Using "Omics" and Integrated Multi-Omics Approaches to Guide Probiotic Selection to Mitigate Chytridiomycosis and Other Emerging Infectious Diseases

Eria A. Rebollar[1]*, Rachael E. Antwis[2,3,4], Matthew H. Becker[5], Lisa K. Belden[6], Molly C. Bletz[7], Robert M. Brucker[8], Xavier A. Harrison[3], Myra C. Hughey[6], Jordan G. Kueneman[9], Andrew H. Loudon[10], Valerie McKenzie[9], Daniel Medina[6], Kevin P. C. Minbiole[11], Louise A. Rollins-Smith[12], Jenifer B. Walke[6], Sophie Weiss[13], Douglas C. Woodhams[14] and Reid N. Harris[1]

[1] Department of Biology, James Madison University, Harrisonburg, VA, USA, [2] Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa, [3] Institute of Zoology, Zoological Society of London, London, UK, [4] School of Environment and Life Sciences, University of Salford, Salford, UK, [5] Center for Conservation and Evolutionary Genetics, Smithsonian Conservation Biology Institute, National Zoological Park, Washington, DC, USA, [6] Department of Biological Sciences, Virginia Tech, Blacksburg, VA, USA, [7] Zoological Institute, Technische Universität Braunschweig, Braunschweig, Germany, [8] Rowland Institute, Harvard University, Cambridge, MA, USA, [9] Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA, [10] Department of Zoology, Biodiversity Research Centre, University of British Columbia, Vancouver, BC, Canada, [11] Department of Chemistry, Villanova University, Villanova, PA, USA, [12] Department of Pathology, Microbiology and Immunology and Department of Pediatrics, Vanderbilt University School of Medicine, Department of Biological Sciences, Vanderbilt University, Nashville, TN, USA, [13] Department of Chemical and Biological Engineering, University of Colorado at Boulder, Boulder, CO, USA, [14] Department of Biology, University of Massachusetts Boston, Boston, MA, USA

Emerging infectious diseases in wildlife are responsible for massive population declines. In amphibians, chytridiomycosis caused by *Batrachochytrium dendrobatidis*, *Bd*, has severely affected many amphibian populations and species around the world. One promising management strategy is probiotic bioaugmentation of antifungal bacteria on amphibian skin. *In vivo* experimental trials using bioaugmentation strategies have had mixed results, and therefore a more informed strategy is needed to select successful probiotic candidates. Metagenomic, transcriptomic, and metabolomic methods, colloquially called "omics," are approaches that can better inform probiotic selection and optimize selection protocols. The integration of multiple omic data using bioinformatic and statistical tools and *in silico* models that link bacterial community structure with bacterial defensive function can allow the identification of species involved in pathogen inhibition. We recommend using 16S rRNA gene amplicon sequencing and methods such as indicator species analysis, the Kolmogorov–Smirnov Measure, and co-occurrence networks to identify bacteria that are associated with pathogen resistance in field surveys and experimental trials. In addition to 16S amplicon sequencing, we recommend approaches that give insight into symbiont function such as shotgun metagenomics, metatranscriptomics, or metabolomics to maximize the probability of finding effective probiotic candidates, which can then be isolated in culture and tested in persistence and clinical trials. An effective mitigation strategy to ameliorate chytridiomycosis and other emerging infectious diseases is necessary; the advancement of omic methods and the integration of multiple omic data provide a promising avenue toward conservation of imperiled species.

Keywords: probiotics, emerging diseases, metagenomics, transcriptomics, metabolomics, amphibians

INTRODUCTION

Emerging infectious diseases (EIDs) in wildlife pose a grave threat to biodiversity (Wake and Vredenburg, 2008; Blehert et al., 2009; Fisher et al., 2009, 2012; Price et al., 2014; Schrope, 2014). Examples of EIDs caused by fungal pathogens include white-nose syndrome in bats (Blehert et al., 2009) and chytridiomycosis in amphibians (Berger et al., 1998). The latter, caused by *Batrachochytrium dendrobatidis* (*Bd*), is considered the greatest disease threat to biodiversity at the current time (Wake and Vredenburg, 2008). Recently, a newly described chytrid fungal species, *B. salamandrivorans* (*Bsal*) has been identified as the causal agent of chytridiomycosis in salamanders and is causing many salamander populations declines in Europe (Martel et al., 2013, 2014; Yap et al., 2015). Several strategies have been proposed to contend against EIDs in amphibians (Fisher et al., 2012; Woodhams et al., 2012; McMahon et al., 2014; Langwig et al., 2015) including vaccination, selective breeding and the use of probiotic bioaugmentation (Harris et al., 2006, 2009; Woodhams et al., 2007; Stice and Briggs, 2010; McMahon et al., 2014; Hoyt et al., 2015). Successful implementation of the later approach for the conservation of wild populations will benefit from further laboratory and field-testing, particularly when informed by integrated multi-omics methods.

There is growing evidence that probiotic therapy in particular could be a promising approach to mitigating disease in a variety of organisms, including human, plant crop, and wildlife systems (Harris et al., 2009; Sánchez et al., 2013; Akhter et al., 2015; Forster and Lawley, 2015; Hoyt et al., 2015; Papadimitriou et al., 2015). A relevant case comes from studies on plant-microbial interactions, which have identified several bacterial taxa involved in protection of plant crops against pathogens and extreme environmental stressors as well as in nutrient availability (Berlec, 2012). Some of these microorganisms have been widely and successfully used as bio-fertilizers or bio-controls in plant agriculture (Bhardwaj et al., 2014; Lakshmanan et al., 2014). In amphibians, the approach that is currently being developed is probiotic bioaugmentation, which is the establishment and augmentation of protective microbes that are already naturally occurring on at least some individuals in a population or community (Bletz et al., 2013). Bioaugmentation has prevented morbidity and mortality otherwise caused by *Bd* during laboratory-based and field-based trials for some amphibian species (Harris et al., 2006, 2009, Bletz et al., 2013). However, application of probiotics has been ineffective in other amphibian species (Becker et al., 2011, 2015a; Küng et al., 2014). The mixed success of probiotics could in part be caused by the selection of ineffective probiotic candidates because knowledge about the diversity of the microbiota and the ecological interactions occurring within these communities was lacking. For example, initial "training" of the immune system by early symbiotic colonists during development and priority effects of the microbial community, may exert strong influences on community resilience and colonization potential of probiotics (Reid et al., 2011; Hawkes and Keitt, 2015).

In an effort to improve the chances of a positive outcome from the use of amphibian probiotics, a protocol that filters out ineffective candidates has been proposed (Bletz et al., 2013). This method was designed to identify successful probiotics for disease mitigation and species survival based on culture-dependent data. In addition to the Bletz et al. (2013) filtering protocol, a mucosome assay, which aims to measure the protective function of the skin mucus, has recently been developed and applied to test potential probiotics (Woodhams et al., 2014).

