), fish (El-Rhman et al., 2009), and shellfish (Van Hai and Fotedar, 2009). In addition to bacteria, *D. leucomelas* was enriched in *Bd*-inhibiting fungi from the families Chaetomiaceae, Lasiosphaeriaceae, and the genus *Pestalotiopsis*, suggesting a potential role for these taxa in *Bd* defense. The persistence of *Bd*-inhibiting taxa on amphibian skin, as well cosmopolitan distribution of many of these taxa across biomes suggests they could be potential probiotic candidates for treating chytridiomycosis.

Our results demonstrated a potential link between amphibian-associated fungi and host defense against *Bd* infection. While we were limited to a small study using the endangered midwife toad, probiotic application of *P. expansum* to midwife toads did not significantly alter host immune or stress levels, while bacterial probiotics did. Applications of the probiotic bacterium *J. lividum* were protective against chytridiomycosis for several species of amphibians (Becker et al., 2009; Harris et al., 2009; Kueneman et al., 2016). Further, the viability of *Bd* zoospores was significantly reduced after exposure to mucus from frogs treated with the bacterium *F. johnsoniae* and the fungus *P. expansum* (Woodhams et al., 2014). However, a recurrent problem with probiotic applications is the colonization resistance of hosts (Becker et al., 2011; Küng et al., 2014), such that augmented bacteria fail to establish in the skin, particularly in the absence of an environmental reservoir for the probiotic bacteria (Kueneman et al., 2016). Additionally, Küng et al. (2014) showed that some probiotic treatments may stress hosts or cause an immune reaction in amphibians that prevents establishment of the probiotic. Our results suggest that fungal taxa such as *Penicillium*, that are common across amphibians and environments, may be potential candidates for amphibian probiotic therapy, as has been previously shown in agricultural systems (Fox, 2015). Fungal probiotics may not induce a host stress responses or repress the host's mucosal peptide response, although the generality of this finding among hosts and potential probiotics is not known. Instead, fungal probiotics may produce antimicrobial metabolites while inducing host defenses that target foreign fungi, including *Bd*.

CONCLUSIONS

We show species-specific bacterial and fungal communities associated with *Dendrobates* frogs that are distinct from their environment. The distinct microbiome (including the mycobiome) on host skin is, in part, due to host recruitment of potentially anti-microbial taxa that may help promote host health. Frog-associated bacterial communities possessed a significant portion of *Bd*-inhibitory taxa and the fungal communities were dominated by *Bd* inhibitory taxa, suggesting fungi may play a greater role in host protection than bacteria

in amphibians. Our results suggest that host-associated fungi should be a greater focus of future efforts to develop probiotic therapies for the treatment of chytridiomycosis and attempts at manipulation and probiotic experiments are needed to fully elucidate this relationship. When considering the host immune priming function provided by microbiota (Kurtz and Scharsack, 2007), and host resistance to bacterial colonization (Küng et al., 2014), fungi may provide key probiotics needed for disease management in amphibians.

DATA AVAILABILITY

Fungal isolate sequences can be found in NCBI under accession numbers KY114967-KY115101. High-throughput sequencing data can be found in the NCBI Sequence Read Archive under accession numbers PRJNA354614 and PRJNA354619.

AUTHOR CONTRIBUTIONS

PK, JLB, MT, and DW designed the poison arrow frog experiment. PK oversaw the students that performed all sequencing related activities. SF and PK performed the qPCR, *Bd* inhibition assays, and isolation of fungal cultures. SF-B, CG, JB, and DW performed midwife toad experiments. PK performed all sequence and statistical analyses. PK, JLB, MT, and DW wrote the paper with contributions from SF, SF-B, CG, and JB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02494/full#supplementary-material

REFERENCES

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Aust. Ecol.* 26, 32–46. doi: 10.1111/j.1442-9993.2001.01070.pp.x

Aronesty, E. (2011). *ea-utils: Command-Line Tools for Processing Biological Sequencing Data*. Available online at: https://expressionanalysis.github.io/ea-utils/

Bálint, M., Bartha, L., O'Hara, R. B., Olson, M. S., Otte, J., Pfenninger, M., et al. (2015). Relocation, high-latitude warming and host genetic identity shape the foliar fungal microbiome of poplars. *Mol. Ecol.* 24, 235–248. doi: 10.1111/mec.13018

Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N., and Minbiole, K. P. (2009). The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Appl. Environ. Microbiol.* 75, 6635–6638. doi: 10.1128/AEM.01294-09

Becker, M. H., Harris, R. N., Minbiole, K. P., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., et al. (2013). Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. *Methods Ecol. Evo.* 4, 914–919. doi: 10.1111/2041-210X.12073

Biddle, J. F., Fitz-Gibbon, S., Schuster, S. C., Brenchley, J. E., and House, C. H. (2008). Metagenomic signatures of the Peru Margin subseafloor biosphere show a genetically distinct environment. *Proc. Natl. Acad. Sci. U.S.A.* 105: 10583–10588. doi: 10.1073/pnas.0709942105

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bletz, M. C., Rosa, G. M., Andreone, F., Courtois, E. A., Schmeller, D. S., Rabibisoa, N. H., et al. (2015). Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. *Sci. Rep.* 26:8633. doi: 10.1038/srep08633

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat. Meth.* 10, 57–59. doi: 10.1038/nmeth.2276

Bowen, J. L., Byrnes, J. E., Weisman, D., and Colaneri, C. (2013). Functional gene pyrosequencing and network analysis: an approach to examine the response of denitrifying bacteria to increased nitrogen supply in salt marsh sediments. *Front. Microbiol.* 42:342. doi: 10.3389/fmicb.2013.00342

Bowen, J. L., Ward, B. B., Morrison, H. G., Hobbie, J. E., Valiela, I., Deegan, L. A., et al. (2011). Microbial community composition in sediments resists perturbation by nutrient enrichment. *ISME J.* 5, 1540–1548. doi: 10.1038/ismej.2011.22

Butts, C. T., Acton, R. M., Hipp, J. R., and Nagle, N. N. (2012). Geographical variability and network structure. *Soc. Net.* 34, 82–100. doi: 10.1016/j.socnet.2011.08.003

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Meth.* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., et al. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4516–4522. doi: 10.1073/pnas.1000080107

Cho, I., and Blaser, M. J. (2012). The human microbiome: at the interface of health and disease. *Nat. Rev. Gen.* 13, 260–270. doi: 10.1038/nrg3182

Choi, J., and Kim, S. (2017). A genome tree of life for the fungi kingdom. *Proc. Natl. Acad. Sci. U.S.A.* 114, 9391–9396. doi: 10.1073/pnas.1711939114

Czeczuga, B., Muszyńska, E., and Krzemińska, A. (1998). Aquatic fungi growing on the spawn of certain amphibians. *Amphibia-Reptilia* 19, 239–251. doi: 10.1163/156853898X00151

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13, 414–430. doi: 10.1111/j.1364-3703.2011.00783.x

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

El-Rhman, A. M. A., Khattab, Y. A., and Shalaby, A. M. (2009). *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. *Fish. Shellfish Immun.* 27, 175–180. doi: 10.1016/j.fsi.2009.03.020

Fierer, N. (2017). Embracing the unknown: disentangling the complexities of the soil microbiome. *Nat. Rev. Microbiol.* 10, 579–590. doi: 10.1038/nrmicro.2017.87

Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–194. doi: 10.1038/nature10947

Fox, J. L. (2015). Agricultural probiotics enter spotlight. *Nat. Biotech.* 33, 122–122. doi: 10.1038/nbt0215-122

Frank, W. (1976). "Mycotic infections in amphibians and reptiles," in *Wildlife Diseases*, ed. L. A. Page (Boston, MA: Springer), 73–88.

Fruchterman, T. M. J., and Reingold, E. M. (1991). Graph drawing by force-directed placement. *Softw. Pract. Exp.* 21, 1129–1164. doi: 10.1002/spe.4380211102

Gabor, C. R., Fisher, M. C., and Bosch, J. (2013). A non-invasive stress assay shows that tadpole populations infected with *Batrachochytrium dendrobatidis* have elevated corticosterone levels. *PLoS ONE* 8:e56054. doi: 10.1371/journal.pone.0056054

Givens, C. E., Ransom, B., Bano, N., and Hollibaugh, J. T. (2015). Comparison of the gut microbiomes of 12 bony fish and 3 shark species. *Mar. Ecol. Prog. Ser.* 518, 209–223. doi: 10.3354/meps11034

Griffin, A. S., West, S. A., and Buckling, A. (2004). Cooperation and competition in pathogenic bacteria. *Nature* 430, 1024–1027. doi: 10.1038/nature02744

Gugnani, H. C., and Okafor, J. I. (1980). Mycotic flora of the intestine and other internal organs of certain reptiles and amphibians with special reference to characterization of basidiobolus isolates: die pilzflora des darmes und anderer innerer organe von bestimmten reptilien und amphibien unter besonderer berücksichtigung der charakterisierung von basidiobolus-isolaten. *Mycoses* 23, 260–268. doi: 10.1111/j.1439-0507.1980.tb02605.x

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Hirokawa, K., Innis, C., Frasca, S. Jr., and Nyaoke, A. (2008). *Paper Presented at the 33rd Eastern Fish Health Workshop* (Atlantic Beach).

Holmström, C., and Kjelleberg, S. (1990). Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Ecol.* 30, 285–293. doi: 10.1016/S0168-6496(99)00063-X

Huffnagle, G. B., and Noverr, M. C. (2013). The emerging world of the fungal microbiome. *Trends Microbiol.* 21, 334–341. doi: 10.1016/j.tim.2013.04.002

Kembel, S. W., Cowan, P. D., Helmus, M. R., Cornwell, W. K., Morlon, H., Ackerly, D. D., et al. (2010). Picante: R tools for integrating phylogenies and ecology. *Bioinformatics* 26, 1463–1464. doi: 10.1093/bioinformatics/btq166

Kirk, P. M., Cannon, P. F., Minter, D. W., and Stalpers, J. A. (2008). *Dictionary of the Fungi, 10th Edition*. Wallingford: CABI.

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and McKenzie, V. J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E., and Woodhams, D. C. (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS ONE* 9:e87101. doi: 10.1371/journal.pone.0087101

Kurtz, J., and Scharsack, J. P. (2007). Resistance is skin-deep: innate immunity may help amphibians to survive a deadly fungus. *Anim. Conserv.* 10, 422–424. doi: 10.1111/j.1469-1795.2007.00149.x

Lee, O. O., Wang, Y., Yang, J., Lafi, F. F., Al-Suwailem, A., and Qian, P. Y. (2011). Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea. *ISME J.* 5, 650–664. doi: 10.1038/ismej.2010.165

Letunic, I., and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127–128. doi: 10.1093/bioinformatics/btl529

Lozupone, C., Hamady, M., and Knight, R. (2006). UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 7:371. doi: 10.1186/1471-2105-7-371

McMurdie, P. J., and Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comp. Biol.* 10:e1003531. doi: 10.1371/journal.pcbi.1003531

Micallef, S. A., Shiaris, M. P., and Colón-Carmona, A. (2009). Influence of Arabidopsis thaliana accessions on rhizobacterial communities and natural variation in root exudates. *J. Exp. Bot.* 60, 1729–1742. doi: 10.1093/jxb/erp053

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Picard, C., and Bosco, M. (2008). Genotypic and phenotypic diversity in populations of plant-probiotic *Pseudomonas* spp. colonizing roots. *Naturwissenschaften* 95, 1–6. doi: 10.1007/s00114-007-0286-3

Porras-Alfaro, A., and Bayman, P. (2011). Hidden fungi, emergent properties: endophytes and microbiomes. *Ann. Rev. Phyto.* 49, 291–315. doi: 10.1146/annurev-phyto-080508-081831

R Core Team (2012). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033

Strobel, G., and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* 67, 491–502. doi: 10.1128/MMBR.67.4.491-502.2003

Thompson, J. D., Gibson, T. J., and Higgins, D.G. (2003). Multiple sequence alignment using ClustalW and ClustalX. *Cur. Prot. Bioinform.* Chapter 2:Unit 2.3. doi: 10.1002/0471250953.bi0203s00

Van Hai, N., and Fotedar, R. (2009). Comparison of the effects of the prebiotics (Bio-Mos® and ß-1, 3-D-glucan) and the customised probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture* 289, 310–316. doi: 10.1016/j.aquaculture.2009.02.001

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326, 582–585. doi: 10.1126/science.1176765

Wake, D. B., and Vrendenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11466–11473. doi: 10.1073/pnas.0801921105

Walke, J. B., and Belden, L. K. (2016). Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog.* 12:e1005796. doi: 10.1371/journal.ppat.1005796

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., et al. (2016). Improved Bacterial 16S rRNA Gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems* 1:e00009-15. doi: 10.1128/mSystems.00009-15

West, S. A., and Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial parasites. *Proc. R. Soc. B Biol. Sci.* 270, 37–44. doi: 10.1098/rspb.2002.2209

White, T. J., Bruns, T., Lee, S. J. W. T., and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols:* 18, 315–322.

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96, 595–599. doi: 10.1890/14-1837.1

Woodhams, D. C., Bletz, M., Kueneman, J., and McKenzie, V. (2016). Managing amphibian disease with skin microbiota. *Trend Microbiol.* 24, 161–164. doi: 10.1016/j.tim.2015.12.010

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS ONE* 9:e96375. doi: 10.1371/journal.pone.0096375

Wright, K. M., and Whitaker, B. R. (2001). *Amphibian Medicine and Captive Husbandry*. Malabar, FL: Krieger Publishing Company.

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Temperature-Dependent Effects of Cutaneous Bacteria on a Frog's Tolerance of Fungal Infection

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Defense against pathogens is one of many benefits that bacteria provide to animal hosts. A clearer understanding of how changes in the environment affect the interactions between animals and their microbial benefactors is needed in order to predict the impact and dynamics of emerging animal diseases. Due to its dramatic effects on the physiology of animals and their pathogens, temperature may be a key variable modulating the level of protection that beneficial bacteria provide to their animal hosts. Here we investigate how temperature and the makeup of the skin microbial community affect the susceptibility of amphibian hosts to infection by *Batrachochytrium dendrobatidis (Bd)*, one of two fungal pathogens known to cause the disease chytridiomycosis. To do this, we manipulated the skin bacterial communities of susceptible hosts, northern cricket frogs (*Acris crepitans*), prior to exposing these animals to *Bd* under two different ecologically relevant temperatures. Our manipulations included one treatment where antibiotics were used to reduce the skin bacterial community, one where the bacterial community was augmented with the antifungal bacterium, *Stenotrophomonas maltophilia*, and one in which the frog's skin bacterial community was left intact. We predicted that frogs with reduced skin bacterial communities would be more susceptible (i.e., less resistant to and/or tolerant of *Bd* infection), and frogs with skin bacterial communities augmented with the known antifungal bacterium would be less susceptible to *Bd* infection and chytridiomycosis. However, we also predicted that this interaction would be temperature dependent. We found a strong effect of temperature but not of skin microbial treatment on the probability and intensity of infection in *Bd*-exposed frogs. Whether temperature affected survival; however, it differed among our skin microbial treatment groups, with animals having more *S. maltophilia* on their skin surviving longer at 14 but not at 26°C. Our results suggest that temperature was the predominant factor influencing *Bd*'s ability to colonize the host (i.e., resistance) but that the composition of the cutaneous bacterial community was important in modulating the host's ability to survive (i.e., tolerate) a heavy *Bd* infection.

Keywords: *Acris crepitans*, amphibian chytridiomycosis, antifungal, bioaugmentation, host–pathogen interactions, skin microbes, *Stenotrophomonas maltophilia*

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INTRODUCTION

That there exist mutualistic and even symbiotic relationships between animals and microbes has long been understood, yet it is only recently that we have come to appreciate how common and influential these relationships can be for ecological processes that play out across taxa and environments (reviewed in McFall-Ngai et al., 2013). While effects of temperature on microbial growth and community structure in soil and other environmental samples have been well-documented (e.g., Jansson and Tas, 2014; Piquet et al., 2016), how temperature variation affects animal–microbe interactions is less well-understood. Empirical data exist for a few hosts and their bacterial symbionts (e.g., sponges: Webster et al., 2008; Fan et al., 2013; sea anemone: Fraune et al., 2016), yet the range of taxa and types of interactions under which this relationship with temperature has been explored remains small (Carey and Duddleston, 2014). Studies that broaden this range of hosts and ecological contexts are needed to clarify how temperature affects animal interactions with microbes, and how these effects may impact wildlife responses to environmental stressors such as climate change and habitat modification.

Defense against infection is one important service that microbes can provide for their animal hosts (McFall-Ngai et al., 2013; Clavel et al., 2017) and changes in temperature can affect the potential of microbes to cause disease or help their hosts resist infections (Daskin and Alford, 2012). For example, elevated ocean temperatures increase the expression of virulence genes in the bacterial pathogen *Vibrio shiloi*, which induces bleaching in the coral *Oculina patagonica* (Rosenberg and Ben-Halm, 2002). On the flip side, the ability of ascidians to defend themselves against pathogens is likely impacted by effects of temperature on their community of symbiotic microbes (Tianero et al., 2015); bacterial symbionts provide these animals with their diverse repertoire of defensive secondary metabolites, some of which have antimicrobial and antiviral properties (Paul et al., 1990). Temperature may be a key player in determining the health benefits that symbiotic microbes bestow upon their animal hosts, especially for ectotherms (Kohl and Yahn, 2016; Ferguson, 2017), though clear empirical examples appear to be limited to handful of invertebrates (Tianero et al., 2015; Ferguson, 2017). Amphibians are another taxon useful for investigating the effects of temperature on microbial symbioses, as several aspects of the amphibian immune system are known to function in a temperature-dependent manner (Maniero and Carey, 1997; Rollins-Smith and Woodhams, 2012) and the cutaneous bacteria of amphibians are known to be important to their defense against other skin microbes, including pathogenic fungi in the genus *Batrachochytrium* (Harris et al., 2009a).

Chytridiomycosis, the disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans*, has been implicated in global amphibian declines (Berger et al., 1998; Lips et al., 2006; Rachowicz et al., 2006; Martel et al., 2013). Because *Batrachochytrium salamandrivorans* has only recently been described (Martel et al., 2013), less is known about its potential consequences for host populations (but see Martel et al., 2014). *Bd*, however, is known to disrupt electrolyte transport across frog skin, which can cause cardiac arrest, the mechanism of mortality (Voyles et al., 2009). Not all amphibians are equally at risk of infection. Hosts found in consistently cool, wet habitats in both temperate and tropical regions appear particularly vulnerable to *Bd*-related declines (Berger et al., 2016). *Bd* infection dynamics have also been correlated with climate and seasonality (Berger et al., 2004; Woodhams and Alford, 2005; Bishop et al., 2009; Rohr and Raffel, 2010), with infections often peaking in early spring (Kriger and Hero, 2007; Longcore et al., 2007; Rothermel et al., 2008). Variation in susceptibility to chytridiomycosis also exists within and among host species (Tobler and Schmidt, 2010; Martel et al., 2014), with some species requiring a higher pathogen load in order to become sick than others (Berger et al., 2004). This could be caused by differences among strains of *Bd*, as some are more pathogenic than others (Retallick and Miera, 2007; Farrer et al., 2011). Differences in susceptibility among hosts (Woodhams et al., 2007a) and populations (Savage and Zamudio, 2011) could also reflect intrinsic or temperature-driven differences in host immunity (reviewed in Rowley and Alford, 2010; Rollins-Smith and Woodhams, 2012).

Amphibian hosts have several potential lines of defense against *Batrachochytrium* pathogens. Antimicrobial peptides (AMPs), produced in the granular glands of the skin of some amphibians and secreted in mucus, have been shown to inhibit the growth of *Bd in vitro* (Rollins-Smith et al., 2006; Ramsey et al., 2010). Antibody and lymphocyte production is also stimulated by *Bd* exposure in some host species, suggesting the potential for an acquired immune response to this pathogen (Ramsey et al., 2010; McMahon et al., 2014). Variation in body temperature among individuals has also been correlated with the probability of *Bd* infection (Richards-Zawacki, 2010; Rowley and Alford, 2013; Roznik et al., 2015). Amphibians may also elevate their body temperature above normal by selecting warmer microhabitats, thereby inducing a behavioral fever (Sherman and Stephens, 1998; Woodhams et al., 2003; Sherman, 2008; Richards-Zawacki, 2010). This elevated temperature presumably enhances the immune response (Maniero and Carey, 1997; Rollins-Smith and Woodhams, 2012) though the effectiveness of such a fever in combatting *Bd* infection has not been demonstrated empirically.

Another component of amphibian defense against pathogens is the skin microbiome. The mucus on frog skin is home to a rich community of bacteria (McKenzie et al., 2012; Kueneman et al., 2013). While there is also variation within frog species (Lauer et al., 2008), cutaneous bacterial communities have been found to differ more among amphibian species than among individuals and/or environments (McKenzie et al., 2012; Becker et al., 2014). This suggests that innate differences in skin bacterial communities among species could contribute to differences in susceptibility to chytridiomycosis (McKenzie et al., 2012). Some members of amphibian skin bacterial communities are known to produce substances with antifungal capabilities. This was first demonstrated in two North American salamander species by Harris et al. (2006), who isolated skin bacteria and challenged *Bd* to grow in the presence of those isolates *in vitro*. Since then, a large and growing number of bacteria found on amphibian skin have been shown to inhibit the growth of *Bd* (Woodhams et al., 2015). It is believed to be metabolites produced by these bacteria

that inhibit *Bd* growth and confer resistance and/or tolerance to infection in amphibian hosts (Brucker et al., 2008a,b; Harris et al., 2009a; Becker et al., 2015). Support for this idea comes from *in vitro* studies where the metabolites produced by a variety of bacteria isolated from frog mucus inhibit the growth of *Bd* (Daskin et al., 2014; Woodhams et al., 2015).

Laboratory exposure studies provide further evidence that bacteria found on frog skin can contribute to variation in susceptibility to chytridiomycosis (Harris et al., 2009b; Woodhams et al., 2014). For example, adding *Janthinobacterium lividum*, a bacterium that produces the *Bd*-inhibitory metabolite violacein, to the skin of *R. muscosa* decreased the risk of mortality after *Bd* exposure (Harris et al., 2009a). Correlations between skin bacterial communities and susceptibility to chytridiomycosis have been documented in wild populations as well (Woodhams et al., 2007b). Given this, efforts are underway to develop probiotics that could be applied to frogs to protect them from *Bd* in the wild (Bletz et al., 2013). Probiotic approaches may prove to be the most plausible solution for *in situ* conservation of species that are threatened with extinction due to *Bd*, but their effectiveness may be dependent upon environmental conditions, including temperature (Woodhams et al., 2014).

Temperature is known to affect the growth (Gaddad and Rodgi, 1987; Pietikainen et al., 2005) and antifungal metabolite production (Noaman et al., 2004; Ripa et al., 2009; Kariluoto et al., 2010) of bacteria. There is also some evidence that the bacteria found on frog skin produce anti-*Bd* metabolites better at some temperatures than others. Daskin et al. (2014) found that the cell free supernatants from frog skin bacteria cultured at cooler temperatures were less effective at inhibiting the growth of *Bd in vitro*. Robak (2016) also found that antifungal metabolites produced by frog skin bacteria were more effective at inhibiting *Bd* growth at higher temperatures, but in this study, the temperature at which the metabolites were produced was less important than the temperature at which the growth challenge assay was performed. If this temperature dependence of anti-*Bd* activity is a general phenomenon, it could contribute to the observed correlations between frog body temperatures and *Bd* infection (Richards-Zawacki, 2010; Rowley and Alford, 2013; Roznik et al., 2015) and between climatic variation and chytridiomycosis (Berger et al., 2004; Woodhams and Alford, 2005; Rohr and Raffel, 2010). It also suggests that development of a successful probiotic treatment for *Bd* infected animals will require information on how the chosen bacteria would function in natural environments, where conditions such as temperature vary in space and time. In the Robak (2016) study, one bacterium of interest, *Stenotrophomonas maltophilia,* was found to produce metabolites that inhibit the growth of *Bd in vitro* at 14, 20, and 26°C, although the extent of inhibition was greatest at 20°C. While its products effectively inhibit *Bd* growth across a range of ecologically relevant temperatures in culture, it is not known whether (1) *S. maltophilia* presence on the skin would protect frogs from *Bd* infection and/or chytridiomycosis, or whether (2) the relationship between temperature and protection on hosts would mirror what was seen for *Bd* growth *in vitro*.

In this study, we examined the effect of temperature on the ability of skin microbes to protect an amphibian host against *Bd* infection and chytridiomycosis. To do this, we manipulated the skin bacterial communities of northern cricket frogs (*Acris crepitans*), a species known to be susceptible to chytridiomycosis (Zippel and Tabaka, 2008; Sonn et al., 2017) and either exposed them to *Bd* or sham-exposed them and housed half of each group at 14°C and the other half at 26°C. These temperatures were chosen as they are within the range of body temperatures that this host experiences during times of the year when they are infected with *Bd* in the wild (Sonn, 2016) and because susceptibility to chytridiomycosis in this host has been shown to differ between these two temperatures (Sonn et al., 2017). Within each temperature and exposure group (*Bd* vs. sham), frog skin bacterial communities were either (1) maintained intact, (2) reduced with antibiotics, or (3) augmented by inoculation with *S. maltophilia* (family Xanthomonadaceae, order Xanthomonadales). This Gram-negative bacterium found on frog skin, as well as in water, soil, and plant samples from a wide variety of environments and geographic regions (Denton and Kerr, 1998), has been demonstrated to inhibit *Bd* growth *in vitro* (Robak, 2016). We predicted that *Bd*-exposed frogs at the lower temperature would be more susceptible to chytridiomycosis, which we defined as having a greater *Bd* load (Voyles et al., 2009), decreased survival (Voyles et al., 2009), a higher prevalence of *Bd* infection (Vredenburg et al., 2010), or a lower body condition (Retallick and Miera, 2007; Murphy et al., 2011). We also predicted that frogs with their bacterial communities reduced would be more susceptible, and frogs with *S. maltophilia* added would be less susceptible to chytridiomycosis than frogs with intact skin microbial communities (Harris et al., 2009a), but that the effect of *S. maltophilia* on *Bd* susceptibility would be temperature dependent.

MATERIALS AND METHODS

Animal Husbandry

In February 2016, we collected 122 *A. crepitans* frogs from Tulane University's F. Edward Hebert Riverside Research Center near Belle Chasse, LA, United States (WGS84: 29.8852489, -89.9694904) and placed them individually into cylindrical plastic enclosures (15 cm tall, 11 cm diameter with ventilated lids) containing a 2.5 cm depth of filtered tap water. While the previous infection history of these individual frogs was not known, *Bd* had been detected, sometimes at greater than 50% prevalence, in this population (Brannelly et al., unpublished data). To clear any potential *Bd* infections, we heat-treated the animals by holding them at 30°C in an environmental chamber (Conviron, Adaptis; 12 h light/dark cycle) for 10 days (Chatfield and Richards-Zawacki, 2011). Given the high prevalence of *Bd* in this population and our observation that individuals frequently gain and lose infections in the wild (Brannelly et al., unpublished data), we assumed that most or all of these animals were likely exposed to *Bd* prior to this study, and if any immunoprotective effects of prior-exposure existed, they did not preclude animals from becoming re-infected. However, our study design did not permit us to control for immunoprotective

effects of prior *Bd*-exposure explicitly. After heat treatment, we tested the frogs for *Bd* following the swabbing and quantitative polymerase chain reaction (qPCR) assay protocols described below. After heat-treatment and during bacterial manipulations, we housed the frogs at 20°C. To get to this temperature, we lowered the temperature gradually over a period of 28 h. We assigned animals haphazardly to temperature, bacterial manipulation, and exposure groups, with each combination of temperature, bacterial manipulation, and exposure containing either 9, 10, or 11 animals. After bacterial manipulation, frogs were housed at either 14 or 26°C and were either inoculated with *Bd* or sham inoculated (**Table 1**). We fed the frogs *ad libitum* on 2 week-old crickets and provided them with a clean enclosure and fresh water every 7 days. We cleaned the enclosures with a 10% bleach solution and allowed them to dry completely before reuse. We wore a clean pair of nitrile gloves when handling each frog. We carried out this study in accordance with the recommendations of Tulane University's Institutional Animal Care and Use Committee (IACUC, Protocol No. 0391R2).

Animal Monitoring

We monitored frogs daily for the following clinical signs of chytridiomycosis: lethargy, inappetence, loss of righting reflex, excessive skin sloughing, abnormal posture, and cutaneous erythema (Berger et al., 2005). To test for *Bd* infection and the presence of *S. maltophilia* on frog skin, we rinsed the frogs in filtered tap water and then swabbed the skin by rubbing a rayon tipped swab (MWE 113, Medical Wire and Equipment, Co., United Kingdom) five times over the dorsum, venter, each side of the body, and the bottom of each foot. This was done once each week starting on day 6. Snout-vent length (SVL), measured to the nearest 0.1 mm with a dial calipers, and mass, measured with a scale to the nearest 0.01 g, were recorded weekly, starting on day −1, the day prior to the first round of *Bd* inoculations (**Table 2**). We used residual mass as our index of body condition and we calculated this using the line of best fit from a linear regression between SVL and body mass for all frogs on day −1 (Supplementary Figure S1). The predicted mass of each frog, based on this pre-experiment regression was then subtracted from the actual mass each week to get a residual value, which reflects body condition relative to the mean for a frog of that size prior to *Bd* exposure (Jakob et al., 2011). Frogs were euthanized by bath in tricaine methane sulfonate (MS-222, pH 7) at the conclusion of the experiment.

TABLE 1 | Treatment groups.

n	Bacteria	Inoculation	Temperature (°C)
10	Reduced	Sham	14
10	Reduced	Sham	26
10	Reduced	*Bd*	14
10	Reduced	*Bd*	26
9	Intact	Sham	14
10	Intact	sham	26
10	Intact	*Bd*	14
11	Intact	*Bd*	26
10	Added	sham	14
10	Added	sham	26
11	Added	*Bd*	14
11	Added	*Bd*	26

n = number of frogs; bacteria = bacterial manipulation; inoculation = inoculated with Bd or sham inoculated; and temperature = temperature frogs were housed at after bacterial manipulations.

TABLE 2 | Summary timeline for bacterial manipulations.

Day	Treatment
−5	Provosoli bath
−4	Antibiotic (BCR* and SMA) or filtered water (BCI) bath
−3	Fresh antibiotic (BCR and SMA) or filtered water (BCI) bath
−2	Filtered water (BCR), Provosoli bath (BCI), or Provosoli bath with added *S. maltophilia* (SMA)
−1	Returned to enclosure
0	*Bd* or sham exposures
1	Returned to enclosure

**Treatment groups: BCR, bacterial community reduced; SMA, S. maltophilia added; and BCI, bacterial community intact.*

Skin Microbe Quantification

We extracted genomic DNA from skin swabs using the Qiagen DNeasy Blood and Tissue kit, following the protocol for animal tissues with two modifications: (1) we incubated swabs for just 1 h, vortexing and spinning them in a centrifuge after 30 and 60 min of incubation; (2) we eluted samples twice with 100 μL of elution buffer instead of once with 200 μL. We then used a qPCR assay, performed on an Applied Biosystems 7500 system, to quantify the amount of *Bd* [in plasmid equivalents (PEs)]. We followed the protocol of Boyle et al. (2004) with the following modifications: (1) 0.7 μL of bovine serum albumin (Applied Biosystems) was added to each well prior to amplification (Garland et al., 2010) and (2) a sevenfold dilution series of *Bd* plasmid standards (Pisces Molecular, Boulder, CO, United States) was included in each run. For *S. maltophilia*, we quantified colony forming units (cfus) per swab using the same qPCR reaction cycling conditions and reagent concentrations as for *Bd*, but with primers and probes from Rios-Licea et al. (2010). For *S. maltophilia*, we generated a sevenfold dilution series of cfu standards by making serial dilutions of DNA extracted from a sample containing 5×10^6 cfus of *S. maltophilia*. We ran qPCRs on all swab samples in singlicate and considered animals positive for *Bd* if the qPCR result indicated that one or more copies of *Bd* DNA (i.e., ≥1 PE) were present in the reaction. Animals were considered to have become infected if they tested positive for *Bd* on one or more weekly swab samples. We converted *Bd* and *S. maltophilia* loads per 5 μL reaction volume to whole swab loads and then log-transformed these values prior to statistical analysis.

Bacterial Manipulations

Our skin bacterial community manipulations took place over 4 days and ended on the day prior to the first *Bd* (or sham) exposure (see **Table 2**). On day −5, we placed frogs in a bath of

30 mL modified Provosoli medium (Wyngaard and Chinnappa, 1982) for 24 h to collect native cutaneous bacteria. On day −4, we removed the frogs from their Provosoli baths and placed these baths in a refrigerator. Animals in the "reduced" bacterial community and "*S. maltophilia* added" treatments were moved directly into a second bath, this time containing 30 mL of an antibiotic cocktail that targets both Gram-negative and Gram-positive bacteria (24 mg/L cephalexin, 14.5 mg/L sulfamethazine, 2.9 mg/L trimethoprim, 100 mg/L streptomycin, and 10^5 I.U./L penicillin) for 24 h (following Holden et al., 2015). Frogs in the "intact" bacterial community treatment were instead moved into a bath containing 30 mL of filtered tap water for 24 h. On day −3, we placed the frogs into either a fresh antibiotic cocktail bath with the same composition as the first ("reduced" and "*S. maltophilia* added" treatments) or into a filtered water bath ("intact" treatment) for 24 h. On day −2, we rinsed all frogs with filtered tap water and frogs in the "reduced" treatment group were placed in a bath containing 30 mL of filtered water for 24 h. Frogs in the "intact" treatment were placed back in their Provosoli baths (from day −4). Frogs in the "*S. maltophilia* added" treatment were placed back in their Provosoli baths also, but only after adding 2×10^8 *S. maltophilia* to the bath. We left the frogs in these baths for 24 h. On day −1, we rinsed all frogs with filtered tap water and placed them in clean enclosures.

Bd Exposure

To prepare inoculum for *Bd* exposures, we grew *Bd* (JEL412 isolated from a *Sachatamia ilex* frog in Panama in 2005 and provided by Dr. Joyce Longcore) on 1% tryptone agar plates for 7 days, at which point we harvested zoospores by flooding plates with 5 mL of deionized water. We then exposed frogs individually by placing them in a bath of 2×10^6 zoospores suspended in 30 mL filtered tap water for 12 h. We carried out sham exposures in the same way except that 1% tryptone plates without *Bd* were flooded. We exposed frogs in this way weekly throughout the experiment, starting on day 0.

Data Analysis

We used generalized linear mixed models (GLMMs) to test for significant effects of bacterial manipulations, temperature, and an interaction between temperature and bacterial manipulation on the log-transformed *Bd* and S. *maltophilia* loads on *Bd*-exposed frogs. Our model contained fixed effects of temperature, bacterial community manipulation ("reduced," "intact," or "*S. maltophilia* added"), and the interaction between those variables with day as a repeated measure. For these and all following GLMMs, we used a first-order autoregressive covariance type for repeated effects and residuals, assumed a non-normal distribution, and used a Satterthwaite approximation for degrees of freedom. A second GLMM with these same fixed effect plus exposure group (*Bd*- vs. sham-exposed) allowed us to test for significant main and interactive effects of temperature, bacterial manipulation, and *Bd* exposure on body condition. We used a third GLMM to test whether temperature, bacterial treatment, or their interaction affected the probability that *Bd*-exposed frogs became infected (yes/no). This model used a binary logistic distribution and the events/trials syntax, with infection as the event and day as the trial. To test for significant differences in survival among treatments (bacterial manipulations, temperatures, and exposure groups), we used a Cox regression with log-transformed *Bd* load included as a covariate. For significant effects, we used a Kaplan–Meier survival analysis to compute Tarone–Ware pairwise comparisons among groups.

As an alternative analytical approach, since *S. maltophilia* loads did not always differ among our bacterial community manipulation groups (see section "Results"), we repeated the analyses for *Bd* load, body condition, probability of infection (yes/no), and survival described above, but this time replacing the fixed effect of bacterial community manipulation ("reduced," "intact," or "*S. maltophilia* added") with the covariate log-transformed *S. maltophilia* load (as determined by qPCR). These models included only *Bd*-exposed animals. When we had significant interaction effects with temperature in our survival analysis, we used separate Cox regressions for each experimental temperature (with Bonferroni-corrected *p*-values) for *post hoc* comparisons since Kaplan–Meier survival analyses cannot handle continuous factors like *S. maltophilia* load. All analyses were performed in IBM SPSS Statistics (v 23).

RESULTS

Stenotrophomonas maltophilia was detected on all frogs from all three bacterial manipulation groups and both temperatures throughout the experiment, with the exception of two frogs at 26°C that each tested negative in one weekly sample: one frog from the "*S. maltophilia* added" group tested negative on day 41 and one from the "intact" bacteria group tested negative on day 55 (Supplementary Figures S2A,B). The amount of *S. maltophilia* on the skin differed among temperature (GLMM: $F_{1,239} = 30.608$, $P < 0.001$) and bacterial manipulation (GLMM: $F_{2,239} = 5.538$, $P = 0.004$) groups, but the interaction between temperature and bacterial manipulation was not significant (GLMM: $F_{2,239} = 1.618$, $P = 0.200$). Frogs at 14°C had greater *S. maltophilia* loads than frogs at 26°C and frogs in the "intact" bacterial treatment had lower *S. maltophilia* loads than frogs in the "reduced" and "*S. maltophilia* added" groups (Tukey LSD: $t_{239} \geq 2.960$, $P \leq 0.003$, Supplementary Figure S3). There was no significant difference in *S. maltophilia* load between frogs in the "reduced" and "*S. maltophilia* added" treatments (Tukey LSD: $t_{239} = 0.047$, $P = 0.962$), suggesting that our cocktail of antibiotics did not reduce *S. maltophilia* abundance on frog skin.

All animals in *Bd*-exposed treatment groups had a positive qPCR result for *Bd* on at least one week of the experiment (Supplementary Figures S2C,D) and were therefore considered to have become infected. However, at 26°C, our *Bd*-exposed frogs tended to test positive for *Bd* only once or twice during the initial weeks of the experiment and then clear their infections. None of the sham-exposed animals ever tested positive for *Bd*. *Bd*-exposed frogs at 14°C had a greater probability of testing positive for *Bd* on any given swab than did *Bd*-exposed frogs at 26°C (GLMM: $F_{1,106} = 90.731$, $P < 0.001$; **Figure 1A**). There was no significant effect of bacterial manipulation (GLMM:

$F_{2,106} = 0.097$, $P = 0.908$) and no significant interaction between temperature and bacterial manipulation (GLMM: $F_{2,106} = 0.151$, $P = 0.860$) on the weekly probability of *Bd* infection. When *S. maltophilia* load replaced bacterial manipulation in our model, the result was similar: neither *S. maltophilia* load (GLMM: $F_{1,184} = 1.174$, $P = 0.280$) nor the interaction between *S. maltophilia* load and temperature (GLMM: $F_{1,193} = 1.528$, $P = 0.218$) was significant predictors of *Bd* infection in weekly swab samples.

