SYMBIOTIC RELATIONSHIPS AS SHAPERS OF BIODIVERSITY

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SYMBIOTIC RELATIONSHIPS AS SHAPERS OF BIODIVERSITY

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EDITORIAL
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Editorial: Symbiotic Relationships as Shapers of Biodiversity

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Keywords: symbiosis, biodiversity, climate change, microbes, coral

Editorial on the Research Topic

Symbiotic Relationships as Shapers of Biodiversity

Symbiosis—the living together of unlike organisms (de Bary, 1878)—is exceedingly common in nature. Symbiotic relationships modify the physiology and influence the ecological dynamics and evolutionary processes of interacting partners, ultimately altering the distribution of species across the planet (Douglas, 2010; Chomicki et al., 2019). This is particularly true with microbial symbiosis. To date, research has demonstrated that symbionts play a pivotal role in shaping biodiversity at ecological and evolutionary scales. However, many facets of the relationship between symbiosis and the generation and maintenance of biodiversity remain unexplored. In the face of unprecedented climatic changes, biodiversity studies incorporating symbiotic relationships will be key to understanding how species will endure changing environmental conditions.

In this Research Topic, we have assembled a collection of articles that broaden our understanding of how symbiotic relationships between hosts and microbes alter biodiversity patterns at ecological and evolutionary scales and as a result of environmental change.

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SYMBIOSES MODULATE RESPONSES TO ENVIRONMENTAL CHANGE

This Research Topic addresses how and when symbioses shape species' responses to environmental changes using a variety of systems and approaches.

Maher et al. showed that coral microbiomes exhibit flexibility and resilience following thermal stress. The authors performed an experiment during a 2016 heat stress event on Moorea Island, testing how heat stress, with the addition of two forms of nitrogen, affects the coral microbiome. The authors found that heat stress overwhelms the system, masking the effect of nitrogen. Following heat stress, the coral microbiome became dominated by a bacterial clade known as *Endozoicomonas*, a signature that persisted following the heat stress event. Because *Endozoicomonas* is a likely beneficial symbiont, thermal stress-mediated remodeling of the coral microbiome may play a key role in coral reef resilience to climate change.

Fuess et al. highlighted how an increase in algal symbiont density, as a result of nitrogen water pollution, drives an increased coral host susceptibility to disease via a reduced immunity. Thus, low water quality can increase coral mortality indirectly, by driving up symbiont density which in turn reduces the expression of immunity genes.

Thurber et al. reviewed the state-of-the-art in coral disease dynamics. They argued that disease outbreaks are largely driven by changing environments, and suggest an integrative approach that includes evaluating the impacts of changing environments on the host, its microbiome, and the environment in tandem.

Nourabadi and Nichiguchi asked how a fluctuating environment affects the recruitment and success of environmentally-acquired symbionts, using the symbiosis between Hawaiian bobtail squids and their bioluminescent bacterial symbionts. Using experimental evolution, they showed that *Vibrio* strains adapted to low pH have both more efficient colonization rate and higher competence (bioluminescence) than ancestral strains. Their results suggest that environmental change can drastically affect the functioning of laterally-acquired symbioses.

Soto and Nishiguchi reviewed the current understanding of how environmental stress drives *Vibrio* symbiont diversity. The authors discuss the mechanisms enabling *Vibrio* to cope with environmental stress in both their free-living and symbiotic stages, and how this in turn affects their diversity.

Work by van't Padje et al. tested how mycorrhizal symbioses are affected by extreme weather events. In particular, they tested how phosphorus is up-taken and transferred to the plant host under extreme heat stress or flooding—relying on a recently developed technique based on quantum-dot tagging of nutrients, which allows to track nutrients along the fungal network using fluorescence (Whiteside et al., 2019). Phosphorus transfer, as well as plant and fungal survival, were deeply affected by flooding, but less so by heat treatment. Their innovative approach opens new avenues to test how different plant-host combinations are impacted by extreme weather events.

SPECIFICITY AND DEPENDENCE AS KEY FACTORS OF SYMBIOTIC RELATIONSHIPS

The level of dependence (facultative to obligate) and specialization/specificity (generalist: many partners to specialist: few specific partners) is pivotal in driving stability of symbiotic mutualisms across ecological and evolutionary scales (Chomicki et al., 2020). Contribution to this Research Topic demonstrates how specificity and dependence are key to predict species' response to climate change, pinpointing a number of essential areas for future research.

Ginete and Goodrich-Blair reviewed state-of-the-art research on the specificity of host-symbiont systems. They argue that recognizing below species level strain variation is critical to understand symbiotic specificity. The authors recognized two main factors driving specificity: (1) factors that contribute a required activity in support of the association and (2) factors involved in antagonistic interactions with organisms outside of the association. Identifying these factors across host-symbiont partnerships is the key understanding the drivers of specificity in microbial symbioses.

Goulet and Goulet reviewed current data on dependence and specificity in the large array of Cnidarian symbioses from coral reefs. Because many Cnidarian symbioses are obligate and show high specificity, they are likely to be strongly impacted by the effects of climate change. Thus, the robustness and potential plasticity of the host-symbiont association will determine the survival of the symbioses following environmental change. Understanding the dependence, specificity as well as performance and plasticity of host symbiont combinations in Cnidarian symbiosis is therefore critical to forecast the effect of climate change on these extremely biodiverse communities.

SYMBIOSES AS DRIVERS OF ECOLOGICAL COMMUNITY DYNAMICS

Microbial symbioses are ubiquitous interactions in nature. As such, they play key roles in structuring and modulating ecological communities. Contributions to this Research Topic show how symbiosis can drive species composition within communities, but also how it can promote species coexistence. This in turn, can feed back on the functioning of the symbiosis.

Sanders-Smith et al. studied the leaf surface microbiome of the seagrass *Zostera marina*, a foundation species which sustains key temperate marine ecosystems in British Columbia, Canada. The authors found distinct microbiome communities on young and old leaves. Older leaves had richer leaf microbiomes and showed more geographic microbiome differentiation a, suggesting host filtering and environmental influence, with core microbial taxa suggesting functional roles. Understanding how the leaf microbiome affects the growth, epiphyte control, and detoxification of seagrass waste product is key to unlocking the factors maintaining complex marine ecosystems.

Herder et al. asked how the interplay of intrinsic host vs. extrinsic environmental factors shapes the fecal microbiome, using Anna's hummingbird as a model system. They found that the greatest explanatory variable was intrinsic, but the environment had a substantial effect on the abundance of bacterial lineages. The authors argue that understanding how the environment shapes microbiome-host symbiosis has profound consequences for species conservation.

Runte et al. investigated how symbiosis predicts fungal communities in mixed conifer-hardwood communities. They found that the turnover of ectomycorrhizal and saprotrophic communities was differentially predicted by the availability of canopy species, though both were driven by similar soil properties. At the study scale, rRNA was a much better predictor than DNA. This work reveals the importance of ectomycorrhizal symbiosis in structuring soil fungal communities.

Hsu and Moeller tested whether the metabolic symbiosis between *Paramecium bursaria* and the endosymbiotic green algae *Chlorella* impacts species coexistence. Specifically, they tested whether the symbiosis with Chlorella influences the coexistence of *P. bursaria* and its non-symbiotic competitor *Colpidium*. Their study reveals light-dependent priority effects modulating competition between both bacterivorous species, which pinpoints the pivotal role that mutualisms can play as modulators of species coexistence (Chomicki et al., 2019).

Leclair et al. tested whether the endosymbiont communities of different pea aphid biotypes had a protective effect on their hosts under natural conditions and whether endosymbiotic associations and parasitoid communities associated with the pea aphid complex were linked. The authors confirmed the protective effects of endosymbionts on their hosts in nature, and found a positive correlation between the α and β diversities of the endosymbiont and parasitoid communities, indicating interactions between these two guilds. Their work emphasizes the importance of field studies of symbioses.

OUTLOOK

Altogether, this Research Topic broadens our understanding of the diverse ways in which microbial symbiosis can alter and be altered by biodiversity changes across scales. It pinpoints the need to give a central place to symbiotic partnerships in the study of species response to climate change. It also highlights several future research avenues aiming at understanding how symbiotic associations shape—and are shaped by—biodiversity.

AUTHOR CONTRIBUTIONS

GC wrote first draft with edits from RB, CP, KR, and MW. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Coral Microbiomes Demonstrate Flexibility and Resilience Through a Reduction in Community Diversity Following a Thermal Stress Event

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Thermal stress increases community diversity, community variability, and the abundance of potentially pathogenic microbial taxa in the coral microbiome. Nutrient pollution, such as excess nitrogen can also interact with thermal stress to exacerbate host fitness degradation. However, it is unclear how different forms of nitrogen (nitrate vs. ammonium/urea) interact with bleaching-level temperature stress to drive changes in coral microbiomes, especially on reefs with histories of resilience. We used a 13-month field experiment spanning a thermal stress event in the Austral summer of 2016 on the oligotrophic fore reef of Mo'orea, French Polynesia to test how different forms of nitrogen (nitrate vs. urea) impact the resistance and resilience of coral microbiomes. For *Acropora*, *Pocillopora*, and *Porites* corals, we found no significant differences in diversity metrics between control, nitrate-, and urea-treated corals during thermal stress. In fact, thermal stress may have overwhelmed any effects of nitrogen. Although all three coral hosts were dominated by the bacterial clade *Endozoicomonas* which is a proposed beneficial coral symbiont, each host differed through time in patterns of community diversity and variability. These differences between hosts may reflect different strategies for restructuring or maintaining microbiome composition to cope with environmental stress. Contrary to our expectation, post-stress microbiomes did not return to pre-stress community composition, but rather were less diverse and increasingly dominated by *Endozoicomonas.* The dominance of *Endozoicomonas* in microbiomes 10 months after peak sea surface temperatures may suggest its ability to utilize host metabolic products of thermal stress for a sustained competitive advantage against other microbial members. If *Endozoicomonas* is a beneficial coral symbiont, its proliferation after warm summer months could provide evidence of its ability to mitigate coral holobiont dysbiosis to thermal stress and of resilience in coral microbiomes.

Keywords: climate change, symbiosis, nutrient pollution, coral reef, *Endozoicomonas*, microbial community dynamics

INTRODUCTION

Coral reef ecosystems are exceptionally vulnerable to rapid increases in sea surface temperatures. Driven by climate change, coral bleaching events are increasing in frequency and intensity, inspiring extensive efforts to understand the breakdown of the symbiotic association between the coral host and its photosynthetic dinoflagellate endosymbionts of the family Symbiodiniaceae (Bourne et al., 2008; Hughes et al., 2018; Sully et al., 2019). Similarly, bacterial members of the coral holobiont are sensitive to changing environmental conditions but have been evaluated less extensively under increasing seawater temperatures. Research on bacterial community dynamics under temperature stress demonstrates shifts to more disease-associated states, increases in community variability, compromised function of beneficial microbiota, and selection for potentially pathogenic bacteria (Ritchie, 2006; Bourne et al., 2008; Thurber et al., 2009; Mouchka et al., 2010; Maher et al., 2019). Given that coral microbiota are thought to play an important role in nutrient cycling and antimicrobial protection (Ritchie, 2006; Wegley et al., 2007), it is important to understand how their response to thermal stress events can mitigate or exacerbate host survival and ecosystem resilience.

Nutrient enrichment resulting from human activities is an important contributor to coral reef decline (Szmant, 2002; Fabricius, 2011). Elevated inorganic nutrients (i.e., nitrogen and phosphorus) can induce profound changes in the benthic communities of coastal ecosystems, fostering the growth of macroalgae and increasing the prevalence of coral diseases (McCook et al., 2001; Burkepile and Hay, 2006; Vega Thurber et al., 2014). In addition, nutrient enrichment may affect coral physiological traits, such as growth and reproductive effort and may impair coral thermal tolerance to bleaching (Wooldridge, 2009; Cunning and Baker, 2013; D'Angelo and Wiedenmann, 2014). That said, recent studies indicate these effects may depend on the chemical form (i.e., nitrate, ammonium, urea) and source of nitrogen, as well as on the stoichiometry of the N:P (Wiedenmann et al., 2013; Shantz and Burkepile, 2014). Both laboratory and field experiments show negative effects of elevated nitrate levels derived from anthropogenic sources on coral growth rate, bleaching prevalence and duration—especially when coupled with low levels of phosphorus (Wiedenmann et al., 2013; Ezzat et al., 2015; Burkepile et al., 2019). In contrast, fish-derived nutrients such as ammonium and urea have either neutral or beneficial effects on coral growth, photosynthesis, and bleaching tolerance (Béraud et al., 2013; Shantz and Burkepile, 2014; Ezzat et al., 2015; Allgeier et al., 2017; Burkepile et al., 2019; Ezzat et al., 2019b).

While the effects of excess nitrogen levels on coral physiology have been well-documented, less is known about their potential to alter coral-associated bacterial communities, especially when combined with stressors such as ocean warming. For corals maintained in aquaria, both nitrate and ammonium were sufficient to destabilize the coral-associated bacterial community, although ammonium-treated corals remained more similar compositionally to controls than nitrate-treated corals (Maher et al., 2019; Rice et al., 2019). In the Florida Keys, nitrogen and phosphorous enrichment made corals more susceptible to mortality from predation, above-average seawater temperatures, and bacterial opportunism (Zaneveld et al., 2016). In fact, during that multi-year experimental enrichment, nutrient loading increased both the prevalence and severity of coral disease and bleaching (Vega Thurber et al., 2014).

To understand the interacting effects of nutrients and bleaching on coral microbiomes in a coral depauperate reef, we previously enriched corals with nutrients during a 2014 bleaching event in the Florida Keys (Wang et al., 2018). The Florida Keys, like many Caribbean reefs, have experienced deterioration since the 1980s leading to phase shifts from coral- to algal-dominated reefs that show little evidence of reversibility or recovery (Rogers and Miller, 2006; Maliao et al., 2008). From *Siderastrea siderea* coral metagenomes, we found that nutrient enrichment alone increased microbial community beta diversity throughout the bleaching event but had no interacting effects with temperature. This supports mounting evidence that microbial community diversity increases with stress (McDevitt-Irwin et al., 2017) potentially reflecting microbiome destabilization or dysbiosis (van Oppen and Blackall, 2019). In sharp contrast to the Florida Keys which remain in a state of low total coral cover, the fore reef of Mo'orea, French Polynesia, has recovered from numerous landscape-scale perturbations within about a decade with total coral cover reaching ∼50% in 2019 (Adjeroud et al., 2002, 2009; Berumen and Pratchett, 2006; Penin et al., 2007; Adam et al., 2011, 2014; Trapon et al., 2011; Holbrook et al., 2018). Mesocosm experiments on *Pocillopora meandrina* microbiomes in Mo'orea showed that while nutrients and the interaction between nutrient enrichment and high temperature had an effect on individual members of the microbiome, temperature alone had the strongest effect on alpha and beta diversity overall (Maher et al., 2019). However, the temperature stress applied in the experiment was not sufficient to induce bleaching or mortality (Rice et al., 2019). To extend this previous work to a natural system, we assessed the response of coral microbiomes to combined nutrient and thermal stress *in situ* on the historically resilient fore reef of Mo'orea.

This study investigated how the availability of different types of nitrogen (nitrate vs. urea) influenced the community composition of coral microbiomes during a bleaching event on the oligotrophic fore reef in Mo'orea, French Polynesia. We included coral genera susceptible to thermal stress, *Acropora* and *Pocillopora*, and a more resistant genus, *Porites* (Burkepile et al., 2019). Over 13 months, we sampled members of each species from plots that were either maintained as controls or continuously enriched with nitrate or urea. We used high taxonomic resolution based on sub-operational taxonomic units to assess the compositional variability of *Acropora*, *Pocillopora*, and *Porites* microbiomes before, during, and after a bleaching event in the 2016 Austral summer. The goal of our study was to evaluate how different nitrogen sources interact with seawater warming to drive changes in bacterial community dynamics. We hypothesized that stress would lead to dysbiosis of the microbial community resulting in increased diversity and between-sample variability, and that communities would demonstrate resilience by returning to their initial state after the stress event. Additionally, we expected nitrate to exacerbate community dysbiosis induced by increased seawater temperatures compared to ambient nutrient conditions, while urea would have no interacting effects with temperature.

MATERIALS AND METHODS

Design of the Nutrient Enrichment Experiment

To test how temperature stress interacted with nitrate and urea enrichment to reorganize coral microbiomes, we conducted a 13-month enrichment experiment at 10 m depth on the north shore of Mo'orea, French Polynesia (17°30′S, 149°50′W) (Burkepile et al., 2019). Mo'orea is a high-relief volcanic island at the eastern end of the Society Island archipelago with a well-developed lagoon and barrier reef formation. Conditions on the fore reef are relatively oligotrophic (0.28 ± 0.19 μM DIN (mean ± SE); 0.14 ± 0.05 μM SRP; Alldredge, 2019) with coral cover approaching 50% at the study site when our experiment began (Holbrook et al., 2018). The coral community was dominated by *Acropora* spp. (primarily *Acropora retusa*, *Acropora hyacinthus*, *Acropora globiceps*), *Pocillopora* spp. (primarily *Pocillopora verrucosa*, *Pocillopora meandrina*, and *Pocillopora eydouxi*), and *Porites lobata* complex; therefore we set out to examine the impacts of enrichment on the microbiome of representative corals from each of these three genera.

In January 2016, we enriched small sections of the benthos around individual focal corals with polymer coated, slow-release nitrate (Multicote 12-0-44, Haifa Chemicals Ltd.) or urea (Apex 39-0-0, JR Simplot Company) fertilizers. To achieve localized enrichment, we created "nutrient diffusers" by drilling holes in 4 cm diameter PVC tubes which we then wrapped in window screen and filled with either 200 g of nitrate fertilizer or 62 g of urea fertilizer. Different amounts of each fertilizer were used to standardize the total amount of N delivered in both treatments. Nutrient diffusers were secured to the bottom within 15 cm of focal corals with cable ties attached to stainless steel all-thread posts or eyebolts drilled into the reef framework and epoxied in place. Empty diffusers containing no fertilizer were also deployed next to control colonies to account for any effects the diffusers may have had that were unrelated to the fertilizer. To ensure continuous enrichment, diffusers were exchanged every 10–12 weeks from January 2016 to September 2017. As described in Burkepile et al. (2019), nitrogen concentrations of enrichment treatments were quantified each week over a 10-week period following the deployment of a fresh nutrient diffuser at a subset ($n = 5$) of control, nitrate, and urea plots.

Plots for enrichment were haphazardly selected between 10 and 12 m depth by identifying areas where *Porites, Pocillopora,* and *Acropora* were all growing within a 0.5 m radius of a central point where a diffuser could be deployed. However, because not all of the plots contained all three genera of corals, the total replication for our treatments differed by genera. For *Pocillopora*, replication was $n = 70$ for nitrate, $n = 63$ for urea, and $n = 67$ for controls. *Acropora* colonies were present in $n = 35$ nitrate plots, $n = 32$ urea plots, and $n = 40$ control plots. *Porites* colonies were present in $n = 59$ nitrate, $n = 55$ urea, and $n = 65$ control plots. To facilitate re-sampling, focal corals were marked by epoxying stainless steel, numbered cattle tags at the base of each colony. All diffusers were separated by at least 1–2 m and spread over approximately 11,000 m^2. Sea water temperature was recorded every 2 min via two thermistors deployed at opposite ends of the site.

Tissue Sampling for Microbial Communities

To track changes in the coral microbiome we collected tissue samples from a subset of the study's focal corals in January, March, May, and July of 2016 as well as January of 2017. For *Pocillopora* and *Acropora* spp., divers used bone cutters to clip off ~1 cm sections of branches from each focal coral. For massive *Porites,*~1 cm^2 sections of tissue and skeleton were removed from focal colonies with a hammer and chisel or leather punch. Samples were collected underwater in individually labeled, sterile whirlpaks and transferred to the boat. On board the boat, the water was drained from each whirlpak and the samples were placed on ice, transported ~10 min to shore, and stored at −80°C until analysis.

Sample Selection, DNA Extraction, 16S Library Preparation and Sequencing

For library preparation and sequencing, a subset of the focal coral samples was chosen to include only individual corals sampled at all five time points and within each nutrient treatment. Therefore, this subset only included corals with no observed mortality either due to bleaching or some stochastic process for the duration of the experiment (280 samples total). See **Supplementary Table 1** for replication by treatment. Subsamples of frozen fragments were taken and preserved in individual bead-beating garnet tubes from MoBio PowerSoil® DNA Isolation Kit (now QIAgen PowerSoil® DNA Isolation Kit). DNA was extracted from each sample according to the MoBio PowerSoil® DNA Isolation Kit protocol. To target bacterial and archaeal communities, the V4 region of the hypervariable16S rRNA gene was amplified via 2-step PCR coupling forward and reverse primers 515F (5′-GTG YCA GCM GCC GCG GTA A-3′) (Parada et al., 2016) and 806R (5′-GGA CTA CNV GGG TWT CTA AT-3′) (Apprill et al., 2015). First-step reactions (12.5 μl reaction volume) included 6.25 μl AccuStart II ToughMix (2X), 1.25 μl forward primer (10 μM), 1.25 μl reverse primer (10 μM), 0.5 μl sample DNA, and 3.25 μl PCR-grade water. Sample DNA concentrations ranged widely from 0.07 to 10.0 μg/mL. Thermocycler reaction protocol was performed with 3 min denaturation at 94°C; 35 cycles of 45 s at 94°C, 60 s at 50°C, and 90 s at 72°C; followed by 10 min at 72°C and a 4°C hold. Amplified products were run on a 1.5% agarose gel and manually excised. Following gel purification using Wizard® SV Gel and PCR Clean-Up System (Promega), products were barcoded with dual indices with custom multiple amplicon adapters in a 12-cycle PCR reaction (12.5 μl AccuStart II ToughMix (2X), 9.5 μl PCR-grade water, 1 μl (10 μM) each of forward and reverse barcodes, 1 μl of gel-purified DNA). After pooling amplicons in equivolume ratios, we used Agencourt® AMPure XP beads in a final clean-up step on the single resulting pool. Libraries were sequenced at Oregon State University (OSU) by the Center for Genome Research and Biocomputing (CGRB) with v.3 reagent 2 × 300 bp read chemistry on Illumina MiSeq.

Quality Control, and Initial Data Processing

A total of 280 samples were sequenced, quality filtered, and run through the Deblur workflow (**Supplementary Table 1**). Raw reads were first demultiplexed using the fastq-multx tool from ea-utils[1] resulting in a total of 12,079,654 reads. Then reads were trimmed of primers and adapters using Cutadapt v1.12 (Martin, 2011). The following quality control steps were conducted using VSEARCH v2.8.1 (Rognes et al., 2016). Sequences were truncated at the first position having a quality score ≤ 10, and paired-end reads were merged resulting in 5,989,931 reads. Next, sequences with a total expected error >1 per base or with >1 N were discarded. The resulting 5,388,863 reads underwent the Deblur workflow to trim quality-controlled sequences to 250 base pairs, to identify exact sequences with single-nucleotide resolution, and to filter *de novo* chimeras (Amir et al., 2017). The Deblur workflow is a novel method for obtaining sequences that describe community composition at the sub-operational taxonomic unit (sOTU) level using Illumina error profile (Amir et al., 2017). A total of 1,110,070 reads remained across the 280-sample dataset with 2,016 unique sequences from the Deblur workflow. The loss of ∼80% of reads in the workflow likely reflects the large proportion of host coral mitochondrial sequences (<250 base pairs) amplified by the primers, which is a known issue in using the 515F-806R primers on coral tissues.

The resulting sOTU table from the Deblur workflow was processed in QIIME 2 2019.7 (Bolyen et al., 2019). Taxonomy was assigned with the q2-feature-classifier plugin (Bokulich et al., 2018) which employs the classify-sklearn naïve Bayes taxonomy classifier against the Silva 132 99% OTUs reference sequences from the 515F/806R region (Quast et al., 2012). Next, sOTUs were removed from the dataset if they annotated as mitochondrial or chloroplast sequences or were only present in a single sample further reducing the number of reads per sample to a median value of 1,210 with a variance of 1.2.

The remaining sOTUs were aligned with mafft (Katoh et al., 2002) (via q2-alignment) and used to construct a phylogeny with fasttree2 (Price et al., 2010) (via q2-phylogeny). Alpha rarefaction curves were visualized using the q2-diversity plugin to pick a minimum frequency of 881 reads per sample as a sufficient rarefying depth (**Supplementary Table 1** and **Supplementary Figure 1**). The sOTU table was rarefied resulting in 159 remaining samples with unbalanced replication across treatments and coral hosts (**Table 1**) using the package *phyloseq* (v1.28.0) (McMurdie and Holmes, 2013). Alpha diversity metrics including Faith's phylogenetic diversity (Faith, 1992), Chao1 statistic (Chao and Chiu, 2016), and Simpson's diversity index (Heip et al., 2001), and beta diversity metrics including weighted UniFrac (Lozupone et al., 2007), unweighted UniFrac (Lozupone and Knight, 2005), Binary Jaccard distance, and Bray-Curtis dissimilarity were calculated after log-transformation in *phyloseq*.

Statistical Analyses

To improve normality of alpha diversity metrics, Chao1 and Faith's phylogenetic diversity were square root-transformed, while Simpson's index was arcsine-transformed. Experimental group effects on each alpha diversity metric were assessed with linear mixed effect models (LMM) using *lme4* (v1.1.21) (Bates et al., 2014) with month, coral genus, and nutrient treatment as fixed effects and factorial interaction terms and individual colony as a random effect. Multiple comparisons were performed with estimated marginal means (EMMs) using the *emmeans* (v1.4) package. For beta diversity metrics, Permutational Analyses of Variance (PERMANOVA; Anderson, 2001) were conducted to test differences in bacterial community compositions between groups and group factorial interactions. In addition, Permutational Analyses of Multivariate Dispersions (PERMDISP; Anderson, 2006) were used to test for homogeneity of multivariate dispersions between groups. PERMANOVA and PERMDISP were performed using the functions adonis and betadisper in the package *vegan* (v2.5.5) followed by a pairwise analysis of variance with *pairwiseAdonis* (v0.01) and permutest in *vegan*, respectively, with FDR adjusted *p*-values. The betadisper command also was used to calculate the distance to centroid for each sampling group.

All analyses were initially conducted on all microbiome data controlling for host taxa so that patterns of change driven by time and treatment were assessed across all samples with coral genus (*Porites*, *Acropora*, or *Pocillopora*) as an independent variable. When there was a significant interaction between treatment and coral genus, analyses were repeated for each individual host genus to discern differences in main effects between coral genera that may have been masked when all genera were combined. Due to the opportunistic nature of field sampling, replication across coral genera, treatment, and month vary widely with *Acropora* corals having the highest replication and *Porites* corals having no samples from January 2016 (**Table 1**). Samples from January 2016 were collected pre-treatment and were therefore analyzed as controls.

Additionally, changes in the abundance of different bacterial genera across month and treatment in all three corals combined, and within each coral genus were assessed with analysis of composition of microbiomes (ANCOM) with controls for false discovery rate (Mandal et al., 2015). For differential abundance analysis with ANCOM, an unrarefied sOTU table was used including samples with 881 or more reads. While treatment and the interaction between month and treatment were assessed in ANCOM models, significant differentially abundant taxa were only identified in the ANCOM model with month as a single predictor and individual colony as a random effect.

RESULTS

Sea Surface Temperatures, Thermal Stress, and Nitrogen Exposure

The 2015/2016 El Niño event increased the probability that corals would experience thermal stress and bleaching, providing us with

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[1] http://code.google.com/p/eautils/

TABLE 1 | Sample sizes and mean daily sea surface temperature (SST) with standard error across months, coral hosts, and nutrient treatments.

Month	SST Mean and SE	Treatment	*Acropora*	*Pocillopora*	*Porites*
January 2016	28.4 ± 0.15°C	Control	6	4	0
March 2016	29.0 ± 0.08°C	Control	7	6	2
		Nitrate	6	6	3
		Urea	6	3	3
May 2016	28.4 ± 0.02°C	Control	6	1	5
		Nitrate	6	0	3
		Urea	7	2	5
July 2016	26.9 ± 0.02°C	Control	6	7	2
		Nitrate	5	4	2
		Urea	4	4	4
January 2017	28.9 ± 0.05°C	Control	5	3	4
		Nitrate	5	4	3
		Urea	5	2	3

an opportunity to test the effects of nutrient enrichment and bleaching on the coral microbiome. As reported in Burkepile et al. (2019), the daily average sea surface temperature (SST) at our experimental site peaked in late March at 29.7°C, and remained at or above 29°C through May of 2016. These temperature thresholds correlate with thermal stress and coral bleaching in Mo'orea (Pratchett et al., 2013). Thus, for a total of 45 days, including 37 consecutive days from mid-March to mid-April, corals at our site experienced thermal stress sufficient to cause bleaching. Average monthly SST is reported in **Table 1** and a graph of average daily temperatures during the experiment can be found in **Supplementary Figure 2**. Of the corals analyzed here, only seven colonies bleached (*Acropora*: $n = 6$, *Porites*: $n = 1$) in May 2016 all of which had no signs of bleaching in July 2016 (**Supplementary Table 1**). Due to this low sample size of bleached corals and the absence of bleaching-induced mortality in the dataset, bleaching was not included in statistical analyses.

Over a 10-week period, nutrient diffusers in nitrate and urea plots increased the concentrations of nitrogen in the surrounding seawater compared to control plots (Burkepile et al., 2019). Analysis of concentrations in Burkepile et al., 2019 showed that nitrogen exposures in nitrate and urea plots were similar and significantly distinct from control plots, and treatments were consistent throughout the 10-week diffuser deployment. Total water-column nitrogen concentrations ranged from approximately 1–3 μM, 3–8 μM, and 3–11 μM Nitrogen for control, urea, and nitrate plots, respectively, over the 10-week period (see Figure 2 in Burkepile et al., 2019).

Bacterial Community Composition Varied Over Time

The dominant bacterial taxon in the dataset ($n = 159$) belonged to the genus *Endozoicomonas* (mean relative abundance 0.448 ± 0.033 SEM); this genus was present in all but 21 samples (**Figure 1**). The next most abundant taxa across the dataset belonged to the genera *Vibrio* (0.060 ± 0.011), *Acinetobacter* (0.059 ± 0.008), *Pseudomonas* (0.049 ± 0.006), and *Candidatus Amoebophilus* (0.038 ± 0.011). Generally, *Endozoicomonas* relative abundance was lowest in March (0.277 ± 0.050) and July 2016 (0.186 ± 0.047) and highest in January 2017 (0.817 ± 0.058) for all corals combined. Despite low replication for *Pocillopora* samples in May of 2016 (**Table 1**), coral samples from all three genera had high relative abundance of *Endozoicomonas*. The decrease in relative abundance of *Endozoicomonas* in March, May, and July coincided with an increase in the relative abundance of minor taxa including *Vibrio*, *Pseudomonas*, *Staphylococcus*, and *Halobacteriovorax*, all of which decreased or disappeared in January 2017 (**Figure 1**). In contrast, other taxa such as *Acinetobacter*, *Candidatus Amoebophilus*, and *Corynebacterium* were present throughout the sampling period. **Figure 1** does not reflect the high variance in relative abundance across samples, for instance, taxa such as *Spiroplasma*, *Halomonas*, and *Tenacibaculum* dominated a single sample within a genus/treatment/month combination (**Supplementary Figure 3**).

Patterns in Microbiome Alpha Diversity Differed Among Coral Host Genera During Thermal Stress

Analyses of bacterial species richness and evenness suggested seasonal variation in alpha diversity, although the patterns varied by coral host genus (**Figure 2**). Pooled by coral genus, *Porites* corals had the highest Chao1 diversity index (mean 33.983 ± 2.88 SEM, $n = 39$) and Faith's phylogenetic diversity (4.289 ± 0.302) compared to *Acropora* (27.910 ± 2.211 and 3.643 ± 0.211, respectively, $n = 74$) and *Pocillopora* (21.085 ± 1.806 and 3.118 ± 0.225, respectively, $n = 46$). By contrast, *Acropora* had the highest Simpson's diversity index (0.679 ± 0.027) compared to *Pocillopora* (0.580 ± 0.050) and *Porites* (0.557 ± 0.046). In LMMs with nutrient treatment, month, and coral genus as fixed effects, nutrient treatment was not a significant predictor for Chao1 [$p = 0.470$, $F_{(2,64.7)} = 0.765$], Simpson's diversity [$p = 0.085$, $F_{(2,122)} = 2.516$], or Faith's phylogenetic diversity [$p = 0.694$, $F_{(2,58.3)} = 0.368$] (**Supplementary Table 2**). Instead, the interaction between month and coral genus was a significant predictor for Chao1 [$p < 0.01$, $F_{(7,109.4)} = 3.133$], Simpson's diversity [$p < 0.01$, $F_{(8,122)} = 3.469$], and Faith's phylogenetic

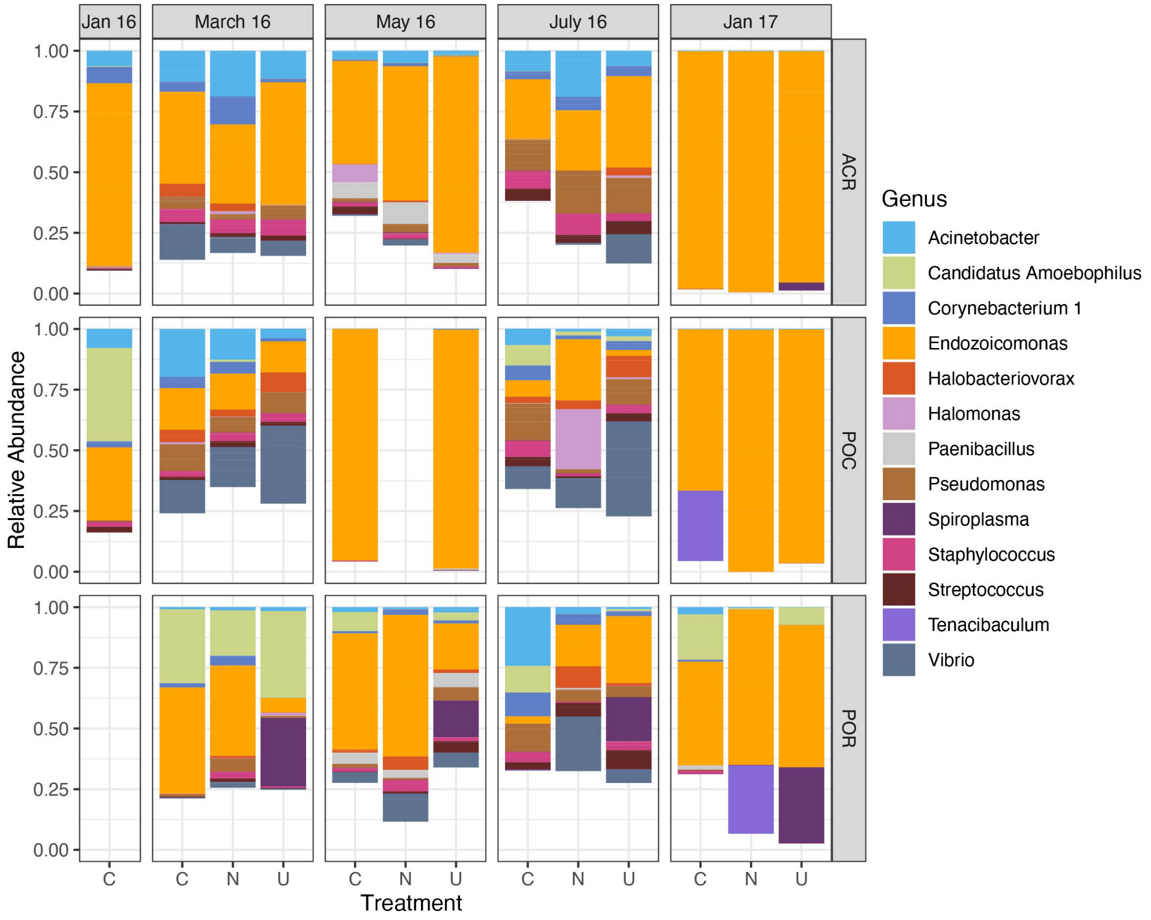

FIGURE 1 | Relative abundance of dominant microbial genera varies over time across all corals. Data are organized by month, coral host (ACR: *Acropora*, POC: *Pocillopora*, POR: *Porites*), and nutrient treatment (C, Control; N, Nitrate; U, Urea). Only genera with a mean relative abundance greater than 0.10 are included. Sample sizes are reported in **Table 1**.

diversity [$p < 0.01$, $F_{(7,106.8)} = 3.108$], suggesting that patterns across time differed between coral genera (**Figure 2**). For this reason, we evaluated patterns of alpha diversity across time with LMMs within each coral genus. Due to loss of samples during bioinformatic filtering, replication varied widely between time points (**Table 1**).

Temporal Patterns in Alpha Diversity Were Similar to Patterns in the Relative Abundance of *Endozoicomonas*

In *Acropora* samples, month was a significant predictor of Chao1 [$p < 0.001$, $F_{(4,57.9)} = 18.476$], Simpson's diversity [$p < 0.001$, $F_{(4,69)} = 7.483$], and Faith's phylogenetic diversity [$p < 0.001$, $F_{(4,57.5)} = 23.265$, **Supplementary Table 3**]. In pairwise comparisons, the last time point, January 2017, was significantly lower than March, May, and July of 2016 for both Chao1 and Simpson's diversity and was significantly lower than all other time points for Faith's phylogenetic diversity (**Figure 2**). Initially, in January 2016, before bleaching, *Acropora* samples had a mean Chao1 of 19.724 ± 2.671 which increased significantly to 40.844 ± 4.275 in May 2016 and decreased significantly to 9.586 ± 1.045 in January 2017 (**Figure 2A**). Similarly, *Acropora* samples had the lowest Simpson's diversity in January 2017 (0.465 ± 0.048), although January, May, and July 2016 were variable with some low diversity samples (**Figure 2B**). These patterns closely mirrored the temporal pattern in relative abundance of *Endozoicomonas* in *Acropora* samples where initial mean relative abundance of 0.747 ± 0.143 decreased to 0.277 ± 0.079 in July 2016 and increased to 0.972 ± 0.011 in the final sampling point (**Figure 1**).

Porites samples showed similar patterns to *Acropora* with month as a significant predictor of Chao1 [$p < 0.05$, $F_{(3,35)} = 4.354$], Simpson's diversity [$p < 0.05$, $F_{(4,26.2)} = 5.105$], and Faith's phylogenetic diversity [$p < 0.001$, $F_{(3,35)} = 8.203$, **Supplementary Table 4**]. All three diversity metrics significantly decreased from May or July to January 2017 in *Porites* (**Figure 2**). Similar to the pattern displayed by *Acropora*, the highest relative abundance of *Endozoicomonas* was during the month with lowest diversity in January 2017 (0.533 ± 0.136) (**Figure 1**).

In contrast to *Acropora* and *Porites*, *Pocillopora* exhibited low alpha diversity in May 2016 as well as January 2017

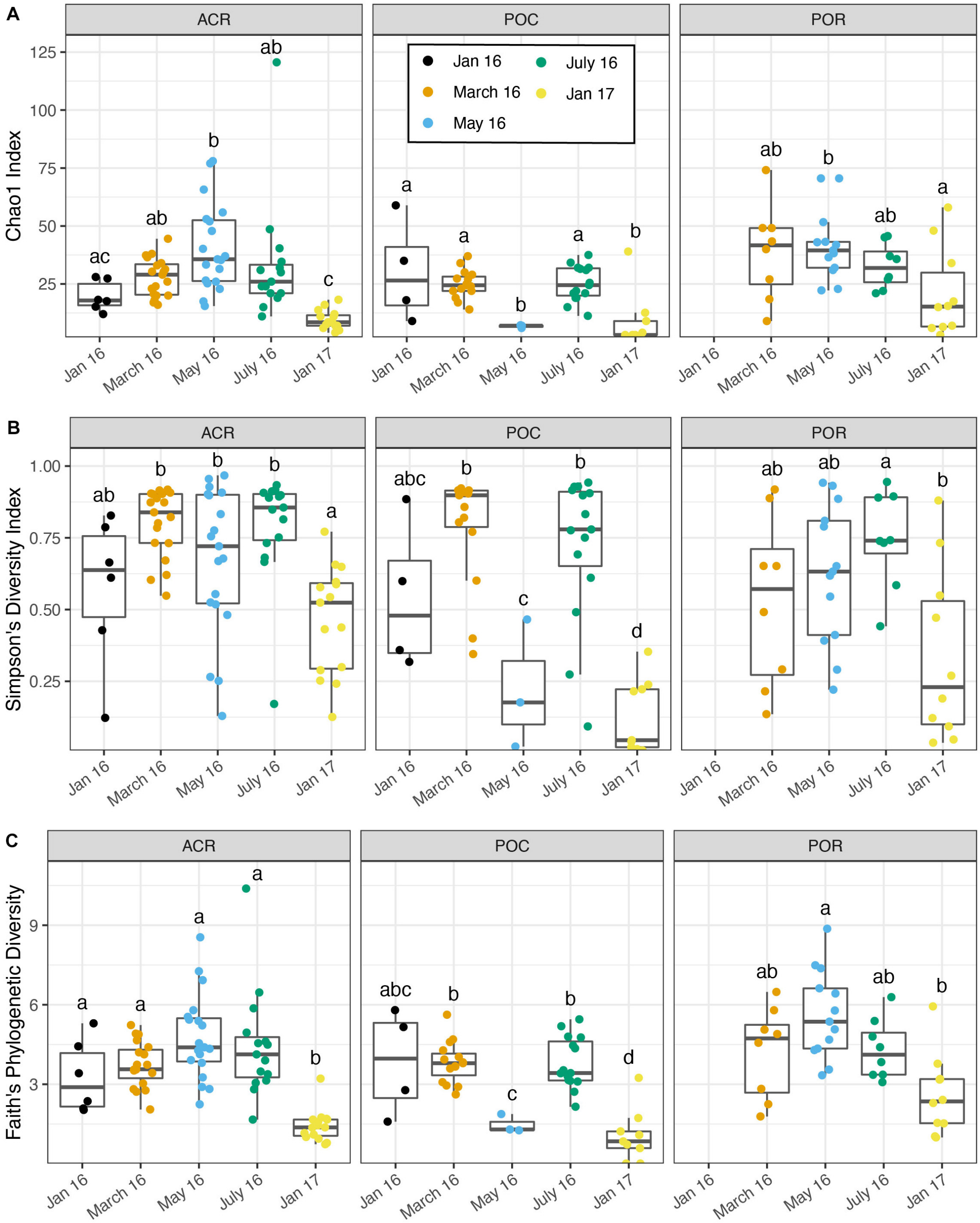

FIGURE 2 | Microbiome alpha diversity varies by time and between coral genera. Due to a significant interaction effect between month and genus, significant differences were determined between months within each coral genus using linear mixed-effects models and pairwise comparisons. Boxes sharing a letter are not significantly different from one another and are only comparable within genus. **(A)** Chao1 index vs. month. **(B)** Simpson's diversity index vs. month. **(C)** Faith's phylogenetic diversity vs. month.

(**Figure 2**). Month was a significant predictor of Chao1 [$p < 0.001$, $F_{(4,41)} = 11.724$], Simpson's diversity [$p < 0.001$, $F_{(4,41)} = 17.740$], and Faith's phylogenetic diversity [$p < 0.001$, $F_{(4,35.85)} = 18.986$, **Supplementary Table 5**]. For all three measures, alpha diversity significantly decreased from March to May, increased from May to July, and decreased from July to January 2017 (**Figure 2**). The low replication in May compared to March and July for *Pocillopora* samples may contribute to this

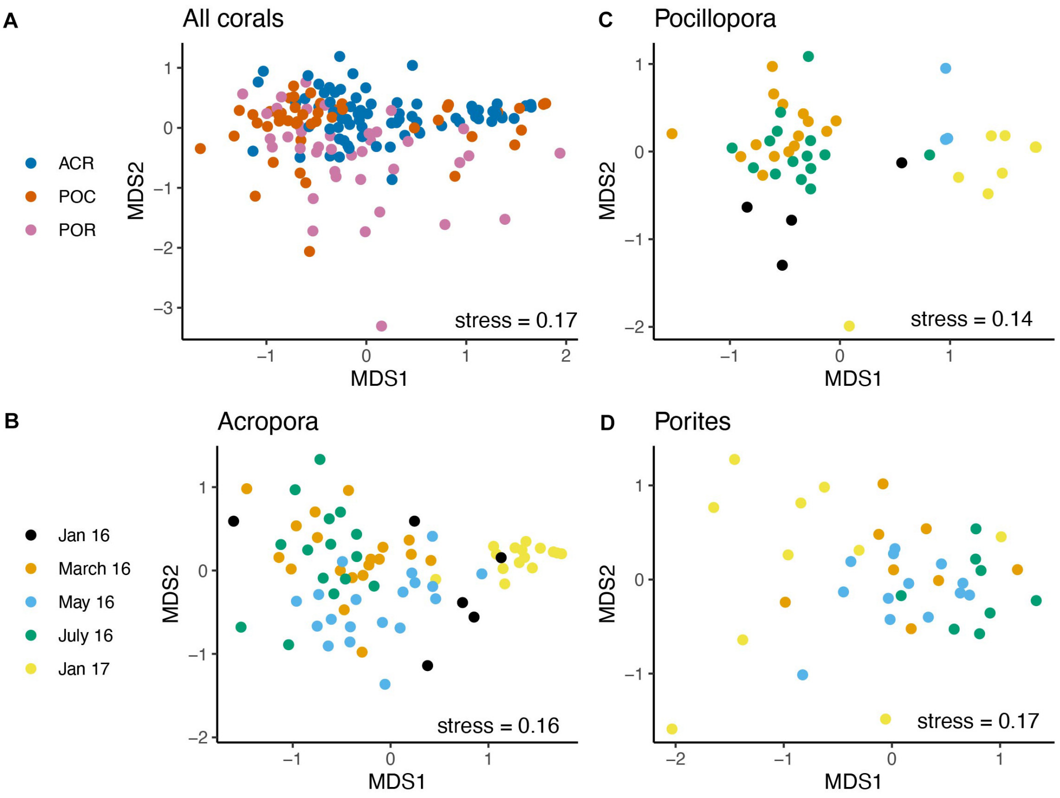

FIGURE 3 | Coral microbiomes are distinct across month and between coral genera. The bacterial community data were first log-transformed and dissimilarity was calculated using weighted UniFrac. NMDS ordination of **(A)** All corals combined by genus, **(B)** *Acropora* samples by time, **(C)** *Pocillopora* samples by time, and **(D)** *Porites* samples by time.

pattern. However, all three samples from May were consistently dominated by *Endozoicomonas* (0.960 ± 0.026) as in January 2017 (0.875 ± 0.109), compared to March (0.143 ± 0.072) and July (0.105 ± 0.064) (**Figure 1**).

Thermal Stress and Recovery Produced Distinct Microbial Communities in All Three Coral Hosts

PERMANOVA results, with month as the predicting factor, showed the presence of distinct microbial communities for all four measures of community dissimilarity. Month explained the most variance using weighted UniFrac distances (PERMANOVA; $p < 0.001$, $R^2 = 0.270$, **Supplementary Table 6**), and pairwise comparisons showed that all months were significantly different from one another. Coral host genus ($p < 0.001$, $R^2 = 0.072$, **Figure 3A**) and the interaction between genus and month ($p < 0.001$, $R^2 = 0.074$) were also significant using weighted UniFrac distances. In fact, all four dissimilarity measures found month, host genus, and their interaction significant for predicting distinct microbial communities. Nutrient treatment did not produce distinct communities for any dissimilarity measure.

For all four dissimilarity measures, month produced distinct communities in *Acropora* corals, while treatment and the interaction between month and treatment did not (**Supplementary Table 7**). Month explained the most variance with weighted UniFrac distances (PERMANOVA; $p < 0.001$, $R^2 = 0.403$), and pairwise comparisons showed that all pairwise comparisons of month were different (**Figure 3B**). Likewise, month produced distinct communities for *Pocillopora* ($p < 0.001$, $R^2 = 0.427$, **Supplementary Table 8**) and *Porites* ($p < 0.001$, $R^2 = 0.249$, **Supplementary Table 9**) samples using weighted UniFrac distances (**Figures 3C,D**). All four months were significantly different from each other for *Porites* samples from pairwise comparisons. For *Pocillopora* samples, January 2016 was not different from May 2016, nor were March and July 2016 or May 2016 and January 2017.

Community Dispersion Varied Among Coral Hosts Over Time

Across all corals, community dispersion was significantly different over time but only for the Binary Jaccard presence/absence measure (PERMDISP; $p < 0.01$, $F = 3.609$). Dispersion varied by coral genus with weighted UniFrac ($p < 0.01$, $F = 4.766$, **Figure 4A**), Bray-Curtis ($p < 0.01$, $F = 4.911$), and Binary Jaccard ($p < 0.01$, $F = 5.880$). Additionally, there were no differences in dispersion by nutrient treatment across all coral hosts and any dissimilarity measure (**Supplementary Table 10**). Dispersion differed

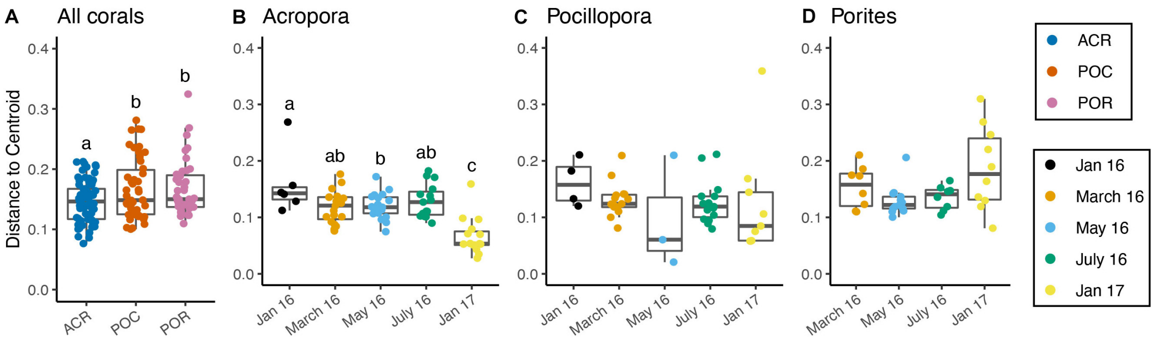

FIGURE 4 | Microbiome dispersion varies by coral host and over time. **(A)** All corals combined by genus, **(B)** *Acropora* corals by time, **(C)** *Pocillopora* corals by time, **(D)** *Porites* corals by time. Boxes sharing a letter are not significantly different from one another. Dispersion was not significant for *Pocillopora* samples, but was significant for *Porites* samples, although there were no significant pairwise differences.

significantly among sampling periods for *Acropora* corals based on the weighted UniFrac distances ($p < 0.001$, $F = 13.009$, **Figure 4B**), but was not significantly different among nutrient treatments ($p = 0.386$, $F = 0.964$, **Supplementary Table 11**). Pairwise comparisons showed that community dispersion in January 2017 was significantly less than in all other months and dispersion in May 2016 was significantly less than in January 2016 ($p < 0.01$). Dispersion did not significantly differ among months for *Pocillopora* samples ($p = 0.671$, $F = 0.591$, **Figure 4C**). Dispersion was also significantly different between months for *Porites* samples ($p < 0.05$, $F = 3.459$, **Supplementary Table 11**) with May having the lowest dispersion and January 2017 having the highest although no pairwise comparisons were significant after correction (**Figure 4D**).

Differentially Abundant Taxa Increased During Thermal Stress and Decreased During Recovery

Differential abundance analysis with ANCOM was performed on *Acropora*, *Pocillopora*, *Porites*, and combined coral samples to assess if specific bacterial genera significantly changed in abundance relative to other genera in the community. There were no differences in taxon abundance by nutrient treatment for combined and individual coral communities. However, there were differentially abundant bacterial genera between months ($p < 0.05$, $W = 0.9$). A total of 14 bacterial genera were significantly differentially abundant in all coral samples combined (**Figure 5A** and **Supplementary Table 12**). *Acropora* corals had 11 differentially abundant taxa with month, while *Pocillopora* and *Porites* corals had 2 and 3 differentially abundant taxa, respectively (**Figures 5B–D**). *Endozoicomonas* was differentially abundant across all corals combined, *Acropora* alone, and *Pocillopora* alone, but not *Porites* corals alone. *Candidatus Amoebophilus* was only differentially abundant within *Pocillopora* samples, while *Streptococcus* was only differentially abundant within *Porites* samples and all corals combined. Interestingly, *Pseudoxanthomonas* was differentially abundant for *Acropora* alone and all corals combined and was found exclusively in May 2016 (**Figures 5A,B**). Based on relative abundance (**Figure 5**), differentially abundant taxa across all samples appear to fall into three categories: (a) moderate decreases in May 2016 and severe decreases in January 2017 compared to March and July (i.e., *Acinetobacter*, *Pseudomonas*, *Corynebacterium*), (b) nearly exclusive occurrence in May 2016 (i.e., *Paenibacillus*, *Alteromonas*, *Reyranella*, *Pseudoxanthomonas*), (c) increased abundance and occurrence in January 2017 (i.e., *Endozoicomonas*).

DISCUSSION

We tracked the composition and stability of microbiomes associated with *Acropora*, *Pocillopora*, and *Porites* corals throughout a thermal stress event under ambient nutrient conditions and nitrogen enrichment. We found that microbiomes varied widely across months, potentially due to the temperature fluctuations that contributed to the stress event. Periods of thermal stress were accompanied by increased alpha diversity and community heterogeneity. Coral microbiomes returned to a state of reduced diversity, dominated by *Endozoicomonas*, some months following the event. Neither nitrate nor urea exposure had any effects on community diversity or abundance of individual taxa despite experimental evidence that nitrate and urea diffusers increase the concentration of nitrogen in the surrounding seawater over a 10-week period (Burkepile et al., 2019). Contrary to our hypothesis, nitrogen did not interact with month, which is inherently connected with seawater temperatures. Instead, temperature likely overwhelmed any effects of nutrients. Although conclusions presented here are limited by reduced sample sizes in some groups (**Table 1**), our data demonstrate the importance of collecting time series datasets across several coral hosts and with sufficient sampling periods to capture the dynamics of microbiome recovery post stress.