As new technologies and methods are being developed, it is desirable to further improve the filtering protocol with additional methods that can be used to facilitate probiotic candidate selection to increase the likelihood of success. In particular, high-throughput molecular techniques, colloquially called "omics" methods, have greatly increased our ability to characterize the taxonomic and genetic structure of bacterial communities, to estimate their functional capabilities and to evaluate their responses to stressors or pathogens (Grice and Segre, 2011; Fierer et al., 2012; Greenblum et al., 2012; Knief et al., 2012; Jorth et al., 2014). Some of the omics methods developed to date are gene amplicon sequencing, shotgun metagenomics, transcriptomics, proteomics, and metabolomics. Several studies and extensive reviews on these high-throughput molecular methods can be found in the literature (Fiehn, 2002; Dettmer et al., 2007; Caporaso et al., 2011, 2012; Stewart et al., 2011; Altelaar et al., 2012; McGettigan, 2013; Franzosa et al., 2015; Loman and Pallen, 2015). Moreover, integrated multi-omics, which we define as the integrative analysis of data obtained from multiple omic methods, has the potential to greatly advance our understanding of ecological interactions occurring in microbial communities (Borenstein, 2012; McHardy et al., 2013; Meng et al., 2014). In this review, we establish an omics and integrated multi-omics framework with the aim of increasing the chances of selecting effective probiotic bacteria and achieving a successful disease mitigation strategy against EIDs. While these principles are applicable to other biological systems, for example in humans (Sánchez et al., 2013; Buffie et al., 2015; Forster and Lawley, 2015), we focus on applying these principles to the amphibian system, emphasizing current omics methods that have been explored in amphibians such as 16S rRNA gene amplicon sequencing (hereafter 16S amplicon sequencing), shotgun metagenomics, transcriptomics, and metabolomics. However, other omics methods such as proteomics could be relevant in future studies to understand the interactions between hosts, pathogens and host-associated microbial communities.
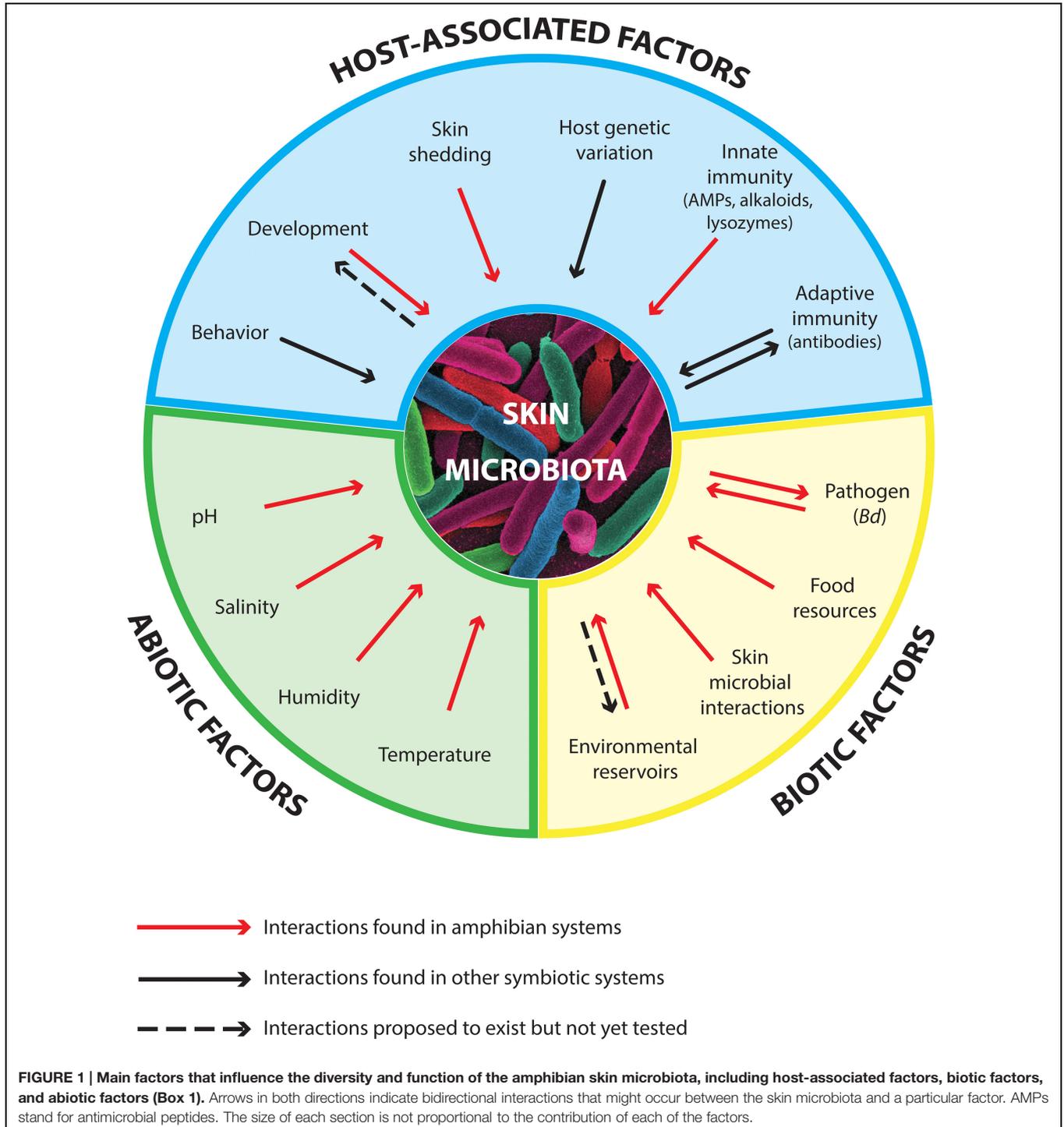
In this review, we will (1) provide relevant knowledge about the skin microbiome in amphibians; (2) proceed with a description of the omics and integrated multi-omics methods that have been or could be applied to the amphibian system; (3) describe how omics and integrated multi-omics approaches can be incorporated into a previously described filtering protocol to identify probiotic candidates (Bletz et al., 2013); (4) provide important considerations and future directions that are relevant to the success of probiotic selection supported by multi-omics data. It is important to note that the omics methods as well as the statistical, modeling and integrative methods mentioned in this review are a subset of the current methods available and additional methods can also be used to identify successful probiotic candidates.

ECOLOGY OF THE AMPHIBIAN SKIN MICROBIOME

The amphibian skin microbiome is defined as the microbiota and its combined genetic material present on the skin. Determining the main drivers of the assembly of the skin microbiome through the use of omic methods and culture-dependent approaches

may greatly enhance our ability to develop successful probiotic treatments and prevent amphibian population declines caused by chytridiomycosis.

The amphibian skin microbiome is determined by the microbiota's interactions with host-associated factors and with abiotic and biotic factors (**Figure 1**, Box 1). Host-associated factors include host genetic diversity and the adaptive and

FIGURE 1 | Main factors that influence the diversity and function of the amphibian skin microbiota, including host-associated factors, biotic factors, and abiotic factors (Box 1). Arrows in both directions indicate bidirectional interactions that might occur between the skin microbiota and a particular factor. AMPs stand for antimicrobial peptides. The size of each section is not proportional to the contribution of each of the factors.

innate immune systems, in addition to host behavior, ecology and development. Biotic factors include ecological interactions between skin symbiotic microbes and the microbial composition of environmental reservoirs, and abiotic factors include environmental conditions such as temperature and humidity. Altogether, the factors that influence the skin microbiome of amphibians determine the chemical composition of the skin mucus and in turn help determine the degree of host susceptibility against pathogens (Searle et al., 2011; Woodhams et al., 2014). Box 1 summarizes the current state of knowledge on the drivers of the amphibian skin microbiome. We now focus on omics and integrative multi-omics methods and how they can be used to address knowledge gaps that are key to developing effective probiotic strategies.

OMICS METHODS TO IDENTIFY PROBIOTIC CANDIDATES

An important first step toward the identification of potential probiotics in amphibians is to determine differences in the structure and function of skin microbial communities in the presence or absence of *Bd*. This approach includes comparing diseased and not diseased individuals after exposure to *Bd* in laboratory trials, as well as examining *Bd*-tolerant or resistant species from localities that have experienced population declines. The assumption is that individuals or species that persist in the presence of *Bd* might harbor protective bacteria that allowed them to survive. Such studies can be done through 16S amplicon sequencing of the whole microbial community (Caporaso et al., 2012). Furthermore, in order to identify key bacterial species responsible for pathogen protection it will be necessary to go beyond taxonomic descriptions and determine the functional capacities of the community through the use of additional techniques such as shotgun metagenomics, metatranscriptomics, and metabolomics. These approaches can be used to identify bacterial strains that contain genes whose function could make them an effective probiotic. For example, based on previous knowledge about bacterial interactions, searching for genes associated with the production of antibiotics (including anti-fungal metabolites) and beneficial host-microbe interactions could increase the chances of selecting good probiotic candidates.

It is important to emphasize that the use of omic approaches to identify probiotics will only be relevant if these are linked with biological assays of culturable bacteria (*sensu* Becker et al., 2015b). Linking culture-independent with culture-dependent data is a fundamental step toward the identification of successful probiotics (Walke et al., 2015). Importantly, if bacterial cultures with inhibitory activities are available, then physiological, metabolic and genomic analyses of these strains can greatly inform omics predictions. Below, we describe currently available omic approaches and their applications for probiotic selection in amphibians.

16S Amplicon Sequencing
Amplicon sequencing is the sequencing of a particular gene or gene fragment of an entire microbial community through

the use of high-throughput sequencing methods (Metzker, 2010). In particular, 16S amplicon sequencing of skin bacterial communities has allowed us to determine the most prevalent and relatively abundant bacterial OTUs (operational taxonomic units) on different amphibian species and populations, and across life-history stages (McKenzie et al., 2012; Kueneman et al., 2014; Loudon et al., 2014a; Walke et al., 2014; Rebollar et al., 2016). By providing information about which bacterial taxa appear to be involved in pathogen protection, 16S amplicon sequencing can help target the isolation of potential probiotic bacteria in pure culture. This can be accomplished using data from both field surveys and laboratory experiments.