For pathogen load, we found a significant effect of temperature (GLMM: $F_{1,175} = 446.463$, $P < 0.001$) but not of bacterial manipulation (GLMM: $F_{2,190} = 1.521$, $P = 0.221$) and the interaction between temperature and bacterial manipulation was not significant (GLMM: $F_{2,190} = 0.254$, $P = 0.776$). *Bd*-exposed animals at 14°C had greater *Bd* loads than those at 26°C (**Figure 1B** and Supplementary Figures S2C,D). When *S. maltophilia* load replaced bacterial manipulation in our model, the effect of temperature remained significant (GLMM: $F_{1,197} = 4.659$, $P = 0.032$) but the main effect of *S. maltophilia* load (GLMM: $F_{1,188} = 4.559$, $P = 0.034$) and the interaction between *S. maltophilia* load and temperature (GLMM: $F_{1,186} = 6.924$, $P = 0.009$) were also significant. *Post hoc* linear regressions (**Figure 2**) showed that the relationship between *S. maltophilia* load and *Bd* load on swabs was positive at 14°C ($R^2 = 0.171$, $B = 0.572$, $F_{1,99} = 20.466$, $P < 0.001$) but non-significant at 26°C ($R^2 = 0.003$, $F_{1,100} = 0.350$, $P = 0.555$).

Neither temperature (GLMM: $F_{1,672} = 2.827$, $P = 0.093$), bacterial manipulation (GLMM: $F_{1,672} = 0.212$, $P = 0.809$), nor exposure group (*Bd* vs. sham, GLMM: $F_{1,672} = 0.083$, $P = 0.773$) had a significant effect on body condition and there were no significant two- or three-way interactions between temperature, bacterial manipulation, and exposure group (GLMM: $F_{2,672} \leq 2.483$, $P \geq 0.084$). Results were similar when *S. maltophilia* load replaced bacterial manipulation in our model (GLMM: all $F_{1,201} \leq 1.702$, all $P \geq 0.194$). Body condition did change over the course of the experiment, though (GLMM, day: $F_{9,672} = 1.950$, $P = 0.043$; Supplementary Figure S4), with frogs increasing in body condition during the initial 3 weeks of the experiment (Tukey HSD: all pairwise $t_{672} \leq 1.834$, $P \geq 0.05$ except for day −1 vs. days 6, 13, and 20, which had $t_{672} \geq 2.464$, $P \leq 0.014$).

Clinical signs of chytridiomycosis and mortality were observed in *Bd*-exposed animals beginning 8 days after the initial *Bd* exposure. By the end of the experiment, 62 days after the first exposure, only five *Bd*-exposed frogs survived. Four of these were from 26°C treatments (one from each of the "intact" and "reduced" bacterial treatments and two from the "*S. maltophilia* added" treatment). The only surviving frog at 14°C was from the "reduced" bacterial community treatment (Supplementary Figures S2E,F). Mortality was low in sham-infected frogs (≤2 deaths per group), no clinical signs of chytridiomycosis were observed, and there were no significant differences in survival with respect to temperature or bacterial treatment (Tarone–Ware: $X_2 \leq 1.108$, $P \geq 0.293$).

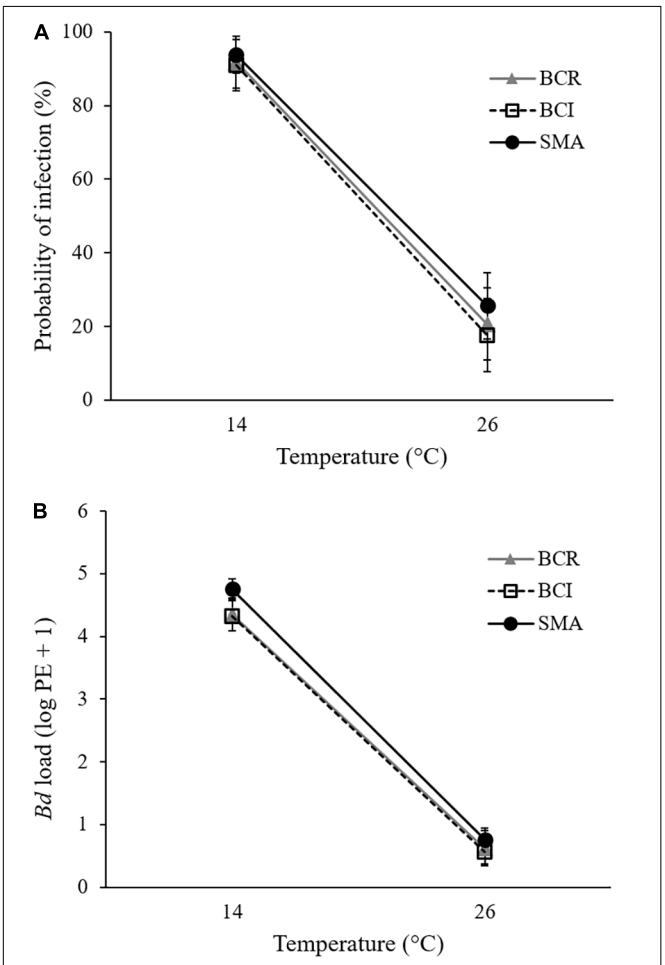

FIGURE 1 | Mean (± SE) of **(A)** probability of infection and **(B)** *Bd* load, measured as $\log_{10}$ of plasmid equivalents (PEs) for *Bd*-exposed frogs at two treatment temperatures and three bacterial manipulations (BCR, bacterial community reduced; BCI, bacterial community intact; and SMA, *S. maltophilia* added) over the 9 weeks of the experiment.

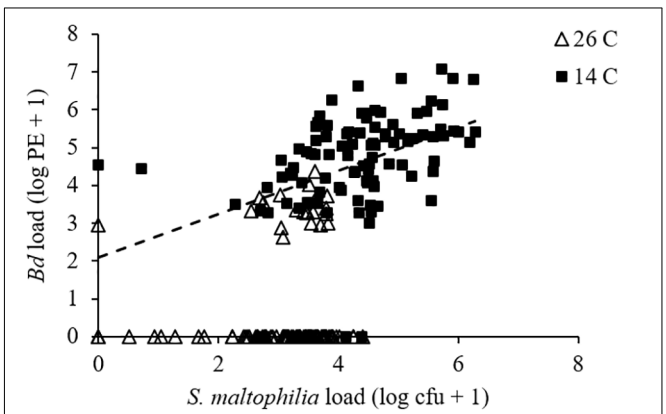

FIGURE 2 | Relationship between *Bd* load, measured as $\log_{10}$ of PEs and *S. maltophilia* load, measured as $\log_{10}$ of colony forming units (cfus) for *Bd*-exposed frogs. Line is the least-squares line of best fit ($y = 0.5722x + 2.0977$; $R^2 = 0.171$) for frogs at 14°C. The relationship was non-significant at 26°C.

Our final Cox regression model (overall model: $\chi^2 = 143.135$, $P < 0.001$) included the effect of exposure group ($Wald_1 = 71.712$, $P < 0.001$), with *Bd*-exposed frogs having lower survival than sham-exposed animals, and the interaction between bacterial manipulation and temperature ($Wald_2 = 7.453$, $P = 0.024$) but not the main effects of temperature ($Wald_1 = 0.122$, $P = 0.727$) or bacterial manipulation ($Wald_2 = 3.393$, $P = 0.183$) as significant predictors of survival. Considering only the *Bd*-exposed animals, *Bd* load was not a significant predictor of survival ($Wald_1 = 0.153$, $P = 0.695$). Frogs in the "*S. maltophilia* added" treatment survived longer than frogs with either "intact" or "reduced" bacterial communities at 26°C (Tarone–Ware: $\chi^2 \geq 5.123$, $P \leq 0.023$). At 14°C, *Bd*-exposed frogs in the "*S. maltophilia* added" treatment survived longer than those in the "intact" bacterial community treatment (Tarone–Ware: $\chi^2 = 11.392$, $P \leq 0.001$), but not significantly longer than frogs in the "reduced" bacteria treatment (Tarone–Ware: $\chi^2 = 0.017$, $P \leq 0.896$; **Figure 3**). When *S. maltophilia* load replaced bacterial manipulation in our Cox regression model, only the interaction between *S. maltophilia* load and temperature was significant ($Wald_1 = 5.852$, $P = 0.016$). Separate Cox regressions for animals at 14 versus 26°C showed that odds of mortality in *Bd*-exposed frogs decreased with increasing *S. maltophilia* load at 14°C ($B = -0.547$, $Wald_1 = 5.731$, corrected $P = 0.034$) but at 26°C, *S. maltophilia* load was not a significant predictor of mortality ($Wald_1 = 2.128$, corrected $P = 0.290$).

DISCUSSION

We examined temperature's effect on the ability of skin microbes to protect a susceptible frog species against *Bd* infection and chytridiomycosis. Given that amphibian immune function is reduced at low temperatures (Rollins-Smith and Woodhams, 2012), and that *Bd* infections and chytridiomycosis are more prevalent in animals with cooler body temperatures (Rowley and Alford, 2013) and in cool climates (Berger et al., 2004; Woodhams and Alford, 2005; Rohr and Raffel, 2010), we predicted that temperature would affect the susceptibility of *A. crepitans* to *Bd* infection and chytridiomycosis in this experiment. Specifically, we predicted that, as in a previous study (Sonn et al., 2017), *Bd*-exposed frogs housed under colder conditions would have higher pathogen loads, a greater likelihood of becoming infected, lower body condition, and/or lower survival. We also predicted that frogs treated with antibiotics prior to *Bd* exposure would be more susceptible, and frogs with the anti-*Bd* bacterium *S. maltophilia* added to their skin would be less susceptible to infection and/or disease than animals with an un-manipulated bacterial community. And finally, we predicted that the effects of *S. maltophilia* on *Bd* susceptibility would be temperature dependent. Some, but not all, of these predictions were upheld.

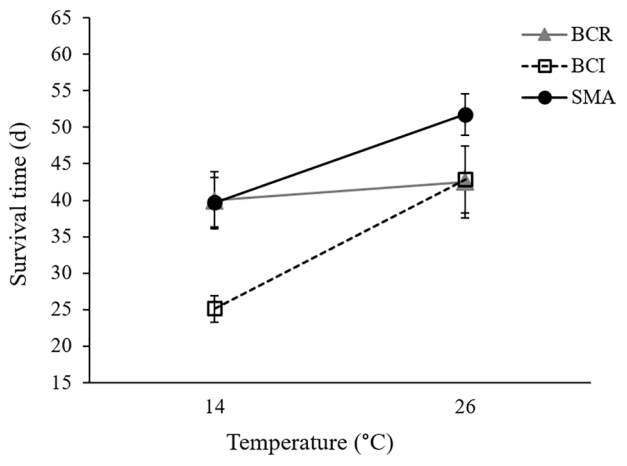

FIGURE 3 | Mean (± SE) survival time for *Bd*-exposed frogs in three bacterial manipulation groups (BCR, bacterial community reduced; BCI, bacterial community intact; and SMA, *S. maltophilia* added) at 14 and 26°C.

Temperature had an effect on the susceptibility of *Bd*-exposed frogs, with animals housed at 14°C having a greater likelihood of infection (**Figure 1A**) and also having greater pathogen loads (**Figure 1B** and Supplementary Figures S2C,D) than frogs housed at 26°C. This is consistent with idea that amphibian immune responses are often improved at higher temperatures (Maniero and Carey, 1997; Rollins-Smith and Woodhams, 2012). It is also consistent with the observations that in nature, (1) individual frogs with higher body temperatures are less likely to be infected with *Bd* (Richards-Zawacki, 2010; Rowley and Alford, 2013) and (2) disease variables and risk of *Bd*-related declines often reach a peak in cool seasons and climates (Berger et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2006; Longcore et al., 2007; Rohr and Raffel, 2010). This result is also consistent with a recent laboratory exposure study where the susceptibility of *A. crepitans* to chytridiomycosis was found to be inversely related to temperature (Sonn et al., 2017). In contrast to that study, however, the present study showed no significant main effect of temperature on the body condition or survival of *Bd*-exposed animals. There were, however, significant interactions between temperature and bacterial manipulation and between temperature and *S. maltophilia* load for survival, suggesting that the effect of temperature on the survival of *Bd*-exposed frogs depended on their cutaneous bacterial community.

While we predicted we would see significant main effects of bacterial manipulation on several indices of susceptibility to *Bd* infection and chytridiomycosis, the only variable we measured that appears to have been affected by our bacterial manipulations was survival, which differed in a temperature-dependent manner across our bacterial manipulation groups. On average, *Bd*-exposed frogs in the "reduced" bacterial treatment survived equally well at 14 and 26°C whereas frogs with "intact" or "*S. maltophilia* added" bacterial communities survived longer at the higher temperature (**Figure 3**). Frogs in the "*S. maltophilia* added" treatment at 26°C had the greatest mean survival time of all *Bd*-exposed groups.

Stenotrophomonas maltophilia has been found not only on frog skin, but also in water, soil, and plant samples from a wide variety of environments and geographic regions (Denton and Kerr, 1998). It is known to inhibit the growth of a broad range of plant (e.g., Elad et al., 1994; Kobayashi et al., 1995; Berg et al., 1996), and even some human (e.g., *Candida* spp., *Aspergillus*

fumigatus; Kerr, 1996) fungal pathogens. *Stenotrophomonas* species have been isolated from amphibian skin on several continents (North America: Woodhams et al., 2007b; South America: Flechas et al., 2012; Australia: Woodhams et al., 2015) and *S. maltophilia* (Flechas et al., 2012; Robak, 2016) and some of its congeners (Woodhams et al., 2015) have been shown to inhibit *Bd* growth *in vitro*. We chose *S. maltophilia* for use in this study because our previous work suggested that *S. maltophilia* inhibits *Bd* growth *in vitro* across a range of temperatures (Robak, 2016). However, despite having higher average *S. maltophilia* loads on their skin during this experiment, we did not see a lower probability of infection or lower pathogen load on *Bd*-exposed animals in our "*S. maltophilia* added" treatment, compared with animals in our "intact" bacterial treatment. Importantly though, we did see greater survival in the "*S. maltophilia* added" treatment, compared to our "intact" treatment animals at both 14 and 26°C. This suggests that augmentation of the cutaneous *S. maltophilia* population may yield benefits for *Bd*-exposed frogs across a range of temperatures.

We attempted to reduce the number and diversity of bacteria present on the skins of frogs in our "reduced" bacterial treatment via bath in a cocktail of antibiotics. However, our qPCR assays suggest that animals in our "reduced" treatments maintained as much *S. maltophilia* on their skins as animals in our "*S. maltophilia* added" treatments. It is possible that our antibiotic baths failed to reduce the bacterial communities on the frogs that received them. However, *S. maltophilia* is known to be naturally resistant to many broad-spectrum antibiotics (Denton and Kerr, 1998), so the large populations of this bacterial species on the skins of frogs in our "reduced" treatments could also be explained by the growth of *S. maltophilia* (and potentially other antibiotic-resistant bacteria) after the removal of their more antibiotic-susceptible competitors. The lower concentrations of *S. maltophilia* maintained by frogs with putatively more diverse skin communities in our "intact" treatments are consistent with this explanation. If *S. maltophilia* does indeed augment the host's ability to tolerate a heavy *Bd* infection, this could explain why frogs in our "reduced" treatment, which maintained high *S. maltophilia* loads on their skin, especially at 14°C, survived as long as frogs in our "*S. maltophilia* added" treatment at that temperature. However, in that case, it is not clear why our "reduced" treatment frogs did not receive the same survival benefit as our "*S. maltophilia* added" frogs at 26°C.

At 14°C, but not at 26°C, frogs with abundant *S. maltophilia* on their skin survived repeated exposures to *Bd* longer than frogs with "intact" skin bacterial communities where *S. maltophilia* was present, but less abundant. Interestingly, though *S. maltophilia* (Denton and Kerr, 1998) and other bacteria isolated from *A. crepitans* grow faster *in vitro* at 26 than at 14°C (Robak, 2016), frogs in all three of our bacterial treatment groups maintained more *S. maltophilia* on their skin at 14 than at 26°C (Supplementary Figure S3). It is not clear what caused this difference between *in vitro* and *in vivo* growth of *S. maltophilia* or whether the pattern holds for other members of the microbial community on amphibian skin. Interestingly, the temperature optimal for *Bd* growth on *A. crepitans* (Sonn et al., 2017) and other amphibian hosts (Cohen et al., 2017) also commonly differs from that of growth in culture.

While augmentation with a known anti-*Bd* bacterium was associated with longer survival in *Bd*-exposed animals at both 14 and 26°C, our results also support our prediction of temperature-dependent effects of beneficial skin microbes. For example, at 14°C, there was a positive relationship between *S. maltophilia* load and the odds of survival in *Bd*-exposed frogs. At this temperature, frogs in the "*S. maltophilia* added" and "reduced" bacterial community treatments, which had greater *S. maltophilia* loads (Supplementary Figure S3), survived *Bd* infections longer than did animals in the "intact" bacterial community treatment (**Figure 3** and Supplementary Figure S2E). We observed this difference in survival, despite the fact that *Bd* loads were similarly high among animals in all three bacterial treatment groups, suggesting that at 14°C, the load of *S. maltophilia* on the skin affected the animals' ability to survive with (i.e., tolerate) a heavy *Bd* infection. In contrast, at 26°C, the relationship between *S. maltophilia* load and survival was not significant.

The mechanism by which tolerance of *Bd* infection is modulated by *S. maltophilia* remains unclear. This bacterium's inhibition of phytopathogenic fungal growth has been linked to its production the antifungal secondary metabolites pyrrolnitrin (Kerr, 1996) and maltophilin (Jakobi et al., 1996). Interestingly, *S. maltophilia* also exhibits chitinolytic activity (Kobayashi et al., 1995). Chitin is an important part of cell wall structure stability for *Bd* and other chytrid fungi, and drugs that interfere with chitin synthesis have been shown to inhibit *Bd* growth *in vitro* (Holden et al., 2014). It seems likely that this chytinolytic activity plays a role in the effect that heavy loads of *S. maltophilia* on the skin had on the survival of our *Bd*-exposed hosts. At 14°C, *S. maltophilia* load was positively associated with survival in our *Bd*-exposed frogs. However, at 26°C, we saw no significant relationship between *S. maltophilia* load and survival. This could be because the *S. maltophilia* loads on our 26°C frogs never reached the levels that they did on animals at 14°C. Perhaps some threshold load of *S. maltophilia* is needed before the benefits of this microbe can be seen and the higher temperature prevented frogs in our 26°C treatments from reaching this threshold? It could also be that heavy *Bd* infections facilitate frogs sustaining large populations of *S. maltophilia* on the skin, as we found a positive relationship between *Bd* load and *S. maltophilia* load at 14°C (**Figure 2**). In this case, the lack of an effect of *S. maltophilia* on the survival of *Bd*-exposed animals at 26°C could be explained by the lower average *Bd* loads these animals experienced (**Figure 1B**).

All but one of the *Bd*-exposed frogs in our 14°C treatments became heavily infected with *Bd* during our experiment and most died after exhibiting clinical signs of chytridiomycosis. Manipulation of the bacterial community appears to have affected the survival time of heavily infected frogs (i.e., tolerance) but not the likelihood of infection (i.e., resistance). However, the infections we observed in *Bd*-exposed frogs at 26°C were generally light and often transient. In many cases, death at this temperature was preceded by one or more weekly skin swabs

that tested negative for the presence of *Bd*. It seems unlikely that these animals at 26°C were dying of chytridiomycosis, though they exhibited similar clinical signs to animals that died with high *Bd* loads in the 14°C treatments. However, survival of the *Bd*-exposed frogs was significantly lower than sham-exposed animals at both temperatures, suggesting that mortality was due to *Bd* exposure and not another pathogen or husbandry-related cause.

While it is not uncommon in *Bd* exposure studies for hosts to remain uninfected or clear *Bd* infections (e.g., Ramsey et al., 2010; Brannelly et al., 2012), we are not aware of other published studies where high mortality was seen in animals with transient and generally low-intensity *Bd* infections. We can think of two plausible explanations for the mortality experienced by our *Bd*-exposed animals at 26°C, both of which are related to our having exposed these animals repeatedly to high concentrations of this pathogen.

First, resisting infection can be costly (Dallas et al., 2016), especially if it involves activation of the immune system and/or stress response. Given that amphibian immune function is often temperature dependent (Rollins-Smith and Woodhams, 2012), the cost of resisting infection may depend upon temperature as well. Though this topic remains understudied, evidence for a cost of resisting *Bd* infection exists for newts (Cheatsazan et al., 2013) and tadpoles (Gabor et al., 2017). If such a cost exists for *A. crepitans*, it could explain the mortality we saw in *Bd*-exposed frogs at 26°C, though in that case, it is perhaps surprising that we did not see a decline in body condition in these animals. On the contrary, both *Bd*- and sham-exposed frogs at 26°C gained body condition over the course of the study and at no point in the experiment, there was a difference in body condition between these two exposure groups (Tukey HSD: $t_{672} \leq 1.895$, $P \geq 0.058$).

Second, *Bd* is known to produce and release toxic factors that cause pathology and mortality in crayfish, even in the absence of infection (McMahon et al., 2013). *Bd* is also known to produce a toxic factor or factors that inhibit immune responses to *Bd in vitro* (Fites et al., 2014) and possibly also *in vivo* (Ellison et al., 2014). While it is not clear whether the pathology and mortality in crayfish and immune inhibition in amphibians are generated by the same or different toxic factors, it seems likely that this fungus, like many others (Bondy and Pestka, 2000), produces toxins capable of affecting the fitness of amphibian hosts, perhaps even in the absence of an active infection in the skin (e.g., in tadpoles: Blaustein et al., 2005). While we cannot definitively attribute the mortality seen in our 26°C treatment to a toxin, if *Bd* does produce a substance capable of causing mortality in amphibians, our methods may have been more likely to produce this effect than the methods of other similar studies. We exposed frogs weekly by bath to small volumes of water containing millions of zoospores whereas other studies have tended to use fewer exposures and lower concentrations of *Bd* (e.g., reviewed in Kilpatrick et al., 2009). Not much is known about the frequency of exposure or the concentration of *Bd* in natural environments, so it is unclear whether our results would be expected to hold in the wild. However, a mark-recapture study of Louisiana *A. crepitans* suggests that repeated exposure and cycles of clearance and re-infection are common (Brannelly et al., unpublished data). We suggest that the potential for mortality due to toxin exposure rather than skin infection in amphibians exposed to *Bd* deserves further study.

Our results demonstrate that both temperature and the makeup of the skin bacterial community can impact the susceptibility of amphibian hosts to chytridiomycosis. Temperature's main effects were on the likelihood (i.e., resistance) and magnitude of infection whereas the skin microbial community affected the host's ability to survive a heavy infection (i.e., tolerance). Frogs at 14°C survived longer, despite large *Bd* burdens, when they harbored large populations of the antifungal bacterium *S. maltophilia* on their skin. Survival of frogs with *S. maltophilia*-enhanced skin communities was also longer at 26°C, though at this temperature, survival was not correlated with *S. maltophilia* load and exposure to, rather than infection with, *Bd* seems to have been the main cause of mortality. Whether this sort of interaction between temperature and the protection that bacteria provide against animal pathogens is common remains to be seen. Given its importance to the physiology of all three players, we predict that temperature may have especially strong impacts on the interactions of ectotherm hosts and their bacterial communities with fungal pathogens (Fisher et al., 2012; Carey and Duddleston, 2014; Daskin et al., 2014).

AUTHOR CONTRIBUTIONS

MR and CR-Z designed the study. MR conducted the lab work and wrote the first draft of the manuscript. CR-Z revised it for publication. Both authors analyzed and interpreted the data and approved the manuscript's content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00410/full#supplementary-material

REFERENCES

Becker, M. H., Richards-Zawacki, C. L., Gratwicke, B., and Belden, L. K. (2014). The effect of captivity on the cutaneous bacterial community of the critically endangered Panamanian golden frog (*Atelopus zeteki*). *Biol. Conserv.* 176, 199–206. doi: 10.1016/j.biocon.2014.05.029

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. Lond. B Biol. Sci.* 282:20142881. doi: 10.1098/rspb.2014.2881

Berg, G., Marten, P., and Balling, G. (1996). *Stenotrophomonas maltophilia* in the rhizosphere of oil-seed rape – occurrence, characterization, and interaction with phytopathogenic fungi. *Microbiol. Res.* 151, 19–27. doi: 10.1016/S0944-5013(96)80051-6

Berger, L., Marantelli, G., Skerratt, L. F., and Speare, R. (2005). Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Dis. Aquat. Organ.* 68, 47–50. doi: 10.3354/dao068047

Berger, L., Roberts, A. A., Voyles, J., Longcore, J. E., Murray, K. A., and Skerratt, L. F. (2016). History and recent progress on chytridiomycosis in amphibians. *Fungal Ecol.* 19, 89–99. doi: 10.1016/j.funeco.2015.09.007

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Berger, L., Speare, R., Hines, H. B., Marantelli, G., Hyatt, A. D., McDonald, K. R., et al. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.* 82, 434–439. doi: 10.1111/j.1751-0813.2004.tb11137.x

Bishop, P. J., Speare, R., Poulter, R., Butler, M., Speare, B. J., Hyatt, A., et al. (2009). Elimination of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* by Archey's frog *Leiopelma archeyi*. *Dis. Aquat. Organ.* 84, 9–15. doi: 10.3354/dao02028

Blaustein, A., Romansic, J., Scheessele, E., Han, B., Pessier, A., and Longcore, J. (2005). Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batrachochytrium dendrobatidis*. *Conserv. Biol.* 19, 1460–1468. doi: 10.1111/j.1523-1739.2005.00195.x

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bondy, G. S., and Pestka, J. J. (2000). Immunomodulation by fungal toxins. *J. Toxicol. Environ. Health B Crit. Rev.* 3, 109–143. doi: 10.1080/109374000281113

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Brannelly, L. A., Chatfield, M. W. H., and Richards-Zawacki, C. L. (2012). Field and laboratory studies of the susceptibility of the green treefrog (*Hyla cinerea*) to *Batrachochytrium dendrobatidis* infection. *PLoS One* 7:e38473. doi: 10.1371/journal.pone.0038473

Brucker, R. M., Baylor, C. M., Walters, R. L., Lauer, A., Harris, R. N., and Minbiole, K. P. C. (2008a). The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 39–43.

Brucker, R. M., Harris, R. N., Schwantes, C. R., Gallaher, T. N., Flaherty, D. C., Lam, B. A., et al. (2008b). Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 34, 1422–1429. doi: 10.1007/s10886-008-9555-7

Carey, H. V., and Duddleston, K. N. (2014). Animal-microbial symbioses in changing environments. *J. Therm. Ecol.* 44, 78–84. doi: 10.1016/j.jtherbio.2014.02.015

Chatfield, M. W. H., and Richards-Zawacki, C. L. (2011). Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. *Dis. Aquat. Organ.* 94, 235–238. doi: 10.3354/dao02337

Cheatsazan, H., de Almedia, A. P., Russell, A. F., and Bonneaud, C. (2013). Experimental evidence for a cost of resistance to the fungal pathogen *Batrachochytrium dendrobatidis* for the palmate newt, *Lissotriton helveticus*. *BMC Ecol.* 13:27. doi: 10.1186/1472-6785-13-27

Clavel, T., Gomes-Neto, J. C., Lagkouvardos, I., and Ramer-Tait, A. E. (2017). Deciphering interactions between the gut microbiota and the immune system via microbial cultivation and minimal microbiomes. *Immunol. Rev.* 279, 8–22. doi: 10.1111/imr.12578

Cohen, J. M., Venesky, M. D., Sauer, E. L., Civitello, D. J., McMahon, T. A., Roznik, E. A., et al. (2017). The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecol. Lett.* 20, 184–193. doi: 10.1111/ele.12720

Dallas, T., Holtackers, M., and Drake, J. M. (2016). Costs of resistance and infection by a generalist pathogen. *Ecol. Evol.* 6, 1737–1744. doi: 10.1002/ece3.1889

Daskin, J. H., and Alford, R. A. (2012). Context-dependent symbioses and their potential roles in wildlife diseases. *Proc. R. Soc. Lond. B Biol. Sci.* 279, 1457–1465. doi: 10.1098/rspb.2011.2276

Daskin, J. H., Bell, S. C., Schwarzkopf, L., and Alford, R. A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians—implications for disease management and patterns of decline. *PLoS One* 9:e100378. doi: 10.1371/journal.pone.0100378

Denton, M., and Kerr, K. G. (1998). Microbial and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. *Clin. Microbiol. Rev.* 11, 57–80.

Elad, Y., Koelh, J., and Fokkema, N. J. (1994). Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic bacteria and fungi. *Eur. J. Plant Pathol.* 100, 315–336. doi: 10.1007/BF01876443

Ellison, A. R., Savage, A. E., DiRenzo, G. V., Langhammer, P., Lips, K. R., and Zamudio, K. R. (2014). Fighting a losing battle: vigorous immune response countered by pathogen suppression of host defenses in the chytridiomycosis-susceptible frog *Atelopus zeteki*. *G3* 4, 1275–1289. doi: 10.1534/g3.114.010744

Fan, L., Liu, M., Simister, R., Webster, N. S., and Thomas, T. (2013). Marine microbial symbiosis heats up: the phylogenetic and functional response of a sponge holobiont to thermal stress. *ISME J.* 7, 991–1002. doi: 10.1038/ismej.2012.165

Farrer, R. A., Weinert, L. A., Bielby, J., Garner, T. W., Balloux, F., Clare, F., et al. (2011). Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18732–18736. doi: 10.1073/pnas.1111915108

Ferguson, L. V. (2017). *Thermal Biology of Insect Immunity and Host-microbe Interactions*. Doctoral dissertation, University of Western Ontario, London, ON.

Fisher, M. C., Hank, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., et al. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature* 484, 186–194. doi: 10.1038/nature10947

Fites, J. S., Reinert, L. K., Chappell, T. M., and Rollins-Smith, L. A. (2014). Inhibition of local immune responses by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infect. Immun.* 82, 4698–4706. doi: 10.1128/IAI.02231-14

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS One* 7:e44832. doi: 10.1371/journal.pone.0044832

Fraune, S., Foret, S., and Reitzel, A. M. (2016). Using *Nematostella vectensis* to study the interactions between genome, epigenome, and bacteria in a changing environment. *Front. Mar. Sci.* 3:148. doi: 10.3389/fmars.2016.00148

Gabor, C., Forsburg, Z., Voros, J., Serrano-Laguna, C., and Bosch, J. (2017). Differences in chytridiomycosis infection costs between two amphibian species from Central Europe. *Amphib. Reptilia* 38, 250–256. doi: 10.1163/15685381-00003099

Gaddad, S. M., and Rodgi, S. S. (1987). The effect of temperature on the growth and biochemical activities of *Escherichia coli* in sewage. *Environ. Pollut.* 43, 313–321. doi: 10.1016/0269-7491(87)90184-9

Garland, S., Baker, A., Phillott, A. D., and Skerratt, L. F. (2010). BSA reduces inhibition in a TaqMan assay for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 92, 113–116. doi: 10.3354/dao02053

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Holden, W. M., Fites, J. S., Reinert, L. K., and Rollins-Smith, L. A. (2014). Nikkomycin Z is an effective inhibitor of the chytrid fungus linked to global amphibian declines. *Fungal Biol.* 118, 48–60. doi: 10.1016/j.funbio.2013.11.001

Holden, W. M., Hanlon, S. M., Woodhams, D. C., Chappell, T. M., Wells, H. L., Glisson, S. M., et al. (2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (*Rana sphenocephala*) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 187, 91–102. doi: 10.1016/j.biocon.2015.04.007

Jakob, E. M., Marrshall, S. D., and Uetz, G. W. (2011). Estimating fitness: a comparison of body condition indices. *Oikos* 77, 61–67. doi: 10.2307/3545585

Jakobi, M., Windelmann, G., Kaiser, D., Kempter, C., Jung, G., Berg, G., et al. (1996). Maltophilin: a new antifungal compound produced by *Stenotrophomonas maltophilia* R3089. *J. Antibiot.* 49, 1101–1104. doi: 10.7164/antibiotics.49.1101

Jansson, J. K., and Tas, N. (2014). The microbial ecology of permafrost. *Nat. Rev. Microbiol.* 12, 414–425. doi: 10.1038/nrmicro3262

Kariluoto, S., Edelmann, M., Herranen, M., Lampi, A., Shmelev, A., Salovaara, H., et al. (2010). Production of folate by bacteria isolated from oat bran. *Int. J. Food Microbiol.* 143, 41–47. doi: 10.1016/j.ijfoodmicro.2010.07.026

Kerr, J. R. (1996). Inhibition of growth of fungi pathogenic to man by *Stenotrophomonas maltophilia*. *J. Med. Microbiol.* 45, 380–382. doi: 10.1099/00222615-45-5-380

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2009). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol. Evol.* 25, 109–118. doi: 10.1016/j.tree.2009.07.011

Kobayashi, D. Y., Guglielmoni, M., and Clark, B. B. (1995). Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turf grass. *Soil Biol. Biochem.* 27, 1479–1487. doi: 10.1016/0038-0717(95)00062-J

Kohl, K. D., and Yahn, J. (2016). Effects of environmental temperature on the gut microbial communities of tadpoles. *Environ. Microbiol.* 18, 1561–1565. doi: 10.1111/1462-2920.13255

Kriger, K. M., and Hero, J. M. (2006). Survivorship in wild frogs infected with chytridiomycosis. *EcoHealth* 3, 171–177. doi: 10.1007/s10393-006-0027-7

Kriger, K. M., and Hero, J. M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *J. Zool.* 271, 352–359.

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2013). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A., and Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J.* 2, 145–157. doi: 10.1038/ismej.2007.110

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Longcore, J. R., Longcore, J. E., Pessier, A. P., and Halteman, W. A. (2007). Chytridiomycosis widespread in anurans of northeastern United States. *J. Wildl. Manage.* 71, 435–444. doi: 10.2193/2006-345

Maniero, G. D., and Carey, C. (1997). Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *J. Comp. Physiol. B* 167, 256–263. doi: 10.1007/s003600050072

Martel, A., Blooi, M., Adriaensen, C., Van Rooij, P., Beukema, W., Fisher, M. C., et al. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science* 346, 630–631. doi: 10.1126/science.1258268

Martel, A., Spitzen-van der Slujis, A., Blooi, M., Bert, W., Ducatell, R., Fisher, M. C., et al. (2013). *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 110, 15325–15329. doi: 10.1073/pnas.1307356110

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Comazet-Loso, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3229–3236. doi: 10.1073/pnas.1218525110

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMahon, T. A., Brannelly, L. A., Chatfield, M. W. H., Johnson, P. T., Joseph, M. B., and McKenzie, V. J. (2013). Chytrid fungus *Batrachochytrium dendrobatidis* has nonamphibian hosts and releases chemicals that cause pathology in crayfish in the absence of infection. *Proc. Natl. Acad. Sci. U.S.A.* 110, 210–215. doi: 10.1073/pnas.1200592110

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., et al. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature* 511, 224–227. doi: 10.1038/nature13491

Murphy, P. J., St.-Hilaire, S., and Corn, P. S. (2011). Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. *Dis. Aquat. Organ.* 95, 31–42. doi: 10.3354/dao02336

Noaman, N. H., Fattah, A., Khaleafa, M., and Zaky, S. H. (2004). Factors affecting antimicrobial activity of *Synechococcus leopoliensis*. *Microbiol. Res.* 159, 395–402. doi: 10.1016/j.micres.2004.09.001

Paul, V. J., Lindquist, N., and Fenical, W. (1990). Chemical defenses of the tropical ascidian *Atapozoa sp*. and its nudibranch predators *Nembrotha spp*. *Mar. Ecol. Prog. Ser.* 59, 109–118. doi: 10.3354/meps059109

Pietikainen, J., Pettersson, M., and Baath, E. (2005). Comparison of temperature effects on soil respiration and bacterial and fungal growth rates. *FEMS Microbiol. Ecol.* 52, 49–58. doi: 10.1016/j.femsec.2004.10.002

Piquet, A. M.-T., Maat, D. S., Confurius-Guns, V., Sintes, E., Herndl, J., van de Poll, W. H., et al. (2016). Springtime dynamics, productivity and activity of prokaryotes in two Arctic fjords. *Polar Biol.* 39, 1749–1763. doi: 10.1007/s00300-015-1866-x

Rachowicz, L. J., Knapp, R. A., Morgan, J. A. T., Stice, M. J., Vredenburg, V. T., Parker, J. M., et al. (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87, 1671–1683. doi: 10.1890/0012-9658(2006)87[1671:EIDAAP]2.0.CO;2

Ramsey, J. P., Reinert, L. K., Harper, L. K., Woodhams, D. C., and Rollins-Smith, L. A. (2010). Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. *Infect. Immun.* 78, 3981–3992. doi: 10.1128/IAI.00402-10

Retallick, R. W. R., and Miera, V. (2007). Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. *Dis. Aquat. Organ.* 75, 201–207. doi: 10.3354/dao075201

Richards-Zawacki, C. L. (2010). Thermoregulatory behavior affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. *Proc. R. Soc. Lond. B Biol. Sci.* 277, 519–528. doi: 10.1098/rspb.2009.1656

Rios-Licea, M. M., Bosques, F. J., Arroliga, A. C., Galindo-Galindo, J. O., and Garza-Gonzalez, E. (2010). Quadruplex real-time quantitative PCR assay for the detection of pathogen related to late-onset ventilator-associated pneumonia. A preliminary report. *J. Microbiol. Methods* 81, 232–234. doi: 10.1016/j.mimet.2010.03.018

Ripa, F. A., Nikkon, F., Zaman, S., and Khondkar, P. (2009). Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp. RUPA-08PR isolated from Bangladeshi soil. *Mycobiology* 37, 211–214. doi: 10.4489/MYCO.2009.37.3.211

Robak, M. J. (2016). *Effects of Temperature on Amphibian Resistance and Susceptibility to Chytridiomycosis*. Doctoral dissertation, Tulane University, New Orleans, LA.

Rohr, J. R., and Raffel, T. R. (2010). Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8269–8274. doi: 10.1073/pnas.0912883107

Rollins-Smith, L. A., and Woodhams, D. C. (2012). "Amphibian immunity: staying in tune with the environment," in *Ecoimmunology*, eds G. E. Demas and R. J. Nelson (Oxford: Oxford University Press), 92–143.

Rollins-Smith, L. A., Woodhams, D. C., Reinert, L. K., Vredenburg, V. T., Briggs, C. J., Nielsen, P. F., et al. (2006). Antimicrobial peptide defense of the mountain yellow-legged frog (*Rana muscosa*). *Dev. Comp. Immunol.* 30, 831–842. doi: 10.1016/j.dci.2005.10.005

Rosenberg, E., and Ben-Halm, Y. (2002). Microbial diseases of corals and global warming. *Environ. Microbiol.* 6, 318–326. doi: 10.1046/j.1462-2920.2002.00302.x

Rothermel, B. B., Walls, S. C., Mitchell, J. C., Dodd, C. K., Irwin, L. K., Green, D. E., et al. (2008). Widespread occurrence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* in the southeastern USA. *Dis. Aquat. Organ.* 82, 3–18. doi: 10.3354/dao01974

Rowley, J. J. L., and Alford, R. A. (2010). "Factors affecting interspecific variation in susceptibility to disease in amphibians," in *Amphibian Biology. Amphibian Decline: Diseases, Parasites, Maladies and Pollution*, Vol. 8, eds H. Heatwole and J. W. Wilkinson (Baulkham Hills, NSW: Surrey Beatty and Sons), 3053–3066.