Alpha Diversity Changes Under Seasonal Thermal Stress Vary Between Coral Genera

Microbiome species richness of Mo'orean corals varied significantly among months and host genus. SST peaked in March

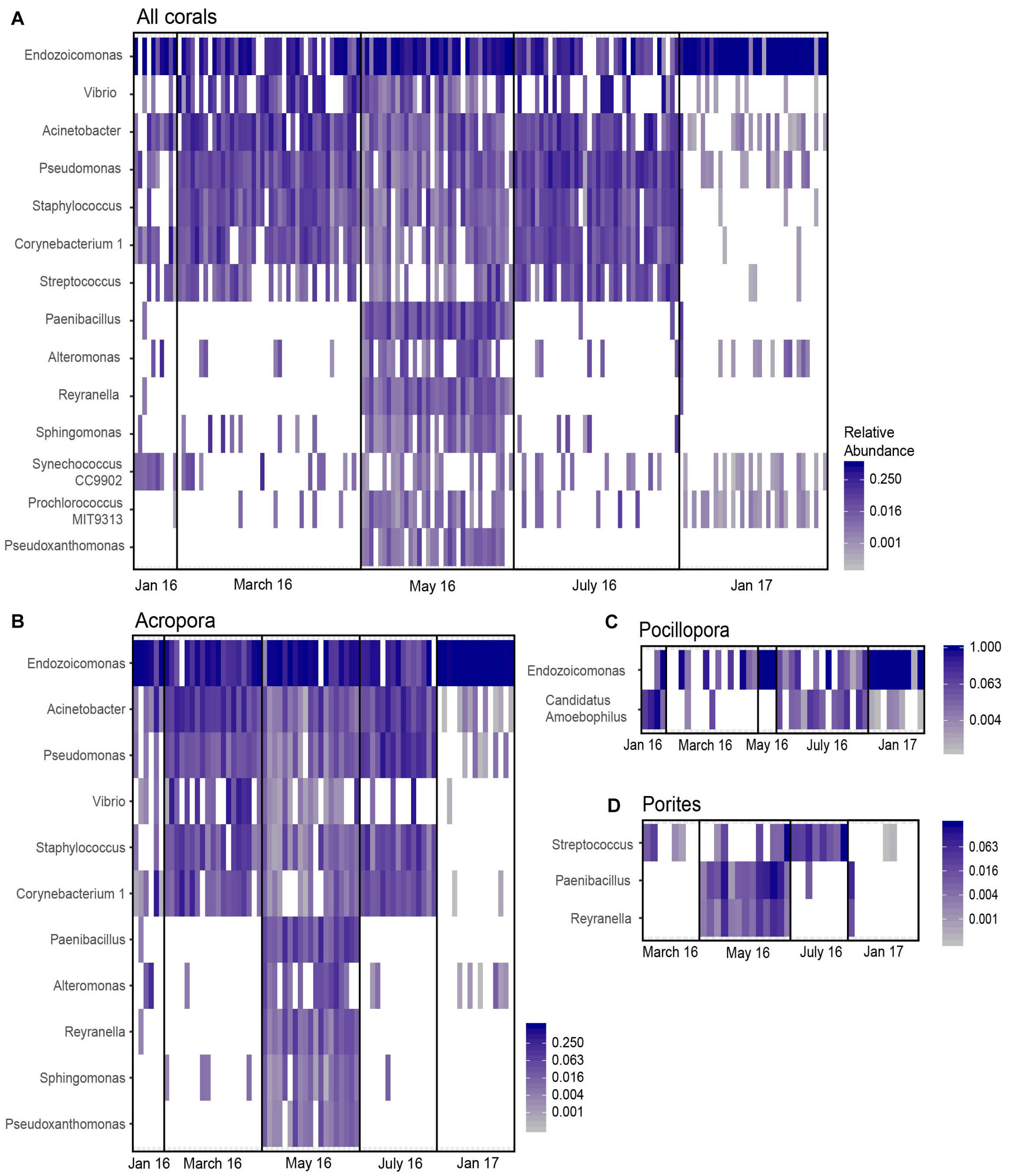

FIGURE 5 | Differentially abundant taxa across month identified by ANCOM. Significant taxa were identified for **(A)** all samples combined, **(B)** *Acropora* samples only, **(C)** *Pocillopora* samples only, and **(D)** *Porites* samples only. Cells are scaled by the relative abundance and white cells indicate an absence of that taxon in the sample.

2016 and decreased slightly but maintained bleaching-level temperatures until May. Interestingly, mean microbial species richness peaked in May 2016 for *Acropora* and *Porites* corals. A similar result was found in a study of *Agaricia* corals in the Florida Keys, where microbial species richness was highest in the month following temperature and bleaching highs (Wang et al., 2018). This could suggest that the colonization or establishment of temperature-sensitive opportunistic taxa into a stressed coral microbiome may be delayed following peak thermal stress. Two putative examples of such opportunistic taxa are *Paenibacillus* and *Reyranella*, which occurred almost exclusively in May in both *Acropora* and *Porites* corals (**Figures 5B,D**). Alternatively, opportunistic taxa may become established stochastically throughout the duration of stress events, with species richness gradually increasing as long as the event lasts. To distinguish between these patterns, future microbial time series will be required which span bleaching events with sufficiently fine-scale repetitive sampling.

Time series should also consider including multiple coral host species, since the patterns in diversity observed in Mo'orea varied by host (**Figures 2**, **3**). Contrary to the response of *Acropora* and *Porites* microbiomes, *Pocillopora* microbiomes experienced a drastic decrease in alpha diversity following peak temperatures (**Figure 2**). The reduction in observed species richness was accompanied by a much higher relative abundance of the putative coral symbiont *Endozoicomonas* (**Figure 5**). This pattern could be the result of an active regulatory response to exclude heat-associated opportunists, possibly mediated by *Endozoicomonas* (Neave et al., 2016). However, it is also possible that drastically increased absolute abundance of *Endozoicomonas* outcompeted the rest of the community (with unknown implications for host health), or even simply overwhelmed signatures of other taxa with relative abundances too low to detect at our sequencing depths. If an active regulatory mechanism were responsible, the increased diversity in July could reflect the eventual failure of this response to exclude or reduce opportunists such as *Vibrio*, *Pseudomonas*, and *Staphylococcus*, which subsequently increased in relative abundance at that time (**Figure 1**). If a drastic increase in *Endozoicomonas* absolute abundance was responsible for the patterns, these opportunists could have been present throughout March, May, and July but gone undetected in May. Distinguishing between these possibilities could be a target of future studies that sequence samples to much greater depths.

Dynamics of *Endozoicomonas* Abundance Drive Community Variability and Resilience

Our results add to mounting evidence supporting the importance of *Endozoicomonas* in shaping coral microbiomes (Neave et al., 2016; McDevitt-Irwin et al., 2017; Pollock et al., 2018; Maher et al., 2019). For both *Acropora* and *Pocillopora* corals, the abundance of *Endozoicomonas* significantly changed over the thermal stress event (**Figures 5B,C**). Most notably, January 2017 samples of both corals were dominated almost exclusively by *Endozoicomonas*. For *Acropora* corals, this was accompanied by a significant reduction in the sample-to-sample variability (**Figure 4B**). *Pocillopora* samples also appear to be less variable during January 2017, although low replication may have prevented us from detecting a response in dispersion (**Figure 4C**) (Anderson and Walsh, 2013). In contrast, *Porites* samples during this time point were highly variable (**Figure 4D**). Interestingly, *Endozoicomonas* abundances in *Porites* did not significantly change over the thermal stress event (**Figure 5D**). Experiments and surveys on *Porites lobata* in Mo'orea have shown a similar community response under various stressors, including mechanical wounding, predation, corallivore feces deposition, and combinations of stressors (Ezzat et al., 2019a, 2020). In these experiments, Hahellaceae (family of *Endozoicomonas*) was a dominant member of the coral microbiome but was generally not differentially abundant with stress. Hahellaceae only decreased significantly 3 h after corals were exposed to feces, but recovered to control levels within 48 h (Ezzat et al., 2019a). This suggests that while the dominant symbiont *Endozoicomonas* fluctuates in abundance during stress for *Acropora* and *Pocillopora* corals, this taxon is generally less variable in *Porites* corals. However, the relative proportion of this taxon did still change in *Porites* samples, particularly in July (**Figure 1**). Thus, despite lower variability, these changes could still result in shifts in the relative contribution of *Endozoicomonas* to microbiome function in *Porites*.

The dynamics of *Endozoicomonas* throughout this experiment combined with evidence of its involvement in holobiont sulfur cycling suggest its potential functional role in microbiome resilience (Bourne et al., 2016). The dominance of *Endozoicomonas* at the final month for *Acropora* and *Pocillopora* corals may be explained by sulfur cycling processes in the coral holobiont. Corals are significant sources of dimethylsulfoniopropionate (DMSP) and dimethylsulfide (DMS) in reef waters (Broadbent and Jones, 2004). Research shows that coral DMSP and DMS production is upregulated during oxidative stress, such as warming events and bleaching (Lesser, 2006; Deschaseaux et al., 2014). Some *Endozoicomonas* species can metabolize DMSP to DMS, using DMSP as a carbon source for growth and survival (Tandon et al., 2020). Increased DMSP production during stress could provide substrate for *Endozoicomonas* to proliferate and confer the taxon a competitive advantage over other coral-associated taxa. This could explain the dominance of *Endozoicomonas* by January 2017 to levels that surpass those of pre-bleaching communities.

The increase in abundance of *Endozoicomonas* during oxidative stress could confer benefits to their coral host that may provide resilience during thermal stress. For instance, the breakdown of DMSP to DMS by *Endozoicomonas* produces carbon (Tandon et al., 2020) which could provide the coral with an alternative carbon source during recovery from thermal stress to partially compensate for the loss of energy-supplying algal symbionts. Furthermore, the coral pathogen *Vibrio coralliilyticus* uses DMSP as a strong cue to find heat-stressed hosts through chemotaxis and chemokinesis (Garren et al., 2014). The increased metabolism of DMSP by growing *Endozoicomonas* populations after thermal stress could reduce the amount of chemoattractant for *Vibrio* spp. to detect, potentially helping to alleviate *Vibrio* infection. However, we did not find any evidence *Vibrio* spp. abundance was influenced by *Endozoicomonas* and the idea that *Endozoicomonas* provide benefits to their coral hosts remains speculative. Future investigation is warranted to determine what role *Endozoicomonas* plays in holobiont sulfur cycling and overall health during temperature stress.

Dynamics of Opportunistic Microbiota Differentiate Hosts' Responses to Stress

The number of bacterial genera that significantly fluctuated throughout the thermal stress event may provide evidence for coral host-specific mechanisms for coping with environmental change. For instance, *Acropora* samples had more differentially abundant bacterial taxa than *Pocillopora* or *Porites*. This could be related to the fact that *Acropora* were also the most sensitive of the three coral genera to bleaching (Burkepile et al., 2019). *Acropora* corals have been described as microbiome conformers by adapting to changing environmental conditions while *Pocillopora* corals were described as microbiome regulators by remaining stable through change (Ziegler et al., 2019). For instance, Ziegler et al. (2019) found the microbiome of *A. hemprichii* to be readily "responding" and variable across different anthropogenic impacts and flexible upon transplantation. It remains to be determined whether microbiome restructuring is a deterministic mechanism for beneficial holobiont adaptation or plasticity or if it is a stochastic response to dysbiosis. However, *Acropora* corals were less variable than *Pocillopora* or *Porites* corals (**Figure 4A**) suggesting that more deterministic changes were driving *Acropora* community dynamics (Zaneveld et al., 2017). Based on our evaluation of the number of individual bacterial taxa that changed in abundance, *Porites* may fall closer to the "microbiome regulator" side of the two proposed stress-response mechanisms. However, differentiating conformers from regulators may require a closer look at the identity and function of those individual bacterial taxa.

The high microbiome flexibility in *Acropora* may leave the host-associated community vulnerable to the loss of important or beneficial symbionts and their corresponding functions or to the acquisition of pathogens. For instance, bacterial genera present in March and/or May 2016 including *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, *Corynebacterium 1*, *Alteromonas*, and *Vibrio* have each been association with various coral stressors including elevated seawater temperature and ocean acidification (Grottoli et al., 2018), hyper-salinity (Röthig et al., 2016), bleaching (Koren and Rosenberg, 2008), bacterial challenge (Wright et al., 2017), and coral disease (Sweet et al., 2013). However, these associations with stress are not always consistent across studies and stressors. For example, *Acinetobacter*, *Corynebacterium 1*, and *Vibrio* have been found both in association and not associated with Dark Spot Syndrome (Sweet et al., 2013; Meyer et al., 2016) and *Acinetobacter* has also been found in high abundance with healthy corals (Cai et al., 2018). Similarly, *Pseudomonas* was found to be positively associated with hyper-salinity but negatively associated with bleaching (Ritchie et al., 1994; Röthig et al., 2016). The coarse classification of bacterial taxa to the genus-level in these studies as well as the study presented here limit our ability to detect finer scale functional differences, for instance at the species or strain level. Although these taxa are associated with thermal stress in this study, future functional analysis at the sOTU level would better discern their potential positive or negative contributions to holobiont health.

In contrast, although taxa changed in relative abundance, we did not detect differentially abundant stress-associated bacterial taxa in *Pocillopora* corals (**Figures 1**, **5**). This may be due to our reduced replication for *Pocillopora* samples in May 2016. Alternatively, this may represent the coral host's or microbiome's ability to strategically maintain a stable and robust microbial community during stress. That said, abundance of the dominant symbiont *Endozoicomonas* changed throughout temperature stress despite evidence that the globally conserved association between *Pocillopora verrucosa* and *Endozoicomonas* remains unchanged during bleaching or mortality (Pogoreutz et al., 2018; Maher et al., 2019). However, evidence from previous studies is based on short-term (<1 month) aquaria experiments that may not reflect microbiome dynamics on the reefs over realistic timescales (Pogoreutz et al., 2018; Maher et al., 2019). Additionally, the abundance of the taxon *Candidatus Amoebophilus* which has been associated with diseased and healthy corals (Apprill et al., 2016) significantly changed in *Pocillopora* with decreases in abundance and occurrence in March and May (**Figure 4**). This taxon is a member of the core microbiome for Australian corals and an intracellular symbiont of eukaryotes with genomic evidence of a symbiotic lifestyle (Schmitz-Esser et al., 2010; Pollock et al., 2018). Its reduction in March and May could reflect an interaction with Symbiodiniaceae within the coral tissue (Apprill et al., 2016) which are then lost during thermal stress. The decrease of putative symbionts in *Pocillopora* corals contrasts sharply with the increase of potential opportunists in *Acropora* and *Porites* corals further supporting differential host responses to thermal stress.

Effects of Temperature May Overwhelm Those of Nutrients

Elucidating the combined effects of nitrogen pollution and thermal stress on corals is critical to predicting how coral reefs will respond to increasing levels of anthropogenic stress. Previously, a superset of the corals evaluated in the present study were surveyed for bleaching response over the mild bleaching event during the austral summer of 2016 in Mo'orea, French Polynesia. This study found that, compared to corals in ambient conditions, *Acropora* and *Pocillopora* corals that were exposed to nitrate exhibited more frequent bleaching, bleached for longer duration, and were more likely to die (Burkepile et al., 2019). In contrast, we found that under combined and prolonged heat and nitrogen stress, enrichment with either ammonium or nitrate had no discernable effect on the composition of the coral microbiome. Previous work supports the hypothesis that the coral host and microbiome have parallel responses under stress (Ziegler et al., 2017). Our selection of samples that survived the 2016 bleaching event may have inadvertently biased our dataset to corals that did not bleach (bleached $n = 7$). This may have prevented us from detecting any effects by nitrogen on the microbiome that parallel the significant interaction between temperature and nitrate and the significant differences between nitrate and urea observed in the coral host response (Burkepile et al., 2019).

Our results suggest that thermal stress likely overwhelmed the coral microbiome such that additional nutrient stress had no measurable effect. We found no significant interactions on microbiome diversity between nitrogen enrichment and increased seawater temperatures. This corroborates work on *Pocillopora meandrina* in tanks in Mo'orea and *Agaricia* spp. on the reef during severe bleaching in the Florida Keys (Wang, 2006; Maher et al., 2019). Importantly, this result is consistent on the coral reefs studied regardless of disturbance history and during both moderate and severe bleaching events. We show that even under a mild thermal stress event, nutrients do not differentially affect the coral microbiome. However, since few bleached corals were included in our study and because we could not control for temperature, we cannot eliminate the possibility that bleaching response itself may impose some stress-exposure threshold that allows for interactions with nutrients and temperature in terms of changing microbial community dynamics.

Future Research Implications

With thermal stress events increasing in severity and frequency, future research should investigate if and how the homogenization of coral microbiomes after thermal stress will prepare coral holobionts for future stress events. After exposure to a warmer, more variable environment, *Acropora* corals in American Samoa were themselves more tolerant to a subsequent acute heat stress in the laboratory, exhibiting a robust and stable microbiome (Ziegler et al., 2017). This suggests that corals surviving one heat stress may have increased tolerance to future heat stress events. Whether tolerance of the host coral is conferred or promoted through microbiome composition remains to be determined (Ziegler et al., 2017). Burkepile et al. (2019) observed nitrate-treated *Acropora* corals in Mo'orea bleaching for longer duration in the more severe 2017 bleaching event. Evaluation of microbiome dynamics in time series over repetitive stress events could help determine if microbiome tolerance can be developed through stress exposure and if an *Endozoicomonas*-dominated community plays a role in microbiome tolerance.

DATA AVAILABILITY STATEMENT

The datasets and code generated for this study can be found in the online repositories. The names of the repositories and accession number can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA627248; https://github.com/maherrl/RAPID-analysis.

AUTHOR CONTRIBUTIONS

RV, DB, SH, and RS conceived and designed the experiment. AS and TA conducted the experiment. ES and SM conducted the labwork. RLM conducted bioinformatic and statistical analysis with assistance from RV, RM, SM, AS. LE, RLM, RV, and DB interpreted the data. RLM wrote the manuscript. All authors reviewed 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/fevo.2020.555698/full#supplementary-material

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Increased Algal Symbiont Density Reduces Host Immunity in a Threatened Caribbean Coral Species, *Orbicella faveolata*

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Scleractinian corals are the principal builders of coral reefs. These megadiverse ecosystems are declining due to coral mortality from a variety of stressors, including disease. Corals are dependent upon symbiotic dinoflagellates in the family Symbiodiniaceae for phototrophic contributions to their energy budgets. However, suppression of host immunity may be necessary to maintain these intracellular symbioses. To explore the consequences of symbiosis on host immunity, we manipulated symbiont density by increasing nitrogen availability. Replicate cores from four colonies of the Caribbean coral, *Orbicella faveolata*, were reared in seawater treated with ammonium for 1 month to increase symbiont density. Corals were then immune-stimulated using lipopolysaccharide and poly I:C. Gene expression was analyzed using RNAseq and symbiont density was quantified (as symbiont:host cell ratio) using quantitative PCR (qPCR). Ammonium treatment had limited positive effects on host immunity. In contrast, increases in symbiont density had large negative effects on host expression of immune-related transcripts. These results suggest links between nutrient enrichment and coral disease may be the result of the effect of increased symbiont density on host immunity, rather than the direct effect of the nutrients. Further study of the trade-offs between symbiont density and immunity may help understand how decreasing water quality and increasing disease will shape future reef communities.

Keywords: coral, immunity, symbiosis, nutrient enrichment, climate change, marine disease

INTRODUCTION

Coral reefs are one of the most important ecosystems on the planet, both in terms of biodiversity (Odum and Odum, 1955; Sebens, 1994; Roberts, 1995; Bellwood et al., 2006) and ecosystem services (Smith, 1978; Spurgeon, 1992). However global climate change and other anthropogenic effects have exposed these ecosystems to numerous stressors, including rising sea surface temperature (Hughes et al., 2003; Hoegh-Guldberg et al., 2007), and epizootic outbreaks (Daszak et al., 1999; Harvell et al., 1999; Sutherland et al., 2004). Increases in outbreaks of coral disease, particularly in the Caribbean, have been one of the largest drivers of coral declines (Harvell et al., 1999; Daszak et al., 2001; Sutherland et al., 2004; Croquer and Weil, 2009; Weil et al., 2009;

Weil and Rogers, 2011). Disease outbreaks have been growing in frequency and severity over the past 50 years, and the number of described coral diseases has rapidly proliferated (Sutherland et al., 2004). There is considerable variation within and between species in disease susceptibility, but understanding of the factors contributing to this variation is limited (Fuess et al., 2017; Wright et al., 2017). This understanding is critical to forecasting reef futures under rapidly declining conditions.

Rapid increases in coral disease outbreaks have necessitated equally rapid improvement in our understanding of coral immunity. Corals are now known to possess a robust immune system with many aspects similar to human innate immunity. This includes functional toll-like signaling pathways (Miller et al., 2007; Shinzato et al., 2011; Poole and Weis, 2014), complement (Miller et al., 2007; Burge et al., 2013; Pinzon et al., 2015), and melanin cascades (Mydlarz et al., 2008; Palmer et al., 2008; Mydlarz and Palmer, 2011). Furthermore, there has been some investigation of how these pathways may contribute to observed variation in disease susceptibility (Pinzon et al., 2014; Fuess et al., 2017). Indeed, different lineages of corals appear to use different types of immunity in response to immune stimulation (Pinzon et al., 2014), and variation in activated pathways may directly contribute to disease susceptibility (Fuess et al., 2017). However little attention has been paid to external factors that may contribute to the observed variation in immune response. For example, symbiosis is well-known to interact with immunity in cnidarians (Mansfield et al., 2017; Mansfield and Gilmore, 2019), but few studies have examined how variation in symbiotic relationships might contribute to observed variation in immune function and disease susceptibility.

Reef-building corals are critically dependent on their symbiotic algae to meet the majority of their nutritional needs. The relationship between host corals and their symbiotic dinoflagellates (Family Symbiodiniaceae) underpins the success of these organisms in nutrient-poor environments (Muscatine and Porter, 1977; Muscatine, 1984, 1990). Both symbiont identity (Rowan, 1998) and density (Fagoonee et al., 1999; Fitt et al., 2000) vary between and within coral species, and microhabitat variation can lead to further intra-colony variation in these associations (Rowan et al., 1997; Cunning et al., 2015b). However, while it is well-known that symbiont identity directly contributes to the susceptibility of the host to thermally induced bleaching (loss of symbionts) (Rowan, 1998, 2004; Berkelmans and van Oppen, 2006), fewer studies have examined the effects of symbiont density on host stress tolerance (Cunning and Baker, 2013, 2014). Furthermore, little is known regarding the effects of variation in symbiotic relationships on host disease susceptibility (Correa et al., 2009; Detournay et al., 2012). Preliminary findings suggest that symbionts may negatively regulate host immunity, through both the TGFβ and sphingosine rheostat pathways, in order to establish and maintain relationships (Detournay and Weis, 2011; Detournay et al., 2012). However these studies have been primarily conducted in model systems such as anemones, and therefore their ecological significance for reef corals is unknown.

In order to examine the effects of variation in symbiont density on host coral immune response, we conducted an experimental manipulation of the Caribbean coral *Orbicella faveolata*, which is listed as Threatened under the U.S. Endangered Species Act, and Endangered by the IUCN Red List. Using ammonium enrichment, symbiont density was manipulated in replicate cores of the same coral colony. Following this manipulation, corals were exposed to a brief immune stimulation. This study details the transcriptomic effects of experimental immune stimulation, ammonium treatment, and variation in symbiont density on host corals, and presents findings that suggest that nitrogen enrichment and symbiont density can have different effects on both host coral gene expression and immunity.

MATERIALS AND METHODS

Coral Collection

O. faveolata colonies were collected in February 2016 from King Neptune reef, West Palm Beach, Florida (*n* = 1) and in May 2016 from Emerald Reef, Key Biscayne, Florida (*n* = 3). Replicate cores (*n* = 16–21 per colony) of 1 cm diameter were obtained using a drill press and attached to labeled ceramic plugs using cyanoacrylate glue. The cores were maintained in outdoor tanks to allow them to recover for 12 days until the beginning of the ammonium treatment.

Ammonium Enrichment

In July 2016, experimental cores were moved to indoor water tables with a 12 h light/dark cycle and PAR intensity ∼250 μM quanta m^{-2} s^{-1}. Coral cores from each colony were evenly split between Control (C) and Nutrient (N) treatments (*n* = 8–11 per colony per treatment) and allocated to one of two replicate 20.8 L glass aquaria. Each aquarium contained filtered seawater from the University of Miami Marine Technology and Life Science Seawater (MTLSS) complex (intake at Bear Cut, Miami) and maintained in temperature-controlled tanks at ∼27°C for 47 days. NH_4Cl was added to each N-treatment aquaria to increase the ammonium concentration by 20 μM, with water changes and aquarium cleaning every 2–3 days (including control aquariums). Ammonium concentrations were selected based on existing literature and the goals of our study (Muscatine et al., 1998; Zhou et al., 2017).

Experimental Design

Following the 12-day acclimation period and 47-day ammonium treatment period, cores were haphazardly allocated to control and immune stimulation treatment groups such that colony and nutrient treatments were evenly split into immune-stimulated and non-immune stimulated groups. The six experimental aquaria, three for each immune treatment group, were then randomly distributed between water tables. Samples were allowed to acclimate to new tanks for approximately 24 h, and each aquarium was continuously aerated and maintained at 27°C for the duration of the experiment.

After the second acclimation period, corals were then subjected to immune stimulation treatments. Treatment cores were injected with a 20 μg/mL PAMP solution (20 μg/mL lipopolysaccharides (LPS) from *Escherichia coli* 0127:B8 (Sigma-Aldrich L3129-100 MG) and 20 μg/mL Poly (I:C) (Sigma-Aldrich P9582) in equal parts). Inoculants were prepared in sterile seawater and warmed to 27°C prior to injection. 25 μL of PAMP solution was injected into four random polyps from each core, for a total injection volume of 100 μL. Injections were performed using a 27G 1 1/4″ needle (BD). Control cores were injected in the same manner with the same sterile seawater used in the preparation of the inoculant.

Following injections, cores were maintained in ambient conditions for 2 h after which a small tissue sample was taken from each core (∼1 polyp) with a razor blade to identify and quantify algal symbionts. Experimental cores were then removed from the tanks and immediately flash-frozen in liquid nitrogen. All samples were shipped on dry ice to the University of Texas at Arlington where they were stored at −80°C until processed.

Symbiont to Host Cell Ratios

Total genomic DNA was extracted from razor blade samples following established protocols (Baker and Cunning, 2016). Extracted DNA was analyzed using qPCR assays. For the coral hosts, we used a SYBR green assay (Life Technologies (Cunning et al., 2015a), which targets a single copy locus in *O. faveolata* (Severance et al., 2004), For the algal symbionts (*Cladocopium* and *Durusdinium*) we used actin-based assays developed for these taxa (Cunning and Baker, 2013; Cunning et al., 2015a). Each coral sample was run in duplicate and amplification was considered positive only when the two technical replicates amplified and there was no amplification in no-template controls.

Symbiont to host (S:H) cell ratios were calculated using StepOneR package for R (Cunning, 2018). Briefly, this repository corrects the C_T values obtained from the qPCR machine for differences in organism ploidy and DNA extraction efficiency, and calculates the symbiont to host call ratio with the formula $2\hat{}(C_T \text{ host} - C_T \text{ symbiont})$. The sum of all symbiont genera ratios (*Cladocopium*:Host + *Durusdinium*:Host) is reported as the Total S:H ratio for each sample. However, most corals (see Results) were found to only host *Durusdinium*.

Differences in S:H cell ratio were evaluated with a two-way ANOVA with nutrient treatment (C vs. N) and colony as independent variables, and S:H as the dependent variable. Prior to statistical tests, homogeneity of variance was assessed using multiple models and showed no significant differences. Since both nutrient and colony had a significant effect on the S:H cell ratio ($p < 0.0001$), we used a *post-hoc* Tukey HSD test (alpha = 0.05) to perform multiple comparisons. All the analyses were performed in R (version 3.5.3) using $\log_{10}$-transformed S:H data to meet assumptions of normality.

RNA Extraction and Sequencing

Full transcriptome sequencing was conducted on a subset of samples. One replicate per colony and treatment group was randomly selected for RNA extraction ($N = 16$). RNA was then extracted for a small fragment of core (∼20 mg tissue) using the RNAqueous with DNAse step kit (Life Technologies AM1914). Extracted RNA quality was determined using an Agilent BioAnalyzer 2100 and samples with RIN numbers (quality values) higher than 7 were sent to the University of Texas Austin Genomic Core Facility, which used llumina TruSeq RNA with Poly-A selection libraries kits (Illumina) to create cDNA libraries prior to sequencing on a single lane with 100 bp single end reads.

Transcript Alignment and Annotation

Following sequencing, the Trimmomatic v. 3 software package was used with default settings (leading = 3, trailing = 3, slidingwindow = 4:15) to remove Illumina adaptors and low quality reads (Bolger et al., 2014). Reads were then aligned to the existing reference *O. faveolata* transcriptome using Tophat (Trapnell et al., 2009; Pinzon et al., 2015). One sample had poor alignment to the reference transcriptome (∼3%), likely due to contamination. This sample, belonging to the CC treatment group was removed from subsequent analyses, reducing sample size for this group to 3. The reference host transcriptome was re-annotated against the UniProtKB/Swiss-Prot database (release-2017_03), using a blastx algorithm (0.0E-5 e-value threshold; Altschul et al., 1990; Camacho et al., 2009) prior to analysis of differentially expressed transcripts.

Differential Expression and Gene Ontology Analyses

Read count matrices were generated using the Cufflinks (Trapnell et al., 2013) and htSeq (Anders et al., 2015) packages. Differential expression analyses were then conducted in the R package, DESeq2 (Love et al., 2014). Prior to modeling low count reads were removed. Differential expression was modeled using treatment combination and S:H cell ratio as additive effects. It should be noted that this model assumes a linear relationship between S:H cell ratio and gene expression. This approach separates the factors and allows for the identification of distinct effects of symbiont density vs. experimental treatments on host gene expression. Genotype was not included in the model. Average $\log_2$fold change per transcript was then generated for all relevant contrasts between treatment combinations (NC vs. CC, CI vs. CC, NI vs. CI, NI vs. NC), and for the effects of S:H cell ratio. Significantly differentially expressed transcripts were identified based on $\log_2$fold change ($padj < 0.05$) across treatments.

Gene Ontology analyses of each list of differentially expressed genes were conducted using the R script GOMWU (Wright et al., 2015) with default parameters. Briefly, the script uses the "stat" value and annotation for all transcripts to generate a rank-based estimate of enriched GO terms. GO terms were considered significant if *padj* was less than 0.05.

Coexpression Network Analyses

In order to identify groups of coexpressed genes that were potentially correlated with treatment and symbiont density, the R package Weighted Gene Correlation Network Analyses (WGCNA) was used to analyze the full host transcriptome (v. 1.66; Langfelder and Horvath, 2008). Normalized read counts for all transcripts were derived using the variance stabilizing transformation in DESeq2 (Love et al., 2014). Using these values for all 15 samples (one outlier removed), a single signed network was built with manual signed blockwise network construction methods (bicor correlations, max block Size = 44,000, soft power = 20, minimum module size = 100, merge cut height = 0.25). Module eigengene values (average expression of all transcripts in a module) of each resulting module were then correlated to a variety of factors (nutrient enrichment treatment, immune treatment, S:H cell ratio) using a bicor correlation. Host genotype was not included in these analyses. Modules with significant correlations were identified as those with $p \leq 0.05$. These modules were then further analyzed using the R script GO-MWU with default parameters for the analysis of WGCNA modules (Wright et al., 2015). Significant GO terms associated with a module were determined as those with $p < 0.05$.

RESULTS

Effect of Ammonium Enrichment on Algal Symbiont Density

Our four colonies of *O. faveolata* predominantly hosted *Durusdinium trenchii*, although symbionts in the genus *Cladocopium* were also detected in 3 of 74 cores sampled in the experiment, two from colony 2 and one from colony 4. However, *Cladocopium* abundance in these cores was <5% of the total symbiont community. Overall, corals exposed to elevated ammonium doubled their symbiont to host (S/H) cell ratios compared to controls ($p < 0.001$; **Figure 1**), despite significant variation by colony in the initial symbiont densities ($p < 0.001$; **Figure 1**).

Host Differential Gene Expression

We obtained a total of 28,343,735 sequencing reads for an average of approximately 1,771,483 reads per sample. On average, roughly 48% of reads per sample aligned to the reference transcriptome. There was no correlation between S:H ratio and either total reads or alignment rate (**Supplementary Table S1**). Transcriptional analysis revealed broad effects of symbiont density on host transcription, coupled with minor effects of ammonium enrichment and immune stimulation. A total of 2,853 transcripts were differentially expressed as the result of one or more treatments. Only seven of these transcripts (0.016%) were affected by ammonium treatment (NC v. CC), of which 4 were annotated, including one heat shock cognate 71 kDa protein, and a peroxidasin homolog, both of which were

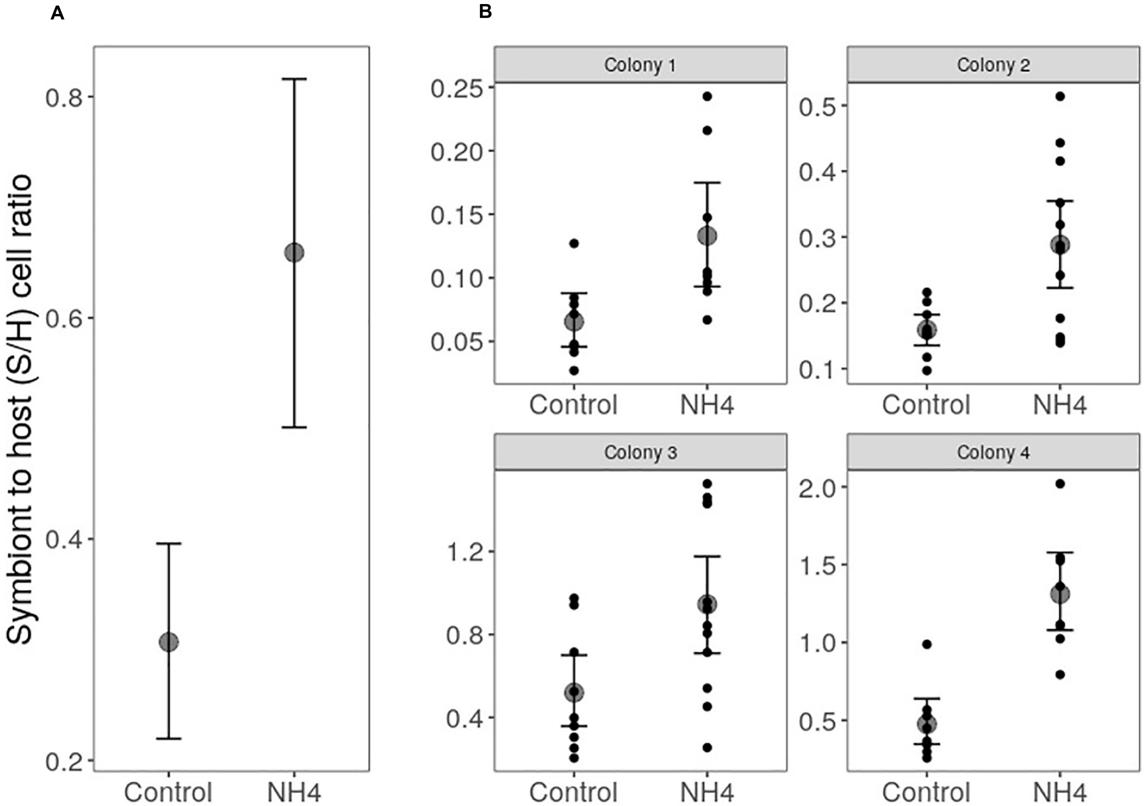

FIGURE 1 | Effect of ammonium enrichment on *O. faveolata* symbiont to host (S/H) cell ratio (mean ± 95 CI) across. Two-way ANOVA revealed higher S/H cell ratios in corals exposed to elevated ammonium ($p < 0.001$; **A**), regardless the differences in the S/H cell ratio among coral colonies ($p < 0.001$; **B**). Letters represent significantly different groups obtained with Tukey's HSD test (alpha = 0.05). Statistical analysis was performed on $\log_{10}$ transformed S/H cell ratios to increase normality of the data, but graphs show untransformed values.

upregulated (**Figure 2**). A total of 18 biological process (BP) GO terms were significantly enriched as a result of ammonium treatment, including two positively enriched terms involved in immune system processes: antigen processing and presentation of peptide antigen via MHC class I and cell activation involved in immune response (**Supplementary Figure S1**). In contrast, 8 transcripts were significantly differentially expressed as a result of immune stimulation under control conditions (CI v. CC), none of which were annotated. Six BP GO terms were significantly enriched as a consequence of immune challenge, the majority of which were positively enriched and involved in translation and RNA processing (**Supplementary Figure S2**).

There were more widespread effects of ammonium treatment on the subsequent immune response of host corals, with 23 transcripts significantly differentially expressed as a result of immune stimulation following ammonium treatment (NI v NC). Of these, 10 were annotated (**Figure 3**), including several putative immune transcripts (GFP-like fluorescent chromoprotein cFP484 and tyrosinase). Additionally, 45 BP GO terms were enriched following immune challenge in ammonium-treated corals (**Supplementary Figure S3**). Finally, corals pretreated with elevated ammonium had different responses to immune challenge than those maintained under control conditions (NI v. CI), with 165 transcripts differentially expressed between these two groups, including transcripts with immune function such as peroxiredoxins, TLR responsive protein deleted in malignant brain tumor protein 1 (Rosenstiel et al., 2007), and tyrosinase (**Supplementary Figure S4**). A total of 44 BP GO terms were significantly enriched between these two groups, including terms involved in ciliary process (expressed higher in NI corals) and terms involved in cell cycle and growth (expressed higher in CI corals) (**Supplementary Figure S5**).

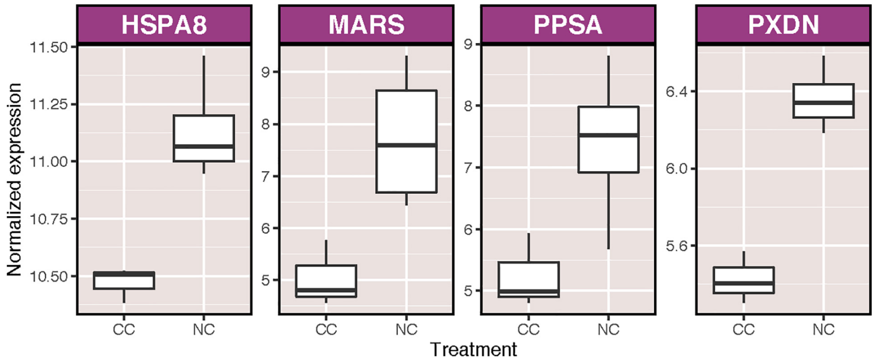

FIGURE 2 | Variance stabilized normalized expression of annotated transcripts that were significantly differentially expressed as a result of the addition of nutrients to coral samples. Genes are listed in alphabetical order. Those displayed are: heat shock protein 70 kDA 8 (HSPA8), methionine-tRNA ligase (MARS), phthiocerol synthesis polyketide synthase type I PpsA (PPSA), and peroxidasin homolog (PXDN).

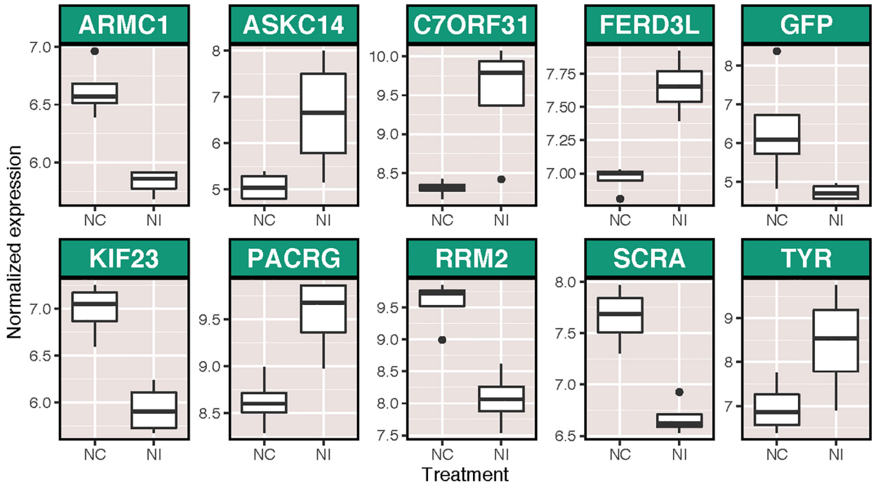

FIGURE 3 | Variance stabilized normalized expression of annotated transcripts that were significantly differentially expressed as a result of immune stimulation in corals pretreated with ammonium treatment. Genes are listed in alphabetical order. Those displayed are: armadillo repeat-containing protein 1 (ARMC1), U-actitoxin-Avd3q (ASKC14), uncharacterized protein C7orf31 (C7ORF31), fer3-like protein (FERD3L), GFP-like fluorescent chromoprotein cFP484 (GFP), kinesin-like protein KIF23 (KIF23), parkin coregulated gene protein homolog (PACRG), ribonucleoside-diphosphate reductase subunit M2 (RRM2), anillin (SCRA), and tyrosinase (TYR).

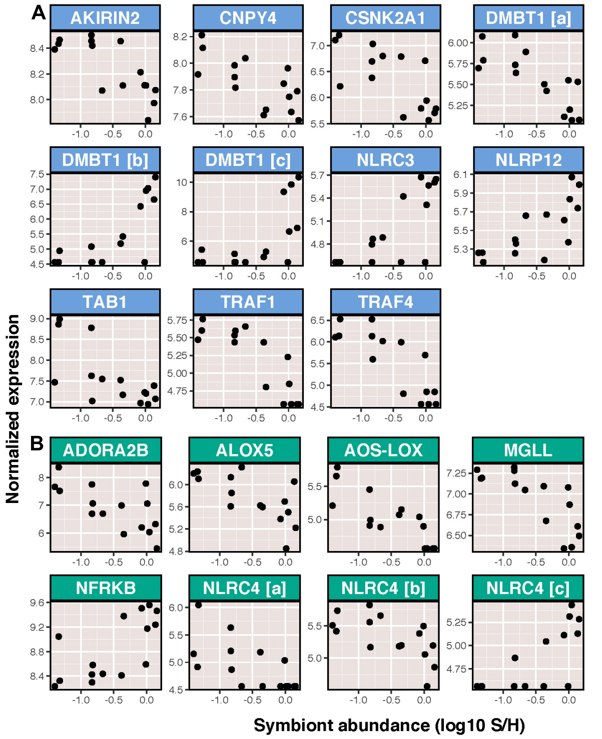

FIGURE 4 | Symbiont abundance vs. variance stabilized normalized expression of significantly differentially expressed transcripts with functions related to or downstream of **(A)** toll like receptor signaling and **(B)** inflammation. Genes are listed in alphabetical order. Those displayed are: **(A)** akirin-2, protein canopy homolog 4 (CNPY4), casein kinase II subunit alpha (CSNK2A1), deleted in malignant brain tumors protein 1 (DMBT1), protein NLRC3, NACHT, LRR, and PYD domains-containing protein 12 (NLRP12), TGF-beta activated kinase 1 (TAB1), TNF-receptor associated factor 1 (TRAF-1), and TNF-receptor associated factor 4; **(B)** adenosine receptor A2b (ADORA2b), arachidonate 5-lipoxygenase (ALOX5), allene oxide synthase-8R-lipoxygenase (AOS-LOX), monoglyceride lipase (MGLL), nuclear factor related to kappa-B binding protein (NFRKB), and NLR family CARD domain-containing protein 4 (NLRC4).

In contrast to the relatively minor effects of ammonium treatment and immune stimulation on host transcription, symbiont density was correlated with a large portion of transcripts (2,686 transcripts or approximately 6% of the transcriptome). This included changes in expression of 73 immune transcripts, the majority of which (48) decreased in expression as a result of increasing symbiont density. This group included transcripts involved in the toll-like receptor signaling pathway and inflammation (**Figure 4**). Despite this, the effects of symbiont density resulted in just four significantly enriched BP GO terms involved in either microtubule process (positively enriched) or translation (negatively enriched).

Host Coexpression Analyses

We then conducted coexpression analyses to identify large groups of genes associated with treatments and/or symbiont density. Coexpression analysis resulted in a network of 18 modules, plus a 19th module (8,376 transcripts) that contained transcripts that could not be placed into any other module (**Supplementary Figure S6**). Modules ranged in size from 197 (module 11) to 7,051 (module 8) transcripts each. After testing for correlation between average expression (eigenvalue) of these modules and our treatment groups as well as symbiont density, we identified six candidate modules (**Supplementary Figure S8**).

Three modules were positively correlated to ammonium treatment or symbiont density. Module 3, which contained 3,933 genes (1,117 of which were annotated), was significantly positively correlated with symbiont density ($r = 0.57$, $p = 0.03$). No significantly enriched BP GO terms were included in this module. Module 4 was significantly positively correlated to ammonium treatment ($r = 0.74$, $p = 0.002$). This module contained 1,988 genes, of which 743 were annotated. Gene ontology analysis revealed significant enrichment of 45 biological terms, mostly involved in ciliary processes (**Supplementary Figure S8**). Finally, module 6 was positively correlated to symbiont density ($r = 0.79$, $p = 5^{e-4}$). This module was not enriched for any BP GO terms.

Three modules were negatively correlated to ammonium treatment, immune challenge, or symbiont density. Module 16 was negatively correlated to immune challenge ($r = -0.59$, $p = 0.02$), and contained 468 genes, of which 216 were annotated. In addition, this module was significantly enriched for 40 BP GO terms, most of which were involved in cell cycle processes and growth (**Supplementary Figure S9**). Module 18 was negatively correlated to ammonium treatment ($r = -0.45$, $p = 0.009$). This module contained 975 transcripts and was enriched for nine BP GO terms (**Supplementary Figure S10**). Finally, module 8, which contained 7,051 transcripts, was negatively correlated with symbiont density ($r = -0.69$, $p = 0.004$). This module was enriched for three BP GO terms positive regulation of type I interferon process, regulation of antigen receptor-mediated signaling, and response to thyroid hormone. The 20 transcripts included in this module contributed to enrichment of two immune-related BP GO terms (**Figure 5**).

DISCUSSION

We used ammonium enrichment to manipulate algal symbiont density in a threatened reef coral species (*O. faveolata*) and then exposed these corals to an immune challenge to test the effect of symbiont density on host coral gene expression and response to a mock immune challenge. We documented widespread effects of these treatments, or resulting variation in symbiont density, on the coral transcriptome (2,853 differentially expressed transcripts, ~6%). Separating the effects of symbiont density from ammonium treatment using our DESeq2 model indicated most changes in gene expression were induced by variation in symbiont density (2,686 transcripts) rather than on the ammonium enrichment itself (7 transcripts, only ~0.016% of the transcriptome). Changes associated with increased symbiont density included negative effects on host expression of immune contigs. Here we discuss the potential ecological importance of variation in coral-algal symbiotic relationships in coral disease dynamics.

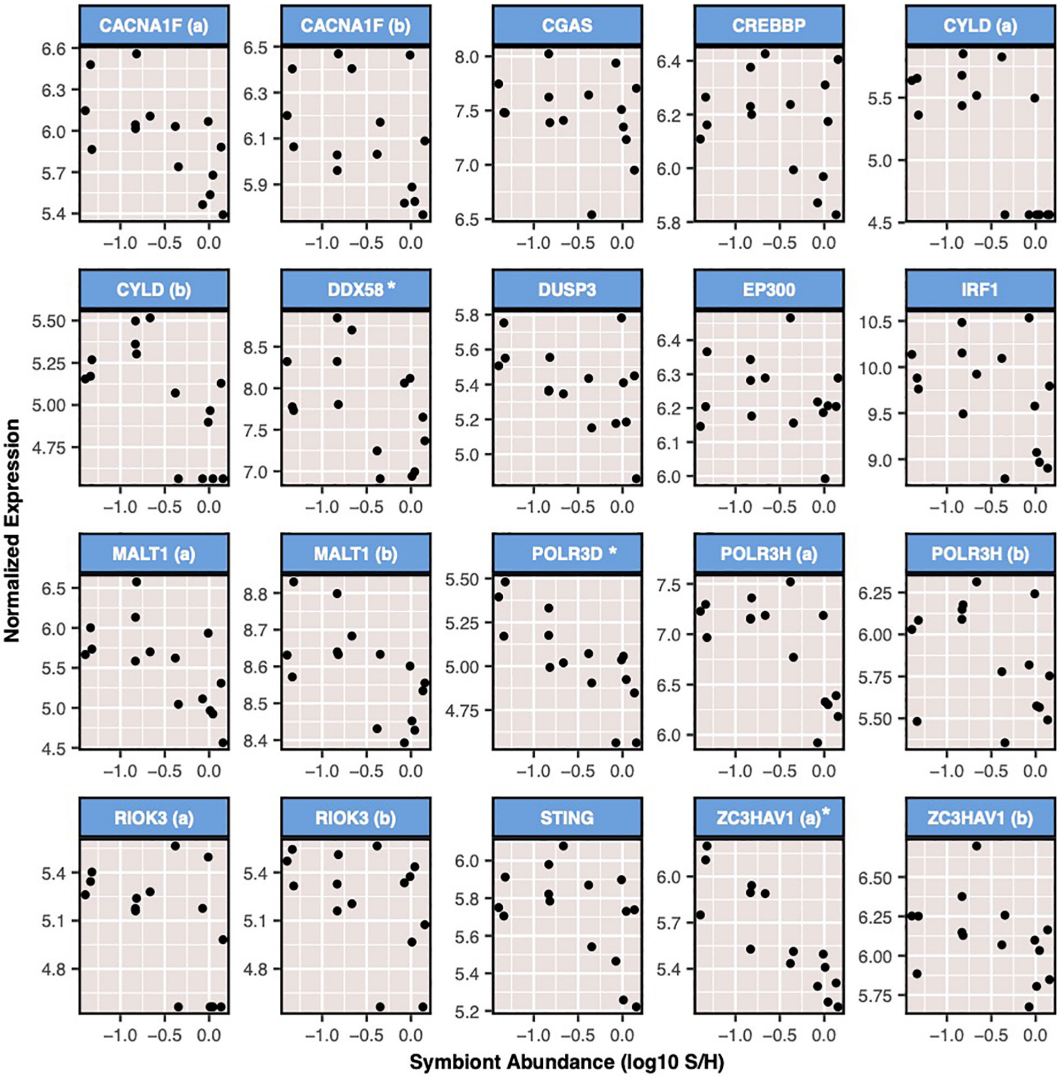

FIGURE 5 | Expression of transcripts from module 8 that were included in significantly enriched BP GO terms related to immune function. Expression is shown for all samples relative to sample S/H (symbiont density). Expression is displayed as variance stabilizing normalized values. * indicates transcripts which were significantly differentially expressed as a result of symbiont density (*Padj* < 0.05). Genes are listed in alphabetical order by gene id. Those displayed are: voltage-dependent L-type calcium channel subunit alpha-1F (CACNA1F), cyclic GMP-AMP synthase (CGAS), CREB-binding protein (CREBBP), ubiquitin carboxyl-terminal hydrolase CYLD, Antiviral innate immune response receptor RIG-I (DDX58), dual specificity protein phosphatase 3 (DUSP3), histone acetyltransferase p300 (EP300), interferon regulatory factor 1 (IRF1), Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), DNA-directed RNA polymerase III subunit RPC4 (POLR3D), DNA-directed RNA polymerase III subunit RPC8 (POLR3H), serine/threonine-protein kinase RIO3 (RIOK3), stimulator of interferon genes (STING), and zinc finger CCCH-type antiviral protein 1 (ZC3HAV1).

Gene ontology analysis of coexpression network correlations revealed that the minimal effects of the ammonium treatment on host transcriptome included an effect on ciliary action and extracellular matrix function. Increases in ciliary action are expected in response to additional nutrients; studies have shown that corals are capable of increasing ciliary action to create vortices and promote nutrient exchange (Shapiro et al., 2014). Thus, observed positive correlations between ammonium treatment and expression of ciliary transcripts may reflect host coral mechanisms to promote nutrient acquisition and waste disposal associated with increased metabolism. Additionally, changes in extracellular matrix are expected as a result of increasing photosynthesis associated with ammonium treatment. Coral mesoglea layers are primarily comprised of ECM components (Tucker and Adams, 2014), and changes in the thickness of the mesoglea may affect light availability within tissues (Dimond et al., 2012). Negative correlations between ammonium treatment and ECM are thus likely are related to thinning of the mesoglea to increase light availability and support increased photosynthesis. In addition, ammonium treatment had a slight positive effect on host coral immunity. Ammonium treatment increased both the basal expression of immune-related transcripts and the host's response to immune stimulation. This observed trend may be attributed to allocation of increased nutrients to enhanced immune defense (Schneeberger et al., 2013). However, previous ecological findings that suggest nutrient enrichment (including increased nitrogen) results in increasing disease prevalence (Bruno et al., 2003; Voss and Richardson, 2006; Vega Thurber et al., 2014), which suggests that either these increases in constitutive immunity are insufficient, or more complex dynamics not modeled in our laboratory experiment drive these observed trends.