Field surveys of amphibians naturally exposed to *Bd* can allow the tracking of changes in the microbial community structure in response to *Bd* infection. For example, recent studies in *Rana sierrae* populations have shown a clear correlation between specific OTUs and *Bd* infection intensity in a field survey (Jani and Briggs, 2014). Field studies can therefore inform us about the bacterial taxa that increase in abundance in the presence of *Bd* and might therefore be involved in a concerted response to the infection (Rebollar et al., 2016). This is an essential step to direct the isolation of potential probiotic bacteria in order to test their ability to inhibit *Bd*.

Field surveys, however, are inadequate to determine causal relationships between OTU presence and pathogen presence. Experimental laboratory *Bd* exposures are essential to determine changes in the microbial structure in response to *Bd* infection so they can provide information about which bacterial taxa could be involved in host defense against pathogens. Variation in susceptibility to *Bd* has been linked to changes in cutaneous bacterial community structure (Becker et al., 2015a; Holden et al., 2015), presence of skin antifungal metabolites (Brucker et al., 2008; Becker et al., 2009, 2015b), function of the mucus components (Woodhams et al., 2014), and Major Histocompatibility Complex (MHC) genotype (Savage and Zamudio, 2011; Bataille et al., 2015). For example, an experiment investigating the use of probiotics to prevent chytridiomycosis in the highly susceptible Panamanian golden frog (*Atelopus zeteki*) demonstrated that individuals that were able to clear *Bd* infection harbored a unique community of bacteria on their skin prior to probiotic treatment (Becker et al., 2015a). Furthermore, the authors identified several bacterial families on surviving frogs that were correlated with clearance of *Bd* (Flavobacteriaceae, Sphingobacteriaceae, Comamonadaceae, and Rhodocyclaceae). In contrast OTUs on individuals that died belonged to the families Micrococcineae, Rhizobiaceae, Rhodobacteraceae, Sphingomonadaceae, and Moraxellaceae (Becker et al., 2015a). To determine if the OTUs associated with survival could prevent chytridiomycosis in *A. zeteki*, the next steps would be to isolate the potentially beneficial OTUs from surviving golden frogs, test the isolates for *Bd* inhibition *in vitro* (Bell et al., 2013) and/or use mucosome assays (Woodhams et al., 2014), and finally test the resistance of inoculated individuals to *Bd* infection (Bletz et al., 2013). In addition, the ability to predict host susceptibility via 16S amplicon sequencing may provide a useful tool for captive population managers to identify individuals that could be used for reintroduction trials.

A number of studies using 16S amplicon sequencing have detected bacterial community members that persist independent of variation in environmental reservoirs (Loudon et al., 2014a), time in captivity (Becker et al., 2015a), and in different developmental stages (Kueneman et al., 2014). These prevalent and persistent community members may be closely associated with their hosts over evolutionary timescales. If important for disease defense, OTUs identified in these studies may also provide probiotic candidates that are effective at persisting on hosts, and even naturally transmitted between hosts or across generations (Walke et al., 2011). Augmenting these bacteria in the habitat may also provide disease mitigation benefits (Muletz et al., 2012). Moreover, amplicon sequencing is not limited to bacterial identification but it can also unravel the diversity of micro-eukaryotes through the sequencing of the 18S rRNA gene. For instance, a recent study characterized the bacterial and fungal composition of amphibian skin communities and determined changes in fungal diversity across different developmental stages (Kueneman et al., 2015).

Shotgun Metagenomics

The sequencing of the total microbial community DNA known as shotgun metagenomics has provided information about the genes present in microbial ecosystems (Barberán et al., 2012b; Knief et al., 2012; Xu et al., 2014). Metagenomic information can allow the identification of genes or genetic pathways associated with specific functions, and therefore it can provide useful information about the potential functional capabilities of microbial communities. For example, metagenomic approaches in marine symbiotic systems have revealed some of the capabilities of bacterial symbionts that are important for interaction with their hosts such as genes involved in nutrient availability and recycling of the host's waste products (Woyke et al., 2006; Grzymski et al., 2008). As mentioned previously, *in vitro* inhibition assays with bacterial isolates cultured from amphibian skin have detected many bacterial strains with antifungal activities (Harris et al., 2006; Holden et al., 2015; Woodhams et al., 2015). Using metagenomics, these antifungal activities, such as the ability to produce extracellular secondary metabolites, can be identified and bacterial species containing these genes could be inferred. However, metagenomic inferences rely on how much information is available in databases and how much we know about antifungal genetic pathways of isolates in culture. Nonetheless, the comparison of shotgun metagenomic data from resistant and/or tolerant frogs will be very helpful for identifying potential bacterial candidates for probiotics. Bacteria whose genomes contain antifungal gene pathways and pathways associated with the ability to colonize and persist can be identified, which can narrow down the number of probiotic candidates.

Metatranscriptomics

Metatranscriptomics is the analysis of the mRNA expression profiles in a community and is relevant for identifying genes or genetic pathways that are up or down regulated in response to a pathogen infection. This method can also unravel functional responses involved in bacterial-host interactions such as the expression of adhesin genes or additional traits associated with bacterial colonization and attachment to eukaryotic hosts (Klemm and Schembri, 2000; Dale and Moran, 2006; Kline et al., 2009; Chagnot et al., 2013). Determining the capacity of different bacteria to colonize the skin of the host is extremely relevant for selecting bacterial probiotic candidates. A metatranscriptome approach has shown differences in gene expression in human oral microbiomes between healthy and diseased individuals, and specific metabolic pathways associated with periodontal disease have been identified (Jorth et al., 2014). Metatranscriptomes of fungi and algae in symbiosis with plants and corals, respectively, have also revealed changes in gene expression in response to stressors and environmental cues (Gust et al., 2014; Liao et al., 2014).

Metatranscriptomics may be a good approach in experimental laboratory settings, in which amphibians are exposed to *Bd*. This will allow for the identification of genes that change expression levels in response to pathogen infection and could be associated with host survival. To our knowledge, no studies have used a metatranscriptome approach to study the amphibian skin microbiome, in part because acquiring enough bacterial mRNA from amphibians skin is difficult. To pursue a metatranscriptome approach in amphibians it will be important to improve sampling strategies and molecular methods that increase the bacterial mRNA yield and reduce the proportion of eukaryotic mRNA from the host and from fungi present on the skin (Stewart et al., 2011; Giannoukos et al., 2012; Jorth et al., 2014).

An additional research avenue would be to conduct transcriptomic studies on amphibian hosts (Ellison et al., 2014; Savage et al., 2014; Price et al., 2015) and in parallel measure changes in the skin microbial structure (using 16S amplicon sequencing) and function (metabolomics) in the context of disease or probiotic application. Understanding the role of the genes expressed in host immune responses in shaping the microbiota that colonize and persist on the host may allow novel insights for disease treatment (Box 1). Moreover recent methods like dual RNA-seq, which aim to determine the expression profiles of both the host and the associated microbiota (including pathogens), may allow us to determine the interactions occurring between skin microbiota, the pathogen and the host (Westermann et al., 2012; Schulze et al., 2015). These interactions may provide useful insights for understanding infection dynamics and informing probiotic design.

Metabolomics

Metabolites are the chemical intermediates and final products of cellular processes, and system-wide attempts to document all chemical species present in a selected biological sample (i.e., metabolomics) have been undertaken for over a decade (Fiehn, 2002; Bouslimani et al., 2015). In amphibians, differences in skin metabolite profiles (representing the sum of host and microbially produced metabolites) across species have been identified (Umile et al., 2014). Metabolomics could also be used to compare species, populations or individuals with varying susceptibility to pathogens like *Bd*. For example, metabolite profiles can be compared between naïve populations and populations that have survived an epidemic of *Bd*, and metabolites that appear among

survivors can be identified. The bacteria that produce these metabolites can then be tested for their inhibitory properties and probiotic potential.

A complementary approach is to experimentally expose amphibians to *Bd* and compare the metabolite profiles of survivors and non-survivors. Individuals of the salamander *Plethodon cinereus* that were exposed to *Bd* and survived had significantly higher concentrations of the metabolite violacein on their skins than did individuals that died (Becker et al., 2009). This metabolite is produced by several species of bacteria, most notably *Janthinobacterium lividum*, which lives on the skin of many amphibian species and inhibits *Bd in vitro* (Harris et al., 2006). Moreover, use of *J. lividum* as a probiotic on *Rana muscosa* decreased morbidity when individuals were exposed to *Bd* (Harris et al., 2009), although extension to another host species, the Panamanian golden frog (*A. zeteki*), failed to provide similar protection (Becker et al., 2011).