Rowley, J. J. L., and Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Sci. Rep.* 3:1515. doi: 10.1038/srep01515

Roznik, E. A., Sapsford, S. J., Pike, D. A., Schwarzkopf, L., and Alford, R. A. (2015). Natural disturbance reduces disease risk in endangered rainforest frog populations. *Sci. Rep.* 5:13472. doi: 10.1038/srep13472

Savage, A. E., and Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16705–16710. doi: 10.1073/pnas.1106893108

Sherman, E. (2008). Thermal biology of newts (*Notophthalmus viridescens*) chronically infected with a naturally occurring pathogen. *J. Therm. Biol.* 33, 27–31. doi: 10.1016/j.jtherbio.2007.09.005

Sherman, E., and Stephens, A. (1998). Fever and metabolic rate in the toad *Bufo marinus*. *J. Therm. Biol.* 23, 49–52. doi: 10.1016/S0306-4565(97)00045-4

Sonn, J. S. (2016). *The Influence of Temperature on Chytridiomycosis in Northern Cricket Frogs (Acris crepitans)*. Doctoral dissertation, Tulane University, New Orleans, LA.

Sonn, J. S., Berman, S., and Richards-Zawacki, C. L. (2017). The influence of temperature on chytridiomycosis *in vivo*. *EcoHealth* 14, 762–770. doi: 10.1007/s10393-017-1269-2

Tianero, M. D. B., Kwan, J. C., Wyche, T. P., Presson, A. P., Koch, M., Barrows, L. R., et al. (2015). Species specificity of symbiosis and secondary metabolism in ascidians. *ISME J.* 9, 615–628. doi: 10.1038/ismej.2014.152

Tobler, U., and Schmidt, B. R. (2010). Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricians*. *PLoS One* 5:e10927. doi: 10.1371/journal.pone.0010927

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326, 582–585. doi: 10.1126/science.1176765

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9688–9694. doi: 10.1073/pnas.0914111107

Webster, N. S., Cobb, R. E., and Negri, A. P. (2008). Temperature thresholds for bacterial symbiosis with a sponge. *ISME J.* 2, 830–842. doi: 10.1038/ismej.2008.42

Woodhams, D. C., and Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conserv. Biol.* 19, 1449–1459. doi: 10.1111/j.1523-1739.2005.004403.x

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96:595. doi: 10.1890/14-1837.1

Woodhams, D. C., Alford, R. A., and Marantelli, G. (2003). Emerging disease of amphibians cured by elevated body temperature. *Dis. Aquat. Organ.* 55, 65–67. doi: 10.3354/dao055065

Woodhams, D. C., Ardipradja, K., Alford, R. A., Marantelli, G., Reinert, L. K., and Rollins-Smith, L. A. (2007a). Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.* 10, 409–417. doi: 10.1111/j.1469-1795.2007.00130.x

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Kupfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Vredenburg, V. T., Simon, M., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007b). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Wyngaard, G. A., and Chinnappa, C. C. (1982). "General biology and cytology of Cyclopoids," in *Developmental Biology of Freshwater Invertebrates*, eds F. W. Harrison and R. R. Crowden (New York, NY: Liss), 485–533.

Zippel, K., and Tabaka, C. (2008). Amphibian chytridiomycosis in captive *Acris crepitans blanchardi* (Blanchard's cricket frog) collected from Ohio, Missouri, and Michigan, USA. *Herpetol. Rev.* 39, 192–193.

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Widespread Elevational Occurrence of Antifungal Bacteria in Andean Amphibians Decimated by Disease: A Complex Role for Skin Symbionts in Defense Against Chytridiomycosis

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Emerging infectious disease is a growing threat to global health, and recent discoveries reveal that the microbiota dwelling on and within hosts can play an important role in health and disease. To understand the capacity of skin bacteria to protect amphibian hosts from the fungal disease chytridiomycosis caused by *Batrachochytrium dendrobatidis* (Bd), we isolated 192 bacterial morphotypes from the skin of 28 host species of frogs (families Bufonidae, Centrolenidae, Hemiphractidae, Hylidae, Leptodactylidae, Strabomantidae, and Telmatobiidae) collected from the eastern slopes of the Peruvian Andes (540–3,865 m a.s.l.) in the Kosñipata Valley near Manu National Park, a site where we previously documented the collapse of montane frog communities following chytridiomycosis epizootics. We obtained isolates through agar culture from skin swabs of wild frogs, and identified bacterial isolates by comparing 16S rRNA sequences against the GenBank database using BLAST. We identified 178 bacterial strains of 38 genera, including 59 bacterial species not previously reported from any amphibian host. The most common bacterial isolates were species of *Pseudomonas, Paenibacillus*, *Chryseobacterium, Comamonas, Sphingobacterium*, and *Stenotrophomonas*. We assayed the anti-fungal abilities of 133 bacterial isolates from 26 frog species. To test whether cutaneous bacteria might inhibit growth of the fungal pathogen, we used a local Bd strain isolated from the mouthparts of stream-dwelling tadpoles (*Hypsiboas gladiator*, Hylidae). We quantified Bd-inhibition *in vitro* with co-culture assays. We found 20 bacterial isolates that inhibited Bd growth, including three isolates not previously known for such inhibitory abilities. Anti-Bd isolates occurred on aquatic and terrestrial breeding frogs across a wide range of elevations (560–3,695 m a.s.l.). The inhibitory ability of anti-Bd isolates varied considerably. The proportion of anti-Bd isolates was lowest at mid-elevations (6%), where amphibian declines have been steepest, and among hosts that are highly susceptible to chytridiomycosis (0–14%). Among non-susceptible species, two had the highest proportion of anti-Bd isolates (40 and 45%), but one common and non-susceptible species

had a low proportion (13%). In conclusion, we show that anti-Bd bacteria are widely distributed elevationally and phylogenetically across frog species that have persisted in a region where chytridiomycosis emerged, caused a devastating epizootic and continues to infect amphibians.

Keywords: 16S rRNA gene, amphibian declines, amphibian skin bacteria, antifungal bacteria, elevational gradient, montane diversity gradient, neotropical, tropical Andes

INTRODUCTION

Emerging infectious disease is a growing threat to global health and is identified as a major factor involved in the current biodiversity crisis (Daszak et al., 1999). Amphibians are considered one of the most threatened group of vertebrates on earth (Stuart et al., 2004; Wake and Vredenburg, 2008; Catenazzi, 2015), and the recently emerged disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd), has decimated species of amphibians in many parts of the world (Berger et al., 1998; Skerratt et al., 2007; Kilpatrick et al., 2010). The microbiota that live on and within hosts can play an important role in health and disease (Harris et al., 2009; Hanada et al., 2010; Hoyt et al., 2015; Ford and King, 2016). To understand the role of skin bacteria in protecting amphibians from chytridiomycosis, we document the distribution of amphibian skin bacteria with antifungal properties in host communities along the eastern slopes of the tropical Andes where amphibian populations have collapsed after Bd epizootics (Catenazzi et al., 2011). This region has the highest amphibian species richness on Earth (Hutter et al., 2017), and along our elevational gradient amphibian richness changes from more than 60 species in the Andean foothills to six species of amphibians in the high-elevation grasslands (Catenazzi et al., 2013). In our previous work (Burkart et al., 2017), we showed that culturable skin bacteria inhibiting Bd growth (henceforth, anti-Bd isolates) is linked to resistance to chytridiomycosis in high-elevation frogs. Here, we extend this approach to the broader elevational gradient, including elevations where species declines have been steepest from 1,250 to 1,750 m a.s.l. (Catenazzi et al., 2011), and relate the presence of anti-Bd bacteria on frogs to host susceptibility to chytridiomycosis (Catenazzi et al., 2017).

Amphibian skin provides an excellent environment for the growth of a wide variety of microorganisms (Kueneman et al., 2014; Bletz et al., 2017; Prado-Irwin et al., 2017). These microbial communities are functionally important for the host, for example in the defense against pathogens, such as Bd (Bettin and Greven, 1986; Harris et al., 2006; Belden and Harris, 2007; Culp et al., 2007; Woodhams et al., 2007b; Lauer et al., 2008; Becker and Harris, 2010; Flechas et al., 2012). Although many amphibian species have succumbed to epizootics of chytridiomycosis, others persist despite infection. Resistance and tolerance to chytridiomycosis has been attributed to the presence of beneficial bacteria in the skin, which can delay or inhibit the growth of the pathogen allowing host survival (Harris et al., 2006, 2009; Woodhams et al., 2007a).

Amphibian skin bacteria are likely to vary across hosts and elevation, as seen in recent studies of other Neotropical amphibian communities (Bresciano et al., 2015; Hughey et al., 2017). A putative source of amphibian skin bacteria, soil bacterial communities are known to change with elevation and slope aspect on mountains (Bryant et al., 2008). We hypothesize two main types of drivers affecting bacterial species composition at our study site, one related to environmental variation and the other to host behavior. We hypothesized that the environment (water, soil, leaf surface, etc.) is the main source of bacteria colonizing the skin of amphibians and that a species' natural history (elevational range, aquatic vs. terrestrial reproduction) influences its encounters with bacterial sources. At our study site the main determinant of environmental change is elevation, which is correlated with changes in temperature, rainfall, oxygen availability, and UV radiation. Bacterial species richness in three habitats (the phyllosphere, the mineral and the organic soil) does not vary consistently along the elevational gradient, but bacterial community composition varies across these three habitats, and within each habitat, across elevation (Fierer et al., 2011; Nottingham et al., unpublished). Thus, it is possible that the species composition of bacterial communities on amphibian skin could vary with elevation.

In addition to environmental factors, strains (or isolates; these two terms are interchangeable throughout the manuscript) colonizing the amphibian skin from the environmental pool may be filtered by host identity and behavior (McKenzie et al., 2012; Kueneman et al., 2014). Possible host-associated factors include skin morphology, the type and diversity of antimicrobial peptides secreted by skin, microhabitat use (e.g., use of microhabitats in close contact with soil; **Figure 1**), reproductive mode (e.g., requiring extended time in water), and hydric and thermoregulatory behaviors (Rollins-Smith and Woodhams, 2011; Bletz et al., 2017). Amphibians differ widely in their reproductive modes (Wells, 2007), which are linked to host affinity with aquatic environments. Adults of species with aquatic eggs and/or tadpoles often congregate in or around water bodies to attract mates, mate, lay eggs, and attend progeny (for species with parental care). In contrast, adults of species that lay eggs on land may rarely approach water bodies, and may be more homogeneously distributed on the forest litter or arboreal vegetation far from ponds or streams.

Traditional, culture-based methods to study bacterial diversity are limited in that only a fraction of the taxa present can be isolated and cultured. The use of next-generation sequencing has improved our understanding of microbial community composition (Qin et al., 2010; Fierer et al., 2011; Russo et al., 2012). Nonetheless, culture-based methods can provide accurate information on the functions of a specific bacterium (Becker et al., 2015b), and relevance of these functions to its symbiotic

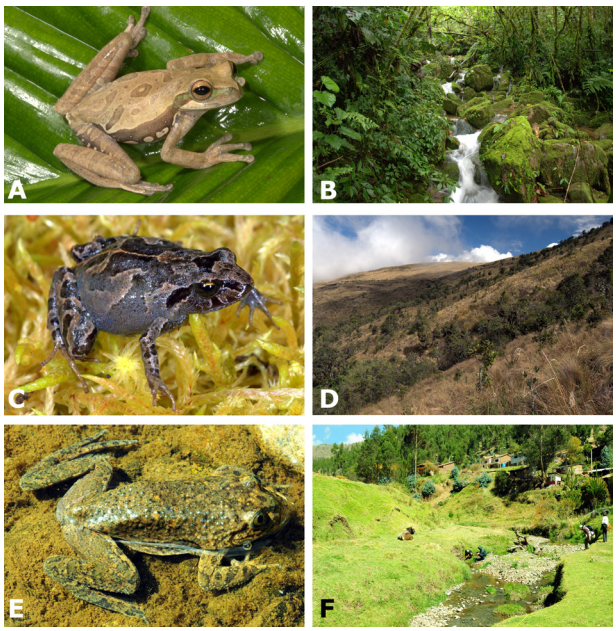

FIGURE 1 | Representative amphibian hosts and their habitats sampled for this study: **(A,B)** *Hypsiboas gladiator* is non-susceptible to chytridiomycosis and lays aquatic eggs in streamside basins along montane streams in the cloud forest; **(C,D)** *Psychrophrynella usurpator* is non-susceptible and lays terrestrial eggs that undergo direct development under mosses in the high-Andean grassland; **(E,F)** *Telmatobius marmoratus* is highly susceptible to chytridiomycosis and lays aquatic eggs in small, high-Andean streams. Photographs by A. Catenazzi.

organism. Many studies have tested the antifungal properties of bacteria harbored on the skin of different amphibian species (Harris et al., 2006; Woodhams et al., 2007b; Lauer et al., 2008; Becker and Harris, 2010) and demonstrated that either a high proportion of anti-Bd bacteria, or bacteria with strong antifungal capacities, might explain the survival of some host species (Flechas et al., 2012; Burkart et al., 2017). Bacterial species belonging to the genera *Pseudomonas*, *Serratia*, and *Janthinobacterium* are among those that have demonstrated great abilities to inhibit Bd growth (Becker et al., 2015a,b; Bresciano et al., 2015), but there is a wide variety of other genera with similar abilities (Woodhams et al., 2015). Thus, there is a need to combine culture-based methods with molecular analyses to explore amphibian skin bacterial communities in relation to Bd resistance and tolerance (Lauer et al., 2008; Flechas et al., 2012; Muletz-Wolz et al., 2017).

We explored amphibian skin bacteria in host communities that experienced a collapse in species richness following epizootics of chytridiomycosis (Catenazzi et al., 2011, 2014). We previously discovered that the impact of Bd on amphibian communities was strongest among stream-breeding species (Catenazzi et al., 2011), and in amphibian communities at mid-elevations where Bd growth is optimal (Catenazzi et al., 2014). Many amphibians persist despite the continuous presence of Bd, but several of these persisting species continue to be susceptible to chytridiomycosis (Catenazzi et al., 2017). In this context, and considering how little is known about amphibian skin bacteria in the tropical Andes (Bresciano et al., 2015; Burkart et al., 2017), the overarching goal of this study is to examine the role of skin bacteria, and their anti-Bd abilities, in protecting amphibian hosts from disease at our study site. Here, we aim to (1) document species composition of bacterial isolates across elevation, host identity and behavior, (2) determine the inhibitory ability of bacterial isolates against the local strain of Bd, and (3) compare the distribution of anti-Bd isolates with respect to host susceptibility, host behavior and elevation. The wide elevational range, large number of hosts (28 total species), high susceptibility (six susceptible out of nine species with known status), and diversity of host reproductive modes (spanning from aquatic eggs and tadpoles to terrestrial viviparous embryos), provide an ideal opportunity to investigate the contribution of skin bacteria to host defense against chytridiomycosis.

MATERIALS AND METHODS

Study System

We worked along the Paucartambo–Pillcopata road traversing the Kosñipata Valley and bordering Manu National Park and its buffer zone in southern Peru (Catenazzi et al., 2011, 2014). This road connects the high-Andean grasslands (puna) of the Cordillera de Paucartambo at the mountain pass of Acjanaco (3,400 m a.s.l.) to the Andean foothills (Pillcopata, 540 m a.s.l.) and Amazonian lowland rainforest of the upper Madre de Dios watershed. Owing to its wide elevational span and diversity of ecosystems, Manu NP protects the highest number of amphibian species of any protected area (Catenazzi et al., 2013). We sampled 131 frogs belonging to 28 species in seven families distributed from 560 to 3,865 m a.s.l. in the submontane and montane forests, montane scrub, and puna of the Kosñipata Valley (**Table 1**) during the dry season (14 June–22 August 2012). Furthermore, we include *Telmatobius marmoratus* (Telmatobiidae) obtained from the drier puna region surrounding the city of Cusco in June and July 2012. This species does not occur in the Kosñipata Valley, but is distributed on the western side of the Cordillera de Paucartambo at short distance from the Acjanaco mountain pass, and is known for having high Bd prevalence and susceptibility to Bd (Catenazzi et al., 2010, 2017; Warne et al., 2016).

Ethics Statement

This work has been approved by the Animal Care and Use Committees of San Francisco State University (Protocol #A12-07) and Southern Illinois University (Protocol #13-027), and by the Peruvian Ministry of Agriculture. The Asociación para la Conservación de la Cuenca Amazónica authorized work at its Wayqecha Biological Station.

Disease Prevalence

We estimated disease prevalence by swabbing 543 frogs captured from 540 to 3,865 m a.s.l. with sterile synthetic rayon swabs (MW113, Medical Wire and Equipment, England). We gently stroked the sterile swab across the skin of each frog a total of 30 times: five strokes on each side of the abdominal midline,

TABLE 1 | Species of anurans and elevations sampled for skin bacteria.

Host species (sample size)	#tes/isl	Elevation (m)	Reproduction [mode in Wells (2007)]
Bufonidae			
Rhinella manu (3)	0/4	1,920–2,100	Terrestrial, direct development (23)
Centrolenidae			
Hyalinobatrachium bergeri (3)	6/6	1,030–1,920	Epiphyllous eggs, lotic tadpoles (25)
Hemiphractidae			
Gastrotheca antoniiochoai (2)	2/2	2,915	Marsupial, direct development (35)
Gastrotheca excubitor (11)	14/15	3,340–3,695	Marsupial, direct development (35)
Gastrotheca nebulanastes (6)	9/9	2,790–2,920	Marsupial, direct development (35)
Gastrotheca testudinea (1)	2/2	2,100	Marsupial, direct development (35)
Hylidae			
Dendropsophus rhodopeplus (2)	2/2	560	Lentic eggs and tadpoles (1)
Hypsiboas gladiator (9)	10/12	1,350–1,450	Streamside basin eggs and tadpoles (4)
Hypsiboas punctatus (3)	2/3	560	Streamside basin eggs and tadpoles (4)
Osteocephalus mimeticus (1)	1/1	1,100	Streamside basin eggs and tadpoles (4)
Scinax ruber (4)	0/4	560–1,410	Lentic eggs and tadpoles (1)
Leptodactylidae			
Adenomera andreae (1)	1/1	540	Terrestrial foam nest, non-feeding tadpoles (32)
Engystomops freibergi (1)	1/1	540	Aquatic foam nest and lentic tadpoles (11)
Strabomantidae			
Bryophryne cophites (1)	2/2	3,865	Terrestrial, direct development (23)
Noblella pygmaea (1)	1/2	2,970	Terrestrial, direct development (23)
Oreobates amarakaeri (1)	1/2	560	Terrestrial, direct development (23)
Pristimantis cf. *cruciocularis* (1)	1/1	930	Terrestrial, direct development (23)
Pristimantis danae (10)	14/15	1,410–2,000	Terrestrial, direct development (23)
Pristimantis cf. *diadematus* (1)	1/1	1,065	Terrestrial, direct development (23)
Pristimantis lindae (1)	1/1	1,920	Terrestrial, direct development (23)
Pristimantis pharangobates (15)	3/28	1,920–2,790	Terrestrial, direct development (23)
Pristimantis pluvialis (1)	2/2	930	Terrestrial, direct development (23)
Pristimantis cf. *platydactylus* (6)	8/10	1,920–1,990	Terrestrial, direct development (23)
Pristimantis salaputium (10)	14/19	1,350–1,450	Terrestrial, direct development (23)
Pristimantis cf. *toftae* (7)	3/10	1,350–1,450	Terrestrial, direct development (23)
Pristimantis sp. (1)	1/1	2,350	Terrestrial, direct development (23)
Psychophrynella usurpator (13)	15/17	2,950–2,975	Terrestrial, direct development (23)
Telmatobiidae			
Telmatobius marmoratus (15)	16/19	3,400	Streamside basin eggs and tadpoles (4)
Total (sample size: 131)	133/192	540–3,865	

[#]*tes = number of morphotypes tested for Bd inhibition (duplicates considered as single morphotype);* [#]*isl = number of morphotypes isolated from host species (after discarding duplicates). Note that not all morphotypes could be sequenced.*

five strokes on the inner thighs of each hind leg, and five strokes on the foot webbing of each hind leg (Catenazzi et al., 2011, 2017). We extracted DNA from swabs with Prepman Ultra (Life Technologies, Carlsbad, CA, United States). Each single swab extract was amplified once following a standard, probe-based quantitative Polymerase Chain Reaction protocol (Boyle et al., 2004; Hyatt et al., 2007) using a 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA, United States), as reported in Catenazzi et al. (2017). This qPCR assay estimates Bd genomic equivalents (GEs) in each sample, and we converted GE values to provide "zoospore equivalents" on each frog by using genomic standards of known zoospore concentrations. We considered frogs to be infected if zoospore equivalents > 0, and non-infected if zoospore equivalents $= 0$. We calculated Bd prevalence (proportion of swabbed frogs infected with Bd) using Bayesian inference with Jeffrey's non-informative priors with the 'binom' package in R (Dorai-Raj, 2014). Bd prevalence data are available online at the Amphibian Disease database[1] at the URL: https://n2t.net/ark:/21547/AXY2.

Bacterial and Bd Isolation

We obtained bacteria directly from skin swabs of frogs. We handled each frog with new nitrile gloves and rinsed them with distilled water prior to swabbing in order to remove transient cutaneous bacteria (Lauer et al., 2007). We sampled bacteria by running a sterile MW113 swab (not the same swab used for determining Bd infection) on the frog's left and right sides and ventral surfaces, hindlimbs, and interdigital membranes for a

[1]https://amphibiandisease.org/

total of 50 strokes (Flechas et al., 2012). We then streaked the swab on a petri dish of nutrient agar and incubated the dish at room temperature (14–18°C) until observing bacterial growth (4–6 days). We defined bacterial morphotypes according to macroscopic characteristics (color, form, elevation, and margin), and transferred each to fresh nutritive agar plates until pure cultures were obtained. We transported each isolate to the laboratory for Bd growth inhibition assays and identification through sequencing of the 16S rRNA gene (Weisburg et al., 1991).

We isolated a local strain of Bd from the mouthparts of *Hypsiboas gladiator* tadpoles collected in creeks of the Kosñipata Valley at 1,350 m a.s.l. We examined the mouthparts for signs of Bd infection, such as depigmentation and missing tooth rows (Fellers et al., 2001; Marantelli et al., 2004; Rachowicz and Vredenburg, 2004). We euthanized individuals with signs of disease by decapitation, their mouthparts dissected and cut into approximately 2 mm × 2 mm squares. We cleaned each piece of mouthpart in water-based agar with antibiotics in order to remove bacteria, yeast, and fungal spores. We then transferred the clean mouthparts to a fresh plate with TGh media (10 g tryptone, 10 g agar, 4 g gelatin hydrolysate, 1,000 mL distilled water). We transferred Bd colonies to fresh plates as soon as they appeared, and these were transported to the laboratory and maintained in culture on TGh agar at 23°C. We cryopreserved the Bd strain following standard procedure (Boyle et al., 2003).

Bacterial DNA Extraction, Sequencing, and Identification

We swabbed agar plates with pure cultures of each bacterial strain, then extracted DNA from these swabs using Prepman Ultra, and amplified DNA by PCR with GoTaq Green Master Mix (Promega Corporation, Madison, WI, United States) and 16S primers 27F and 1492R (Lane, 1991). After verifying PCR products by gel electrophoresis, we sequenced each isolate's DNA (MCLAB, San Francisco, CA, United States). We aligned sequences (only those with >60% high quality base pairs) using Geneious v8.1.9 (Biomatters Limited, Auckland, New Zealand), and compared consensus sequences to microbial DNA sequences on the NCBI database (using BLAST default parameters). Isolates that were >99% similar were considered a match (Burkart et al., 2017).

We could not assay 50 of our bacterial isolates for Bd inhibition (see below). Therefore, we attempted to infer inhibitory status for these isolates from a published dataset (Woodhams et al., 2015). We also included in this comparison our assayed isolates to examine consistency of inhibitory status across studies. In order to compare our sequenced isolates with the published literature, we retained 159 sequences after discarding five duplicate and three triplicate sequences and 19 low quality reads. These 159 sequences (see GenBank accession codes in Supplementary Table S1) were added to the dataset of amphibian skin bacteria of Woodhams et al. (2015), and the combined list of 2102 strains was aligned using MAFFT v7.0 using the default option FFT-NS-2 (Katoh et al., 2017). The online interface we used[2] allows computation of multiple sequence alignment for large datasets (i.e., thousands of sequences), and calculates genetic distances for the aligned sequences. We used 1% 16S genetic distance as our criterion for delimitation of operative taxonomic units (OTUs).

Bd Growth Inhibition Assays

We used agar plate co-cultures to assay the ability of bacterial strains to inhibit Bd growth. We flushed plates with a Bd-enriched broth to produce a homogeneous distribution of Bd colonies. Then we streaked a line of the bacterial isolate on one side of the plate, parallel to a second line of a bacterial strain known to lack anti-Bd properties (*Escherichia coli* strain DH5α) serving as negative control. We assayed bacterial strains in triplicate. After 3 days incubated at 23°C, we visually inspected plates and classified the bacterial strains as either non-inhibitory (no zone of Bd growth inhibition present) or potentially inhibitory (clear zone of Bd growth inhibition present). We examined plates after 3 days because that time is when maximum Bd zoospore production was observed during our protocol optimization procedures. Furthermore, many bacteria grew quickly, and the third day was optimal for quantifying Bd inhibition and comparing values among bacterial isolates.

We quantified the relative distance from the streak of the query bacterium to where 50% of maximum Bd growth occurred, and used this value to compare each isolate's strength at inhibiting Bd (Burkart et al., 2017). Isolates with stronger inhibitory abilities depressed Bd growth over longer distances. We recorded Bd growth by measuring the gray values of standardized photographs of the petri dishes using ImageJ software (Flechas et al., 2012; Burkart et al., 2017). Gray values (hereafter referred to as "growth") are measurements of the intensity of light in a pixel of a black and white image, and by averaging among pixel wide columns across the area between the query bacterium and negative control, we quantified growth over minute distance increments from the query. Distances between the query and the negative control varied slightly between petri dishes, so we expressed them as percentages of the total length (i.e., relative distance) to facilitate comparative analysis. We expressed growth as percentage of the maximum growth recorded to control for any variation between petri dishes. For each replicate, we plotted values on a graph using Microsoft Excel (Microsoft Corporation, Redmond, WA, United States), and determined the distance at which 50% growth occurred using the function of a line fit to the graphed values (Burkart et al., 2017). We report average relative distance and standard error calculated across the three replicates of each isolate.

Four bacterial strains were assayed twice (because strains had not been sequenced prior to assays, and thus morphologically variable strains were thought to be distinct following visual examination), three strains were non-inhibitory across the two trials (*Paenibacillus* sp. 2; *Pseudomonas* sp. 24; *Chryseobacterium lactis*); whereas *Pseudomonas* sp. 2 was inhibitory in the first trial (average inhibition strength of 67%), and non-inhibitory

[2]https://mafft.cbrc.jp/alignment/server/

in the second trial. These four strains were considered to be non-inhibitory for our analyses.

Host Susceptibility Data and Statistical Analyses

We used data from infection experiments conducted as part of another study (Catenazzi et al., 2017) to relate host susceptibility to proportion and strength of anti-Bd bacterial strains. Frogs of eight species sampled in this study (*Gastrotheca excubitor, G. nebulanastes*, *Hypsiboas gladiator*, *Pristimantis danae*, *P.* cf. *platydactylus*, *P. pharangobates*, *P. toftae*, *Psychrophrynella usurpator*) were exposed to highly infected individuals of *Telmatobius marmoratus*. Following infection, we compared survival between groups of Bd-exposed and non-exposed (control) individuals (duration of experiments varied by species). Frogs were cleared of Bd infection before the experiments by immersion in a 1% itraconazole solution for 5 min a day for seven consecutive days. We used Cox's proportional hazards model with censoring to assess the risk of dying for Bd-exposed frogs (Catenazzi et al., 2017). Here, we use the predicted days to death from Cox's proportional hazards models for each species as reported in Catenazzi et al. (2017), and relate this value to the proportion and inhibitory strength of anti-Bd strains. We used analysis of variance to determine whether the proportion of anti-Bd isolates predicts the number of days to death. For descriptive purposes, we consider host species to be Bd-susceptible whenever our survival analysis models in Catenazzi et al. (2017) produced an estimate for number of days to death. We also include data of survival for *T. marmoratus* (not included in Catenazzi et al., 2017) comparing survival between infected and treated frogs (immersion in itraconazole baths).

We examined patterns of bacterial composition at two taxonomic levels: strain (OTU) and phylum. We used analysis of variance to test the significance of linear regression relating frog sample size with number of OTUs. In order to explore the effect of elevation on number of OTUs, skin bacterial composition and proportion of anti-Bd isolates, we used analysis of variance and pooled samples into seven 500 m elevational classes (540–999 m, 1,000–1,499 m, etc. up to 3,500–3,865 m a.s.l.). We also fitted a polynomial, quadratic curve to model the relationship between elevation and the proportion of anti-Bd isolates. In order to explore the effect of host reproductive behavior, we used a χ^2 contingency table test with the categories of reproductive mode proposed by Wells (2007), arranged along a continuum from aquatic eggs and tadpoles, to direct development on land, and viviparity (marsupial frogs), as listed in **Table 1**. For dimensional analyses of skin bacterial communities we used non-metric multidimensional scaling (NMDS). NMDS produces an ordination, i.e., conflates information from multiple dimensions into a few dimensions based on a distance or dissimilarity matrix. NMDS is usually considered as a more robust and flexible technique than other ordination techniques because the original distances are replaced with ranks (Minchin, 1987). While the information on distance magnitude is thus lost, the rank based approach is more robust for data lacking an identifiable distribution. Furthermore, mixed quantitative and qualitative variables can be used. Because there was little overlap of bacterial species and genera among amphibian hosts, we considered data on distribution of phyla for NMDS. We performed analyses in the R package vegan (Oksanen et al., 2015) by using the "metaMDS" function for NMDS, the "rankindex" function to determine the best method for calculating the distance matrix from our data, and the "adonis" function to perform analysis of variance (permutation test with pseudo-F ratios) using the distance matrices and five categorical variables (elevation, reproductive mode, host sample size, and host susceptibility). For the purpose of these analyses of variance, we conflated reproductive modes into two categories (terrestrial eggs and aquatic eggs), and performed the analysis for host susceptibility on a reduced dataset (information on susceptibility only available for nine of the 28 sampled amphibian species). We report averages ± SE.

RESULTS

Composition of Cutaneous Bacteria

We isolated 199 bacterial morphotypes from the skin of 131 frogs distributed across 28 species and seven anuran families, and from 540 to 3,865 m a.s.l. (**Table 1**). We were able to sequence 185 of these 199 morphotypes, and among these 185 sequences, we identified five duplicates and one triplicate, reducing the number of bacterial isolates to 178 OTUs (Supplementary Table S1). These OTUs were distributed across four bacterial phyla: Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes.

The number of OTUs varied with host sample size ($F_{1,26} = 366.3$, $p < 0.001$), and after taking into account this sampling effect, there was no change in the average number of OTUs per host across elevation ($F_{1,5} < 0.01$, $p = 0.977$). There were some changes in the relative proportion of phyla across elevation (**Figure 2**). At all elevations, isolated skin bacteria were dominated by Proteobacteria, followed by Firmicutes, Bacteroidetes, and Actinobacteria, but the relative proportion of Bacteroidetes peaked at 1,000–1,499 m a.s.l., and that of Firmicutes at 2,500–2,999 m a.s.l.

Analyses of variance (permutation test) showed that host sample size ($F_{1,23} = 51.29$; $p = 0.001$) and elevation ($F_{1,23} = 4.13$; $p = 0.034$) significantly affected phyla community composition across amphibian hosts (Supplementary Figure S1), but there were no effects of egg laying site ($F_{1,23} = 0.67$; $p = 0.521$), and host susceptibility ($F_{1,7} = -0.08$; $p = 0.978$).

Distribution of Anti-Bd Bacteria and Host Susceptibility to Chytridiomycosis

We assayed 137 morphotypes, belonging to 133 isolates (four morphotypes were assayed twice), sampled from 26 species of frogs. Six of the 133 strains could not be sequenced ("undetermined bacteria"; Supplementary Table S1), five of which were inhibitory. Therefore, our dataset of assayed strains includes 126 OTUs identified through sequencing of 16S rRNA, and six unidentified isolates.

We found 21 anti-Bd bacterial isolates in nine host species of six genera (*Dendropsophus*, *Gastrotheca*, *Hypsiboas*, *Pristimantis*, *Psychrophrynella*, and *Telmatobius*; **Table 2**), with only two

species, *G. excubitor* and *H. gladiator*, having more than four anti-Bd isolates each. These hosts and their associated anti-Bd bacteria live at elevations from 560 to 3,695 m a.s.l., in habitats that span from foothill tropical rainforest to cloud forest and high-Andean grassland, and hosts that lay eggs in ponds (*Dendropsophus*), streams (*Hypsiboas*, *Telmatobius*), moist terrestrial environments (*Pristimantis*, *Psychrophrynella*), and that retain eggs in specialized dorsal pouches (*Gastrotheca*).

The proportion of anti-Bd isolates ranged from 0 to 14% in six host species that were susceptible to Bd, but there was no relationship between number of days to death and proportion of anti-Bd isolates ($F_{1,4} = 1.46$, $p = 0.293$; **Figure 3A**). Among the three non-susceptible host species, *G. excubitor* and *H. gladiator* had the largest proportions of anti-Bd isolates (40 and 45%, respectively), but *P. usurpator* only had 13% anti-Bd isolates, within the range of Bd-susceptible hosts.

The strength of Bd inhibition averaged 55.0 ± 2.6% across all isolates, and ranged from 32.9 to 78.0% (**Table 2** and **Figure 3B**). Although data points are too few to compute statistical analyses, the inhibition strengths of anti-Bd isolates were generally higher in non-susceptible hosts than they were in Bd-susceptible hosts, with the exception of *P. usurpator*. Only three of the six Bd-susceptible hosts are shown in **Figure 3B**, because the other three species had no anti-Bd isolates. Hosts of unknown susceptibility to Bd had anti-Bd isolates with inhibition strength similar to that of non-susceptible species.

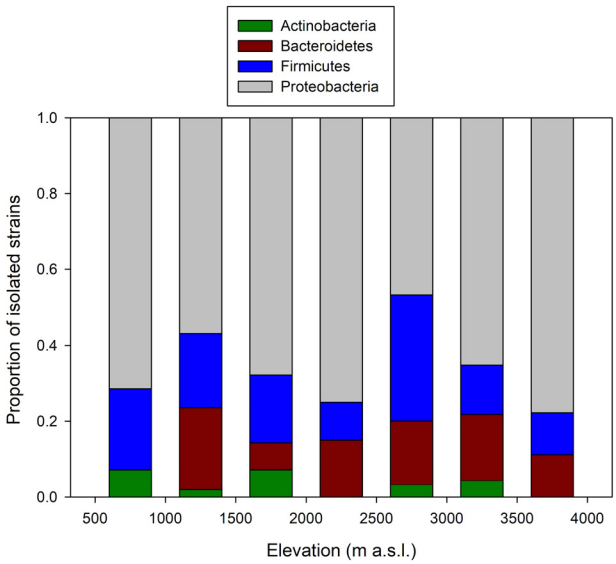

FIGURE 2 | Change in the composition of frog skin bacterial isolates arranged by phyla along the elevational gradient in the Kosñipata Valley near Manu National Park, SE Peru.

TABLE 2 | Distribution and inhibitory strength (as measured by the relative distance from the streak of the query bacterium to point of 50% of max Bd growth; see Materials and Methods) of anti-Bd bacterial isolates across host species.

Host species and isolate	Elevation (m)	Distance	SE
Dendropsophus rhodopeplus			
Pseudomonas entomophila	560	78.04%	2.20%
Serratia marcescens	560	42.43%	14.97%
Gastrotheca excubitor			
Pseudomonas sp. 2	3,340	67.21%	10.39%
Pseudomonas sp. 34	3,340	61.44%	10.77%
Pseudomonas sp. 18	3,340	58.80%	7.67%
Rahnella aquatilis	3,695	50.25%	9.10%
Pseudomonas sp. 1	3,695	47.91%	1.91%
Janthinobacterium lividum	3,695	32.86%	3.08%
Gastrotheca nebulanastes			
Pseudomonas sp. 25	2,850	38.44%	N/A
Hypsiboas gladiator			
Undetermined bacterium 14	1,410	69.98%	6.85%
Undetermined bacterium 13	1,410	61.38%	1.76%
Rahnella sp. 2	1,410	56.37%	4.47%
Undetermined bacterium 1	1,450	49.69%	9.77%
Sphingobacterium faecium	1,350	43.68%	N/A
Pristimantis cf. diadematus			
Paenibacillus sp. 24	1,065	66.64%	1.29%
Pristimantis danae			
Pseudomonas sp. 36	1,920	58.94%	9.96%
Paenibacillus sp. 3	2,000	45.73%	6.93%
Pristimantis salaputium			
Undetermined bacterium 2	1,350	73.50%	10.48%
Psychrophrynella usurpator			
Undetermined bacterium 5	2,970	58.26%	9.31%
Pseudomonas azotoformans	2,975	51.11%	11.02%
Telmatobius marmoratus			
Pseudomonas fluorescens	3,400	41.77%	2.43%

Bacteria are ranked according to their inhibitory abilities.

The proportion of anti-Bd isolates varied with elevation ($F_{2,4} = 36.05$, $p = 0.0028$; **Figure 4**), following a polynomial, quadratic curve that reaches its lowest values at mid-elevations from 1,500 to 2,000 m a.s.l., partially overlapping with elevations from 1,250 to 1,750 m a.s.l. where at least 35% of amphibian hosts became extinct during the Bd epizootics (Catenazzi et al., 2011). These elevational ranges also partially overlapped with the 1,500–2,200 m a.s.l. range where prevalence of Bd infection was highest during our sampling period in the dry season of 2012 (Supplementary Table S2).

Anti-Bd isolates occurred across four types of reproductive modes (**Figure 5**): aquatic eggs and tadpoles in ponds, aquatic eggs and tadpoles in streamside basins, terrestrial breeding with direct development, and marsupial brooding with direct development. There was no effect of reproductive mode on the proportion of anti-Bd isolates ($\chi^2 = 10.372$, df = 6, $p = 0.110$) and, among the four reproductive modes with anti-Bd isolates, no effect on the inhibitory strength of anti-Bd isolates ($F_{3,46} = 0.68$, $p = 0.571$; **Figure 5**).