More significant were the widespread effects of variation in symbiont density on host gene expression. Nearly 6% of the host's transcriptome was affected by variation in symbiont density, rather than ammonium treatment itself, as indicated by our model. Affected transcripts belonged to an exceptionally wide variety of cellular pathways. This is not unexpected, since previous findings have suggested that symbiont density can have significant effects on host stress response, among other processes (Cunning and Baker, 2013, 2014). While corals are dependent on their relationship with Symbiodiniaceae to meet their nutritional needs (Muscatine and Porter, 1977; Muscatine, 1984, 1990), numerous studies have indicated that there may also be cellular-level trade-offs associated with maintaining this symbiotic relationship (Rowan, 1998, 2004; Berkelmans and van Oppen, 2006; Cunning and Baker, 2013, 2014). Consistent with this concept, our results support far-reaching and significant effects of symbiont density on cellular stress and immune response in corals. It should be noted that while the most likely explanation of this variation in gene expression is due to changes in symbiont density. However, as coral colonies naturally vary in symbiont density, some of this effect could be confounded with genotype.

Variation in symbiont density correlated to numerous putative immune transcripts involved in a diversity of pathways. Specifically, nearly 50 immune-related transcripts decreased in expression as a result of increasing symbiont density. The toll-like receptor and inflammatory pathways appeared to be most affected by variation in symbiont density. This included the downstream TLR mediator Akirin2 (Tartey et al., 2014), which was negatively correlated to symbiont density, as well as the negative regulator of TLR signaling, NLRC3 (Schneider et al., 2012), which was positively correlated to symbiont density. Additionally various pro-inflammatory transcripts such as ALOX5 (Herb et al., 2008), and two copies of NLRC4 (Tomalka et al., 2011) were negatively correlated to symbiont density. Finally, coexpression analysis also revealed negative correlations between symbiont density and immune response, specifically anti-viral response. Module 8 was negatively correlated to symbiont density and enriched for two GO terms related to immunity, which were mostly comprised of transcripts involved in antiviral responses. This included antiviral transcripts: ubiquitin carboxyl-terminal hydrolase CYLD (CYLD) (Friedman et al., 2008), probable ATP-dependent RNA helicase DDX58 (DDX58; also known as RIG-I) (Loo and Gale, 2011), DNA-directed RNA polymerase III subunits RPC4 (POLR3D) and RPC8 (POLR3H) (Chiu et al., 2009), and zinc finger ccch-type antiviral protein 1 (ZC3HAV1) (Hayakawa et al., 2010). Several of these (DDX58, POLR3D, and one copy of ZC3HAV1) were significantly differentially expressed as a result of symbiont density.

Together, these patterns of differential expression and co-expression networks suggest a trade-off between symbiont density and multiple types of immunity. Toll-like receptor signaling, inflammation, and antiviral responses comprise a large portion of the innate immune responses of scleractinian corals (Mydlarz et al., 2016). Furthermore, these three pathways play important roles in defense against the three major types of cnidarian pathogen: bacterial (Libro et al., 2013; Libro and Vollmer, 2016; Brennan et al., 2017), fungal (Mydlarz et al., 2008), and viral (Soffer et al., 2014; Sweet and Bythell, 2017). *O. faveolata* has a complete TLR-NF-kB signaling pathway which is responsive to bacterial LPS (Williams et al., 2018); inflammation plays a role in response of the octocoral *Gorgonia ventilina* to *Aspergillus sydowii* (Mydlarz et al., 2008) and in the response of *Acropora cervicornis* to white band disease (Libro et al., 2013; Libro and Vollmer, 2016); antiviral responses may also be an important component of *A. cervicornis* response to white band disease (Libro and Vollmer, 2016). The strong negative effects of symbiont density on these three important coral immune response pathways suggests significant implications for coral disease susceptibility, and justify further study of the relationship between symbiosis and immunity in corals, particularly given increased disease incidence on coral reefs.

While we were able to document significant effects of symbiont density on the immune response of *O. faveolata*, we should note that the mechanism of this relationship remains unclear. Previous studies have suggested that symbiont may exploit host pathways such as TGFβ (Detournay et al., 2012; Berthelier et al., 2017) and the sphingosine rheostat (Detournay and Weis, 2011) to elicit changes in host coral gene expression, particularly as it relates to immunity. Furthermore, new findings suggest that TGFβ is capable of modulating immune response in *O. faveolata* (Fuess et al., 2020). Specifically, both toll-like receptor signaling (Naiki et al., 2005; Lee et al., 2011) and inflammation (Letterio and Roberts, 1998; Huynh et al., 2002) are intimately controlled by TGFβ signaling, and our results document significant negative correlations between symbiont density and these pathways. While these patterns suggest a potential role of TGFβ in mediating observed trends, we were unable to find robust transcriptomic evidence of such a link. However, it is still possible that TGFβ signaling drives observed patterns using mechanisms undetectable with transcriptomic analyses (e.g., post-translational modification, etc.). Thus, robust further investigation of the roles of TGFβ in linking symbiosis and immunity is necessary. Finally, we should note that most of our experimental corals contained one species of algal symbiont (*D. trenchii*). It is possible that other algal symbionts may modify coral immune response in different ways. Thus, both density and identity of algal symbionts may influence disease susceptibility and are worthy of further research. Thus, both density and identity of algal symbionts may influence disease susceptibility and are worthy of further research.

In sum, these findings suggest an important, and ecologically relevant, trade-off in coral-algal symbiosis. Reef coral hosts are dependent on their algal symbionts for nutrition (Muscatine and Porter, 1977; Muscatine, 1984, 1990), but higher symbiont densities may reduce host immune response, potentially increasing disease susceptibility. Since elevated nutrients can increase symbiont densities, this suggests that poor water quality may not only result in corals that are less thermally tolerant (Cunning and Baker, 2013), but may also lead to immune-compromised corals that are more susceptible to disease. In the face of increasing water temperatures and disease prevalence, this trade-off may result in new selective pressures on reef symbioses. Consequently, it is essential to further explore coral-algal symbiosis from the perspective of coral immunity and its potential ecological implications for future reef communities.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA668736.

AUTHOR CONTRIBUTIONS

AP-C and AB planned collections, collected, and maintained the corals from reefs near Miami. AP-C conducted ammonium treatments prior to experimentation and conducted qPCR assays. LF and AP-C planned and conducted immune stimulation experiment. LF and CB processed samples for RNA extraction and sequencing, assembled the transcriptome, and conducted all relevant analyses. LF and LM wrote the manuscript with editorial assistance from AP-C, CB, and AB. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.572942/full#supplementary-material

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Deciphering Coral Disease Dynamics: Integrating Host, Microbiome, and the Changing Environment

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Diseases of tropical reef organisms is an intensive area of study, but despite significant advances in methodology and the global knowledge base, identifying the proximate causes of disease outbreaks remains difficult. The dynamics of infectious wildlife diseases are known to be influenced by shifting interactions among the host, pathogen, and other members of the microbiome, and a collective body of work clearly demonstrates that this is also the case for the main foundation species on reefs, corals. Yet, among wildlife, outbreaks of coral diseases stand out as being driven largely by a changing environment. These outbreaks contributed not only to significant losses of coral species but also to whole ecosystem regime shifts. Here we suggest that to better decipher the disease dynamics of corals, we must integrate more holistic and modern paradigms that consider multiple and variable interactions among the three major players in epizootics: the host, its associated microbiome, and the environment. In this perspective, we discuss how expanding the pathogen component of the classic host-pathogen-environment disease triad to incorporate shifts in the microbiome leading to dysbiosis provides a better model for understanding coral disease dynamics. We outline and discuss issues arising when evaluating each component of this trio and make suggestions for bridging gaps between them. We further suggest that to best tackle these challenges, researchers must adjust standard paradigms, like the classic one pathogen-one disease model, that, to date, have been ineffectual at uncovering many of the emergent properties of coral reef disease dynamics. Lastly, we make recommendations for ways forward in the fields of marine disease ecology and the future of coral reef conservation and restoration given these observations.

Keywords: coral, reefs, disease, microbiome, dysbiosis, climate change, physiology, genotype

INTRODUCTION

Insights into disease mechanisms are being broadly reconsidered (Byrd and Segre, 2016), and investigations into coral disease highlight many of the issues in identifying single pathogens that can reproduce the signs of a specific disease. Coral biologists have cataloged outbreaks on reefs since the 1970s (Antonius, 1973; Garrett and Ducklow, 1975; Mitchell and Chet, 1975; Dustan, 1977; Gladfelter et al., 1977; Gladfelter, 1982), and evidence from paleontological and ecological monitoring suggest that the number and geographic distribution of coral disease epizootics increased in recent years (Richardson, 1998; Precht et al., 2002, 2016; Sutherland et al., 2004; Harvell et al., 2007; Tracy et al., 2019). Global coral disease distributions are both diverse and widespread across all ocean basins, indicating hotspots occur in multiple geographic locations (**Figure 1**).

Yet despite extensive monitoring and exploration, only six described coral diseases have a known pathogen that can repeatedly initiate a consistent disease phenotype. To date, Koch's postulates was fulfilled for few coral diseases, although numerous studies have also questioned these results (**Table 1**). Disease outbreaks often appear in relatively pristine environments, during mild seasons, and in ideal ecological states (e.g., low host densities), altogether countering many theories in traditional disease ecology and confounding pathogen identification. The environmental variables (e.g., temperature, salinity, organic and inorganic nutrient concentrations, benthic competitor density, predator abundance) that influence coral disease outright are numerous for sure, and those that are indirect are likely incalculable. Thus, many controversies about the etiological agents and ecological conditions responsible for coral diseases likely result in: (1) our inability to distinguish coral phenotypes or disease signs, (2) our incomplete understanding of the interactive roles of host phylogeny, genotype, immunology and physiology, (3) animal and reef-associated microbial and viral community dynamics, (4) the contextual roles that a highly variable environment play in coral disease susceptibility, onset, progression and transmission and (5) the limitation on reproducibility or comparability among studies. Although these issues are not unique to coral disease (Egan and Gardiner, 2016; Logan et al., 2016; Apprill, 2017; Levy et al., 2017), the rapidity and severity of coral disease driven decline has brought these issues to the forefront of coral biology research.

Therefore, after over 40 years of work, we argue here that by adopting the assumptions from classic wildlife disease ecology, our paradigms and viewpoints have been clearly insufficient to understanding the dynamics of coral disease ecology—particularly the one pathogen-one disease framework. Therefore, to identify emergent properties of coral reef disease ecology, that will help us preserve these ecosystems in the future, we must adopt and adapt more holistic and modern paradigms that reflect the complexities of our system, including interactions among the three major players associated with most epizootics (**Figure 2**): the host, its microbiome, and the environment. Below we present a discussion of each component of the classic disease triad, how adhering to traditional wildlife disease frameworks limits current understanding of coral diseases, and why expanding our approach to studying each component separately and collectively provides a way forward.

PART I: THE HOST

The host animal is clearly a critical facet in the quest to decipher disease dynamics in any system. However, the sessile, modular nature of corals, and the complexity of their endosymbioses with algae and bacteria introduce unique challenges not typically considered in the development of disease ecology theory in other animals. In addition, subtle but important variation in the evolutionary history, genetics, physiology, and immunity among coral species makes predicting the pathogen or groups of pathogens underlying coral diseases elusive because not all hosts are equally permissive to infections. Here, we describe the main contributors to variability in coral hosts that have hampered our ability to produce reliably validated hypotheses about disease dynamics across the coral tree of life, yet we also make recommendations for ways forward in this area of coral biology research to help us in these endeavors.

Coral Disease Phenotypes and Disease Classification

A major hurdle in the field of coral disease ecology is our reliance on linking characteristic phenotypes of corals to explicit disease signs. Weil et al. (2009) expressed this problem well when asserting that descriptions of many coral diseases are limited and often confounded by the lack of clear diagnostic criteria with no pathological observations, so that similar disease signs may emerge in multiple coral species (Weil, 2004; Work and Aeby, 2006; Raymundo et al., 2008; Work et al., 2008). In other words, ascribing a single pathogen to a disease sign indicative of numerous conditions hampers our ability to monitor the drivers of disease. For example, Couch et al. (2014) found that diffuse subacute tissue loss was the most common phenotype across all species suffering from distress. Similarly, Bourne et al. (2015) highlighted that white syndromes are a collective term for unexplained patterns of coral tissue loss of unknown etiology in Indo-Pacific corals. These examples illustrate a common challenge that arises because tissue loss is a general and widespread clinical sign that may have multiple root causes. Indeed, coral biologists acknowledge that corals may only have a limited number of ways to express visual signs of disease. This has led to a push for systematic, detailed species-specific descriptions to facilitate sharing of disease information, especially when the etiology remains unknown (Work and Aeby, 2006).

Coral Evolutionary History

The long evolutionary history of scleractinian corals has given rise to variability in biological and ecological characteristics both among and within coral species that are central to current difficulties in identifying the etiology of coral diseases. Although simple in design, scleractinian corals are a group of organisms that have evolved over more than 400 million years, a timeline equivalent to the evolution of land plants. Coral have diverged into 21 distinct clades that represent potentially hundreds of

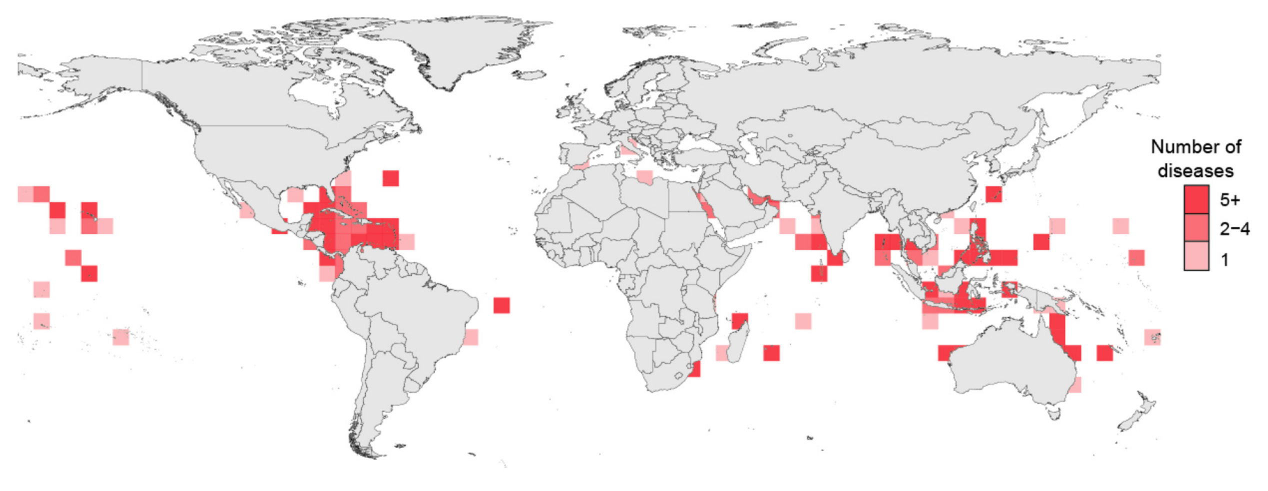

FIGURE 1 | Recorded coral disease richness from surveys published between 1973 and 2020.

different species (Fukami, 2008; Huang, 2012; Huang and Roy, 2013, 2015). This relatively long evolutionary history and high species diversity creates uncertainty in the ongoing efforts to generalize how all coral hosts might respond to various pathogens and environmental conditions.

Differences in disease susceptibility among species illustrates the extent of host variability in corals, as entire coral families experience higher rates and severity of disease than others (Pinzon et al., 2014; Williams et al., 2020). Acroporids and pocilloporids generally show elevated susceptibility to disease (Willis et al., 2004), particularly diseases like white syndromes that affect many coral species (Hobbs et al., 2015). Palmer et al. (2010) attributed broad patterns of differential susceptibility among coral families to differing levels of investment in immune parameters in a meta-analysis of skeletal eroding band disease. Pocilloporids were most susceptible, followed by acroporids and finally poritids (Palmer et al., 2010). These kinds of patterns may have a clear phylogenetic basis. For example, Pinzon et al. (2014) demonstrated that species-specific and genus-specific disease susceptibility in Caribbean corals was higher in modern taxa and lower in corals from earlier lineages such as *Porites* and *Siderastrea* that diverged more than 200 MYA.

TABLE 1 | Coral disease studies confirming Koch's Postulates and work refuting the findings.

Coral disease	Paper confirming pathogen	But see
White band type II	Richie and Smith, 1998	
White plague type II	Richardson et al., 1998	Pantos et al., 2003; Kellogg et al., 2013
Aspergillosis	Nagelkerken et al., 1997; Geiser et al., 1998; Smith et al., 1998	Toledo-Hernández et al., 2008
Acroporid serratiosis	Patterson et al., 2002; Sutherland et al., 2011	Lesser and Jarett, 2014
Bacterial bleaching	Kushmaro et al., 1997; Ben-Haim et al., 2003b	
White syndromes	Sussman et al., 2008; Pollock et al., 2017	

Coral Species Genotypic Diversity

Even among coral species within the same genus, responses to exposure to the same pathogen or environment can differ. The important role that a coral's genotype plays in its resistance and resilience to thermal stress and bleaching has been recognized for some time (Edmunds, 1994; Fitt et al., 2009). Bleached coral colonies can directly neighbor colonies of the same species that do not exhibit bleaching (Edmunds, 1994; Ritson-Williams and Gates, 2020). Even less is known about genotypic variation in disease resistance within coral species, but evidence is mounting that it is important for disease dynamics (Muller et al., 2018). Conceptually, coral genotypes should differ in the same ways

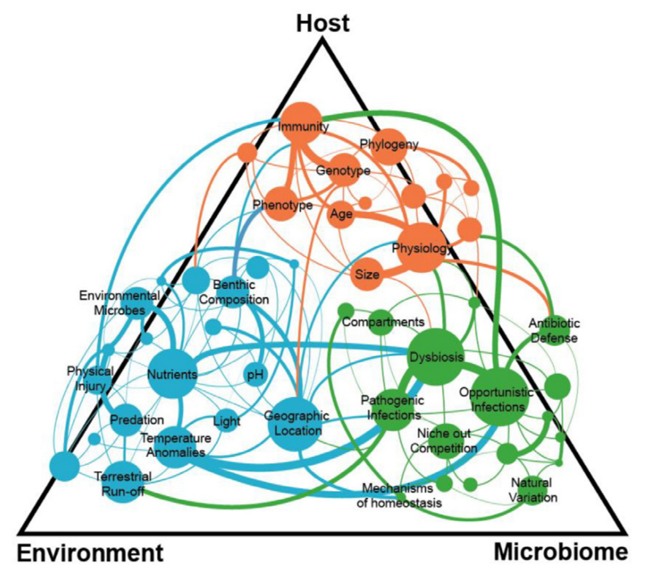

FIGURE 2 | Complex interactions within and among the coral host, environment, and microbiome can lead to a disease state with many connections still unknown.

that coral species differ in four main ways: (1) the signs of disease they display at a certain level of infection, (2) the way they manifest damage, (3) their degree of resistance and (4) degree of tolerance. As an example, genetic diversity among species complicates disease diagnosis. White syndrome lesions in different *Porites* species healed at different rates (Lozada-Misa et al., 2015). In this case, coral morphology is a critical metric of susceptibility as massive and branching *Porites* species showed different white syndrome dynamics. Another example of differences in the level of resistance was found by Mullen et al. (2006), when they detected differential prevalence of a fungal disease in three gorgonian species in the Yucatan, hypothesizing that lower prevalence in *Gorgonia mariae* than in *G. ventalina* resulted from its greater chemical defenses.

Another major knowledge gap is the degree and rate of increased host genotype resistance following an epizootic. Several studies examined whether coral populations become more resistant following a major epizootic. For example, Vollmer and Kline (2008) detected a small fraction of *Acropora cervicornis* genotypes resistant to white band disease in Panama following an outbreak. Currently about 5% of *A. cervicornis* are resistant (Libro and Vollmer, 2016) and genes potentially involved in this process were identified using RNASeq approaches (Libro et al., 2013; Wright et al., 2017). In many cases, there is little understanding of the mechanisms behind these patterns, although candidate genes and immune mechanisms are being correlated to resistance (Fuess et al., 2017; Wright et al., 2017). Recently, Muller and van Woesik (2014) shed light on the role of colony genotype with signs of white pox disease in *Acropora palmata* in the US Virgin Islands. While the size of the colony and elevated water temperatures mattered, white pox susceptibility also depended in part on the number of past infections of the colony. The number of previous infections could function as an integrative metric of susceptibility which was perhaps initially based on colony genotype sensitivity. Interestingly, the distance of a colony from diseased colonies did not determine disease presence, suggesting that white pox disease is likely not a contagious disease *in situ* (Muller and van Woesik, 2014). Furthermore, following a major Caribbean-wide epizootic of aspergillosis of sea fans, Kim and Harvell (2004) suggested natural selection for host resistance as a cause of the end of the epizootic. Support for the host resistance hypothesis, as opposed to a changing environment or changing pathogen virulence, was supported by 10 years of studying marked fans on permanent transects in the Florida Keys and Mexico, revealing large mortality from the epizootic and a large bottleneck in reproduction following peak epizootic years (Bruno et al., 2011). The plausibility of the host resistance hypothesis was tested with a population model showing that host evolution could proceed quickly enough to explain the observed decrease in overall prevalence from ∼30% in 1997 to < 10% by 2003 (Bruno et al., 2011). While most studies show support for host resistance increasing after major epizootics, the only study that controlled for pathogen strain variability (as opposed to host resistance) was in infection trials with *Acropora palmata* and controlled strains of *Serratium marcescens*, the causal agent of acroporid serratiosis (Sutherland et al., 2011). This study demonstrated that the pathogen had changed and was not able to cause the same disease signs as the epizootic that spread through the Florida Keys 10 years prior. While the mechanisms for individual coral colonies to acquire resistance to a particular disease will depend on their ability to maintain nutritional reserves and mount an appropriate immune defense (Gibbin et al., 2019), the resistance of the remaining individuals in a population and their ability to reproduce and grow fast (Fuess et al., 2018). These characteristics may limit acquired resistance as a viable mechanism to maintain coral populations.

Although researchers have recorded these differences in genotype-based disease susceptibility, the underlying genomic mechanisms that contribute to various aspects of disease (e.g., permissiveness, tropism, immunity, tolerance) are not yet well elucidated but are ongoing (e.g., Sato et al., 2017). Several studies on the genetic basis for both coral(e.g., Fuller et al., 2020) and symbiont (e.g., Chakravarti et al., 2020) thermal tolerance could be a model for future experiments linking population genomic features to disease resistance or susceptibility, and the increasingly sophisticated use of comparative genomics (e.g., Kitchen et al., 2019; Rodríguez-Casariego et al., 2020) will accelerate these efforts.

Coral Immune Responses and Their Influences on Disease

Host phenotypic variability also poses significant challenges for identifying the proximate causes of a disease outbreak. Corals rely on the innate immune defense system, and do not have the adaptive arm of immunity as do vertebrates. It was often thought that innate immunity in corals was primitive and led to an oversimplified view that corals all had the same immune capabilities. But, variability in disease presentations, lesion sizes, and color among different hosts when infected by the same pathogen suggest otherwise (Williams et al., 2020) and are likely due to differences in host innate immune systems(Mydlarz and Palmer, 2011). Host cell physiology such as receptor binding sites used by the pathogen, host cellular machinery used for pathogen replication, and apoptosis can also lead to differences in the way diseases manifest themselves in different coral species (Fuess et al., 2017).

The study of coral host physiology and genomics has progressed in recent years, with studies looking at immune responses in naturally diseased corals, or experimentally exposed to other diseased corals and pathogens and immune elicitors, such as lipopolysaccharides (LPS). These studies identify the known repertoires of innate immune responses: self/non-self recognition, signaling, effector responses, and wound healing (Mydlarz et al., 2016; Palmer and Traylor-Knowles, 2018).

Several patterns in the data sets emerge leading to some potential coral immune targets for further study. The gene DMBT-1 is upregulated in several corals exposed to LPS (Connelly et al., 2020), and live bacteria Vibrio challenges (Wright et al., 2017), and importantly is associated with increased survival. DMBT-1 likely maintains coral mucosal immunity and microbial homeostasis (Libro and Vollmer, 2016; Zhou et al., 2019) and is capable of agglutinating bacteria and activating the complement system. Although it is tempting to assign immune competence of a host to one gene or pathway, the response is likely multifaceted. In fact, most transcriptomic studies of corals see changes in GO terms associated with immunity that may allow for more direct comparisons between different coral species and diseases. In several transcriptomic studies the GO terms for Immune Response Regulation and Innate Immunity (and variations within those) are typically enriched (Fuess et al., 2018, 2020; Zhou et al., 2019; Takagi et al., 2020).

The potential for a coral species to exhibit transgenerational plasticity can also lead to variation in disease resistance that can confound coral disease diagnostics (Putnam et al., 2017; Torda et al., 2017). New phenotypes of offspring that arise post-stress of the parental colonies can be a result of transfer of nutrients and hormones, as well as beneficial microbes that could protect against dysbiosis (see section "Part II: The Microbiome"; Torda et al., 2017). Epigenetic processes that regulate gene expression such as gene methylation are also a mechanism for transgenerational plasticity. While gene methylation has not been directly studied in the context of coral disease yet, studies looking at other stressors such as heat and nutrient addition do show methylation of immune-related adhesion and signaling genes in addition to housekeeping genes (Dimond and Roberts, 2016; Liew et al., 2020). It is important to note that these mechanisms will not benefit all coral species equally, and species that are relatively short-lived and brood their embryos may be better candidates for adaptation due to transgenerational plasticity that could lead to disease resistance, such as *Porites astreoides* (Pinzon et al., 2014; Fuess et al., 2017).

Immunity in corals varies with many environmental factors and with exposure to pathogens whether or not disease phenotypes are apparent (Mydlarz et al., 2008, 2009; Pinzón et al., 2015). Some of these responses are to directly mitigate the stress, such as antioxidants, fluorescent proteins and melanin cascade, other have upstream signaling roles such as NFkb and NOD-like receptors (Traylor-Knowles and Connelly, 2017). These environmentally and pathogen induced variations in immunity change through time (Pinzón et al., 2015; Traylor-Knowles and Connelly, 2017), leading to different susceptibilities to diseases and inconsistencies in the development of disease signs. Such persistent natural variation in immune status makes it more difficult and complex to determine the baseline or health status of a coral at any given time.

Knowledge Gaps and Ways Forward in Coral Host Biology

In many of the studies mentioned above, it was not known whether the beginning or end of an epizootic was affected by host resistance and tolerance or changing pathogen virulence or change in some aspect of the environment (see sections below). While we have made progress in understanding the mechanisms of host immunity, we still do not understand the main drivers of variation in immunity both within and between species. Overcoming this knowledge gap is particularly needed as fast spreading coral diseases still are causing losses throughout Caribbean reefs (Aeby et al., 2019).

The emergence of more genomic and proteomic tools will help elucidate the pathways important to coral disease resistance and tolerance (Okamura et al., 2019; Ricci et al., 2019), however, we still need to understand how they vary and why. The "hidden" effects of evolutionary and environmental history and transgenerational plasticity of an individual coral or population result in a reduced ability for direct quantification and links between host factors and disease outbreaks (Torda et al., 2017).

PART II: THE MICROBIOME

For several decades, coral-associated bacteria (and to a lesser degree viruses) were examined in an explicit effort to link some microbes to specific disease phenotypes (**Table 2**). Yet for the vast majority of cases, linkages between a given taxa (or group) and a coral disease are inconclusive. It is now clear that the fundamental properties of the coral microbiome (see below), combined with current standard operating procedures in coral disease ecology, plus conventional assumptions about disease etiology, have contributed to our inability to recapitulate the standard one pathogen-one disease paradigm. Here we provide background on the coral microbiome and present new ideas on how to integrate this information into coral disease ecology studies.

Corals Play Host to Unique and Dynamic Coral Microbiomes

Since the late 1990's it has become increasingly clear that most organisms host a wide variety of microbes that are responsible for aspects of host health and longevity, as well as ecosystem functioning (Dethlefsen et al., 2007; Manor et al., 2016; Apprill, 2017; Burkepile and Thurber, 2019). This concept that the microbes or "microbiome" contribute to the functioning, and even evolution, of marine taxa is now well established (Wilkins et al., 2019). The exact nature of these relationships is often unknown and likely dynamic and host taxon-specific. Luckily, recent methodological advances in tracking microbial and even viral communities with fine resolution are dramatically advancing our knowledge about the membership and function of these host-associated microbiomes.

Corals contain dozens to hundreds of species-specific bacteria that can both contribute to and detract from coral health depending on the state of the host and the environmental context at the time (Blackall et al., 2015; Thompson et al., 2015; Bourne et al., 2016; Pollock et al., 2018; Rosales et al., 2019). In particular, environmental stressors such as climate change, reduced water quality, and habitat exploitation all can affect the composition and function of the coral microbiome (McDevitt-Irwin et al., 2017). Thus, coral microbiomes and viromes dynamically respond to host and environmental perturbations in a variety of ways (Bourne and Webster, 2013; Bourne et al., 2016; Thurber et al., 2017). Yet how the structure and function of these microbiomes relate to coral health and resilience to anthropogenic and natural stressors remains a major area of investigation because most of the linkages between changes in the microbiome and coral health remain correlative.

Drivers of Coral Microbiome Dynamics

As a research community, we have extensively documented how several biotic and abiotic factors contribute to coral

TABLE 2 | Proposed causative agents to coral diseases.

Proposed causal agent	Described disease	Studies
Aspergillus sydowii	Aspergillosis	Alker et al., 2001
Aspergillus sydowii and other fungal pathogens	Aspergillosis	Soler-Hurtado et al., 2016
Vibrio coralliilyticus	Bacterial bleaching	Ben-Haim et al., 2003a,b
Boring cyanobacteria and novel bacteria	Black band	Miller et al., 2011
Consortium	Black band	Cooney et al., 2002; Frias-Lopez et al., 2003, 2004; Sekar et al., 2006, 2008; Barneah et al., 2007; Voss et al., 2007; Sato et al., 2010; Séré et al., 2016
Cyanobacteria	Black band	Frias-Lopez et al., 2003; Voss et al., 2007; Hutabarat et al., 2018
Cyanophyte pathogen	Black band	Rützler and Santavy, 1983
Desulfovibrio	Black band	Viehman et al., 2006; Brownell and Richardson, 2014
Geitlerinema species	Black band	Richardson and Ragoonath, 2008
Novel T4 bacteriophages	Black band	Buerger et al., 2019
Pseudoscillatoria coralii	Black band	Rasoulouniriana et al., 2009
Roseofilum	Black band	Buerger et al., 2016
Scuticociliatia	Black band	Bourne D. G. et al., 2008
Vibrio species	Black band	Arotsker et al., 2009
Arcobacter sp. (JF831360), cilliates: Morph1 (JN626268) and Morph2 (JN626269)	Brown band, white syndrome	Sweet and Bythell, 2012
Porpostoma guamensis	Brown band	Lobban et al., 2011
Corynebacterium (KC190237), *Acinetobacter* (KC190251*),* *Parvularculaceae* (KC19027*), Oscillatoria (*KC190271), two *Vibrio* species	Dark spot	Sweet et al., 2013
Pseudoalteromonas piratica	Montipora white syndrome	Beurmann et al., 2017
Vibrio coralliilyticus	Montipora white syndrome	Ushijima et al., 2014
Vibrio owensii	Montipora white syndrome	Ushijima et al., 2012
Icosahedral virus-like particles	Porites white patch syndrome	Lawrence et al., 2015
Shimia marina and *Vibrio hepatarius*	Porites white patch syndrome	Séré et al., 2013
Vibrio tubiashii	Porites white patch syndrome	Séré et al., 2015
Vibrio harveyi	Rapid tissue necrosis	Luna et al., 2007
Serratia marcescens	Acropora Serriatosis	Sutherland et al., 2011
Vibrio species	Skeletal tumors	Breitbart et al., 2005
Photobacterium damselae and multiple *Vibrio* species	Ulcerated yellow spot syndrome	Cervino et al., 2012
Vibrio species	Ulcerative white spot	Arboleda and Reichardt, 2010
Gram negative bacterium	White band	Peters et al., 1983
Vibronales and Rickettsiales	White band	Gignoux-Wolfsohn and Vollmer, 2015
Bacterial Strain BA-3	White plague	Barash et al., 2005
Nucleocytoplasmic large DNA virus	White plague	Soffer et al., 2014
Thalassomonas loyana	White plague	Thompson et al., 2006
Vibrio species	White plague	Chimetto Tonon et al., 2017
Aurantimonas coralicida	White plague type II	Denner et al., 2003
Serratia marcescens	White pox	Patterson et al., 2002
Vibrio alginolyticus	White syndrome	Zhenyu et al., 2013
Consortium of *Vibrio* species	White syndrome	Sussman et al., 2008
Vibrio harveyi	White syndrome	Luna et al., 2010
Vibrio species	Yellow band (blotch)	Cervino et al., 2004, 2008

microbiome variability. For example, it is clear that the largest contributing factor of microbiome composition is host phylogeny (Rohwer et al., 2002; Chu and Vollmer, 2016; Pollock et al., 2018). But along with host specificity, variation in many co-occurring variables associated with geographic location (e.g., latitude, temperature, and depth) can contribute significantly to differences in the microbiome (Littman et al., 2009; Kvennefors et al., 2010; Pantos et al., 2015; Glasl et al., 2017; Pootakham et al., 2019). Two main factors, host species and geographic variation, can obscure clear patterns during disease outbreaks. For example, the co-occurrences of microbiomes with disease outbreaks may falsely lead researchers to believe that a particular microbial taxon is responsible for a disease outbreak, when in fact the uninfected coral lacks that microbe because of a difference in geographic location (Roder et al., 2014) or is a cryptic species susceptible to the infection (Cuvelier et al., 2014).

Corals are composed of four major compartments each containing unique microbial members (Ainsworth et al., 2015): the surface mucus layer, the tissues (gastroderm and epithelium), the coelenteron (stomach), and the skeleton (Pollock et al., 2018). Many of these compartment-specific bacteria are core members, as defined by a baseline prevalence, while others are considered transients (Ainsworth et al., 2015; Hester et al., 2016; Zaneveld et al., 2016). Membership and environmental responsiveness of the coral microbiome varies across the compartments, with the mucus being the most variable and responsive (Pollock et al., 2018). Even more surprisingly, coral size and whether corals are physically touching other benthic community members (e.g., macroalgae) are major variables that drive differences in coral microbiome diversity and function (Zaneveld et al., 2016).

On top of all these biotic factors, abiotic factors such as nutrient pollution, anomalous temperature, PCO_2 levels, and sedimentation can act alone or interact to cause disruptions in the microbiome that can either preclude an outbreak or directly lead to one. However, a recent meta-analysis found that patterns can be found in how different members of the microbiome respond to these local and global stressors (McDevitt-Irwin et al., 2017). It was found that almost all stressors induced three kinds of changes in the microbiome: (1) shifts in alpha diversity, (2) shifts in beta diversity, and the (3) loss of one major group of bacteria, the Endozoicomonas clade, hypothesized to be involved in nutrient cycling in corals.

Challenging the "One Disease, One Pathogen" Hypothesis

When evaluating coral disease, researchers must be aware of potentially misleading, but common, baseline assumptions about the properties and patterns of microbial prevalence, abundance, and function in the host. Several papers concluded that due to the persistent presence of some bacterial taxa (i.e., high prevalence) in apparently healthy animals that such members cannot be the source of a disease (Casas et al., 2004; Kline and Vollmer, 2011). Yet commensal and even mutualist bacteria can become pathogenic under various conditions (Seyedsayamdost et al., 2011). For example, a recently discovered yet common bacterial parasite, *Candidatus* Aquarickettsia, is commonly found in corals but proliferates when exposed to elevated nutrients reducing coral growth and increasing tissue loss and mortality (Shaver et al., 2017; Klinges et al., 2019). What's more, this parasite has been found to be associated with disease susceptibility in some coral genotype backgrounds (Klinges et al., 2020). Pathogenesis also may initiate from either an extrinsic, invading pathogen or from an intrinsic member of the microbiome sometimes referred to as a "pathobiont" (Sweet and Bulling, 2017) or "opportunistic." The increase in abundance or virulence activity of this pathobiont response to some perturbation may be the trigger that begins the process of disease onset. An example in corals is, *Vibrio coralliilyticus*, a thermally sensitive pathogen that requires elevated temperatures to induce chemotaxis toward coral and to express virulence cassettes for infection, with higher rates of tissue lysis and disease progression when seawater temperatures rise above 29–30°C (Ben-Haim et al., 2003b; Cervino et al., 2008; Séré et al., 2015; Tout et al., 2015; Garren et al., 2016). The pathobiont concept was developed based on evidence that gastrointestinal inflammatory diseases are often caused by bacterial species found in healthy hosts, including *Clostridium difficile* and *Heliobacter pylori* (Chow et al., 2011). Similarly, the Rhodobacteraceae increased fourfold in coral white syndrome lesions compared to healthy tissues (Pollock et al., 2017; Rosales et al., 2020) and are implicated in Stony Coral Tissue Loss Disease that is currently devastating Caribbean reefs. Therefore, it is possible that some coral diseases are caused by pathobionts rather than environmentally acquired pathogenic agents.

Other standard assumptions contributing to our inability to identify clear patterns of disease etiology are that one must: (1) isolate a given strain that upon application leads to a recapitulation of the disease (Koch's postulates), (2) observe a strictly distinct microbial alternative state, or (3) identify statistically more abundant taxa that coincide with the apex phenotype of the disease. Unfortunately, due to these assumptions, attempts have been generally unfruitful in linking a pathogen to a coral disease. This is also true in other complex host-microbiome systems (but see exceptions described below). It has become apparent that the etiology of many diseases that initiate in mucosal membranes, including periodontitis, lung infections, bacterial vaginosis, and gastro-intestinal inflammatory diseases, do not involve monocultures of bacteria, but rather heterogeneous communities of organisms (Nelson et al., 2012; Lamont and Hajishengallis, 2015). Pathogenesis of disease can arise from polymicrobial synergy that results in disruption of microbiome homeostasis and normal host immune function (Nelson et al., 2012; Vayssier-Taussat et al., 2014; Byrd and Segre, 2016). For example, although some model pathogens infect corals, such infections lead to downstream alterations in the microbiome that may contribute to the ultimate death of the host (Welsh et al., 2017). When *V. coralliilyticus* is added to naïve corals, the infection can be transient while opportunist bacteria such as Rhodobacterales and Cytophagales become more abundant and begin to dominate the coral system.

This invasion by opportunists during an epizootic is likely a common feature of coral diseases. This is evident by the observation that several common Caribbean coral diseases, including yellow-band disease, dark-spot syndrome, white pox disease, and white plague, all of which do not display typical transmission dynamics characteristic of contagious diseases (Muller and van Woesik, 2012, 2014; Klinges et al., 2020), suggesting intrinsic properties of the holobiont may play a large role in disease initiation and progression. In these cases, it is important to examine host organismal traits that affect disease susceptibility and environmental thresholds that serve as tipping points for disruption of microbiome homeostasis and disease induction rather than focusing on transmission dynamics of pathogens.

Dysbiosis as a Hallmark of Coral Disease

Another major hurdle in our attempts to identify the bacteria that are strongly linked to disease may be a lack of accurate paradigms for diseases in the marine system. One such paradigm is that diseased animals always exhibit microbial communities that are distinct from apparently healthy congeners. Recently Zaneveld et al. (2017) argued that under real-life environmental conditions, coral microbiomes do not always demonstrate clear shifts in bacterial community stable states. Instead, increases in overall dispersion of the community are major features of stressed corals, where high variability in the community likely represents the host's inability to regulate the microbiome. This quantitatively defined "dysbiotic state" is strongly correlated with coral disease, tissue loss, and mortality and may be a prominent feature of coral disease progressions (Zaneveld et al., 2016; Ezzat et al., 2019; Maher et al., 2019). In fact, although first reported in corals, this feature of microbiome disruption as a feature of disease (in contrast to true alternative stable states) has been described but not formalized in many host systems, including chimpanzees infected with Simian Immunodeficiency Virus (SIV) and the lung microbiomes of smokers (Zaneveld et al., 2017).

Knowledge Gaps and Ways Forward on the Coral Microbiome

Adherence to the "one microbe—one disease" as well as other standard disease ecology paradigms have centralized our efforts on identifying and describing the role of particular microbial taxa. A more appropriate focus should be on understanding successional stages in the coral disease process (**Figure 3**). For example, the development of black band disease pathogenesis in corals was linked to successional changes in the dominant cyanobacterium associated with lesions, followed by heterotrophs and the development of anoxia and sulfide in the microbial mat, which enhances colonization by a variety of bacterial groups and Archaea that intensifies tissue loss (Sato et al., 2016). It is likely that the role of blooming secondary opportunists (or accessory pathogens) during dysbiosis is as critical as the primary and elusive pathogen and therefore should not be discounted. For example, a low-abundance keystone pathogen may disrupt normal host immune function, allowing microbiome members that are otherwise commensal to take on a pathogenic role, exacerbating inflammatory tissue breakdown and the disease process (Hajishengallis et al., 2012). Furthermore, blooming opportunists or accessory pathogens may promote further host health declines, increase virulence of other members of the microbiome via horizontal gene transfer of important virulence factors (e.g., antibiotic resistance, toxin production, flagellar motility, and sensing behavior), or modify the mucosal environment to such as degree that the host cannot return to normal homeostasis (Stecher et al., 2013; Rice et al., 2019).

One of the defining characteristics of the field of coral microbiology is that it is traditionally conducted in the field, with all of the accompanying environmental and contextual complexities that come with studying disease ecology in *in situ* studies. The coral microbiome is also often sampled in a state of dysbiosis following disturbance (environmental factors or pathogenesis), therefore the timing of sampling often lacks necessary resolution. Rather than directly addressing questions of pathogen dynamics, the opportunistic nature of outbreaks often means that sampling more likely characterizes the wake of pathogen disturbance in the microbiome. At the same time, the current body of literature suggests that we often do not sample at sufficiently frequent intervals to capture the dynamics of pathogenesis, and are more often blending our analyses of pathogenesis and dysbiosis. Thus, we should aim to adjust our approaches and paradigms, develop our models, and refocus our approaches to testing critical questions in coral disease ecology that are more in step with what we know about animal microbiome dynamics.

PART III: THE ENVIRONMENT

All interactions between corals and pathogens are intimately governed by varying environmental parameters. Being sessile and existing as a thin layer of tissue in constant contact with the environment, corals have limited options for avoiding unfavorable environmental change that may directly or indirectly lead to disease. The changing nature of the environment in the Anthropocene is likely a major factor causing coral microbiome dysbiosis, leading to the onset of disease, and may go a long way

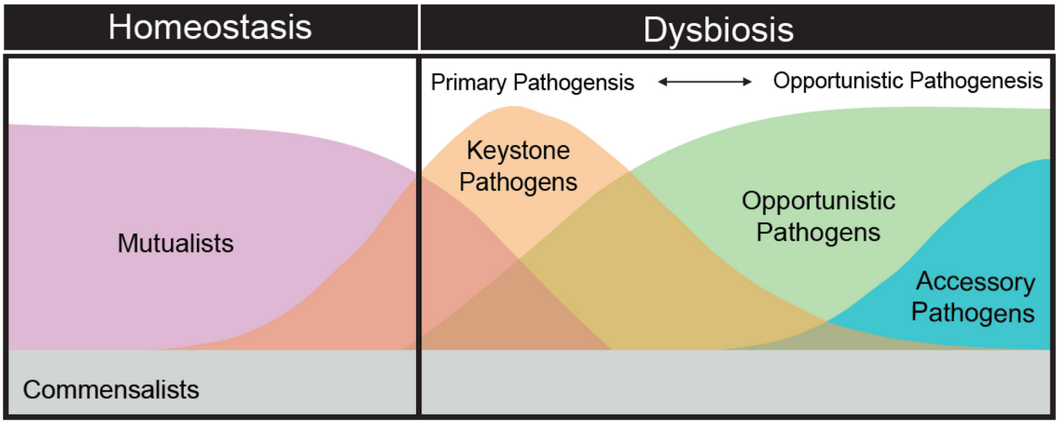

FIGURE 3 | The theoretical succession of the composition and diversity of microbial organisms as the coral microbiome shifts from homeostasis to dysbiosis.

toward explaining recent rises in the prevalence of coral diseases globally (Mera and Bourne, 2018).

Environmental stressors affect both host resistance and pathogen virulence (see above). Similarly, changes in the environment impacts population dynamics, spatial distributions of hosts or pathogens or both, providing greater opportunities for host-pathogen interactions that are favorable for the emergence or re-emergence of disease. It is well established that many coral diseases are connected to multiple environmental parameters. These parameters can be abiotic (e.g., temperature, light, nutrients) or biotic (e.g., contact with macroalgae) and can affect disease directly (as when warmer temperatures induce virulence factors in pathogens) and indirectly through a cascade of events. In some cases, predictive models were successfully developed to describe the relationship between disease dynamics and environmental drivers (Bruno et al., 2007; Williams et al., 2010; Maynard et al., 2011, 2015). However, many coral disease studies are initiated in response to disease outbreaks, where long-term environmental data do not exist, leading to difficulties in determining the role of potential environmental drivers after the fact (Brandt et al., 2012). Below we discuss our understanding of how the environment affects host-microbiome relationships with emphasis on the complexities and multiple stressors interactions that corals and their microbiomes experience.

Thermal Stress

Several bacterial, fungal and protozoan diseases of corals are linked to seawater temperatures, including black band disease (Muller and van Woesik, 2012, 2014), yellow band disease (Cervino et al., 2004; Harvell et al., 2009), and white syndromes (Bruno et al., 2007; Heron et al., 2010; Maynard et al., 2011, 2015). There were rare instances where the specific link driving this relationship was identified, as when warmer spring temperatures allow for the adhesion of *Vibrio shiloi* to the coral *Oculina patagonica* leading to bleaching (Kushmaro et al., 2001). However, in most cases it is unknown what is driving the link between temperature and coral disease, whether it is a positive impact of increasing temperature on the growth and virulence of the pathogen(s), negative impacts to the host leading to greater vulnerability (i.e., the compromised host hypothesis), or both.

Temperature also interacts with light intensity to effect disease. For instance, while many field studies correlated black band disease with seasonally warmer temperatures (Kuta and Richardson, 2002; Voss and Richardson, 2006; Sato et al., 2009), a laboratory experiment tested the interaction between temperature and light on black band disease progression rates and found that light, not temperature, was the driving factor influencing disease progression. The high light treatments were also associated with negative impacts to the algal symbionts (Sato et al., 2011). Similarly, Muller and Van Woesik (2009) demonstrated in a field experiment that reducing irradiance on corals affected by the disease white plague led to a reduction in disease progression rates. However, a separate study (Muller and Van Woesik, 2011) also found that shading black band disease in the field resulted in faster progression rates, possibly because the dominant cyanobacterium component of the pathogenic band experienced reduced photosynthetic pressure.

Projected climate change-related increases in sea surface temperatures range from 1°C (under RCP 4.5) to 3°C (under RCP 8.5) by 2100 (Stocker et al., 2013). This increase in sea surface temperature will likely increase overall disease risk for corals (Maynard et al., 2017). Thermal stress as a result of abnormally high temperatures leads to shifts in coral-associated microbial communities (Bourne D. et al., 2008; Littman et al., 2011), a breakdown in the symbiosis between the coral animal and its endosymbiotic algae, and stressful physiological state for the coral. Numerous studies report links between thermal bleaching events and subsequent increases in disease outbreaks globally (Brandt and McManus, 2009; Cróquer and Weil, 2009; Miller et al., 2009; Weil et al., 2009; Muller et al., 2018; but see Ban et al., 2013), with a dramatic example being widespread outbreaks following the 2005 Caribbean mass bleaching event (Eakin et al., 2010). Outbreaks following that region-wise event encompassed multiple diseases, host species, and habitats, and resulted in devastating losses in coral cover (Cróquer and Weil, 2009; Miller et al., 2009; Weil et al., 2009). Coral colonies followed through time during this event showed that the bleaching-compromised state of the host, vs. environmental impacts on pathogens, was the likely driver of disease incidence and severity during this event (Muller et al., 2008; Brandt and McManus, 2009).

The abnormally high temperatures that drove the 2005 mass bleaching were driven by human-induced climate change (Donner et al., 2007). Record seawater temperatures have triggered several global-scale coral bleaching events since mass bleaching was first documented in the 1980s (Hughes et al., 2017). Adding to this complexity, other studies show negative associations between thermal stress and coral disease (Aeby et al., 2011b), while others suggest cooler winter conditions prior to summer thermal events could reduce disease outbreak likelihoods as a result of pathogen "knockback" (Heron et al., 2010).

Ocean acidification is also considered a significant environmental impact affecting reef-building corals, with experimental studies showing potential shifts in coral-associated bacterial communities toward a more disease-associated state with decreasing pH (Thurber et al., 2009; Meron et al., 2011; Webster et al., 2013; Morrow et al., 2015). However, explicit links between ocean acidification and coral disease currently do not exist, thus efforts in this area are sorely needed.

There are a number of other environmental perturbations that influence coral disease, such as reductions in salinity (Haapkylä et al., 2011), influxes of terrestrial pollutants or nutrients (Bruno et al., 2003; Vega Thurber et al., 2014; Lamb et al., 2016; Klinges et al., 2019; Rice et al., 2019), overfishing (Zaneveld et al., 2016) and storms and cyclones (Brandt et al., 2013; Beeden et al., 2015). These environmental factors are all linked to human development and resource use. This introduces additional complexity into determining the role of the environment on coral disease due to a suite of anthropogenic stressors that may act synergistically with climate-related stressors. For example, several anthropogenic factors outside climate related activities are implicated in disease outbreaks and rising prevalence levels such as those involved in coastal development, tourism, farming, and resource extraction (**Table 3**).

TABLE 3 | Anthropogenic factors associated with increased coral disease levels.

Anthropogenic stressors promoting disease	References
Proximity to human population centers	Sandin et al., 2008; Aeby et al., 2011a; Gutiérrez-Ruiz et al., 2011; Guilherme Becker et al., 2013
Coastal land alteration	Haapkylä et al., 2011
Dredging, terrestrial runoff, of sediment or agricultural herbicides	Sheridan et al., 2014; Aeby et al., 2016; Lamb et al., 2016; Pollock et al., 2016
Sewage release	Redding et al., 2013; Wear and Thurber, 2015
Release of human enteric microorganisms	Sutherland et al., 2011
Increases in nutrient concentrations	Bruno et al., 2003; Voss and Richardson, 2006; Vega Thurber et al., 2014
Aquaculture and fish farms	Garren et al., 2009; Sabdono et al., 2019
Reduction in the diversity of reef fish assemblages	Raymundo et al., 2009
Overfishing	Zaneveld et al., 2016
Injuries from fishing line	Lamb et al., 2015, 2016
Tourism	Lamb and Willis, 2011; Lamb et al., 2014
Sunscreens	Danovaro et al., 2008
Plastic waste	Lamb et al., 2018
Damage from ship grounding	Raymundo et al., 2018

Interactive Influences of Local and Global Stressors on Coral Disease

Diseases of corals are influenced by changing climate and increasing levels of anthropogenic activities, but these relationships are often complex and interactive (Harvell et al., 2002; Altizer et al., 2013). Not only are these drivers of coral disease likely to be both spatially and temporally heterogeneous, pathogen reservoirs in the environment further complicate our understanding of environmental influences of coral disease. These include airborne African dust, which has been shown to contain fungal spores associated with aspergillosis in gorgonian sea fans (Shinn et al., 2000); sewage outfalls off the coast of Florida, which harbor *Serratia marcescens,* a human gut microbiota that has been associated with white pox in acroporid corals (Patterson et al., 2002); and reef crevices for microbial communities associated with black band disease (Kuta and Richardson, 2002). Management actions to reduce anthropogenic stress are needed at locations with high or very high anthropogenic stress (Burke et al., 2011), and are particularly urgent given the expected increases in sea surface temperature (Maynard et al., 2015) that will likely drive disease dynamics in the coming decade.

IV: BEYOND THE HOLOBIONT: PROBLEMS AND SOLUTIONS FOR MANAGING CORAL DISEASE IN A VARIABLE ENVIRONMENT

Marine Reserves and Protected Areas

Marine reserves and protected areas serve to protect existing natural space while simultaneously supplementing non-reserve areas with marine resources. Evidence from studies testing the efficacy of marine reserves as management tools for preventing disease in coral populations varies. For example, no-take marine reserves were shown to reduce coral disease levels through mitigation of tissue injury associated with fishing activities and derelict gear (Lamb et al., 2015, 2016) or sustaining functionally diverse fish assemblages (Raymundo et al., 2009). High densities of herbivorous fish within protected areas could limit algal growth (Bellwood et al., 2003), which have been implicated as reservoirs of pathogens on reefs in the Caribbean and Indo-Pacific (Nugues et al., 2004; Smith et al., 2006). Exclusion of activities that damage corals inside marine reserves that directly damage corals (e.g., Asoh et al., 2004; Yoshikawa and Asoh, 2004) and high-intensity tourism (e.g., Lamb and Willis, 2011; Lamb et al., 2014), is likely to mitigate disease by reducing entry points for opportunistic coral pathogens (Page and Willis, 2008; Nicolet et al., 2013; Katz et al., 2014; Lamb et al., 2014). Environmental influences that permeate reserve borders (e.g., Coelho and Manfrino, 2007; McClanahan et al., 2009; Page et al., 2009) have been shown to limit reserve effectiveness. It is also plausible that protected areas facilitate the spread of disease by increasing the number of susceptible coral hosts (McCallum et al., 2005; Bruno et al., 2007; Myers and Raymundo, 2009), or fishes that act as vectors for coral pathogens through feeding injuries (Aeby and Santavy, 2006; Raymundo et al., 2009).