One challenge in metabolomics is that metabolites vary enormously in chemical structure and reactivity, making the use of a single analytical tool to create a "chemical master inventory" nearly impossible. High-resolution mass spectrometry, often coupled with a separation technique such as high-performance liquid chromatography (LCMS), has led to significant strides in this arena (Dettmer et al., 2007). Since LCMS does not automatically provide molecular structure, further analysis is required, which can involve comparison to molecular databases. Free-access compendia of metabolite data have been published as early as 2005, in the first metabolomics web database METLIN, as well as the subsequent Human Metabolome Database (HMDB; Wishart et al., 2007, 2009, 2013). Another challenge arises from the sheer number of data points generated by such analyses, the visualization of which can be daunting, although multivariate statistical analyses and analytical methods have been presented to address this chemometric challenge (Sharaf et al., 1986; Patti et al., 2013; Bouslimani et al., 2015).

STATISTICAL TOOLS TO IDENTIFY PROBIOTIC CANDIDATES AND INTEGRATE MULTI-OMICS DATA

Identifying Key Bacterial Species Associated with Amphibian Survival Against *Bd*

There are several statistical tools that can be used to identify OTUs that are driving differences at the community level between two or more groups (e.g., susceptible and non-susceptible individuals). For example, indicator species analysis (Dufrene and Legendre, 1997) provides a method to identify indicator OTUs based on the relative abundance and relative frequency of each OTU in predefined groups. In this analysis, each OTU is given an indicator value ranging from one to zero. An OTU that is observed in all the frogs of one group and absent from the other would be designated an indicator value of one. In contrast, an OTU that is equally distributed across both groups would have an indicator value of 0. Statistical significance of each value is

then calculated with Monte Carlo simulations. Indicator species analysis can be performed with the IndVal function in the *laBdsv* package (Roberts, 2007) of the R statistical software (R Core Team, 2014).

An additional statistical technique is the Kolmogorov–Smirnov (K–S) Measure (Loftus et al., 2015), which is an extension of the K–S test statistic (Kolmogorov, 1933; Smirnov, 1936). While the K–S test statistic has long been used to assess differences in empirical distribution functions between two groups, the K–S Measure was designed to assess differences in the distributions of the relative abundances of individual OTUs among K > 2 groups. For a given OTU, empirical relative abundance distribution functions are assembled for each group using the data for all individuals assigned to that group. The K–S Measure simultaneously assesses the magnitude of the differences between the distributions, using the weighted sum of the K–S statistics for all pairwise comparisons of distributions defined by K groups. The K–S Measure ranges from zero to one, where values closer to one imply greater differences between the K distributions than values closer to zero (Loftus et al., 2015).

The linear discriminant analysis (LDA) effect size method, LEfSe, can also be an informative method (Segata et al., 2011). LEfSe can be used to compare among groups that are biologically relevant and determine which features (organisms, clades, OTUs, genes, or functions) are significantly different (Albanese et al., 2015; Clemente et al., 2015; Zeng et al., 2015). LEfSe determines the factors that most likely explain differences between classes by coupling standard tests for statistical significance (Kruskal–Wallis and Wilcoxon non-parametric tests) with additional discriminant tests that estimate the magnitude of the effect (LDA score).

Another promising analysis technique is DESeq2, which offers higher power detection for smaller sample sizes (less than 20 samples per group) compared to traditional non-parametric tests based on Kruskal–Wallis and Wilcoxon rank-sum approaches (McMurdie and Holmes, 2014; Weiss et al., 2015). While the non-parametric tests do not assume a distribution, DESeq2 assumes a negative binomial distribution to obtain maximum likelihood estimates for a feature's (gene, OTU, etc.) log-fold change between two groups (Anders and Huber, 2010; Love et al., 2014). Bayesian shrinkage is then used to reduce the log-fold change toward zero for those OTUs of lower mean count and/or with higher dispersion in their count distribution. These shrunken log-fold changes are tested for significance with a Wald test. If the average number of sequences per sample between the two sample groups differs greatly (>3x), it is better to use a Kruskal–Wallis type approach such as LEfSe for lower type 1 error. All methods, indicator species, K–S Measure, LEfSe and DESeq2, take into account the relative abundance and prevalence of each OTU with the latter two methods allowing for a stratified statistical design with biologically relevant classes and subclasses.

We suggest that all or some of these statistical methods can be used in parallel to identify taxa involved in protection against pathogens. Importantly, some of these statistical tools can be used to identify OTUs based on 16S amplicon sequencing but they can also be used to identify genes and metabolites associated with pathogen protection based on metagenomics,

metatranscriptomics, and metabolomics data (Segata et al., 2011; Love et al., 2014; Loftus et al., 2015; Weiss et al., 2015).

Defining Interactions and Networks Involved in Protection Against Pathogens

One inherent challenge of omic data is interpreting the complex interactions present within the data collected. Many microbial datasets can have more than 5,000 features (e.g., OTUs in the case of 16S amplicon sequencing), so this implies almost 12.5 million possible two-feature correlations. Also, it is expected that within these complex microbial communities three or more feature interactions will occur. Furthermore, omic datasets exhibit diverse challenges, including only providing relative abundances based on a fixed total number of sequences rather than absolute abundances, or the abundance and spatial distribution of zeroes in a data matrix (compositionality; Aitchison, 1986; Lovell et al., 2010; Friedman and Alm, 2012). Data sets with many zeroes, due to incomplete sampling or to ecological interactions (e.g., parasitism, commensalism, etc.), further complicates statistical analysis (Reshef et al., 2011; Friedman and Alm, 2012). However, despite the challenges, computation is possible in terms of time and expense as compared with evaluating more than 12.5 million microbial interactions in the laboratory. Also, the mathematical and statistical approaches for analyzing community data are improving. One technique for inferring microbial interactions from sequencing data is correlation network analysis. Networks consist of "nodes" (OTUs, genes, metabolites, integrated omics) and "edges," based on the strength of the interaction between nodes, and which imply a biologically or biochemically meaningful relationship between features (Imangaliyev et al., 2015). Interaction values between nodes are commonly referred to as co-occurrence patterns (Faust and Raes, 2012).