Comparison With Published Literature

The alignment produced through MAFFT v7.0 generated shorter genetic distances than those calculated for our isolates in Geneious. Despite these shorter distances, 59 of our 159 sequenced isolates had no matches (our OTUs defined as those

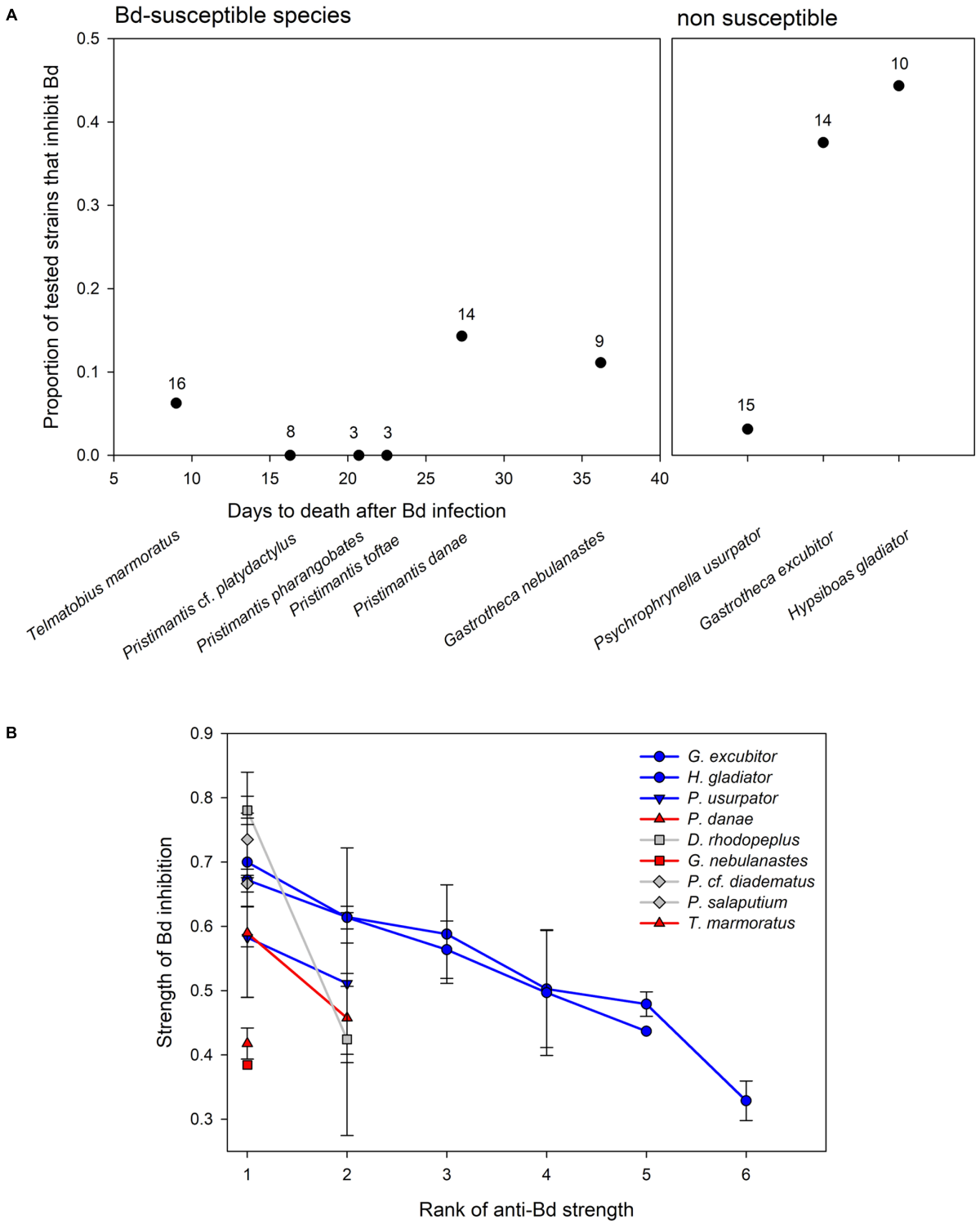

FIGURE 3 | (A) Variation in proportion of anti-Bd bacterial isolates in Bd-susceptible and non-susceptible amphibian hosts. In susceptible species, days to death after Bd infection are calculated from survival analysis (Catenazzi et al., 2017). Number of tested bacterial morphotypes reported above each data point. **(B)** Average strength (±SE) of Bd inhibition of anti-Bd isolates (as measured by the relative distance from the streak of the query bacterium to point of 50% of max Bd growth; see Materials and Methods) ranked by inhibitory strength (from strongest to weakest inhibitor) in three Bd-susceptible hosts (in red), three non-susceptible hosts (in blue), and three hosts of unknown Bd susceptibility (i.e., not tested, in gray). Bacterial strains vary by frog host species.

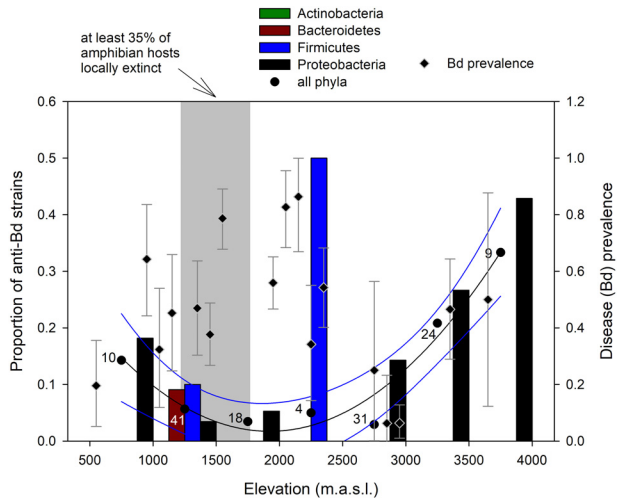

FIGURE 4 | Change in proportion of anti-Bd bacterial strains within phyla (bars) and across all phyla (circles; numbers indicate host sample size) along the elevational gradient in the Kosñipata Valley near Manu National Park, SE Peru. Diamonds indicate disease (Bd) prevalence with Bayes credible intervals in amphibian hosts, and the shaded area indicates elevations at which at least 35% of amphibian species have disappeared following the Bd epizootics (Catenazzi et al., 2011).

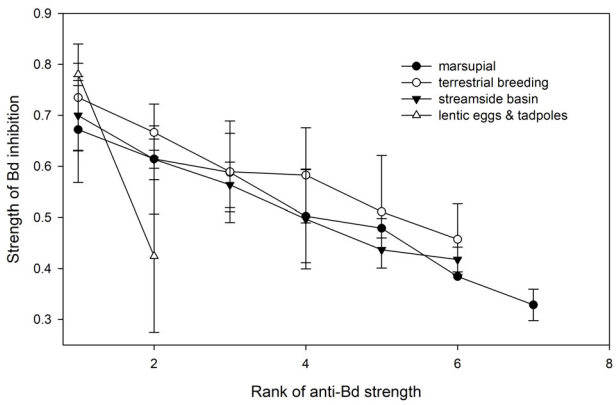

FIGURE 5 | Variation in average strength (±SE) of Bd inhibition of anti-Bd isolates (ranked from strongest to weakest inhibitor) across host reproductive modes.

having 16S genetic distance <1%) among the 1,944 previously published isolates listed in Woodhams et al. (2015). The remaining 100 isolates produced 445 matches with isolates in the published dataset, with some isolates of *Pseudomonas* generating up to 100 (*Pseudomonas entomophila*), 81 (*Pseudomonas* sp. 7), and 74 matches (*Pseudomonas* sp. 2 and *Pseudomonas* sp. 24). There was little consistency in inhibitory status among OTUs. None of the OTUs we identified as inhibitory were inhibitory across all studies summarized in the published dataset. Likewise, none of the OTUs we identified as non-inhibitory were non-inhibitory across all studies summarized in the published dataset. Therefore, we decided not to use OTU as a predictor of inhibitory status for the isolates that we could not assay against Bd.

DISCUSSION

We show that bacterial isolates inhibiting the fungal disease chytridiomycosis are distributed across a wide range of environmental conditions (temperature and rainfall, as they vary along elevation), host families and genera, host susceptibility status, and microhabitat associated with host reproductive behavior. Our findings suggest anti-Bd isolates may play a role in host defense against chytridiomycosis, maintenance of host community composition, and the elevational pattern of Bd infection prevalence. We found the highest proportion and inhibition strengths of anti-Bd isolates in two non-susceptible species, while Bd-susceptible species had low proportions (<15%) of weakly inhibitory bacterial isolates. Intriguingly, we also recorded low proportion, and weak inhibitory strength in a common, non-susceptible species. Our attempt at inferring inhibitory status of bacterial isolates by matching their 16S sequences with sequences from previous studies was unsuccessful, suggesting that OTU identity is a poor predictor of inhibition strength.

Our findings acquire relevance in the context of our study system, which consists of species-rich amphibian communities that have been decimated by chytridiomycosis. This highly virulent disease infects the skin and interferes with the organ's functions, eventually leading to death in many amphibian species (Voyles et al., 2009; Kilpatrick et al., 2010). We have previously documented the collapse of montane forest amphibian communities at our study site (Catenazzi et al., 2011, 2014), following epizootics of chytridiomycosis that reached southern Peru in the early 2000s (Catenazzi and von May, 2014). During these epizootics nearly 20 out of 60 species of amphibians were extirpated from the Kosñipata Valley, and many more declined in abundance or disappeared from part of their previous elevational range. While the host/Bd dynamics have shifted from epizootic to enzootic (Catenazzi et al., 2017), and communities of surviving species do not seem to undergo further declines, we recently demonstrated that many surviving host taxa continue to be susceptible to chytridiomycosis (Catenazzi et al., 2017). Therefore, host-specific factors, or the dynamics of the enzootic host/Bd system, currently prevent Bd from causing widespread host mortality and further population declines. With this study we have explored one such factor, host skin defense provided by symbiotic bacteria.

Our primary interest was to examine any relationship between distribution of anti-Bd isolates and host susceptibility in the context of amphibian communities ravaged by chytridiomycosis. We compared the elevational distribution of anti-Bd isolates with the elevational range corresponding to the sharpest declines in amphibian diversity (where at least 35% of frog species have been extirpated), and with elevational variation in Bd infection prevalence. Difference in elevational resolution among these datasets prevented more rigorous statistical analyses; data on proportion of declining amphibian species and Bd

prevalence are available for 100 m elevational classes (Catenazzi et al., 2011), whereas data on anti-Bd isolates were pooled into 500 m elevational classes to minimize differences in sample size among classes. Assuming the observed patterns are representative, our data suggest that the lowest proportion of anti-Bd isolates overlap with regions of high Bd prevalence and high host extirpation, which creates a conundrum. One would expect the opposite pattern to occur if Bd was selecting for host resistance. If symbiotic bacteria were contributing to skin defenses, then hosts that survived Bd epizootics in disease-ravaged communities should have greater proportions of anti-Bd bacteria than survivors in communities that had not been strongly diminished by disease. Another possibility is that most frogs at mid-elevations lacked abundant beneficial symbionts, explaining why so many species were extirpated during epizootics, and that surviving species continue to lack symbionts, but are able to persist for other reasons. Other skin defenses, such as antimicrobial peptides, play important roles in inhibiting Bd growth and delaying the lethal effects of chytridiomycosis (Rollins-Smith et al., 2002; Melzer and Bishop, 2010; Holden et al., 2015). Moreover, diminished frog density and lower number of host species following epizootic outbreaks, as well as the transition of the host/Bd dynamics from epizootic to enzootic likely reduces rates of transmission and the availability of environmental disease reservoirs (Briggs et al., 2010), allowing host species to persist and even recover (Knapp et al., 2016).

An alternative hypothesis to the conundrum posed by the U-shaped curve in elevational distribution of anti-Bd isolates is that high prevalence of Bd, and thus high probability of infection in hosts, is disrupting skin bacterial communities (Jani et al., 2017), and disproportionally affecting anti-Bd bacteria relative to co-occurring bacteria. This alternative hypothesis would again imply that other factors, unrelated to skin symbionts, are preventing high Bd virulence in these hosts. Continuous exposure to Bd in regions of high prevalence, for example, could strengthen acquired immunity in hosts (McMahon et al., 2014), although our experimental data (Catenazzi et al., 2017) and findings from other studies show limited support for immunoprotective effects in many species of amphibians (Stice and Briggs, 2010; Cashins et al., 2013; Hudson et al., 2016; Geiger et al., 2017).

Support for a role of symbiotic bacteria in frog skin defense is provided through our comparison of the inhibitory strength and proportion of anti-Bd isolates in hosts that were experimentally infected with Bd. With the exception of one common and non-declining frog species (*P. usurpator*), we found that non-susceptible species had the highest proportion of strongly inhibitory anti-Bd isolates, whereas Bd-susceptible species had a low proportion of weakly inhibitory anti-Bd isolates. We previously associated variation in the host response to Bd infection in marsupial frogs to the presence of bacteria with antifungal properties (Burkart et al., 2017). We confirmed this association along the broader elevational gradient and across nine frog species from four families. For the case of *P. usurpator,* a species that persisted and even increased in abundance despite the presence of Bd (Catenazzi et al., 2017), the proportion of anti-Bd bacteria is very low and similar to what we found for Bd-susceptible species, and for species found at mid-elevations. Bd prevalence and infection loads in *P. usurpator* are generally lower than in sympatric frog species (Catenazzi et al., 2011, 2017), suggesting that this species might avoid infection, or that its skin might excel at preventing zoospore penetration, or that its adaptive immune system is more effective at resisting chytridiomycosis (Savage and Zamudio, 2011). This frog breeds terrestrially and does not use aquatic habitats where Bd zoospores occur at highest densities, nor does it congregate for reproduction, thus limiting potential for Bd transmission from both environmental reservoirs and highly infected individuals. Behavioral effects and independence from water bodies may also explain why other terrestrial-breeding species, such as *P. toftae* which occurs at mid-elevation in regions of high Bd prevalence, can continue to persist without protection from anti-Bd bacteria.

Comparisons of our strains with a published dataset, after alignment of 16S sequences and using 1% genetic distance as criterion for OTU delimitation, revealed that OTU identity is a poor predictor of inhibition status and strength, to the extent that it thwarted our attempt to determine inhibition strength for isolates that we could not assay against Bd. Although bacterial genera such as *Jantinobacterium*, *Serratia*, *Stenotrophomonas*, *Aeromonas,* and *Pseudomonas* are frequently associated with anti-Bd capacity (Harris et al., 2009; Becker et al., 2015b; Woodhams et al., 2015), emergent traits of bacterial communities, including community structure and species richness (Woodhams et al., 2014; Becker et al., 2015a; Piovia-Scott et al., 2017), might be more predictive of host resistance to chytridiomycosis than data from co-culture assay testing single bacterial isolates. These findings are important to inform probiotic treatments aimed at mitigating the effects of chytridiomycosis on amphibian hosts (Bletz et al., 2013; Kueneman et al., 2016).

Our investigation, which was performed by using culturable bacteria, may provide a limited representation of overall bacterial diversity yet has the advantage of producing isolates that can be tested for anti-Bd capacity (Becker et al., 2015b). Previous work has demonstrated that most dominant skin bacteria can be isolated and cultured (Walke et al., 2015), so the undetected bacteria may not constitute an extensive group. Furthermore, culturable isolates were needed to determine isolate-specific inhibition strength against the local strain of Bd. These strain-specific inhibition data were especially informative because we previously determined host susceptibility to the same local Bd strain *in situ* (Catenazzi et al., 2017), and because inhibitory capacity of bacteria appears to vary when different Bd strains are assayed (Antwis et al., 2015). By sampling bacteria directly from wild frogs, testing them against a local strain of Bd, and relating our findings to known patterns of disease prevalence and susceptibility, we aim to minimize external factors that would interfere with our interpretation regarding the role of symbiotic bacteria as a line of defense against fungal disease.

The environment has been hypothesized as the main source of symbiotic bacteria in frogs (Muletz et al., 2012), although there is evidence that the core of host bacterial communities may occur independently of environment (Wiggins et al., 2011; Loudon et al., 2014). Vertical transmission of bacteria to offspring has also been suggested as an alternative route for species with parental

care (Walke et al., 2011; Burkart et al., 2017). Among our sampled species, four species of *Gastrotheca* have direct development with embryos enclosed in a sealed dorsal pouch until hatching, when they emerge through the opening of the brooding pouch. The pouch is made of modified skin, and even allows for nutrient provisioning by the brooding mother (Warne and Catenazzi, 2016). It is reasonable to imagine that such intimate contact between mother and progeny promotes vertical transmission of symbiotic bacteria at birth (Bresciano et al., 2015; Burkart et al., 2017). In another sampled species, *Hyalinobatrachium bergeri*, males attend nests of epiphyllous eggs, and could transfer skin microbes to developing embryos (Walke et al., 2011). Despite this natural history, which might provide an explanation for differences in the relative proportion of core, vertically transmitted and possibly transient, environmentally acquired bacteria, our analyses at the coarse level of phyla did not detect any effect of host reproductive mode. A more comprehensive characterization of bacterial diversity using next-generation sequencing will improve examination of sources of bacterial symbionts in our amphibian species.

Host reproductive mode might also affect skin bacterial composition because amphibians breed in a variety of aquatic, terrestrial and arboreal environments, thus exposing the skin to different environmental source pools of microbes. Although our coarse, phylum level analyses did not reveal any variation in skin bacterial composition, nor variation in the proportion and strength of anti-Bd isolates among host reproductive modes, it is possible that more comprehensive and finer analyses of bacterial diversity (i.e., at the species level) may detect an effect of host behavior. Furthermore, future research that quantifies relative abundances of the different symbiotic bacteria, for example by using count plates (Bresciano et al., 2015) or quantitative PCR (Piovia-Scott et al., 2017) from skin swab samples, would help us understand the complexity of bacterial communities on amphibian skin.

Among environmental factors influencing bacterial diversity, elevation can be used as a proxy for temperature, which decreases at a rate of ∼0.55°C every 100 m in elevation, and for rainfall, which ranges from over 4 m at the foothill of the Andes to less than 1 m on the western side of the Cordillera de Paucartambo (Catenazzi et al., 2014). These changes in temperature and rainfall may act directly on soil, rock and leaf bacteria living in the environment, or indirectly on skin symbionts through the body temperature of amphibian hosts. Furthermore, ecological interactions among skin symbionts, fungal pathogen, and other symbiotic microbes and parasites may influence bacterial composition along the elevational gradient. The patterns of cutaneous bacterial diversity, as well as their role in skin defense, likely result from the interplay of multiple forces: temperature and moisture effects on pathogen (Piotrowski et al., 2004; Stevenson et al., 2013), temperature and moisture effects on host (Raffel et al., 2006, 2015; Catenazzi et al., 2014) and bacteria (Fierer et al., 2011; Nottingham et al., unpublished), and ecological interactions among co-occurring microbes (Woodhams et al., 2014; Piovia-Scott et al., 2017).

CONCLUSION

We found that anti-Bd bacteria are widely distributed across bacterial phyla and genera, occur along a wide elevational range in the Amazon to Andes transition, and are found on amphibian hosts that use aquatic, terrestrial and arboreal environments. The pattern of elevational distribution of anti-Bd isolates, and the association of high proportion of anti-Bd isolates of high inhibitory strength with low host susceptibility to disease, support the idea that symbiotic bacteria play a functional role in amphibian skin defense. Yet this association does not consistently explain the fate of amphibian hosts along the elevational gradient, suggesting complex interactions among bacterial symbionts, hosts, and environmental factors in determining frog persistence in a region of high disease prevalence.

AUTHOR CONTRIBUTIONS

AC, SVF, and VTV designed the study, developed the methodology, and collected the field data. AC and VTV acquired the funding. AC, SVF, DB, NH, and JT conducted the lab work. AC supervised the data curation and conducted the analyses. AC and SVF wrote the original draft. All authors reviewed and edited drafts.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00465/full#supplementary-material

REFERENCES

Antwis, R. E., Preziosi, R. F., Harrison, X. A., and Garner, T. W. J. (2015). Amphibian symbiotic bacteria do not show a universal ability to inhibit growth of the global panzootic lineage of *Batrachochytrium dendrobatidis*. *Appl. Environ. Microbiol.* 81, 3706–3711. doi: 10.1128/AEM.00010-15

Becker, M. H., and Harris, R. N. (2010). Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One* 5:e10957. doi: 10.1371/journal.pone.0010957

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015a). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B Biol. Sci.* 282:20142881. doi: 10.1098/rspb.2014.2881

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015b). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Belden, L. K., and Harris, R. N. (2007). Infectious diseases in wildlife: the community ecology context. *Front. Ecol. Environ.* 5, 533–539. doi: 10.1890/060122

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rainforests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Bettin, C., and Greven, H. (1986). Bacteria on the skin of *Salamandra salamandra* (L) (Amphibia, Urodela) with notes on their possible significance. *Zool. Anz.* 216, 267–270.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Bletz, M. C., Perl, R. G. B., Bobowski, B. T. C., Japke, L. M., Tebbe, C. C., Dohrmann, A. B., et al. (2017). Amphibian skin microbiota exhibits temporal variation in community structure but stability of predicted Bd-inhibitory function. *ISME J.* 11, 1521–1534. doi: 10.1038/ismej.2017.41

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Boyle, D. G., Hyatt, A. D., Daszak, P., Berger, L., Longcore, J. E., Porter, D., et al. (2003). Cryo-archiving of *Batrachochytrium dendrobatidis* and other chytridiomycetes. *Dis. Aquat. Organ.* 56, 59–64. doi: 10.3354/dao056059

Bresciano, J. C., Salvador, C. A., Paz-Y-Mino, C., Parody-Merino, A. M., Bosch, J., and Woodhams, D. C. (2015). Variation in the presence of anti-*Batrachochytrium dendrobatidis* bacteria of amphibians across life stages and elevations in Ecuador. *Ecohealth* 12, 310–319. doi: 10.1007/s10393-015-1010-y

Briggs, C. J., Knapp, R. A., and Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9695–9700. doi: 10.1073/pnas.0912886107

Bryant, J. A., Lamanna, C., Morlon, H., Kerkhoff, A. J., Enquist, B. J., and Green, J. L. (2008). Microbes on mountainsides: contrasting elevational patterns of bacterial and plant diversity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11505–11511. doi: 10.1073/pnas.0801920105

Burkart, D., Flechas, S. V., Vredenburg, V. T., and Catenazzi, A. (2017). Cutaneous bacteria, but not peptides, are associated with chytridiomycosis resistance in Peruvian marsupial frogs. *Anim. Conserv.* 20, 483–491. doi: 10.1111/acv.12352

Cashins, S. D., Grogan, L. F., Mcfadden, M., Hunter, D., Harlow, P. S., Berger, L., et al. (2013). Prior infection does not improve survival against the amphibian disease chytridiomycosis. *PLoS One* 8:e56747. doi: 10.1371/journal.pone.0056747

Catenazzi, A. (2015). State of the world's amphibians. *Annu. Rev. Environ. Resour.* 40, 91–119. doi: 10.1146/annurev-environ-102014-021358

Catenazzi, A., Finkle, J., Foreyt, E., Wyman, L., Swei, A., and Vredenburg, V. T. (2017). Epizootic to enzootic transition of a fungal disease in tropical Andean frogs: are surviving species still susceptible? *PLoS One* 12:e0186478. doi: 10.1371/journal.pone.0186478

Catenazzi, A., Lehr, E., Rodriguez, L. O., and Vredenburg, V. T. (2011). *Batrachochytrium dendrobatidis* and the collapse of anuran species richness and abundance in the upper Manu National Park, southeastern Peru. *Conserv. Biol.* 25, 382–391. doi: 10.1111/j.1523-1739.2010.01604.x

Catenazzi, A., Lehr, E., and Von May, R. (2013). The amphibians and reptiles of Manu National Park and its buffer zone, Amazon basin and eastern slopes of the Andes, Peru. *Biota Neotrop.* 13, 269–283. doi: 10.1590/S1676-06032013000400024

Catenazzi, A., Lehr, E., and Vredenburg, V. T. (2014). Thermal physiology, disease and amphibian declines in the eastern slopes of the Andes. *Conserv. Biol.* 28, 509–517. doi: 10.1111/cobi.12194

Catenazzi, A., and von May, R. (2014). Conservation status of amphibians in Peru. *Herpetol. Monogr.* 28, 1–23. doi: 10.1655/HERPMONOGRAPHS-D-13-00003

Catenazzi, A., Vredenburg, V. T., and Lehr, E. (2010). *Batrachochytrium dendrobatidis* in the live frog trade of *Telmatobius* (Anura: Ceratophryidae) in the tropical Andes. *Dis. Aquat. Organ.* 92, 187–191. doi: 10.3354/dao02250

Culp, C. E., Falkinham, J. O., and Belden, L. K. (2007). Identification of the natural bacterial microflora on the skin of eastern newts, bullfrog tadpoles and redback salamanders. *Herpetologica* 63, 66–71. doi: 10.1655/0018-0831(2007)63[66:IOTNBM]2.0.CO;2

Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E., and Speare, R. (1999). Emerging infectious diseases and amphibian population declines. *Emerg. Infect. Dis.* 5, 735–748. doi: 10.3201/eid0506.990601

Dorai-Raj, S. (2014). *Binomial Confidence Intervals for Several Parameterizations*. Vienna: R Foundation for Statistical Computing.

Fellers, G. M., Green, D. E., and Longcore, J. E. (2001). Oral chytridiomycosis in the mountain yellow-legged frog (*Rana muscosa*). *Copeia* 2001, 945–953. doi: 10.1643/0045-8511(2001)001[0945:OCITMY]2.0.CO;2

Fierer, N., Mccain, C. M., Meir, P., Zimmermann, M., Rapp, J. M., Silman, M. R., et al. (2011). Microbes do not follow the elevational diversity patterns of plants and animals. *Ecology* 92, 797–804. doi: 10.1890/10-1170.1

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. *PLoS One* 7:e44832. doi: 10.1371/journal.pone.0044832

Ford, S. A., and King, K. C. (2016). Harnessing the power of defensive microbes: evolutionary implications in nature and disease control. *PLoS Pathog.* 12:e1005465. doi: 10.1371/journal.ppat.1005465

Geiger, C. C., Bregnard, C., Maluenda, E., Voordouw, M. J., and Schmidt, B. R. (2017). Antifungal treatment of wild amphibian populations caused a transient reduction in the prevalence of the fungal pathogen, *Batrachochytrium dendrobatidis*. *Sci. Rep.* 7:5956. doi: 10.1038/s41598-017-05798-9

Hanada, R. E., Pomella, A. W. V., Costa, H. S., Bezerra, J. L., Loguercio, L. L., and Pereira, J. O. (2010). Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuaçu) trees and their potential for growth promotion and biocontrol of black-pod disease. *Fungal Biol.* 114, 901–910. doi: 10.1016/j.funbio.2010.08.006

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of Amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Holden, W. M., Reinert, L. K., Hanlon, S. M., Parris, M. J., and Rollins-Smith, L. A. (2015). Development of antimicrobial peptide defenses of southern leopard frogs, *Rana sphenocephala*, against the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*. *Dev. Comp. Immunol.* 48, 65–75. doi: 10.1016/j.dci.2014.09.003

Hoyt, J. R., Cheng, T. L., Langwig, K. E., Hee, M. M., Frick, W. F., and Kilpatrick, A. M. (2015). Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans*, the causative agent of white-nose syndrome. *PLoS One* 10:e0121329. doi: 10.1371/journal.pone.0121329

Hudson, M. A., Young, R. P., Lopez, J., Martin, L., Fenton, C., Mccrea, R., et al. (2016). In-situ itraconazole treatment improves survival rate during an amphibian chytridiomycosis epidemic. *Biol. Conserv.* 195, 37–45. doi: 10.1016/j.biocon.2015.12.041

Hughey, M. C., Pena, J. A., Reyes, R., Medina, D., Belden, L. K., and Burrowes, P. A. (2017). Skin bacterial microbiome of a generalist Puerto Rican frog varies along elevation and land use gradients. *PeerJ* 5:e3688. doi: 10.7717/peerj.3688

Hutter, C. R., Lambert, S. M., and Wiens, J. J. (2017). Rapid diversification and time explain amphibian richness at different scales in the tropical Andes, earth's most biodiverse hotspot. *Am. Nat.* 190, 828–843. doi: 10.1086/694319

Hyatt, A. D., Boyle, D. G., Olsen, V., Boyle, D. B., Berger, L., Obendorf, D., et al. (2007). Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 73, 175–192. doi: 10.3354/dao073175

Jani, A. J., Knapp, R. A., and Briggs, C. J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proc. R. Soc. B Biol. Sci.* 284:20170944. doi: 10.1098/rspb.2017.0944

Katoh, K., Rozewicki, J., and Yamada, K. D. (2017). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform.* doi: 10.1093/bib/bbx1108 [Epub ahead of print]

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol. Evol.* 25, 109–118. doi: 10.1016/j.tree.2009.07.011

Knapp, R. A., Fellers, G. M., Kleeman, P. M., Miller, D. A. W., Vredenburg, V. T., Rosenblum, E. B., et al. (2016). Large-scale recovery of an endangered amphibian despite ongoing exposure to multiple stressors. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11889–11894. doi: 10.1073/pnas.1600983113

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and Mckenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc. B Biol. Sci.* 283:20161553. doi: 10.1098/rspb.2016.1553

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Techniques in Bacterial Systematics*, eds E. Stackbrandt and M. Goodfellow (New York, NY: Wiley), 115–175.

Lauer, A., Simon, M. A., Banning, J. L., Andre, E., Duncan, K., and Harris, R. N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 2007, 630–640. doi: 10.1643/0045-8511(2007)2007[630:CCBFTE]2.0.CO;2

Lauer, A., Simon, M. A., Banning, J. L., Lam, B. A., and Harris, R. N. (2008). Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J.* 2, 145–157. doi: 10.1038/ismej.2007.110

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., Mckenzie, V., et al. (2014). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Marantelli, G., Berger, L., Speare, R., and Keegan, L. (2004). Distribution of the amphibian chytrid *Batrachochytrium dendrobatidis* and keratin during tadpole development. *Pac. Conserv. Biol.* 10, 173–179. doi: 10.1071/PC040173

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

McMahon, T. A., Sears, B. F., Venesky, M. D., Bessler, S. M., Brown, J. M., Deutsch, K., et al. (2014). Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. *Nature* 511, 224–227. doi: 10.1038/nature13491

Melzer, S., and Bishop, P. J. (2010). Skin peptide defences of New Zealand frogs against chytridiomycosis. *Anim. Conserv.* 13, 44–52. doi: 10.1111/j.1469-1795.2009.00340.x

Minchin, P. R. (1987). An evaluation of relative robustness of techniques for ecological ordinations. *Vegetatio* 69, 89–107. doi: 10.1007/BF00038690

Muletz, C. R., Myers, J. M., Domangue, R. J., Herrick, J. B., and Harris, R. N. (2012). Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. *Biol. Conserv.* 152, 119–126. doi: 10.1016/j.biocon.2012.03.022

Muletz-Wolz, C. R., DiRenzo, G. V., Yarwood, S. A., Campbell Grant, E. H., Fleischer, R. C., and Lips, K. R. (2017). Antifungal bacteria on woodland salamander skin exhibit high taxonomic diversity and geographic variability. *Appl. Environ. Microbiol.* 83:e00186-17. doi: 10.1128/AEM.00186-17

Oksanen, J., Blanchet, F. G., Roeland Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., et al. (2015). *vegan: Community Ecology Package. R Package Version 2.3-0*. Available at: https://cran.r-project.org/package=vegan

Piotrowski, J. S., Annis, S. L., and Longcore, J. E. (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia* 96, 9–15. doi: 10.1080/15572536.2005.11832990

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., Mckenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

Prado-Irwin, S. R., Bird, A. K., Zink, A. G., and Vredenburg, V. T. (2017). Intraspecific variation in the skin-associated microbiome of *Ensatina*, the terrestrial ring-species salamander. *Microb. Ecol.* 74, 745–756. doi: 10.1007/s00248-00017-00986-y

Qin, J. J., Li, R. Q., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Rachowicz, L. J., and Vredenburg, V. T. (2004). Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. *Dis. Aquat. Organ.* 61, 75–83. doi: 10.3354/dao061075

Raffel, T. R., Halstead, N. T., Mcmahon, T. A., Davis, A. K., and Rohr, J. R. (2015). Temperature variability and moisture synergistically interact to exacerbate an epizootic disease. *Proc. R. Soc. B Biol. Sci.* 282:20142039. doi: 10.1098/rspb.2014.2039

Raffel, T. R., Rohr, J. R., Kiesecker, J. M., and Hudson, P. J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Funct. Ecol.* 20, 819–828. doi: 10.1111/j.1365-2435.2006.01159.x

Rollins-Smith, L. A., Doersam, J. K., Longcore, J. E., Taylor, S. K., Shamblin, J. C., Carey, C., et al. (2002). Antimicrobial peptide defenses against pathogens associated with global amphibian declines. *Dev. Comp. Immunol.* 26, 63–72. doi: 10.1016/S0145-305X(01)00041-6

Rollins-Smith, L. A., and Woodhams, D. C. (2011). "Amphibian immunity," in *Ecoimmunology*, eds G. E. Demas and R. J. Nelson (Oxford: Oxford University Press), 92–143.

Russo, S. E., Legge, R., Weber, K. A., Brodie, E. L., Goldfarb, K. C., Benson, A. K., et al. (2012). Bacterial community structure of contrasting soils underlying Bornean rain forests: inferences from microarray and next-generation sequencing methods. *Soil Biol. Biochem.* 55, 48–59. doi: 10.1016/j.soilbio.2012.05.021

Savage, A. E., and Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16705–16710. doi: 10.1073/pnas.1106893108

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., Mcdonald, K. R., Phillott, A. D., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* 4, 125–134. doi: 10.1007/s10393-007-0093-5

Stevenson, L. A., Alford, R. A., Bell, S. C., Roznik, E. A., Berger, L., and Pike, D. A. (2013). Variation in thermal performance of a widespread pathogen, the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *PLoS One* 8:e73830. doi: 10.1371/journal.pone.0073830

Stice, M. J., and Briggs, C. J. (2010). Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *J. Wildl. Dis.* 46, 70–77. doi: 10.7589/0090-3558-46.1.70

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W. F., Dinudom, A., et al. (2009). Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. *Science* 326, 582–585. doi: 10.1126/science.1103538

Wake, D. B., and Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11466–11473. doi: 10.1073/pnas.0801921105

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2015). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *Appl. Environ. Microbiol.* 81, 6589–6600. doi: 10.1128/AEM.01486-15

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A., and Woodhams, D. C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–400. doi: 10.1111/j.1744-7429.2011.00787.x

Warne, R. W., and Catenazzi, A. (2016). Pouch brooding marsupial frogs transfer nutrients to developing embryos. *Biol. Lett.* 12:20160673. doi: 10.1098/rsbl.2016.0673

Warne, R. W., Labumbard, B., Lagrange, S., Vredenburg, V. T., and Catenazzi, A. (2016). Co-infection by chytrid fungus and Ranaviruses in wild and harvested frogs in the tropical Andes. *PLoS One* 11:e0145864. doi: 10.1371/journal.pone.0145864

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991

Wells, K. D. (2007). *The Ecology and Behavior of Amphibians*. Chicago, IL: University of Chicago Press. doi: 10.7208/chicago/9780226893334.001.0001

Wiggins, P. J., Smith, J. M., Harris, R. N., and Minbiole, K. P. C. (2011). Gut of red-backed salamanders (*Plethodon cinereus*) may serve as a reservoir for an antifungal cutaneous bacterium. *J. Herpetol.* 45, 329–332. doi: 10.1670/10-231.1

Woodhams, D. C., Alford, R. A., Antwis, R. E., Archer, H., Becker, M. H., Belden, L. K., et al. (2015). Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96:595. doi: 10.1890/14-1837.1

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Kuepfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Rollins-Smith, L. A., Alford, R. A., Simon, M. A., and Harris, R. N. (2007a). Innate immune defenses of amphibian skin: antimicrobial peptides and more. *Anim. Conserv.* 10, 425–428. doi: 10.1111/j.1469-1795.2007.00150.x

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007b). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

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Host and Aquatic Environment Shape the Amphibian Skin Microbiome but Effects on Downstream Resistance to the Pathogen *Batrachochytrium dendrobatidis* Are Variable

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Symbiotic microbial communities play key roles in the health and development of their multicellular hosts. Understanding why microbial communities vary among different host species or individuals is an important step toward understanding the diversity and function of the microbiome. The amphibian skin microbiome may affect resistance to the fungal pathogen *Batrachochytrium dendrobatidis* (Bd). Still, the factors that determine the diversity and composition of the amphibian skin microbiome, and therefore may ultimately contribute to disease resistance, are not well understood. We conducted a two-phase experiment to first test how host and environment shape the amphibian skin microbiome, and then test if the microbiome affects or is affected by Bd infection. Most lab experiments testing assembly of the amphibian skin microbiome so far have compared sterile to non-sterile environments or heavily augmented to non-augmented frogs. A goal of this study was to evaluate, in an experimental setting, realistic potential drivers of microbiome assembly that would be relevant to patterns observed in nature. We tested effects of frog genetic background (2 source populations) and 6 natural lake water sources in shaping the microbiome of the frog *Rana sierrae*. Water in which frogs were housed affected the microbiome in a manner that partially mimicked patterns observed in natural populations. In particular, frogs housed in water from disease-resistant populations had greater bacterial richness than frogs housed in water from populations that died out due to Bd. However, in the experiment this difference in microbiomes did not lead to differences in host mortality or rates of pathogen load increase. Frog source population also affected the microbiome and, although none of the frogs in this study showed true resistance to infection, host source population had a small effect on the rate of pathogen load increase. This difference in infection trajectories could be due to the observed differences in the microbiome, but could also be due to other traits that differ between frogs from the two populations. In addition to examining effects of the microbiome on Bd, we tested the effect of Bd infection severity on the microbiome. Specifically, we studied a time series of the microbiome over the course of infection to test if the effects of Bd on the microbiome are dependent on Bd infection severity. Although limited to a small subset of frogs, time series analysis suggested that

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relative abundances of several bacterial phylotypes changed as Bd loads increased through time, indicating that Bd-induced disturbance of the *R. sierrae* microbiome is not a binary effect but instead is dependent on infection severity. We conclude that both host and aquatic environment help shape the *R. sierrae* skin microbiome, with links to small changes in disease resistance in some cases, but in this study the effect of Bd on the microbiome was greater than the effect of the microbiome on Bd. Assessment of the microbiome differences between more distantly related populations than those studied here is needed to fully understand the role of the microbiome in resistance to Bd.

Keywords: microbiome, community assembly, *Batrachochytrium dendrobatidis*, defensive symbiosis, chytridiomycosis, *Rana sierrae*

INTRODUCTION

Symbiotic bacterial communities are ubiquitous inhabitants of multicellular organisms and play important roles in the health and development of their hosts (Dethlefsen et al., 2007; Grice and Segre, 2012; Engel and Moran, 2013; Philippot et al., 2013). A great deal of research has focused on the microbiome of the human gut, but recent studies have begun to explore the microbiome of the skin, which in many animals is the largest organ in the body and a primary line of defense against pathogens (Abdallah et al., 2017). Shifts in the skin microbiome of humans are linked to psoriasis, atopic dermatitis, acne, and *Demodex*-associated rosacea, although it is unclear whether microbiome shifts are a cause or effect of these diseases (Grice, 2014). In a mouse model, skin-associated bacteria influenced skin-specific immune responses and severity of infection by the protozoan parasite *Leishmania major* (Naik et al., 2012). The links between microbiome composition and host health highlight the importance of understanding the factors that shape symbiotic communities. Understanding why bacterial communities differ can help identify the causes of imbalances (dysbioses) in the microbiome, and advance our ability to mediate disease susceptibility by facilitating the maintenance of healthy microbial communities.