Well-managed marine reserves may help assist adaptation to impacts of climate change (Roberts et al., 2017), however, there is mounting evidence that climate-related stressors can undermine coral resistance to disease afforded by reserve protection. For example, although marine reserves were found to mitigate coral disease following a severe cyclone, they were found to be ineffective in moderating disease when sites were exposed to higher than average levels of terrestrial runoff from a degraded river catchment (Lamb et al., 2016). This is further supported by Hughes et al. (2017), which recently reported that water quality and marine reserves had no influence on the unprecedented bleaching on the Great Barrier Reef in 2016, suggesting that local protection may provide little or no value to coral diseases associated with temperature.

Satellite Imagery and Predictive Modeling

The implications of climate-driven and anthropogenic outbreaks of disease on services provided to people will require preemptive solutions and mitigation. Early warning systems form an important component of potential solutions. For example, high risk areas for malaria outbreaks were predicted using global atmospheric and ocean climate models in order to initiate early mitigation strategies in Botswana (Thomson et al., 2006). Forecasting is well-established in managing diseases of agricultural crops, leading to improved deployment of planting strategies that lower disease risk though precise pesticide timing (Schaafsma and Hooker, 2007). On coral reefs, accurate forecasting programs to predict bleaching have become essential to marine resilience programs (Eakin et al., 2010) and are leading to the development of climate-driven, coral disease–forecasting algorithms (Maynard et al., 2011, 2015). More recently, the first satellite-derived water quality data were critical in assessing the drivers of disease following chronic exposure to terrestrial run-off from a degraded river catchment (Lamb et al., 2016) and sediment exposure from seafloor dredging (Pollock et al., 2014), offering the potential for identifying and forecasting locations that are at increased risk of outbreaks from poor water quality. Linking forecasting models to trait-based models (Laughlin et al., 2012) in corals may help identify the specific coral individuals or species that are at risk when environmental conditions favor disease outbreaks.

Human-Assisted Evolution and Active Intervention for Reef Recovery

Although it is critical to increase efforts to understand and reduce environmental threats influencing coral disease, recent reviews and commentaries are increasingly considering alternative strategies that involve more active interventions. There is discussion about the feasibility of developing coral stocks with enhanced disease and stress tolerance through the acceleration of naturally occurring processes, an approach known as (human)-assisted evolution (van Oppen et al., 2015). For selective breeding approaches to be successful, the resistance to disease would need to be clearly identified and exhibit significant heritability. At the same time, for microbial symbionts, vertical or horizontal transmission of the mutualist taxa would likely promote plasticity or benefits to fight disease while transmission of parasites or pathogens would potentially prevent success of such approaches. Evidence for genetic-based heritability exists in the coral host (Meyer et al., 2009) and their symbiotic algae (Csaszar et al., 2010) in response to thermal stress, however, little is known about other environmental conditions of heritability (van Oppen et al., 2015). Damjanovic et al. (Damjanovic et al., 2019) showed that inoculation of *Acropora tenuis* and *Platygyra daedalae* recruits with cocktails of bacterial cultures influenced the coral microbiome, demonstrating that the host bacterial community may be manipulated for the purposes of enhancing coral resilience. Other probiotic studies have shown that bacterial addition to corals prior to stress events may mitigate some of the negative effects; what the mechanisms behind these effect are remains unknown (Peixoto et al., 2017). Similarly, following a coral disease outbreak, *in situ* treatment could include the therapeutic use of bacteriophages. For instance, Atad et al. (2012) found that phage-treated corals experienced much lower levels of tissue loss compared to non-treated corals. Furthermore, by phage-treating diseased corals, the disease transmission rates to surrounding healthy coral colonies were much lower. Although these intervention examples are promising and have the potential to treat or increase resistance to disease expansive areas, there is considerable public resistance and unknown environmental risk. Lastly, the scalability of these approaches may be too limited to adequately address global and long reaching stressors such as climate change.

CONCLUSION AND WAYS FORWARD

It is now becoming very clear from nearly four decades of research that many coral diseases do not conform to standard assumptions and paradigms in disease ecology. Here, we recommend the development of new concepts to integrate what we now know about coral species genotype diversity, physiological or immunological variability, the role of the microbiome, and complex nature and dynamics of the marine environment. We recommend using a multi-layered approach when undertaking coral disease research that specifically integrates the host, microbiome, and the environment. Experiments where environmental parameters are tracked or manipulated and then holobiont genotype, phenotype, and the microbiome are evaluated at appropriate time scales will be our best way forward. We recognize that these are not small asks, and the methods to integrate such disparate and computationally intensive data streams are not fully developed. Nevertheless, we foresee that such approaches can help not only explain the causes and mechanisms behind disease outbreaks but also predict disease susceptibility in different corals and locations. We envision that by re-developing theory from the vast amounts of empirical and observation-based data collected thus far, we can invigorate our community and ultimately reveal new principles that govern coral disease ecology and biology, allowing us to better combat and mitigate current and future coral disease outbreaks.

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All authors contributed to the conceptual development, writing and revisions of the manuscript.

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Host-Specificity and Core Taxa of Seagrass Leaf Microbiome Identified Across Tissue Age and Geographical Regions

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The seagrass *Zostera marina* is a widespread foundational species in temperate coastal ecosystems that supports diverse communities of epiphytes and grazers. Bacteria link the production of seagrass to higher trophic levels and are thought to influence seagrass biology and health. Yet, we lack a clear understanding of the factors that structure the seagrass microbiome, or whether there is a consistent microbial community associated with seagrass that underpins functional roles. We sampled surface microbiome (epibiota) from new and old growth seagrass leaves and the surrounding seawater in eight meadows among four regions along the Central Coast of British Columbia, Canada to assess microbiome variability across space and as leaves age. We found that the seagrass leaf microbiome differs strongly from seawater. Microbial communities in new and old growth leaves are different from each other and from artificial seagrass leaves we deployed in one meadow. The microbiome on new leaves is less diverse and there is a small suite of core OTUs (operational taxonomic units) consistently present across regions. The overall microbial community for new leaves is more dispersed but with little regional differentiation, while the epiphytes on old leaves are regionally distinct. Many core OTUs on old leaves are commonly associated with marine biofilms. Together these observations suggest a stronger role for host filtering in new compared to old leaves, and a stronger influence of the environment and environmental colonization in old leaves. We found 11 core microbial taxa consistently present on old and new leaves and at very low relative abundance on artificial leaves and in the water column. These 11 taxa appear to be strongly associated with *Z. marina.* These core taxa may perform key functions important for the host such as detoxifying seagrass waste products, enhancing plant growth, and controlling epiphyte cover.

Keywords: core microbiome, 16S rRNA gene, eelgrass (*Zostera marina*), biodiversity, symbiosis

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INTRODUCTION

Seagrass meadows are highly productive coastal ecosystems that provide numerous ecosystem functions and services (Duarte and Chiscano, 1999; Fourqurean et al., 2012). Bacteria link seagrass productivity to higher trophic levels by metabolizing organic carbon and nitrogen released by the host, suggesting a symbiotic relationship between the seagrass and associated microbes (McRoy and Goering, 1974; Kirchman et al., 1984). The seagrass microbiome is diverse, with 10 to 100 s of taxa identified per leaf sample (Ugarelli et al., 2017; Crump et al., 2018) and shaped by seagrass species identity (Crump et al., 2018), space (Ugarelli et al., 2017) and local environment (Bengtsson et al., 2017). A better understanding of the factors that structure the seagrass microbiome and a catalog of symbionts tightly associated with seagrass is needed to improve our knowledge of the complex interactions between seagrass and their symbionts in a changing world.

Microbial symbionts influence the growth and health of a wide range of hosts (Fitzpatrick et al., 2018; Longford et al., 2019). For example, isolated beneficial bacteria have been shown to improve resistance of corals to bleaching under high temperatures (Rosado et al., 2019). Similarly, seagrass symbionts are thought to influence host health by consuming toxic by-products (i.e., methanol and ethanol) and by producing agarases which limit epiphyte overgrowth (Crump et al., 2018). We use the broad definition of symbiont, microbes that live in association with a host, as the fitness consequence of most microbes is unknown and often variable in different contexts (Leung and Poulin, 2008; Wilkins et al., 2019). On the other side of the symbiotic spectrum, the pathogenic protist *Labyrinthula zosterae* is the best studied symbiont in seagrass ecosystems, putatively causing the seagrass wasting disease responsible for extensive declines in *Zostera marina* meadows in the Atlantic Ocean in the past (Short et al., 1987) and still of concern today.

One path toward making sense of the vast diversity in the seagrass microbiome and identifying candidate symbionts that may be essential to host functioning is investigating the core microbes that are tightly associated with the host (Shade and Handelsman, 2012; Wilkins et al., 2019; Risely, 2020). The definition of core taxa varies (Risely, 2020), but typically only a few microbial symbionts are consistently found at high prevalence on host populations across regions (Hernandez-Agreda et al., 2018).

At the level of the whole community, better understanding of how symbiont communities are assembled can provide foundational insights such as whether the microbiome is likely to shift or remain stable in changing environments. In marine ecosystems, hosts are submerged in a sea of microbes and horizontal transmission (acquisition of microbes from the environment) is likely the main route (Turon et al., 2018; Russell, 2019). Environmental acquisition might lead to microbial communities on marine hosts that mirror those from the surrounding environment, as is reported in one global study of seagrass leaves (Fahimipour et al., 2017). But, much more often selective processes lead to host microbial communities that are distinct from their surroundings and specific to host species (Wilkins et al., 2019). These patterns are observed for diverse seaweeds (Roth-Schulze et al., 2016; Lemay et al., 2018a), kelps (Lemay et al., 2018b; Weigel and Pfister, 2019), seagrass (Crump et al., 2018), sponges (Thomas et al., 2016), and others. In fact, seaweeds can select or deter microorganisms that colonize from the environment via their exudates (Lam et al., 2008; Salaün et al., 2012). Seagrass leaves also release exudates that attract microbes as food sources, such as methanol and cellulose, and others that deter colonization. Seagrass leaves are highly defended by phenols (Harrison, 1982) and other compounds that have been shown to modulate settlement and growth of microbes in lab settings (Papazian et al., 2019).

We sampled microbial communities from new and old seagrass leaves and the surrounding seawater in eight meadows across four regions along the Central Coast of British Columbia, Canada, to test host specificity and identify core seagrass taxa that are consistently present. If host selectivity is strong, we expect: (1) seagrass leaf microbiome to differ from the microbiome of the surrounding environment, (2) seagrass leaf microbiome to be similar across regions with different source pools, and (3) a consistent core assemblage found across tissue ages and regions. We further compare the role of selective processes influencing microbial biofilm communities using data from artificial seagrass leaves made of plastic and shaped like natural seagrass that were deployed in one of the regions. We used these artificial leaves to assess the similarity between the biofilm communities on this passive surface and natural seagrass shoots.

METHODS

Sampling

The Hakai Lúxvbálís conservancy encompasses 120,000 acres of land and sea and is the largest provincial marine protection area on the Coast of British Columbia. The conservancy hosts relatively low human impacted *Zostera marina* meadows. We collected 54 *Zostera marina* shoots and 35 seawater samples by scuba diving among eight paired meadows nested within four geographical regions (**Figure 1**), within two weeks during the summer of 2015.

For seagrass leaf samples, we collected one shoot just outside each quadrat and placed it inside a sterile Ziploc bag underwater at the location of collection. New leaves of *Zostera marina* shoots are encompassed within a protective leaf sheath as they develop from a basal meristem. The sheath bundles all the leaves of an individual shoot by surrounding the blades at the shoot base and minimizes exposure to the environmental pool of microbes for new leaves. We accessed the new leaf growth by carefully opening the sheath from its upward edge and locating the youngest leaf tip within the sheath bundle. The old growth (emerging beyond the sheath) was standardized across replicates by sampling a 10 cm length of the mid-section of the third oldest leaf close to the sheath (frequently the longest leaf), in an area with minimal macro-epibiota such as bryozoan species and the red algal epiphyte, *Smithora naiadum*. Both new and old growth leaves, from the same shoot, were rinsed with 0.22 μm filtered seawater for 10 seconds to remove passive microbes and then

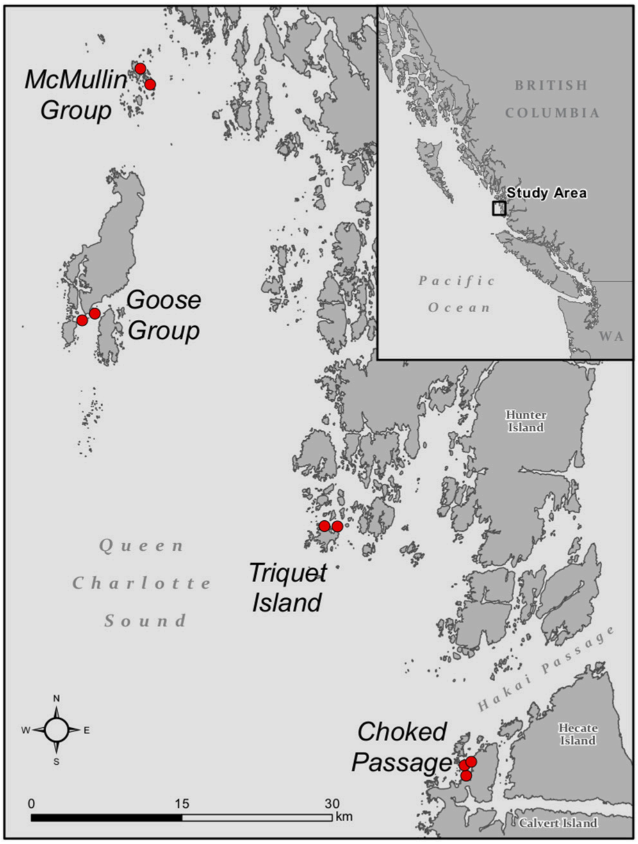


FIGURE 1 | Map showing the sampled seagrass meadows across four regions along the Central Coast of BC. A total of 54 *Zostera marina* shoots and 35 seawater samples were collected.

sampled for another 10 seconds with a Puritan® sterile swab and transferred to a sterile cryovial (VWR). Seawater samples (500 mL; 3-5 reps) were collected by scuba diving in Nalgene water bottles from each meadow at seagrass collection depth (3–5 meters) within ∼ 1 meter of the eelgrass canopy before the seagrass sampling. All samples were stored on ice in the field and transferred to a −80°C freezer upon return. Water samples were filtered using 0.22 µm membrane filters using a peristaltic pump the same day of collection.

In order to further explore biological and physical environmental filtering processes in seagrass ecosystems we sampled microbial communities from artificial seagrass units deployed in the Choked region. A single unit consisted of a 15 cm^2 plastic grid for securing the artificial plants on which were attached 4 seagrass "shoots" (1 centimeter wide strips of high density polyethylene) each consisting of two 50 centimeter long "blades." This provided a blade width, length and shoot density within the range observed in the same location in July 2014 (unpublished data, Hakai Institute). Artificial seagrass units were anchored to the substrate within the eelgrass meadow along a transect such that they were 40 meters apart and 10 m away from the meadow edge. When retrieved, a single blade on one shoot of each artificial seagrass unit was sampled 1 week after deployment and processed the same way as seagrass samples.

Molecular Methods

DNA was extracted from swabs and water filters using the MoBio PowerSoilVR –htp 96 well DNA extraction kit (Carlsbad, CA) following the manufacturers recommended protocol. The V4 region of 16S rRNA gene in Bacteria and Archaea was targeted for amplification using redesigned versions of the primers 515f/806r (Caporaso et al., 2012): 515f: 5'–GTGYCAGCMGCC GCGGTAA−3', 806r: 5'–GGACTACNVGGGTWTCTAAT−3' as previously described by Lemay et al. (2018a). Equal amounts (25 ng) of each sample were pooled and then purified using the MoBio UltaCleanVR PCR clean-up kit. Pooled library quantitation and paired-end Illumina MiSeq sequencing (2 × 300 bp) was carried out at the Integrated Microbiome Resource facility in the Center for Genomics and Evolutionary Bioinformatics at Dalhousie University (Halifax, Canada).

Sequence Data Analysis

Raw sequencing reads were demultiplexed using the Split Libraries function from the Quantitative Insights into Microbial Ecology (QIIME v.1.9) analysis pipeline (Caporaso et al., 2010b). Demultiplexed reads were quality trimmed, then truncated to a uniform length of 250 bp with a quality score of 20 using FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and processed into taxonomic units using the Minimum Entropy Decomposition (MED) method (Eren et al., 2015). MED does not rely on sequence similarity thresholds such as a standard 97% OTU, but instead uses Shannon entropy to separate out meaningful patterns of nucleotide diversity from sequencing noise and partition the data into MED nodes, which in practice are roughly analogous to ASVs or ≥99% OTUs. This analysis was carried out with the minimum substantive abundance parameter (–M) set at 500 reads while other parameters were run with default settings. Taxonomy was assigned to the resulting MED-nodes as implemented in the Assign Taxonomy function of QIIME v.1.9 using SILVA 128 database (Quast et al., 2013) clustered at 99% similarity. OTUs annotated to either chloroplast or mitochondrial sequences were removed as host contamination. Additionally, we removed OTUs with fewer than three reads per sample and fewer than 250 total reads, and samples with low read count (fewer than 1000 reads). After filtering, the final dataset consisted of 1206 OTUs with sequences/sample counts ranging from 3,130 to 103,313. Representative sequences for the remaining OTUs were aligned with PyNAST v.1.2.2 (Caporaso et al., 2010a), and a tree was constructed using FastTree (Price et al., 2010) within Qiime v 1.9. Alpha and beta diversity analyses were performed after rarefying the data to 3,000 reads/sample.

Statistical Analyses

We calculated OTU richness using the non-parametric Chao1 index (Chao, 1984) and Pielou's evenness (Pielou, 1966). We used Analysis of Variance (ANOVA) to test for differences in alpha diversity metrics among sample types (new and old seagrass leaf growth, seawater) with geographical regions as a random effect, using function "lm" in the stats R package (R Core Team, 2020). Additionally, we ran an ANOVA with seagrass individual as a random effect to confirm differences between new and old seagrass leaves after accounting for sampling from the same plant. Because artificial seagrass samples were only taken in the Choked region, comparisons with real seagrass and seawater were restricted to samples from this region only. We performed pairwise comparisons using Tukey HSD correction for multiple comparisons. In cases where we found heterogeneity in the data, we randomly subsampled the sample types to get equal sample sizes, so that *F*-tests are robust (Keppel, 1991). The rarefaction curves for new and old seagrass leaves and artificial seagrass are all saturated. Several seawater samples do not appear to have reached saturation; therefore, richness comparisons with seawater samples were not discussed in depth.

To further compare microbial community composition, we visualized the similarity of sample types across regions with a non-metric multidimensional scaling (NMDS) using weighted UniFrac distance (phylogenetic relatedness of the species) (Lozupone and Knight, 2005) and used Permutational Multivariate Analysis of Variance (PERMANOVA) to test for differences in community structure between sample types for all regions, using both the weighted UniFrac distance and the Bray-Curtis dissimilarity index (abundance data). We should find similar patterns in both metrics when communities are composed of distantly related species, whereas when communities have a large share of closely related species, we should find a higher overlap in the UniFrac distances compared to Bray Curtis dissimilarities (Ortmann and Ortell, 2014). We further used Jaccard dissimilarity (presence-absence data) on PERMANOVA to test if differences were also related to community composition. PERMANOVA was performed using function "adonis2" in the vegan R package (Oksanen et al., 2018) to account for marginal effects of regions in each sample type (new and old seagrass leaf growth, seawater), and for Choked region including comparisons with artificial seagrass. We further compared new and old growth seagrass leaves to account for the effect of the individuals, since they were sampled from the same plant. Results of PERMANOVA analyses are potentially sensitive to heterogeneity in dispersions among treatments when there is an unbalanced design (Anderson and Walsh, 2013). Therefore, when our data was heterogeneous, we ran PERMANOVA tests by randomly subsampling the sample types to get a balanced design. We also tested for differences in the microbial community of each sample type among regions. Pairwise comparisons with Benjamini and Hochberg correction were conducted using function "adonis.pair" in the EcolUtils package (Salazar, 2019). Test statistics were generated using 10,000 permutations. Moreover, we calculated the percentage of taxa shared between sample types and regions, based on OTUs found in at least two samples of each sample type or region to avoid accounting for noise.

To determine the core microbes consistently associated with both new and old growth leaves of *Zostera marina*, we used Indicator Species Analysis (IndVal) (Dufrêne and Legendre, 1997), with the function "multipatt" in the Indicspecies R package (De Caceres and Legendre, 2009). IndVal combines both specificity (i.e., if a species is found in higher relative abundances in a particular habitat) and fidelity (i.e., if a species is prevalent across samples from that habitat) to determine indicator species (Legendre, 2013). This approach has been used to identify habitat specialists of coral compartments (Li et al., 2014) and of the mucus and surrounding habitats of corals (Glasl et al., 2016). We considered OTUs as core taxa if they were significant according to the permutation tests showing high association values (IndVal statistics > 0.7) (Demircan et al., 2018; Kruger, 2020), present in over 50% of the samples from that sample type, and present in all regions. This means that for an OTU to be considered core of *Zostera marina*, for example, it would have to be found in relatively higher abundance in both new and old leaves when compared to seawater and artificial seagrass, be present in all regions and across most of the seagrass samples. We ran this analysis including seawater and artificial seagrass samples to determine the core seagrass microbes that were not generally found in either seawater or other marine surfaces. Significance ($P < 0.05$) was tested by 10,000 random permutations.

All data statistical analyses were performed in R version 3.6.3 (R Core Team, 2020). Graphs were constructed in R using package ggplot2 (Wickham, 2016).

RESULTS

Seagrass Microbiome in Comparison to the Environment

Microbial communities associated with seagrass differ in diversity from the surrounding seawater. Both richness and evenness differed between new and old growth leaves and seawater [ANOVA Richness $F_{(2,99)} = 31.51, p < 0.0001$; Evenness $F_{(2,99)} = 30.34, p < 0.0001$] (**Figure 2A**; **Supplementary Table 1**). New and old leaves were sampled from each plant, but differences in richness and evenness remain significant even after controlling for individual (**Supplementary Table 1**). Microbial communities in new growth leaves showed the lowest OTU richness and highest dominance (a few species were highly abundant, and most were rare). Richness in old growth leaves was the highest, and the abundance was more evenly distributed between species than in new growth leaves. Artificial seagrass was similar to old growth leaves in terms of richness and evenness (**Figure 2B**; **Supplementary Table 1**).

PERMANOVA analyses showed a clear distinction in microbial community structure between seawater, new and old growth seagrass leaves [weighted UniFrac distance; $F_{(2,99)} = 70.75$, $P < 0.0001$, $R^2 = 0.55$] (**Figure 2C**, **Supplementary Table 2**). These community distinctions are underlain by differences in relative abundance as well as composition as the differences are also significant when using non-phylogenetic Bray-Curtis and Jaccard dissimilarity indices (**Supplementary Table 2**). Differences in microbial community structure between new and old growth leaves persist after accounting for individuals as a random effect [PERMANOVA weighted UniFrac distance; $F_{(1,43)} = 49.82$, $P < 0.0001$, $R^2 = 0.29$; **Supplementary Table 2**]. Almost 50% of all taxa were shared among *Zostera marina* and seawater samples across

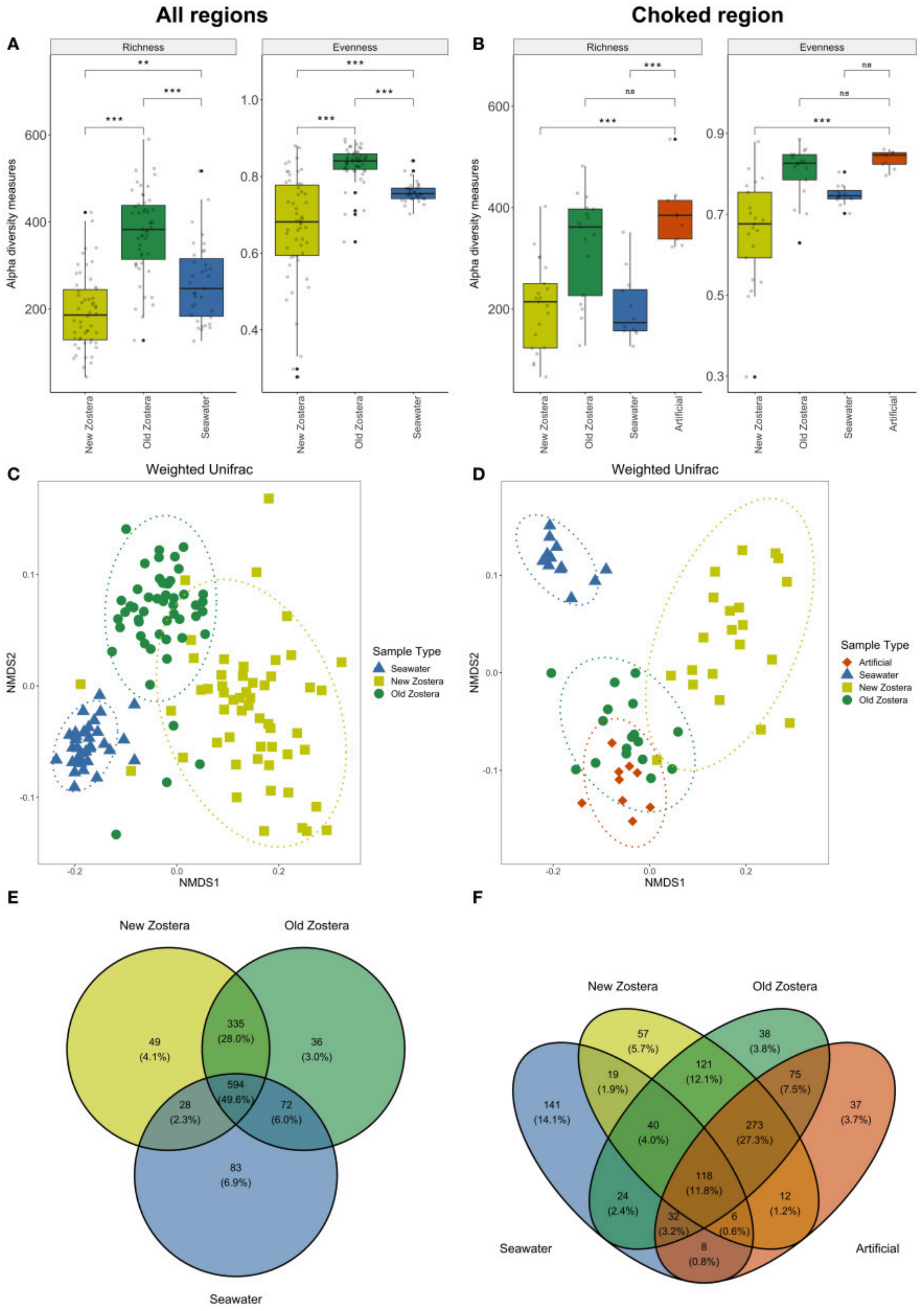


FIGURE 2 | Alpha diversity metrics, NMDS plots based on weighted UniFrac distances, and Venn diagrams of shared taxa of microbial communities on Z. marina new growth leaves, old growth leaves, and seawater for all regions **(A,C,E)**, and for Choked region including artificial seagrass **(B,D,F)**. Asterisks in boxplots represent significant difference, where *indicates $p > 0.05$, **indicates $p > 0.01$, and ***indicates $p > 0.001$; ns, not significant. Ellipses in NMDS plots represent ordination confidence intervals (95%).

all regions, though shared taxa where generally much more abundant in one sample type, and new and old growth leaves shared around 28% of taxa (**Figure 2E**). We used data for the Choked region to compare microbiome on natural (new and old growth) and artificial seagrass leaves. We found that all sample types were distinct from each other [**Supplementary Table 2**: PERMANOVA weighted UniFrac $F_{(3,32)} = 18.98$, $P < 0.0001$, $R^2 = 0.64$], but artificial seagrass appear more similar to old growth (**Figure 2D**). Artificial seagrass shared the vast majority of OTUs with seagrass, with only 3.7% unique OTUs and <1% shared only with water (**Figure 2F**). Still, old and new leaves share more OTUs than do old and artificial (**Figure 2F**).

Spatial Variation in Seagrass Microbiome

Microbial community structure varied among regions on seagrass but not in the water column, with the most pronounced differences observed in old seagrass leaves (**Figure 3**). Microbiome significantly differ by region using both taxon and phylogenetic-based metrics for old growth [PERMANOVA Bray Curtis $F_{(3,40)} = 5.87$, $P < 0.0001$, $R^2 = 0.30$; Weighted UniFrac $F_{(3,40)} = 8.41$, $P < 0.0001$, $R^2 = 0.38$] and new growth [PERMANOVA Bray Curtis $F_{(3,50)} = 2.78$, $P < 0.0001$, $R^2 = 0.14$; Weighted UniFrac $F_{(3,50)} = 2.35$, $P = 0.0018$, $R^2 = 0.12$] leaves. However, pairwise comparisons using weighted UniFrac distances indicated greater regional overlap in microbial communities for new growth leaves (**Figures 3A,D**; **Supplementary Table 3**); this contrast between Bray Curtis and Weighted Unifrac results indicate that new leaves have a large share of closely related species across regions (Ortmann and Ortell, 2014). Old growth leaves remained quite distinct (**Figures 3B,E**; **Supplementary Table 3**). There was also a lower degree of taxa sharing between regions, with 19.7 and 31.7% of taxa shared among regions for new and old growth, respectively (**Figure 3**). Microbial community structure in seawater samples was somewhat distinct across regions when we considered both Bray-Curtis and UniFrac distance (**Figures 3C,F**; **Supplementary Table 3**), and around 32.1% of taxa were shared among all regions (**Figure 3I**).

Core Bacteria of Seagrass

We used indicator species analysis (Dufrêne and Legendre, 1997) to define core bacteria, with the criteria that the core are significantly enriched on seagrass compared to seawater and artificial seagrass, at 50% or greater prevalence, and present across all regions. We identified core taxa across *Zostera marina* in general and separately on new and old growth leaves to better understand persistent taxa and changes with tissue age.

Zostera marina core: We found 11 core OTUs belonging to six families on *Zostera marina* leaves (enriched and prevalent in both new and old growth leaves; **Figure 4**). Six core OTUs come from the Methylophilaceae family, genus *Methylotenera*. One core OTU (genus *Marinomonas*) was undetected in seawater samples (**Figure 4**). *Alteromonas* and *Paraglaciecola* (Alteromonadaceae), *Rubidimonas* (Saprospiraceae), and Rhodobacteraceae round out the core (**Figure 4**).

New growth core: For new growth seagrass leaves, we found a small and taxonomically consistent set of 16 core OTUs. The new growth core includes several representatives from *Methylotenera* (Methylophylaceae) and *Marinomonas posidonica* (Oceanospirillaceae), the Marine Methylotrophic group 3 in the Piscirickettsiaceae family, and *Rhizobium* (**Figure 4**; the complete figure showing all core taxa can be found in the **Supplementary Figure 1**).

Old growth core: For old growth seagrass leaves we found 57 core OTUs. Core taxa for old growth leaves come from many different clades common on marine surfaces, including Granulosicoccaceae, Rhodobacteraceae, and Planctomycetaceae (**Figure 4**; **Supplemenatary Figure 1**).

Artificial seagrass core: The core genera for artificial seagrass include taxa typical of marine biofilms such as Saprospiraceae, as well as *Cocleimonas* (Thiotrichaceae) and taxa that are likely degrading these strips of plastic bags *Oleibacter* (Oceanospiralles).

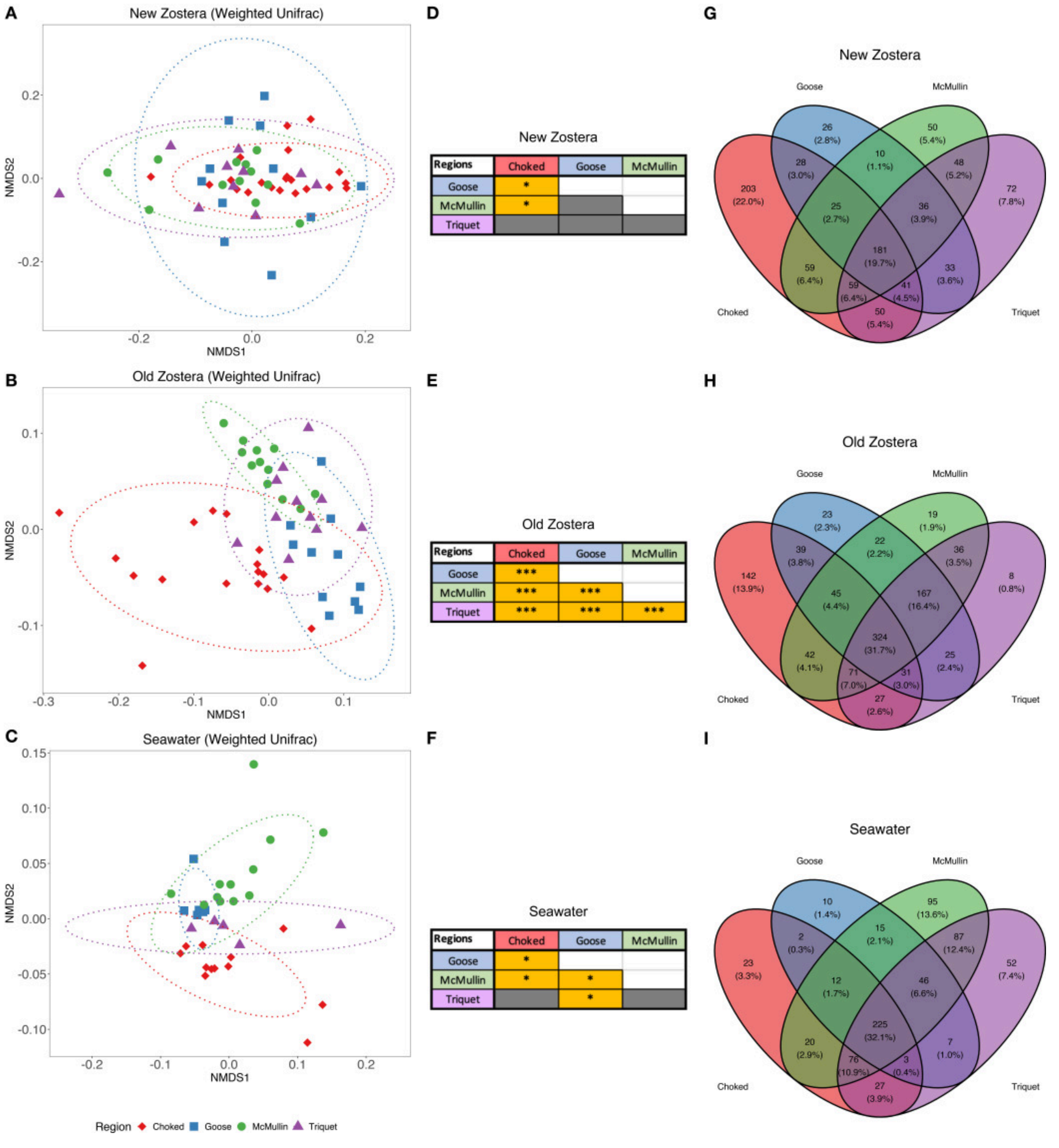
Detailed information on indicator species analysis statistics, taxonomy, and sequences of the core taxa can be found in the **Supplementary Table 4**.

DISCUSSION

Seagrass meadows are highly productive ecosystems. The microbes associated with seagrass likely influence seagrass health and cycling of carbon and other nutrients through the ecosystem. We investigated the specificity of seagrass microbiome compared to the water column and artificial seagrass, and the consistency of the microbiome across plant tissues of different age and across regions with different environmental conditions. We identified a core community associated with *Z. marina*. We hypothesized that if host selectivity is strong, we would find: (1) seagrass microbiome that differ from the microbiome of the surrounding environment, (2) seagrass microbiome that are similar across regions with different source pools, and (3) a consistent core assemblage across tissue ages and regions. We found good evidence for predictions 1 and 3: the seagrass leaf microbiome is distinct from their surrounding environment (**Figure 2**) and is colonized by a consistent suite of core taxa (**Figure 4**). Evidence for prediction 2 is mixed: we detected differences across regions in old leaves but little differentiation across regions for new leaves (**Figure 3**), that had a larger share of closely related species across regions. This points to seagrass leaves being more selective in newer tissues that are more highly defended. As seagrass blades age the species pool of colonizers available in each region may more strongly influence the leaf microbiome.

Seagrass Leaf Microbiome Are a Selective Habitat, but Are Largely Influenced by the Available Species Pool

Seagrass leaves harbor microbial communities that are distinct from seawater. The strong differences in microbiome composition hold using taxonomic and phylogenetic dissimilarity measures (**Figure 2**; **Supplementary Table 2**), and different bacterial lineages are dominant on seagrass compared to seawater. This result is consistent with previous studies reporting distinct microbial communities on a range



New Zostera

Regions	Choked	Goose	McMullin
Goose	*		
McMullin	*		
Triquet			

Old Zostera

Regions	Choked	Goose	McMullin
Goose	***		
McMullin	***	***	
Triquet	***	***	***

Seawater

Regions	Choked	Goose	McMullin
Goose	*		
McMullin	*	*	
Triquet		*	

FIGURE 3 | NMDS plots based on weighted UniFrac distances, pairwise PERMANOVA results table and Venn diagrams of shared taxa of microbial communities of *Z. marina* new growth leaves **(A,D,G)**, old growth leaves **(B,E,H)**, and seawater **(C,F,I)** samples among regions. Pairwise PERMANOVA grids colored in gray are not significant, whereas grids colored in yellow are significant, where * indicates $p > 0.05$ and *** indicates $p > 0.001$. Ellipses represent ordination confidence intervals (95%).

of marine hosts compared to the surrounding seawater communities, including seaweed (Michelou et al., 2013; Lemay et al., 2018a), sponges (Thomas et al., 2016), and seagrass (Bengtsson et al., 2017; Crump et al., 2018; Ugarelli et al., 2019). Our results contrast with a global study of seagrass that found similar microbiome on seagrass leaves using different

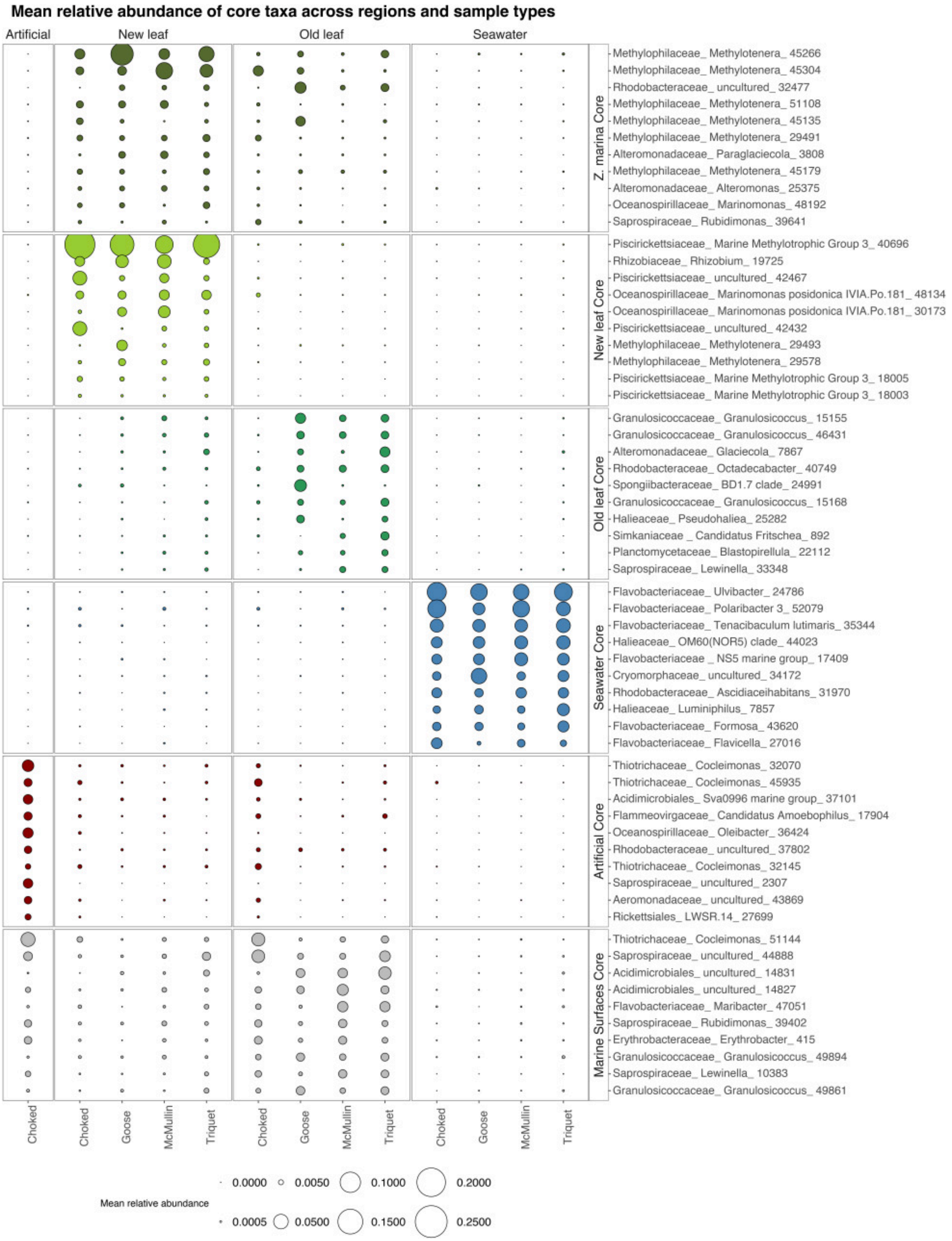


FIGURE 4 | Bubble plot showing the mean relative abundance of the 10 most abundant core taxa across regions and sample types: New growth leaves, Old growth leaves, *Zostera marina* (core in both new and old growth, here all 11 core taxa are shown), seawater, artificial and marine surfaces (core in both old leaves and artificial seagrass). We considered core taxa the ones significant in Indval analysis, present in at least 50% of the samples and present in all regions. Taxa are labeled by Family names along with the lowest taxonomy level identified.

methods (Fahimipour et al., 2017), as discussed in (Crump et al., 2018).

Differences between seagrass and seawater microbiome point to the importance of selective mechanisms in microbial community assembly. The physicochemical properties of seagrass leaves and seagrass exudates enrich for or select against particular taxa from the surrounding environment. Seagrass surfaces are chemically defended by compounds such as flavonoids, phenolic compounds, and carboxylic acids (Papazian et al., 2019). Seagrass also exudes nutrients and dissolved organic carbon, including methanol, that support abundant bacterial production (Penhale and Thayer, 1980; Kirchman et al., 1984). Experimental work in seagrass shows that different cultured isolates grow on seagrass blades compared to abiotic surfaces (Kurilenko et al., 2007; Papazian et al., 2019), supporting the notion that seagrass leaves are selective surfaces. Metatranscriptomic analysis highlights matches between seagrass leaf bacterial metabolism and the plant chemistry/exudates (Crump et al., 2018).

Almost half of the OTUs in our study were shared between seagrass and seawater (**Figure 2E**) and most of the seagrass core taxa are found at low relative abundance, but still present, in the water column (**Figure 4**). Thus, seagrass leaf microbiome seems to be primarily acquired horizontally via transmission through the water column, as is also the case for sponges (Turon et al., 2018), seaweeds, and other marine surfaces (Russell, 2019). Studies of the microbiome in seeds and/or shoots grown from seed in sterile conditions are needed to investigate vertical transmission. Transmission of microbes is bidirectional; some of the microbes detected in seawater, which was sampled just above the seagrass meadow canopy, surely originated on seagrass. An additional layer of complication arises because seagrass and seaweed exudates can influence the microbial composition of the water column (Lam et al., 2008; Lamb et al., 2017; Chen and Parfrey, 2018).

Our data is consistent with initial colonization of a handful of seagrass specialists that feed on the metabolic by-products of seagrass—particularly methanol and 1-carbon compounds such as *Methylotenera* (Kalyuhznaya et al., 2009) and Piscirickettsiaceae Methylotrophic group 3 (Krolicka et al., 2017), as well as a potential seagrass symbiont *Marinomonas posidonica* (Lucas-Elió et al., 2011) and predicted nitrogen fixing *Rhizobium* (Avis et al., 2008). We expected new leaves to represent a more selective microbial environment because they are more chemically defended and have lower microbial biomass (Harrison, 1982; Kurilenko et al., 2001). Further, new leaves were fully within the protective sheath at the time of sampling, and thus in much reduced contact with the water column microbial source pool. Consistent with expectations, new leaves of *Zostera marina* harbor reduced microbial diversity and their microbiome composition is distinct from old leaves (**Figure 2**).

As seagrass blades age, they accumulate higher bacterial loads (Harrison, 1982) and are colonized by diverse micro- and macroalgae. These biofilms support a diverse array of bacteria and heterotrophic grazers such as ciliates, copepods, and rotifers (Segovia et al., 2020) that feed on metabolic by-products or directly on microbial or algal biomass. Although only 11 core taxa are shared between new and old growth leaves, many more are shared between old growth leaves and artificial seagrass (**Supplementary Figure 1**). Our data suggest that as *Zostera marina* leaves age, seagrass microbiome composition shifts to include more generalist biofilm taxa, along with seagrass specialists. In contrast with Ettinger et al. (2017), we found similarities between seagrass leaf- and seaweed microbiome even at lower taxonomic ranks, since the genera *Blastopirellula* and *Granulosicoccus*, both core to old growth leaves (**Figure 4**), are also commonly found on seaweeds (Bondoso et al., 2017; Lemay et al., 2018a; Weigel and Pfister, 2019). Research on influence of plant age in terrestrial plants also show that leaves harbor distinct communities over time, but contrary to our findings, they seem to resemble the surrounding (i.e., air and sediment) communities during the first stages while converging to more unique communities as they age (Maignien et al., 2014; Copeland et al., 2015). This difference could be due to new leaves in seagrass being protected within the sheath and/or more chemically defended, as mentioned above.

Many taxa overlap between old growth and artificial leaves (**Figure 4** Marine surfaces core), including representatives of Saprospiraceae, Flavobacteriaceae and additional *Granulosicoccus* OTUs, all of which are widespread on marine surfaces (Burke et al., 2011; Florez et al., 2017; Van De Water et al., 2018; James et al., 2020). The increase in generalists is likely due to decreased selectivity of the leaves with age (Harrison, 1982), coupled with the formation of a biofilm that enables generalists to colonize (Datta et al., 2016). It is likely that other marine surfaces are an important source pool for seagrass leaves as they age, as many core genera present in old growth leaves are shared with other hosts.

Additional research, particularly experimental manipulations, are necessary to uncover the relative importance of host selectivity and the environment in the microbiome assembly of seagrass leaves.

Several Core Seagrass Taxa Likely Perform Important Functions for the Host

We identified core bacteria as those found in more than 50% of samples for a given sample type, found across regions and enriched in relation to other sample types. Only a few taxa associated with seagrass met these criteria and represent the *Zostera* core microbiome (**Figure 4**). Corals also have a small core microbiome (Hernandez-Agreda et al., 2018) and this is likely a common phenomenon. Below we discuss potential functions and distribution of select members of the core.

Only 11 taxa are consistently part of the *Zostera* core for both new and old leaves. Most of these core taxa belong to methylotrophic clades within the Methylophilaceae (genus *Methylotenera*) and Piscirickettsiaceae, while *Rhizobium*, is usually involved in nitrogen fixation and is known for promoting plant growth (Avis et al., 2008). Methylophilaceae are non-methane oxidizing methylotrophs—which use single-carbon compounds such as methanol and methylamine as carbon and energy sources. They have been suggested to use the methanol released by the methane oxidizing methanotrophs (Chistoserdova and Kalyuzhnaya, 2018) and by the seagrass itself—which is a toxic by-product for the plant—and were found to be an important component of *Z. marina* leaf microbiome (Crump et al., 2018). Other methylotrophic bacteria—Gammaproteobacteria, family Piscirickettsiaceae—encompassed half of all core taxa found in new leaves. *Pseudoalteromonas*, a common marine genus that is part of the core of new leaves (**Supplementary Figure 1**), shows antibacterial and antialgal activity, and thus may modulate the establishment of other species (Lage and Bondoso, 2014). The microbes present in new leaves could influence the assembly of communities in old leaves.

Marinomonas appears to be a true associate of seagrass, and we find *Marinomonas* in the new growth and overall *Zostera* core (**Figure 4**). This genus has been recently isolated from *Zostera marina* leaf tissues from Bodega Bay, California (Ettinger and Eisen, 2020). Several species of *Marinomonas* have also been isolated from the seagrass *Posidonia oceanica* on the coast of Spain (Espinosa et al., 2010; Lucas-Elió et al., 2011), including *M. posidonica,* which is in the core of new leaves here (**Figure 4**). *Marinomonas posidonica* was shown to enhance leaf growth in *P. oceanica* likely due to the release of metabolites and nutrients (Celdrán et al., 2012). It has also been registered in leaves of the seagrass *Enhalus acoroides* in Papua New Guinea (Hassenrück et al., 2015). This indicates that this taxon may have an affinity for seagrass leaf microbiomes.

Several taxa we identified in the core for old growth and *Zostera* overall are common colonizers of marine plants and algae (**Figure 4**). The genus *Granulosicoccus* (Gammaproteobacteria) is part of the *Z. marina* core, as well as the marine surfaces core, encompassing most of the old growth leaves core taxa. *Granulosicoccus* was also pervasive across leaf microbiomes of two *Zostera* species on the coast of Oregon, US (Crump et al., 2018), and has been commonly found in association with seaweeds in the Calvert Island region where we sampled (Lemay et al., 2018a). Rhodobacteraceae are frequently the first colonizers of marine surfaces (Dang et al., 2008) and are found here on both new and old leaves. Several taxa from this family are in seagrass leaf microbiomes across different regions [German Baltic Sea coast: (Bengtsson et al., 2017); Bodega Bay, CA: (Ettinger et al., 2017); coast of Oregon, US: (Crump et al., 2018)]. Similarly, *Blastopirellula* (Planctomycetes) is commonly found on macroalgae across the globe (Bondoso et al., 2017) and in the core for old leaves here.

Interestingly, some core OTUs found in old growth leaves and artificial seagrass are endosymbionts of protists and invertebrates. This suggests their eukaryotic hosts must also be common in the seagrass microbiome, demonstrating that exchange with the microbiome of other organisms also contributes to the overall diversity of seagrass microbial communities. For instance, OTUs assigned to "*Candidatus Fritschea*" in the order Chlamydiales (family Simkaniaceae) are among the core of old leaves. *Candidatus Fristchea* are obligatory intracellular endosymbionts first found living in the gut of terrestrial insects (Thao et al., 2003), while two closely related Chlamydiales symbionts have been identified from marine environments living in association with the marine worm *Xenoturbella westbladi* and the gills of Atlantic salmon (Israelsson, 2007; Everett, 2014). *Caedibacter caryophilus*, core to artificial seagrass, are endosymbionts of the protist *Acanthamoeba* (Horn et al., 1999).

Further comparative studies including samples from seagrass roots, nearby sediments and other marine surfaces are needed to identify the true habitat range of these core taxa and their influence on host health.

CONCLUSION

Differences between the seagrass leaf microbiome and the surrounding seawater and artificial seagrass, together with the consistent core assemblage across tissue age and regions, all point to host selectivity being an important process in the microbiome assembly in seagrass leaves. Microbiome community structure on new and old growth leaves is distinct; new leaves are also less diverse with a smaller suite of core, and show little regional differentiation, in contrast with old leaves that are spatially more distinct. This points to seagrass leaves being more selective in newer tissues that are more highly defended. As seagrass blades age the species pool of colonizers available in each region more strongly influences the leaf microbiome. Despite those differences, we found 11 core microbial taxa that persisted throughout development, showing a strong association with *Z. marina*. These core taxa may perform key functions important for the host such as detoxifying seagrass waste products, enhancing plant growth, and controlling epiphyte cover. The functional role of these core taxa should be further investigated in follow up studies. These findings provide baseline knowledge of the seagrass microbiome that will inform our understanding of marine microbiome in this foundational species.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: https://www.ebi.ac.uk/ena/browser, PRJEB25267. More details about the code and figure construction can be found in GitHub: https://github.com/biatsegovia/Core_Z.marina_leaf_microbiome.git; a copy of this repository is archived at Zenodo: https://doi.org/10.5281/zenodo.4287628.

AUTHOR CONTRIBUTIONS

RS-S analyzed the data and wrote the first draft of the manuscript. BS analyzed the data, interpreted the results, and led the writing. EM performed bioinformatic analysis. CF, MO'C, and MH-L collected the host data. RS-S, ML, and LP collected and generated the microbial data. LP, MO'C, and MH-L designed and conceived the study. All authors contributed to manuscript corrections and improved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.605304/full#supplementary-material

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Metabolic Symbiosis Facilitates Species Coexistence and Generates Light-Dependent Priority Effects

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Metabolic symbiosis is a form of symbiosis in which organisms exchange metabolites, typically for mutual benefit. For example, acquired phototrophs like *Paramecium bursaria* obtain photosynthate from endosymbiotic green algae called *Chlorella.* In addition to facilitating the persistence of *P. bursaria* by providing a carbon source that supplements *P. bursaria*'s heterotrophic digestion of bacteria, symbiotic *Chlorella* may impact competitive interactions between *P. bursaria* and other bacterivores, with cascading effects on community composition and overall diversity. Here, we tested the effects of metabolic symbiosis on coexistence by assessing the impacts of acquired phototrophy on priority effects, or the effect of species arrival order on species interactions, between *P. bursaria* and its competitor *Colpidium*. Our results suggest light-dependent priority effects. The acquired phototroph benefited from metabolic symbiosis during sequential arrival of each organism in competition, and led to increased growth of late-arriving *Colpidium*. These findings demonstrate that understanding the consequences of priority effects for species coexistence requires consideration of metabolic symbiosis.