Many different techniques have been developed for assessing correlations and constructing interaction networks. Some classic correlation techniques are the Pearson correlation coefficient (Pearson, 1909), which assess linear relationships, or the Spearman correlation coefficient (Spearman, 1904), which measures ranked relationships. Both Pearson and Spearman correlation are very useful (e.g., Arumugam et al., 2011; Barberán et al., 2012a; Buffie et al., 2015), however, neither was developed specifically for the challenges of sequencing data, e.g., compositionality. Of the two, Spearman is less adversely affected by these challenges. Other correlation methods that have been developed include CoNet (Faust et al., 2012), MENA, or Molecular Ecological Network Analysis (Zhou et al., 2011; Deng et al., 2012), Maximal Information Coefficient (MIC; Reshef et al., 2011), Local Similarity Analysis (LSA; Ruan et al., 2006; Beman et al., 2011; Steele et al., 2011; Xia et al., 2013), and Sparse Correlations for Compositional Data (SparCC; Friedman and Alm, 2012). Network visualizations are often performed in the igraph package in R (R Core Team, 2014) or in Cytoscape (Shannon et al., 2003).
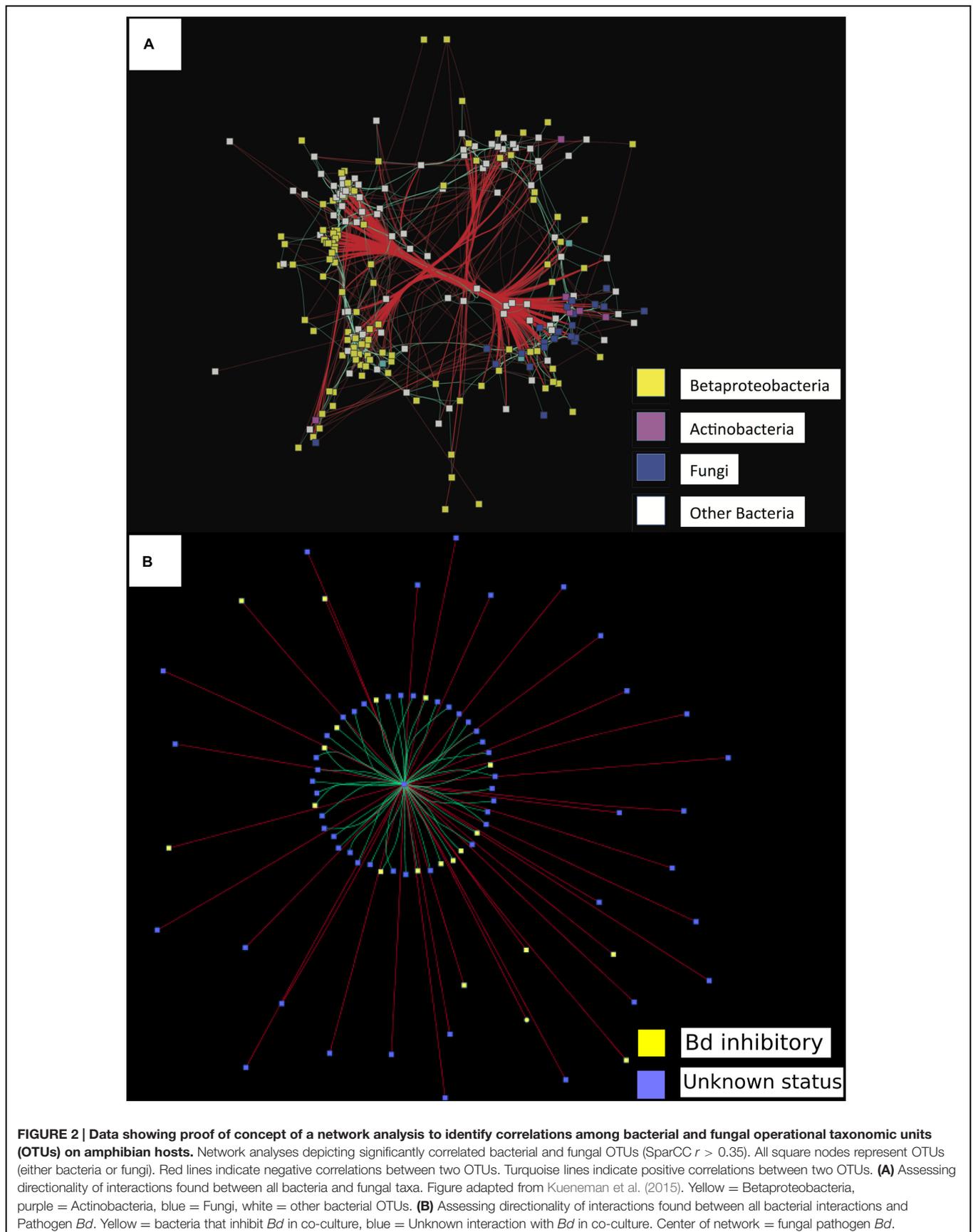
For probiotic selection, the construction and analysis of networks can infer which taxa occur together in natural communities, and can attempt to identify the direction of interactions between taxa or groups of highly connected taxa

(Barberán et al., 2012a). For example, correlation networks in human and mouse models helped identify *Clostridium scindens* as exhibiting a negative correlation pattern with the pathogen *C. difficile*. Transfer of *C. scindens,* either alone or with other bacteria identified by the correlation networks, was then experimentally shown to increase resistance to *C. difficile* infection in mouse models (Buffie et al., 2015). In the case of amphibians, networks that integrate bacterial and fungal omics data taken from hosts, can inform our understanding of interactions occurring between diverse bacterial and fungal taxa (**Figure 2A**). Determining the negative or positive correlations that shift in the presence of a pathogen like *Bd* in experimental trials could help distinguish groups of microbes (mainly bacteria and fungi) involved in resistance against pathogens (**Figure 2B**).

In order to identify potential probiotics against *Bd* in amphibians, correlation networks can be used to compare individual or group interactions in omic data between (1) *Bd*-positive and *Bd*-negative populations in the field, (2) *Bd*-infected and uninfected hosts in experimental trials, (3) hosts with differential *Bd* infection intensity in the field or in experiments and (4) hosts from different life stages. However, caution is warranted when inferring a mechanism of interaction based solely on patterns of correlation (Levy and Borenstein, 2013). Targeted culturing of taxa identified by networks may additionally be used to inform probiotic selection and test their ability to inhibit *Bd* singly or jointly (see Using Omics to Predict if Probiotic Candidates Should be Tested Individually or in Combination), as there may be synergistic *Bd* inhibition (Loudon et al., 2014b). These taxa can be the basis for forming specific hypotheses that can be explored in experimental studies such as a probiotic treatment to determine if the addition of these species to amphibian skin can establish, persist, and increase the anti-*Bd* function of the microbial community of susceptible species.

Integrating Multi-Omics Data to Identify Anti-Fungal Genetic or Metabolic Pathways

Metagenomics, metatranscriptomics, and metabolomics are important tools to determine molecular pathways present in microbial ecosystems. One of the main goals is integrating these multiple massive data sets to distinguish community patterns associated with a specific function such as host disease resistance. Protection against *Bd* in amphibians is likely achieved by a combination of functional pathways present in the skin microbiome in concert with the host's immune system. Therefore, the integration of multiple high dimensional datasets using predictive computational approaches such as bioinformatic predictive tools, multi-omic correlations and *in silico* models are key to predict functional outcomes within the skin microbiome (Borenstein, 2012; Langille et al., 2013; McHardy et al., 2013; Meng et al., 2014). One approach termed Reverse Ecology offers a promising way to use high-throughput genomic data to infer ecological interactions from complex biological systems (Levy and Borenstein, 2012, 2014). It involves predicting the metabolic capacity of a biological system (including symbiotic systems) based on metagenomic data

FIGURE 2 | Data showing proof of concept of a network analysis to identify correlations among bacterial and fungal operational taxonomic units (OTUs) on amphibian hosts. Network analyses depicting significantly correlated bacterial and fungal OTUs (SparCC r > 0.35). All square nodes represent OTUs (either bacteria or fungi). Red lines indicate negative correlations between two OTUs. Turquoise lines indicate positive correlations between two OTUs. **(A)** Assessing directionality of interactions found between all bacteria and fungal taxa. Figure adapted from Kueneman et al. (2015). Yellow = Betaproteobacteria, purple = Actinobacteria, blue = Fungi, white = other bacterial OTUs. **(B)** Assessing directionality of interactions found between all bacterial interactions and Pathogen *Bd*. Yellow = bacteria that inhibit *Bd* in co-culture, blue = Unknown interaction with *Bd* in co-culture. Center of network = fungal pathogen *Bd*.
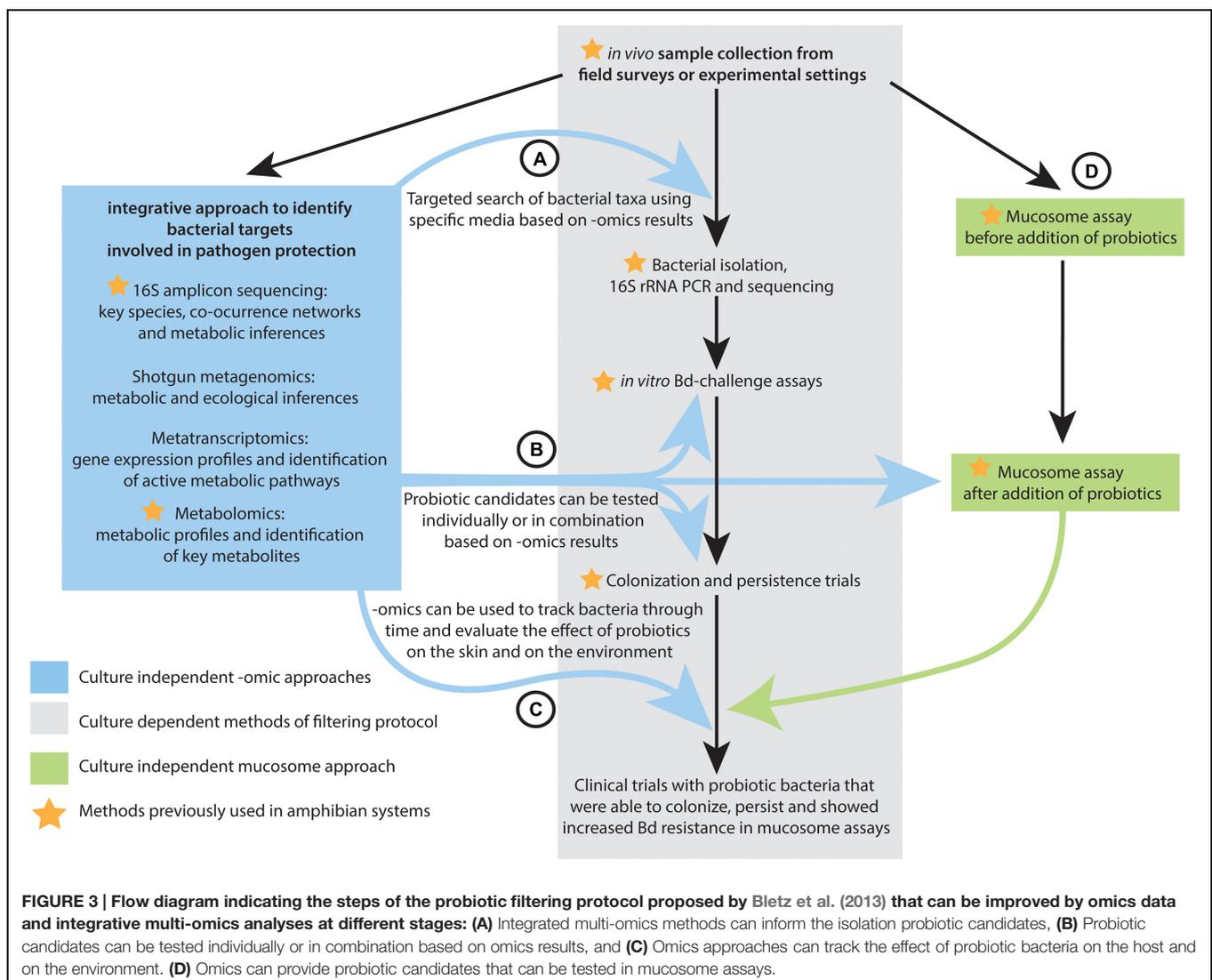
through the use of graph-theory based algorithms and genome-scale metabolic networks (Borenstein et al., 2008; Borenstein and Feldman, 2009; Freilich et al., 2009; Levy and Borenstein, 2012; Manor et al., 2014). To date, the amphibian skin microbiome has mainly been described using culture-dependent techniques and 16S amplicon sequencing. The use of these additional techniques may greatly improve our understanding of this microbial system and could allow us to identify fundamental metabolic pathways and ecological networks associated with defense against pathogens like *Bd*. For example, multi-omic correlations of 16S amplicon sequencing and metabolomics (McHardy et al., 2013) may allow us to determine bacterial taxa and metabolites associated with *Bd* inhibition in *Bd*-tolerant species and in individuals exposed to *Bd* in experimental trials.