In amphibians, understanding the assembly and dynamics of the skin-associated microbiome has implications for management of *Batrachochytrium dendrobatidis* (Bd), a fungal pathogen that infects the skin of amphibians and causes the potentially lethal disease chytridiomycosis. Bd has been implicated in amphibian declines worldwide (Berger et al., 1998; Lips et al., 2006; Briggs et al., 2010; Crawford et al., 2010; Kilpatrick et al., 2010; Vredenburg et al., 2010), and currently there are no practical methods to control the disease in wild populations. Recent research has raised the possibility that symbiotic bacteria present on amphibian skin might affect resistance to chytridiomycosis. Differences in the skin microflora of wild amphibians coincide with differences in apparent disease resistance (Woodhams et al., 2007, 2014; Rebollar et al., 2016; Jani et al., 2017). Probiotic treatments have been effective in some cases (Harris et al., 2009; Muletz et al., 2012; Kueneman et al., 2016), but not others (Becker et al., 2011, 2015; Woodhams et al., 2012; Kueneman et al., 2016). This variation in the efficacy of probiotic treatments is probably the product of multiple factors, a full discussion of which is beyond the scope of this article, but drivers of microbial community assembly are likely important. For example, factors shaping microbial communities may affect probiotics by determining the abundance of symbiotic microbes that suppress or facilitate the growth of the probiotic or alter its behavior. In addition, because probiotics are themselves microbes, their ability to colonize and persist is likely to be affected by the drivers shaping microbial community structure, stability and resilience. Better understanding of the factors controlling the amphibian skin microbiome, in both the presence and absence of Bd, are critical to refining microbially based approaches to mitigate the spread and impact of Bd.

A few recent studies have begun to examine the factors shaping the amphibian skin microbiome. Microbiomes differ between amphibian species, and this is at least partly host-controlled (McKenzie et al., 2012; Kueneman et al., 2014; Walke et al., 2014). Microbiome differences among populations of a given amphibian species (intraspecific differences) have also been observed (Kueneman et al., 2014; Walke et al., 2014; Belden et al., 2015; Costa et al., 2016; Rebollar et al., 2016; Jani et al., 2017), but the role of host and environment in driving those intraspecific differences is largely unknown. The skin of amphibians selects for a microbiome that is distinct from the environment (Jani and Briggs, 2014; Walke et al., 2014; Jani et al., 2017), but that is also influenced by environmental factors. Salamanders housed in sterile or non-sterile environments develop different microbiomes (Loudon et al., 2013; Knutie et al., 2017). The composition of aquatic environmental bacterial communities predicted the composition of the *R. sierrae* microbiome (Jani et al., 2017), and transplanting fire salamanders between aquatic habitats shifted their microbiomes (Bletz et al., 2016). We also know that Bd infection alters the microbiome (Jani and Briggs, 2014; Walke et al., 2015). In summary, we know that the microbiome differs among host species and is affected by dramatic changes in the environment (e.g., sterile versus non-sterile environment) or infection by Bd. However, we lack understanding of how microbiome variation that is specifically linked to host or environment subsequently influences resistance to disease.

The Sierra Nevada yellow-legged frog (*Rana sierrae*) is a species for which understanding microbiome assembly and

interaction with Bd is particularly important. These frogs are severely threatened by Bd (Rachowicz et al., 2006; Vredenburg et al., 2007, 2010). However, *R. sierrae* populations exhibit variation in their response to Bd infection: many populations have rapidly been driven extinct by the pathogen ("die-off populations"), but some populations appear to resist large-scale lethal disease ("persistent populations") (Briggs et al., 2010; Vredenburg et al., 2010; Knapp et al., 2011, 2016; Jani et al., 2017). Persistent and die-off populations harbor different bacterial communities (Woodhams et al., 2007; Jani et al., 2017). Understanding the drivers of microbiome variation and microbiome – Bd interactions in *R. sierrae* may inform efforts to conserve the species. Here, we present a study that experimentally tested the roles of host and environment in shaping the *R. sierrae* microbiome in the presence and absence of Bd. We then link host and environmental effects on the microbiome with subsequent response to Bd exposure. We address four overarching questions about the bacterial component of the microbiome (hereafter simply "microbiome"): (1) Do innate (e.g., genetic) differences in the host shape intraspecific variation in the microbiome? (2) Does the aquatic habitat shape intraspecific variation in the microbiome? (3) Does the microbiome variation that results from environmental and host differences affect downstream resistance to Bd? (4) What is the evidence for the microbiome altering Bd infection dynamics, as opposed to Bd altering the microbiome?

MATERIALS AND METHODS

Collection of *R. sierrae* and Lake Water

All equipment, including nets and shoes, that was likely to come into contact with frogs or lake water was disinfected (incubated with 0.1% quaternary ammonium solution for at least 5 min) before beginning work in any field site. Small or sensitive equipment was disinfected with 70% ethanol. *R. sierrae* were from existing laboratory colonies, originally collected as eggs or tadpoles from two populations in the Sierra Nevada. All *R. sierrae* collections were made during the 2010 field season under research permits from the National Park Service and United States Forest Service, and subsequently housed at the University of California, Santa Barbara (UCSB) in facilities certified by the UCSB Institutional Animal Care and Use Committee (IACUC). United States Fish and Wildlife permits were not applicable at the time of this study, which was prior to the 2014 listing of *R. sierrae* as endangered by the United States Fish and Wildlife Service. One population was located in a lake of Humphrey's Basin (Sierra National Forest, collected as tadpoles), and the other population was located in Dusy Basin (Kings Canyon National Park, collected as eggs). Both populations have experienced Bd-induced die-offs to the point of population extirpation. We targeted "stranded" eggs for collection, meaning that the eggs were found in microhabitats where they were unlikely to survive to metamorphosis due to high risk of desiccation or predation. At the time of this experiment, the frogs from Humphreys Basin were larger than those from Dusy Basin ($P < 0.0001$), and we cannot definitively distinguish effects of genetic background from possible effects of frog age or size. We chose to work with these existing colonies despite the age difference rather than collect new individuals of a threatened species from the wild. However, we do not expect the age difference to have measurable effects because previous work found no difference in the microbiomes of juvenile and adult *R. sierrae* and *Rana cascadae* (Jani and Briggs, 2014; Kueneman et al., 2014). Furthermore, in the current study the age difference was small, and all individuals were juveniles (post-metamorphosis, but pre-reproductive).

Lake water to be used as aquatic habitat treatments in the experiment was collected from six Sierra Nevada lakes. Three of the water collection sites were inhabited by persisting *R. sierrae* populations. The other three sites had been previously inhabited by *R. sierrae* populations that had declined due to Bd one to 3 years prior. We refer to these as "persistent" and "die-off" populations, respectively. Water was collected in 2.5-gallon (∼10 l) collapsible polyethylene jugs (Cubitainer), which were washed with 0.1% quaternary ammonium solution 128, triple-rinsed with tap water, soaked in tap water for at least 15 min, then rinsed again. Jugs were additionally triple-rinsed in lake water at the collection site before being used for lake water collection. Because most *R. sierrae* populations are located in back-country areas far from roads, water was transported from field sites on foot. Lake water was stored at 4°C at the Sierra Nevada Aquatic Research Laboratory for up to 8 days, then transported by automobile, with water jugs held in insulated containers with ice, to UCSB where it was stored at 4°C until use. The experiment was initiated 3 days after arrival at UCSB. Prior to being added to experimental frog tanks, all lake water was filtered (1.2 μm pore size) to remove any Bd cells that may naturally occur in lake water while retaining bacteria in the lake water. Bd zoospores are 3–5 μm in diameter and sporangia are much larger. Based on published size distributions of freshwater bacteria (Šimek and Chrzanowski, 1992), we estimate that at least 80% of planktonic bacteria in our lake water should pass through 1.2 micron pores and remain in the water after filtration.

Preparation of Bd and Sham Inocula

Four Bd strains (TST75, CBJ4, CJB5, CJB7) isolated from *R. muscosa* or *R. sierrae* (which were formerly classified as the same species) were used in this experiment. A mixed-strain inoculum was used to minimize any strain-specific biases in infection dynamics. Bd cultures from cryo-archived stocks were grown in 1% (w/v) tryptone liquid medium. Once viability was confirmed, cultures were passaged to agarose tryptone media in petri plates (10 g L^{-1} tryptone, 10 g L^{-1} agar). Tryptone plates without Bd added were prepared in parallel with cultures as a sham inoculum. Cultures and sham plates were harvested after 4–6 days of growth by flooding plates with sterile water for 45 min to induce release of zoospores from sporangia and then collecting the zoospore suspension. To avoid introducing Bd culture medium to frog tanks along with Bd inoculum, zoospores and sham inocula were rinsed three times by gently pelleting (500 G, 5 min) in 50 ml conical tubes, drawing off the supernatant, and resuspending cells (or sham inoculum) in 35 ml sterile water. This is important because culture medium

could affect microbial communities in the experiment. After rinsing, cells were counted visually at 200X magnification on a compound light microscope using a hemocytometer. The four strains were pooled, with an equal concentration of each strain, and the cell suspension was diluted to 200,000 cells mL^{-1} and used immediately.

Experimental Design

We conducted a two-phase experiment to test determinants of microbiome composition and downstream effects on resistance to Bd infection. The experiment was conducted September – December 2011. A previous publication (Jani and Briggs, 2014) included data from the second phase of this experiment to test if Bd infection (as a binary variable) alters the microbiome, to test effects of captivity on the microbiome, and to compare dynamics in the laboratory and field. The former study did not analyze variation among infected frogs in the experiment, analyze time series data, test if the microbiome predicts Bd infection, or analyze any of the community assembly data from Phase I of the experiment. An outline of the experiment timeline is provided in Supplementary Figure S1. *Phase 1 – microbiome assembly:* The first phase of the experiment tested effects of aquatic environments and host genetic background on the *R. sierrae* skin microbiome. To vary host population, forty-two frogs from each of the two *R. sierrae* populations were included in the study. To vary the aquatic habitat, each frog was housed in an individual tank with water from one of seven Water Sources, which included natural water collected from six different lakes and one sterile water treatment. We chose lake water as our focus because *R. sierrae* are aquatic amphibians most often found either in lake water or basking on adjacent rocks. The high elevation Sierra Nevada lakes where the species is found are rocky, oligotrophic habitats with relatively little soil or vegetation, so lake water is a dominant feature of these frogs' environment. Furthermore, we previously showed that the bacterial communities present in lake water are correlated with the bacterial communities found on *R. sierrae* (Jani et al., 2017). In addition to the six lake water treatments, one sterile water treatment was prepared by autoclaving bottled drinking water (Arrowhead); sterility was confirmed by plating cooled 100 μL aliquots onto R2A and LB agar plates. We use the term "Water Sterility" to refer to whether water was living lake water or sterile bottled water. Lake water was collected from six Sierra Nevada lakes, of which three were inhabited by persistent *R. sierrae* and three were once inhabited by die-off populations. We use the term "Lake Water Type" to refer to the type of population site from which lake water was collected: "persistent" or "die-off" Lake Water Type indicates water collected from the geographic site of a persistent or die-off population, respectively. Note that "Lake Water Type" and "Water Source" (described above) are distinct variables: there are 7 Water Sources; six of them are from lake water, and these six are evenly divided among persistent and die-off Lake Water Types. We used a fully crossed design with 14 treatments (2 Frog Sources × 7 Water Sources), and six replicate frogs assigned to each treatment. For 2 weeks prior to beginning the experiment, animals from both populations were co-housed in three large common-garden tanks to standardize any pre-experiment environmental effects (Jani and Briggs, 2014). Non-sterile, bottled drinking water was used to provide an aquatic habitat in the pre-experiment common-garden tanks. Immediately before beginning the experiment, each frog was treated with 3% hydrogen peroxide (50 ml in a 100 ml container) for 30 s, and then rinsed thoroughly with sterile water (two 100-ml sterile water baths lasting 2 and 8 min, respectively), in an attempt to reduce and standardize the bacterial community present on the skin of frogs (e.g., Harris et al., 2009). (Hydrogen peroxide baths have been used in previous laboratory studies of the amphibian skin microbiome, however, in the current study we found that the treatment caused stress for *R. sierrae*. We therefore advise caution in the use of this method.) To initiate the experiment, twelve frogs (six from each population) were randomly assigned to each of the seven Water Sources. All frogs were housed in individual tanks, each containing 250 ml of water from one of the seven Water Sources, for the duration of the experiment. Tanks were positioned at a slant so that the tank floor was partially submerged in water, offering aquatic and basking space. Each frog was offered 7 crickets once per week, and tank water was changed after feeding to minimize the establishment of food-associated bacteria in experimental tanks. All tank water changes were done using the assigned experimental water source for each frog. Tanks were randomly assigned positions in environmental chambers maintained at 17°C with a 12 h photoperiod. Phase 1 was allowed to run for 3 weeks, based on our assumption that this would be ample time for effects of the water treatments on the microbiome to be detectable. *Phase 2 – Bd challenge:* Three weeks (21 days) after beginning the experiment, 42 of the frogs (3 frogs from each Frog Source × Water Source treatment) were challenged with Bd (200,000 zoospores per frog for three consecutive days). The dose of 200,000 zoospores is similar to previous studies (e.g., Stice and Briggs, 2010). The remaining 42 frogs served as Bd-free controls and received a sham inoculum. Frogs were monitored daily throughout the experiment. At 60 days post-exposure (82 days after initiating the experiment), the experiment was concluded and surviving frogs were cleared of Bd infection by treatment with Itraconazole (1.5 mg/L bath, 7 min daily for 11 days). Infection status for all frogs was confirmed by quantitative PCR analysis of skin swabs (described below in "Quantifying Bd Load").

To minimize the use of *R. sierrae* for experiments while maximizing information gained, we designed this experiment to address multiple research questions: (1) How do host and environment affect the microbiome? This question is addressed in the first 3-week phase of the experiment. (2) How does variation in the skin microbiome affect resistance to Bd? This question is addressed by examining how variation in the microbiome present at the end of phase 1 (just prior to Bd challenge) is correlated with downstream severity of Bd infection. (3) Does the severity of Bd infection drive changes in the microbiome? To address this question, we test if the *R. sierrae* microbiome changes as Bd infection severity increases through time. (4) Does Bd infection *per se* (infected versus uninfected) alter the microbiome? This question

is addressed by comparing the microbiomes of Bd-infected and Bd-free (control) frogs after Bd challenge. The first three research questions are the focus of the current article, while the fourth question is addressed elsewhere (Jani and Briggs, 2014).

Data Collection

Microbes present on frog skin (including bacteria and Bd) were sampled from all frogs immediately prior to placing them in experimental tanks (at the initiation of the experiment), and at least once weekly thereafter. In addition, frogs were swabbed twice weekly during Phase 1, before Bd challenge. A subset of frogs was also swabbed twice prior to beginning the experiment: immediately before (subset of frogs) and after (all frogs) hydrogen peroxide treatment. New nitrile gloves were worn for each frog handled, and frogs were rinsed twice with 60 ml sterile water before swabbing the skin using a sterile synthetic swab (Medical Wire and Equipment Company) following standard protocols (Briggs et al., 2010; Jani and Briggs, 2014). Swab buds were immediately placed in sterile microcentrifuge tubes on ice, and then frozen within 1 h of collection. We monitored symptoms of chytridiomycosis, including weight loss, inappetence, and excessive shedding of skin. We recorded snout-to-vent length and weight of all frogs before infection and at 6 and 8 weeks post-exposure. We counted the number of crickets eaten by each frog weekly and scored the amount of shed skin present in tank water using a qualitative 3-level rating system: no shed skin, moderate amount, or copious amount of shed skin observed in tank water. Prior to adding lake water to frog tanks, bacteria present in lake water were sampled by filtering 250 ml from each lake Water Source through a 0.22 μm pore polyethersulfone filter (Sterivex-GP; Millipore). Filters with samples of aquatic bacteria were frozen immediately.

DNA Extraction

Total DNA (including bacterial and Bd DNA) from frog skin swabs was prepared for PCR using Prepman Ultra as described previously (Jani and Briggs, 2014). Prepman Ultra minimizes the amount of DNA lost in the extraction process and has been used effectively in other studies (Jani and Briggs, 2014; Jani et al., 2017). DNA from aquatic bacterial samples was extracted following Nelson (2009). Tubes with extraction reagents, but without sample, were included as extraction negative controls.

Quantifying Bd Load

We quantified Bd loads from all swabs collected at the beginning of the experiment, immediately prior to Bd challenge, and weekly thereafter until the end of the experiment. Bd load (also referred to as Bd infection intensity) was measured by quantitative PCR (qPCR) applied to skin swab DNA samples as described previously (Boyle et al., 2004), with Bd standards provided by the laboratory of Alex Hyatt (CSIRO, Australia). Bd load represents the number of Bd cells in a swab sample, and when combined with a standardized swabbing method represents the total pathogen load of each frog. No-template controls and extraction negative controls were included in all PCR runs. All frogs in the Bd- treatment remained free of Bd, with the exception of one frog, which became contaminated with Bd and was excluded from analyses.

Selection of Samples for Bacterial 16S Sequencing

Due to the labor requirements and cost of sequencing, it was not feasible to analyze bacterial communities from all samples collected in the experiment (84 animals sampled weekly for up to 11 weeks). Instead, we analyzed bacterial communities across all frogs at three time points: (1) Immediately before beginning the experiment, to confirm that no differences exist between experimental water treatments before beginning the experiment. This is referred to as day 0 of the experiment. (2) After 21 days of inhabiting the various water treatments, but immediately before Bd challenge. This time point is referred to as day 21 of the experiment or 0 days post-exposure (PE). (3) After Bd infection (day 43 of the experiment, or 21 days PE). Samples of aquatic bacteria in stored lake water were also analyzed on three dates roughly corresponding to time periods when frog skin bacteria were analyzed. The 21 days PE time point was chosen for two reasons. First, Bd loads at 3 weeks PE were representative of loads observed during epidemics in the wild (Jani and Briggs, 2014; Jani et al., 2017). Second, mortality began between the sampling dates at 3 weeks and 4 weeks PE, so 3 weeks PE is the latest sampling point at which analyses are not biased by missing individuals. To analyze progressive effects of Bd loads as they increased through time while minimizing variation due to Water Source and Frog Source, we also sequenced weekly samples of 6 frogs: 3 Bd-infected and 3 unexposed animals, all from the Marmot Frog Source and with the same Water Source.

Bacterial Community Sequencing and Bioinformatic Processing

The bacterial communities present on frog skin and in lake water were characterized by sequencing a portion of the 16S gene, as described in detail in Jani and Briggs (2014). Briefly, the V1–V2 region of the 16S gene was amplified using barcoded primers with sequencing adapters, and PCR products were purified, pooled in equimolar quantities, and sequenced on a Roche/454 GS FLX instrument using Titanium chemistry. Negative controls and no-template controls were included. The program mothur v 1.30 (Schloss et al., 2009) was used to quality-filter sequences, align them to a non-redundant representative subset of the SILVA v111 SSU Ref 16S curated alignment database (Nelson et al., 2014), and cluster sequences into 95% sequence identity operational taxonomic units (OTUs) and phylotypes at the most specific classification level available, which is generally the "genus" level. We grouped OTUs at the 95% sequence identity level because 95% identity across the sequenced V1–V2 region of the 16S rRNA gene best approximates 97% identity across the entire 16S gene, a standard benchmark for assigning bacterial taxa (Schloss, 2010). Phylotype refers to a grouping of sequences based on taxonomic classification rather than percent identity.

Sequences were classified using the Bayesian classifier of Wang et al. (2007) and each OTU was assigned a consensus taxonomy

from SILVA v111 (Pruesse et al., 2007; Quast et al., 2013). Weighted Unifrac distance (Lozupone and Knight, 2005) was calculated from OTU relative abundance data to quantify the degree of phylogenetic difference among bacterial communities from different samples. To estimate bacterial community richness and diversity, the number of observed OTUs (S_{OBS}), Chao's richness estimate (Chao, 1984), Shannon diversity, and Shannon evenness were calculated after subsampling to 500 sequences per sample. Unifrac distances and community diversity were calculated based on 95% identity OTUs. To identify specific bacterial taxa that were correlated with variables of interest (Frog Source, Water Sterility, Lake Water Type, or Bd infection or load) we conducted tests on bacterial phylotypes rather than 95% identity OTUs because we have found that comparisons of specific bacterial groups among studies is better facilitated by phylotype classification (e.g., genus) than percent identity OTU. We note that results from phylotype and 95% identity OTU analyses were consistent (Supplementary Table S1). DNA sequence data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA, accession number SRR1598944).

Testing Effects of Frog Source and Aquatic Environment on the Microbiome

We tested for effects of Water Source and Frog Source on the *R. sierrae* microbiome in the absence of Bd by examining bacterial communities from frogs sampled after 21 days in the water source treatments (before Bd exposure). Our response variable was Unifrac distance, a measure of phylogenetic dissimilarity between bacterial communities. We tested the effects of Frog Source (Dusy Basin or Humphreys Basin) and Water Source (7 sources) as fixed factors using permutational multivariate ANOVA (PERMANOVA). The Frog × Water interaction was not significant and was dropped from the model.

In addition to testing effects of the seven Water Sources, we tested two specific hypotheses about how the aquatic environment might affect the *R. sierrae* microbiome. Based on field observations (Jani et al., 2017) we hypothesized that the aquatic habitat helps shape differences in the microbiome between *R. sierrae* populations that persist or die out due to Bd. We predicted that frogs housed in water from lakes inhabited by persistent populations would harbor different bacterial communities than frogs housed in water from lakes where die-offs occurred. We used PERMANOVA with Frog Source and Lake Water Type (water from a persistent or die-off population) as main effects and Water Source nested in Lake Water Type to account for the fact that each class of water (persistent or die-off) encompasses three different Water Sources. We also tested if the frog skin microbiome differed between frogs housed in sterile water and frogs housed in non-sterile lake water. For this test we used PERMANOVA with Frog Source and Water Sterility ("sterile" or "live") as main effects and Water Source nested in Water Sterility to account for the fact that the live water treatment encompasses six distinct Water Sources. Analogous parametric (ANOVA) models were used to test effects of water treatments on bacterial diversity, using the four diversity metrics described in the previous section ("*Bacterial Community Sequencing and Bioinformatic Processing*").

We repeated tests of effects of Frog Source, Water Sterility, and Lake Water Type on the microbiome using data collected on day 43 of the experiment (21 days PE). The objective here was to test if Bd disturbance overrides the effects of host and aquatic environment in shaping the microbiome. For analyses of data after Bd exposure, Bd infection status (Bd+, Bd−) was included in ANOVA and PERMANOVA models to account for the effect of Bd on microbiome diversity and composition, respectively.

To confirm that no effect of water treatments existed before the water treatments were applied, the effects of Water Source and Frog Source on the microbiome were also tested using samples collected at the beginning of the experiment (21 days pre-infection, immediately before placing frogs in their respective experimental tanks with lake water).

Testing Effects of Frog Source and Water Source on Bd Infection Severity

Bd load data collected from swabs before Bd challenge and weekly thereafter were used to examine infection trajectories through time. Only Bd load data from the 42 frogs in the Bd+ treatment (not the Bd− treatment) were analyzed for Bd infection trajectories because we were interested in effects of experimental treatments on variation in Bd trajectories given that frogs were exposed to Bd. We used repeated measures ANOVA (RM-ANOVA) to test for differences among experimental treatments in the rate of increase of Bd load through time. Data for RM-ANOVA were restricted to swabs collected between 0 and 21 days PE because 21 days PE was the latest time point for which all frogs were still alive. As with tests for treatment effects on the microbiome described above, we tested effects of Frog Source and Water Source and then followed up with specific hypothesis tests for effects of Water Sterility and Lake Water Type.

Testing Direct Correlations Between Bacterial Communities and Bd Infection Severity

An objective of this study was to tease apart the effects of Bd on the microbiome from effects of the microbiome on Bd infection. For this analysis we included only frogs in the Bd+ treatment, since we are interested in how the microbiome affects the severity of infection given that a frog is exposed to the pathogen. We used Mantel tests to calculate Spearman rank correlation coefficients between the distance matrices based on the microbiome and the distance matrix based on differences in Bd load (3 weeks PE). The Mantel test quantifies the correlation between two distance matrices using permutation methods, therefore Bd load data were converted to pairwise (among-sample) Euclidean distances for Mantel tests. Bacterial community composition is already in the form of the Unifrac distance matrix. To test if the microbiome affects disease resistance, we tested for a correlation between the bacterial distance matrix prior to infection (at 0 days PE) and the Bd distance matrix 3 weeks after exposure (21 days PE). To test if Bd infection severity affects bacterial communities, we tested for a correlation between the Bd load matrix and the bacterial distance

matrix, both measured at 21 days PE. The strength of the two effects (Bd effect on microbiome and vice versa) was examined by comparing *p*-values and correlation coefficients of the two Mantel tests. We used parametric tests to determine univariate correlations between diversity metrics and Bd load.

Identifying Bacterial Phylotypes Associated With Host, Environment, or Bd Infection

The above analyses test for whole-community patterns in the microbiome. We also identified individual bacterial phylotypes that are associated with frog source or water treatments using parametric ANOVA with structure analogous to the corresponding PERMANOVA models described above, as follows: To test effects of Frog Source and Lake Water Type in the absence of Bd (using data from 0 days PE), we used ANOVA with Frog Source, Lake Water Type, and Water Source (nested in Lake Water Type) as the predictors, and phylotype relative abundance as the response. To test the effect of Water Sterility, we included Water Sterility, Water Source (nested in Water Sterility), and Frog Source as predictors and phylotype relative abundance as the response. To test the effect of Bd load (continuous variable) on phylotype relative abundance, using swabs from day 21 PE and only from frogs that were exposed to Bd, we included Frog Source and Water Source as random variables, Bd load as the predictor and phylotype relative abundance as the response. To test effects of initial abundance of each phylotype on subsequent Bd infection severity, using only data from frogs exposed to Bd, we included Frog Source and Water Source as random variables, phylotype relative abundance on day 0 PE as the predictor, and Bd load on day 21 PE as the response.

Time Series Data to Test Effect of Bd Load on Bacteria

We further tested for effects of Bd on the microbiome by analyzing weekly samples through time, while eliminating variation due to Frog Source and Water Source. To do this, we selected one Frog Source × Water Source group, consisting of the 6 frogs from the Marmot population that were housed in water from a single lake. Three of the frogs were Bd-exposed and three were unexposed. The choice of which Water Source × Frog Source treatment to sequence was arbitrary because, prior to the 16S sequencing run, we had no data to suggest that any particular lakewater-frog treatment would be more informative than others. However we did avoid the sterile water treatments because those were expected to be less representative of wild frogs than the lake water treatments. We sequenced weekly samples from the 6 frogs from 0 to 28 days PE. (In this subset of frogs, mortality began between 4 and 5 weeks PE, hence this analysis extends to 28 days PE rather than the 21 day PE time point used when analyzing all frogs together). This complete time series analysis was not performed for the whole set of 84 frogs due to the cost of sequencing so many samples. By analyzing frogs from the same frog source and water source, we reduced variation due to frog and water source, allowing a clearer analysis of variation due to Bd infection. We tested for change over time individually for each phylotype that was represented by at least 10 reads across the 6 frogs. We first conducted repeated measures ANOVA to test for an interaction between Bd infection and time (Bd*time effect). However, all tests resulted in $P > 0.05$ for the Bd*time effect, possibly because of the low sample size relative to the number of time points (3 frogs per treatment, 5 time points). Therefore, as a rough test to identify phylotypes that are likely affected by Bd, we also conducted a simple correlation between time (day) and relative abundance of each phylotype. This simple correlation does not account for non-independence of an individual frog on different days as repeated measures ANOVA does, and we present the results as suggestive trends.

Statistical Details

Parametric statistical analyses (ANOVA, repeated measures ANOVA) were performed in JMP (SAS Institute Inc., Cary, NC, United States, 1989–2007). Non-metric multidimensional scaling (NMDS) ordination and permutation-based analyses of bacterial community composition, including PERMANOVA and permutational Mantel tests, were conducted in Primer-e v6 (Clarke and Gorley, 2006). Bd load data were $\log_{10}(X+1)$ transformed for all analyses. For analyses of abundances of individual phylotypes, relative abundance data were arcsine(square root) transformed, and only phylotypes represented by at least 10 reads in the sequencing run were analyzed (177 phylotypes). For time series analysis, we included only phylotypes with at least 10 reads across the 6 frogs for which we had time series microbiome data (95 phylotypes). Significance of analyses of relative abundances of individual phylotypes was determined after accounting for the false discovery rate (FDR) using the program *Q*-value (Storey, 2002), with a threshold of $P < 0.05$ and $Q < 0.05$ (Storey and Tibshirani, 2003). In some cases we discuss results with $P < 0.05$ and $0.05 \leq Q < 0.1$, which we annotate as marginally significant.

Pooling of Data for Graphical Display

We use NMDS ordination to visualize multidimensional data (e.g., Unifrac distances). In NMDS, the stress associated with an ordination provides a measure of the distortion of the data incurred when multidimensional data are represented in fewer (usually 2) dimensions. In this study, NMDS stress for most ordinations was high (up to 0.22). This may be due to very high variability in the data. To reduce stress in the ordinations, we pooled data across the three replicate frogs within each treatment and day. Ordination plots therefore display data pooled within treatment and day, while all statistical analyses were conducted on the unpooled data, as specified in descriptions of the statistical models. Ordinations are for data visualization only and do not affect statistical results.

RESULTS

Frog Source and Water Source Contributed to Microbiome Composition

At 0 days PE (before Bd challenge but 21 days after initiation of Water Source and Frog Source experimental

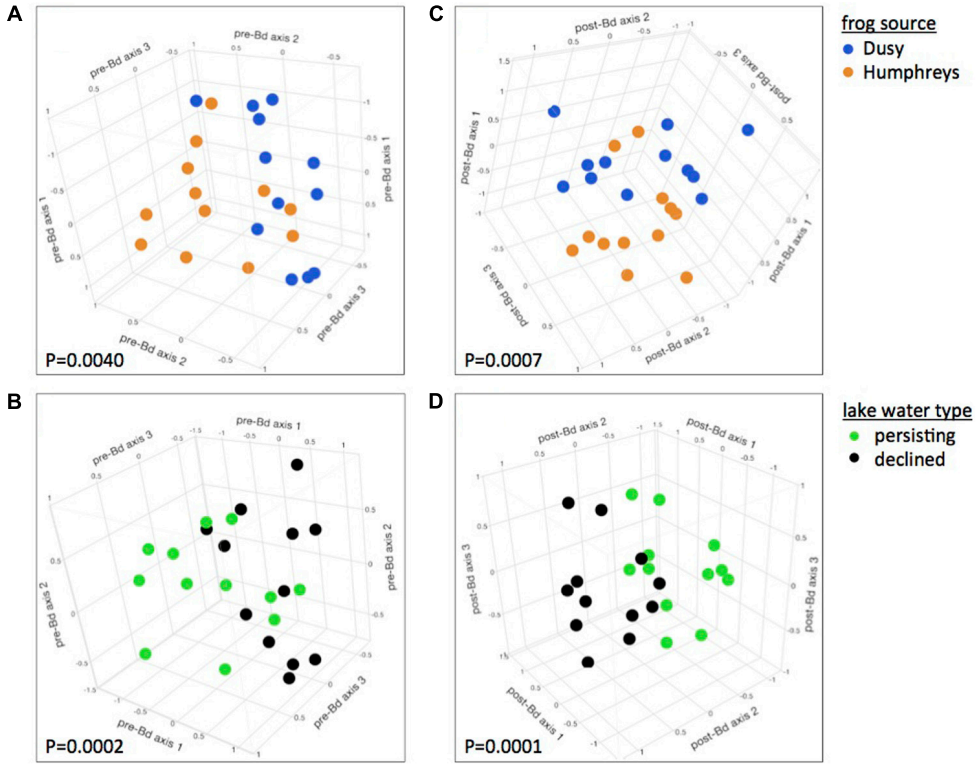

FIGURE 1 | Effects of host and aquatic environment on the skin microbiome. Skin bacterial communities were significantly different between frogs from the two source populations prior to Bd infection **(A)** and differences persisted after Bd infection **(C)**. Frogs housed in water from persistent and die-off field sites had different microbiomes, **(B)** and these differences persisted after Bd infection **(D)**. Plots are 3-dimensional NMDS ordinations of *R. sierrae* skin-associated bacterial communities, pooled by treatment, sampled on day 21 of the experiment (after 3 weeks of water treatments, and before Bd infection, left panels), and day 43 (21 days after Bd exposure, right panels). Plots are rotated to display treatment differences. Marker colors indicate Frog Source (top) or Lake Water Type (bottom). NMDS stress: **(A,B)** 0.06; **(C,D)** 0.1.

treatments), microbiome composition was significantly affected by both Water Source and Frog Source. Frogs from the two source populations harbored significantly different bacterial communities, and this was true regardless whether all treatments or only lake water treatments were considered (PERMANOVA for all treatments: Pseudo-$F_{1,75}$ = 2.92, P = 0.0166, and for only lake water treatments: Pseudo-$F_{1,65}$ = 4.03, P = 0.0040, **Figure 1A**). *R. sierrae* microbiome composition differed based on Lake Water Type (population persistence or die-off at the field site from which water was collected, Pseudo-$F_{1,65}$ = 7.18, P = 0.0002, **Figure 1B**). In analyses focused on water sterility, frogs housed in sterile water harbored different bacterial communities than frogs housed in live water (Pseudo-$F_{1,75}$ = 4.52, P = 0.0009, Supplementary Figure S2).

Microbiome composition of samples collected at the start of the experiment (immediately before placing frogs in their respective water treatments) showed no effect of Water Source ($P > 0.05$), confirming that the effects of Water Source observed after application of experimental treatments was indeed due to the treatments. In contrast, the composition of microbial communities did differ between the two Frog Sources at the start of the experiment (Pseudo-$F_{1,75}$ = 5.30, P = 0.0035), indicating that effects of Frog Source on the microbiome are at least partially robust to normalizing forces such as shared aquatic environments created by the common-garden pre-experiment tanks or the pre-experiment hydrogen peroxide treatments employed in this study. When analyzing each phylotype individually, we identified a number of phylotypes for which relative abundance was associated with Frog Source, Lake Water Type, or Water Sterility (Supplementary Table S2).

Three weeks after Bd infection, skin bacterial communities still differed based on Frog Source and Water Source. Bacterial community composition differed between frogs from the two source populations (Pseudo-$F_{1,64}$ = 4.38, P = 0.0007, **Figure 1C**) as well as between frogs housed in water from field sites with different disease dynamics, i.e., population persistence or die-off (Pseudo-$F_{1,64}$ = 7.95, P = 0.0001, **Figure 1D**). Microbiomes also differed between sterile and live water treatments (Pseudo-$F_{1,74}$ = 3.92, P = 0.0007, Supplementary Figure S2).

Bd Infection Trajectories and Frog Survival Were Subtly Affected by Water Source and Frog Source

Frogs from Humphreys Basin showed more rapid rates of Bd load increase than frogs from Dusy Basin (repeated

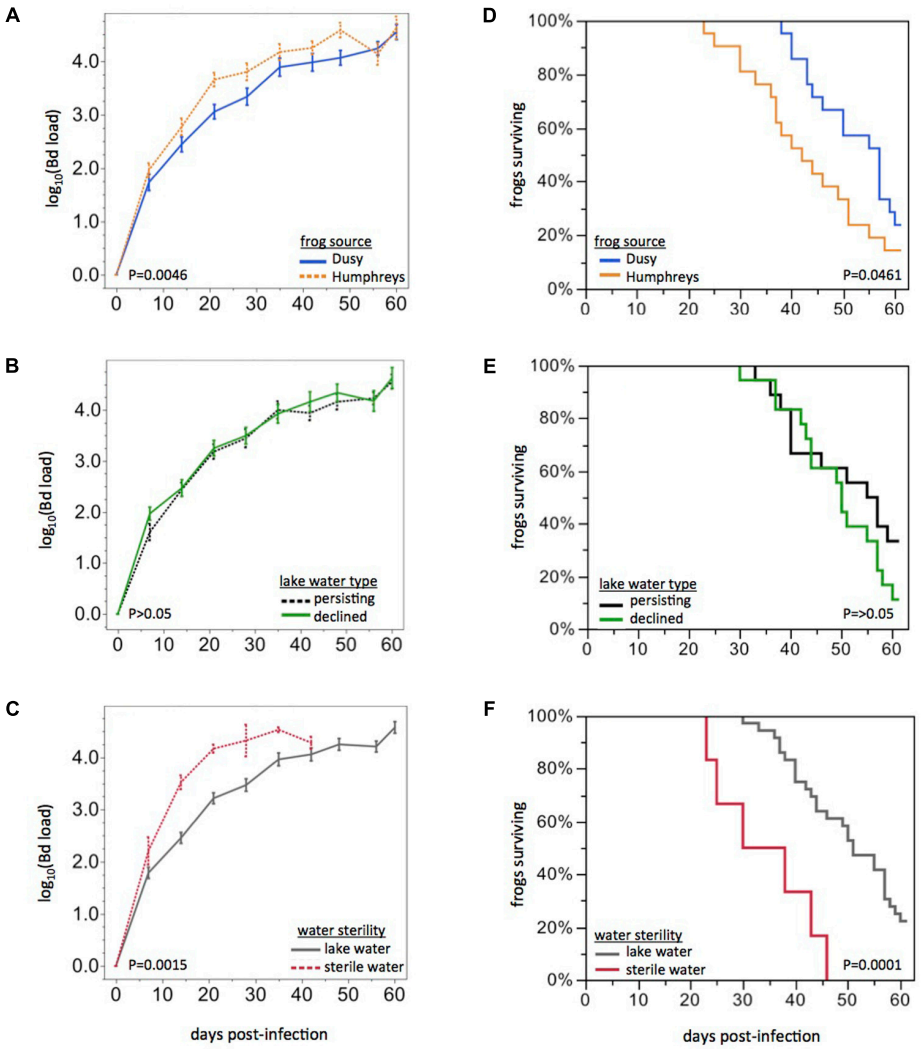

FIGURE 2 | Effects of frog source and water source on disease dynamics. Bd load trajectories and survival of frogs, grouped by Frog Source (top), Lake Water Type (middle), or Water Sterility (bottom). Rates of Bd load increase and survival curves were affected by Frog Source **(A,D)** and Water Sterility **(C,F)** but not Lake Water Type **(B,E)**. Statistical tests are based on the first 21 days post-exposure only because of missing data (due to mortality) beyond that time span. All frogs eventually developed high Bd loads.

measures ANOVA: $F_{1,34}$ = 7.43, P_{Frog} = 0.0100, $F_{3,32}$ = 5.25, $P_{\text{Frog x Time}}$ = 0.0046, **Figure 2A**). Water Source also affected Bd load trajectories ($F_{6,34}$ = 3.18, $P_{\text{Water Source}}$ = 0.0139, Wilk's Lambda approximate $F_{18,91}$ = 2.06, $P_{\text{Water Source x Time}}$ = 0.0135). The significant Water Source effect was primarily driven by a difference between sterile water and lake water: if the sterile water treatment was excluded, Water Source and the Water Source x Time effects were no longer significant. The disease dynamics of field sites from which water was collected (Lake Water Type: persistent or die-off) had no effect on Bd load trajectories ($P > 0.05$, **Figure 2B**). Bd load dynamics did differ between frogs housed in sterile water compared with frogs housed in live lake water ($F_{1,34}$ = 15.99, $P_{\text{Water Sterility}}$ = 0.0003, $F_{3,32}$ = 6.47, $P_{\text{Water Sterility x Time}}$ = 0.0015, **Figure 2C**). Patterns in frog survival were consistent with Bd load trajectories: Kaplan Meier survival curves differed based on Frog Source (Chi-square: log rank test, P = 0.0461; Wilcoxon test, P = 0.0108) but not Lake Water Type ($P > 0.05$, **Figures 2D,E**). Water Sterility affected survival curves (log rank, $P < 0.0001$; Wilcoxon, $P < 0.0001$, **Figure 2F**). Notably, any differences we observed in Bd load trajectories or survival curves were only quantitative: the rate of Bd load increase or time to mortality differed slightly but all Bd-exposed frogs became infected and eventually developed Bd loads in the lethal range. Mortality of infected animals would likely have been 100% if we had not intervened with antifungal treatment.