Keywords: acquired photosynthesis, ciliates, *Colpidium*, *Paramecium bursaria*, protist microcosm

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INTRODUCTION

Metabolic symbiosis is a form of symbiosis in which organisms exchange metabolites, typically for mutual benefit. In some cases, metabolic partners are capable of integrating metabolic pathways across genomes to produce shared metabolites, such as amino acids and vitamins, for mutual survival (Husnik et al., 2013), and other organisms can directly use metabolites produced by a partner organism for energy or nutrient sources (Estrela et al., 2012). Symbiotic mycorrhizal fungi benefit plant hosts by increasing the efficiency of nutrient acquisition in exchange for carbohydrates (Marschner and Dell, 1994). Similarly, planktonic ciliates, such as *Paramecium bursaria,* host algae in exchange for photosynthates (Stoecker et al., 2009; Iwai et al., 2019). Symbiotic relationships, such as mutualisms that benefit the two organisms involved in the symbiosis, can also have important implications for community dynamics by promoting diversity, population stability (Pachepsky et al., 2002; Mougi and Kondoh, 2012), and even co-evolution (Guimarães et al., 2011). Metabolic symbiosis is context-dependent: environmental conditions are known to shift mutually beneficial relationships into host-parasitism when costs begin to outweigh benefits of symbiosis (Bronstein, 1994; Johnson et al., 1997; Lowe et al., 2016).

When organisms receive metabolic benefits from a symbiont, this symbiosis can increase competitive ability and facilitate coexistence between competitors. In plant and mycorrhizal symbiosis, fungal partners may increase competitive abilities of host plant species through differential benefits (Callaway et al., 2001), and may also mediate competition through common mycorrhizal networks within a plant population (Francis and Read, 1984; Grime et al., 1987). Acquired phototrophs, such as *P. bursaria*, can also become a better competitor by gaining access to a new resource via acquisition of photosynthetic machinery through their endosymbionts (Moeller et al., 2016). However, the extent of these benefits can vary. The growth rate of *P. bursaria* with endosymbiotic algae depends on temperature (Salsbery and DeLong, 2018) and light level (Pado, 1965; Weis, 1974). Here, we study the role of metabolic symbiosis on species coexistence under different community assemblage scenarios.

The effects of metabolic symbiosis on competitive outcomes may also be impacted by the particular timing and order in which species join a community, a phenomenon known as priority effects (Chase, 2003; Fukami, 2015). In other words, the competitive ability of a species can change based on its arrival order relative to its competitor. Priority effects have been studied across taxonomic domains between yeast and bacteria (Fukami, 2015), between functional groups in plants (Weidlich et al., 2018), and under various environmental conditions (Vannette and Fukami, 2014). The outcomes of priority effects are highly variable across species and ecosystems (Fukami, 2015). When priority effects are present, there are two possible outcomes: facilitation, in which the early arriving species positively influences the growth of the late-arriving species, or inhibition, where the opposite is true (Fukami, 2015). The impact of a metabolic symbiosis on the strength of priority effects has not been extensively investigated.

In this study, we used two bacterivorous ciliate competitors, *Paramecium bursaria*, and *Colpidium* sp., to test for priority effects. *Colpidium* is a strict heterotroph while *P. bursaria* engages in metabolic symbiosis with its algal endosymbiont, *Chlorella*. *Paramecium bursaria* almost always host *Chlorella*, yet is known to harbor endosymbionts that come from at least five different species, including *Coccomyxa simplex* and *Micractinium conductrix* (Hoshina and Imamura, 2008; Johnson, 2011; Pröschold et al., 2011). In the presence of light, endosymbiotic *Chlorella* undergoes photosynthesis and provides sugar metabolites for *P. bursaria* presumably in exchange for protection (Karakashian, 1963; Pado, 1965; Weis, 1974). *Paramecium bursaria* also provides essential nitrogen by prey cell digestion for *Chlorella's* nutritional requirement, and *P. bursaria* cells with endosymbionts have increased bacterial ingestion (Albers and Wiessner, 1985; Johnson, 2011). To understand the role of metabolic symbiosis on species coexistence, we tested the strength of priority effects between *P. bursaria* and *Colpidium*. We manipulated light level to control the contribution of photosynthesis on *P. bursaria*'s growth. We hypothesized that as light level increased, *Chlorella* would also increase its photosynthetic capacity, thereby providing more metabolites for *P. bursaria*'s growth. In darkness, hosting photosynthetic endosymbionts becomes too costly and confers no energetic benefits, so *Chlorella* would likely be expelled and *P. bursaria* would rely entirely on heterotrophy (Lowe et al., 2016). In our study, we did not observe bleached *P. bursaria*, suggesting that expulsion, if it was occurring, proceeded at slower timescales than death of *P. bursaria*. The two species were inoculated sequentially to mimic differential species assemblage, and the strength of priority effects were measured by comparing species densities across treatments. Our results suggested light-dependent priority effects. The acquired phototrophs persisted in the presence of metabolic symbiosis with endosymbiotic algae when the competitors assembled sequentially, and even facilitated the growth of late-arriving *Colpidium*. Our findings demonstrated that understanding the consequences of priority effects for species coexistence and overall maintenance of biodiversity requires consideration of metabolic symbiosis.

METHODOLOGY

Cultures of *Colpidium* sp., *P. bursaria*, and bacterial species (see below) were obtained from Carolina Biological Supply company. To allow *Colpidium* and *P. bursaria* to adjust to the light environments corresponding to the experimental conditions, the laboratory stock cultures were maintained at 50, 100, and 200 μmol quanta m^{-2} s^{-1} in an incubator at 24°C under controlled light conditions (12 h light: 12 h dark) for at least 2 weeks prior to experimentation.

Initially sterilized protozoan pellet media (Carolina Biological Supply Company) was inoculated with bacteria 2 days prior to the start of experimentation to control for bacterial prey availability in experimental microcosms. We inoculated treatments with three species of bacteria: (1) *Serratia marcescens* and the dominant bacterial species from purchased protist cultures of (2) *P. bursaria* and (3) *Colpidium*. The purchased protist cultures were plated onto Nutrient Agar (Research Products International), and we isolated the most abundant bacterial morphotype to identify the dominant bacterial morphospecies co-occurring with *P. bursaria* and *Colpidium*. These bacterial morphospecies were transferred from the agar plates to liquid media culture to be inoculated into treatment microcosms. Bacterizing experimental media with the same three bacterial morphospecies ensured that each treatment culture was primed with the same key bacterial prey community members.

To test how photosynthetic symbiosis impacted the strength of priority effects, we sequentially inoculated *P. bursaria* and *Colpidium* into experimental microcosms at four different light levels (0, 50, 100, 200 μmol quanta m^{-2} s^{-1}) in triplicate. The two introduction treatments were: (i) "*P. bursaria*-first" sequential inoculations, in which *P. bursaria* was introduced first on Day 0, and *Colpidium* on Day 14; (ii) "*Colpidium*-first," in which *Colpidium* was introduced first on Day 0, and *P. bursaria* on Day 7. The intrinsic growth rate and carrying capacity of both species were determined by conducting single-ciliate control treatments. In single-species treatments, *Colpidium* had a much higher intrinsic growth rate, and stopped growing exponentially by Day 7, while *P. bursaria* required 14 days for its populations to stabilize. Because, in our priority effects experiments, we wanted *P. bursaria* and *Colpidium* to grow to their maximum population sizes before the introduction of the second species, we decided to allow *P. bursaria* 14 days of growth before

Colpidium inoculation for *P. bursaria*-first treatments, and 7 days for *Colpidium* in *Colpidium*-first treatments. Thus the total experiment contained 48 microcosms: 2 priority effect treatments (*P. bursaria*-first, *Colpidium*-first) × 4 light levels (0, 50, 100, 200 μmol quanta m^{-2} s^{-1}) × 3 replicates, plus 2 single-species controls (*P. bursaria* only, *Colpidium* only) × 4 light levels × 3 replicates.

At the start of each experiment, we inoculated 50 ciliates/mL into the experimental microcosms with 40 mL of liquid media. For priority effects treatments, we subsequently inoculated 50 ciliates/mL of the second species on Day 7 (*Colpidium*-first) or Day 14 (*P. bursaria*-first) of the experiment. We quantified population densities of each ciliate three times per week over the duration of the experiment (40 days). During sampling, an aliquot containing at least 50 ciliate cells (50–1,000 μL) was removed from each microcosm, and the ciliates were individually counted using a dissecting microscope. We used a range of aliquot sample volumes because the population sizes throughout the duration of the experiment (40 days) often differed up to two orders of magnitude (**Supplementary Figure S1**). More specifically, during the first week, we removed 1 mL from each experimental flask for enumeration, during the second and third weeks we removed 200 μL, and during the subsequent weeks we removed 50 μL.

We quantified a priority effects metric (modified from Vannette and Fukami, 2014) by comparing the maximum population sizes achieved by each species when introduced first [$D(i)_{ij}$] to maximum population when introduced last [$D(i)_{ji}$]:

$$P_{ij} = \ln\left[D\left(i\right)_{ji} / D\left(i\right)_{ij}\right] \quad (1)$$

The order of the subscripts i and j indicate arrival order of the species. By taking the natural log of this ratio of population sizes, we achieve a metric for which positive values indicate positive priority effects (e.g., lower population sizes when introduced first, indicating that the early presence of the other species benefited the later arriving species) and negative values indicate negative priority effects (e.g., lower population sizes when introduced last, indicating that the early presence of the other species inhibited growth of the later arriving species).

To confirm that observed priority effects were not due to the presence of free-living *Chlorella* cells, we visually inspected stock cultures and experimental cultures using light microscopy for the presence of *Chlorella* colonies. We also performed chlorophyll-*a* extractions on unspent media (media that had never been inoculated with *P. bursaria*) as well as spent media from experimental cultures at all light levels. We obtained this spent media by passaging 1 mL of culture volume through an 8.0 mm transwell membrane filter (Corning Incorporated, Corning, NY, United States), and confirming using light microscopy that the filtrate did not contain any *P. bursaria* cells. We then collected this filtrate on a GF/F filter (Whatman, SigmaAldrich, St. Louis, MO, United States), placed the filter in 5 mL of a 90% acetone solution in water, and incubated overnight at -20°C. The following day, we measured chlorophyll-*a* content in relative fluorescence units using a Trilogy fluorometer with a 450 nm LED (Turner Designs, San Jose, CA).

We performed all data analyses and plotting using the open-source software package R (R Core Team, 2020). Our analysis script and data files can be found at https://doi.org/10.5281/zenodo.4342275.

RESULTS

Paramecium bursaria was negatively affected by early arriving *Colpidium*, with *P. bursaria* populations visibly lower in all *Colpidium*-first treatments and nearly competitively excluded at the end of 40 days (**Figures 1A–D**). Conversely, *Colpidium* grew to higher population sizes when *P. bursaria* was introduced first, and density differences between *Colpidium*-first and *P. bursaria*-first treatments at each light level increased with increasing irradiance (**Figures 1E–H**). The calculated strength of priority effects using the metric defined by Eq. 1 supported these observations (**Figure 2** and **Supplementary Table S1**). *P. bursaria* experienced negative priority effects, which indicates that early arriving *Colpidium* inhibited the growth of *P. bursaria* (**Figure 2A**). **Figure 2B** shows the strength of priority effects by light level, and at 0 μmol quanta m^{-2} s^{-1}, *P. bursaria* populations died off independent of *Colpidium* (**Figures 1**, **2B**). Yet increasing light level (from 50 to 200 μmol quanta m^{-2} s^{-1}) did not enhance the negative affect of *Colpidium* on *P. bursaria* (**Figure 2B**). Conversely, *P. bursaria* had a positive priority effect on *Colpidium* (**Figure 2A**), and this positive priority effect strengthened with increasing light (**Figure 2B**). *Colpidium* populations at 100 and 200 μmol quanta m^{-2} s^{-1} both benefited from pre-established *P. bursaria*, with significantly higher positive priority effects at 200 μmol quanta m^{-2} s^{-1} (**Figure 2B**).

We obtained maximum population sizes within the 40-day duration of the experiment (**Figures 3A,B**) and growth rates captured the initial exponential growth period before reaching carrying capacity, calculated from the first 10 days for *Colpidium*, and 20 days for *P. bursaria* (**Figures 3C,D**). Comparison of priority effect treatments with single-species controls demonstrated that presence of any competitor, regardless of arrival order, inhibited *P. bursaria* growth (**Figure 1**). In high light (100 and 200 μmol quanta m^{-2} s^{-1}), *P. bursaria* maximum population abundances were significantly lower in the presence of *Colpidium* competitors (ANOVA $F = 47.14$, $p < 0.01$, **Supplementary Table S2**), and were visibly lower when *Colpidium* was introduced first (**Figure 3A**). Even when *P. bursaria* had pre-established populations before *Colpidium* was introduced in *P. bursaria*-first treatments, the later introduction of *Colpidium* resulted in lower maximum population sizes of *P. bursaria* (**Figure 3A**). The initial growth rates were not significantly different between the *P. bursaria* controls and *P. bursaria*-first treatments (**Figure 3C**), so lower populations sizes were not explained by initial growth rates. When *P. bursaria* was introduced second, however, the priority effect was strong enough to inhibit initial growth rates (**Figure 3C**) and ultimately, population sizes (**Figure 3A**).

Colpidium population densities were generally greater in the presence of *P. bursaria,* especially when *P. bursaria* arrived first

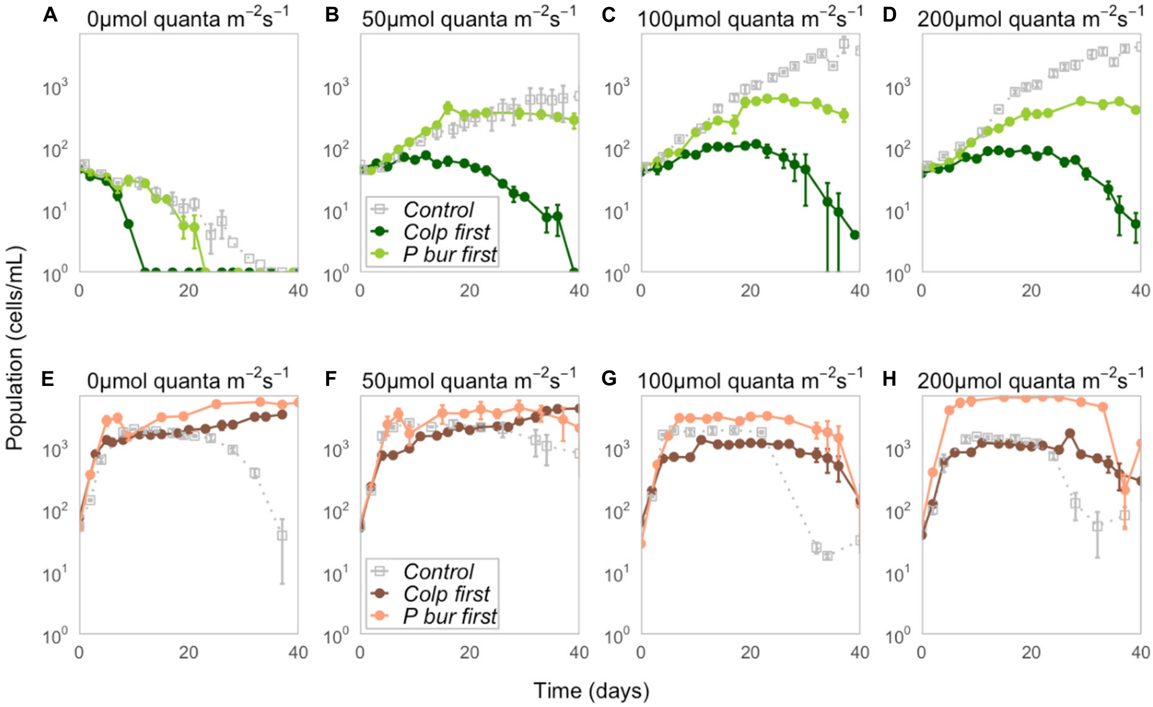

FIGURE 1 | Top row **(A–D)** Population dynamics of *P. bursaria* for control (gray), *Colpidium*-first (dark green), and *P. bursaria*-first (light green) treatments at four different light levels (0, 50, 100, and 200 μmol quanta m^{-2} s^{-1}). Time is measured in days since inoculation, rather than experimental day, so that *t* = 0 represents the date on which the focal (plotted) species was inoculated. (see **Supplementary Figure S1** for population size as a function of experimental day). Bottom row **(E–H)** Population dynamics of *Colpidium* for control (gray), *Colpidium*-first (dark coral), and *P. bursaria*-first (light coral) treatments at four different light levels (0, 50, 100, and 200 μmol quanta m^{-2} s^{-1}). Note that population densities were recorded on a logarithmic scale, and error bars represent ± one standard error.

(**Figure 3B**). However, there was an exception in *Colpidium*-first treatments at 100 and 200 μmol quanta m^{-2} s^{-1}, in which early arriving *Colpidium* did not benefit from the subsequent introduction of *P. bursaria* (**Figure 3B**). Effects on *Colpidium* were generally not mediated by initial growth rate, suggesting alleviation of later resource limitation was responsible for priority effects (**Figure 3D**). We found no evidence of free-living *Chlorella* cells in either microscope assays or through increases in chlorophyll-*a* fluorescence (**Supplementary Figure S2**).

DISCUSSION

Our results highlight the importance of acquired metabolism to community assembly. In our study, *P. bursaria*, whose photosynthetic symbionts provide a secondary source of energy, facilitated the later arrival of the strict heterotroph, *Colpidium* (a positive priority effect). This facilitative priority effect of *P. bursaria* on *Colpidium* is absent in direct competition (Hsu et al., unpublished) and is unlikely due to free-living endosymbiotic algae *Chlorella*. Although *Chlorella* can escape host cells and grow independent of *P. bursaria* (Lowe et al., 2016), there was no significant increase in chlorophyll-*a* from free-living *Chlorella* across light levels (**Supplementary Figure S2**) perhaps due to the presence of *Chlorella* viruses in our cultures or lack of regulatory factors to survive on its own (Kato and Imamura, 2009; Johnson, 2011). Facilitative priority effects are often explained by niche modification, in which the early arriving species alters the type of niches available in the local environment, consequently shaping the species that are capable of colonization (Fukami, 2015). One mechanistic explanation could be that photosynthetic activity by early arriving *P. bursaria* increased available organic material in the system, increasing the bacterial resources and, ultimately, the maximum population size of *Colpidium*. Because *Chlorella* are endosymbiotic, such a release of organic matter would have to occur through either excretion of waste products by *P. bursaria*, or lysis of *P. bursaria* cells and subsequent remineralization of this organic matter by bacteria. Positive priority effects have also been observed when certain plant species increase nutrient availability and alter soil chemistry, facilitate the establishment of mutualistic mycorrhizal networks, or leave behind nutrient-rich litter (D'Antonio and Vitousek, 1992; van de Voorde et al., 2011). Species can also change the physical conditions of the environment to alter community succession. For example, grass invasions can promote seed germination of other alien grasses by changing soil temperature and moisture (D'Antonio and Vitousek, 1992).

In contrast, early arrival by *Colpidium* accelerated competitive exclusion of *P. bursaria* (a negative priority effect). In a previous study, Müller et al. (2012) cultured *P. bursaria* in *Colpidium* conditioned media, and found that allelochemicals produced by *Colpidium* did not inhibit *P. buraria* growth. Thus, the negative priority effects of *Colpidium* on *P. bursaria* may have been driven by competition for common resources,

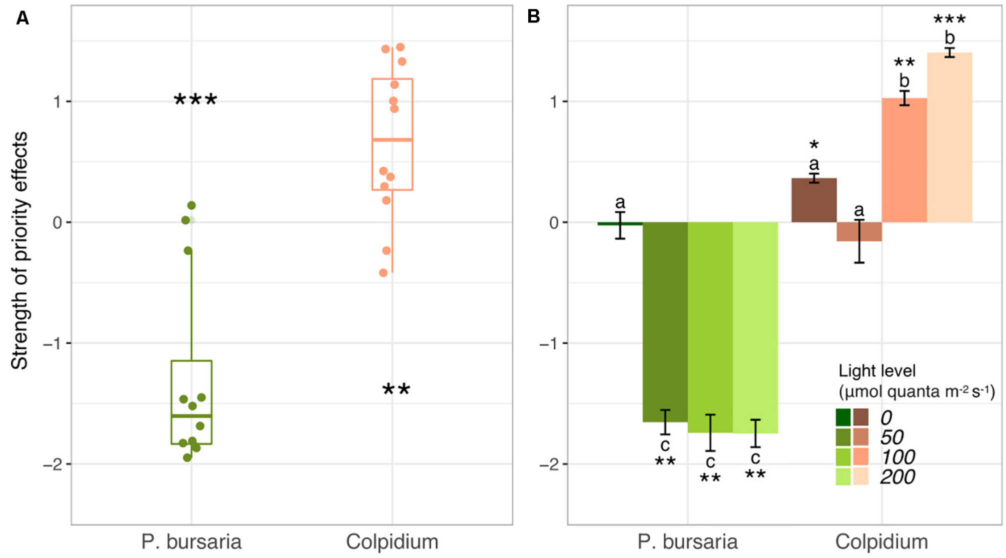

FIGURE 2 | Priority effects metric (Eq. 1) between *Colpidium* and *P. bursaria* (Vannette and Fukami, 2014) generated from population dynamics shown in **Figure 1**. **(A)** Boxplots of priority effects metric demonstrate that *Colpidium* was positively affected by early arriving *P. bursaria*, and *P. bursaria* was negatively affected by early arriving *Colpidium*. **(B)** The strength of priority effects by light level. Different letters represent statistically significant differences at the $p < 0.05$ level (Tukey's HSD, analysis of variance comparing all light × species interactions, see summary of initial ANOVA tests **Supplementary Table S1**). On all panels, error bars represent ± one standard error. Stars indicate significant differences from zero (*T*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

such as bacterial prey, though we did not collect data on bacterial abundance as part of our study. *Paramecium bursaria* needs to maintain bacterial ingestion to provide important nutrients, such as nitrogen, essential for both *P. bursaria* and its endosymbiont, *Chlorella* (Albers and Wiessner, 1985; Johnson, 2011). This competition may be enhanced when *Colpidium* populations are pre-established. Indeed, niche preemption, in which the early arriving species reduces the resources available (Fukami, 2015), is a widely known phenomenon observed in other microbial systems (Peay et al., 2012; Ng et al., 2013; Vannette and Fukami, 2014), as well as between competing plants (Grman and Suding, 2010; Kardol et al., 2013) and animals (Geange and Stier, 2009).

The availability of benefits from metabolic symbiosis shapes the strength and direction of priority effects. In our study, the photosynthetic contribution of *Chlorella* was limited by light availability: In light environments (>0 µmol quanta m^{-2} s^{-1}) when symbiotic benefits were greatest, *P. bursaria* persisted for longer than in dark environments (0 µmol quanta m^{-2} s^{-1}). In other systems, the presence or absence of symbiotic partners has a similar effect. For example, in grassland systems where pioneer species preempt light and space niches from subsequent community members, the inclusion of soil mutualists (such as mycorrhizal fungi) reduced the strength of priority effects and allowed for the later establishment of native grass (Burkle and Belote, 2015).

In our study system, the metabolic value of symbiosis (acquired phototrophy) should scale with light availability. We found that the strength of positive priority effects (facilitation of *Colpidium*) increased with increasing light, which supports our proposed mechanism: that *Colpidium* is facilitated by increased bacterial growth supported by *P. bursaria* symbiont photosynthetic activity. Because photosynthetic rates (and population sizes of *P. bursaria* in control environments, **Figure 3A**) increase with increasing light levels, the supply of organic matter should also increase. However, the negative effect of *Colpidium* on *P. bursaria* did not scale with light. We found that increasing light (and, presumably, increasing photosynthetic benefits from acquired metabolism) did not alleviate competitive effects of *Colpdium* on *P. bursaria*, perhaps because *P. bursaria* initial growth (**Figure 3C**) and establishment were inhibited by the presence of established *Colpidium* populations, which are not light-dependent. This phenomenon may be explained by niche preemption, a mechanism of priority effects in which the early arriving species limits the resources available for successive species (Fukami, 2015). Pre-established *Colpidium* could have reduced bacterial densities, which limited *P. bursaria* population sizes across all light levels due to limited access to essential nitrogen. In this situation, additional photosynthate benefits of *Chlorella* were apparently insufficient to compensate for limited bacterial supply, especially since bacterial ingestion by *P. bursaria* is also a primary source of nitrogen for *Chlorella* (Lowe et al., 2016).

Although symbioses, such as metabolic symbiosis of *P. bursaria,* provide access to new resources and increased competitive ability, there are tradeoffs that constrain the survival in certain environments. *Paramecium bursaria* does not dominate *Colpidium* in low light environments, perhaps because it experiences tradeoffs between heterotrophy and photosynthesis (e.g., spatial constraints within its cytoplasm, where space is shared between digestive vacuoles and vacuoles containing *Chlorella* photosymbionts) that prevent *P. bursaria* from

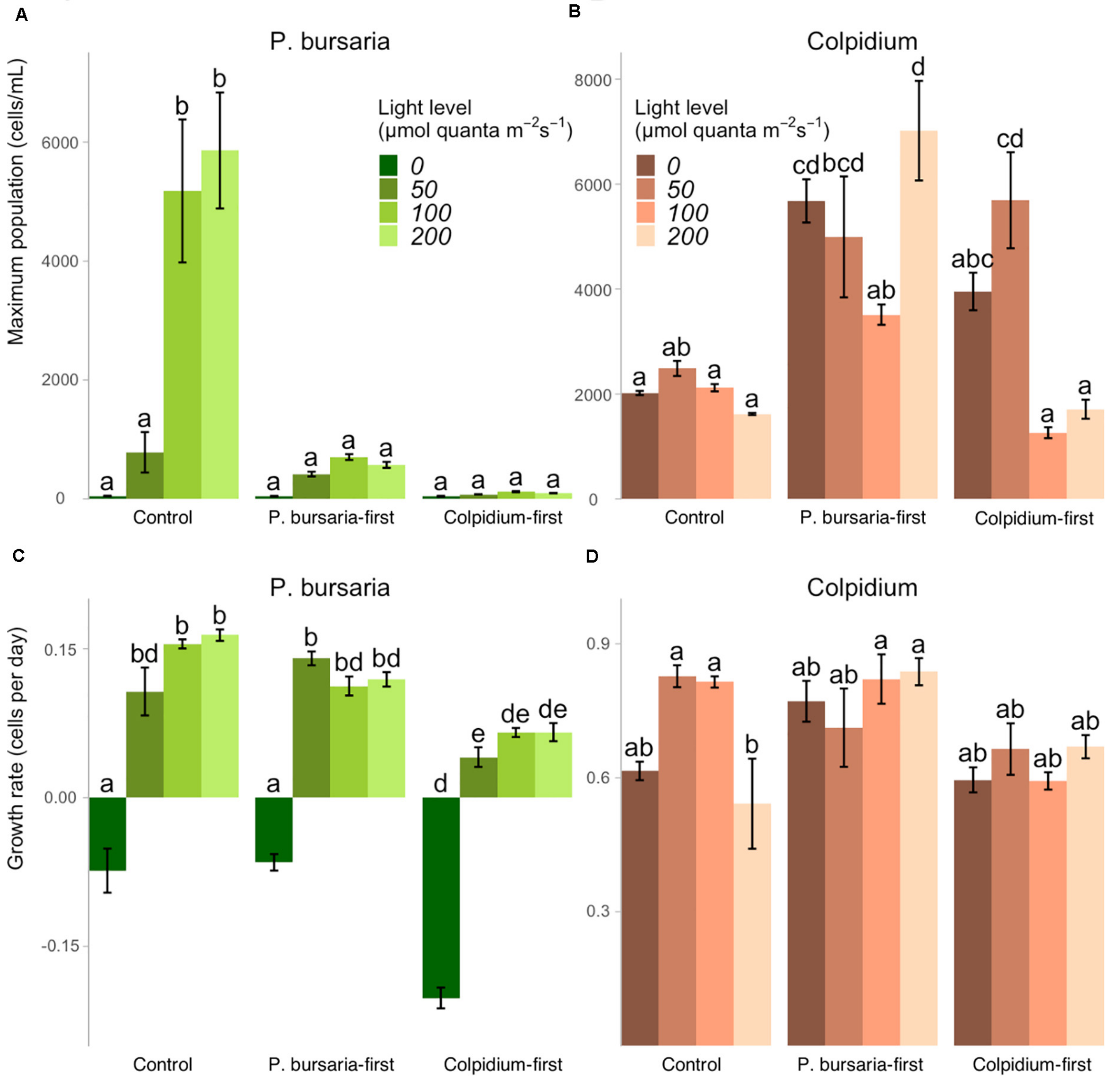

FIGURE 3 | The effect of light levels on maximum population size and growth rates for single-celled controls, *P. bursaria*-first and *Colpidium-first* treatments, using the species' observed light level response curves shown in **Figure 1**. Maximum population sizes for **(A)** *P. bursaria* demonstrates strong inhibition by early and late-arriving *Colpidium,* while maximum population sizes of **(B)** *Colpidium* generally benefited from presence of *P. bursaria.* The growth rates of **(C)** *P. bursaria* are significantly lower when *Colpidium* arrives first, while growth rates of **(D)** *Colpidium* are generally unaffected across treatments. Different letters represent statistically significant differences at the $p < 0.05$ level (Tukey's HSD within each panel, analysis of variance comparing all treatments × light levels, see summary of initial ANOVA tests **Supplementary Tables S2, S3**), and error bars represent ± one standard error.

persisting on heterotrophy alone (Salsbery and DeLong, 2018; Flynn et al., 2019). A previous model found that mixotrophs are most successful by being mostly photosynthetic and supplementing nutritional needs with heterotrophy (Crane and Grover, 2010), which could explain why the *P. bursaria* in our experiments were light-dependent. In darkness, endosymbiotic *Chlorella* cells may also become parasitic because they no longer provide photosynthates, and these costs may contribute to the accelerated declines in *P. bursaria* populations (Lowe et al., 2016). In other systems in which hosts rely on acquired photosynthesis, hosts have become obligately reliant on photosynthesis. For example, the acquired phototroph *Mesodinium rubrum* requires light for growth (Moeller et al., 2011), and zooxanthellate corals (which depend on symbiotic dinoflagellates for photosynthates) have decreased growth and survival without the symbiont (Brown, 1997; Douglas, 2003). Similarly, insects can have thermally sensitive bacterial partners that constrain the insects adaptation to elevated temperatures (Wernegreen, 2012).

Metabolic symbiosis allows species to access new resources that were previously unavailable. However, these benefits require energetic trade-offs that would become disadvantageous when environmental conditions change.

Because metabolic symbiosis alters competitive abilities and priority effects, it has cascading effects on the maintenance of diversity in ecosystems. In our system, acquired photosynthesis can facilitate the subsequent invasion by a second, heterotrophic species, and facilitate long-term coexistence of *P. bursaria* and *Colpidium* (Hsu et al., unpublished). By using a stylized model system—protist microcosms whose light levels mimic a range of natural freshwater pond settings yet which contain a constrained subset of species—we are able to more clearly identify the direction and strength of species interactions, including the facilitative effects of symbiosis. Previous work in grassland systems has shown that microbial symbionts (e.g., mycorrhizae) can increase grassland biodiversity and ecosystem functioning via facilitative priority effects (Burkle and Belote, 2015). Long term studies show that wood-decaying fungi, metabolic symbionts of some insects, can mediate positive priority effects of rare, wood-living beetles, therefore maintaining biodiversity of beetle species in boreal forests in Sweden (Weslien et al., 2011). Thus, accounting for metabolic symbiosis allows us to better predict the outcomes of competition, strength of priority effects, and success of restoration projects. The results of our study demonstrate the importance of metabolic symbiosis on competition outcomes under different community assemblage scenarios, and should encourage future work on priority effects and symbiosis.

DATA AVAILABILITY STATEMENT

All datasets and code used in this study can be accessed at https://doi.org/10.5281/zenodo.4342275. Further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

VH and HM conceived the ideas, designed methodology, analyzed the data, drafted the manuscript, contributed to subsequent revisions, and gave final approval for publication. VH collected the experimental data. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.614367/full#supplementary-material

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Elevation Correlates With Significant Changes in Relative Abundance in Hummingbird Fecal Microbiota, but Composition Changes Little

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The microbial communities living on and in vertebrate hosts have myriad effects on their hosts, potentially including fitness and speciation. Microbiomes are influenced by both intrinsic (from the host) and extrinsic (from the environment) factors, but the relative contributions of each are unknown for most non-model species. Abiotic environmental factors can influence the microbiome directly but it is less clear how abiotic gradients shape microbiome communities in the wild. Here, we captured eight wild Anna's hummingbirds from three different elevations along their elevational distribution in California and moved them directly to a middle ("Within Range") elevation. After some time at this elevation, the birds were moved in captivity to an "Above Range" elevation, and two birds were later moved back to the Within Range elevation. Fecal and food samples were collected longitudinally and the V4 region of the 16S rRNA gene analyzed. The most abundant phyla in all samples were Fusobacteria, Firmicutes, Actinobacteria, and Proteobacteria. Individual Bird ID explained the greatest amount of microbiome variation at 27.5%, signifying some amount of stability in the Anna's hummingbird fecal microbiome. Sample elevation explained 19.6% ($p = 0.001$) of the variation using weighted UniFrac, but only 2.0% ($p = 0.047$) using unweighted UniFrac, implying a change in abundance of bacterial lineages in the microbiome but not in the presence or absence of the microbes. Additionally, Fusobacteria were 7.0x more abundant in the Above Range elevation samples while Firmicutes were 0.3x lower. A thorough understanding of how the environment can shape the microbiome may assist in conservation efforts and a general understanding of host-microbiome relationships in an era of rapid and global environmental change.

Keywords: *Riemerella*, avian, altitude, *Streptobacillus*, *Calypte*, microbiome, 16S rRNA gene

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INTRODUCTION

A microbiome is the community of microorganisms in a specified location (Bäckhed et al., 2005) and the microbiomes of vertebrates have myriad effects on their hosts, from digestion (Oliphant and Allen-Vercoe, 2019) and behavior (Borre et al., 2014) to lifespan (Smith et al., 2017) and brain development (Heijtz et al., 2011). Microbiomes are important for the health of hosts and can affect the onset, duration, and severity of diseases, including intestinal diseases, metabolic diseases, obesity, cardiovascular diseases, and others (Kinross et al., 2011; Nicholson et al., 2012; Tu et al., 2020). Most knowledge of vertebrate-associated microbiomes derives from mammalian hosts (Bleich and Fox, 2015; Colston and Jackson, 2016; Davenport et al., 2017; Sharpton, 2018). Host genetics was originally identified as the main influence on the human microbiome composition (Goodrich et al., 2014), but human environment (i.e., living conditions and behavior, including diet) may be the larger driver (Rothschild et al., 2018). Whether mammalian microbiome results apply broadly is unknown (Hird, 2020; Song et al., 2020) and species-level microbiome information for non-model taxa provide important basic and contextual information about host-associated microbiomes.

Birds are important contributors to our environment, economy, and food sources, though few studies have experimentally investigated what influences their microbiome. The role of the external environment on the avian microbiome appears to vary across studies and systems. For example: in brown-headed cowbirds, sampling location is a larger correlate of microbiome diversity than host taxonomy or ecology (Hird et al., 2014). Similarly, the location in which the adult woodlark and skylark reside shapes their microbiome (van Veelen et al., 2017). Birds living in urban environments have more diverse microbiomes than those in rural environments (Phillips et al., 2018). Together these show the importance of the environment on the microbiome. Conversely, when 59 neotropical bird species were sampled, the environment was not shown to be a statistically significant correlate to microbial diversity (Hird et al., 2015). Nest parasitic birds have significantly different microbiomes than non-parasitic young raised in a shared nest, indicating a greater role for genetic than environmental influence (Ruiz-Rodríguez et al., 2009). Cloacal microbiomes of free-living rufous-collared sparrows were different between the different sexes and among the different seasons; male and female birds showed different cloacal microbial communities but both changed throughout the breeding season (Escallón et al., 2019). Contrastingly, sex has been not significant in other avian microbiome studies (Hird et al., 2015, 2018). While these studies show that intrinsic and extrinsic factors may affect the avian microbiome, causal studies are needed to determine the extent to which each does.

Hummingbirds (family: Trochilidae) have extremely small body sizes and high metabolic requirements (Beuchat and Chong, 1998). They live almost exclusively in states of hyperglycemia so extreme that similar conditions would likely cause severe tissue damage or death in mammals (Beuchat and Chong, 1998). Additionally, hummingbirds can live at high elevations with highly variable environmental conditions. Hummingbirds are well equipped to deal with most challenges of high elevation, like low ambient temperature, decreased air density, and low oxygen availability (Altshuler and Dudley, 2006). However, there are also physiological costs. At higher elevations, hummingbirds reduce their translational velocities, accelerations and decelerations, and rotational velocities while also making less frequent complex turns, showing behavioral compensation for the costs of living at high altitude (Segre et al., 2016). Anna's hummingbirds (*Calypte anna*) disperse upslope during the summer months, extending their elevational range from sea level to approximately 2,500 m above sea level, but all move to lower elevations in the winter during the breeding season. At the higher elevations, Anna's hummingbirds experience reduced competition, predation, and parasitism (Jankowski et al., 2013). There is also evidence that this species has only recently lived in high elevation habitats in the summer months, as historical data from the early 20th century in California indicates that the species was then only found in low-to-mid elevations throughout the year (Grinnell and Miller, 1986; Tingley et al., 2012).

Other systems indicate elevation can affect microbiomes. Coqui frogs (*Eleutherodactylus coqui*) sampled along an elevation gradient in eastern Puerto Rico show no significant alpha diversity changes in the microbiome, but beta diversity metrics consistently differed across the elevation changes (Hughey et al., 2017). How microbiomes may change with current and expected future elevational shifts is currently unknown, nor how that will affect the bird physiology.

The goal of our study was to assess how elevation affects microbiome richness and taxonomic composition in an experimental and semi-wild captive experiment. Anna's hummingbirds were captured from three elevations across their natural range in the summer of 2018 in CA, United States, moved to a middle "Within Range" elevation, and later moved to a higher "Above Range" elevation while the fecal microbiome was sampled longitudinally (**Figure 1**). Fecal microbiome samples were collected at the two elevations allowing us to track the changes within individuals and thus infer causality.

MATERIALS AND METHODS

Sampling

Anna's hummingbirds are a medium-sized hummingbird native to Western North America (Clark and Russell, 2020). In the summer of 2018, we captured Anna's hummingbirds at three different elevations along their elevational distribution in the Central Valley and Sierra Nevada of CA, United States: 0 m (sea level, "Low"), 1,219 m ("Medium"), and 2,438 m ("High"). Eight birds were captured from each elevation and transported immediately, while fed every 45 min, to Big Pine, CA, United States (elevation: 1,219 m above sea level; 37.1649°N, 118.2895°W, "Within Range"). All hummingbirds were housed individually in wooden cubicles (0.75 m × 0.75 m × 0.75 m) with one single mesh wall to allow for natural air and lighting. Hummingbirds were provided *ad libitum* access to NEKTON-Nektar-Plus (NEKTON GmbH, Keltern, Germany)–a hummingbird-specific food that provides carbohydrates, proteins, and vitamins–as well as perches and water. Hummingbirds were kept at this "Within Range" site for a variable amount of time due to risks from inclement weather at high elevations and concern for animal well-being. Birds were kept Within Range for an average of 13 days (**Figure 1**) and a range of 3–20 days. The birds were subsequently moved to an elevation above their known natural range at Barcroft Station (elevation: 3,657 m above sea level; 37.5835°N, 118.2369°W,

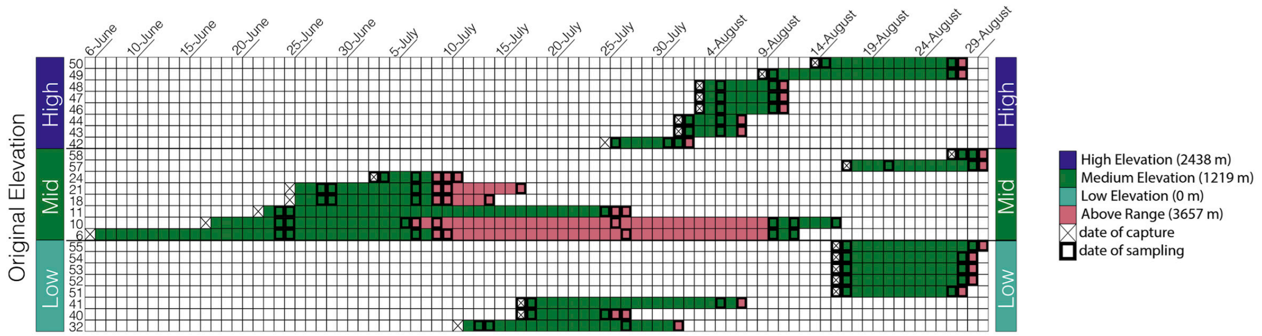

FIGURE 1 | Sampling scheme: Each bird is represented by one row and each day within the study is represented as a separate column starting from June 6, 2018 and ending at August 30, 2018. The birds are grouped by capture elevation.

"Above Range"), in the White Mountains of California, and kept there for an average of 3 days. The longest amount of time spent at the Above Range elevation was 20 days and the shortest amount of time spent at the Above Range elevation was 1 day. Following the Above Range elevation, two birds were moved back to the Within Range elevation. One bird was kept there for 3 days and one bird was kept there for 7 days.

To collect fecal samples, wax paper was sterilized with a 10% bleach solution and left to air dry. Birds were housed individually and enclosures were lined with fresh wax paper daily. Every 24 h, the wax paper was removed and sterile toothpicks were used to transfer all of the fecal material from the wax paper to a sterile micro centrifuge tube. Samples were frozen at −20°C and shipped to the University of Connecticut for further processing. Additionally, 15 food samples were taken from the Within Range elevation over the course of the study and six food samples were taken from the Above Range elevation over the course of the study. All hummingbirds were captured and retained under United States Fish and Wildlife Service Permits MB087454-0 and MB087454-2 and California Fish and Wildlife Service Permit SC 006598. All methods were approved under University of Connecticut Institutional Animal Care and Use Committee Protocols A16-012 and A19-013.

Extraction

A subset of all fecal samples were selected for DNA extraction, targeting the first two samples and the last sample at each elevation (i.e., Within Range and Above Range) for each bird. This was to capture rapid shifts in the microbiome and to determine if the fecal microbiomes reached a stable microbiome prior to moving the birds to a different elevation. This resulted in a minimum of four samples per bird and up to nine samples per bird. Food samples were taken frequently at both sites. Six food samples were selected for DNA extraction: the first sample, middle sample, and last sample taken from each site. DNA was extracted using the QIAamp PowerFecal DNA (Qiagen, Hilden, Germany) kit. The manufacturer's instructions were followed except for a 5 min extension of the bead beating step, to produce a final volume of 100 μl. A "trial" set of 14 samples was initially extracted and sequenced with its own DNA extraction control. The remainder of the samples were extracted together with their own DNA extraction control.

Sequencing

The samples were sequenced at the Microbial Analyses, Resources, and Service (MARS) facility at the University of Connecticut, Storrs, CT, United States on the Illumina MiSeq platform. The V4 region of the 16S rRNA gene was sequenced using the Earth Microbiome Project (EMP) primers and protocols (Caporaso et al., 2012). The "trial" set was sequenced with its one sequencing control and the remainder of the samples were divided across four sequencing runs, each with their own sequencing controls. Following standard protocol at the University of Connecticut Microbial Analysis, Resources, and Services, the Quant-iT PicoGreen kit was used to quantify the DNA extracts (Invitrogen, ThermoFisher Scientific). GoTaq (Promega) was used to amplify, in triplicate, 15 μL reactions of a variable region of the bacterial 16S rRNA genes [V4, 0.8 picomole each 515F and 806R with Illumina adapters and eight basepair dual indices (Kozich et al., 2013)] with the addition of 10 μg BSA (New England BioLabs). As most primers do not match the template priming site, 0.1 femtomole 515F and 806R that does not have barcodes and adapters was added to overcome initial primer binding inhibition. The PCR reaction began with a 95°C incubation step for 2 min followed by 30 cycles of 95°C for 30 s, 50°C for 60 s, and 72°C for 60 s. The final extension was completed at 72°C for 10 min. After pooling, the PCR products were visualized through the QIAexcel DNA Fast analysis (Qiagen) and normalized using the concentration of DNA from 350-420 base pairs. The QIAgility liquid handling robot was used to pool the DNA. Mag-Bind RxnPure Plus (Omega Bio-tek) was used to clean the pooled PCR products according to the manufacturer's protocol. The MiSeq was used to sequence the cleaned pool using the v2 2 × 250 base pair kit (Illumina, Inc.).

Sequence Processing

The sequences were processed using DADA2 in R version 3.6.1 (Callahan et al., 2016; R Development Core Team, 2019). DADA2 stringently quality controls raw sequences and then identifies every unique amplicon sequence variants (ASVs) as an operational taxonomic unit. The sequences were aligned to the Silva reference database (v. 132) and assigned taxonomy with the RDP's Naive Bayesian Classifier (Pruesse et al., 2007; Wang et al., 2007). Mitochondrial and chloroplast sequences were removed and the DECIPHER package in R was used to make a multiple alignment (Wright, 2015). The phangorn package version 2.5.5 was used to create a phylogenetic tree (Schliep, 2011). The decontam package in R was used to identify potential sequence contaminants in the negative extraction and PCR controls (Davis et al., 2018). The samples were divided into their respective sequencing runs when using the decontam package so that each run had the extraction and sequencing control that was used with those samples; all ASVs identified as likely contaminants were then removed. Following decontam, the phyloseq objects were re-merged into one phyloseq object (McMurdie and Holmes, 2013).

Analyses

R was used for all statistical analyses (R Development Core Team, 2019). For alpha (within sample) and beta (between samples) diversity analyses, all samples were rarefied to an equal sequencing depth of 7,675 sequences. We calculated alpha diversity using the Observed number of ASVs (richness) and the Shannon diversity index (Shannon and Weaver, 1949); these were calculated using the phyloseq package (McMurdie and Holmes, 2013). Rarefaction curves from the vegan package (Jari Oksanen et al., 2018) were used to assess whether the samples were sequenced in sufficient depth. Additionally, we conducted a sensitivity analysis to determine whether a single rarefied set of samples would adequately propagate uncertainty arising from the rarefaction process. We subsequently created 1,000 rarefied versions of our dataset and calculated that uncertainty arising from rarefaction was trivial–on average, only 0.06% of variation in Shannon diversity between samples could be attributed to rarefaction, and only 0.5% of variation in ASV richness of samples could be attributed to rarefaction. Consequently, our subsequent analyses were conducted on only a single rarefied set of samples.

The phyloseq and ggplot2 packages were used for microbial analyses and visualization of results (Wickham, 2009; McMurdie and Holmes, 2013). Non-metric Multidimensional Scaling (NMDS) visualized Bray–Curtis, unweighted UniFrac, and weighted UniFrac distances (Lozupone and Knight, 2005). Bray–Curtis is a count-based dissimilarity metric. Unweighted (sequence presence/absence) and weighted (sequence-abundance weighted) UniFrac distances are phylogenetic metrics. We chose these metrics to assess complementary aspects of the microbiome. Statistical significance of the following variables was determined using the adonis2 function (PERMANOVA) from the vegan package (McArdle and Anderson, 2001; Jari Oksanen et al., 2018): the elevations at time of sampling; the elevations on the date of capture; the sample taken on the date of capture; the first sample taken for each bird; the first sample at each elevation; the last sample at each elevation; each sex; the first, second, or last sample taken at each elevation; the sample types (fecal vs. food); and the sequencing runs.

For each bacterial taxon, we statistically tested for whether detection depended on elevation of capture, elevation at time of sampling, and days in captivity. Consequently, we used the rarefied sample abundances in a Generalized Linear Mixed Modeling (GLMM) framework to test for the effects of these variables for each taxa. Because only two birds were returned to the Within Range elevation after being moved to the Above range elevation, the samples taken from those birds at the Within Range elevation after Above Range elevation were excluded from this analysis. Given power limitations of small samples, we further limited analyses to only taxa that were detected in at least 15 different samples and had a total number of reads (i.e., abundance) greater than or equal to 20. These data requirements limited GLMM analyses to six phyla and 23 genera. We fit taxon-specific models using a (type 2) negative binomial distribution and a log link, with all three covariates as fixed effects. We additionally included individual bird IDs as a random effect, to account for the pseudoreplication of repeatedly sampling the same bird at different times. Many taxa were additionally zero-inflated, so for taxa where $>10\%$ of all sample reads were zeroes (21 of 29 taxa), we additionally added a hierarchical zero-inflation component to our model, where the frequency of zeroes was modeled with a single intercept. For two taxa (*Asaia* and *Neokomagataea*), the effect of testing elevation lacked identifiability, as the taxa were never found in samples from the High elevation site. In these two cases only, a single High elevation datapoint was randomly changed to the non-zero mean number of reads from all other samples of the taxa in order to make the model identifiable. Finally, given the large number of parameters estimated across all taxa and the risk of false discoveries, "significant" *p*-value thresholds were adjusted using the Benjamini–Hochberg procedure with a false discovery rate of 0.05 (Benjamini and Hochberg, 1995). Model fitting was conducted using the *R* package glmmTMB (Brooks et al., 2017).

To determine if bacterial taxa were differentially abundant the DESeq2 package in *R* was used (Love et al., 2014). Within the birds captured at the Low elevations, three of the birds were captured about 4.5–5.5 weeks before the remaining five birds were captured. Therefore, to test whether time of capture separately affects the microbiome, the first samples collected from these eight birds were compared in this package. Prior to further analyses, these eight samples were rarified to 14,289 sequences, the lowest number of sequences contained by one of the eight birds. Using DESeq2, plots were created that showed the logarithmic differences between the two groups of samples in the phyla and genera that were present using an alpha cutoff of 0.01.

RESULTS

Sequencing and Initial Data Processing

A total of 7,197,010 reads came from 126 samples and seven controls; 7,171,655 were from samples only. After quality control, 6,364,521 high-quality reads remained; 6,354,811 from samples only. Using decontam, only one run, containing 72 total samples, was identified to have possible contamination. The four possible contaminated sequences were removed from all samples in the run. Following all filtering steps and decontam, there is an average of 52,339 ± 43,123 reads per sample. Samples were rarefied to 7,675 reads, the number of reads the sample with the lowest had after quality filtering steps (**Supplementary Figure 1**). Sequencing run was not significantly correlated with microbiome diversity (**Table 1**).

Microbiome Taxonomic Composition

There were a total of 742 ASVs present in the samples. Of those, 691 ASVs were present only in the fecal samples and 15 were present only in the food samples with 36 ASVs shared between the types of samples. The 36 ASVs found in both hummingbirds and food comprised 65.8% of the hummingbird microbiome and were primarily Firmicutes and Proteobacteria (**Figure 2**). The hummingbird fecal samples were dominated by four phyla: Actinobacteria (35.6%), Firmicutes (26.7%), Proteobacteria (21.8%), and Fusobacteria (15.2%) (**Figures 2**, **3A**). These four phyla accounted for 99.28765% of all of the sequences and were consistently seen regardless of Bird ID or sampling elevation (**Figure 2**). There were 213 genera present in the samples, 10 genera had abundances greater than 1% of all sequences in the samples: *Corynebacterium* (32.1%), *Leuconostoc* (18.6%), *Streptobacillus* (13.0%), *Klebsiella* (10.3%), *Fructobacillus* (9.6%), *Rothia* (3.8%), *Lactococcus* (2.7%), *Enterococcus* (2.0%), *Weissella* (1.9%), and *Escherichia/Shigella* (1.8%).

Mixed-effects models were run on six phyla and 23 genera that met data requirements (see Methods). Of the resulting 29 GLMM models, one showed a lack of numerical convergence (Pseudomonas), likely due to low sample sizes. Consequently, we present results on the remaining 28 taxa and taxonomic groups with adequate model convergence (**Supplementary Table 1**).

Mixed-effects modeling showed significant differences (correcting for false discoveries, see Methods) between the fecal microbiomes sampled at the Within Range and Above Range elevations, and, to a lesser extent, as a function of how long birds had been in captivity (**Figure 4**). The phylum Fusobacteria was 7.0x more abundant at Above Range elevations while Firmicutes was 0.3x lower (**Figures 3B,C**, **4**). These effects were predominantly caused by genera that were also significantly associated with one of the two elevations. In particular, *Streptobacillus* relative abundance was significantly higher in Above Range samples (6.9x; **Figure 3D**), as was–to a lesser (and non-significant) extent–*Riemerella* (2.4x). A number of taxa were significantly associated with the Within Range elevation. Two genera, *Asaia* and *Neokomagataea* were only found in samples from birds tested Within Range and were entirely absent from samples Above Range. In addition, *Fructobacillus*, *Gluconobacter*, *Klebsiella*, *Lactococcus*, *Leuconostoc*, *Rothia*, and *Weissella* all were significantly associated with the Within Range elevation (**Figure 4**).

Our results also showed statistically significant changes in the microbiome as a result of how long birds stayed in captivity (**Figure 4**). In particular, the phylum Fusobacteria increased by 1.1x for each day the birds were kept in captivity. Several genera also increased significantly over time (**Figure 4** and **Supplementary Table 1**), including the genera *Bergeyella* (1.1x), *Coenonia* (1.2x), *Rothia* (1.05x), *Streptobacillus* (1.06x), and *Suttonella* (1.08x). No taxa showed a significant decrease in relative abundance over time in captivity.