Moreover, the bacterial taxa that produce the metabolites could be determined by statistical methods that associate metabolite presence with bacterial species' presence. For example, using random forest with machine learning one can rank microbes by relative contribution (importance; Knights et al., 2011a,b,c; Ditzler and Rosen, 2014). Random forest is an accurate machine-learning multi-category classification algorithm for linking abundances of microbial taxa to physiological states such as metabolite production or immune function (Statnikov et al., 2013). Bacterial species that produce one or more anti-*Bd* metabolites that are associated with survival in a *Bd*-positive environment would be excellent probiotic candidates for bioaugmentation in at-risk populations.

USING OMICS AND INTEGRATING MULTI-OMICS DATA TO INFORM PROBIOTIC SELECTION THROUGH A FILTERING PROTOCOL

Bletz et al. (2013) recently outlined sampling strategies and screening protocols for identifying ideal probiotics for amphibians (**Figure 3**). The framework involves (1) collecting

FIGURE 3 | Flow diagram indicating the steps of the probiotic filtering protocol proposed by Bletz et al. (2013) that can be improved by omics data and integrative multi-omics analyses at different stages: (A) Integrated multi-omics methods can inform the isolation probiotic candidates, **(B)** Probiotic candidates can be tested individually or in combination based on omics results, and **(C)** Omics approaches can track the effect of probiotic bacteria on the host and on the environment. **(D)** Omics can provide probiotic candidates that can be tested in mucosome assays.

and culturing skin microbes from selected host species; (2) isolating all morphologically distinct colonies into pure culture; (3) testing each isolate for its ability to inhibit *Bd in vitro* (Bell et al., 2013); (4) testing highly inhibitory isolates for their ability to colonize and persist on amphibian skin; (5) and for those isolates that persist testing their ability to protect the host against *Bd* infection in clinical trials in the laboratory, followed by field trials (Bletz et al., 2013). In the case of the skin of some amphibian species, the dominant members of the microbiota are readily cultured (Walke et al., 2015), whereas some rare but prevalent members identified by 16S amplicon sequencing have been difficult to isolate in culture (Loudon et al., 2014a). Specialized media may be necessary to target microbes identified by omics approaches including not only bacteria but also fungi. Even though most of the probiotic search in amphibians has focused on bacterial candidates, the filtering protocol proposed by Bletz et al. (2013) could be also used to target potential fungal probiotics. Omic datasets and the integration of multi-omic analyses can facilitate the selection of the probiotic candidates that progress through this sampling and screening protocol. Below we describe the steps of the filtering protocol (Bletz et al., 2013) and the mucosome assay (Woodhams et al., 2014) that can be improved by omics and the integration of multi-omic approaches (**Figure 3**).

Using Omics Data to Inform the Isolation of Probiotic Candidates

A probiotic approach typically requires culturing and isolation of microbial species in order to test their antifungal functions and use only those species with desired properties. Given the taxonomic diversity of the amphibian skin microbiome (Kueneman et al., 2014, 2015; Loudon et al., 2014a; Walke et al., 2014; Becker et al., 2015a), it would be useful to reduce the number of microorganisms that one is trying to isolate and test for inhibition. Omics methods can streamline the isolation process by identifying promising probiotic taxa, which can then be isolated using media and culture conditions that favor or enrich for specific bacterial or fungal groups (Watve et al., 2000; Connon and Giovannoni, 2002; Rappé et al., 2002; Zengler et al., 2002; Vartoukian et al., 2010).

Through the integration of multi-omics data, microbial community members that are associated with surviving amphibian populations in the field and in laboratory experiments can be identified. We suggest using several methods such as indicator species analysis, the K–S Measure, LEfSe and co-occurrence networks in parallel to identify probiotic candidates. The main goal of using several methods is to obtain a list of OTUs that are congruent among methods. OTUs suggested as probiotic candidates by these culture-independent methods can be matched to bacterial isolates in pure culture identified with 16S rRNA Sanger sequencing (Woodhams et al., 2015) or to bacterial strains whose whole genome has been sequenced. These isolates would then proceed to testing for inhibition against *Bd* using *in vitro* challenge assays (Bell et al., 2013) or mucosome assays (Woodhams et al., 2014).

Using Omics to Predict if Probiotic Candidates should be Tested Individually or in Combination

Data obtained by omics methods can be useful for determining if single species or combinations of species are optimal for probiotic inoculation. Isolates can be chosen based on co-occurrence networks and genetic or metabolic pathways enriched in hosts that survived in the presence of *Bd* or that cleared *Bd* infections.

Single isolate probiotics have been successful in some systems such as the probiotic bacterium *J. lividum* in experimental trials with the host *R. muscosa* experimental trials (Harris et al., 2009). However, research in several symbiotic systems has shown that bacterial mixtures are necessary to exert a protective effect against pathogens and a restorative effect on hosts (Lawley et al., 2012; Fraune et al., 2014). For example, in a mouse model of *C. difficile* infection, a six-species probiotic mixture led to a community reset *and* recovery from *C. difficile* infection (Lawley et al., 2012). The authors speculated that the six species in combination were successful due to their phylogenetic distinctiveness, which allowed them to more effectively fill available niche space. Importantly each species alone was not curative, but each species was necessary in the mixture for the treatment to be effective (Lawley et al., 2012).

We currently do not know when a one-species probiotic or when a mixture will be more effective against *Bd* in amphibians. Omics data might offer insight into why single probiotics have failed in some cases. In addition, the integration of multi-omic data could be used to choose sets of isolates that might work in concert based on the presence of facilitative interactions among them from co-occurrence networks or based on the existence of complementary components of genetic or metabolic pathways.

Using Omics Data to Track the Effectiveness of Probiotic Bacteria in Laboratory and Field Trials

Omics can be used to determine if a probiotic will be able to colonize, persist and/or trigger antifungal pathways in the symbiotic community. This is key to its success as a probiotic (Bletz et al., 2013). We hypothesize that this can be accomplished if the community reaches an alternative stable state once the candidate taxon is applied and community structure begins to shift in response (Faust and Raes, 2012; Fierer et al., 2012). The new stable state of the community must have antifungal functions and sufficient competitive abilities against invading pathogens to protect the host. Co-occurrence networks could be helpful to track whether bacterial interactions remain stable or shift through time after probiotic application (Rosvall and Bergstrom, 2010). In addition, these approaches could be useful for understanding whether probiotics applied during one life stage persist and remain effective in subsequent life stages (e.g., through metamorphosis).