No Direct Evidence for Microbiome Affecting Bd Infection

Differences in skin bacterial communities prior to Bd challenge were not correlated with Bd infection intensity at

21 days PE (Mantel test: $P > 0.05$). In addition, bacterial diversity prior to Bd exposure was not correlated with downstream Bd load. Nor did individual analyses of phylotypes identify any phylotypes for which relative abundance prior to Bd infection significantly predicted downstream Bd load.

Bacterial Communities Were Predicted by Intensity of Bd Infection

Differences in frog skin microbiomes at 21 days PE were correlated with Bd infection intensity measured on the same day (21 days PE), indicating that the intensity of Bd infection, not just Bd infection in a binary sense, influences bacterial communities (Mantel test: $P = 0.0037$, Spearman's rank correlation coefficient = 0.15). Across all infected frogs on that same day, linear mixed models testing the effects of Bd load on relative abundance of each bacterial phylotype yielded no significant results after adjusting for multiple tests. The lack of individual phylotypes correlated with Bd load does not contradict the significant whole-community correlation with Bd load (Mantel test). Instead, it may simply indicate that the whole-community correlation with Bd load is due to small changes in many OTUs, rather than dramatic changes in a few OTUs. The lack of significant change in individual phylotypes could also be due to high intra-individual variation due to the many experimental treatments (7 Water Sources × 2 Frog Sources). Indeed, when we eliminated variation due to Frog Source and Water Source by analyzing a subset of frogs from one treatment through time, we found that relative abundances of several individual phylotypes changed as Bd loads increased over time (**Figure 3** and Supplementary Table S3). The identities of these phylotypes were highly consistent with the OTUs that responded to Bd infection when analyzed as a binary variable (infected versus uninfected, Jani and Briggs, 2014 and Supplementary Tables S2, S3). For many of these phylotypes, dramatic changes in relative abundance occurred only after Bd loads were quite high (mean above 1,000 Bd cells/swab, **Figure 3**). None of these phylotypes were correlated with time in the Bd-free frogs.

Bacterial Diversity Was Affected by Lake Water Type

Bacterial diversity was not affected by Frog Source or Water Sterility (all $P > 0.05$). In contrast, bacterial diversity was affected by Lake Water Type: after 3 weeks in their respective lake water treatments, frogs housed in Persistent water had higher bacterial richness than frogs housed in Die-off water ($F_{1,60} = 7.41$, S_{OBS} $P = 0.0083$, $F_{1,60} = 10.29$, Chao's richness $P = 0.0021$, $F_{1,60} = 8.30$, Shannon diversity $P = 0.0054$, $F_{1,60} = 5.32$, Shannon evenness $P = 0.0243$; **Figure 4**), seeming to mimic patterns observed in the wild (Jani et al., 2017). The difference in richness between frogs in the two types of water was due to richness declining over time in the lab more rapidly for frogs housed in die-off water than in persistent water. The decline in richness was not due to transition from the wild to captivity, as these frogs were housed in captivity for a year prior to this study. The decline might have been due to the transition from common garden tanks

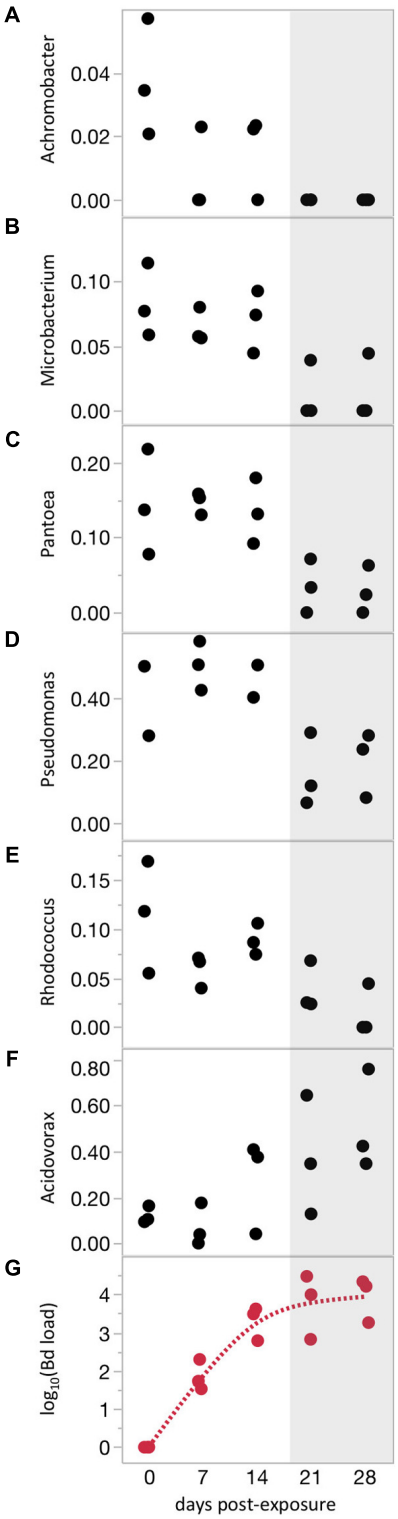

FIGURE 3 | Bacterial phylotypes shift with increasing Bd severity. **(A–F)** Relative abundances of phylotypes with significant correlations with time over the course of Bd infection. **(G)** Bd load trajectory. Shaded area highlights region of highest Bd loads. All correlations shown are statistically significant ($P < 0.05$, $Q < 0.05$). Relative abundance data are arcsine(square root) transformed.

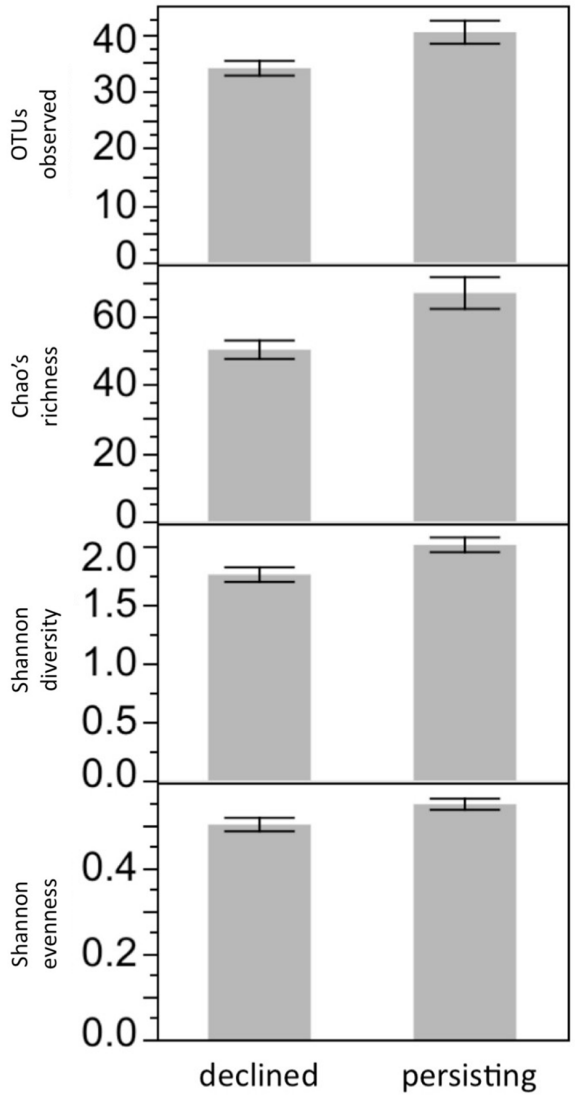

FIGURE 4 | Microbiome diversity is affected by Lake Water Type. After 3 weeks of exposure to water treatments, bacterial diversity was higher on *R. sierrae* housed in water that had been collected from lakes inhabited by persisting frog populations, compared with frogs housed in water collected from lakes that had been inhabited by populations that died out due to Bd. $P < 0.05$ for all four richness or diversity metrics.

prior to the experiment to solitary tanks used in the experiment, if for example contact among frogs replenishes the microbiome. Another 3 weeks later, after Bd exposure, richness no longer differed between these groups. This loss of the difference in richness between frogs in Persistent and Die-off water was not explained by Bd infection: there was no significant interaction between Bd infection and Lake Water Type. Nor was bacterial diversity at 21 days PE correlated with Bd load.

Symptoms Caused by Bd Infection

All frogs in the Bd+ treatment became infected with Bd, and Bd loads increased rapidly with time (Supplementary Figure S3a). Increases in Bd load were accompanied by weight loss: Frogs in the Bd- group appeared to gain weight over the course of the experiment, while frogs in the Bd+ group lost weight (repeated measures ANOVA with Bd treatment, Frog Source, and Water Source as explanatory variables; $P_{\text{BdxTime}} < 0.0001$, Supplementary Figure S3b). All frogs in the Bd- group survived the experiment, but the Bd+ group experienced considerable mortality (Supplementary Figure S3c). We used ANOVA models to formally test effects of Bd infection on frog appetite (number of crickets eaten per week) and skin shedding measured (based on an ordinal 3-level rating system) at 6 and 8 weeks post infection. Frog Source and Water Source were included as additional explanatory variables in the model. Bd infection led to reduced appetite ($P < 0.0001$ at 6 and 8 weeks PE, Supplementary Figure S4a) and increased skin sloughing ($P < 0.0001$ at both 6 and 8 weeks PE, Supplementary Figure S4b).

Bacterial Communities in Stored Lake Water

Water collected from field sites maintained consistent differences due to Water Source despite storage (Pseudo-$F_{5,12} = 5.23$, $P_{\text{Water Source}} = 0.0001$) and was consistent through time (Supplementary Figure S5). Richness and composition of bacterial communities in water did not differ by Lake Water Type (persistent or die-off, $P > 0.05$ for all tests). However, one measure of richness was marginally higher in persistent water ($P_{\text{SOBS}} = 0.0860$), and lack of significance in other metrics is not conclusive because power to detect differences was low (Supplementary Figure S5).

DISCUSSION

Summary

Wild *R. sierrae* populations exhibit distinct disease dynamics, either persisting or experiencing catastrophic declines in response to Bd infection. These differences in disease dynamics in wild populations coincide with differences in the composition of skin-associated bacterial communities (Jani et al., 2017). To understand the biological meaning and conservation relevance of these patterns, we need to tease apart cause and effect in interactions between Bd and the microbiome and improve understanding of what shapes the microbiome in the first place. With the current experiment, we aimed to identify factors that shape the *R. sierrae* skin microbiome, and to clarify causal Bd-bacteria relationships. Our results demonstrate that both host background and the aquatic environment affect the skin microbiome. We found that innate (i.e., inherent) differences between conspecific populations led to differences in the skin microbiome. We also found that lake water to which frogs are exposed can shape differences in the skin microbiome, even in the absence of additional environmental factors such as sediment, vegetation, or contact with other amphibians. Host background also predicted variation in Bd load increase and time to mortality. However, we found no direct evidence that the microbiome confers resistance to Bd, at least in this laboratory setting. Thus, although our experimentally altered microbiomes were

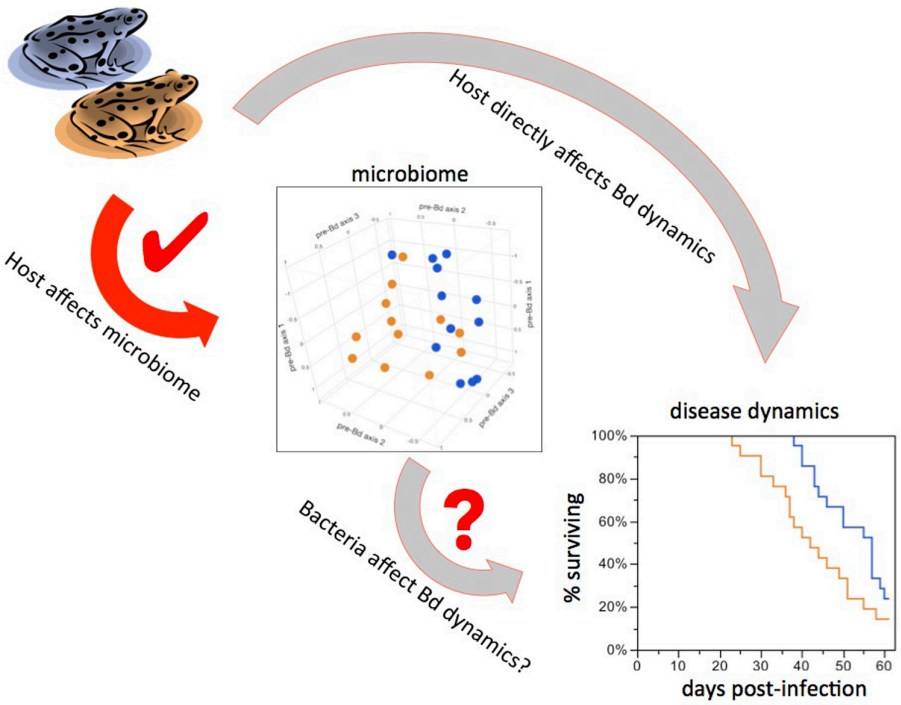

FIGURE 5 | Teasing apart cause from correlation. Possible causal links between experimental treatments and Bd infection outcomes. Frog sources led to different bacterial communities and differences in downstream Bd dynamics (red arrow). This could mean that bacteria mediate disease dynamics (short gray arrow). It is also possible that the host influences both the microbiome and disease dynamics independently of each other (long gray arrow), such that the variation in bacteria is not the driver of differences in Bd dynamics.

correlated with downstream Bd dynamics, we cannot conclude that the microbiome was causally linked to Bd dynamics.

Frog Population Affects Skin Microbiome and Bd Infection Trajectory

Previous work showed differences between microbiomes of amphibian species sharing a common lake environment (McKenzie et al., 2012; Kueneman et al., 2014; Walke et al., 2014). Here, we show that within-species variation in the microbiome is at least partly controlled by innate (most likely genetic) differences between host populations. Furthermore, the effect of Frog Source was significant both in the absence of Bd and after Bd infection, indicating that the disturbance to the microbiome caused by Bd infection does not completely override host effects on the microbiome. We also showed that the rate of increase in Bd loads in the experiment differed between frogs from the two populations, demonstrating that host-controlled microbiome variation is associated with host response to Bd. Thus there is potential for a cascade of effects in which host background influences the microbiome, which in turn affects infection dynamics. However, it is also possible that frog population background or age affects disease dynamics through unknown mechanisms independent of the skin microbiome (**Figure 5**). Isolating the effect of the microbiome is extremely difficult – particularly in cases such as this, when we are interested in subtle differences representative of natural variation – and presents an important challenge for future research.

Using a controlled, factorial experiment enables us to isolate the role of the host in shaping the microbiome. We included frogs from two different source populations to test how different genetic backgrounds affect the microbiome. However, in this study, the frogs from the two populations were of slightly different ages, therefore we cannot completely rule out the possibility that age played a role in microbiome differences. However, previous studies of *R. sierrae* and its close relative *Rana cascadae* found no difference in the microbiomes of juveniles compared with adults (Jani and Briggs, 2014; Kueneman et al., 2014). In the current study the age variation was relatively small, with all frogs being juveniles, therefore we do not expect the age difference to be the cause of the observed differences in microbiomes between the two population sources. We conclude that in this study genetic differences are a more likely driver of the microbiome differences between the two frog populations.

In this study, initial rates of Bd load increase varied, but no frogs fully resisted or cleared Bd infection, and all frogs challenged with Bd eventually developed Bd loads in the lethal range. Thus, none of the experimental treatments conferred true resistance to infection or disease. While we had hoped that exposing frogs to aquatic bacteria from persistent sites would confer some resistance to Bd, it is not surprising that neither of the frog populations exhibited inherent resistance to disease given that wild populations in both Dusy Basin and Humphreys Basin have collapsed due to Bd. However, differences

in rates of Bd load increase may have practical importance even in the absence of complete disease resistance. In recent years, researchers have attempted interventions using antifungal drugs or probiotics to curb the devastating effects of Bd on *R. sierrae* populations (Knapp et al., unpublished). These efforts depend critically on researchers and managers having sufficient time to mobilize intervention efforts before frogs become too heavily infected with Bd. Thus, even without complete resistance, variation in the rate of increase in Bd infection intensity can have practical conservation implications. In the current study we found frogs from Dusy Basin and Humphreys Basin populations harbor different bacterial communities, and also exhibit subtly different rates of increase in Bd loads. These results highlight the importance of further research to understand host population level differences, both in terms of host genetics and associated symbiotic microbes. The populations in the current study were closely related, both belonging to the same *R. sierrae* clade (Vredenburg et al., 2007) and both suffering the same fate due to Bd in the wild. We speculate that laboratory comparisons of more distantly related populations (e.g., persisting northern populations compared with declining southern populations) would reveal more dramatic differences in microbiome composition and disease resistance.

Lake Water Type Affects Skin Microbiome But Not Bd Infection Trajectory

A key finding of our study is that housing frogs in water from different lakes was sufficient to mimic some microbiome patterns in the field: namely, differences between persistent and die-off *R. sierrae* populations. In the field, *R. sierrae* populations that persist with Bd harbor different bacterial communities than populations that died out due to Bd. Similarly, in the current laboratory experiment, frogs developed different bacterial communities depending on whether they were housed in water collected from field sites inhabited by persistent or die-off populations. This result is remarkable given the limitations of our mesocosms: the mesocosms consisted simply of tanks with water from different lakes. No vegetation or sediment from field sites was added. Despite their limitations, our mesocosms induced differences in the bacterial communities on *R. sierrae* skin that were consistent with field patterns. Notably, all frogs in this experiment were from die-off populations, which is important because frog source and water source are not confounded and we can conclude that the differences observed between different water treatments are indeed due to those water treatments. In contrast, in field surveys we cannot conclude whether differences in the microbiomes of persistent and die-off populations is due to the environment or frog genetic background, or both. Notably, in field surveys the difference between microflora of persistent and die-off populations is more dramatic than in the current experiment, indicating that not all of the variation observed in the field is captured by our experiment. We think it is most likely that, in addition to the aquatic environment, host genetic background contributes to differences between die-off and persistent populations in the field, although additional environmental variables not tested in our experiment (such as lake sediment) likely also play a role. Our results demonstrate that water is an important environmental driver of variation in the microbiome, and differences in water sources alone can explain some of the variation in microbiomes between persistent and die-off *R. sierrae* populations in the field.

Although frogs housed in water from persistent and die-off water developed different microbiomes, this did not translate to any difference in the rate of Bd load increase. Thus, to the extent that our mesocosms represent environmental variation, we found no evidence supporting the hypothesis that environmentally mediated differences in the microbiome determine *R. sierrae* populations persistence or decline due to chytridiomycosis. We therefore speculate that host genetic differences may be more important than environmental drivers in determining *R. sierrae* population response to Bd infection.

Water Sterility Affects the Microbiome and Downstream Bd Dynamics

This experiment also demonstrated differences in the overall composition of microbiomes of frogs housed in sterile compared with non-sterile aquatic environments. These results are consistent with previous studies (Loudon et al., 2013; Knutie et al., 2017). In addition to affecting the *R. sierrae* microbiome, the experimental aquatic environment affected Bd infection trajectories. Increases in Bd loads were more rapid in frogs housed in sterile water than frogs housed in live lake water. As with differences in frog genetic background, the tentative conclusion of a cascade of effects from aquatic environment to skin microbiome to disease dynamics is only one possible interpretation of the data, and it is important to consider alternative explanations. For example, in addition to harboring different bacterial communities, water sources may vary in water chemistry, which may affect the *R. sierrae* microbiome. Another possible explanation for why Bd dynamics differed between live and sterile water treatments is that Bd survival in the aquatic habitat is directly affected by Water Sterility. Re-infection of a frog by zoospores released from its own skin to the water probably contributed to Bd infection dynamics in this experiment. If water bacterial communities or chemistry affect Bd zoospore survival, this could lead to differences in the density of infective zoospores in the aquatic environment, affecting the rate of Bd load increase on frogs. In the latter case, Water Sterility affects the frog microbiome and also independently affects disease dynamics, without there necessarily being a causal link between the frog microbiome and disease dynamics. Studies have found that grazing by aquatic crustaceans affects Bd zoospore densities (Hamilton et al., 2012; Searle et al., 2013; Kagami et al., 2014). In the current study, we filtered all macro-organisms from lake water, but bacteria present in the water may interact with Bd, and presence of organic matter, albeit minimal in these oligotrophic lakes, may provide resources for Bd growth or

survival. Thus we cannot conclude with certainty that the differences in disease trajectories between live and sterile water treatments were caused by a cascade of effects from aquatic bacteria to frog microbiome to disease dynamics. Additional studies examining growth and survival of Bd in different aquatic environments may help clarify the effects of aquatic bacteria on Bd.

Does Microbiome Diversity Increase Disease Resistance?

The source of lake water in this experiment affected the richness of the *R. sierrae* skin microbiome. Notably, these effects mimicked patterns observed in the field: frogs housed in water from persistent (northern) populations harbored higher bacterial richness than frogs housed in water from die-off (southern) populations (Jani et al., 2017). However, in the current experiment the higher richness on frogs in Persistent water did not prevent or lessen Bd infection when frogs were subsequently exposed to Bd. In contrast, previous experimental work showed that greater bacterial diversity reduces Bd growth *in vitro* (Piovia-Scott et al., 2017). In addition, field observations showed that northern (persistent) *R. sierrae* populations have greater diversity than southern populations, which generally suffer die-offs due to Bd infection. Based on these previous findings we hypothesized that microbiome richness may play a protective role in *R. sierrae*. Our results in the current study fail to support that conclusion. However, there are at least four plausible explanations for our results. The results may mean that richness is in fact not protective, and that the richness – persistence relationship in the field is not causal. A second possibility is that diversity does increase disease resistance in the wild, but that the effect is not due to diversity *per se* but rather due to the increased likelihood of key taxa being present. If those key taxa are uncommon in the laboratory environment, then richness may not confer protection in the lab, even if it does in the field. A third hypothesis is that richness needs to reach a minimum threshold to provide protection. Richness in the field was much higher than in the current laboratory study, so it is possible that even the highest richness observed in the lab was insufficient to confer protection. (Mean Chao's richness in persistent water was 66 in current experiment and ∼250 in field, see Figure 4c in Jani et al., 2017.) Finally it is possible that the magnitude of the difference in richness in the current experiment was not great enough to result in differences in disease resistance between the experimental groups. In the field, frogs in northern sites had about twice the richness of southern frogs, while frogs in persistent water in the current study had only about a 30% increase in richness compared with frogs in die-off water. Thus, the current experiment does not support a protective role for bacterial diversity, but does not rule it out either. We have observed that microbiome diversity patterns are less consistent than patterns based on composition, suggesting that the identity of bacteria is important, rather than simply the number of species and evenness of their abundances. Repeating studies that examine microbiome diversity will be important for forming firm conclusions.

Bd Disturbance Depends on Bd Load

Analyses of time series data over the course of increasing Bd infection identified several phylotypes that appeared to be affected by increasing Bd loads, suggesting that certain bacteria respond to the severity of Bd infection, not simply to the presence or absence of infection. Notably, for several phylotypes, the effects of Bd was most pronounced after Bd loads reached high levels (**Figure 3**). Previous studies have found effects of Bd infection on the microbiome (Jani and Briggs, 2014; Longo et al., 2015; Jani et al., 2017), while another case found no effect (Becker et al., 2015). The latter study tested for effects of Bd on the microbiome early in infection when loads were low. Our current finding that the effect of Bd on bacteria depends on Bd load suggests that discrepancies between previous studies could be in part due to the fact that studies examine Bd effects at different Bd loads.

Strengths and Limitations of Laboratory Experiments

Laboratory experiments cannot reproduce natural settings. Laboratory storage of lake water alters aquatic microbial communities, and captivity alters the *R. sierrae* skin microbiome. However, microbial community composition was more strongly predicted by sample type (*R. sierrae* versus water) than by location (lab versus field). *R. sierrae* microbiomes from the field and lab were more similar to each other than to aquatic microbial communities (Supplementary Results and Supplementary Figure S1 in Jani and Briggs, 2014). In contrast, in other study systems, the shift between captive and wild microbiomes can be more dramatic. For example, microbiomes of terrestrial salamanders in the wild were more similar to field soil bacterial communities than to the microbiomes of the same animals in captivity (Loudon et al., 2013). In general, laboratory studies cannot be assumed to exactly reproduce field phenomena. The strengths of laboratory experiments lie in the ability to randomize and assign treatments, thereby clarifying cause and effect and disentangling factors that often co-vary in the field. Field studies have shown that persistent *R. sierrae* populations have different microbiomes from die-off populations, but wild populations differ in both genetic background and environmental conditions (Jani et al., 2017). The current experiment complements prior field results by separating host and environmental drivers of microbiome variation and directly testing links to disease outcomes.

Teasing Apart Cause and Effect in Correlations Between Bd and the Microbiome

Surveys of wild populations of *R. sierrae* and its close relative *Rana muscosa* have found correlations between population response to Bd (persistence or decline) and skin-associated bacterial communities (Woodhams et al., 2007; Jani et al., 2017). Bd has also been shown to disturb the microbiome (Jani and Briggs, 2014). It is therefore impossible to definitively determine from field surveys of infected populations whether

correlations between bacteria and Bd load are due to variation in protective effects of bacteria or Bd-induced disturbance of the microbiome. In the current study, we used a controlled experiment to tease apart cause and effect. We found that the severity of Bd infection is significantly correlated with overall composition of the microbiome after Bd infection, but not before Bd infection. These results indicate that in this experiment the effect of Bd infection on the microbiome was stronger than the effect of the microbiome on Bd infection. However in this study variation in response to disease was limited: despite variation in rates of disease progression, all frogs in this study were susceptible to chytridiomycosis. Similar experiments comparing populations or species that show greater distinction in resistance to Bd would provide valuable additional insight to the role of the amphibian microbiome in disease resistance.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Institutional Animal Care and Use Committee of the University of California, Santa Barbara (UCSB IACUC). All animal procedures were approved by the UCSB IACUC.

AUTHOR CONTRIBUTIONS

AJ and CB designed the study. AJ performed the experiments and analyses and wrote the paper with revisions from CB.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00487/full#supplementary-material

REFERENCES

Abdallah, F., Mijouin, L., and Pichon, C. (2017). Skin immune landscape: inside and outside the organism. *Mediators Inflamm.* 2017:5095293. doi: 10.1155/2017/5095293

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2011). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Becker, M. H., Walke, J. B., Cikanek, S., Savage, A. E., Mattheus, N., Santiago, C. N., et al. (2015). Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc. R. Soc. B Biol. Sci.* 282:20142881. doi: 10.1098/rspb.2014.2881

Belden, L. K., Hughey, M. C., Rebollar, E. A., Umile, T. P., Loftus, S. C., Burzynski, E. A., et al. (2015). Panamanian frog species host unique skin bacterial communities. *Front. Microbiol.* 6:1171. doi: 10.3389/fmicb.2015.01171

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Bletz, M. C., Goedbloed, D. J., Sanchez, E., Reinhardt, T., Tebbe, C. C., Bhuju, S., et al. (2016). Amphibian gut microbiota shifts differentially in community structure but converges on habitat-specific predicted functions. *Nat. Commun.* 7:13699. doi: 10.1038/ncomms13699

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Briggs, C. J., Knapp, R. A., and Vredenburg, V. T. (2010). Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9695–9700. doi: 10.1073/pnas.0912886107

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* 11, 265–270.

Clarke, K., and Gorley, R. (2006). *Primer v6: UserManual/Tutorial.* Plymouth: PRIMER-E.

Costa, S., Lopes, I., Proença, D. N., Ribeiro, R., and Morais, P. V. (2016). Diversity of cutaneous microbiome of *Pelophylax perezi* populations inhabiting different environments. *Sci. Total Environ.* 572, 995–1004. doi: 10.1016/j.scitotenv.2016.07.230

Crawford, A. J., Lips, K. R., and Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13777–13782. doi: 10.1073/pnas.0914115107

Dethlefsen, L., McFall-Ngai, M., and Relman, D. A. (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* 449, 811–818. doi: 10.1038/nature06245

Engel, P., and Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol. Rev.* 37, 699–735. doi: 10.1111/1574-6976.12025

Grice, E. (2014). The skin microbiome: potential for novel diagnostic and therapeutic approaches to cutaneous disease. *Semin. Cutan. Med. Surg.* 33, 98–103. doi: 10.12788/j.sder.0087

Grice, E. A., and Segre, J. A. (2012). The human microbiome: our second genome*. *Annu. Rev. Genomics Hum. Genet.* 13, 151–170. doi: 10.1146/annurev-genom-090711-163814

Hamilton, P. T., Richardson, J. M. L., and Anholt, B. R. (2012). Daphnia in tadpole mesocosms: trophic links and interactions with *Batrachochytrium dendrobatidis*: daphnia in tadpole mesocosms. *Freshw. Biol.* 57, 676–683. doi: 10.1111/j.1365-2427.2011.02731.x

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jani, A. J., Knapp, R. A., and Briggs, C. J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proc. R. Soc. B Biol. Sci.* 284:20170944. doi: 10.1098/rspb.2017.0944

Kagami, M., Miki, T., and Takimoto, G. (2014). Mycoloop: chytrids in aquatic food webs. *Front. Microbiol.* 5:166. doi: 10.3389/fmicb.2014.00166

Kilpatrick, A. M., Briggs, C. J., and Daszak, P. (2010). The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends Ecol. Evol.* 25, 109–118. doi: 10.1016/j.tree.2009.07.011

Knapp, R. A., Briggs, C. J., Smith, T. C., and Maurer, J. R. (2011). Nowhere to hide: impact of a temperature-sensitive amphibian pathogen along an elevation gradient in the temperate zone. *Ecosphere* 2, 1–26. doi: 10.1890/ES11-00028.1

Knapp, R. A., Fellers, G. M., Kleeman, P. M., Miller, D. A., Vredenburg, V. T., Rosenblum, E. B., et al. (2016). Large-scale recovery of an endangered amphibian despite ongoing exposure to multiple stressors. *Proc. Natl. Acad. Sci. U.S.A.* 113, 11889–11894. doi: 10.1073/pnas.1600983113

Knutie, S. A., Wilkinson, C. L., Kohl, K. D., and Rohr, J. R. (2017). Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. *Nat. Commun.* 8:86. doi: 10.1038/s41467-017-00119-0

Kueneman, J. G., Parfrey, L. W., Woodhams, D. C., Archer, H. M., Knight, R., and McKenzie, V. J. (2014). The amphibian skin-associated microbiome across species, space and life history stages. *Mol. Ecol.* 23, 1238–1250. doi: 10.1111/mec.12510

Kueneman, J. G., Woodhams, D. C., Harris, R., Archer, H. M., Knight, R., and McKenzie, V. J. (2016). Probiotic treatment restores protection against lethal fungal infection lost during amphibian captivity. *Proc. R. Soc. B Biol. Sci.* 283, 20161553. doi: 10.1098/rspb.2016.1553

Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., et al. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc. Natl. Acad. Sci. U.S.A.* 103, 3165–3170. doi: 10.1073/pnas.0506889103

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Loudon, A. H., Woodhams, D. C., Parfrey, L. W., Archer, H., Knight, R., McKenzie, V., et al. (2013). Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (*Plethodon cinereus*). *ISME J.* 8, 830–840. doi: 10.1038/ismej.2013.200

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

McKenzie, V. J., Bowers, R. M., Fierer, N., Knight, R., and Lauber, C. L. (2012). Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *ISME J.* 6, 588–596. doi: 10.1038/ismej.2011.129

Muletz, C. R., Myers, J. M., Domangue, R. J., Herrick, J. B., and Harris, R. N. (2012). Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by Batrachochytrium dendrobatidis. *Biol. Conserv.* 152, 119–126. doi: 10.1016/j.biocon.2012.03.022

Naik, S., Bouladoux, N., Wilhelm, C., Molloy, M. J., Salcedo, R., Kastenmuller, W., et al. (2012). compartmentalized control of skin immunity by resident commensals. *Science* 337, 1115–1119. doi: 10.1126/science.1225152

Nelson, C. E. (2009). Phenology of high-elevation pelagic bacteria: the roles of meteorologic variability, catchment inputs and thermal stratification in structuring communities. *ISME J.* 3, 13–30. doi: 10.1038/ismej.2008.81

Nelson, C. E., Carlson, C. A., Ewart, C. S., and Halewood, E. R. (2014). Community differentiation and population enrichment of Sargasso Sea bacterioplankton in the euphotic zone of a mesoscale mode-water eddy. *Environ. Microbiol.* 16, 871–887. doi: 10.1111/1462-2920.12241

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., McKenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., et al. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res.* 35, 7188–7196. doi: 10.1093/nar/gkm864

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rachowicz, L. J., Knapp, R. A., Morgan, J. A., Stice, M. J., Vredenburg, V. T., Parker, J. M., et al. (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87, 1671–1683. doi: 10.1890/0012-9658(2006)87[1671:EIDAAP]2.0.CO;2

Rebollar, E. A., Hughey, M. C., Medina, D., Harris, R. N., Ibáñez, R., and Belden, L. K. (2016). Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. *ISME J.* 10, 1682–1695. doi: 10.1038/ismej.2015.234

Schloss, P. D. (2010). The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA Gene-Based Studies Eisen JA (ed). *PLoS Comput. Biol.* 6:e1000844. doi: 10.1371/journal.pcbi.1000844

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Searle, C. L., Mendelson, J. R., Green, L. E., and Duffy, M. A. (2013). Daphnia predation on the amphibian chytrid fungus and its impacts on disease risk in tadpoles. *Ecol. Evol.* 3, 4129–4138. doi: 10.1002/ece3.777

Šimek, K., and Chrzanowski, T. H. (1992). Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. *Appl. Environ. Microbiol.* 58, 3715–3720.

Stice, M. J., and Briggs, C. J. (2010). Immunization is ineffective at preventing infection and mortality due to the amphibian chytrid fungus *Batrachochytrium dendrobatidis*. *J. Wildl. Dis.* 46, 70–77. doi: 10.7589/0090-3558-46.1.70

Storey, J. D. (2002). A direct approach to false discovery rates. *J. R. Stat. Soc. Ser. B Stat. Methodol.* 64, 479–498. doi: 10.1111/1467-9868.00346

Storey, J. D., and Tibshirani, R. (2003). Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9440–9445. doi: 10.1073/pnas.1530509100

Vredenburg, V. T., Bingham, R., Knapp, R., Morgan, J. A. T., Moritz, C., and Wake, D. (2007). Concordant molecular and phenotypic data delineate new taxonomy and conservation priorities for the endangered mountain yellow-legged frog. *J. Zool.* 271, 361–374. doi: 10.1111/j.1469-7998.2006.00258.x

Vredenburg, V. T., Knapp, R. A., Tunstall, T. S., and Briggs, C. J. (2010). Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9689–9694. doi: 10.1073/pnas.0914111107

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Cormier, G., Jensen, R. V., et al. (2014). Amphibian skin may select for rare environmental microbes. *ISME J.* 8, 2207–2217. doi: 10.1038/ismej.2014.77

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P. C., et al. (2015). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus Fisher MC (ed). *PLoS One* 10:e0139848. doi: 10.1371/journal.pone.0139848

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial Taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Küpfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin

mucosome predict disease risk and probiotic effectiveness Navas CA (ed). *PLoS One* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Geiger, C. C., Reinert, L. K., Rollins-Smith, L. A., Lam, B., Harris, R. N., et al. (2012). Treatment of amphibians infected with chytrid fungus: learning from failed trials with itraconazole, antimicrobial peptides, bacteria, and heat therapy. *Dis. Aquat. Organ.* 98, 11–25. doi: 10.3354/dao02429

Woodhams, D. C., Vredenburg, V. T., Simon, M.-A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

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Increased Numbers of Culturable Inhibitory Bacterial Taxa May Mitigate the Effects of *Batrachochytrium dendrobatidis* in Australian Wet Tropics Frogs

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Symbiotic bacterial communities resident on amphibian skin can benefit their hosts. For example, antibiotic production by community members can control the pathogen *Batrachochytrium dendrobatidis* (*Bd*) and it is possible for these community members to be used as probiotics to reduce infection levels. In the early 1990s, the emergence of *Bd* caused declines and disappearances of frogs in the Australian Wet Tropics; the severity of its effects varied among species and sites. Some species have since recolonized despite enzootic *Bd* within their populations. This variation in history among species and sites provided an opportunity to investigate the role of anti-fungal cutaneous bacteria in protecting frogs against *Bd* infection. We collected cutaneous swab samples from three species of frogs at two upland and two lowland sites in the Wet Tropics, and used *in vitro* challenge assays to identify culturable *Bd*-inhibitory bacterial isolates for further analysis. We sequenced DNA from cultured inhibitory isolates to identify taxa, resulting in the classification of 16 *Bd*-inhibitory OTUs, and determined whether inhibitory taxa were associated with frog species, site, or intensity of infection. We present preliminary results showing that the upper limit of *Bd* infection intensity was negatively correlated with number of inhibitory OTUs present per frog indicating that increased numbers of *Bd*-inhibiting taxa may play a role in reducing the intensity of *Bd* infections, facilitating frog coexistence with enzootic *Bd*. One upland site had a significantly lower prevalence of *Bd* infection, a significantly higher proportion of frogs with one or more culturable *Bd*-inhibitory OTUs, a greater number of inhibitory bacterial genera present per frog, and statistically significant clustering of individual frogs with similar *Bd*-inhibitory signatures when compared to all other sites. This suggests that *Bd*-inhibitory taxa are likely to be particularly important to frogs at this site and may have played a role in their ability to recolonize following population declines. Our findings suggest that the use of multi-taxon *Bd*-inhibitory probiotics to support at-risk amphibian populations may be more effective than single-taxon alternatives.