Comparatively, taxa were infrequently associated with capture elevation. Relative to birds captured at High elevations, there were no significant differences in the relative abundance of any taxa for birds captured at Low elevations (**Supplementary Table 1**). For birds captured at middle elevations, Cyanobacteria was significantly higher (6.4x), and the genera *Coenonia* (0.01x) and *Weissella* (0.04x) were significantly lower (**Supplementary Table 1**).

When comparing the first samples of the Low capture elevation birds in July to those captured in August, only two genera were differentially associated with an alpha of 0.01 (**Supplementary Figure 2**). *Weissella* was associated at >5-fold higher levels with the birds captured in August and *Fructobacillus* was associated at >10-fold higher levels with the birds caught in July.

Alpha Diversity

Across all fecal samples, the average rarefied ASV richness per sample is 30.7 ± 20.1 (range: 9.9–181.7; **Supplementary Figure 3**). The average rarefied ASV Shannon diversity per sample is 1.66 ± 0.50 (rangeI: 0.14–2.56).

Beta Diversity

Food samples modestly clustered away from the fecal samples and were statistically distinct (weighted UniFrac: $r^2 = 0.05731$, $p = 0.002$; **Figure 5**). The food samples were removed for the remaining analyses.

Bird ID, Capture Elevation, the sample taken on the date of capture, and the first sample taken at each elevation were all significant correlates to microbiome diversity ($p < 0.05$) for two of the three tested metrics: Bray–Curtis and weighted UniFrac. However, there were substantial differences in effect size and p-value depending on distance metric for each variable (**Table 1**). Sample elevation, the first sample taken from each bird, the last sample at each elevation, and the sample type (Fecal or Food) were significant for all three of the distance metrics. Individual Bird ID explained the most variation at 27.511% ($p = 0.009$; **Supplementary Figure 4**) using weighted UniFrac. The variable that explained the second highest amount of variation was Sample Elevation at 19.583% ($p = 0.001$; **Figure 6A**). Sample Elevation also had the largest discrepancy between different metrics as it explained 19.583% ($p = 0.001$) using weighted UniFrac but only 1.961% ($p = 0.047$) using unweighted UniFrac with Bray–Curtis falling between explaining 11.01% ($p = 0.001$; **Figure 6**).

The sequencing run, sex, and whether the sample was the first, second, or last taken at the elevation were not significant ($p > 0.05$) for the three metrics tested (**Supplementary Figures 5A,B**).

DISCUSSION

The microbiome is an important aspect of vertebrate health and fitness, yet how the microbiome assembles, persists and changes through time are largely unknown. Additionally, how external forces affect microbiomes, and particularly avian microbiomes,

TABLE 1 | Adonis2 results for sample variables using each beta-diversity matrix.

Sample variable	Bray–Curtis		Unweighted UniFrac		Weighted UniFrac	
	R^2	P	R^2	P	R^2	p
Bird ID	0.32682	0.001	0.239	0.083	0.27511	0.009
Sample elevation	0.11008	0.001	0.01961	0.047	0.19583	0.001
Elevation on date of capture	0.05178	0.001	0.02261	0.197	0.05707	0.001
Sample taken on date of capture	0.02741	0.007	0.01526	0.097	0.05201	0.002
First sample in study for each bird	0.03178	0.001	0.0235	0.028	0.05976	0.002
First sample at each elevation	0.01892	0.039	0.00551	0.753	0.02321	0.044
Last sample at each elevation	0.02501	0.008	0.02369	0.015	0.03578	0.012
Sequencing run	0.04244	0.186	0.04888	0.092	0.03669	0.382
Sex	0.00869	0.45	0.01792	0.056	0.00533	0.635
First, second, or last sample at each elevation	0.02823	0.068	0.01658	0.497	0.02808	0.139
Fecal or food sample*	0.05326	0.001	0.08325	0.001	0.05731	0.002

**Fecal or food sample variable includes the six food samples.*
Significant ($p < 0.05$) indicated by green boxes.

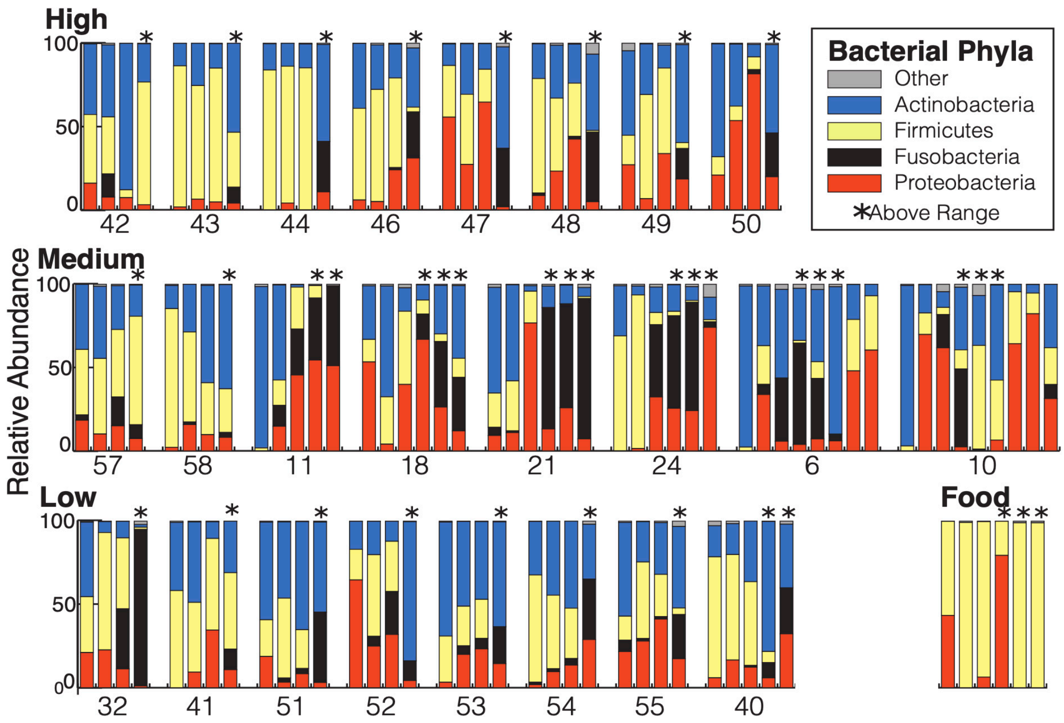

FIGURE 2 | Bacterial composition of the Anna's hummingbird fecal microbiome: All samples from each bird are grouped together and displayed chronologically. Birds are grouped by capture elevation. Samples taken at above range elevation have asterisks above them.

is poorly understood but could be significant to the health of the host. Due to the rising temperatures associated with anthropogenic climate change, many species are expected to shift their ranges to higher elevations (Tingley et al., 2012). Understanding how this shift affects the microbiome, and how microbiome changes affect the host, will allow us to better predict if future elevation shifts will be harmful to the birds.

This study shows that elevation can directly affect the Anna's hummingbird fecal microbiome (**Figures 2–4**, **6** and **Table 1**). Many birds only have one sample at Above Range elevation, so we can not say that this is a long-term or permanent change in the microbial diversity. Comparing the phylum-level average relative abundances from Within Range and Above Range, the percentages of Actinobacteria and Proteobacteria remained relatively stable. However, the percentages of Firmicutes and Fusobacteria changed significantly. The Within Range average contained 0.3x as much Firmicutes while the Above Range average contained 7.0x as much Fusobacteria (false discovery rate of 0.05, **Figures 3A–C**, **4**). The Within Range and Above Range samples cluster apart from each other in the weighted UniFrac

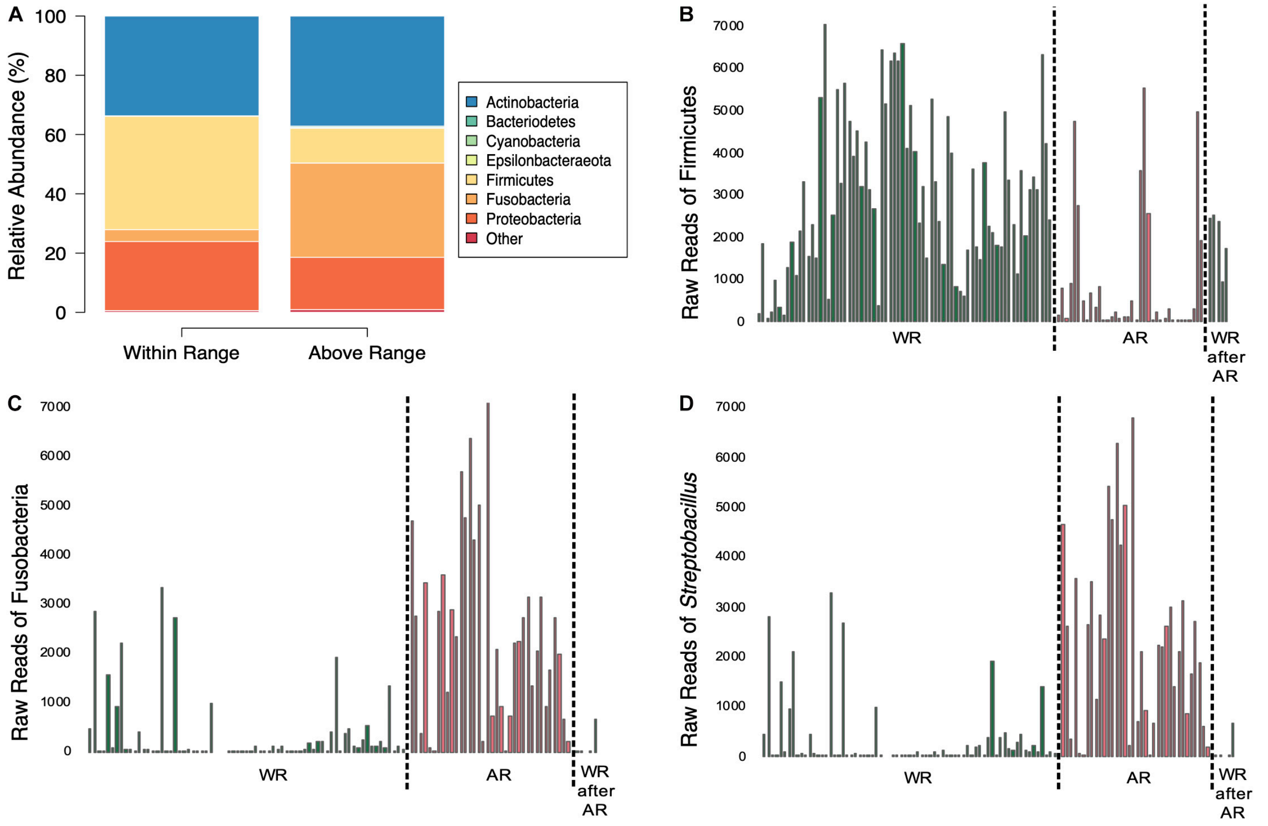

FIGURE 3 | Comparisons of bacterial composition between sampling elevations. **(A)** Average relative abundances of samples taken at the two elevations. **(B–D)** Amount of sequences of Firmicutes **(B)**, Fusobacteria **(C)**, and *Streptobacillus* **(D)** in the samples separated by sampling elevation (dotted line); WR, within range; AR, above range; WR after AR, within range after above range.

NMDS plot with this statistic explaining 19.583% (p = 0.001) of the variation (**Figure 6A**). This is a 10-fold higher percent explained than the unweighted UniFrac, explaining only 1.961% (p = 0.047) of the variation (**Figure 6B**). Because unweighted UniFrac does not take abundance into account and weighted UniFrac does, this indicates that Within Range and Above Range samples contain overlapping ASVs, but their relative abundances shift due to elevation.

Firmicutes are negatively associated with the higher elevation. Firmicutes are diverse in their oxygen requirements, including some species that are anaerobic while others are obligate or facultative aerobes. Fusobacteria, the bacteria that are more relatively abundant at the higher elevation, are typically anaerobic. The genus *Streptobacillus* was specifically associated with the Above Range elevation and is typically microaerophilic. This was the only genus of Fusobacteria that showed any significant changes and is predominantly responsible for the increase in relative abundance of Fusobacteria at the higher elevation. Because of the lower effective oxygen concentrations available at the higher elevations, one hypothesis to explain our results is that Firmicutes may not be able to thrive at high elevations, and are outcompeted by Fusobacteria, specifically *Streptobacillus*. Experimental corroboration of this would need to be performed. To formally test this, competition assays could be performed in a laboratory setting and gut oxic levels could be monitored within the hummingbirds. Additionally, quantifying absolute abundance of Firmicutes, Fusobacteria, and *Streptobacillus* would be necessary to determine changes in population size.

One genus of Bacteroidetes, *Riemerella,* was detected at a higher level in the Above Range elevation (2.4x higher, **Figure 4**). This genus contains only three known species, two of which are considered avian pathogens: *Riemerella anatipestifer*, a pathogen that causes septicemia, and *Riemerella columbina*, a pathogen that causes a respiratory disease (Segers et al., 1993; Vancanneyt et al., 1999; Rubbenstroth et al., 2013). While hummingbirds have not been reported to contract these diseases, they did experience physiological challenges at the Above Range elevation that could potentially be due to, or facilitate invasion by, one of these pathogens.

Capture elevation explained 5.7% of the microbial diversity in the fecal microbiomes (weighted UniFrac, p = 0.001; **Table 1** and **Supplementary Figure 5C**), showing that the elevations in which the birds were living have an effect on the relative abundances of phylogenetic lineages within microbiomes. Capture elevation was also significant using Bray–Curtis (R^2 = 0.05178, p = 0.001), but not unweighted UniFrac (R^2 = 0.02261, p = 0.197). This shows that capture elevation explains 5% of the variation when

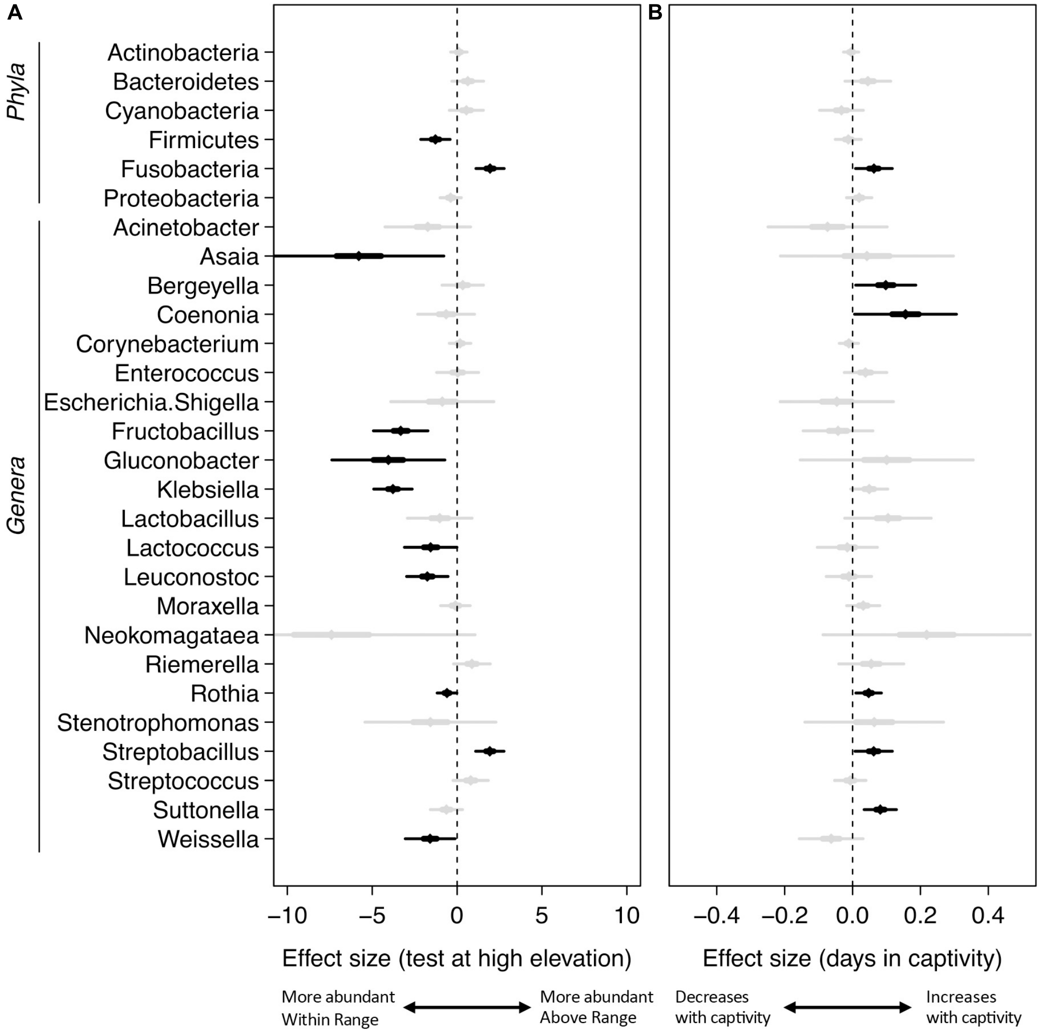

FIGURE 4 | Mixed-effects modeling of sampling elevation **(A)** and days in captivity **(B)**. The central diamond is the mean estimate, the thicker line shows the 50% confidence interval, and the thin line shows the 99% confidence interval. Black indicates significant results using a Benjamini–Hochberg adjustment with a false discovery rate of 0.05, gray are not significant at this value.

using simple counts of the taxa, but when phylogeny is taken into account, the counts are no longer significant until the abundances of those taxa are incorporated. Birds captured at middle elevation had significantly more Cyanobacteria (6.4x) and significantly less *Coenonia* (0.01x) and *Weissella* (0.04x) compared to birds captured at High elevation (**Supplementary Table 1**). Among Cyanobacteria, *Coenonia*, and *Weissella*, only *Weissella* also showed significant differences between Within Range and Above Range sampling, however, it is more abundant in Within Range samples and less abundant at Medium capture elevation samples. Therefore, living at a higher elevation within the species natural range does not appear to precondition the microbiome for living at an Above Range elevation. Together with the high abundance of *Riemerella* at the Above Range elevation, these data give rise to a new question: What are the physiological limits imposed on the host by the microbiome?

All birds were first transported to and housed at the Within Range elevation before being housed at the Above Range elevation; thus, (1) all birds underwent the same initial conditions and (2) time in captivity could be responsible for shifts in phylum and genus abundances. Due to the differences in time birds spent at the Within Range and Above Range elevations, controlling for the time spent in captivity was possible in our analyses. No genera or phyla responsible for shifts in sample elevation were as highly associated with time in captivity (**Figure 4**). Both Fusobacteria and *Streptobacillus* increased significantly per day, but only at 1.1x and 1.06x, respectively. This indicates that the time spent in captivity was not the driver of the changes shown in sample elevations.

Beyond identifying the differences in the Anna's hummingbird fecal microbiome due to elevation changes, our results characterize the hummingbird fecal microbiome and the longitudinal changes within individuals. We show that the

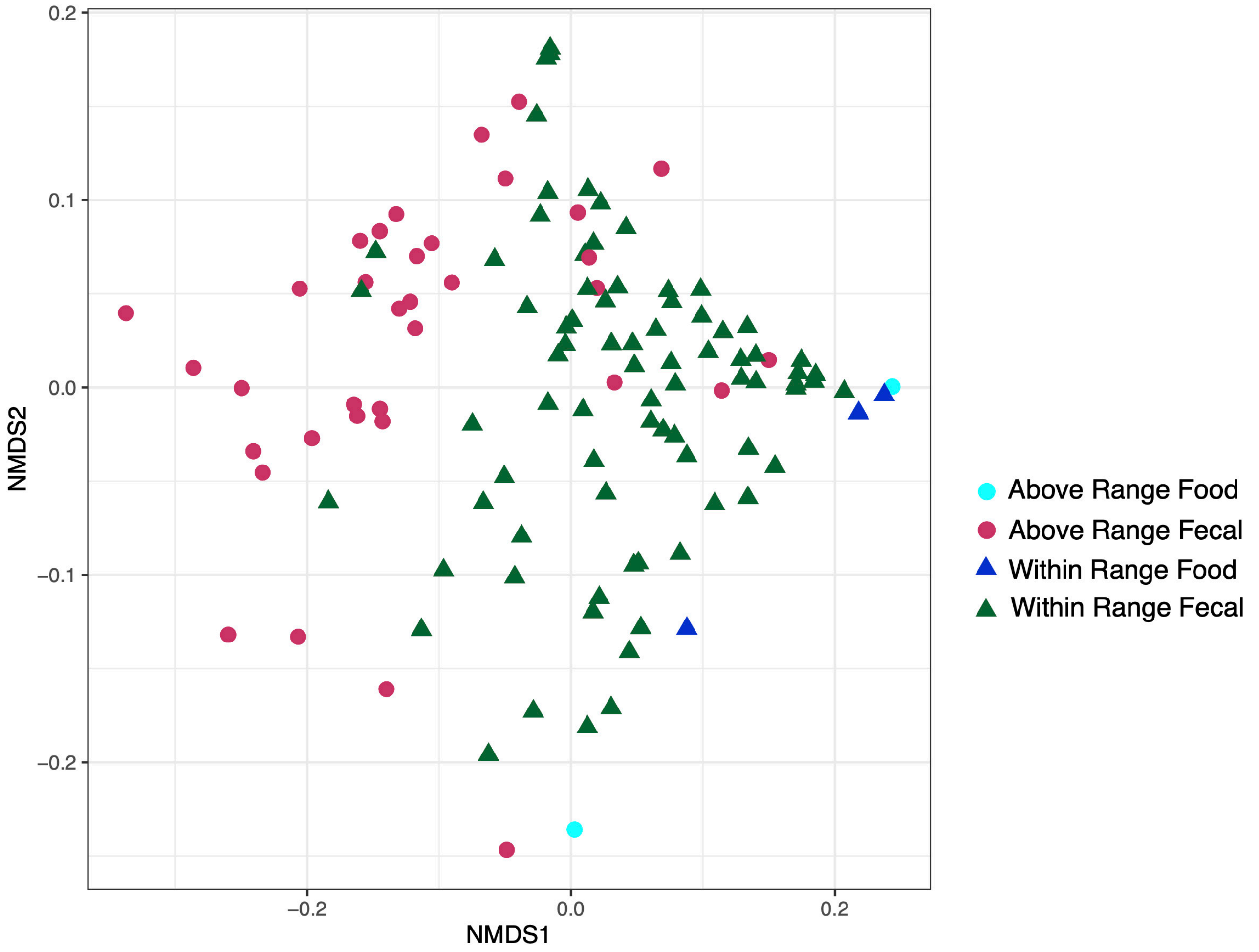

FIGURE 5 | Non-metric multidimensional scaling ordination constructed from weighted UniFrac of fecal and food microbiome samples at the within range and above range elevations.

hummingbird fecal microbiome is relatively consistent with respect to the four main phyla: Actinobacteria, Firmicutes, Proteobacteria, and Fusobacteria (**Figures 2**, **3**). Across many species, the gastrointestinal tract of birds has been shown to be predominantly Firmicutes and Proteobacteria with lower amounts of Bacteroidetes and Actinobacteria (e.g., Waite and Taylor, 2014; Grond et al., 2018; Capunitan et al., 2020), including in hummingbirds (Hird et al., 2015). The dominant phyla in Anna's hummingbirds are commonly seen in other avian species, but with higher abundances of Actinobacteria and Fusobacteria than is commonly seen. The phyla break down into only ten genera represented at more than 1% in all of the samples: *Corynebacterium*, *Leuconostoc*, *Streptobacillus*, *Klebsiella*, *Fructobacillus*, *Rothia*, *Lactococcus*, *Enterococcus*, *Weissella*, and *Escherichia/Shigella*. *Corynebacterium* was the most dominant at 32.1% of all sequences in all of the samples, a result shown in other avian microbiome studies (García-Amado et al., 2018). While elevation explained a high amount of variation in the samples, Bird ID, or from which bird the sample actually came, explained the most variation in two of the three metrics used for beta diversity (**Table 1** and **Supplementary Figure 4**). Using Bray–Curtis, Bird ID explained 33% ($p = 0.001$) and using weighted UniFrac, Bird ID explained 28% ($p = 0.009$). When using unweighted UniFrac, Bird ID was not statistically significant between the two groups, potentially showing that similar bacteria were present in the samples.

Sex did not explain the differences in the fecal microbiome of these samples in any of the three beta diversity distance metrics used (**Table 1** and **Supplementary Figure 3A**), a result that has been found in other bird species (Hird et al., 2015, 2018). However, this may not be a consistent result throughout the year, as birds undergo significant physiological changes throughout the year and this sampling was conducted during the non-breeding season.

Diet is frequently an important driver of avian intestinal microbiomes (Grond et al., 2018). To determine the relationship between the supplied food and the hummingbird fecal microbiome, we sampled the food given to the hummingbirds in the study three times at each elevation the birds were housed and compared their microbial diversity to that of

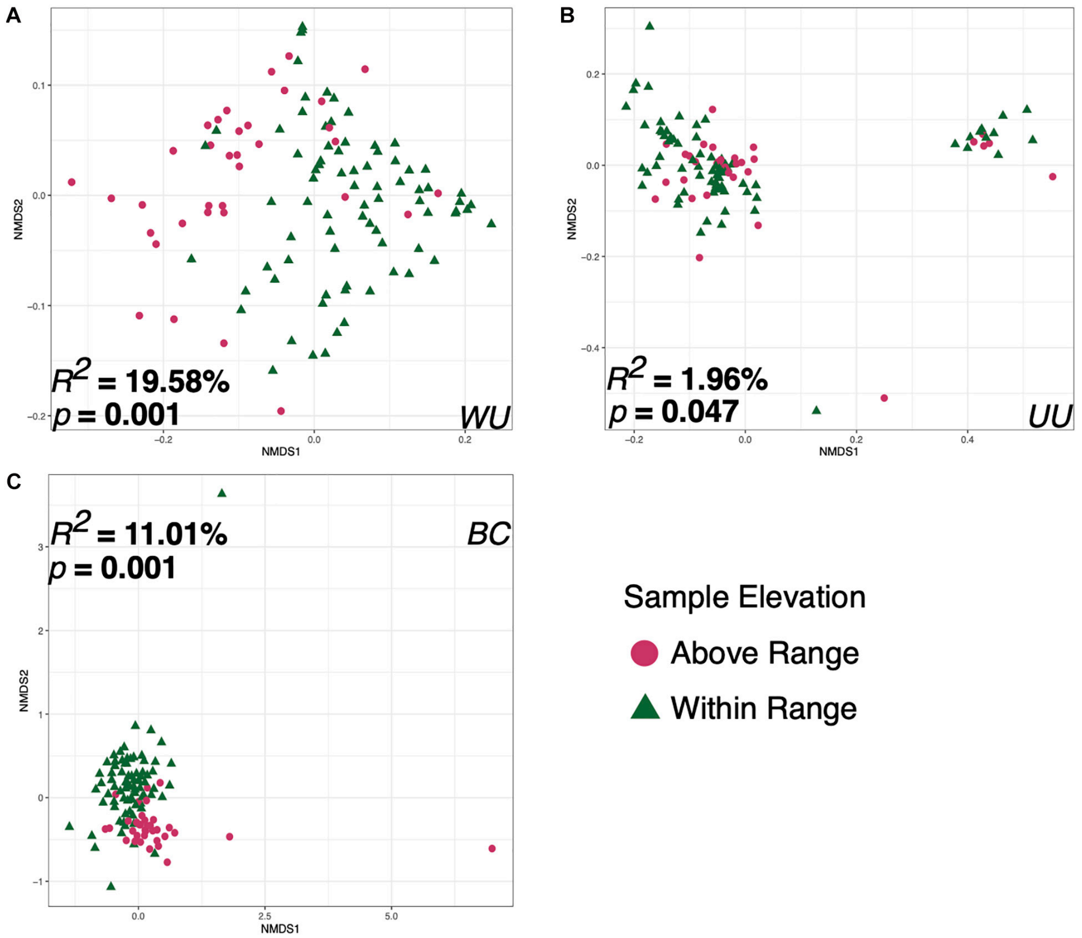

FIGURE 6 | Non-metric multidimensional scaling ordination constructed from weighted UniFrac **(A)**, unweighted UniFrac **(B)**, and Bray–Curtis **(C)** matrices of fecal microbiomes collected from Anna's hummingbirds in captivity at two elevations. Colors represent the two different sample elevations.

the fecal samples. 99.6% of the food microbiomes belonged to Firmicutes and Proteobacteria (**Figure 2**). There were no Fusobacteria found in any food samples. Thirty six of the ASVs present in the food samples were present in the fecal samples as well. Only 15 ASVs were present in the food samples and not present in the fecal samples, but 691 ASVs were unique to the fecal samples. Roughly 65.8% of the fecal microbiome sequences are from ASVs shared with food samples so there is high similarity between the fecal samples and the food samples. However, 34.2% of fecal microbiome sequences were not in the food samples, meaning more than one third of the fecal microbiome is not derived from the birds' current food sources. The food that an Anna's hummingbird eats is actively moved through the digestive tract for <15 min and is retained (including non-moving time) for 48 min on average (Karasov et al., 1986), the number of ASVs shared between their food and the fecal microbiome may be unsurprising. Instead, the greater than one-third of the fecal samples that come from unshared ASVs indicates Anna's hummingbirds host a resident microbiome that does not solely come from the food immediately passing through them.

One of the main strengths of this study was that we experimentally manipulated elevation in wild animals under natural (semi-captive) conditions. However, because of the realities of field studies, there were variables outside of our control. There may be treatment-independent factors affecting the microbiomes in our study. One such factor is that these were wild birds that were captured at different times. This study may only be showing a portion of the variables affecting the Anna's hummingbird fecal microbiome. One variable that could have a factor is date of capture. To determine if capture date did affect the samples, we compared the microbiomes of the first samples of the birds caught at Low elevation as three of the birds were caught approximately 1 month before the other five birds. Only two Firmicutes genera were differentially abundant due to when the birds were captured, showing that 1 month difference in sampling time has a small role in the microbiome samples, but more information is needed to show to what extent time of capture changes a microbiome.

Overall, this study showed elevation causes a change in the Anna's hummingbird microbiome while birds also maintain some stability in their microbiome over time. Additional experiments are needed to assess the physiological outcomes and the specific limitations that the microbiomes imposes on the birds. Understanding these limitations posed by how external factors, like elevation, shape the biodiversity of the Anna's hummingbird microbiome could lead to predictions of the consequences of future potential range shifts due to climate change.

DATA AVAILABILITY STATEMENT

The sequences have been deposited to the NCBI Short Read Archive accession number PRJNA659540.

ETHICS STATEMENT

All hummingbirds were captured and retained under United States Fish and Wildlife Service Permits MB087454-0 and MB087454-2 and California Fish and Wildlife Service Permit SC 006598. All methods were approved under University of Connecticut Institutional Animal Care and Use Committee Protocols A16-012 and A19-013.

AUTHOR CONTRIBUTIONS

EH participated in the conceptualization and methodology, conducted the investigation and formal analysis, created visualizations, and wrote the manuscript. AS participated in the conceptualization and methodology, conducted the investigation and resource collection, created visualizations, and acquired funding. MT participated in the formal analysis, supervision, and project administration (particularly within respect to permitting and data acquisition), in addition to writing. SH participated in the conceptualization, visualization, supervision, funding acquisition, and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.597756/full#supplementary-material

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From Binary Model Systems to the Human Microbiome: Factors That Drive Strain Specificity in Host-Symbiont Associations

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Microbial symbionts are ubiquitous and can have significant impact on hosts. These impacts can vary in the sign (positive or negative) and degree depending on the identity of the interacting partners. Studies on host-symbiont associations indicate that subspecies (strain) genetic variation can influence interaction outcomes, making it necessary to go beyond species-level distinction to understand host-symbiont dynamics. In this review, we discuss examples of strain specificity found in host-symbiont associations, from binary model systems to the human microbiome. Although host and bacterial factors identified as mediators for specificity could be distinct at the molecular level, they generally fall into two broad functional categories: (1) those that contribute a required activity in support of the association and (2) those involved in antagonistic interactions with organisms outside of the association. We argue here based on current literature that factors from these two categories can work in concert to drive strain specificity and that this strain specificity must be considered to fully understand the molecular and ecological dynamics of host-symbiont associations, including the human microbiome.

Keywords: host-symbiont associations, strain specificity factors, bacterial strain variation, model systems, human microbiome, antagonistic interactions

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Drive Strain Specificity in
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INTRODUCTION

Microbes form associations with diverse eukaryotic hosts from protists to humans (McFall-Ngai et al., 2013). These microbial associations can impact host fitness in positive (mutualistic) or negative (antagonistic) directions that in turn influence host diversity and evolution. For example, the notable spread in North America of *Drosophila neotestacea* flies is associated with the bacterial symbiont *Spiroplasma* that protects the fly host from parasitic competitors (Jaenike et al., 2012; Cockburn et al., 2013). Organisms that engage in associations can be generalists capable of associating with many potential partners, or specialists that are restricted to associations with one or a few partners (Chomicki et al., 2020). In this review we limit our discussion to associations in which a host partner exhibits specialization for interaction with one or a few specific subspecies of a microbial symbiont. Host specialization for specific subspecies of symbionts with particular traits can confer consequences on the symbiotic outcome. Host associations with non-native partners from the same microbial species as the native partner can result in lower net fitness benefits, and even a switch from net fitness benefit (mutualistic) to net fitness cost to the host (antagonistic). To explore the molecular traits and processes that promote the presence of a particular symbiont subspecies within an individual host, we discuss here examples of systems in which these processes have been experimentally tested. We do not delve deeply into the evolutionary or ecological theory of specificity, but refer readers to recent publications on these topics (Heath and Stinchcombe, 2014; Shapiro and Polz, 2014; Foster et al., 2017; Batstone et al., 2018; Chomicki et al., 2020).

One of the earliest examples of host-symbiont strain specificity comes from studies of binary associations between one host and one microbial partner. There is a rich history of symbiosis research on the nodules of leguminous plants that harbor nitrogen-fixing rhizobia bacterial symbionts (Oldroyd, 2013). Rhizobia strain-level differences discernable through numerical taxonomy, nucleic acid hybridization, and 16S rRNA analyses can dictate nodule formation with specific legume hosts (as reviewed in van Rhijn and Vanderleyden, 1995). Strain specificity also occurs in the well-studied symbiosis between the squid animal host *Euprymna scolopes* and *Vibrio fischeri* bacteria. In this association, the squid host provides nutrients for bacterial growth while *V. fischeri* bioluminesce in a specialized host structure called the light organ (Nyholm and McFall-Ngai, 2004). Bioluminescence is thought to provide counterillumination that helps camouflage and protect the host from predation by matching environmental down-dwelling light (Jones and Nishiguchi, 2004; Haddock et al., 2010). Strains of *V. fischeri*, categorized by having >95% single-gene (*gapA*) sequence identity, varied in competitive colonization proficiency, with the native strain outcompeting non-native strains (Lee and Ruby, 1994; Nishiguchi et al., 1998). Recently, the association between soil-dwelling insect-parasitic *Steinernema* nematode species and *Xenorhabdus* bacterial species has proven to be a powerful system to reveal the impact of bacterial strain identity on overall fitness of a symbiotic pairing. *Steinernema* nematode spp. from two distinct phylogenetic clades (I and III) naturally associate with different *X. bovienii* strains, classified based on >96% average nucleotide identity of 1,893 sets of orthologous genes (Murfin et al., 2015). Cross-pairing studies using six *Steinernema* nematode hosts and nine *X. bovienii* bacterial strains showed that the fitness of the association differed among *X. bovienii* strains, despite their genome-wide sequence similarity (Murfin et al., 2015; McMullen et al., 2017). More strikingly, the fitness of a non-native pairing negatively correlated with the phylogenetic distance of the non-native *X. bovienii* strain to the native *X. bovienii* strain (Murfin et al., 2015; McMullen et al., 2017). Together, the findings from these three well-studied model systems provide ample evidence that bacterial strain identity influences overall fitness of host-symbiont associations.

The varying impacts of bacterial strains on hosts is not limited to binary associations, but also occurs in complex systems that include more than one microbial partner, such as the well-studied *Apis mellifera* (honeybees) gut microbiome that provides immunity against pathogens and nutrients through metabolism of complex carbohydrates (Kwong and Moran, 2016). It consists of up to nine bacterial species including well-characterized symbionts *Snodgrassella alvi* and *Bifidobacterium asteroides* (Kwong and Moran, 2016). 16S rRNA and metagenomic sequencing and bioinformatics revealed strain variation among honeybee gut microbiome members (Engel et al., 2012; Moran et al., 2012; Ellegaard and Engel, 2019) with functional consequences to the host. For example, an *S. alvi* strain isolated from honeybees has a competitive advantage over *S. alvi* strains isolated from *Bombus* spp. (bumblebees) for colonization of honeybee hosts (Kwong et al., 2014). Similarly, metabolomics comparisons of bee gut colonized by distinct strains of *B. asteroides* revealed differences in the abundance of metabolites such as arabinose, galactose, and xylose, suggesting that bacterial strain-level variation can alter available nutrients (Zheng et al., 2019).

Strain differences also impact human interactions with microbial partners, which in the gut alone consist of up to 1,000 bacterial species (Sekirov et al., 2010). More recent studies that couple metagenomics with techniques to differentiate strains (as reviewed in Brito and Alm, 2016; Niu et al., 2018) revealed strain variation in the human-associated microbiome. Although multiple strains of a single species can be found in the gut microbiota of an individual human, one strain is typically stably dominant in abundance (Schloissnig et al., 2013; Costea et al., 2017; Truong et al., 2017; Garud and Pollard, 2019). In one estimate, a single strain can account for >80% of the strain composition in an individual's gut microbiota (Truong et al., 2017). However, the identity of the dominant strain can vary among individuals (Schloissnig et al., 2013; Costea et al., 2017; Lloyd-Price et al., 2017; Truong et al., 2017). Compared to the gut, inter-individual microbial strain variation is even greater in other body sites such as the oral cavity, nose, and vagina (Lloyd-Price et al., 2017). The broad impacts of the microbiota on human health such as in development, immunity, and nutrient acquisition (Sekirov et al., 2010; Mohajeri et al., 2018) can correlate with bacterial strain variation within the microbiota. For example, the strain-variable copy number of a toxin secretion component encoded by the gut bacterium *Bacteroides uniformis* is positively correlated with inflammatory bowel disease (Greenblum et al., 2015). Similarly, the presence of *Staphylococcus epidermidis* strains that encode virulence factors such as the secretory antigen SsaA correlates with the skin disease psoriasis (Tett et al., 2017).

Overall, strain-level differences in bacterial symbionts can dictate the degree and the sign of interaction outcomes between hosts and microbes. In the next section, we discuss molecular factors that mediate strain specificity in host-symbiont associations. To showcase general trends, we included molecular factors described in the literature as having some level of strain specificity, though we note that the definition of "strain" varies among researchers. Identified factors generally fall into two broad categories: (1) those that fulfill direct functional roles in activities specialized for the symbiosis and (2) those that mediate antagonistic interactions that indirectly shape the symbiosis.

STRAIN SPECIFICITY FACTORS THAT FULFILL FUNCTIONAL ROLES IN HOST-SYMBIONT ASSOCIATIONS

Signals mediating the initiation of rhizobia-legume host associations have been well-characterized with respect to

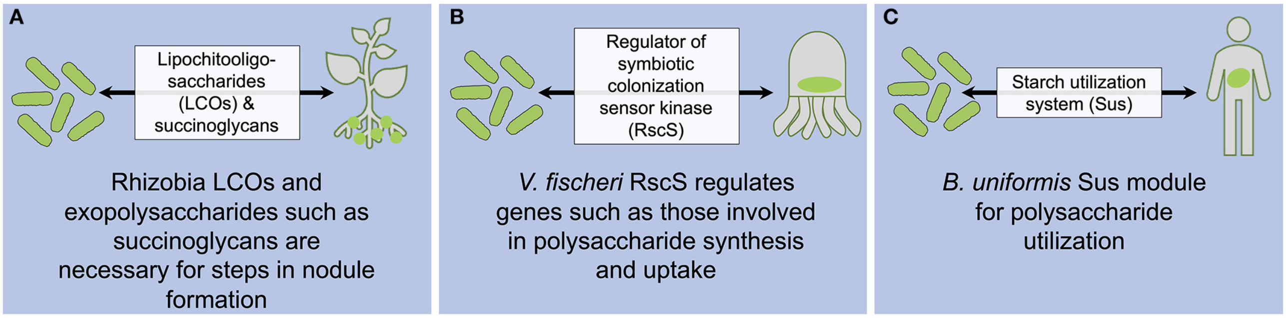

FIGURE 1 | Strain specificity factors that fulfill a functional role in hosts in rhizobia-legume **(A)**, *V. fischeri*-squid **(B)**, and *B. uniformis-human* **(C)** associations. LCOs indicate lipochitooligosaccharides, RscS indicates regulator of symbiotic colonization sensor kinase, and Sus indicates starch utilization system.

their role in strain specificity. When leguminous plants need nitrogen, they elicit rhizobial expression and secretion of nodulation factors: lipochitooligosaccharides (LCOs) (Oldroyd, 2013). When plants perceive LCOs they activate the symbiosis signaling pathway triggering plant restructuring and bacterial invasion through infection threads to form nodules (Oldroyd, 2013). *Rhizobium leguminosarum* LCOs mediate the host range specificity of individual strains (**Figure 1A**). Specifically, *R. leguminosarum* bacteria are classified into biovars (bv): *viciae*, *trifolii*, and *phaseoli*, and each biovar forms nodules with different legume hosts (as reviewed in van Rhijn and Vanderleyden, 1995; Dénarié et al., 1996). A seminal study showed that bv. *trifolii* and bv. *viciae* differ in the gene sequence encoding nodulation factor E (NodE). When *nodE* from bv. *viciae* was introduced to a bv. *trifolii nodE* mutant, it was sufficient to alter the host range of bv. *trifolii* to that of bv. *viciae* (Spaink et al., 1989). Sequence differences between bv. *viciae* and bv. *trifolii nodE* result in distinctive LCO structure, composition, and hydrophobicity, which in turn dictates host responsiveness (Spaink et al., 1991, 1995; Bloemberg et al., 1995).

Nodulation between *Sinorhizobium meliloti* bacteria and *Medicago truncatula* legumes also can vary depending on the bacterial and host identity (Snyman and Strijdom, 1980). When certain *S. meliloti* strains are paired with specific *M. truncatula* cultivars, incompatible interactions can arise that result in pseudonodules that unlike nodules are small, do not fix nitrogen and display sensescence (Tirichine et al., 2000; Simsek et al., 2007). Further, bacteria in pseudonodules are eventually lysed (Wang et al., 2017; Yang et al., 2017). In this legume-bacteria association, exopolysaccharide succinoglycans mediate strain specificity (**Figure 1A**). The production and succinylation of these molecules are necessary for infection thread formation (Leigh and Walker, 1994; Jones et al., 2007; Simsek et al., 2007; Mendis et al., 2016). *S. melioti* strains with compatible or incompatible interactions with *M. truncatula* vary in their succinoglycans trimeric oligosaccharide succinylation patterns (Simsek et al., 2013). Introduction of succinoglycan biosynthetic genes from a compatible to an incompatible *S. meliloti* bacterial strain is sufficient to confer compatibility and alters the succinylation pattern to resemble that of the compatible strain (Simsek et al., 2007). In sum, investigations of diverse legume symbiosis have established that host-symbiont specificity is dictated by the ability of a bacterial strain to produce specific signaling factors (LCOs or succinoglycans) that are recognized by the host (**Figure 1A**).

Similarly, specificity of the *V. fischeri* bacteria-*E. scolopes* squid host association is defined by a strain variable molecular factor. A mutant screen for genes necessary for squid colonization revealed a regulator of symbiotic *c*olonization *s*ensor kinase or RscS (Visick and Skoufos, 2001). RscS regulates the *syp* locus encoding regulatory proteins and structural proteins involved in polysaccharide synthesis and export (Yip et al., 2005, 2006; Shibata et al., 2012). Initial analyses indicated that a specific allelic form of RscS is necessary and sufficient for squid host colonization among *V. fischeri* strains in the paraphyletic group "B" (Mandel et al., 2009), indicating that RscS is a factor for strain specificity in host-microbe association (**Figure 1B**). Intriguingly, follow-up studies revealed that in other groups of *V. fischeri* strains (group "A" and "C"), although the *syp* locus is still necessary for host colonization, RscS is not (Rotman et al., 2019). The current hypothesis explaining this difference is that these other *V. fischeri* strains have distinct mechanisms for regulating the *syp* locus (Rotman et al., 2019) suggesting that *V. fischeri*-squid strain specificity is based on functional differences in regulatory pathways of the conserved *syp* locus, rather than in variation of the *syp*-encoded host-interaction molecules themselves.

In human infants, the bacterial *s*tarch *u*tilization *s*ystem (Sus) is implicated in the strain specificity of the gut bacterium *B. uniformis* (**Figure 1C**) (Yassour et al., 2018). Sus is composed of multiple proteins that bind, uptake, and degrade glycans, with different modules specific to different glycans (Martens et al., 2009). Using metagenomics and single-nucleotide variants in species-specific markers, Yassour and others identified strains that were transmitted between mother-infant pairs within 3 months of birth (Yassour et al., 2018). In their analysis, two patterns of transmission emerged: primary and secondary strain transmission. In primary strain transmission, the bacterial strain dominant in the mother is also the dominant strain in the infant, but in the secondary strain transmission, a non-dominant bacterial strain in the mother is the dominant strain in the infant (Yassour et al., 2018). In mother-infant pairs displaying primary *B. uniformis* strain transmission, the dominant maternal strains encode a Sus module while in mother-infant pairs that displayed secondary strain transmission, the non-dominant maternal strains, and not the dominant maternal strains, encode a Sus module (Yassour et al., 2018). These results provide evidence that Sus module is selected in the infant gastrointestinal tract and may be a driver of strain-specific association of human infants with *B. uniformis* (**Figure 1C**). The authors hypothesized that this strain specificity is driven by unique glycans in mother's breast milk that select for *B. uniformis* strains capable of utilizing these glycans for colonization (Yassour et al., 2018). The role of Sus module in host colonization is supported by findings that *Bacteroides* Sus-like proteins are necessary for colonization in the murine host (Lee et al., 2013).

Overall, these examples showcase that strain-specific associations can arise due to selection for regulation or sequence of molecular factors that fulfill specific positive roles in host-symbiont associations (**Figure 1**). However, strain-specific associations can also be due to molecular factors that are involved in antagonistic interactions between hosts and microbes or among microbes within host-associated communities. In the next section, we showcase host factors that antagonize non-native microbes and microbial factors that antagonizes non-native hosts or other microbes, which mediate strain-specific associations.

STRAIN SPECIFICITY FACTORS THAT ARE INVOLVED IN ANTAGONISTIC INTERACTIONS

M. truncatula legumes exhibit specificity for certain *S. meliloti* bacterial strains and antagonize other strains. The *M. truncatula* *Mtsym6* allele contributes to this strain incompatibility (Tirichine et al., 2000). *Mtsym6* encodes at least two nodule-specific cysteine rich (NCR) peptides (Wang et al., 2017, 2018; Yang et al., 2017). NCRs have bactericidal activity against select *S. meliloti* strains (Wang et al., 2017, 2018; Yang et al., 2017) (**Figure 2A**). Since *S. meliloti* succinoglycans mediate strain specificity (**Figure 1A**), it is intriguing to hypothesize that *M. truncatula* NCRs may select for specific *S. meliloti* strains based on the structure of their exopolysaccharide succinoglycans (**Figure 2A**). Though this hypothesis has not been directly tested, there is strong support for it in the literature. When exposed to sublethal NCR concentrations, *S. meliloti* upregulate succinoglycan synthesis genes (Penterman et al., 2014), and mutants defective in succinoglycan biosynthesis are sensitive to NCRs and display increased membrane permeability, branching, and bloating compared to wild-type *S. meliloti* (Arnold et al., 2017; Montiel et al., 2017). A recent study showed that (1) succinoglycans protect *S. meliloti* against NCR activity, (2) such protection depends on succinylation patterns, (3) there is a direct interaction between NCRs and succinoglycan, and (4) succinoglycan protection is specific to NCRs (and not other cationic peptides) (Arnold et al., 2018) (**Figure 2A**).

Strain-variable antagonism also occurs in the association of *X. bovienii* with *Steinernema* nematode spp. from two distinct phylogenetic clades (I and III). Cross-pairing studies revealed that while *X. bovienii* from clade I nematode hosts (clade I *X. bovienii* strains) are compatible with clade I nematode hosts, they are incompatible with clade III *Steinernema* nematode hosts (Murfin et al., 2015; McMullen et al., 2017). This incompatibility is not due to the inability of clade I *X. bovienii* strains to support clade III *Steinernema* nematode host growth. Rather, it is caused by the toxicity of those particular strains of *X. bovienii* to these non-native nematode hosts (Murfin et al., 2019). Recent work indicates that a Shiga toxin subunit 1 A homolog (StxA) is necessary for a clade I *X. bovienii* bacterial symbiont to kill non-native host clade III *S. feltiae* nematodes (**Figure 2B**) (Ginete, 2020). StxA-encoding clade I *X. bovienii* bacterial symbionts exhibit ribosome-inactivation activity against clade III *S. feltiae* nematodes (Ginete, 2020). When the *stxA* gene encoding the toxin is deleted through mutation, a clade I bacterial symbiont can support clade III *S. feltiae* development and reproduction (Ginete, 2020). These results suggest that clade I bacterial symbionts are capable of forming mutualistic associations with clade III *S. feltiae* nematodes, but their

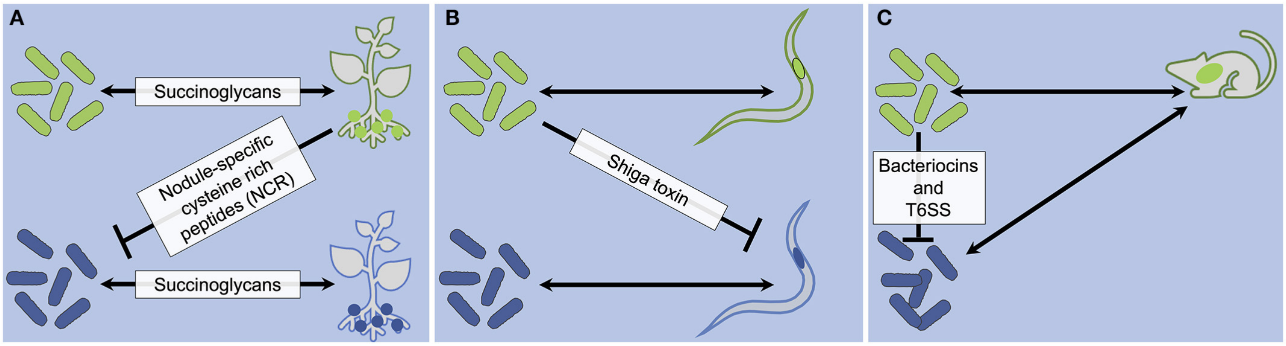

FIGURE 2 | Strain specificity factors that are involved in antagonistic interactions in *S. meliloti-M. truncatula* **(A)**, *X. bovienii-Steinernema* **(B)**, and bacteria-mice **(C)** associations. Strain-specific associations are indicated by matching colors in microbes and hosts.

toxicity hinders or prevents these non-native associations (Ginete, 2020) (**Figure 2B**).

Microbial factors that target other microbes can drive strain-specific host associations (**Figure 2C**). Bacteriocins are microbial-encoded toxins that kill closely related organisms (García-Bayona and Comstock, 2018), and this target specificity impacts colonization between bacterial strains in host environments (**Figure 2C**). In murine host guts, introduction of an *Enterococcus faecalis* strain encoding the bacteriocin Bac-21 eliminates an endogenous vancomycin-resistant *E. faecalis* strain (Kommineni et al., 2015). Another bacteriocin, microcin, is necessary for the probiotic *E. coli* strain Nissle 1917 to reduce colonization levels of another mouse-commensal *E. coli* strain, but only when the intestine is inflamed (Sassone-Corsi et al., 2016), suggesting that environmental conditions such as the presence of inflammation may influence impact of bacteriocins on microbe-microbe competition. Finally, *Bacteroides fragilis* strains expressing the BSAP-1 bacteriocin can eliminate another *B. fragilis* strain in the mouse intestine after 1 week of post-co-inoculation (Roelofs et al., 2016). BSAP-1-mediated elimination depends on the version of a specific outer membrane protein encoded by the target strain, with resistant strain variants expressing OmpR and sensitive variants expressing OmpS (Roelofs et al., 2016). An assessment of human gut metagenomics data set indicates that while *ompR* sequences are present in 98% of metagenomes that were positive for BSAP-1, no *ompS* sequences were found in the BSAP-1 positive metagenomes (Roelofs et al., 2016). These results indicate that variation in both bacteriocins and their receptors can shape the microbiome membership within host environments, which in turn will result in apparent host-symbiont strain specificity.