One of the ultimate goals of probiotic bioaugmentation is for it to be used to reintroduce *Bd*-susceptible amphibian species back into their natural habitats. Thus, candidate probiotics

must persist on the host and function appropriately not only in laboratory settings, but also in the host organism's natural environment. In addition, an ideal probiotic would not disturb other microbial systems, including those of non-target host organisms, upon introduction. This is particularly important to consider if the planned mode of delivery or maintenance of the probiotic is via the soil or water (Muletz et al., 2012). Similar to laboratory trials, it will be important to collect and evaluate "before and after" metagenomic, metatranscriptomic, and metabolomic data to better understand the responses of both the host organism and microbial systems in the surrounding environment to probiotic application.

Using Omics Approaches to Inform Probiotic Testing in Mucosome Assays

The integrated defenses of the amphibian skin mucus, including antimicrobial peptides, mucosal antibodies, lysozymes and alkaloid secretions and the skin microbiota, are called the mucosome. A mucosome assay developed by Woodhams et al. (2014) can be used to predict the infection prevalence of *Bd*-exposed populations and the survival outcome upon exposure. Briefly, a mucosome assay consists of placing individuals in a bath that collects their mucosal secretions. The secretions are used for *in vitro* viability assays, in which they are tested for their ability to kill *Bd*. This assay accurately predicts adult amphibian survival upon *Bd* exposure (Woodhams et al., 2014).

Importantly, the mucosome assay can be used to measure and predict the effectiveness of probiotic treatments. Probiotic candidates identified by omics analyses need to be isolated through culturing methods and then added to amphibian skins to evaluate their effectiveness using a mucosome assay. This is accomplished by comparing the mucosome function before and after the addition of a probiotic bacterium or a group of probiotic bacteria. Probiotic candidates that pass the preliminary assay screen would then be ready for persistence and clinical trials (**Figure 3D**). The advantage of using the mucosome assay is that it could minimize the need to expose amphibians to *Bd* in clinical trials, which is particularly relevant in the case of endangered species or species that are naïve to the disease.

IMPORTANT CONSIDERATIONS AND FUTURE DIRECTIONS

To facilitate the identification of successful probiotic candidates, we recommend using an interdisciplinary approach. The interaction and collaboration of scientists who have different expertise as well as interaction with natural resource managers can greatly improve the outcomes of probiotic research.

In addition to probiotic therapy for the reintroduction of species currently being held in captivity, one important challenge is to identify probiotic candidates for species that are still naïve to pathogen infections. Two relevant cases from highly diverse regions are frogs in regions of Madagascar that have not been exposed to *Bd* (Bletz et al., 2015) and North American salamanders that are so far naïve to *Bsal* (Martel et al., 2013, 2014; Yap et al., 2015).

Omic methods, along with mucosome assays and culture-dependent methods, may greatly improve our knowledge of the capacity of amphibians (and their microbiomes) to contend against pathogenic infections. In addition, other non-omic techniques such as real-time PCR (qPCR), fluorescence *in situ* hybridization (FISH) and mass spectrometry of culturable communities may inform probiotic discovery since they can increase our understanding of the absolute abundances and spatial dynamics of the dominant microbial members in the amphibian skin microbiome (Watrous et al., 2012; Barea, 2015).

Based on previous research on the amphibian system, this review has mainly focused on bacterial probiotics. However, future research may benefit by considering the micro-eukaryotic and viral components of the skin community. Indeed, fungi are important components of mammalian and amphibian skin (Underhill and Iliev, 2014; Kueneman et al., 2015), and viruses have been linked to dysbiosis in the oral cavity (Edlund et al., 2015). Several studies have examined the importance of non-bacterial microbiota in host health (Parfrey et al., 2014; Rizzetto et al., 2015). Indeed, the co-occurrence of diverse microbiota can drive conflicting immune responses and cause trade-offs (Susi et al., 2015).

In addition to altering the microbiota with probiotic bioaugmentation, prebiotics, which are non-digestible carbohydrates, may also have beneficial effects (Patel and Denning, 2013). Prebiotics can alter nutrient sources to selectively favor targeted microbes as in the case of the prebiotics applied in aquaculture systems (Ringø et al., 2010; Akhter et al., 2015). This line of research has only being applied in the intestinal system (Gourbeyre et al., 2011; Patel and Denning, 2013). Thus further research is needed to determine how prebiotics and the combination of probiotics and prebiotics (synbiotics) could be applied to the amphibian system to favor the colonization and growth of antifungal microbes. Moreover, the use of bacterial metabolic products from probiotic microorganisms (postbiotics, Patel and Denning, 2013) and the addition of non-replicating probiotics may lead to effective therapeutics against pathogens.

CONCLUSION

Omic methods provide us with the opportunity to thoroughly describe microbial symbiont communities and to determine their structure and functionality. In particular, the skin microbiome in amphibians can be elucidated through the integration of multi-omics data to identify potential key beneficial microbiota and the antifungal genetic and metabolic pathways involved in protection against *Bd* or *Bsal*. Disease mitigation through bioaugmentation can be and has been applied to other biological systems, such as bats fighting against white nose syndrome disease, as well as in cattle raising, agricultural and aquacultural systems (Kesarcodi-Watson et al., 2008; Bhardwaj et al., 2014; Lakshmanan et al., 2014; Hoyt et al., 2015; Papadimitriou et al., 2015; Uyeno et al., 2015). These systems share similar concerns and also have the difficulties that we have described here in finding mitigation solutions, so they could benefit from this omics approach. We have a clear framework for selecting an ideal probiotic (Bletz

et al., 2013); however, integrative multi-omics can prioritize candidates and facilitate selection of candidates to move to the next steps in the filtering protocol. Finding effective probiotics has the potential to reduce the large losses of biodiversity from EIDs such as chytridiomycosis.

Box 1. Factors Influencing the Amphibian Skin Microbiome

Host-Associated Factors: Genetic and Immune System Diversity

Due to the essential function of amphibian skin in protection of the host against desiccation and pathogens, the skin mucus is a niche with unique chemical properties. Thus, one would predict that only a limited subset of bacterial species would be able to become established on the host (habitat filtering). However, the extent to which amphibian host factors dictate the selection, diversity, and stability of the skin microbiota remains poorly understood. Moreover, we still lack knowledge about how much variation in microbial community structure can be supported by host amphibian genotypes. In other animals, it is clear that many host-specific factors can regulate the assembly of their microbial communities. For example, previous studies in humans and laboratory mice have shown that different genotypes support different microbiota (reviewed in Spor et al., 2011). Likewise, in *Nasonia* wasps, ants and freshwater *Hydra*, species-specific microbiota emerge in "phylosymbiotic" patterns that parallel speciation and ancestry (Brucker and Bordenstein, 2012, 2013; Franzenburg et al., 2013; Sanders et al., 2014). In other animal systems genetic variation of immune associated genes has been associated with differences in symbiotic microbiota. For example, the gut microbial community structure of sticklebacks (*Gasterosteus aculeatus*) is correlated with the diversity of the individual's MHC Class IIb genes (Bolnick et al., 2014). The MHC is a major set of adaptive immune genes that code for molecules that regulate recognition of foreign antigens and pathogens; however, the role of the MHC in regulating microbial communities is poorly understood. A proposed mechanism for MHC control of microbiota is that some MHC molecules may vary in their capacity to recognize the microbial motifs needed to mount an immune response against particular bacterial taxa (Bolnick et al., 2014). Microbes or their microbial antigens are taken up by antigen presenting cells such as dendritic cells and macrophages. The processed antigens are then presented as small peptides complexed with MHC to T lymphocytes. The T lymphocytes release cytokines that recruit other effector cells, and they assist the development of antibodies.

In amphibians, several studies have demonstrated that the host microbiota in amphibians can vary by population (McKenzie et al., 2012; Kueneman et al., 2014; Walke et al., 2014), and it is possible that these differences correlate with population-level differences in immunogenetic diversity. Because the mucus of amphibians contains several classes of antibodies (Ramsey et al., 2010), it is likely that the antibodies expressed in the mucus would play a role in controlling which microbial species are allowed to colonize (Colombo et al., 2015).