Keywords: amphibian, cutaneous bacteria, *Batrachochytrium dendrobatidis*, microbiota, cell-free supernatant, inhibitory bacteria, chytridiomycosis, disease mitigation

INTRODUCTION

Antibiotic-producing bacterial symbionts can protect their hosts from disease (Currie et al., 1999; Haas and Défago, 2005; Scott et al., 2008; Mao-Jones et al., 2010; Mattoso et al., 2012). For example, a high proportion of bacteria isolated from healthy coral can produce antibiotic compounds active against coral pathogens (Ritchie, 2006; Mao-Jones et al., 2010; Zhang et al., 2013). However, the best-studied mutualisms exist between insects and their bacterial symbionts. Both fungus-farming ants and pine beetles house antibiotic-producing bacteria in specialized cuticular compartments to control pathogens that threaten their food supply (Currie et al., 1999, 2006; Scott et al., 2008). In addition, solitary digger wasps use antibiotic-producing bacteria to protect their cocooned larvae against pathogens (Kroiss et al., 2010), and fungus-farming ants maintain a bacterial biofilm on their cuticles to control a fungal pathogen (Mattoso et al., 2012). Variation in the bacterial strain present can cause differential morbidity in fungus-farming ants (Poulsen et al., 2010), demonstrating that antibiotic production by symbionts is important for host protection.

Antibiotic production by symbiotic bacteria is also important in vertebrates. In two bird species, the European Hoopoe and the Green Woodhoopoe, nestlings harbor antibiotic-producing bacteria in their uropygial glands to control feather-degrading pathogens (Soler et al., 2008; Martín-Vivaldi et al., 2010). Symbiotic bacterial communities resident on amphibian skin can also produce, antibiotic compounds that contribute to the control of *Batrachochytrium dendrobatidis* (*Bd*; Becker et al., 2009, 2015; Harris et al., 2009a,b; Lam et al., 2010; Kueneman et al., 2016), a pathogenic fungus responsible for worldwide amphibian declines (Berger et al., 1998; Stuart et al., 2004; Skerratt et al., 2007; Wake and Vredenburg, 2008).

One aspect of community ecology theory predicts that complex communities, with higher numbers of species and hence more potential interactions, are generally more resistant to invasion than simple communities with fewer species (Robinson and Valentine, 1979). This has been demonstrated in soil bacterial communities (Matos et al., 2005; van Elsas et al., 2012; Hol et al., 2015; Hu et al., 2016), and also in the locust gut (Dillon et al., 2005) where higher numbers of inoculated bacterial isolates have been associated with greater resistance against pathogens.

Studies of amphibian skin microbiota have produced similar results. *In vitro* studies have demonstrated the potential importance of multi-species bacterial communities in resistance to *Bd* (Loudon et al., 2014; Piovia-Scott et al., 2017). Co-cultured synthetic multi-species communities were more effective against *Bd* than single species alone (Piovia-Scott et al., 2017). While there are several possible explanations for this effect, the presence of greater numbers of *Bd*-inhibitory metabolites following co-culture of bacterial isolates can have a synergistic effect (Loudon et al., 2014). While no field studies have reported a reduction of *Bd* load with increased *Bd*-inhibitory isolate richness, increased Proteobacteria phylotype richness on the skin of *Eleutherodactylus coqui* frogs was correlated with reduced *Bd* infection loads (Longo et al., 2015). A large portion of *Bd*-inhibitory isolates described to date fall within this phylum (Woodhams et al., 2007; Lam et al., 2010; Walke et al., 2011; Flechas et al., 2012; Bell et al., 2013; Daskin et al., 2014; Becker et al., 2015; Holden et al., 2015; Madison et al., 2017).

Patterns of frog decline and recovery in the Australian Wet Tropics following the arrival of the pathogen *Bd* in the late 1980s (Laurance et al., 1996; Berger et al., 1999; McDonald and Alford, 1999) differed among species and sites. Frog populations of all species at lowland sites (below 400 m) did not appear to experience declines, while several species, including *Litoria nannotis* (waterfall frog) and *Litoria rheocola* (common mist frog), suffered either declines or local extirpation at all upland rainforest sites (above 400 m; Richards et al., 1993; McDonald and Alford, 1999). Some populations of these species have subsequently reappeared at some but not all sites from which they disappeared (McDonald et al., 2005; Woodhams and Alford, 2005; Skerratt et al., 2010; McKnight et al., 2017). This suggests multiple, potentially independent appearances of resistance to *Bd*, or loss of virulence by the pathogen (McKnight et al., 2017). Another species, *Litoria serrata* (green-eyed tree frog) appeared to suffer temporary population declines at upland sites, followed by recovery within a few years (Richards et al., 1993; McDonald and Alford, 1999; Richards and Alford, 2005).

The mechanisms allowing population recovery or recolonization are likely to vary among species and sites (McKnight et al., 2017). There is substantial genetic separation among Wet Tropics frog populations (Schneider et al., 1998; Cunningham, 2001; Hoskin et al., 2005; Richards et al., 2010; Bell et al., 2012); variation among populations in both innate and acquired immune defenses due to selection pressures imposed by the pathogen may affect host resistance to *Bd* and ultimately survival (Rollins-Smith et al., 2002, 2011; Woodhams et al., 2010; Savage and Zamudio, 2011; Fites et al., 2012, 2014; Bataille et al., 2015; McKnight et al., 2017; Voyles et al., 2018). Pathogen virulence may also vary among regions (Berger et al., 2005; Farrer et al., 2011; Becker et al., 2017). Therefore, resistance to *Bd* infection is likely to have been acquired independently at these sites.

Symbiotic cutaneous bacteria are considered to be an important component of the amphibian innate immune defense system (Woodhams et al., 2007, 2014; Harris et al., 2009a). It is likely that the suite of cutaneous bacteria present on amphibian skin can evolve broad antimicrobial activity against invading pathogens much faster than adaptation of the host's innate and acquired defenses (Rosenberg et al., 2007; King et al., 2016; Rosenberg and Zilber-Rosenberg, 2016). Therefore it is possible that at least some of the recolonized frogs in the Wet Tropics uplands have developed resistance to *Bd* through acquisition or evolution of more effective cutaneous bacterial symbionts.

The patterns of frog reappearances in the Wet Tropics uplands provide an opportunity to investigate whether the anti-fungal cutaneous bacterial microbiota have played a role in the ability of frogs to recolonize upland sites. We describe the culturable anti-*Bd* cutaneous bacterial microbiota of three species of rainforest frogs from four sites in northern Queensland, Australia, and assess how its composition may contribute to the health and population recovery of frogs at sites with different histories of pathogen-related population declines.

MATERIALS AND METHODS

Field Sites and Species

We collected cutaneous swab samples from the stream-dwelling rainforest frogs *L. serrata, L. nannotis*, and *L. rheocola* at four rainforest field sites in the Wet Tropics bioregion where enzootic *Bd* infection exists, with one upland and one lowland site in each of two latitudinally separated national parks (**Table 1**). *L. nannotis* are the most, and *L. serrata* the least, strongly associated with water, and *L. serrata* are the least susceptible to population fluctuations as a result of *Bd* infection (McDonald and Alford, 1999; Woodhams and Alford, 2005). The upland sites were subject to extensive frog population declines in the early 1990s, while frog populations at the lowland sites persisted. Sampling was conducted in winter, when *Bd* infection is typically more prevalent (Berger et al., 2004; Woodhams and Alford, 2005; Kriger and Hero, 2007; Sapsford et al., 2013), to maximize chances of including *Bd*-infected frogs in the study. This study was conducted in compliance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004, the Queensland Animal Care and Protection Act, 2001 and the Queensland Nature Conservation Act, 1992. Approval was granted from James Cook University Animal Ethics Committee (A1316 and A1420) and the Queensland government (scientific purposes permit number WITK05922209).

Collection of Bacterial Samples

We hand-captured frogs from vegetation and rocks bordering the creeks, in the winters of 2009 and 2010. All handling was carried out with a new plastic bag and pair of vinyl gloves for each animal to prevent contact with bacteria from our skin, and to preclude the transfer of pathogens or symbionts between animals. We gently restrained each animal by hand and rinsed it twice with a stream of sterile distilled water from a wash bottle to remove transient bacteria, which can differ from the resident microbiota (Lauer et al., 2007). We then swabbed over the dorsal and ventral skin from knee to neck five times using sterile rayon swabs (MW112, MW&E, Bath UK) moistened with sterile distilled water, and immediately transferred the swab's contents to a low-nutrient agar plate (R2A, BD, Franklin Lakes, NJ, United States) by rotating the swab on the plate surface. Agar plates were sealed with parafilm (Parafilm "M," Pechiney Plastic Packaging, Inc., Chicago, IL, United States), held at ambient temperature (10–25°C) and returned to the laboratory within 72 h. We collected an additional swab sample (MW100, MW&E, Bath UK) by rotating the swab over the abdomen, hands, feet, and thighs twice for analysis of *Bd* infection status.

Isolation, Purification, and Storage of Microbial Cultures

Inverted R2A agar plates were incubated for 48–72 h at ambient temperature (22–25°C) in the laboratory until we observed microbial culture growth. We examined plates daily for 5 days using a dissecting microscope and selected all colonies with different morphological characteristics for isolation to pure (axenic) culture using standard microbiological techniques (Salle, 1961). When initial agar plates generated from swabs plated in the field did not yield any viable bacterial colonies, possibly due to media choice or the presence of potent antimicrobial peptides on the frogs, we excluded samples from those frogs from further analysis. Of a total of nine initial plates that produced no viable cultures, five were from *L. rheocola*, and four were from *L. nannotis*. In contrast, R2A agar plates originating from *L. serrata* at the upland and lowland sites in Wooroonooran National Park produced very large numbers of viable bacterial colonies making isolation difficult and time-consuming; all of these plates appeared visually very similar. Because time and available effort constrained the number of these plates we could fully sample, we selected agar plates haphazardly from only four *L. serrata* at each of the Wooroonooran sites (**Table 1**). For all other combinations of site and species, all plates with bacterial colonies present were included.

Diagnosis of *Batrachochytrium dendrobatidis* Infection

DNA was extracted from skin swabs with PrepMan^{TM} Ultra (Applied Biosystems, Scoresby, VIC, Australia) and quantitative

TABLE 1 | Locations, species and *Batrachochytrium dendrobatidis* infection status of frogs sampled for each survey site, and overall *Bd* infection prevalence by site.

Site	Species			*Bd*
	LS	LN	LR	prevalence
Windin Creek North (Upland), Wooroonooran National Park 17°22′01.6″ S 145°42′58.3″ E	2 (4)	9 (7)	5 (8)	0.53
Frenchmans Creek (Lowland), Wooroonooran National Park 17°18′32.8″ S 145°55′04.2″ E	2 (4)	3 (9)	5 (9)	0.33
Kirrama Bridge 11 Creek (Upland), Murray Upper National Park 18°12′49.9″ S 145°47′52.9″ E	1 (10)	0 (10)	–	0.05
Kirrama Bridge 8 Creek (Lowland), Murray Upper National Park 18°11′44.8″ S 145°52′05.4″ E	1 (10)	4 (10)	0 (8)	0.17

Numbers indicate the number of infected individuals present from the 10 frogs of each species sampled at each site and, in parentheses, the number of individuals from which the isolates used in this study were obtained. Species are Litoria serrata (LS), L. nannotis (LN), and L. rheocola (LR).

PCR (Real-time TaqMan® assay) used to diagnose *Bd* infection status as per Boyle et al. (2004) with the addition of BSA (Garland et al., 2010). We considered frogs to be infected with *Bd* when at least two of the three technical PCR replicates were positive. *Bd* intensity was calculated as the mean of the technical replicates.

Challenge Assays

We conducted challenge assays on cell free supernatants from axenic isolates, and categorized them as totally inhibitory, partially inhibitory, or non-inhibitory according to their effects on *Bd* growth as described by Bell et al. (2013). Briefly, cell-free supernatants for each isolate were obtained by centrifuging and filtering bacterial cultures incubated in TGhL medium (8 g tryptone, 1 g hydrolysed gelatin, 2 g lactose, to 1 L deionised water, autoclaved) at 23°C for 48 h. *Bd* isolate "Gibbo River, L. Les, 06-LB-1" was incubated on TGhL agar plates (as above recipe with 10 g bacteriological agar) at 23°C for 3 days. Zoospores were flushed from the plate with sterile TGhL medium, filtered to remove sporangia and resuspended in sterile TGhL at 2×10^6 zoospores ml^{-1}. We conducted challenge assays in 96-well microplates (Costar 3595, Corning) with five replicates of each sample (50 μl cell-free supernatant and 50 μl of *Bd* zoospores), positive, negative, and medium only controls. Plates were incubated at 23°C for approximately 7 days until maximum growth in the positive controls was observed. Daily absorbance readings were collected at 492 nm using a spectrophotometer (Multiskan Ascent, Thermo Scientific) and from these we calculated the proportion of inhibition observed in each sample on its maximum growth day relative to the positive control. Bacterial isolates that produced cell-free supernatants exhibiting total inhibition against *Bd* were selected for taxonomic identification by DNA sequencing. Due to financial constraints it was not possible to sequence other isolates.

Bacterial DNA Extraction

We used a sterile toothpick to inoculate each axenic isolate into a 1.7 ml sterile microtube containing 400 μl molecular grade water. Samples were vortexed to create a cell suspension and subjected to three freeze-thaw cycles (70°C/-80°C; 10 min each), and then centrifuged at 7500 × *g* for 5 min to pellet cell debris. We used the supernatant directly as template in the DNA amplification reaction, but if this was unsuccessful, DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Doncaster, VIC, Australia) as per the manufacturer's protocol.

Amplification of 16S rRNA Gene

We amplified the 16S rRNA gene from pure bacterial isolates by PCR on Bio-Rad C1000/S1000 thermal cyclers (Bio-Rad, Hercules, CA, United States) with the bacteria-specific primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR reaction mix contained 0.2 μM of each primer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.2 mg ml^{-1} BSA, 1.25 U HotStar Taq polymerase (Qiagen, Doncaster, VIC, Australia) with 1× buffer and <1 ug template DNA. The thermocycling parameters were: 95°C for 15 min followed by 35 cycles of 94°C for 1 min, 48°C for 1 min, 72°C for 1.5 min, and a final extension for 10 min at 72°C.

DNA Sequencing and Analysis

PCR product purification and Sanger DNA sequencing were conducted by Macrogen, Inc. (South Korea) using both forward and reverse primers described above. We aligned forward and reverse nucleotide sequences in Geneious Pro (Biomatters, Ltd.; Drummond et al., 2010) to create a consensus sequence of approximately 1400 bp. Following alignment of sequences, we compiled a FASTA file containing a single representative of each unique (one or more bp difference from any other sequence) consensus sequence and defined operational taxonomic units (OTUs) using mothur (v1.36.1; Schloss et al., 2009). Sequences were clustered at 96% sequence similarity to create genus-level OTUs and taxonomy was assigned using the SILVA reference database v128 (Quast et al., 2013; Yilmaz et al., 2014). When three or more sequences were present in a given OTU, the sequence used to assign taxonomy was the sequence that was the minimum distance to the other sequences in the OTU. Clustering was based on 96% sequence similarity to group the isolates into unique genera because our goal was to examine the diversity of cultured taxonomic groups likely to have different mechanisms of inhibiting the growth of *Bd*. Although some genera could comprise multiple OTUs that each produce different antifungal compounds, it is likely that some subgeneric OTUs would have the same mechanisms. For example, multiple *Pseudomonas* species can produce the antifungal compound 2,4-diacetylphloroglucinol (Gutierrez-Garcia et al., 2017). Splitting genera into multiple OTUs may overestimate the diversity of inhibitory mechanisms, so we chose the more conservative approach of leaving genera intact. We examined how well our sequenced data were likely to reflect the total number of culturable inhibitory bacterial OTUs present across all frog taxa at all of our sampling sites by constructing a species accumulation curve using the program EstimateS (Colwell, 2013). Because our study was primarily aimed at examining how the composition of culturable bacterial assemblages on individual frogs affected their *Bd* infection status, we also examined whether the isolates we cultured from each individual were likely to have captured all culturable totally inhibitory OTUs present on their skins. We did this by plotting number of totally inhibitory OTUs found on each frog against the total number of cultured isolates (inhibitory and non-inhibitory) obtained from each frog, and fitting a species accumulation curve to this relationship.

We added additional 16S sequences from closely related bacterial type strains (SILVA reference database v123; Quast et al., 2013; Yilmaz et al., 2014) to our FASTA file sequences and conducted a SINA alignment (Pruesse et al., 2012). From this alignment, we constructed a neighbor-joining phylogenetic tree in Geneious Pro (Biomatters, Ltd.; Drummond et al., 2010) and visualized this in Figtree v1.4.3 (Rambaut, 2016).

Data Manipulation and Analysis

As data were not normally distributed, and attempts at normalization via transformation were unsuccessful, we used non-parametric statistical tests where applicable. All statistical analyses were carried out using R (v3.2.4)[1] and figures produced using *ggplot2* (Wickham and Chang, 2016). We excluded *L. rheocola* from most of the analyses to balance species across sites, but included them in the graphics when appropriate. By including only the two species that were present at all sites, we minimized the possibility that site effects could reflect differences between species.

We used a generalized linear model (GLM) with a binomial response to compare prevalence of infection in *L. serrata* and *L. nannotis* (the species that occurred at all sites) across sites. We used Tukey's *post hoc* pairwise comparisons (Tukey, 1949) to examine differences among pairs of sites using the function "glht" in the package *multcomp* (Hothorn et al., 2017), and adjusted *p*-values using the false discovery rate method (Benjamini and Hochberg, 1995). We assessed significance of the effects in this and other GLMs using the "ANOVA" function from the package *car* (Fox et al., 2017). To determine whether the ability of bacterial cell-free supernatants to inhibit *Bd* differed with infection status, species and site (*L. serrata* and *L. nannotis* only), we used permutational multivariate analysis of variance (PERMANOVA) on the proportions of isolates from each frog (*L. serrata* and *L. nannotis* only) that were in each of two challenge assay result categories; totally inhibitory and partially inhibitory. For this, we used the function "adonis2" with marginal terms (variables were assessed in the model together to show their impact when added last) in the community ecology package *vegan* (Oksanen et al., 2017). Tests were performed with 10000 permutations using Euclidean distance as the distance measure. We used the function "pairwise.adonis" (Martinez Arbizu, 2016) with Benjamini and Hochberg corrected *p*-values to examine differences among site pairs.

Following OTU classification, when totally inhibitory isolates were present on *Bd*-infected frogs, we used quantile regression at the 90th percentile level to examine the relationship between the intensity of *Bd* infection and the number of OTUs present on each frog using the package *quantreg* (Koenker et al., 2017) with 1000 bootstrap replications, and calculated pseudo-R^2 (Koenker and Machado, 1999). We used quantile regression because it is recommended for use when examining relationships in which the independent variable sets a boundary for the dependent variable, but the value the dependent variable takes within that boundary is affected by many other factors (Cade and Noon, 2003). This is the case for *Bd* infection intensity, which can be affected by time since exposure, recent history of body temperature, and effects of innate and inducible immune responses not related to bacteria, among other things. We used the 90th percentile because we were interested in whether inhibitory bacteria set an upper boundary, and the 90th percentile was the highest we could practically examine given the number of observations in our data set. Frogs that did not carry any totally inhibitory isolates were excluded from our quantile regressions because the goal of these analyses was to examine how the upper limit of intensity of infection responds as number of inhibitory taxa increases. Frogs with no inhibitory OTUs will necessarily have no upper limit set by their microbiota, and are thus in a separate class, not comparable for our purpose to frogs with one or more inhibitory OTUs.

If inhibitory bacterial assemblages have evolved in response to *Bd*, they might differ across sites due to the differential availability of candidate sets of bacteria. Similarly, each frog species is likely to provide a different substrate for microbes to colonize. However, we would expect them to be similar across individuals within sites and species. Therefore, to determine if individual frogs clustered according to the taxonomy of their *Bd*-inhibitory bacterial microbiota within sites, frog species or with their interaction, we used PERMANOVA as described above with the Bray–Curtis distance measure (Bray and Curtis, 1957) with 10000 permutations. We excluded *L. rheocola* to ensure that species were balanced across sites. When the interaction term was not significant we repeated the analysis using only the two main effects. We checked for heterogeneity of multivariate dispersion using function "betadisper" in package *vegan* (Oksanen et al., 2017) with 10000 permutations. We used the function "pairwise.adonis" (Martinez Arbizu, 2016) with Benjamini and Hochberg corrected *p*-values to examine differences among pairs of sites. When clustering was observed, we visualized results with non-metric multidimensional scaling (nMDS) using the function "metaMDS." When the nMDS failed to converge, we identified samples that were disconnected using the function "disconnected." As a result, we removed three samples that each had just one unique *Bd*-inhibitory OTU from the nMDS analyses.

We used a GLM, specifying that the response came from a Poisson distribution, to examine species and site-specific differences in the number of *Bd*-inhibitory cutaneous bacterial OTUs present on frogs. We used Tukey's *post hoc* pairwise comparisons (Tukey, 1949) to examine differences among site pairs as above with *p*-values adjusted using the false discovery rate method (Benjamini and Hochberg, 1995). For this, we excluded all data from *L. rheocola* to ensure that species were balanced across sites, and analyzed data as species-site pairs to test whether one species was responsible for any site-specific effects observed. Finally, we used a Fisher's Exact Test to examine the association between the proportion of frogs with *Bd*-inhibitory OTUs and field site; we did not use a GLM to examine the effects of site and species simultaneously because numbers of individuals from which any OTUs were isolated was as low as 4 for some species-site combinations, so that the statistical power of a full analysis would have been near zero. Because this analysis excluded "species" as a factor, any species effects could have been confounded with site effects, since the numbers of each species from which OTUs were isolated differed among sites. To examine this possibility, we conducted a separate Fisher's Exact Test to determine whether there were across-site effects of species (*L. nannotis* and *L. serrata* only, as these were the species in the site analysis) on the proportion of frogs with *Bd*-inhibitory OTUs.

[1] www.r-project.org

RESULTS

Infection Status of Frogs

Thirty-two of the 110 frogs (29%) sampled were infected with *Bd* (**Table 1**). Our GLM on data for the two species (*L. nannotis* and *L. serrata*) that were present at all sites indicated that the prevalence of infection differed significantly between those species (ANOVA: $\chi^2 = 7.77$, df = 1, $p = 0.005$) and among sites (ANOVA; $\chi^2 = 14.98$, df = 3, $p = 0.0002$); the interaction effect was not significant (ANOVA; $\chi^2 = 7.50$, df = 3, $p = 0.058$). *Post hoc* comparisons using Benjamini and Hochberg adjusted *p*-values showed that the Wooroonooran upland and Kirrama upland sites differed significantly ($p = 0.019$). Two of the five remaining pairwise comparisons were suggestive (Wooroonooran upland vs. Kirrama lowland and Wooroonooran upland vs. Wooroonooran lowland, $p = 0.0892$ for both cases). The remaining pairwise comparisons were not significantly different ($p > 0.100$ in all cases). Prevalence in *L. nannotis* and *L. serrata* combined (the species common to all sites) was 5% at Kirrama Uplands, 25% at Kirrama Lowlands, 25% at Wooroonooran Lowlands, and 55% at Wooroonooran Uplands.

We could not statistically compare intensity of infection among species within each site due to the low numbers of infected frogs. However, of the 29 infected frogs, 13 had infections of 10 zoospore equivalents or less indicating relatively low infection levels. Higher infection intensities occurred almost exclusively in *L. rheocola* at the Wooroonooran sites.

Culture and Challenge Assays

We sampled bacterial isolates from a total of 89 frogs (**Table 1**). We obtained a total of 1005 bacterial isolates across all frog species and sites, with a mean of 11.3 cultured bacterial isolates per frog (Range = 1–36, *SD* = 8.3). This is broadly in line with the number of isolates reported in other studies (Woodhams et al., 2007; Walke et al., 2011, 2015a; Becker et al., 2015). Challenge assay results showed that 68.5% of frogs from which isolates were obtained had one or more isolates with some activity against *Bd*; 55% had totally inhibitory isolates and 58% had partially inhibitory isolates [following the definition of Bell et al. (2013); **Figure 1**].

Our PERMANOVA analysis of data for *L. nannotis* and *L. serrata*, the species that occurred at all sites, showed that the proportion of isolates in each category of inhibition (totally inhibitory and partially inhibitory) did not depend significantly upon infection status or species (PERMANOVA: infection status, pseudo-$F_{1,58} = 0.478$, $p = 0.628$, species, pseudo-$F_{1,58} = 0.880$, $p = 0.422$), but differed significantly among sites (**Figure 1**; PERMANOVA; pseudo-$F_{3,58} = 6.189$, $p < 0.001$). *Post hoc* comparisons among sites using Benjamini and Hochberg adjusted *p*-values showed that frogs at the Kirrama upland site had a significantly greater proportion of *Bd*-inhibitory isolates than those at all other sites ($p < 0.01$ in all cases). Frogs at the Wooroonooran upland and Kirrama lowland sites also differed significantly ($p = 0.0285$). The remaining site-pairs were not significantly different from each other ($p > 0.100$ for both cases).

Identification of Bacterial Isolates

From a total of 156 totally inhibitory isolates sequenced, we identified 105 unique 16S rRNA sequences. These comprised 16 OTUs at the 96% sequence similarity level, each representative of one genus (**Figure 2** and **Table 2**). 75% of OTUs fell within the Proteobacteria phylum with small contributions from the

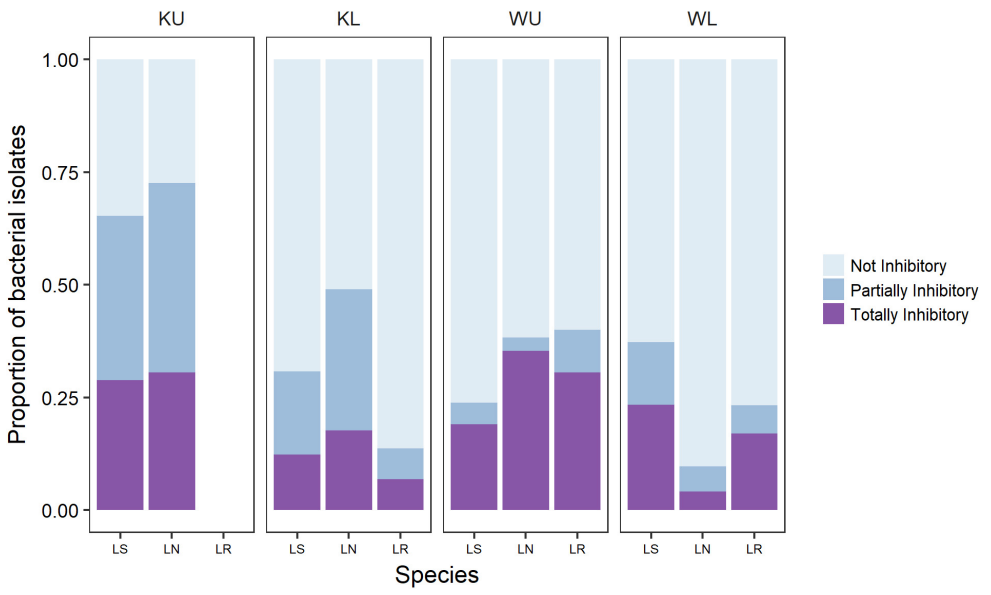

FIGURE 1 | The proportion of culturable isolates as categorized by challenge assay results for each frog species at each site. Category definitions follow Bell et al. (2013). Species are *Litoria serrata* (LS), *L. nannotis* (LN), and *L. rheocola* (LR). Sites are Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), and Wooroonooran lowland (WL).

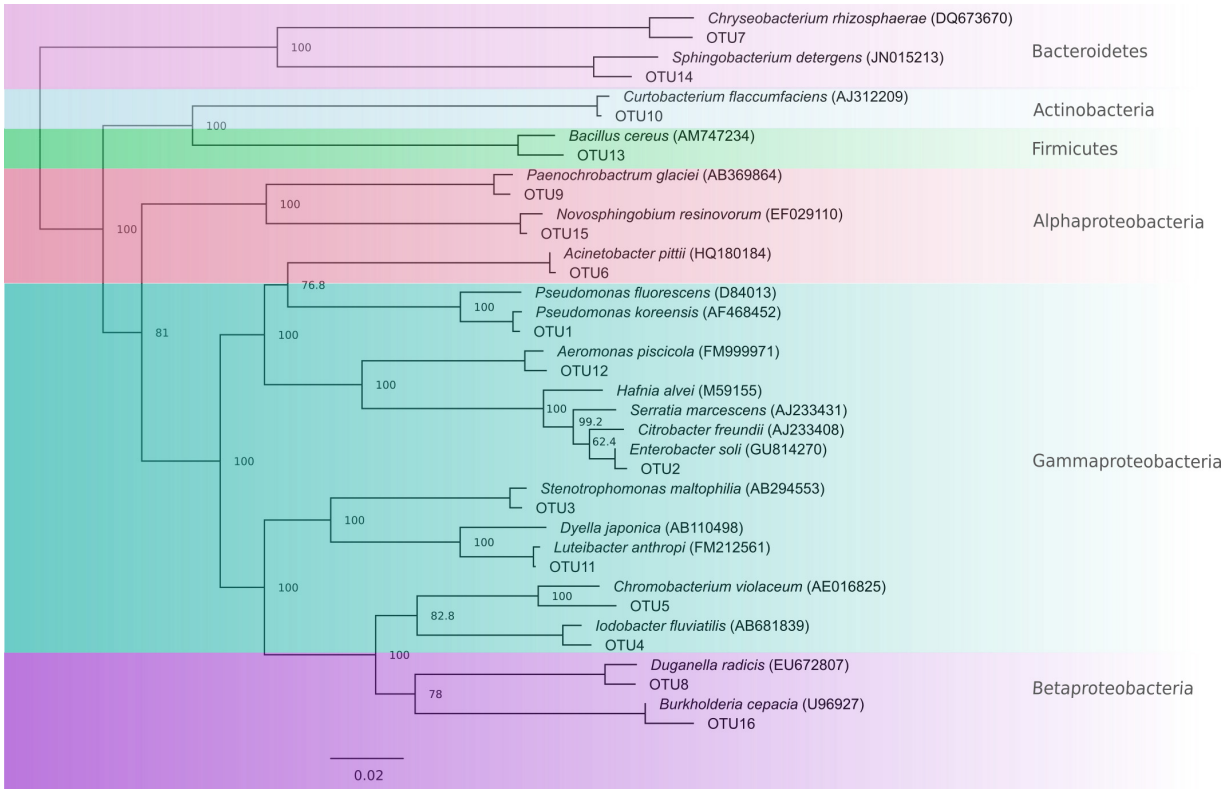

FIGURE 2 | Neighbor-joining tree showing OTUs with representative type species for comparison. Nodes are labeled with percentage bootstrap support values. Labels with 100% bootstrap support were removed from some nodes to improve visualization.

phyla Bacteriodetes, Actinobacteria, and Firmicutes, which is in line with the findings of other studies (Harris et al., 2006; Woodhams et al., 2007; Lam et al., 2010; Walke et al., 2011, 2015a; Flechas et al., 2012; Bell et al., 2013; Daskin et al., 2014; Becker et al., 2015; Holden et al., 2015; Madison et al., 2017). 16S rRNA gene sequences have been lodged in Genbank with accession numbers KJ191368–KJ191378, KJ191380–KJ191390, and MG491528–MG491661.

Analysis of OTU Diversity Across All Sites and Species and Upon Individual Frogs

Figure 3A summarizes the relationship between total number of cultured inhibitory isolates sequenced and expected number of totally inhibitory OTUs obtained for our data aggregated across all species and sites. It shows that our sampling effort is not ideal because we typically sampled less than 30 isolates from any individual (**Figure 3B**). However, as we often sampled the majority of colonies that grew on the plates and in all cases we sampled all morphologically different isolates present, it is a limitation of culturing studies in general that it is impossible to attain adequate sampling effort. **Figure 3B** summarizes our analysis of the relationship between the total number of isolates we found on each individual frog and the number of unique cultured totally inhibitory OTUs included in those isolates. It provides additional information as it includes bacterial isolates that were sampled from agar plates that were not inhibitory. However, as our sampling effort is less than ideal due to the nature of culturing studies, our OTU-based analyses and results should be considered as preliminary findings.

Number of Totally Inhibitory OTUs and *Bd* Infection Intensity

Quantile regression at the 90th percentile level demonstrated a preliminary significant negative relationship between the number of *Bd*-inhibitory OTUs present per frog and the upper limits of *Bd* infection intensity (**Figure 4**; n = 14, t = -2.818, pseudo-R^2 = 0.369, p = 0.0155). No frogs with highly intense *Bd* infections and high numbers of inhibitory bacteria were found during this study.

Preliminary Species and Site-Specific Analyses

Our PERMANOVA examining the effects of species and site on the composition of *Bd*-inhibitory cutaneous bacterial OTUs showed no significant interaction between species and site (pseudo-$F_{3,31}$ = 1.237, p = 0.221) and the effect of species was also not significant (pseudo-$F_{1,34}$ = 0.661, p = 0.721), however, the effect of site was significant (pseudo-$F_{3,34}$ = 1.780,

TABLE 2 | Taxonomic classification of 16S rRNA OTUs by frog species and site.

			Number of totally inhibitory isolates within each OTU by species and site										
			KU		KL			WU			WL		
Taxonomy/representative genus		Sequences	LS	LN	LS	LR	LN	LS	LR	LN	LS	LR	LN
Proteobacteria													
Gammaproteobacteria													
OTU1	*Pseudomonas*	69	11	14	2	3	6	4	9	2	7	10	1
OTU2	*Enterobacter*	35	14	13	2	–	1	–	–	1	3	1	–
OTU3	*Stenotrophomonas*	21	1	6	2	–	1	3	3	–	3	2	–
OTU6	*Acinetobacter*	7	2	5	–	–	–	–	–	–	–	–	+
OTU12	*Aeromonas*	2	–	–	–	–	2	–	–	–	–	–	–
OTU11	*Luteibacter*	1	–	–	–	–	–	–	–	–	1	–	–
Betaproteobacteria													
OTU5	*Chromobacterium*	4	–	–	1	–	2	–	–	–	–	1	–
OTU4	*Iodobacter*	6	–	–	–	–	2	–	1	2	–	1	–
OTU8	*Duganella*	3	–	3	–	–	–	–	–	–	–	–	–
OTU16	*Burkholderia*	1	–	1	–	–	–	–	–	–	–	–	–
Alphaproteobacteria													
OTU9	*Paenochrobactrum*	1	–	–	–	–	1	–	–	–	–	–	–
OTU15	*Novosphingobium*	1	–	–	–	1	–	–	–	–	–	–	–
Bacteroidetes													
Flavobacteriia													
OTU7	*Chryseobacterium*	2	–	1	–	–	–	–	–	1	–	–	–
Sphingobacteriia													
OTU14	*Sphingobacterium*	1	–	1	–	–	–	–	–	–	–	–	–
Actinobacteria													
OTU10	*Curtobacterium*	1	–	–	–	–	–	–	–	–	1	–	–
Firmicutes													
OTU13	*Bacillus*	1	–	–	–	–	–	–	–	–	–	–	1
		Total	28	44	7	4	15	7	13	6	15	15	2

Species are Litoria serrata (LS), L. nannotis (LN), and L. rheocola (LR). Sites are Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), and Wooroonooran lowland (WL).

$p = 0.024$). The multivariate dispersion of data using the Bray–Curtis distances used by the PERMANOVA was not significantly heterogeneous among sites (ANOVA; $F_{3,35} = 2.541$, $p = 0.072$). Inhibitory taxa present on *L. nannotis* and *L. serrata* from the Kirrama uplands clustered more tightly than those on frogs at any other site (**Figure 5**; *post hoc* Benjamini and Hochberg adjusted *p*-values: Kirrama upland – Kirrama lowland, $p = 0.024$: Kirrama upland – Wooroonooran upland, $p = 0.046$ and Kirrama upland – Wooroonooran lowland, $p = 0.046$, all other pairwise comparisons, $p > 0.05$).

The outcome of our GLM examining the number of *Bd*-inhibitory OTUs present on frogs as a Poisson-distributed response showed a significant interaction effect, indicating that site and species had effects that were not independent (ANOVA; $\chi^2 = 44.938$, df = 7, $p < 0.0001$). We therefore performed a *post hoc* analysis comparing 28 pairs of combinations of species and site (Tukey, 1949), using Benjamini and Hochberg adjusted *p*-values. This revealed that the differences among species by site were largely driven by *L. nannotis* at the Kirrama upland site. *L. nannotis* from the Kirrama upland site had significantly greater numbers of *Bd*-inhibitory bacterial OTUs than *L. nannotis* at all three other sites (for each site, $p < 0.05$) and significantly greater numbers of *Bd*-inhibitory bacterial OTUs than *L. serrata* at Kirrama lowland and upland sites ($p < 0.05$). There was also a suggestion of greater OTU numbers in *L. nannotis* from the Kirrama upland site compared with Wooroonooran upland *L. serrata* ($p = 0.061$). A full set of pairwise comparisons are provided as Supplementary Table 1. *Bd*-inhibitory members of the genera *Acinetobacter* and *Duganella* were unique to frogs at the Kirrama upland site (**Figure 6**).

As well as having significantly more *Bd*-inhibitory bacterial OTUs on *L. nannotis*, the Kirrama upland site also had a significantly higher proportion of frogs with one or more *Bd*-inhibitory OTUs than other sites (**Figure 7**; Fisher's Exact Test; $p = 0.0497$; *L. nannotis* and *L. serrata* only due to the absence of *L. rheocola* from the Kirrama upland site). When the Kirrama upland site was removed from the analyses, all other sites did not differ significantly from each other (Fisher's Exact Test; $p = 0.861$). The proportion of frogs with *Bd*-inhibitory OTUs did not differ significantly between species (Fisher's Exact Test; $p = 0.797$; *L. nannotis* and *L. serrata* only). This suggests that the site effect we detected was not a confounded effect of species.

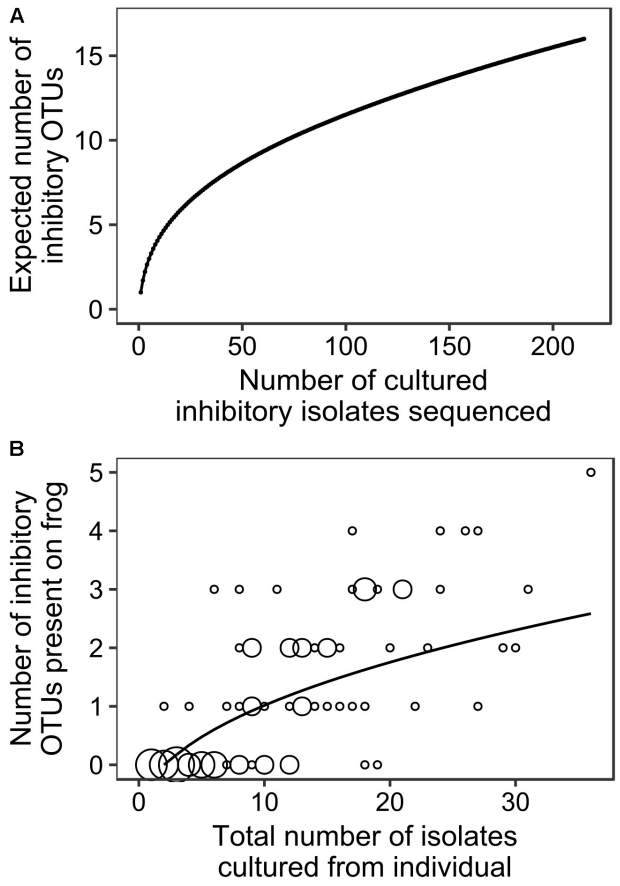

FIGURE 3 | The relationships between **(A)** the number of totally inhibitory bacterial isolates identified and sequenced and the expected number of totally inhibitory OTUs (EstimateS; Colwell, 2013), and **(B)** the total number of isolates obtained from each individual frog and the number of totally inhibitory OTUs detected. The size of circles corresponds to the number of frogs at each combination of number of isolates and number of OTUs, with the smallest indicating one individual and the largest indicating eight. The best fit accumulation curve is a power curve fitted to the number of OTUs plus 1 ($y = 0.717x^{0.449}$ with $R^2 = 0.505$, $p = 0.001$).