Present in ∼25% of gram-negative bacterial species, the Type VI secretion systems (T6SS) are composed of a membrane complex with a base plate, a contractile sheath surrounding an Hcp tube, and a tip complex (Coulthurst, 2019; Allsopp et al., 2020). Bacteria utilize T6SS to secrete effectors ranging in activity from nutrient scavenging to causing cellular death (Allsopp et al., 2020). Recent studies show that T6SS can mediate strain specificity in host-symbiont associations (**Figure 2C**). In the *V. fischeri*-squid model system, the squid can harbor multiple *V. fischeri* strains (Nishiguchi et al., 1998; Wollenberg and Ruby, 2009) with different strains occupying distinct light organ sites (Wollenberg and Ruby, 2009; Sun et al., 2016). When T6SS is deleted in *V. fischeri*, two different strains can occupy the same site, indicating that T6SS may mediate exclusion of other strains from each light organ niche (Speare et al., 2018; Guckes et al., 2019). In mice, the presence of T6SS can impact levels of host colonization by *B. fragilis* strains (**Figure 2C**) (Chatzidaki-Livanis et al., 2016; Hecht et al., 2016; Wexler et al., 2016). For example, a commensal *B. fragilis* strain reduces colonization of an enterotoxigenic *B. fragilis* strain in a T6SS-dependent manner (Hecht et al., 2016). This inhibition decreases cecal injury, inflammation, and ulcerations in the murine host (Hecht et al., 2016), indicating that T6SS-mediated microbial competition can have consequences to host health.

A recent metagenomics analysis revealed that there is an enrichment for T6SS-encoding strains in the human infant intestinal microbiota, suggesting that T6SS are important for colonization at this developmental stage (Verster et al., 2017). *B. fragilis* bacterial strains that encode immunity proteins against T6SS effectors displayed resistance to T6SS-dependent inhibition in murine hosts (Chatzidaki-Livanis et al., 2016; Hecht et al., 2016; Wexler et al., 2016). This indicates that the presence of T6SS, variation in the T6SS effector repertoire, and variation in T6SS effector immunity combine to shape overall *B. fragilis* strain composition in the host by ultimately selecting for *B. fragilis* strains that encode specific immunity proteins. The influence of *B. fragilis* T6SS-effector-mediated selection may extend beyond *Bacteriodes,* since *B. fragilis* T6SS presence or absence results in distinct microbial community composition and anti-*B. fragilis*-T6SS-effectors have been found in other gut microbiome members (Verster et al., 2017; Ross et al., 2019). These findings indicate that T6SS-mediated antagonism selects for certain strains within a microbiome, such that the T6SS-encoding or resistant strains dominate the genotypes available for host interactions. Overall, these findings from diverse symbioses showcase that host and microbial antagonistic factors mediate strain-specific associations (**Figure 2**).

CONCLUSION

Diverse symbiotic microbiomes have been analyzed using 16S rRNA amplicon sequencing that identify individual community members to the species level. This approach is useful for defining broad characteristics of a microbiome and for predicting individual symbiont function based on knowledge of its taxonomic placement. However, studies that employ strategies such as molecular genetic manipulation of laboratory models of symbiosis, and meta-genomic sequencing efforts, are revealing that symbiotic associations can be dramatically influenced by strain-level variation that is not revealed by standard sequencing practices. In particular, binary host-symbiont associations enable cross-pairing experiments that led to early evidence for the impact of bacterial strain variation on hosts. While assessment of consequences of strain variation on host fitness remains difficult in complex systems, simplification by focusing on individual microbial symbionts or on naturally "simple" systems has revealed that strain variation can have consequences for host associations, physiology, and fitness.

Strain-level specificity in host-symbiont associations occurs in a broad range of symbioses, including those in the plant, invertebrate, and mammalian systems. While much remains to be learned regarding the molecular basis of strain specificity of symbioses, two general categories emerge from the examples presented here: those with variable symbiont-encoded factors necessary for association with the host and those with variability in antagonistic behaviors that modulate associations between particular hosts and microbes. The former type of strain specificity commonly involves the presence, recognition, and utilization of polysaccharides: *S. meliloti* and *V. fischeri* bacterial exopolysaccharides necessary for symbiosis with legumes and

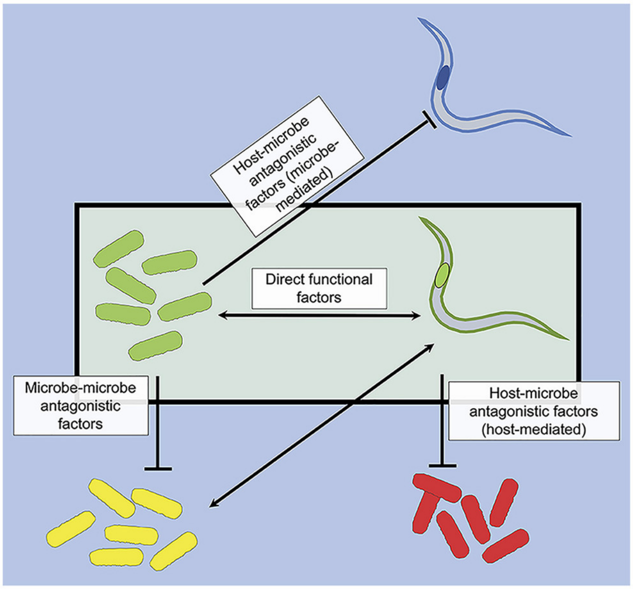

FIGURE 3 | Strain specificity factors in host-symbiont associations can drive selection for specific host-symbiont associations (boxed) through positive selection (direct functional factors) and negative selection (antagonistic factors). Strain-specific association is indicated by matching color in microbes and hosts.

squid, respectively, and bacterial utilization of host-derived polysaccharides in the mammalian gut. The latter involves either host-microbe antagonism or microbe-microbe antagonism.

While these classifications may appear simplistic, they provide a generalized framework to understand factors that contribute to strain specificity in host-symbiont associations beyond those mentioned in this review. For example, field studies indicate that symbiont strain variation even occurs widely in nature (Valette et al., 2013; Parkinson et al., 2015; Russell et al., 2017; Guyomar et al., 2018; Perez and Juniper, 2018; Ellegaard and Engel, 2019; Porter et al., 2019; Ravenscraft et al., 2020). One notable example is the strain-specific associations of wild-sampled *Bathymodiolus* mussels and their gill-localized endosymbionts (Ansorge et al., 2019). There is a positive correlation between the geochemical characteristics of the host environment (e.g., hydrogen levels) and the predicted function of strain-specific genes (e.g., hydrogenases) (Ansorge et al., 2019). The authors of this study hypothesize that strain specificity in this association occurs through metabolic efficiency selection conferred by variable hydrogenase enzymes (Ansorge et al., 2019), similar to Sus-mediated selection among *Bacteroides* strains (Lee et al., 2013; Yassour et al., 2018). From the aforementioned framework, these hydrogenase enzymes can be viewed as potential functional factors involved in host adaptation to environmental nutrient availability that facilitate strain specific association.

Functional and antagonistic factors likely work together to drive the persistence of certain host-symbiont associations (**Figure 3**). Functional factors can act as positive selection for specific host-symbiont associations, initiating and/or maintaining such association over host generations. On the other hand, antagonistic factors can act as negative selection against other host-microbe associations, hindering their formation and/or contributing to their breakdown. Through these selection mechanisms, we posit that they may facilitate the stability and persistence of certain host-symbiont associations over evolutionary periods. In support of this hypothesis, a previous study suggests that antagonism between actinomycetous bacterial symbionts from different ant isolates may facilitate strain specificity of ant-symbiont associations (Poulsen et al., 2007). For future studies, it will be important to contextualize these molecular factors for strain specificity in the ecology and evolution of host-symbiont associations.

As strain variation can dictate the degree and the sign of interaction outcomes between hosts and microbes, it is important to continue investigating ways in which symbiont strains vary genotypically and phenotypically, particularly by identifying strain variable factors driving associations with hosts. In addition, it is essential to elucidate selective pressures that drive evolution of microbial strain variation as well as host-microbe strain specificity. This knowledge will allow a more comprehensive understanding of the molecular and ecological dynamics of host-symbiont associations, and to effectively use this knowledge to our benefit, such as for minimizing host susceptibility to pathogens or maximizing host benefits from agriculturally or medically relevant mutualisms.

AUTHOR CONTRIBUTIONS

DRG: conceived of topic and scope, wrote text, and designed figures. HG-B: contributed to topic refinement, literature review, and editing of text and figures. All authors contributed to the article and approved the submitted version.

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pH Adaptation Drives Diverse Phenotypes in a Beneficial Bacterium-Host Mutualism

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Abiotic variation can influence the evolution of specific phenotypes that contribute to the diversity of bacterial strains observed in the natural environment. Environmentally transmitted symbiotic bacteria are particularly vulnerable to abiotic fluctuations, given that they must accommodate the transition between the free-living state and the host's internal environment. This type of life history strategy can strongly influence the success of a symbiont, and whether adapting to changes outside the host will allow a greater capacity to survive in symbiosis with the host partner. One example of how environmental breadth is advantageous to the symbiosis is the beneficial association between *Vibrio fischeri* and sepiolid squids (Cephalopoda: Sepiolidae). Since *Vibrio* bacteria are environmentally transmitted, they are subject to a wide variety of abiotic variables prior to infecting juvenile squids and must be poised to survive in the host light organ. In order to better understand how a changing abiotic factor (e.g., pH) influences the diversification of symbionts and their eventual symbiotic competence, we used an experimental evolution approach to ascertain how pH adaptation affects symbiont fitness. Results show that low pH adapted *Vibrio* strains have more efficient colonization rates compared to their ancestral strains. In addition, growth rates had significant differences compared to ancestral strains (pH 6.5–6.8, and 7.2). Bioluminescence production (a marker for symbiont competence) of pH evolved strains also improved at pH 6.5–7.2. Results imply that the evolution and diversification of *Vibrio* strains adapted to low pH outside the squid improves fitness inside the squid by allowing a higher success rate for host colonization and symbiotic competence.

Keywords: symbiosis, pH, adaptation, vibrio, beneficial

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INTRODUCTION

pH is biologically a potential environmental stress, which can influence the behavior and subsequent physiological adaptations of bacteria to changing environments. For most microorganisms, the ability to change both phenotypically and genetically to abiotic stress and evolve new adaptive mechanisms is crucial (Hoffmann and Sgro, 2011; Cremer et al., 2012; Casacuberta and González, 2013; Lenski et al., 2015; Aussel et al., 2016). Variation in environmental factors such as low pH is likely to induce new physiological changes that impact microbial fitness and induce mechanisms to cope with this challenge (Casacuberta and González, 2013; Soto and Nishiguchi, 2014; Payne et al., 2016). Most of the time environmental stress provides a trigger for bacterial adaptation and diversification by enhancing their ability to respond quickly and persist throughout those conditions (Lenski et al., 1991; Barrick and Lenski, 2013; Dragosits and Mattanovich, 2013; Brunke and Hube, 2014; Liu et al., 2015). In some cases, adaptation and the subsequent genetic changes can be observed within a few hundred generations, with substantial consequences for fitness of those bacteria. Recently, experimental evolution studies have become a powerful tool to understand functional and structural adaptive changes in bacteria that occur in a short amount of time (Kawecki et al., 2012; Barrick and Lenski, 2013). Microbes are an ideal experimental evolution subject due to their short generation times and large population sizes that can be observed under various conditions simultaneously (Cordero and Polz, 2014). Direct measurements of the competitive fitness of an evolved descendant can be compared to its ancestor *in vivo* and *in vitro* and quantitatively assessed. Additionally, the continual generation of evolved strains are stored frozen (fossil records) and later compared to their respective ancestors- an advantage of using microbes for monitoring evolution progress under various selective pressures that would normally lead to greater diversity in the natural environment (Hindré et al., 2012; Dragosits and Mattanovich, 2013; Wiser et al., 2013).

The beneficial association between sepiolid squids and their *Vibrio* bacteria provides an experimentally tractable model system for the study of the experimental evolution, adaptation, and diversification of these symbionts as they transition between their free-living and symbiotic state (Schuster et al., 2010; Soto et al., 2012, 2014; Soto and Nishiguchi, 2014). During the symbiosis, *Vibrio* bacteria are exposed to periodic acidification while inside the adult host crypts of the light organ during the night cycle due to the high oxygen consumption from bioluminescence activity (Pan et al., 2015; Schwartzman et al., 2015). This change in light organ pH represents a physiological challenge for *V. fischeri* while it resides inside the squid host. *Vibrio* bacteria that can accommodate this change must be able to have a responsive acid-adaptation mechanism for the symbiotic association to be maintained as well as in new *Vibrio* infections. Given the importance of how adaptation to stressful conditions in the surrounding habitat can be co-opted to increase fitness for other cellular functions while in symbiosis, we investigated whether *V. fischeri* that are pre-adapted to low pH outside the squid are better able to colonize and persist in squid light organs. We subjected three different strains of *V. fischeri* for 600 generations at different pH levels and monitored the physiological changes (bioluminescence, production, and growth) in 10 independent evolving lines grown at their respective pH levels. Ancestral strains (control) were compared to evolved strains in *vitro* to determine their fitness prior to animal colonization. These evolved strains were then subjected to juvenile squid colonization assays to determine whether *in vitro* adaptation to low pH affected the ability of *V. fischeri* to perform better when inside squid light organs.

MATERIALS AND METHODS

Experimental Evolution

Three strains of symbiotic *V. fischeri* were experimentally evolved under various pH concentrations: *V. fischeri*, ES114 (version: ES114-L; host *Euprymna scolopes*, from Kaneohe Bay, Hawaii), *V. fischeri* ET401 (host *E. tasmanica*, from Townsville, QLD, Australia), and *V. fischeri* EM17 (host *E. morsei* from Tokyo Bay, Japan) at pHs of 6.5, 6.8, 7.2, 7.6, and 8 for 600 generations (**Table 1**). Based on a pilot study, *V. fischeri* at pH 5.5 and 6 were unable to survive *in vitro* and were not examined further. pH levels for this study were chosen to represent acidic conditions of the light organ (6.5–7.6) and a control pH of 8.0 (seawater pH). For biological significance, 10 independent lines of clonal isolates from each strain were experimentally evolved simultaneously. Chromosomal insertion of the chloramphenicol resistance marker Cam^R into the genomes of *V. fischeri* ET401 and EM17 were made with a mini-Tn*7* cassette. The Cam^R cassette was used as a selective agent for discerning between ancestral (WT-control, no Cam^R) and evolved strains (carrying Cam^R). *V. fischeri* JRM200, an isogenic strain of *V. fischeri* ES114-L with the mini-Tn7 Cam^R, was obtained from earlier studies (McCann et al., 2003; Soto et al., 2012). Briefly, *E. coli* strain CC118λ*pir* carrying pEVS104 and another strain of *E. coli* BW23474 carrying pUX-BF13 (Visick and Ruby, 1997; McCann et al., 2003) were combined with the recipient *V. fischeri* strain in a tri-parental mating-bacterial conjugation step and incubated for ∼12 h at 28°C. The conjugation mixture was then re-suspended in SWT [seawater tryptone; per liter composition: 700 mL of seawater (30 g of Instant Ocean and 10 g of Marine Mix, 300 mL of dH_2O), 5 g tryptone, 3 g yeast extract, 3.75 mL 80% glycerol] media and spread onto SWT-agar plates (15 g agar/L) with chloramphenicol (20 μg/mL). *V. fischeri* JRM200, ET401 (Tn7 Cam^R), and EM17 (Tn7 Cam^R) were then streaked onto SWT agar plates media with chloramphenicol (20 μg/mL), then incubated at 28°C for 18–24 h. Individual clonal isolates (single colonies) were transferred to 5 mL of SWT with chloramphenicol (20 μg/mL) liquid medium with the selected pH levels and placed in a 28°C shaking incubator set at 225 rpm for 8–12 h. When growth reached $OD_{600nm} = 0.5$, 10 μL of each culture was transferred (1:500 dilution) to 5 mL of fresh SWT media with chloramphenicol (20 μg/mL) at their respective pH. Experimental evolution was sustained in 18 × 150 mm glass test tubes kept in an Innova® 43R incubator shaker (New Brunswick, Enfield, CT) at 28°C, 225 rpm.

Previous studies using experimental evolution on *E. coli* (Wielgoss et al., 2013) illustrated how most mutations under selective pressure occur during the exponential phase of bacterial growth; therefore, all 10 lines of the clonal isolates from the three strains at all pH levels were transferred at the end of log phase. For consistent timing and ease of calculations, culture transfers were completed every 12 h at a 1:500 dilution, correlating to the end of log phase, but prior to stationary phase. Generations of evolving bacteria were determined by calculating the specific generation times of *V. fischeri* JRM200, ET401 (Tn7 Cam^R), EM17 (Tn7 Cam^R). Calculations were completed assuming that bacterial growth is a first-order chemical reaction, where $\mu =$

TABLE 1 | *Vibrio fischeri* strains used in the study.

Strains	Description	Host Squid	Location
Ancestor/Wild-type strains (WT)			
ES114[a]-WT	Ancestor (control)	*Euprymna scolopes*	USA (Kaneohe Bay, O'ahu, Hawaii)
ET401-WT	Ancestor (control)	*Euprymna tasmanica*	Australia (Tasmania)
EM17-WT	Ancestor (control)	*Euprymna morsei*	Japan (Tokyo Bay)
Evolved strains with selective marker Tn7 and Cam^R			
JRM200[b]	Mini-Tn7 *Cam^R	Previously constructed[c]	
ET401-Tn7	Mini -Tn7 *Cam^R	Previously constructed[c]	
EM17-Tn7	Mini-Tn7 *Cam^R	Previously constructed[c]	

**Cam^R, Chloramphenicol Resistance.*

[a] *Version: ES114-L.*

[b] *Derived from ES114-L.*

[c] *(McCann et al., 2003).*

(lnN–lnN$_0$) (t–t$_0$) and g = ln2/μ, where μ represents the growth rate, N is the number of cells at time t, N$_0$ is the number of cells at t$_0$, and g is the generation time (Soto et al., 2009). Using the previously calculated generation time of each strain for all five pH levels, evolutionary "frozen fossil records" were generated by freezing down cultures with 20% glycerol final volume every 100 generations, and storing each in 2 mL cryo-vials (Wheaton, NJ) at −80°C. To check for contamination and experimental progress, 50 μL of each culture that was frozen was also spread on SWT-Cam agar plates and examined for the viability of strains through growth studies the following day. All cultures were continually transferred to fresh SWT-Cam (20 μg/mL) at their respective pH levels. Frozen fossil records were collected at 0, 100, 200, 300, 400, 500, and 600 generations. For the experimental calculations we used three replicates from each of the 10 lines of frozen fossil records of the evolved strains from each 100th generation. Each pH treatment had 90 samples (three replicates × 10 lines × three strains).

Growth and Bioluminescence Assays

All ten lines of evolved bacterial strains at 100, 200, 300, 400, 500, and 600 generations and the ancestral (control) strains were grown in SWT liquid media with pH levels adjusted to of 6.5, 6.8, 7.2, 7.6 and 8 by acetic acid (99.7+% A.C.S reagent, Sigma-Aldrich, St. Louis, MO) and measured with an Orion PerpHecT pH Meter (Model 330—Thermo Scientific, Waltham, MA). pH had no negative affect on the antibiotic used throughout this study for all evolved isolates.

Cultures were shaken at 225 rpm at 28°C overnight, then sub- cultured at a 1:500 dilution into new SWT media for all strains at all pH levels measured. All samples were grown to OD$_{600nm}$ of 0.3 at 28°C and shaken at 225 rpm in an Innova® 43R incubator shaker (New Brunswick, Enfield, CT). Cells were inoculated in 5 mL SWT media at 1:10 dilution in triplicate (3 independent bacteriological test tubes (glass, 18 × 150 mm) per each time point/pH levels) for a 12-h growth study. Reading of all triplicate samples were taken every hour by Spectramax spectrophotometer (Molecular Devices, Sunnyvale, CA). The term Relative Light Unit-RLU for bioluminescence measurement is used since luminometers typically do not yield a measurement directly in units of photons. During the measurements, the Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA) was set to one light unit and all other measurements were made relative to the set value. Bioluminescence (Relative light units, RLUs) was measured simultaneously every hour with a Luminoskan Ascent luminometer (Thermo Scientific, Waltham, MA). Growth rates of samples were determined by calculating the generation time of each bacterial strain during each growth study (Soto and Nishiguchi, 2014). The factors of Relative Light Unit (RLU) measurement per generation at specific pH levels for each strain were used in a two-way ANOVA analysis for statistical analysis of the bioluminescence assay. GraphPad Prism (8.0.0) software was used for all analysis ($p < 0.001$, $N = 10$).

For growth assays, the two-way ANOVA analysis (GraphPad Prism, 8.0.0) were conducted using values of the growth rate per strains (evolved and ancestral bacteria) in various pH levels at different generations ($p < 0.001$, $N = 10$).

Host Co-ionization Assays

To determine whether pre-adaptation to low pH impacts animal host colonization compared to non-evolved strains, infection assays were performed as previously described (Nishiguchi, 2000; Chavez-Dozal et al., 2014). For single and competition infection experiments, overnight cultures of the 600 generation wild-type (control) ES114, EM17, ET401 and evolved JRM200, ET401-Tn7, EM17-Tn7strains were regrown in 5 mL of fresh SWT medium at their respective evolved pH until they reached an OD$_{600nm}$ of 0.3. Cultures were then diluted to ∼1 × 10^3 CFU/mL in 5 mL of sterile seawater and added to glass scintillation vials where newly hatched juvenile squids were placed (one individual/vial). Three independent plates of the same inoculation seawater with each respective strain were plated for all samples, and CFUs were counted the next day for the accuracy of the initial inoculum. Seawater (pH = 8) was changed with fresh non-sterile seawater (without bacteria) every 12 h over a period of 48 h. Animals were maintained on a light/dark cycle of 12/12. After 48 h colonization (48 h = standard colonization assay time), animals were sacrificed and homogenized, and the diluted homogenate was plated onto SWT agar plates for both wild-type and evolved *V. fischeri*, and SWT with chloramphenicol (20 μg/mL) for the *V. fischeri* evolved strains. A second set of animals were selected for competition studies, where juvenile squids were co-infected with the ancestral strain (WT-control) and their respective evolved strain, sacrificed after 48 h, homogenized, and the diluted homogenate plated onto SWT and SWT with chloramphenicol (20 μg/mL) agar plates. The concentration of CFUs was the same as the prior set of studies as well as in the competition studies, with the combined total being the same CFUs as the single infections (5000 CFUs total). Colony forming units (CFUs) were counted the next day to determine colonization efficiency of each strain. For calculation accuracy, the plates containing SWT with chloramphenicol (20 μg/mL) were subtracted to quantify evolved strains colonies from wild-type (control). This was due to the fact that only the evolved strains have the capacity to grow on agar plates containing chloramphenicol (20 μg/mL) whereas both ancestral and evolved will grow on SWT without chloramphenicol. A total of 15 animals/strain were used for each competition assay, and five non-infected (aposymbiotic) juveniles were used as negative controls for each sample run. We chose evolved strains at pH 7.2 since these had the most significant change compared to all other pH concentrations measured, and due to our limited supply of animals for the study. Results of single colonization and competition study were analyzed using two-way ANOVA analysis with GraphPad Prism (8.0.0). The factors of analysis for this study are calculated CFU/Light Organ values per strains for both single colonization and competition studies ($p < 0.001$ and $N = 15$).

RESULTS

Growth Rate of Ancestor and Evolved Strains Under Various pH Conditions

Evolved *V. fischeri* strains JRM200, ET401-Tn7, and EM17-Tn7 at 600 generations demonstrated significantly different growth rates at pH 6.5, 6.8, and 7.2 when compared to their respective ancestral strains ($T = 0$) ES114-WT, ET401-WT, and EM17-WT at the same pH (**Figures 1A–F**). The two-way *post hoc* ANOVA analysis demonstrated significant effects for pH levels 6.5, 6.8, and 7.2 among all bacterial strains. The *post-hoc* ANOVA interaction analysis between various pH levels for different generations of bacterial strain shown significant differences ($p < 0.001$, $N = 10$). pH 7.2 shown significant differences at 400, 500, and 600 generations in comparison to pH 6.5, 6.8, 7.2, and 8. pH 7.6 and pH 8 shown no significant differences among each other, but there were significantly different with other pH levels. pH 6.5 and 6.8 were shown no significant differences among each other, but there were significantly differ among other pH levels at different generations ($p < 0.001$, $N = 10$).

Bioluminescence

Bioluminescence increased from 300 to 600 generations with significantly higher luminescence at 600 generations in relative light units for evolved *V. fischeri* strains JRM200, ET401-Tn7, EM17-Tn7 at pH 6.5, 6.8, and 7.2 when compared to their ancestral strains at the same pH (**Figures 2A–C**). The two-way *post hoc* ANOVA analysis demonstrated significant effects for all pH levels among all bacterial strains with respect to the Relative Light Unit (RLU) of bioluminescence. The *post-hoc* ANOVA interaction analysis between various pH levels for different RLU measurements per generation of each bacterial strains showed significant differences. pH 7.2 showed significant differences at 400, 500, and 600 generations in comparison to pH 6.5, 6.8, 7.2, and pH 8. pH 7.6 and 8 showed no significant difference among each other, but there were significantly different with other pH levels. pH 6.5 and 6.8 had no significant difference among each other, but they were significantly different among other pH levels at different generations ($p < 0.001$, $N = 10$).

Animal Studies

Single Infection Assays

To examine the effect of pH adaptation prior to symbiosis, colonization studies between ancestral *V. fischeri* ES114-WT, ET401-WT, and EM17-WT and evolved JRM200, ET401-Tn7 (Cam^R), and EM17-Tn7 (Cam^R) strains at 600 generations at pH 7.2 were completed in the Hawaiian bobtail squid *E. scolopes*. All strains demonstrated significant differences between the ancestor and evolved strain ($p < 0.001$; **Figure 3A**). Native Hawaiian *V. fischeri* ES114 exhibited higher colonization rates than non-native strains ET401 and EM17 in *E. scolopes* for both ancestral and evolved strains. Evolved strains ET401-Tn7 and EM17-Tn7 at 600 generations have the ability to colonize non-native *E. scolopes* light organs, but are significantly lower than either ancestor or evolved native ES114 strains (**Figure 3A**). Overall, single strain infections indicate that native strains (both ancestor and evolved) colonize their specific squid host preferentially, but non-native *V. fischeri* strains (EM17 and ET401 ancestor and evolved) are also capable of colonizing a non-native squid host (**Figure 3A**). All aposymbiotic Hawaiian bobtail squid *E. scolopes* had zero CFUs during colonization study. For ancestral and evolved strains, two-way *post hoc* analysis of variance (ANOVA) for factors of CFU/light organ and strains (evolved and ancestral at 600 generations/pH 7.2) showed significant effect of strain ($p < 0.001$, $N = 15$).

Animal Competition Assays

Competition studies of ancestral *V. fischeri* ES114-WT, ET401-WT, and EM17-WT and evolved JRM200, ET401-Tn7 (Cam^R), and EM17-Tn7 (Cam^R) of *V. fischeri* at 600 generations (pH 7.2) showed significant differences between all three strains examined (**Figure 3B**). Evolved native Hawaiian strain JRM200 and ET401-Tn7 (Cam^R) at 600 generations had higher colonization rates than non-native evolved strain EM17-Tn7 (Cam^R) at 600 generations in *E. scolopes*. Additionally, each of the evolved strains outcompeted their ancestral strain (JRM 200, ET401-WT, and EM17-WT; **Figure 3B**). *V. fischeri* EM17-WT had the lowest colonization rate of all strains examined during competition with its ancestor (**Figure 3B**). All aposymbiotic Hawaiian bobtail squid *E. scolopes* had zero CFUs during colonization study. For all ancestral and evolved strains, a two-way analysis of variance (ANOVA) for factors of CFU/light organ and bacterial strains (evolved and ancestral at 600 generations/pH 7.2) showed significant effects of strain type ($p < 0.001$, $N = 15$).

DISCUSSION

In this study, pH adapted strains of *V. fischeri* were evolved to 600 generations in order to examine how pH adaptation would influence colonization and subsequently symbiotic fitness. Adaptation of *V. fischeri* JRM200, ET401-Tn7, and EM17-Tn7 was not significant until 400 and up to 600 generations for all evolved strains. Since there were no specific measurements of acid resistance to the mildly acidic environment during the study, these results only represent adaptation under various pH growth conditions *in vitro* and subsequently when these evolved isolates were challenged inside the squid light organ *in vivo*. Our results suggest that *V. fischeri* strains that are evolved under low pH conditions *in vitro* gained the ability to better colonize juvenile squids *in vivo*, increasing their overall fitness for successful host colonization, indicating that increased symbiont diversity

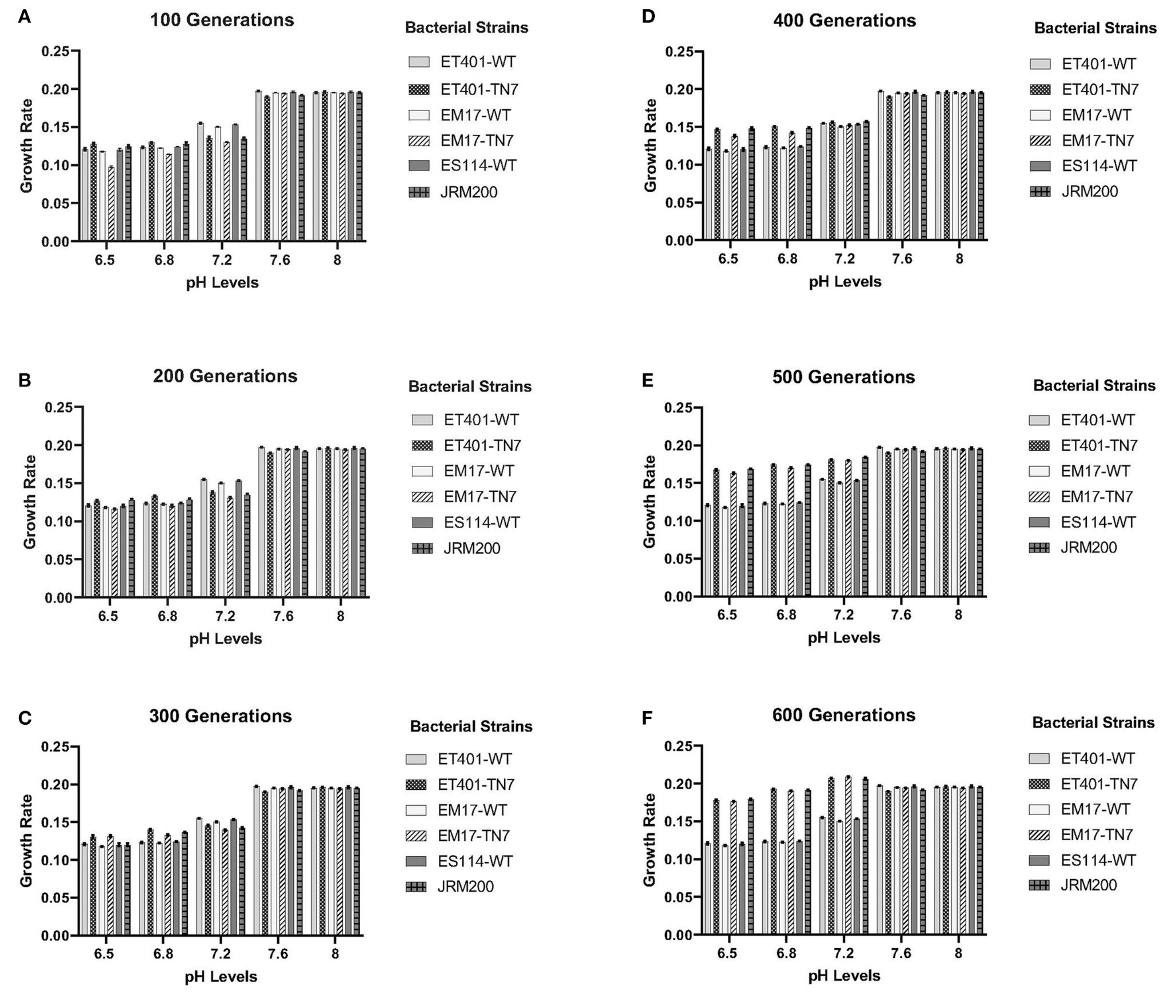

FIGURE 1 | (A–F) Growth rates of ancestor and evolved strains from 100 to 600 generations for pH levels of 6.5–8, respectively. See **Table 1** for strain designations.

offers new avenues for symbiotic associations to exploit. Previous studies using evolved *V. fischeri in situ* demonstrate that they are amenable to experimental evolution in a novel squid host, and were observed to be just as competitive as their ancestral strain as early as 400 generations (Soto et al., 2014). Such symbiont driven benefits can be influenced by environmental factors (Hussa and Goodrich-Blair, 2013), and in some cases abiotic variables may alter factors that enable *V. fischeri* to not only adapt and survive under those conditions, but indirectly provide advantages for host colonization (Riggins et al., 2013). Perhaps increased growth rate and bioluminescence activity, two influential factors important for symbioses, are influenced by both physiological and genetic changes that regulate squid-host selectivity during experimental evolution and provide a competitive advantage during colonization. Earlier work using experimentally evolved bacteria has shown that specific phenotypic characteristics give one bacterial genotype a better competitive advantage under certain environmental conditions (Lenski et al., 1991; Barrick and Lenski, 2013; Zaman et al., 2014). This adaptability may be translated into specific genetic mutations to create a novel strain that is more fit than its predecessor (Lenski et al., 2015; Ribeck and Lenski, 2015). For example, resilience to low pH in various host enteric environments by *Escherichia coli*, *Shigella sonnei*, *Salmonella enterica*, and *Bifidobacterium animalis* has provided evidence that these bacteria have evolved mechanisms to combat acid stress such as the acid-inducible tolerance response (Wielgoss et al., 2013) and expression of acid stress proteins to accommodate low pH (ASPs; (Sanchez et al., 2007; Ramos-Morales, 2012; González-Rodríguez et al., 2013; Lund et al., 2014)). Oftentimes, the ability of mutualistic and pathogenic *Vibrio* species to persist in aquatic environments is highly correlated with the diversity of populations (Johnson,

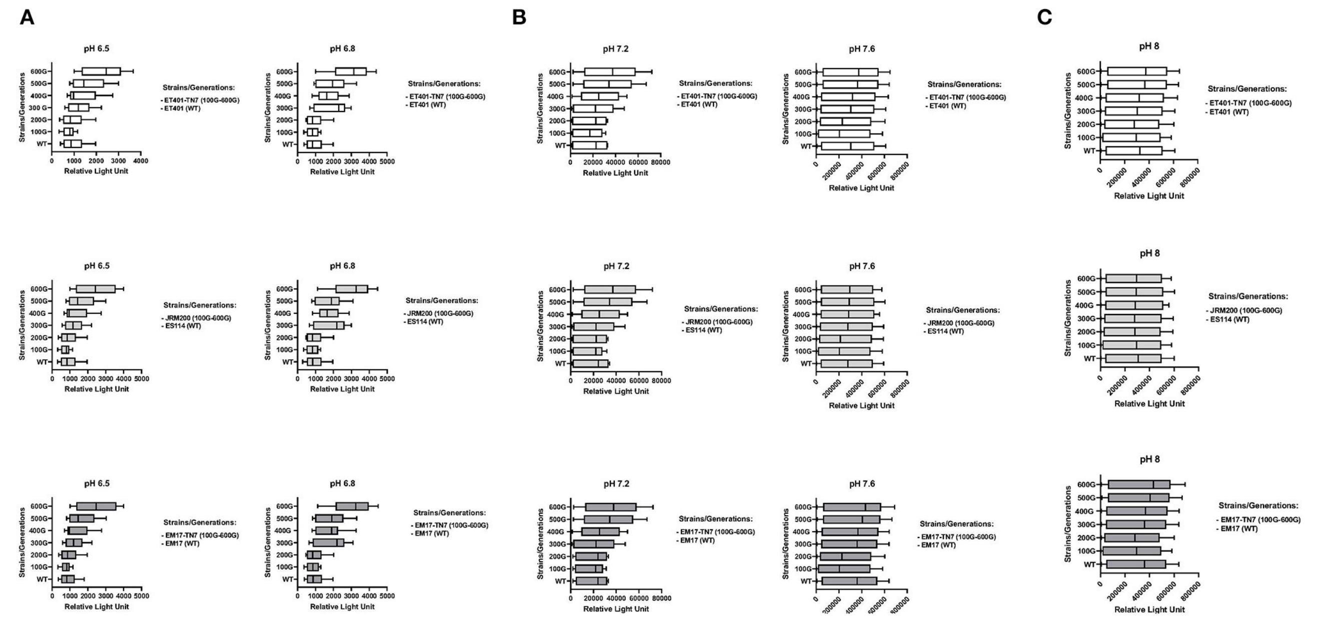

FIGURE 2 | Bioluminescence (relative light units) during growth of ancestor and their evolved strains from 100 to 600 generations at **(A)** pH = 6.5 and pH = 6.8 ET401, JRM200, and EM17, **(B)** pH = 7.2 and pH = 7.6 ET401, JRM200, and EM17 **(C)** pH = 8 ET401, JRM200, and EM17. See **Table 1** for strain designations.

2013; Ceccarelli and Colwell, 2014; Haley et al., 2014; Shaw et al., 2014). Both mutualistic and pathogenic vibrios have developed complex regulatory networks that ensure successful colonization and expression of their colonization/virulence genes while exposed to low pH (de la Fuente-Núñez et al., 2013; Otto, 2014; Saleem, 2015). Bacteria such as *V. cholerae* can subsist in very diverse and different ecological niches when not in their host, yet maintain the ability to colonize the human intestine when the opportunity arises (Runft et al., 2014). Our study demonstrates that infection, colonization, and persistence by both native and non-native experimentally evolved *V. fischeri* strains (ES114-JRM200, ET401-Tn7, and EM17-Tn7) was enhanced when these strains were evolved to low pH prior to exposure to juvenile squids. The potential of *V. fischeri* variants to quickly evolve to environmental fluctuations may explain how genetically related strains are found in multiple hosts across large geographic distances (Jones and Nishiguchi, 2006; Soto et al., 2012; Coryell et al., 2018). Recent studies demonstrate how well one strain of *V. fischeri* (EM17) can adapt to pH stress with respect to their growth, bioluminescence, and host colonization through an *in-vitro* evolution process up to 2,000 generations, where significant differences were observed between acid (pH 6.0) and alkaline (pH 10.) stress (Cohen et al., 2020). Given this wide ecological breadth, *V. fischeri* is a perfect example of how environmental adaptation can influence both host specificity and symbiont biogeography.

Bioluminescence

Bioluminescence results show that relative light units (RLUs) of low pH adapted *V. fischeri* strains ES114, ET401, and EM17 at pH 6.5, 6.8, and 7.2 increased from 400 to 600 generations (**Figure 2**). These results suggest that adaptation to lower pH may also enhance the bioluminescence activity of evolved *V. fischeri* strains, which would entail changes in *lux* operon functionality and alternations to the metabolic changes that influence input/output from associated light producing proteins (Wier et al., 2010; Wilson, 2013). Bacteriogenic bioluminescent animals such as squid and fish have evolved organs specifically dedicated to host luminescent bacteria (e.g., *V. fischeri, V. logei*), whose bioluminescence is used during certain behaviors such as hunting and counterillumination (Jones and Nishiguchi, 2004; Haddock et al., 2010; Bose et al., 2011). The observed increase in bioluminescence activity of evolved strains in this study suggests that maintenance of this important symbiotic feature gives the cell an evolutionary advantage over ancestral strains (Stabb and Visick, 2013). Given that luminescence is important in establishing and maintaining the symbiosis, it is not unlikely that biofilm production is also affected by this phenomenon. Recent proteomic studies on biofilm production between free-living and symbiotic states in *V. fischeri* have detected a bioluminescence regulatory protein which is responsible for regulating the quorum sensing cascade and subsequently increasing exopolysaccharide production in the biofilm matrix (Chavez-Dozal et al., 2015). Although differences in biofilm formation between ancestral and evolved strains were not examined in this study, biofilm production may be affected similarly to bioluminescence and other symbiotic loci when *V. fischeri* is evolved at low pH. Previous research has demonstrated that the day-night oscillation of *V. fischeri's* metabolic pathways shift from aerobic respiration to fermentation, and the concomitant expression of genes from catabolic cycles suggest variation from a neutral to an acidic pH level in the

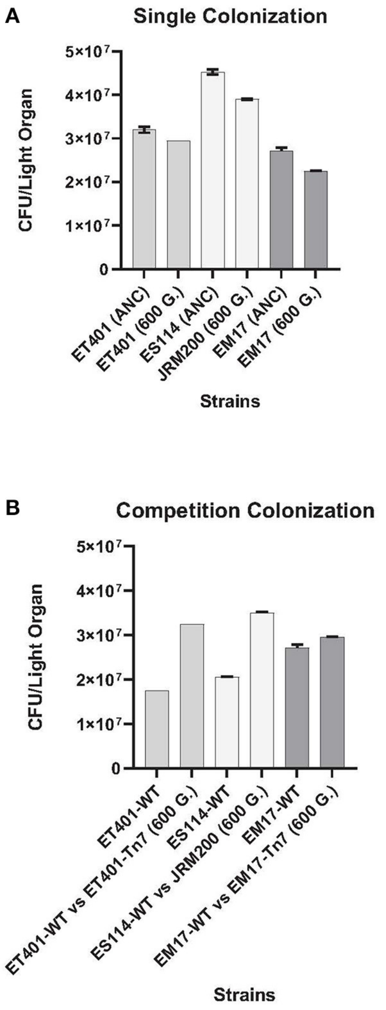

FIGURE 3 | (A) Animal colonization assays at 600 generations with single strains and competitions between ancestor and evolved (pH 7.2) strains in *E. scolopes* juveniles. CFU, colony forming units. See **Table 1** for strain designations. **(B)** Animal competition assays 48-h post-infection of juvenile *Euprymna scolopes* between ancestor (control) and evolved strains of *Vibrio fischeri* at 600 generations (pH 7.2). CFU, colony forming units. See **Table 1** for strain designations.

light organ crypt (Wier et al., 2010; McFall-Ngai, 2014). Thus, enhanced bioluminescence activity that is dependent upon high bacterial cell density in the light organ can be impacted when *V. fischeri* is resistant to low pH (Jones and Nishiguchi, 2006; Guerrero-Ferreira and Nishiguchi, 2010). Future studies will focus on the regulation of bioluminescence and biofilm related proteins both in ancestral and evolved strains to determine how adaptation to low pH influences these specific symbiotic loci. Additionally, understanding the synergism of low pH adapted genes to other regulatory networks responsible for bacterial physiological behavior during infection and colonization will further our knowledge on the genetic diversity of *V. fischeri* to such conditions in the wild.

Animal Studies

Symbiotically competent *V. fischeri* that are free-living in the environment have evolved to form a close and persistent association with host squids (McFall-Ngai, 2014; Schwartzman and Ruby, 2016). Likewise, these symbiotic *V. fischeri* must be able to coexist with the host and be recognized as "beneficial" by the squid immune system, while maintaining features that allow a successful colonization (Sachs et al., 2011). The evolution of specific recognition, colonization, and persistence mechanisms both in the symbiont and host are therefore a combination of environmental and host specific factors that enable the success of such beneficial associations (Sachs et al., 2011; Soto et al., 2012, 2014; Guerrero-Ferreira et al., 2013). For example, cooperation between *Rhizobia* and leguminous plants occurred due to the benefit of nitrogen fixation, which is a very costly process (Sachs and Simms, 2008). In the squid-*Vibrio* model system, similar phenomena occur when the bacteria produce bioluminescence for the host, while in return the host provides a nutrient rich environment for growth (Wier et al., 2010; Wernegreen, 2013; McFall-Ngai, 2014; Soto and Nishiguchi, 2014; Pepper et al., 2015). The squid host maintains the symbiosis and controls cell density by venting 95% of its *Vibrio* symbiont to the surrounding environment at dawn. Thus, the host has evolved a mechanism to cull and maintain only the "best fit" bacteria that can produce luminescence for counterillumination (Jones and Nishiguchi, 2004; Wier et al., 2010; Wernegreen, 2013; McFall-Ngai, 2014; Soto and Nishiguchi, 2014; Pepper et al., 2015). *V. fischeri* that have evolved to outcompete and survive this diel cycle of venting have been "selected" through multiple generations as in an experimental evolution scenario. Since light production is a costly metabolic process, oxygen levels in the light organ slowly decrease throughout the night causing this drop in pH (Schwartzman et al., 2015; Schwartzman and Ruby, 2016). Whether pH adapted vibrios are better able to accommodate this change, thereby impacting the repopulation efficiency to positively influence the number of pH adapted symbionts may explain how environmental conditions complement *V. fischeri's* success as a beneficial microbe. Since our pH adapted *V. fischeri* strains were shown to be more fit than their ancestral lines both *in vitro* and *in vivo*, this may be indicative of specific loci that are beneficial for both pH accommodation and host colonization and persistence. Additionally, symbiotic loci in *V. fischeri* have been found to be more conserved than homologs in free-living strains (Howard et al., 2015). One example is the mannose sensitive hemogglutinin proteins (MshA) that are often induced by environmental factors. MshA is important for light organ colonization (Llorca, 2008; Ariyakumar and Nishiguchi, 2009; Chavez-Dozal et al., 2014) but also for inducing cell-cell communication at low pH (Llorca, 2008). Perhaps proteins such as MshA from evolved *V. fischeri* strains have been impacted due to experimental evolution at low pH, thereby changing the colonization efficiency such that evolved strains outcompete ancestral strains during host competition studies. Future studies will examine *msh* loci and other symbiotic loci of both ancestral and evolved strains to determine whether subtle genetic changes are correlated to the evolution at low pH.

CONCLUSIONS

This study emphasized the broad view of whether *V. fischeri* can respond to selective pressures "outside the light organ of the host" (e.g., pH) and subsequently increase their fitness in their symbiotic state. During symbiosis, *V. fischeri* constantly experience shifts between the light organ milieu and the surrounding aquatic environment. Given that *V. fischeri* can adapt quickly to changing pH stress concentrations under laboratory conditions indicates that this bacterium has the plasticity and breadth that extends far beyond its life history in the ocean (Cohen et al., 2020). Since *V. fischeri* is cosmopolitan in nature, the subtle differences between free-living strains (those that cannot infect sepiolid squids) and symbiotic isolates can offer clues to how host selection and environmental can influence the dynamics between these two life history strategies, and render the pathway toward bacterial speciation among closely related vibrios.

Future research work will expand on examining the transcriptomes of both ancestral and evolved lines in order to determine whether pH adaptation does indeed have pleiotrophic effects in symbiotic *V. fischeri*. Examining changes in biofilm formation of evolved strains may also elude to whether loci regulating structure/matrix proteins and luminescence production are also affected by low pH, and whether such mutations effect luciferase activity. Future proteomic studies on biofilm production between evolved and ancestral strains of *V. fischeri* can eventually lead us to identify unique regulatory protein(s) related to pH stress and their function. This may help to better understand the evolutionary impact on fitness and successful symbiosis under low pH stress at the molecular level.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by New Mexico State University Institutional Animal Care and Use Program.

AUTHOR CONTRIBUTIONS

MN conceived of the project idea. NN ran all the experiments and analyzed the data. NN and MN wrote the manuscript. All authors contributed to the article and approved the submitted version.

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A Link Between Communities of Protective Endosymbionts and Parasitoids of the Pea Aphid Revealed in Unmanipulated Agricultural Systems

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In the last decade, the influence of microbial symbionts on ecological and physiological traits of their hosts has been increasingly recognized. However, most of these effects have been revealed under laboratory conditions, which oversimplifies the complexity of the factors involved in the dynamics of symbiotic associations in nature. The pea aphid, *Acyrthosiphon pisum*, forms a complex of plant-adapted biotypes, which strongly differ in the prevalence of their facultative endosymbionts. Some of the facultative endosymbionts of *A. pisum* have been shown to confer protection against natural enemies, among which *Hamiltonella defensa* is known to protect its host from parasitoid wasps. Here, we tested under natural conditions whether the endosymbiont communities of different *A. pisum* biotypes had a protective effect on their hosts and whether endosymbiotic associations and parasitoid communities associated with the pea aphid complex were linked. A space-time monitoring of symbiotic associations, parasitoid pressure and parasitoid communities was carried out in three *A. pisum* biotypes respectively specialized on *Medicago sativa* (alfalfa), *Pisum sativum* (pea), and *Trifolium* sp. (clover) throughout the whole cropping season. While symbiotic associations, and to a lesser extent, parasitoid communities were stable over time and structured mainly by the *A. pisum* biotypes, the parasitoid pressure strongly varied during the season and differed among the three biotypes. This suggests a limited influence of parasitoid pressure on the dynamics of facultative endosymbionts at a seasonal scale. However, we found a positive correlation between the α and β diversities of the endosymbiont and parasitoid communities, indicating interactions between these two guilds. Also, we revealed a negative correlation between the prevalence of *H. defensa* and *Fukatsuia symbiotica* in co-infection and the intensity of parasitoid pressure in the alfalfa biotype, confirming in field conditions the protective effect of this symbiotic combination.

Keywords: protective symbioses, insect parasitoids, parasitism pressure, symbiont communities, field approach, *Acyrthosiphon pisum*, *Hamiltonella defensa*

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INTRODUCTION

Antagonistic interactions such as host–parasite or prey–predator are major drivers of ecological and evolutionary processes and affect more globally biodiversity patterns and ecosystem functioning. Antagonistic interactions usually involve arms race between protagonists whereby the enemy evolves new weapons to counteract victim's defensive strategies (Abrams, 2000). Selection of these adaptive responses acts on variation encoded by enemy or victim's genomes and relying on different mechanisms such as toxins released by pathogens into the infected host or immunity host defenses triggered upon parasite attacks. More recently, evidence accumulated that some microbial symbionts hosted by eukaryotes can also actively participate in antagonistic interactions by extending the arsenal of enemies or the defensive strategies of victims (Flórez et al., 2015).

Protective microbial symbionts have been particularly well-studied in insects with the best examples being the *Spiroplasma* bacterial endosymbiont which protects a mushroom-feeding fly *Drosophila neotestacea* from parasitic nematodes (Jaenike et al., 2010) or the *Hamiltonella defensa* bacterial endosymbiont which confers protection to aphids against hymenopteran parasitoids (Oliver et al., 2003). In both cases, the mechanisms of the symbiont-mediated protection involve the production of microbial toxins active against the parasite (Oliver and Perlman, 2020). The infection with these protective symbionts confers an immediate fitness advantage to the host when enemy pressure exists. However, hosting protective symbionts may come with some fitness costs (Oliver et al., 2008; Simon et al., 2011; Vorburger and Perlman, 2018). This cost/benefit balance has been repeatedly invoked to explain intermediate frequencies of protective symbionts in natural populations of their hosts (Oliver et al., 2014). In addition, these bacterial symbionts being transmitted occasionally through horizontal transfer events, they may rapidly spread across both host populations and species, leading to rapid adaptations and invasion processes (Jaenike et al., 2010; Himler et al., 2011). Finally, these protective symbionts may modify the diversity, structure and function of ecological networks that link their hosts to lower or higher trophic levels, through bottom-up and top-down effects (Mclean and Godfray, 2015; Rothacher et al., 2016; Ye et al., 2018; Mclean, 2019). Most studies on protective symbionts in insects have been carried out in laboratory conditions or in simplified field experiments. Although these studies have been crucial in investigating the costs and benefits in hosting protective symbionts in presence or absence of parasitism, only few assessed the response of host–symbiont associations to varying pressure intensities of natural enemies and by extent, the link between symbiont communities and parasitoid communities in real and complex natural environments (Smith et al., 2015; Ye et al., 2018).

The pea aphid *Acyrthosiphon pisum* is a good model to study how symbiotic associations are influenced by the parasitism exerted on their hosts and in return, how symbionts affect diversity and community structure of their host's natural enemies. This sap-feeding insect feeds on legumes and forms at least 15 biotypes differentiated by host plant utilization (Peccoud et al., 2009, 2015; Ferrari et al., 2012). A large variation in symbiotic associations is found in natural populations of pea aphids with seven heritable facultative endosymbionts being hosted alone or in co-infections in addition to the primary symbiont *Buchnera aphidicola* (Gauthier et al., 2015; Guyomar et al., 2018). These endosymbionts can have different phenotypic effects on their hosts, including protection from natural enemies. For example, *H. defensa, Fukatsuia symbiotica* (also named PAXS for Pea Aphid X-type Symbiont), and *Serratia symbiotica* have been reported as protective against parasitoids (Oliver et al., 2003; Ferrari et al., 2004; Guay et al., 2009; Leclair et al., 2016), while *Regiella insecticola, Rickettsiella viridis, Rickettsia* sp., *Spiroplasma* sp., and *F. symbiotica* confer protection against fungal pathogens (Scarborough et al., 2005; Łukasik et al., 2013; Heyworth and Ferrari, 2015) and *R. viridis* against predators (Polin et al., 2015). Beyond the phenotype induced by one endosymbiont, other phenotypes are observed when multiple symbionts coexist in a single host (Leclair et al., 2017; Mclean et al., 2018). For example, multiple infections with *H. defensa* and *F. symbiotica* can give stronger protection against parasitoids than each endosymbiont alone (Guay et al., 2009; Heyworth and Ferrari, 2015; Leclair et al., 2016), although this may not be always the case (Doremus and Oliver, 2017). This variation in phenotypic effects induced by the different endosymbionts could result from strain differences as showed in earlier works (Oliver et al., 2003; Cayetano et al., 2015; Leclair et al., 2016).