In addition to the genetic diversity of genes involved in the adaptive immune system, amphibians produce a diverse array of innate immune defenses including antimicrobial peptides (AMPs), lysozymes and alkaloids (Macfoy et al., 2005; Conlon, 2011). A diverse array of AMPs are produced in amphibian's granular glands such as brevinins, ranatuerins, and magainins that are encoded by polymorphic genes that generate variation in peptide profiles among individuals (Tennessen and Blouin, 2007; Tennessen et al., 2009; Conlon, 2011; Daum et al., 2012). In comparison with the genetic diversity of MHC molecules, AMP genes and expressed peptides are much less diverse (Tennessen and Blouin, 2007). However, apparent gene duplications allow for gradual genetic changes that appear to be positively selected in response to pathogens (Tennessen and Blouin, 2007). Defensive AMPs appear to be released constitutively into the mucus at low concentrations, but they can be increased significantly when the amphibian hosts are alarmed or injured (Pask et al., 2012) and can be affected by environmental stressors (Katzenback et al., 2014). However, some amphibians appear to lack the capacity to produce conventional cationic AMPs (Conlon, 2011). Species that lack AMPs may be more dependent on other chemical factors present in the mucus (bacterial antifungal metabolites, lysozymes, and antibodies) in order to mount a defense against skin pathogens.

In summary, all of the host mucosal chemical defenses (AMPs, lysozyme, alkaloids, and antibodies) have the potential to affect survival of some members of the community of skin bacteria. The interplay between chemical defenses in the mucus and microbial communities is not well understood. Future research is needed to understand to what extent microbes shape the immune compartment and how the immune compartment shapes the microbiome.

Biotic Factors

Hosts are in constant contact with environmental microbial communities that serve as reservoirs. In the case of amphibian skin microbiota, environmental reservoirs may provide an important source of bacterial colonizers, which are needed since amphibian cutaneous microbial communities are frequently disturbed by skin shedding (Meyer et al., 2012). In humans, bacteria are found in deep epidermal layers, not only on the skin external layer (Nakatsuji et al., 2013), thus providing a reservoir for re-inoculating the skin after disturbance. This has not yet been demonstrated for amphibians; however, the salamander gut has been shown to be a reservoir for the anti-fungal cutaneous bacterium *J. lividum* (Wiggins et al., 2011) and bacteria residing in gland openings may also serve as a reservoir (Lauer et al., 2007).

Nonetheless, environmental reservoirs appear to be necessary to maintain the diversity of skin symbiotic bacteria (Loudon et al., 2014a). For example, salamanders (*P. cinereus*) without an environmental bacterial reservoir showed a 75% decrease in bacterial richness, and their bacterial communities became uneven with some OTUs becoming dominant in the majority of the individuals. In contrast, salamanders that were housed with a soil reservoir maintained a greater bacterial diversity that was more similar to communities found in nature (Loudon

et al., 2014a). In the case of red-eyed tree frogs (*Agalychnis callidryas*), individuals housed with plants had a greater richness and abundance of skin bacteria than those housed without plants (Michaels et al., 2014). These studies demonstrate that environmental reservoirs are necessary to maintain the bacterial diversity typically found in nature. In terms of probiotics, Muletz et al. (2012) demonstrated that *P. cinereus* can acquire the beneficial bacterium *J. lividum* from soil, and salamanders that were able to acquire *J. lividum* from the environment were less likely to be infected with *Bd* (Muletz et al., 2012).

The skin microbiota also interacts with invading skin pathogens such as *Bd*. In amphibians the skin mucus contains a suite of microorganisms that may play a beneficial symbiotic role for the host (Harris et al., 2006). Anti-*Bd* secretions from skin bacteria have been found on free-living hosts in concentrations that inhibit *Bd in vitro* (Brucker et al., 2008; Becker et al., 2009). Furthermore, some bacterially produced metabolites interact synergistically and additively to inhibit *Bd* (Myers et al., 2012; Loudon et al., 2014a). Recent work has demonstrated that the composition and structure of amphibian skin bacterial communities can change in response to *Bd* infection (Jani and Briggs, 2014). However, it is still not well understood if changes in the diversity of the microbiota are accompanied by changes in function (i.e., increases in the number of beneficial anti-*Bd* symbionts and therefore an increased protective role of the skin microbiota).

A number of different *Bd* lineages have been identified and isolated from amphibian skin (Farrer et al., 2011; Schloegel et al., 2012; Bataille et al., 2013), including the globally distributed and hypervirulent global panzootic lineage (*Bd*GPL) that has been associated with mass mortalities and rapid population declines of amphibians (Farrer et al., 2011, 2013). Within the *Bd*GPL lineage there is considerable genetic variation, as well as significant differences in virulence between isolates (Farrer et al., 2011, 2013). Many bacterial strains isolated from amphibian skin have the ability to inhibit the growth of *Bd in vitro* (Harris et al., 2006; Woodhams et al., 2014; Becker et al., 2015b; Holden et al., 2015). However, it was recently demonstrated that bacteria differ in their capacity to inhibit different *Bd*GPL isolates, and only a small proportion of bacteria show broad scale inhibition across the genetic variation exhibited by *Bd*GPL (Antwis et al., 2015). This, coupled with the variation in host response to different isolates of *Bd*GPL (Farrer et al., 2011), means that potential probiotics will need to account for differing virulence of *Bd* or that probiotics that show broad scale inhibition must be identified and used.

In addition to the influence of environmental microbes and pathogens, the ecological interactions among skin microbes would be expected to play a relevant role in structuring the skin microbiota. Bacteria engage in the full breadth of ecological interactions, from antagonistic to facilitative (reviewed by Faust and Raes, 2012). Many of these bacterial interactions are chemically mediated by secondary metabolites, which can contribute to mutualistic interactions, such as cross-feeding or syntrophy, in which two species benefit from each other's metabolic products (Woyke et al., 2006; Faust and Raes, 2012; Loudon et al., 2014b). Secondary metabolite production is also influenced by the composition of the bacterial community

(Onaka et al., 2011), and therefore changes in the community composition of the host (for example through environmental variation or diet) may intrinsically lead to changes in the secondary metabolite profile of the total community. In addition to mutualistic interactions, competition is common among microbes, and can occur via antibiotic production (Kelsic et al., 2015). Other forms of competition can range from occupying space and therefore inhibiting attachment of colonizing species to more efficient consumption of shared resources.

Abiotic Factors

The skin microbiome in vertebrates is highly sensitive to changes in humidity and temperature (Grice and Segre, 2011; Kueneman et al., 2014). Therefore, the skin microbial community structure might be modified by exposure of the skin to different microclimates. This is particularly relevant for ectotherms like amphibians, in which habitat-mediated thermoregulation can expose the host (and its microbial symbionts) to a wide variety of microclimatic conditions over very short time periods (Huey, 1991). Moreover, seasonal variation may influence host behavior by increasing host body temperature (Rowley and Alford, 2013) and this could in turn modify the skin microbial structure. Warmer temperatures can increase the skin sloughing frequency of anurans, thus reducing the abundance of bacteria on the skin through frequent disturbance (Meyer et al., 2012; Ohmer et al., 2014). In addition, thermal conditions influence the activity and production of antifungal metabolites by symbiotic microbes on amphibian skin (Woodhams et al., 2014). For example, high temperatures can limit the production of antimicrobial metabolites, such as violacein and prodigiosin produced by *J. lividum* strains (Schloss et al., 2010; Woodhams et al., 2014). However, for other bacterial probiotics, cooler temperatures may limit the production of antimicrobial products (Daskin et al., 2014). The combined influences of environmental variation on microbiome stability are poorly understood, and they likely vary among species from different habitats and ecosystems.

Moreover, temperature might also impact the skin microbiome by altering the interaction between the amphibian immune system and invading pathogens. Immunity in ectotherms is strongly affected by temperature (Raffel et al., 2006; Rollins-Smith et al., 2011; Rollins-Smith and Woodhams, 2012). In general, low temperatures (4–10°C) are predicted to favor *Bd* (Woodhams et al., 2008; Voyles et al., 2012), and under these conditions amphibian immune defenses are delayed or diminished (Rollins-Smith et al., 2011; Rollins-Smith and Woodhams, 2012). In contrast, higher temperatures (25–30°C), nearer to the maximum for *Bd* survival (Piotrowski et al., 2004; Stevenson et al., 2013), are predicted to favor the amphibian host, enabling them to develop a more effective immune response (Rowley and Alford, 2013). Similarly, *Bsal* infections can be cleared by host exposure to 25°C for 10 days (Blooi et al., 2015). Thus, thermal preference of the host is associated with lower probabilities for *Bd* or *Bsal* infection (Rowley and Alford, 2013). In this respect, the skin microbiome may also be affected by pathogen

invasions based on the host's and the pathogen's thermal preferences.

AUTHOR CONTRIBUTIONS

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