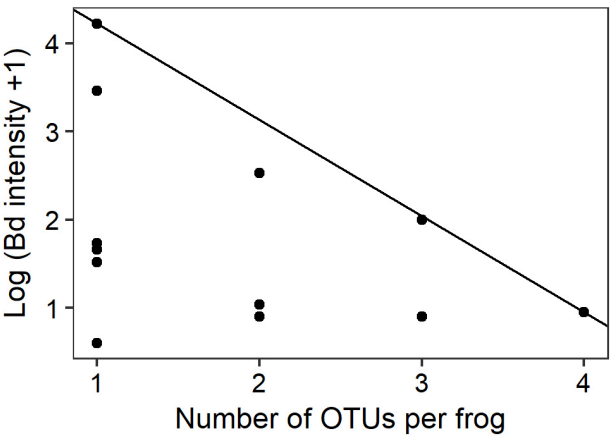

FIGURE 4 | Intensity of *Bd* infection decreased as the number of inhibitory OTUs increased. The ninetieth percentile regression line shows the negative relationship between the number of OTUs per infected frog and the upper limits of *Bd* infection intensity ($n = 14$, $t = -2.818$, $p = 0.0155$).

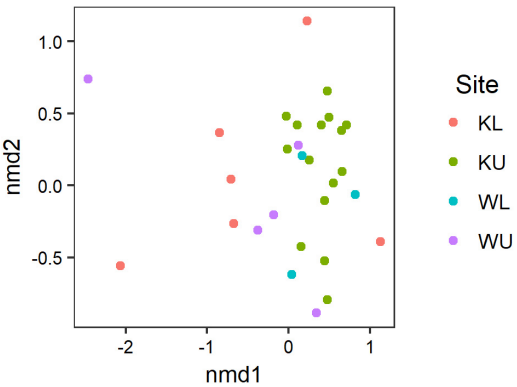

FIGURE 5 | Results of an nMDS illustrating how the OTU signatures of frogs cluster by site. Stress = 0.124. Sites are KL, Kirrama lowland; KU, Kirrama upland; WL, Wooroonooran lowland; WU, Wooroonooran upland.

DISCUSSION

We investigated the potential role that *Bd*-inhibitory cutaneous bacterial microbiota of Australian Wet Tropics frogs may have played in the reappearance of upland frog populations following population declines in the early 1990s and found that frogs from the Kirrama upland site differed from frogs at other sites in the presence and nature of their *Bd*-inhibitory bacteria. There was also a significant negative relationship between the upper limit of *Bd* infection intensity and the number of totally inhibitory bacterial genera on infected frogs. This suggests that greater inhibitory potential is linked to a reduction in the intensity of *Bd* infection, and that it therefore is possible that greater numbers of *Bd*-inhibitory bacterial genera may contribute to tolerance of *Bd* infection.

While our sampling effort was not ideal, it is often impossible for studies based on cultured isolates to achieve adequate sampling effort. For example, **Figure 3A** suggests that approximately 30 cultured totally inhibitory isolates per frog would be adequate. On average, our study found that 22% of the cultured isolates that we tested were totally inhibitory to *Bd*. Therefore, this would have required us to sample and test about 130 isolates from each frog/agar plate (more than 11,500 isolates from the 89 frogs in this study) in order to find 22% inhibitory. In almost all cases, there were considerably less colonies present on the agar plates than this and in many cases we sampled the majority of colonies that grew on the plate. In every case, we sampled all colony morphotypes present on the agar plates in an attempt to capture the diversity present. From our sequencing results, we often obtained identical sequences from individual frogs indicating that we probably over-sampled our agar plates, rather than under-sampled them. For these reasons, it is highly likely that we have captured the majority of cultured inhibitory OTUs present. The use of multiple culture media types may have

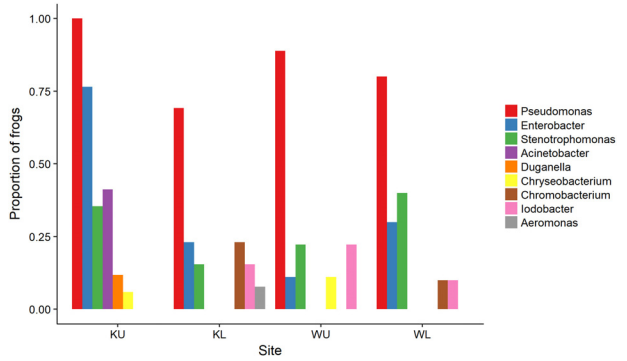

FIGURE 6 | Proportion of frogs with isolates in each OTU by site. For ease of visualization, OTUs that occurred on fewer than two frogs at each site have been excluded. KU, Kirrama upland; KL, Kirrama lowland; WU, Wooroonooran upland; WL, Wooroonooran lowland.

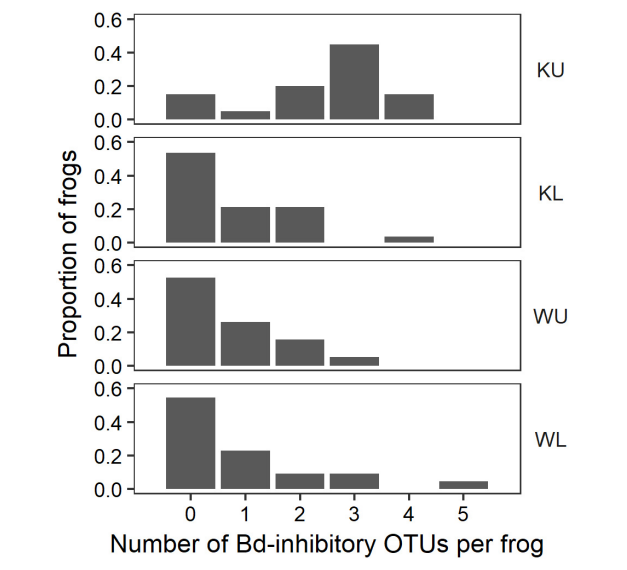

FIGURE 7 | The proportion of frogs with *Bd*-inhibitory OTUs for each field site. KU, Kirrama upland; KL, Kirrama lowland; WU, Wooroonooran upland; WL, Wooroonooran lowland.

resulted in the discovery of more OTUs, but it is likely that only a small fraction of all bacteria present on the frogs could ever be captured by culturing.

Many recent studies have used next generation sequencing techniques to investigate the diversity of amphibian cutaneous bacteria (reviewed by Jimenez and Sommer, 2017), but there is generally little evidence for a negative relationship between uncultured bacterial species richness and *Bd* infection load. However, uncultured proteobacteria phylotype richness was negatively correlated with *Bd* load in *E. coqui* frogs in Puerto Rico (Longo et al., 2015) and the relative abundance of certain taxa was negatively correlated with *Bd* infection load in *Rana sierrae* in California (Jani and Briggs, 2014). There are two possible reasons for negative relationships between *Bd* infection intensity and *Bd*-inhibitory OTU numbers. Frogs with naturally lower numbers of inhibitory bacterial taxa could be more susceptible to *Bd* or conversely, more intense *Bd* infection may cause a reduction in the numbers of inhibitory bacterial taxa.

Bd exposure can change the microbiome's composition (Jani and Briggs, 2014; Walke et al., 2015b; Bataille et al., 2016; Jani et al., 2017). Studies have demonstrated that some OTUs can respond positively or negatively to *Bd* infection (Jani and Briggs, 2014; Familiar López et al., 2017; Longo and Zamudio, 2017) and that higher microbiome richness is associated with host persistence against *Bd* (Jani et al., 2017). However, increasing *Bd* load has not been demonstrated to lead to decreases in overall bacterial diversity or in the diversity of *Bd*-inhibitory bacteria on hosts. Laboratory experiments *in vivo* are therefore needed to ascertain whether a progressing *Bd* infection can cause a reduction in the richness of symbiotic bacterial communities, especially those inhibitory to *Bd*.

In vitro challenge assays have shown that combinations of multiple cultured bacterial strains are more effective at inhibiting *Bd* than are individual strains alone through additive and synergistic effects (Loudon et al., 2014; Piovia-Scott et al., 2017). This provides strong supporting evidence to our observations that increased numbers of inhibitory genera are associated with reduced susceptibility to *Bd*. Similar additive and synergistic effects have led to stronger fungal inhibition in hydra (Fraune et al., 2015), and soil Pseudomonads (Jousset et al., 2014). Three principal *Bd*-inhibitory genera (*Pseudomonas*, *Enterobacter*, and *Stenotrophomonas*) were present at all of our sites. The diverse secondary metabolites that can be produced by these principal genera might perform complementary roles. For example, one compound may act on the zoospore cell membrane while another interferes with transcription of DNA and therefore cell replication (Ghannoum and Rice, 1999; Kathiravan et al., 2012). It therefore seems possible that frogs in our study with greater numbers of *Bd*-inhibitory genera had more synergistic interactions among bacterial community members, leading to the production of additional antimicrobial metabolites and a reduction in either or both the probability of infection by *Bd* and the upper limits of infection load if infected (**Figure 4**).

Ideally, laboratory inoculation-infection experiments are needed to determine whether artificially increasing the number of *Bd*-inhibitory bacterial taxa on frog skin alters susceptibility to, or intensity of *Bd* infections. Similar experiments have been conducted on both plants and locusts and have demonstrated reduced pathogen density when a greater number of bacterial symbionts was present (Dillon et al., 2005; Matos et al., 2005; Hol et al., 2015) indicating the protective effect of a more diverse microbiome.

Our results from the Kirrama upland site showed that frogs at this site differed from all other sites in a number of ways, and together, provide support for protection against *Bd* infection by increased number of *Bd*-inhibitory bacterial genera. Frogs at this site had a significantly lower prevalence of infection than those at the Wooroonooran upland site, a significantly higher proportion of frogs with one or more *Bd*-inhibitory OTUs than other sites (though this could in part

have been influenced by species effects), a higher proportion of inhibitory isolates than their conspecifics at other sites, significantly more *Bd*-inhibitory bacterial OTUs per frog in *L. nannotis* (suggesting increased synergistic anti-*Bd* potential), and statistically significant clustering of individual frogs with similar *Bd*-inhibitory taxonomic signatures (suggesting local selective pressure on skin microbiota). Some of the patterns we observed at the Kirrama upland site were clearly driven by *L. nannotis*. However, given that *L. serrata* only suffered temporary upland declines, it seems likely that the acquisition of greater numbers of *Bd*-inhibitory genera by *L. nannotis* at the Kirrama upland site may have facilitated their recolonization. A higher proportion of Kirrama upland frogs had *Bd*-inhibitory bacteria from the genera *Pseudomonas* and *Enterobacter* than at any other site, and the genera *Acinetobacter* and *Duganella* were unique to frogs from this site. Together, these differences suggest that Kirrama upland frogs, under pressure from enzootic *Bd* infection, have actively or passively acquired a greater number of *Bd*-inhibitory genera than frogs elsewhere, and that this may facilitate a reduction in the prevalence of *Bd* at this site.

All three species of frogs in this study have recolonized the Wooroonooran upland site despite having a lower proportion of *Bd*-inhibitory isolates than frogs at the Kirrama upland site and a lower proportion of frogs with *Bd*-inhibitory bacteria. Given the substantial genetic separation between frogs in the two regions (Schneider et al., 1998; Cunningham, 2001; Hoskin et al., 2005; Richards et al., 2010; Bell et al., 2012), it is likely that acquisition of resistance to *Bd* infection has evolved independently at the two upland sites. Therefore, factors other than symbiotic bacteria have probably played a part in aiding recolonization of the Wooroonooran upland site. These could include host immune defense, behavior, and environmental factors (Rollins-Smith et al., 2011; Savage and Zamudio, 2011; Rowley and Alford, 2013; Jani et al., 2017; McKnight et al., 2017; Voyles et al., 2018).

Bacteria that can inhibit the growth of *Bd* have received much attention in recent years, as it is possible that they could be cultured and used as probiotics to support at-risk populations of frogs through disease outbreaks (Becker et al., 2012; Bletz et al., 2013; Küng et al., 2014). Therefore the need to culture bacterial isolates to assess inhibitory potential against *Bd* remains essential to facilitate probiotic development, because it is not yet possible to accurately predict the functional capability of a community from 16S amplicon sequence data alone (Bletz et al., 2013; Becker et al., 2015). Fortunately, many of the more common OTUs described in 16S amplicon studies also appear to be culturable (Walke et al., 2015a) giving confidence that the functional capacity of the dominant community members was likely to be captured in our study. Future use of shotgun metagenomics, metatranscriptomics and metabolomics will enable insight into the functional capacity of bacterial communities without the need for culture (Rebollar et al., 2016) and help to elucidate the mechanisms by which individuals acquire and maintain communities of cutaneous bacteria that are inhibitory to *Bd*.

To our knowledge, we provide the first evidence that a greater number of *Bd*-inhibitory genera is correlated with lower *Bd* infection loads in wild frogs. This highlights the necessity for the creation of appropriate multiple-isolate combinations to promote potential synergistic interactions when developing probiotic candidates for supplementation of at-risk amphibian populations.

AUTHOR CONTRIBUTIONS

SB and RA designed the study, conducted the data analysis, and produced the first draft of the manuscript. SB completed the fieldwork. SB and SG processed samples in the laboratory. All authors edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01604/full#supplementary-material

REFERENCES

Bataille, A., Cashins, S. D., Grogan, L., Skerratt, L. F., Hunter, D., Mcfadden, M., et al. (2015). Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proc. R. Soc. B Biol. Sci.* 282:20143127. doi: 10.1098/rspb.2014.3127

Bataille, A., Lee-Cruz, L., Tripathi, B., Kim, H., and Waldman, B. (2016). Microbiome variation across amphibian skin regions: implications for chytridiomycosis mitigation efforts. *Microb. Ecol.* 71, 221–232. doi: 10.1007/s00248-015-0653-0

Becker, C. G., Greenspan, S. E., Tracy, K. E., Dash, J. A., Lambertini, C., Jenkinson, T. S., et al. (2017). Variation in phenotype and virulence among enzootic and panzootic amphibian chytrid lineages. *Fungal Ecol.* 26, 45–50. doi: 10.1016/j.funeco.2016.11.007

Becker, M. H., Brucker, R. M., Schwantes, C. R., Harris, R. N., and Minbiole, K. P. C. (2009). The bacterially produced metabolite violacein is associated with

survival of amphibians infected with a lethal fungus. *Appl. Environ. Microbiol.* 75, 6635–6638. doi: 10.1128/AEM.01294-09

Becker, M. H., Harris, R. N., Minbiole, K. P. C., Schwantes, C. R., Rollins-Smith, L. A., Reinert, L. K., et al. (2012). Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. *Ecohealth* 8, 501–506. doi: 10.1007/s10393-012-0743-0

Becker, M. H., Walke, J. B., Murrill, L., Woodhams, D. C., Reinert, L. K., Rollins-Smith, L. A., et al. (2015). Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol. Ecol.* 24, 1628–1641. doi: 10.1111/mec.13135

Bell, R. C., Mackenzie, J. B., Hickerson, M. J., Chavarria, K. L., Cunningham, M., Williams, S., et al. (2012). Comparative multi-locus phylogeography confirms multiple vicariance events in co-distributed rainforest frogs. *Proc. R. Soc. B Biol. Sci.* 279, 991–999. doi: 10.1098/rspb.2011.1229

Bell, S. C., Alford, R. A., Garland, S., Padilla, G., and Thomas, A. D. (2013). Screening bacterial metabolites for inhibitory effects against *Batrachochytrium dendrobatidis* using a spectrophotometric assay. *Dis. Aquat. Organ.* 103, 77–85. doi: 10.3354/dao02560

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate - A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B Methodol.* 57, 289–300.

Berger, L., Marantelli, G., Skerratt, L. L., and Speare, R. (2005). Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. *Dis. Aquat. Organ.* 68, 47–50. doi: 10.3354/dao068047

Berger, L., Speare, R., Daszak, P., Green, D. E., Cunningham, A. A., Goggin, C. L., et al. (1998). Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9031–9036. doi: 10.1073/pnas.95.15.9031

Berger, L., Speare, R., Hines, H. B., Marantelli, G., Hyatt, A. D., McDonald, K. R., et al. (2004). Effect of season and temperature on mortality in amphibians due to chytridiomycosis. *Aust. Vet. J.* 82, 434–439. doi: 10.1111/j.1751-0813.2004.tb11137.x

Berger, L., Speare, R., and Hyatt, A. (1999). "Chytrid fungi and amphibian declines: overview, implications and future directions," in *Declines and Disappearances of Australian Frogs*, ed. A. Campbell (Canberra: Biodiversity Group Environment Australia), 23–33.

Bletz, M. C., Loudon, A. H., Becker, M. H., Bell, S. C., Woodhams, D. C., Minbiole, K. P. C., et al. (2013). Mitigating amphibian chytridiomycosis with bioaugmentation: characteristics of effective probiotics and strategies for their selection and use. *Ecol. Lett.* 16, 807–820. doi: 10.1111/ele.12099

Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A., and Hyatt, A. D. (2004). Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Dis. Aquat. Organ.* 60, 141–148. doi: 10.3354/dao060141

Bray, J. R., and Curtis, J. T. (1957). An ordination of the upland forest communities of southern Wisconsin. *Ecol. Monogr.* 27, 325–349. doi: 10.2307/1942268

Cade, B. S., and Noon, B. R. (2003). A gentle introduction to quantile regression for ecologists. *Front. Ecol. Environ.* 1, 412–420. doi: 10.1890/1540-92952003001[0412:AGITQR]2.0.CO;2

Colwell, R. K. (2013). *EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples, Version 9.1.0*. Available at: http://purl.oclc.org/estimates

Cunningham, M. (2001). *Vicariance, Speciation and Diversity in Australopapuan Rainforest Frogs*. Ph.D. thesis, University of Queensland, Brisbane.

Currie, C. R., Poulsen, M., Mendenhall, J., Boomsma, J. J., and Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311, 81–83. doi: 10.1126/science.1119744

Currie, C. R., Scott, J. A., Summerbell, R. C., and Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398, 701–704. doi: 10.1073/pnas.0812082106

Daskin, J. H., Bell, S. C., Schwarzkopf, L., and Alford, R. A. (2014). Cool temperatures reduce antifungal activity of symbiotic bacteria of threatened amphibians - implications for disease management and patterns of decline. *PLoS One* 9:e100378. doi: 10.1371/journal.pone.0100378

Dillon, R. J., Vennard, C. T., Buckling, A., and Charnley, A. K. (2005). Diversity of locust gut bacteria protects against pathogen invasion. *Ecol. Lett.* 8, 1291–1298. doi: 10.1111/j.1461-0248.2005.00828.x

Drummond, A. J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., et al. (2010). *Geneious v5.3. Biomatters Ltd.* Available at: http://www.geneious.com

Familiar López, M., Rebollar, E. A., Harris, R. N., Vredenburg, V. T., and Hero, J. M. (2017). Temporal variation of the skin bacterial community and *Batrachochytrium dendrobatidis* infection in the terrestrial cryptic frog *Philoria loveridgei*. *Front. Microbiol.* 8:2535. doi: 10.3389/fmicb.2017.02535

Farrer, R. A., Weinert, L. A., Bielby, J., Garner, T. W. J., Balloux, F., Clare, F., et al. (2011). Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18732–18736. doi: 10.1073/pnas.1111915108

Fites, J. S., Ramsey, J. P., and Rollins-Smith, L. A. (2012). Paralysis of amphibian lymphocyte functions by products of the chytrid fungus, *Batrachocytrium dendrobatidis*. *Integr. Comp. Biol.* 52:E58.

Fites, J. S., Reinert, L. K., Chappell, T. M., and Rollins-Smith, L. A. (2014). Inhibition of local immune responses by the frog-killing fungus *Batrachochytrium dendrobatidis*. *Infect. Immun.* 82, 4698–4706. doi: 10.1128/IAI.02231-14

Flechas, S. V., Sarmiento, C., Cardenas, M. E., Medina, E. M., Restrepo, S., and Amezquita, A. (2012). Surviving chytridiomycosis: differential anti-*Batrachochytrium dendrobatidis* activity in bacterial isolates from three lowland species of Atelopus. *PLoS One* 7:e44832. doi: 10.1371/journal.pone.0044832

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S., et al. (2017). *Companion to Applied Regression, Package 'car'. Version 2.1-6*. Available at: https://cran.r-project.org/web/packages/car/car.pdf

Fraune, S., Anton-Erxleben, F., Augustin, R., Franzenburg, S., Knop, M., Schröder, K., et al. (2015). Bacteria–bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance. *ISME J.* 9, 1543–1556. doi: 10.1038/ismej.2014.239

Garland, S., Baker, A., Phillott, A. D., and Skerratt, L. F. (2010). BSA reduces inhibition in a TaqMan (R) assay for the detection of *Batrachochytrium dendrobatidis*. *Dis. Aquat. Organ.* 92, 113–116. doi: 10.3354/dao02053

Ghannoum, M. A., and Rice, L. B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* 12, 501–517.

Gutierrez-Garcia, K., Neira-González, A., Pérez-Gutiérrez, R. M., Granados-Ramírez, G., Zarraga, R., Wrobel, K., et al. (2017). Phylogenomics of 2,4-diacetylphloroglucinol-producing *Pseudomonas* and novel antiglycation endophytes from *Piper auritum*. *J. Nat. Prod.* 80, 1955–1963. doi: 10.1021/acs.jnatprod.6b00823

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319. doi: 10.1038/nrmicro1129

Harris, R. N., Brucker, R. M., Walke, J. B., Becker, M. H., Schwantes, C. R., Flaherty, D. C., et al. (2009a). Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J.* 3, 818–824. doi: 10.1038/ismej.2009.27

Harris, R. N., James, T. Y., Lauer, A., Simon, M. A., and Patel, A. (2006). Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of Amphibian species. *Ecohealth* 3, 53–56. doi: 10.1007/s10393-005-0009-1

Harris, R. N., Lauer, A., Simon, M. A., Banning, J. L., and Alford, R. A. (2009b). Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. *Dis. Aquat. Organ.* 83, 11–16. doi: 10.3354/dao02004

Hol, W. H. G., Garbeva, P., Hordijk, C., Hundscheid, M. P. J., Gunnewiek, P., Van Agtmaal, M., et al. (2015). Non-random species loss in bacterial communities reduces antifungal volatile production. *Ecology* 96, 2042–2048. doi: 10.1890/14-2359.1

Holden, W. M., Hanlon, S. M., Woodhams, D. C., Chappell, T. M., Wells, H. L., Glisson, S. M., et al. (2015). Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (*Rana sphenocephala*) against the frog-killing fungus. *Batrachochytrium dendrobatidis. Biological Conservation* 187, 91–102. doi: 10.1016/j.biocon.2015.04.007

Hoskin, C. J., Higgie, M., McDonald, K. R., and Moritz, C. (2005). Reinforcement drives rapid allopatric speciation. *Nature* 437, 1353–1356. doi: 10.1038/nature04004

Hothorn, T., Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A., and Scheibe, S. (2017). *Silmultaneous Inference in General Parametric Models Package 'multcomp'. Version 2.4-5*. Available at: https://cran.r-project.org/web/packages/multcomp/multcomp.pdf

Hu, J., Wei, Z., Friman, V. P., Gu, S. H., Wang, X. F., Eisenhauer, N., et al. (2016). Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *mBio* 7:e01790-16. doi: 10.1128/mBio.01790-16

Jani, A. J., and Briggs, C. J. (2014). The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5049–E5058. doi: 10.1073/pnas.1412752111

Jani, A. J., Knapp, R. A., and Briggs, C. J. (2017). Epidemic and endemic pathogen dynamics correspond to distinct host population microbiomes at a landscape scale. *Proc. R. Soc. B Biol. Sci.* 284:20170944. doi: 10.1098/rspb.2017.0944

Jimenez, R. R., and Sommer, S. (2017). The amphibian microbiome: natural range of variation, pathogenic dysbiosis, and role in conservation. *Biodivers. Conserv.* 26, 763–786. doi: 10.1007/s10531-016-1272-x

Jousset, A., Becker, J., Chatterjee, S., Karlovsky, P., Scheu, S., and Eisenhauer, N. (2014). Biodiversity and species identity shape the antifungal activity of bacterial communities. *Ecology* 95, 1184–1190. doi: 10.1890/13-1215.1

Kathiravan, M. K., Salake, A. B., Chothe, A. S., Dudhe, P. B., Watode, R. P., Mukta, M. S., et al. (2012). The biology and chemistry of antifungal agents: a review. *Bioorg. Med. Chem.* 20, 5678–5698. doi: 10.1016/j.ymeth.2016.11.014

King, K. C., Brockhurst, M. A., Vasieva, O., Paterson, S., Betts, A., Ford, S. A., et al. (2016). Rapid evolution of microbe-mediated protection against pathogens in a worm host. *ISME J.* 10, 1915–1924. doi: 10.1038/ismej.2015.259

Koenker, R., and Machado, J. A. F. (1999). Goodness of fit and related inference processes for quantile regression. *J. Am. Stat. Assoc.* 94, 1296–1310. doi: 10.1080/01621459.1999.10473882

Koenker, R., Portnoy, S., Ng, P. T., Zeileis, A., Grosjean, P., and Ripley, B. D. (2017). *Quantile Regression Package 'quantreg'. Version 5.33*. Available at: https://cran.r-project.org/web/packages/quantreg/quantreg.pdf

Kriger, K. M., and Hero, J. M. (2007). Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *J. Zool.* 271, 352–359.

Kroiss, J., Kaltenpoth, M., Schneider, B., Schwinger, M.-G., Hertweck, C., Maddula, R. K., et al. (2010). Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nat. Chem. Biol.* 6, 261–263. doi: 10.1038/nchembio.331

Kueneman, J. G., Woodhams, D. C., Van Treuren, W., Archer, H. M., Knight, R., and Mckenzie, V. J. (2016). Inhibitory bacteria reduce fungi on early life stages of endangered Colorado boreal toads (*Anaxyrus boreas*). *ISME J.* 10, 934–944. doi: 10.1038/ismej.2015.168

Küng, D., Bigler, L., Davis, L. R., Gratwicke, B., Griffith, E., and Woodhams, D. C. (2014). Stability of microbiota facilitated by host immune regulation: informing probiotic strategies to manage amphibian disease. *PLoS One* 9:e87101. doi: 10.1371/journal.pone.0087101

Lam, B. A., Walke, J. B., Vredenburg, V. T., and Harris, R. N. (2010). Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. *Biol. Conserv.* 143, 529–531. doi: 10.1016/j.biocon.2009.11.015

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt and M. Goodfellow (Chichester: John Wiley & Sons), 115–175.

Lauer, A., Simon, M. A., Banning, J. L., Andre, E., Duncan, K., and Harris, R. N. (2007). Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 3, 630–640. doi: 10.1643/0045-8511(2007)2007[630:CCBFTE]2.0.CO;2

Laurance, W. F., McDonald, K. R., and Speare, R. (1996). Epidemic disease and the catastrophic decline of Australian rain forest frogs. *Conserv. Biol.* 10, 406–413. doi: 10.1046/j.1523-1739.1996.10020406.x

Longo, A. V., Savage, A. E., Hewson, I., and Zamudio, K. R. (2015). Seasonal and ontogenetic variation of skin microbial communities and relationships to natural disease dynamics in declining amphibians. *R. Soc. Open Sci.* 2:140377. doi: 10.1098/rsos.140377

Longo, A. V., and Zamudio, K. R. (2017). Environmental fluctuations and host skin bacteria shift survival advantage between frogs and their fungal pathogen. *ISME J.* 11, 349–361. doi: 10.1038/ismej.2016.138

Loudon, A. H., Holland, J. A., Umile, T. P., Burzynski, E. A., Minbiole, K. P., and Harris, R. N. (2014). Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Front. Microbiol.* 5:441. doi: 10.3389/fmicb.2014.00441

Madison, J. D., Berg, E. A., Abarca, J. G., Whitfield, S. M., Gorbatenko, O., Pinto, A., et al. (2017). Characterization of *Batrachochytrium dendrobatidis* inhibiting bacteria from amphibian populations in Costa Rica. *Front. Microbiol.* 8:290. doi: 10.3389/fmicb.2017.00290

Mao-Jones, J., Ritchie, K. B., Jones, L. E., and Ellner, S. P. (2010). How microbial community composition regulates coral disease development. *PLoS Biol.* 8:e1000345. doi: 10.1371/journal.pbio.1000345

Martinez Arbizu, P. (2016). *"How can I do perMANOVA Pairwise Contrasts in R? Response to the Question Posted*. Available at: www.researchgate.net

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J. M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M., et al. (2010). Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proc. R. Soc. B Biol. Sci.* 277, 123–130. doi: 10.1098/rspb.2009.1377

Matos, A., Kerkhof, L., and Garland, J. L. (2005). Effects of microbial community diversity on the survival of *Pseudomonas aeruginosa* in the wheat rhizosphere. *Microb. Ecol.* 49, 257–264. doi: 10.1007/s00248-004-0179-3

Mattoso, T. C., Moreira, D. D., and Samuels, R. I. (2012). Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus* subterraneus protect workers from attack by entomopathogenic fungi. *Biol. Lett.* 8, 461–464. doi: 10.1098/rsbl.2011.0963

McDonald, K., and Alford, R. (1999). "A review of Declining Frogs in Northern Queensland," in *Declines and Disappearances of Australian Frogs*, ed. A. Campbell (Canberra: Biodiversity Group Environment Australia), 14–22.

McDonald, K. R., Méndez, D., Müller, R., Freeman, A. B., and Speare, R. (2005). Decline in the prevalence of chytridiomycosis in upland frog populations in North Queensland, Australia. *Pac. Conserv. Biol.* 11, 114–129. doi: 10.1071/PC050114

McKnight, D. T., Alford, R. A., Hoskin, C. J., Schwarzkopf, L., Greenspan, S. E., Zenger, K. R., et al. (2017). Fighting an uphill battle: the recovery of frogs in Australia's Wet Tropics. *Ecology* 98, 3221–3223. doi: 10.1002/ecy.2019

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., et al. (2017). *Community Ecology Package 'vegan'. Version 2.4-4*. Available at: https://cran.r-project.org/web/packages/vegan/vegan.pdf

Piovia-Scott, J., Rejmanek, D., Woodhams, D. C., Worth, S. J., Kenny, H., Mckenzie, V., et al. (2017). Greater species richness of bacterial skin symbionts better suppresses the amphibian fungal pathogen *Batrachochytrium dendrobatidis*. *Microb. Ecol.* 74, 217–226. doi: 10.1007/s00248-016-0916-4

Poulsen, M., Cafaro, M. J., Erhardt, D. P., Little, A. E., Gerardo, N. M., Tebbets, B., et al. (2010). Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. *Environ. Microbiol. Rep.* 2, 534–540. doi: 10.1111/j.1758-2229.2009.00098.x

Pruesse, E., Peplies, J., and Glöckner, F. O. (2012). SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28, 1823–1829. doi: 10.1093/bioinformatics/bts252

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. doi: 10.1093/nar/gks1219

Rambaut, A. (2016). *FigTree. Version 1.4.3*. Available at: http://tree.bio.ed.ac.uk/software/figtree/

Rebollar, E. A., Antwis, R. E., Becker, M. H., Belden, L. K., Bletz, M. C., Brucker, R. M., et al. (2016). Using "omics" and integrated multi-omics approaches to guide probiotic selection to mitigate chytridiomycosis and other emerging infectious diseases. *Front. Microbiol.* 7:68. doi: 10.3389/fmicb.2016.00068

Richards, S. J., and Alford, R. A. (2005). Structure and dynamics of a rainforest frog (Litoria genimaculata) population in northern Queensland. *Aust. J. Zool.* 53, 229–236. doi: 10.1071/ZO03036

Richards, S. J., Hoskin, C. J., Cunningham, M. J., McDonald, K., and Donnellan, S. C. (2010). Taxonomic re-assessment of the Australian and New Guinean green-eyed treefrogs *Litoria eucnemis*, *L. genimaculata* and *L. serrata* (Anura: Hylidae). *Zootaxa* 2391, 33–46.

Richards, S. J., McDonald, K. R., and Alford, R. A. (1993). Declines in populations of Australia's endemic tropical rainforest frogs. *Pac. Conserv. Biol.* 1, 66–77. doi: 10.1071/PC930066

Ritchie, K. B. (2006). Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar. Ecol. Prog. Ser.* 322, 1–14. doi: 10.3354/meps322001

Robinson, J. V., and Valentine, W. D. (1979). The concepts of elasticity, invulnerability and invadability. *J. Theor. Biol.* 81, 91–104. doi: 10.1016/0022-5193(79)90083-3

Rollins-Smith, L. A., Carey, C., Longcore, J. E., Doersam, J. K., Reinert, L. K., Boutte, A., et al. (2002). Antimicrobial peptide defenses against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. *FASEB J.* 16, A291–A291.

Rollins-Smith, L. A., Ramsey, J. P., Pask, J. D., Reinert, L. K., and Woodhams, D. C. (2011). Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integr. Comp. Biol.* 51, 552–562. doi: 10.1093/icb/icr095

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., and Zilber-Rosenberg, I. (2007). The role of microorganisms in coral health, disease and evolution. *Nat. Rev. Microbiol.* 5, 355–362. doi: 10.1038/nrmicro1635

Rosenberg, E., and Zilber-Rosenberg, I. (2016). Microbes drive evolution of animals and plants: the hologenome concept. *mBio* 7:e01395-15. doi: 10.1128/mBio.01395-15

Rowley, J. J. L., and Alford, R. A. (2013). Hot bodies protect amphibians against chytrid infection in nature. *Sci. Rep.* 3:1515. doi: 10.1038/srep01515

Salle, A. J. (1961). *Laboratory Manual on Fundamental Principles of Bacteriology*. London: McGraw-Hill.

Sapsford, S. J., Alford, R. A., and Schwarzkopf, L. (2013). Elevation, temperature, and aquatic connectivity all influence the infection dynamics of the amphibian chytrid fungus in adult frogs. *PLoS One* 8:e82425. doi: 10.1371/journal.pone.0082425

Savage, A. E., and Zamudio, K. R. (2011). MHC genotypes associate with resistance to a frog-killing fungus. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16705–16710. doi: 10.1073/pnas.1106893108

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schneider, C. J., Cunningham, M., and Moritz, C. (1998). Comparative phylogeography and the history of endemic vertebrates in the Wet Tropics rainforests of Australia. *Mol. Ecol.* 7, 487–498. doi: 10.1016/j.ympev.2011.10.010

Scott, J. J., Oh, D. C., Yuceer, M. C., Klepzig, K. D., Clardy, J., and Currie, C. R. (2008). Bacterial protection of beetle-fungus mutualism. *Science* 322:63. doi: 10.1126/science.1160423

Skerratt, L. F., Berger, L., Speare, R., Cashins, S., McDonald, K. R., Phillott, A. D., et al. (2007). Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* 4, 125–134. doi: 10.1007/s10393-007-0093-5

Skerratt, L. F., McDonald, K. R., Hines, H. B., Berger, L., Mendez, D., Phillott, A. D., et al. (2010). Application of the survey protocol for chytridiomycosis to Queensland, Australia. *Dis. Aquat. Organ.* 92, 117–129. doi: 10.3354/dao02272

Soler, J. J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A. M., Martínez-Bueno, M., et al. (2008). Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. *Funct. Ecol.* 22, 864–871. doi: 10.1111/j.1365-2435.2008.01448.x

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L., et al. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783–1786. doi: 10.1126/science.1103538

Tukey, J. (1949). Comparing individual means in the analysis of variance. *Biometrics* 5, 99–114. doi: 10.2307/3001913

van Elsas, J. D., Chiurazzi, M., Mallon, C. A., Elhottovā, D., Krištůfek, V., and Salles, J. F. (2012). Microbial diversity determines the invasion of soil by a bacterial pathogen. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1159–1164. doi: 10.1073/pnas.1109326109

Voyles, J., Woodhams, D. C., Saenz, V., Byrne, A. Q., Perez, R., Rios-Sotelo, G., et al. (2018). Shifts in disease dynamics in a tropical amphibian assemblage are not due to pathogen attenuation. *Science* 359, 1517–1519. doi: 10.1126/science.aao4806

Wake, D. B., and Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11466–11473. doi: 10.1073/pnas.0801921105

Walke, J. B., Becker, M. H., Hughey, M. C., Swartwout, M. C., Jensen, R. V., and Belden, L. K. (2015a). Most of the dominant members of amphibian skin bacterial communities can be readily cultured. *Appl. Environ. Microbiol.* 81, 6589–6600. doi: 10.1128/AEM.01486-15

Walke, J. B., Becker, M. H., Loftus, S. C., House, L. L., Teotonio, T. L., Minbiole, K. P., et al. (2015b). Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLoS One* 10:e0139848. doi: 10.1371/journal.pone.0139848

Walke, J. B., Harris, R. N., Reinert, L. K., Rollins-Smith, L. A., and Woodhams, D. C. (2011). Social immunity in amphibians: evidence for vertical transmission of innate defenses. *Biotropica* 43, 396–400. doi: 10.1111/j.1744-7429.2011.00787.x

Wickham, H., and Chang, W. (2016). *Create Elegant Data Visualisations Using the Grammar of Graphics Package 'ggplot2'. Version 2.2.1*. Available at: https://cran.r-project.org/web/packages/ggplot2/ggplot2.pdf

Woodhams, D. C., and Alford, R. A. (2005). Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. *Conserv. Biol.* 19, 1449–1459. doi: 10.1111/j.1523-1739.2005.004403.x

Woodhams, D. C., Brandt, H., Baumgartner, S., Kielgast, J., Kupfer, E., Tobler, U., et al. (2014). Interacting symbionts and immunity in the amphibian skin mucosome predict disease risk and probiotic effectiveness. *PLoS One* 9:e96375. doi: 10.1371/journal.pone.0096375

Woodhams, D. C., Kenyon, N., Bell, S. C., Alford, R. A., Chen, S., Billheimer, D., et al. (2010). Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata*. *Divers. Distrib.* 16, 703–712. doi: 10.1111/j.1472-4642.2010.00666.x

Woodhams, D. C., Vredenburg, V. T., Simon, M. A., Billheimer, D., Shakhtour, B., Shyr, Y., et al. (2007). Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138, 390–398. doi: 10.1016/j.biocon.2007.05.004

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., et al. (2014). The SILVA and "All-species living tree project (LTP)" taxonomic frameworks. *Nucleic Acids Res.* 42, D643–D648. doi: 10.1093/nar/gkt1209

Zhang, X.-Y., He, F., Wang, G.-H., Bao, J., Xu, X.-Y., and Qi, S.-H. (2013). Diversity and antibacterial activity of culturable actinobacteria isolated from five species of the South China Sea gorgonian corals. *World J. Microbiol. Biotechnol.* 29, 1107–1116. doi: 10.1007/s11274-013-1279-3

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