Population cage experiments showed that pea aphids infected with *H. defensa* increased rapidly in frequency when exposed to *Aphidius ervi,* the main parasitoid of *A. pisum,* but declined when parasitoid pressure was removed (Oliver et al., 2008). Recent independent studies exposed laboratory clones carrying or not *H. defensa* to field conditions in order to analyze the temporal dynamics of protected and unprotected aphid clones when facing a wider range of natural enemies (Hrcek et al., 2016; Rothacher et al., 2016). While parasitism rates were lower in protected clones, this advantage did not translate into higher population growth rate. Overall, these results indicate that although symbiont protection is expressed in field conditions, it is mitigated by multiple factors that may include the rate of parasitism, the range of natural enemies and the costs entailed by harboring the protective symbionts. The work of Smith et al. (2015) was the first to analyze the dynamics of protective endosymbionts in relation with parasitism pressures in natural populations of the pea aphid. Important seasonal shifts in symbiont composition were found, possibly driven by changes in parasitism rates. In particular, the frequency of *H. defensa* was related with parasitoid pressure while the frequency of *R. insecticola* was linked with fungal-induced mortality. Additionally, the aforementioned studies highlighted the influence of protective endosymbionts on diversity and structure of parasitoid communities, with potential consequences on food web functioning (Mclean et al., 2016; Mclean, 2019). More recently, in an elegant experimental evolution study (Hafer-Hahmann and Vorburger, 2020), it was discovered that symbiont diversity could be driven by parasitoid diversity, suggesting potential reciprocal influence of these two communities through their shared aphid hosts.

In the present study, we tested under natural conditions whether the endosymbiont communities of different *A. pisum* biotypes had an actual protective effect on their hosts and whether endosymbiont communities and parasitoid communities associated with the pea aphid complex were linked. To this end, we measured parasitoid pressure on various pea aphid populations, examined whether facultative symbiotic associations responded to parasitoid pressures of varying intensity, and analyzed the relationships between symbiotic and parasitoid communities in terms of both α-diversity (i.e., the species diversity in a given sample) and β-diversity (i.e., the change in diversity of species from one sample to another). In essence, our study is similar to that of Smith et al. (2015) but differs in that we performed field surveys in the native range of *A. pisum* and its natural enemies [and not in the introduced range as in Smith et al. (2015)]. We therefore expect a wider diversity in aphid-endosymbiont–enemy interactions that may drive different patterns of protective symbiont dynamics. To address these issues, we carried out a field survey, spanning twelve dates across the growing season, and encompassing three biotypes of *A. pisum*, each specialized to distinct legume crops and harboring or not protective facultative endosymbionts. For each date and crop, we estimated parasitoid pressure exerted on pea aphids using two proxies, determined the structure of the parasitoid community, recorded the frequencies of each facultative endosymbiont in the pea aphid populations and their distribution among host individuals (i.e., symbiont communities), analyzed how the prevalence of symbiont communities influenced parasitism rate within aphid populations, and measured how diversities of both parasitoid and facultative symbiont communities were related. Finally, because parasitoids may also drive symbiont diversity at the population level (Hafer and Vorburger, 2019), we examined in a subsample of our aphid collections whether variation in the strain of the protective endosymbiont *H. defensa* was related to the intensity of parasitism pressures.

MATERIALS AND METHODS

Sampling Sites

Pea aphid individuals were collected in crop fields of alfalfa (*Medicago sativa*), clover (*Trifolium* sp.), and pea (*Pisum sativum*) in Western France (25 km around Rennes, Brittany) between May and October 2014 during the parthenogenetic phase of the aphid life cycle. Locations of the sampled fields and their coding are given in **Supplementary Figure 1** and **Supplementary Table 1**. When possible, three distant fields per legume species were sampled every two weeks starting on May 7 and ending on October 8, 2014, resulting in 12 sampling dates (**Supplementary Table 2**). As in Brittany pea is harvested by the end of July, no sample was available for this legume in August. By chance, some pea regrowth colonized by aphids were found at beginning of September and were sampled until October 8. While we aimed to monitor the same crop fields during our survey, some fields were sometimes not available because of either the absence of insects or the crop harvesting (see **Supplementary Table 1** for details). From here, one sampling refers to an insects' collection made at one date in one field of a given legume species. Overall, we made a total of 34, 31, and 19 samplings for alfalfa, clover, and pea, respectively. At each sampling, alive pea aphid adults and nymphs were collected using beat sampling from plants separated by approximately 5 m to cover a larger spatial/genetic diversity at the field scale. Aphid mummies (i.e., a dead aphid containing an immature parasitoid) were also collected by view in order to extract parasitoid specimens from field.

Estimates of Parasitism Pressure

To estimate the parasitism pressure exerted on aphid populations, we used two different proxies. For the first one, we collected on average 80 adult aphids at each field sampling (see **Supplementary Table 2** for a precise number of sampled adults per field) that were then installed by batch of ten on plants of broad bean (*Vicia faba*), which is used as a host plant by the different pea aphid biotypes (Peccoud and Simon, 2010), under laboratory conditions (20°C and 16hD:8hN regime). Ten days after the sampling, the rate of parasitism was assessed by calculating the ratio between the number of aphid mummies obtained and the total number of installed aphids for each field per date sample. Overall, 3,074 aphid adults from alfalfa, 1,830 from clover and 1,637 from pea were followed for their parasitism status. A second proxy of parasitism pressure was considered in order to reduce the potential estimate bias caused by aphid resistance against parasitoids. For this second proxy, we sampled the field canopy by using a leaf vacuum cleaner during five seconds at three different sites randomly chosen in a given field. The three canopy samples were kept in a plastic bag separately and stored at −20°C. In the laboratory, pea aphids (all instars combined) were counted and parasitoids were numbered and preserved in 70% ethanol for species identification. The second proxy of parasitism pressure consisted on calculating the ratio between the number of parasitoids belonging to the guild using pea aphids as hosts (Starý, 2006) and the number of pea aphids.

Aphid Parasitoid Communities

To estimate the diversity and structure of parasitoid communities, we considered parasitoid specimens emerging from pea aphid mummies collected from the fields or obtained in laboratory. Those parasitoid specimens were determined at species level (when possible) based on morphological criteria (Müller et al., 1999; Starý, 2006). The present study excluded all hyperparasitoids from the analyses (258 specimens were found). Overall, we determined 744 primary parasitoids from alfalfa, 794 from clover, and 373 from pea, respectively. From all these specimens, six parasitoid taxa were identified: *A. ervi*, *Aphidius eadyi*, *Aphidius avenae*, *Praon volucre*, *Praon barbatum*, and *Aphelinus* sp.

Diversity and Relative Abundance of Facultative Endosymbionts in the Pea Aphid

For each sampling, 60 pea aphid nymphs were collected in order to determine the symbiotic composition in the sampled aphid population. Young aphid nymphs (second-third instars) were chosen instead of older stages to reduce the bias induced by differential mortality caused by parasitoids. After sampling, the aphid nymphs were preserved in 95% ethanol. A random subsample of about 30 aphids per sampling was then inspected for their bacterial symbiont composition (see **Supplementary Table 2** for precise number of aphids inspected per field). A total of 993 of aphids from alfalfa, 831 from clover and 509 from pea were screened for the presence of the seven facultative endosymbionts reported in the pea aphid: *S. symbiotica*, *H. defensa*, *R. insecticola*, *F. symbiotica* (or "Pea Aphid X-type Symbiont"), *R. viridis*, *Rickettsia* sp., and *Spiroplasma* sp. Their presence was detected using species-specific PCR primers according to Peccoud et al. (2014) and the detection of the obligatory bacterial endosymbiont *B. aphidicola* was used as a control for DNA extraction. Note that we also searched for *Wolbachia* because of some reports for the pea aphid in the literature (Russell et al., 2013; Wang et al., 2014; Gauthier et al., 2015) but we did not detect any positive aphids. As in the past, we have conducted extensive surveys of the microbial communities associated with the pea aphid, using either targeted or whole-genome metagenomics approaches (Gauthier et al., 2015; Guyomar et al., 2018), which have confirmed the presence of seven facultative endosymbionts considered in our paper, we are confident that we have surveyed the main fraction of the pea aphid symbionts. For each sampling, we thus estimated the prevalence of these seven facultative heritable endosymbionts and their distribution among aphid individuals. The endosymbiont infection status of each aphid individual (i.e., identity and number of endosymbionts) was then determined.

Seasonal Variation in *Hamiltonella* Strains

In order to monitor possible changes in *H. defensa* strain diversity throughout the cropping season that could result from a variation in the intensity of parasitoid pressure, a subsample of 72 pea aphid nymphs from the alfalfa biotype and infected with *H. defensa* singly (30 aphids) or in coinfection with *F. symbiotica* (42 aphids) was selected among the samplings done before (36 aphids) and after the peak (36 aphids) of parasitoids' activity. All aphid individuals were genotyped at seven microsatellite markers following (Peccoud et al., 2008) in order to distinguish aphid clones based on their multilocus genotype. As the parasitism protection due to *H. defensa* infection is associated with the presence of a bacteriophage (APSE), which encodes toxins potentially responsible for parasitoid development arrest (Degnan and Moran, 2008), phage presence was checked by PCR using the primer pair P3 (forward) 5′-TCGGGCGTAGTGTTAATGAC-3′ (reverse) 5′-TTCCATAGCGGAATCAAAGG-3′ and P51 (forward) 5′-AGGTGCGATTACCCTGTTTG-3′ (reverse) 5′-GATAAAACATCGCCGTTTGC-3′ (Mclean and Godfray, 2015). A multilocus sequence-typing (MLST) was then performed for the characterization of *H. defensa* strains with partial DNA sequences of housekeeping genes accD and murE (Henry et al., 2013). Fragments were amplified by PCR using *H. defensa*-specific primers and cycling conditions described in Henry et al. (2013). Amplicons were sent to Genoscreen for Sanger sequencing. Sequences obtained were cleaned and aligned using Geneious® v.7.1.5 (Kearse et al., 2012). For each sample and each gene (accD and murE) sequences were used to build a phylogenetic tree using the Neighbor Joining method (Tamura-Nei distance). Bootstrap values were computed for each branch node ($N = 1,000$).

Statistical Analysis

Parasitism Proxies and Temporal Variation in Parasitism Pressure

The first analysis consisted in assessing the quality of parasitism pressure estimates by calculating the Spearman correlation coefficient between the two proxies. Then, we analyzed the temporal dynamics of parasitoid activity in the three pea aphid biotypes. From the latter analysis, we found three distinct parasitism periods arbitrarily defined as "pre-parasitism," "maximal parasitism," and "post-parasitism." To test whether the overall parasitism rate differed between the three biotypes, we used Generalized Linear Mixed Model (GLMM) (*glmer* function of the lme4 package (Bates et al., 2015) with a binomial error distribution (logit link function). Both the field ID and session number were fit as random factor to include data substructure.

Assessment of α-Diversity of Parasitoid Communities and Aphid Endosymbiont Infection Statuses

For each combination of parasitism period and pea aphid biotype, the occurrence and co-occurrence of parasitoid species and facultative endosymbionts were visualized using the R package Mondrian (Siberchicot et al., 2016). For each combination biotype/session (i.e., we pooled the data obtained in the fields of a given legume crop during one sampling session), the α-diversity of parasitoid communities was estimated using the Shannon Index that accounts for both abundance and evenness of the species. Also, we calculated the α-diversity of the aphid endosymbiont infection statuses using the Shannon Index by considering the relative abundance of each infection status found in a given biotype/session combination. By using a General Linear Models, we tested whether the parasitism period (i.e., three levels factor: "pre-parasitism," "maximal parasitism," and "post-parasitism") or the pea aphid biotype (i.e., three levels factor: "alfalfa," "clover," and "pea" biotypes) affected the Shannon index of parasitoid communities or endosymbiont infection statuses of aphids. Finally, to test whether α-diversities of both parasitoid communities and aphid endosymbiont infection statuses were linked, a Pearson correlation coefficient between their Shannon indexes was calculated.

Assessment of β-Diversity of Parasitoid Community and Aphid Endosymbiont Infection Statuses

We quantified the dissimilarity between the parasitoid communities or the endosymbiont infection statuses of aphids (β-diversity) between all pea aphid samples using the Bray–Curtis distance. The Bray–Curtis dissimilarities between all pairwise combinations of pea aphid samples were calculated, ordinated following a non-metric multidimensional scaling (nMDS) and represented on a scatter graph where the position of an endosymbiont status or a parasitoid community depended on its distance from all other points in the analysis. Then, the effects of the pea aphid biotype (i.e., three levels factor: "alfalfa", "clover," and "pea" biotypes) and the parasitism period (i.e., three levels factor: "pre-parasitism," "maximal parasitism," and "post-parasitism") on endosymbiont infection statuses of aphids or parasitoid community dissimilarity were tested by performing a permutational multivariate analysis of variance (PERMANOVA) on the Bray–Curtis dissimilarity matrix. Pairwise comparisons between levels of factors were performed using pairwise Adonis tests with Bonferroni corrections (Martinez-Arbizu, 2017). Finally, the correlation between the two dissimilarity matrices (β-diversity of endosymbiont infection statuses of aphids vs β-diversity of parasitoid communities) was calculated by using a Mantel test. The Mantel test, nMDS, and PERMANOVA were implemented using the R package *vegan* (Oksanen et al., 2019), and the graphical representation of the nMDS was generated using the R package *ggplot2* (Wickham, 2016).

Prevalent Symbiotic Associations and Parasitism Rates

To search for a link between symbiont associations and parasitoid pressures, the parasitism rate due to a dominant parasitoid species or all parasitoid species was tested against the frequency of the symbiotic associations in each biotype using General Linear Mixed Models (LMM) (*lme* function of the nlme package; Pinheiro et al., 2020). Note that we did not test all the correlations as some infection states had too few observations. As some fields were considered several times during our survey, the field ID was fit as a random factor to include data dependency. Before each statistical modeling, we checked linearity assumption between the response and the explanatory covariate. In case of linearity departure, we used Generalized Additive Mixed Models (GAMM) (*gamm* function of the mgcv package; Wood, 2017). Model assumptions were verified by plotting residuals versus fitted values for each model.

Parasitism and Variation in *H. defensa* Strain Diversity

To analyze the effect of parasitism pressure on the *H. defensa* strain diversity, we compared the frequencies of each *H. defensa* strain found before and after the parasitism peak in alfalfa fields using a χ^2 test.

RESULTS

Parasitism Proxies and Temporal Variation in Parasitism Pressure

The two proxies of parasitism pressure were positively correlated (Spearman correlation: rho = 0.452, $p < 0.001$) and showed similar dynamics during the survey (**Figure 1**): a low parasitism (<0.3) early in the season, then a maximal parasitism in June

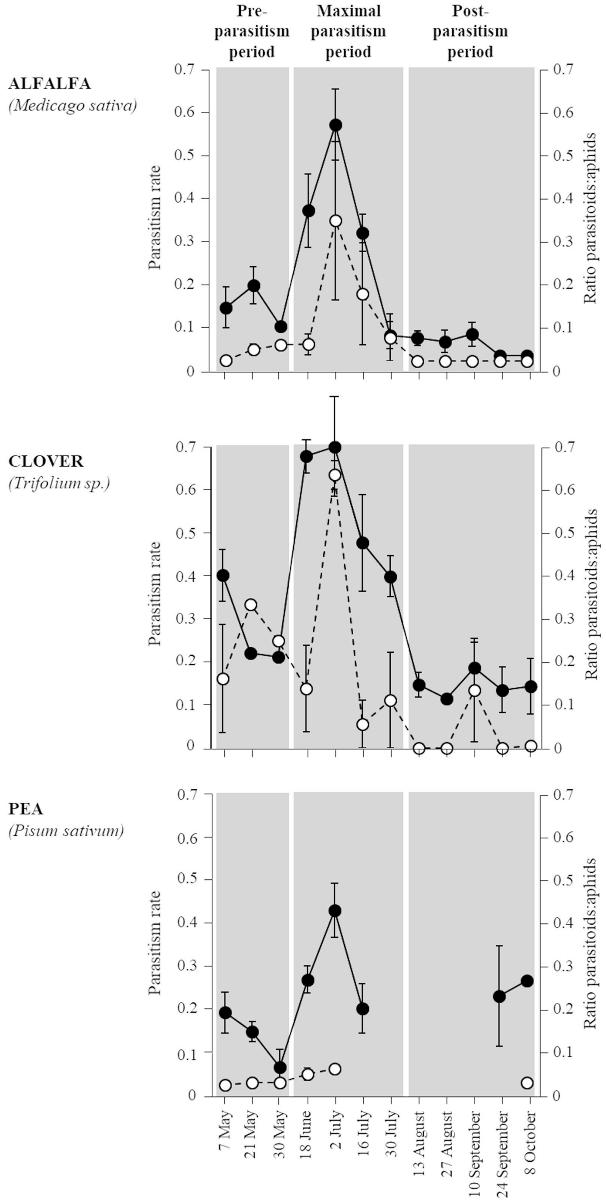

FIGURE 1 | Temporal variation in parasitism pressure in alfalfa, clover, and pea crops estimated using two proxies: parasitism rate (black dots, —) and the ratio of the number of parasitoids/pea aphid individuals (white dots, –). The three shaded areas within each graph correspond to the three parasitism periods arbitrarily defined: "pre-parasitism," "maximum parasitism," and "post-parasitism" periods. Error bars represent standard errors.

and July (up to 0.5) and a decrease below 0.2 from late July to October. The same temporal dynamics of parasitism activity was found in the three legume crops and from these results, three distinct periods were arbitrarily defined as "pre-parasitism," "maximal parasitism" (0.45–0.65 parasitism rate), and "post-parasitism" (**Figure 1**). Overall, the rates of parasitism were the highest in clover fields (30.3%), intermediate in pea (22.4%), and the lowest in alfalfa (15.9%) (GLMM: $\chi^2 = 10.26$, df = 2, $p < 0.005$).

α-Diversity in Parasitoid Communities and Endosymbiont Infection Statuses of Aphids

Overall, *A. ervi* was the dominant parasitoid species in both alfalfa and clover crops (respectively 79.23 and 94.29% of emerging parasitoids over all the season) and the second most abundant species in pea crops (36.44%), after *A. eadyi* (45.48%) and before *A. avenae* (15.16%) (**Figure 2**). Both *P. barbatum* and *Aphelinus* sp. were exclusive to the clover and alfalfa biotypes, with *P. barbatum* accounting for 7.34% of parasitoids in alfalfa crops. The Shannon index in the parasitoid communities varied according to the pea aphid biotype and the parasitism period (LM: interaction term, $F = 4.00$, df = 4, $p = 0.013$). Overall, the α-diversity in parasitoid communities was the lowest in the clover fields, intermediate in alfalfa and the highest in pea. Although the dominant parasitoid species remained the same during the season in alfalfa and clover biotypes, species richness of their parasitoid communities increased throughout the season (**Figure 3**). In pea crops, *A. eadyi* increased in frequency, reaching 82% of parasitoids in post-parasitism period, replacing *A. ervi* as dominant species in the guild and leading to a decline in the Shannon index at this period.

Overall, 94.7% of pea aphids (96.5% for aphids from alfalfa, 94.7% from clover and 91.3% from pea) were infected by at least one facultative bacterial endosymbiont (**Figure 4**). Seven facultative endosymbiont species were detected with prevalence varying strongly between *A. pisum* biotypes. On average aphid individuals harbored 1.8, 1.1, and 1.4 facultative endosymbionts in alfalfa, clover and pea crops, respectively. In alfalfa, coinfection of *H. defensa* with *F. symbiotica* was the most frequent symbiotic association across the field survey (accounting for 43.70% of aphids) followed by *H. defensa* in monoinfection (12.28%). Two other associations involving *H. defensa* were also noted in alfalfa fields: coinfection with *R. insecticola* (8.96%) and triple infection with *R. viridis* and *F. symbiotica* (7.25%). Aphids free of secondary endosymbionts in alfalfa represented only 3.5%. In clover fields, *R. insecticola* in monoinfection represented 74.48% of surveyed aphids while individuals free of any facultative endosymbiont represented only 5.29%. In pea fields, infection with *S. symbiotica* singly (37.25%) or in coinfection with *R. viridis* (37.72%) predominated. Also, 5.89% were singly infected with *R. viridis* and 8.64% were deprived of facultative endosymbiont. The α-diversity in aphid endosymbiont infection statuses varied according to the pea aphid biotype only (LM: $F = 26.13$, df = 2, $p < 0.001$): the Shannon index was the highest in the alfalfa fields, intermediate in pea and the lowest in clover (**Figure 3**).

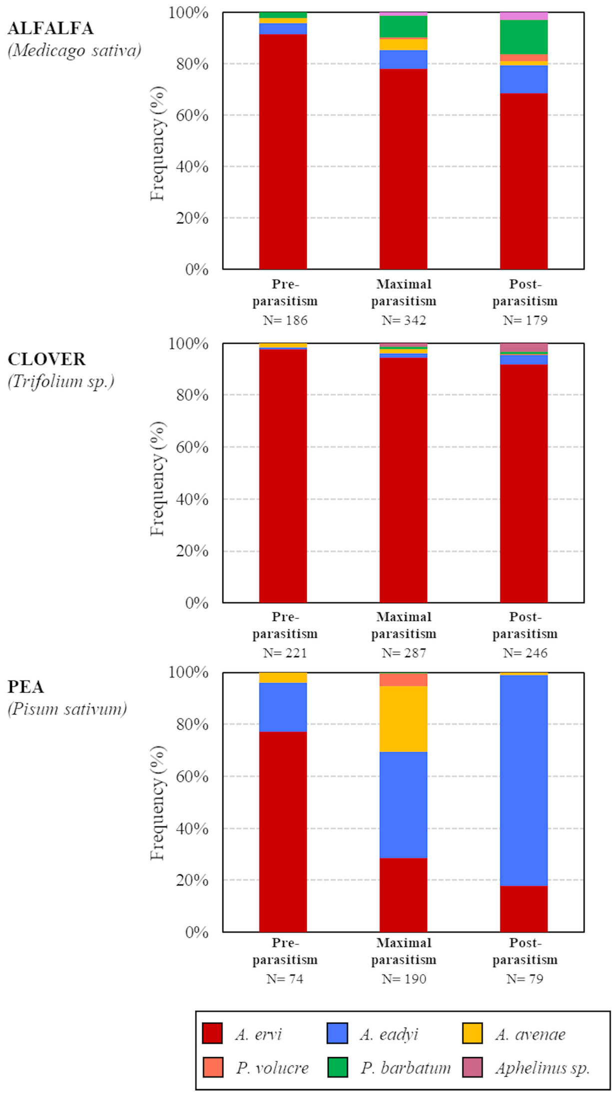

FIGURE 2 | Frequencies of the parasitoid species in the different pea aphid biotypes ("alfalfa," "clover," and "pea") at different parasitism period ("pre-parasitism," "maximal parasitism," and "post-parasitism").

When we analyzed the relationship between the Shannon Index (H) of parasitoid communities and the Shannon Index (H) of endosymbiont infection statuses of aphids, we found a significantly positive covariation (Pearson correlation, $r = 0.499$, $p = 0.004$) in α-diversities (**Figure 3**).

β-Diversity in Parasitoid Communities and Endosymbiont Infection Statuses of Aphids

When the Bray–Curtis distances were calculated between all pairwise combinations of parasitoid communities, we found that they varied significantly in composition between *A. pisum*

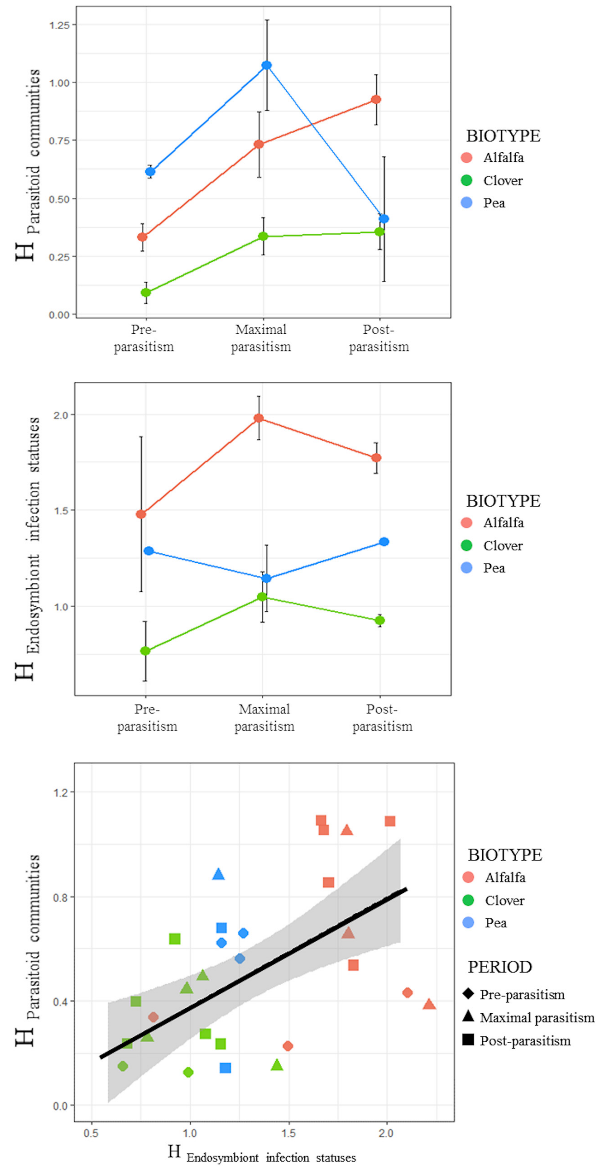

FIGURE 3 | Shannon Index (H) of parasitoid communities and endosymbiont infection statuses, and covariation between both Shannon Indices, considering both the pea aphid biotypes and the parasitism periods.

biotypes (**Figure 5**). PERMANOVA detected a significant effect of aphid biotype on the parasitoid community assemblages ($F = 3.84$, df = 2, $p = 0.003$) and this factor accounted for 21% of the variance in the data. Pairwise comparisons between biotypes showed that the structure of parasitoid communities emerging from pea crops differed from the two other crops. No temporal dynamics in β-diversity in parasitoid communities was found (PERMANOVA, period effect: $F = 1.565$, df = 2, $p = 0.150$).

The Bray–Curtis dissimilarities between endosymbiont infection statuses of aphids presented highly contrasted values and showed that they differed strongly between biotypes while being stable during the season within each biotype (**Figure 5**). PERMANOVA confirmed this pattern since the biotype effect on infection statuses dissimilarity was highly significant ($F = 79.08$, df = 2, $p = 0.001$). This factor accounted for 83% of the variance in the data. No temporal dynamics in β-diversity of endosymbiont infection statuses of aphids was found (PERMANOVA, period effect: $F = 740$, df = 2, $p = 0.596$), confirming their stability throughout the cropping season.

A positive correlation between the two dissimilarity matrices was found (Mantel test: $z_M = 0.186$, $p = 0.002$, see **Supplementary Figure 2**). The dissimilarity between parasitoid communities was therefore correlated with the dissimilarity between endosymbiont infection statuses of aphids.

Parasitism Rates of Pea Aphids in Relation With Their Symbiotic Associations

In both clover and pea crops, the parasitism rate of all parasitoid species or of the dominant ones did not vary according to the prevalence of the most frequent symbiotic associations (**Table 1** and **Supplementary Figure 3**). For the alfalfa biotype, while the prevalence of *H. defensa* in monoinfection did not influence the parasitism rates (**Table 1** and **Supplementary Figure 3**), the coinfection with *H. defensa* and *F. symbiotica* had a significantly negative effect on parasitism rates: when pea aphids from alfalfa fields presented high prevalence of coinfection with both protective endosymbionts, the parasitism rate of *A. ervi* and the overall parasitism rate declined although non-linearly (**Table 1** and **Figure 6**).

Patterns of Temporal Variation in *H. defensa* Strain Diversity

A subsample of 72 *A. pisum* from different alfalfa fields and infected with *H. defensa* singly or co-infected with *H. defensa* and *F. symbiotica* was analyzed to characterize the strain variation of *H. defensa*. Among the pea aphid individuals, 36 aphids were selected before the peak of parasitism and 36 aphids after the peak. The APSE phage associated with *H. defensa* was consistently detected in all aphids. Seven genetically different strains of *H. defensa* were characterized (**Table 2**). Two main haplotypes, representing 73% of the *H. defensa* strains, dominated aphid populations before and after parasitism peak. A greater diversity of strains was observed after the peak of parasitism (0.11 before and 0.19 after) and a similar pattern was observed for the aphid clonal diversity (0.58 before and 0.64 after). However, the frequencies of *H. defensa* strains did not differ significantly before and after the parasitism peak in alfalfa fields (χ^2 test: $\chi^2 = 6.65$, df = 6, $p = 0.645$).

DISCUSSION

Since some microbial symbionts confer a protection against natural enemies to their hosts that can potentially alter food web interactions (Hafer and Vorburger, 2019; Mclean, 2019), our objectives were to test under natural conditions whether the parasitism rate of different *A. pisum* biotypes depended

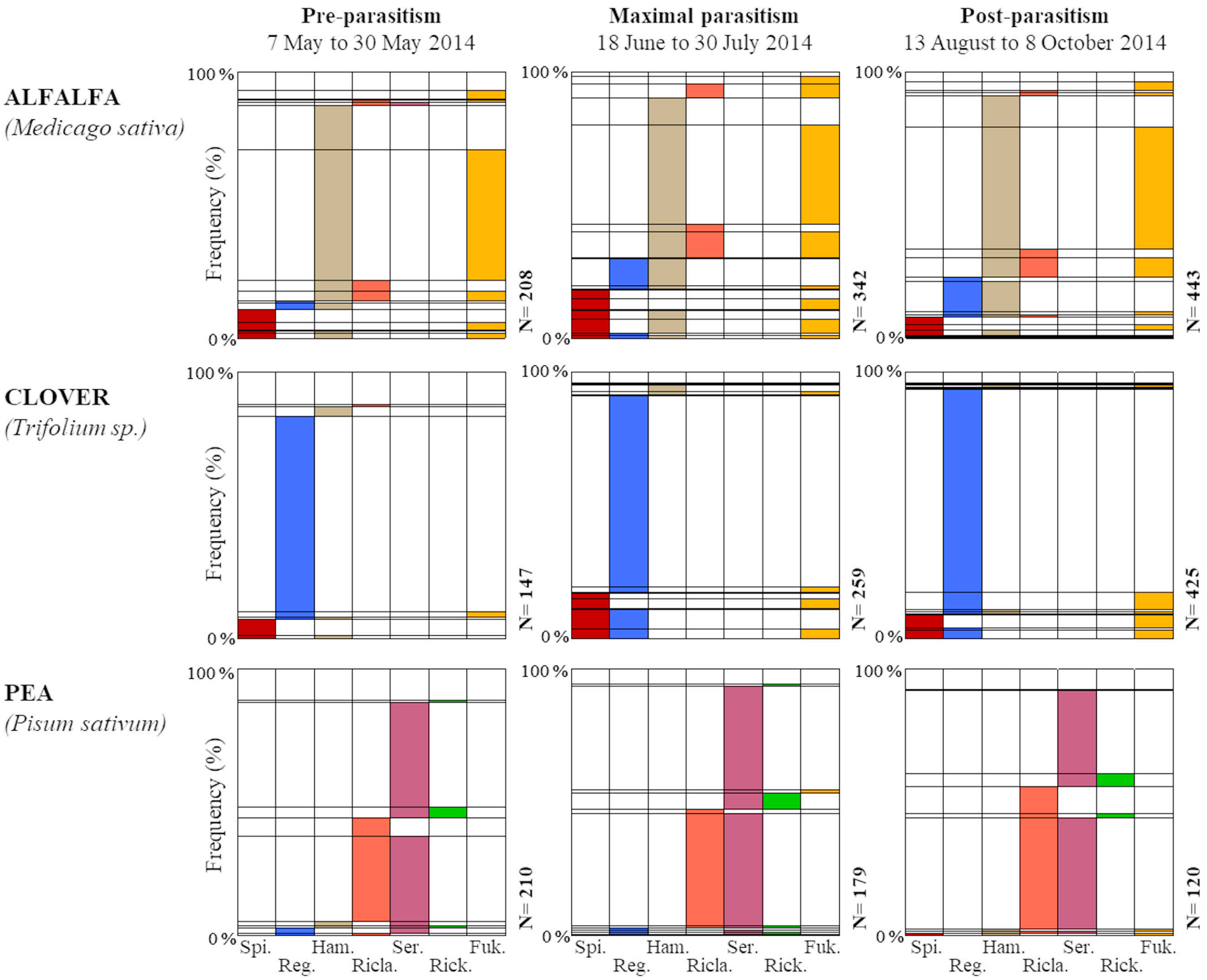

FIGURE 4 | The diversity and relative abundance of endosymbiont infection statuses of pea aphids in relation with aphid biotype ("alfalfa," "clover," and "pea") and parasitism period ("pre-parasitism," "maximal parasitism," and "post-parasitism"). For each column, the colored area corresponds to the percentage of aphid individuals harboring the corresponding symbiont species. When colored areas are present in several columns, it indicates a co-infection with multiple symbiont species. (Spi: *Spiroplasma* sp.; Reg: *Regiella insecticola*; Ham: *Hamiltonella defensa*; Rica: *Rickettsiella viridis*; Ser: *Serratia symbiotica*; Rick: *Rickettsia* sp.; Fuk: *Fukatsuia symbiotica*).

on their endosymbiont communities and whether symbiont communities and parasitoid communities associated with pea aphids were related. We found that facultative endosymbiont communities were highly structured by biotype and stable in time while the parasitoid communities showed moderate differences between pea aphid biotypes and some change in structure over time. At the level of the pea aphid complex, we revealed a correlation between diversities (i.e., α- and β-diversities) of endosymbiont infection statuses of aphids and parasitoid communities. Interestingly, we found a negative correlation between the prevalence of *H. defensa* and *F. symbiotica* in co-infection and the intensity of parasitoid pressure in the alfalfa biotype, confirming in field conditions the protective effect of this symbiotic combination.

The strong associations between endosymbiont communities and pea aphid biotypes are not novel and have been recurrently reported in various studies (Simon et al., 2003; Ferrari et al., 2004; Russell et al., 2013; Henry et al., 2015; Smith et al., 2015). Beyond the absence or the presence of each facultative bacterial endosymbiont in natural populations, each pea aphid biotype presented dominant symbiotic associations during the season: more than 80% of the aphids feeding on clover were singly infected with *R. insecticola*; in alfalfa, the infection with *H. defensa* alone or in coinfection with another facultative symbionts predominated the natural populations; and almost all aphids specialized on pea harbored *S. symbiotica* singly or in coinfection with *R. viridis*. Several hypotheses have been put forward for such differences in symbiotic associations and involve either ecological filters exerting selective pressure on aphid symbioses, symbiont-symbiont interactions or the effect of drift on symbiont associations (Mathé-Hubert et al., 2019).

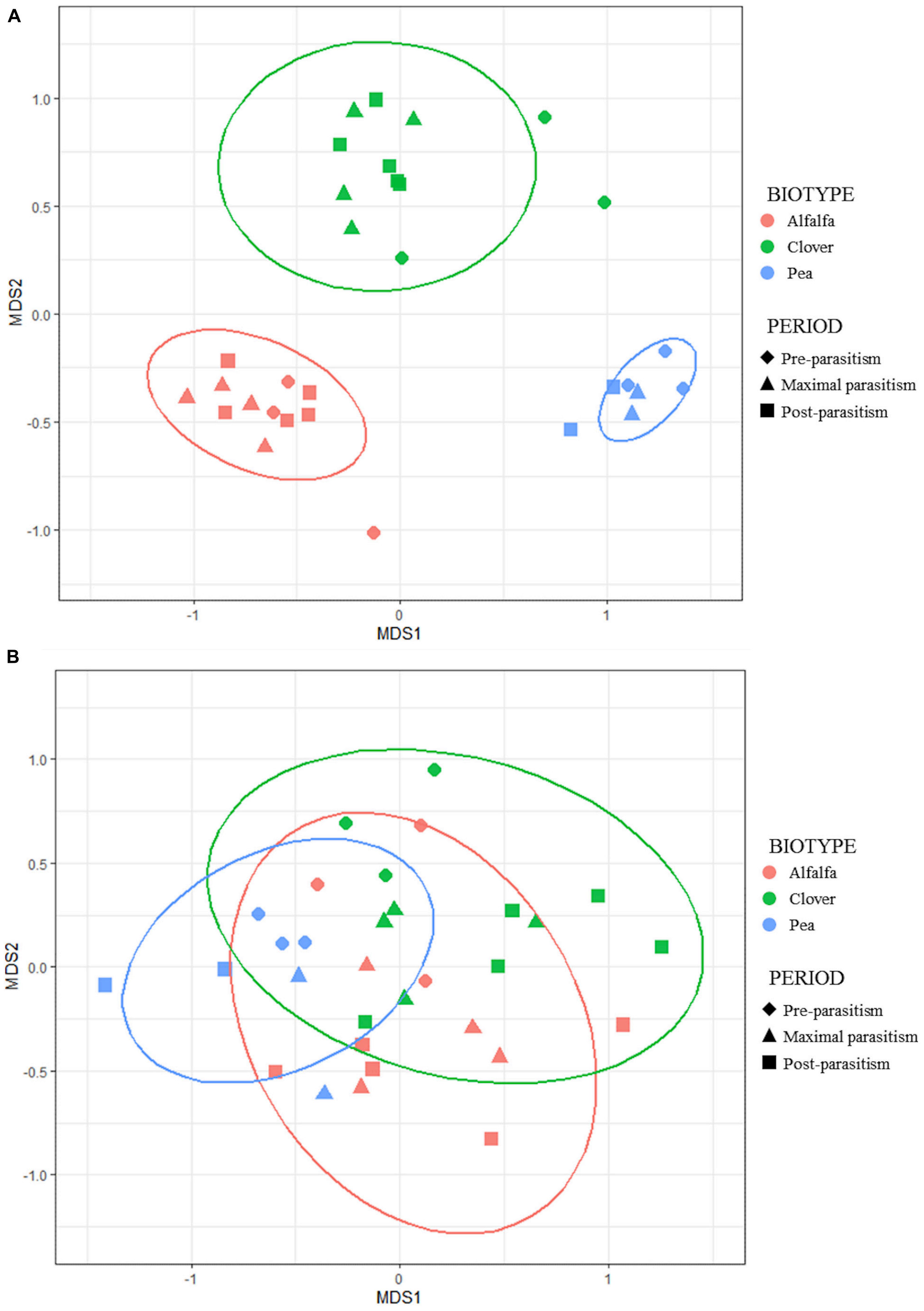

FIGURE 5 | Non-metric MDS ordination plot comparing **(A)** endosymbiont infection statuses and **(B)** parasitoid communities from different pea aphid samples. Each data point represents the **(A)** symbiont/**(B)** parasitoid community identified from aphid samples collected at one date for a given biotype. The shape of the dots refers to the parasitism periods. The Bray-Curtis dissimilarity index was used to rank distances calculated using the abundance community data. Stress of the nMDS: **(A)** symbiont infection status = 0.083; **(B)** parasitoid community = 0.164.

An essential step for linking facultative endosymbionts with parasitoid communities was to reliably assess the parasitism pressure exerted on aphid populations. Here, we used two proxies to reduce the bias of underestimating the parasitoid pressure by measuring only the mummification rate (Oliver et al., 2003). For the three biotypes, both parasitoid pressure proxies were highly

TABLE 1 | Results of statistical models (LMM: General Linear Mixed Models; GAMM: Generalized Additive Mixed Models) testing the effect of the most prevalent symbiotic associations on parasitism rate (for the dominant parasitoid species and all parasitoids).

			Response		
Biotype	**Covariate**		**Parasitism rate of *Aphidius ervi***	**Parasitism rate of *Aphidius eadyi***	**Parasitism rate of all parasitoid species**
Alfalfa	Prevalence of *H. defensa in* monoinfection	Model type	LMM		LMM
		p-value	0.475		0.613
	Prevalence of *H. defensa* and *F. symbiotica* coinfection	Model type	GAMM		GAMM
		p-value	**0.034**		**0.017**
Clover	Prevalence of *R. insecticola* in monoinfection	Model type	LMM		LMM
		p-value	0.539		0.561
Pea	Prevalence of *S. symbiotica* in monoinfection	Model type	LMM	LMM	LMM
		p-value	0.161	0.492	0.846
	Prevalence of *S. symbiotica* and *R. viridis* coinfection	Model type	LMM	LMM	LMM
		p-value	0.275	0.237	0.054

Bold values refer to significant effect.

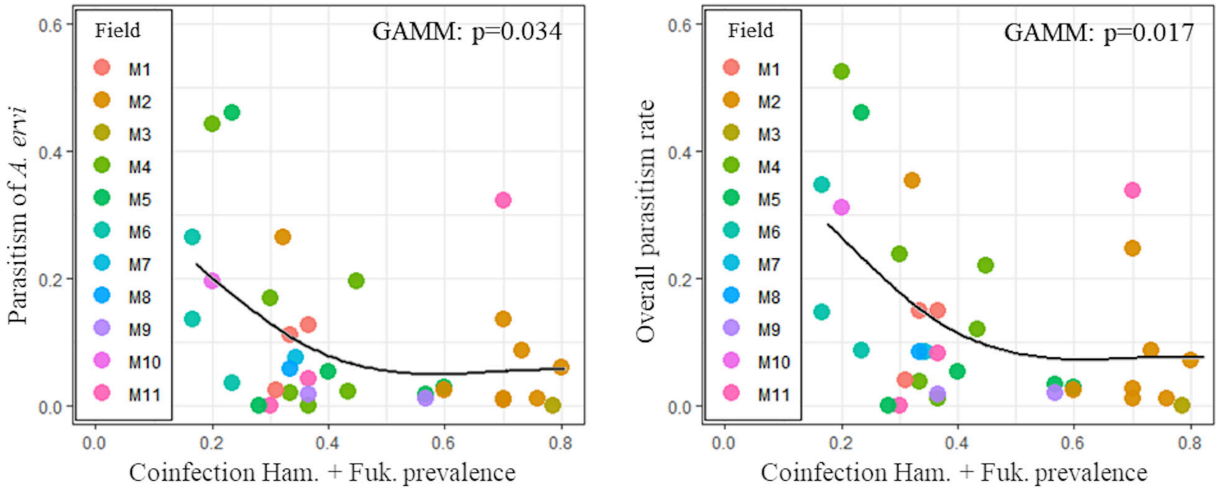

FIGURE 6 | Relationship between *Hamiltonella defensa/Fukatsuia symbiotica* coinfection and parasitism rate (assessed for the dominant parasitoid species, *Aphidius ervi*, and for all parasitoids) in pea aphids from alfalfa fields. ID refers to the alfalfa field. Solid line represents the predicted values from the fitted generalized additive mixed models.

positively correlated and showed the same temporal dynamics, indicating that the actual parasitoid pressure exerted on field populations of the pea aphid in different ecological situations was reliably assessed. Overall, the parasitism pressure over time presented a single peak of high parasitoids' activity in early July. This temporal dynamic was similar between alfalfa, clover and pea biotypes but varied quantitatively: the pressure exerted by parasitoids was the highest in clover, intermediate in pea and the lowest in alfalfa crops. One explanation for these quantitative differences could be that clover is typically grown in a more complex agricultural mosaic than the other crops, which would favor biological regulations. An alternative could be that clover is more attractive to parasitoids.

Despite these temporal dynamics and inter-biotype variations in parasitism pressure, the relative abundance of the endosymbiont infection statuses of aphids changed very little over the season. These results contradict those obtained in a previous study done on both alfalfa and clover biotypes in the United States (Smith et al., 2015). In this earlier work, considerable seasonal shifts were indeed observed in the frequencies of endosymbionts, especially in Pennsylvania fields. This difference may result from the fact that this previous work considered each symbiont species individually while we analyzed them as communities. It could also be due to differences in population and community composition of endosymbionts and parasitoids, which exist between the native and introduced range of *A. pisum*. For example, we found six species of parasitoids in our survey while Smith et al. found only two. In addition, while there is evidence of some protection against parasitoids conferred by *F. symbiotica* in western Europe (Heyworth and Ferrari, 2015; Leclair et al., 2016), this does not appear to be the case in the United States (Doremus and Oliver, 2017), suggesting

TABLE 2 | Aphid clonal diversity, *H. defensa* strain diversity and haplotypes in the "pre-parasitism" and "post-parasitism" periods for pea aphids collected in alfalfa fields.

	Pre-parasitism	**Post-parasitism**
	36 aphids (15 Mono/21 Co)	36 aphids (16 Mono, 20 Co)
***A. pisum* genotypic diversity**	21 genotypes 0.58	23 genotypes 0.64
***H. defensa* strain diversity**	4 strains 0.11	7 strains 0.19
Strain code	**Frequency (Mono/Co)**	**Frequency (Mono/Co)**
Strain 1	2.8% (1/0)	2.8% (1/0)
Strain 2	22.2% (8/0)	30.6% (11/0)
Strain 3	0 (–/–)	2.8% (1/0)
Strain 4	0 (–/–)	2.8% (1/0)
Strain 5	0 (–/–)	2.8% (0/1)
Strain 6	52.8% (5/14)	41.7% (0/15)
Strain 7	22.2% (1/7)	16.7% (2/4)

Aphids were either singly infected with H. defensa or coinfected with F. symbiotica. (Mono/Co: refers to the number of monoinfected/coinfected aphids that harbored the given strain).

strain differences between countries. The temporal stability of symbiotic associations we observed suggests a good fidelity in vertical transmission of symbiont combinations in natural conditions, confirming previous results on field estimates of maternal transmission rate of pea aphid endosymbionts (Rock et al., 2018). This seasonal stability was also observed at the symbiont population level as we did not detect differences in the frequencies of *H. defensa* strains throughout the alfalfa growing season. Temporal fluctuations in symbiotic associations may, however, occur on a longer time scale (between years or beyond), although the same symbiotic associations have been found repeatedly in pea aphid populations in independent studies and in different years (Henry et al., 2013; Rock et al., 2018; Mathé-Hubert et al., 2019).

Interestingly, we found a negative non-linear relationship between the frequency of co-infections with *H. defensa* and *F. symbiotica* and the parasitism rate (estimated for all parasitoid species or for *A. ervi* alone). Surprisingly, the single infection with *H. defensa* alone did not appear related to the rate of parasitism. However, such results are consistent with observations made in controlled conditions. Indeed, it has been shown that the protection conferred by *H. defensa* alone varies greatly according to the host and genotypes of *H. defensa* (Oliver and Higashi, 2019), whereas co-infections with *F. symbiotica* give the aphids a high or even total protection against parasitism (Guay et al., 2009; Leclair et al., 2016), but see Doremus and Oliver (2017). Overall, this endosymbiont protection may be responsible for the lower parasitism rate (between 30 and 50% reduction) observed in the alfalfa biotype compared to the two other biotypes. Negative correlations between protective endosymbionts in pea aphids and parasitoid rates have been reported in earlier field studies (Smith et al., 2015; Hrcek et al., 2016; Rothacher et al., 2016). Our study and the previous ones thus confirm the effectiveness of symbiont-mediated protection in natural environments exploited by complex and diverse parasitoid communities.

One explanation given for the absence of *H. defensa* in clover and pea biotypes is because of a lower parasitoid pressure in natural populations of these biotypes (Oliver et al., 2008). However, our results showed that parasitoid wasps severely attacked these two pea aphid biotypes, with parasitism rates up to 70%. Given this parasitism pressure, an alternative protection against parasitoids may exist in these aphid populations; for instance, another facultative endosymbiont could confer an alternative protection to *H. defensa*. However, we did not observe a relationship between endosymbiont communities in the pea and clover biotypes and the rate of parasitism, although *S. symbiotica* (predominant in the pea biotype) and a strain of *R. insecticola* (the dominant symbiont in the clover biotype) have been reported to confer some parasitoid resistance in several laboratory studies (Oliver et al., 2003; Vorburger et al., 2010; Heyworth and Ferrari, 2015). Also, we showed in controlled conditions that the endosymbiont communities that dominated clover and pea biotypes in our field survey conferred very limited protection to *A. ervi* (Leclair et al., pers. obs.). Other ecological and non-ecological factors would better explain the prevalent symbiotic associations observed in these biotypes as discussed earlier (Mathé-Hubert et al., 2019). For example (Smith et al., 2015) suggested that the high prevalence of *R. insecticola* in the clover biotype was due to the strong incidence of fungal pathogen-induced mortality in clover fields, which would select this symbiont because of the fungal protection it confers (Scarborough et al., 2005). More field works are needed to assess the influence of environmental factors on symbiont composition of the various pea aphid biotypes.

Two earlier field studies showed that protective symbionts could influence the third trophic level by shaping the structure of parasitoid community attacking aphid populations (Hrcek et al., 2016; Rothacher et al., 2016). In addition, an experimental evolution study showed that parasitoid diversity could maintain diversity in protective symbionts (Hafer-Hahmann and Vorburger, 2020). Our study is in line with this body of work linking symbiont and parasitoid diversities. Indeed, we found that both α- and β-diversities of symbionts and parasitoids were correlated, suggesting some interactions between these two communities through their aphid hosts or other environmental factors (i.e., local habitats).

Given the high prevalence of *H. defensa* in the alfalfa biotype and the negative correlation we found only between *H. defensa*-*F. symbiotica* co-infection and parasitoid pressure, one might have expected to find a strong effect of the symbiotic protection found in the alfalfa biotype on the community structure of parasitoids attacking the pea aphid. However, we showed that parasitoid communities varied little between the clover and alfalfa biotypes but more between these two and the pea biotype. *A. ervi* is often cited as the parasitoid species exerting the highest pressure on pea aphid populations (Kavallieratos et al., 2004; Smith et al., 2015). The alfalfa biotype was predominantly attacked by this parasitoid species despite the high prevalence of protective symbioses in this biotype. Previous works showed an evolutionary potential of parasitoids to counteract the symbiont protection conferred by *H. defensa* (Dion et al., 2011; Rouchet and Vorburger, 2014); some genotypes of *A. ervi* may thus have evolved virulence factors against *H. defensa* mediated protection in the wild (Dennis et al., 2020). Our results showed that the parasitoid community associated with the pea biotype strongly differed from the two others, in particular by the abundance of *A. eadyi* and *A. avenae*. Ferrari et al. (2004) have showed that *H. defensa* could provide resistance to *A. eadyi* in *A. pisum* populations. However, this endosymbiont was not present in the pea biotype in our survey and it is not known whether *S. symbiotica* or *R. viridis*, which, on the other hand, were both well represented in *A. pisum* from pea, confer resistance to *A. eadyi*. More work is needed to test whether the most prevalent symbiotic combination in each biotype confers an optimal protection to the corresponding parasitoid communities.

In conclusion, this study showed a temporal stability in symbiont populations and communities, in sharp contrast with a strong seasonality in parasitoid activities. This weak response of symbiont communities to parasitoid pressures could be explained by the limited costs of carrying protective symbionts on this timescale. However, further field surveys are needed to determine whether the composition in protective symbionts is maintained over longer period or is rather driven by an ecological-evolutionary dynamics resulting from selection for or against resistance, as suggested earlier (Smith et al., 2015) and recently demonstrated in a manipulated agricultural system (Ives et al., 2020). We also showed that the three pea aphid biotypes, despite their distinct endosymbiont composition, were exposed to similar range of parasitism pressures, suggesting that other protective alternatives than hosting *H. defensa* alone or with *F. symbiotica*, and involving symbiont or host mechanisms, may be used by the various biotypes of the pea aphid complex, which merits further work. Finally, we detected a link between communities of parasitoids and symbionts, suggesting interactions through shared resources or other environmental filters. The study of other communities of natural enemies of aphids (e.g., predators, pathogens) could reveal more such links and allow to better measure the importance of symbionts in food webs.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed in this article are available in Zenodo at https://doi.org/10.5281/zenodo.4548282.

AUTHOR CONTRIBUTIONS

ML, J-CS, and YO conceived and designed the field works and experiments and managed the field samplings. FM and ML performed both molecular studies and symbiont detection. CB performed species identification. YO analyzed the data. YO and J-CS contributed to the supervision of this study. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.618331/full#supplementary-material

Supplementary Figure 1 | Location of the sampled fields. The color and the shape of the dots represent the legume species ("Alfalfa," "Clover," "Pea") on which pea aphids and parasitoids were collected.

Supplementary Figure 2 | Bray-Curtis dissimilarity matrix of (a) endosymbiont infection statuses and (b) parasitoid communities in the different pea aphid biotypes ("Alfalfa," "Clover," "Pea") at different parasitism period (P1: "pre-parasitism," P2: "maximum parasitism," and P3: "post-parasitism").

Supplementary Figure 3 | Effect of the most prevalent symbiotic associations on the parasitism rate (dominant parasitoid species and all parasitoid together) in pea aphid individuals from the alfalfa, clover and pea biotypes. ID refers to the field crop sampled. For all these panels, no significant relationship was found.

Supplementary Table 1 | Sampling information: code, location and sampling frequency of the fields considered in our study. For a given legume crop, the field code assignment depends on the geographical location: we numbered from the westernmost to the easternmost field.

Supplementary Table 2 | Sampling information: dates, field identity (i.e. *field*), number of aphid nymphs used to assess symbiotic composition (i.e. *nymph*), number of adult aphids kept to measure parasitism rate (i.e. *adult*) and its value for each sampling (i.e. *para*) (when "-", no sampling).

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Climate Change Leads to a Reduction in Symbiotic Derived Cnidarian Biodiversity on Coral Reefs

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Symbiotic relationships enable partners to thrive and survive in habitats where they would either not be as successful, or potentially not exist, without the symbiosis. The coral reef ecosystem, and its immense biodiversity, relies on the symbioses between cnidarians (e.g., scleractinian corals, octocorals, sea anemones, jellyfish) and multiple organisms including dinoflagellate algae (family Symbiodiniaceae), bivalves, crabs, shrimps, and fishes. In this review, we discuss the ramifications of whether coral reef cnidarian symbioses are obligatory, whereby at least one of the partners must be in the symbiosis in order to survive or are facultative. Furthermore, we cover the consequences of cnidarian symbioses exhibiting partner flexibility or fidelity. Fidelity, where a symbiotic partner can only engage in symbiosis with a subset of partners, may be absolute or context dependent. Current literature demonstrates that many cnidarian symbioses are highly obligative and appear to exhibit absolute fidelity. Consequently, for many coral reef cnidarian symbioses, surviving changing environmental conditions will depend on the robustness and potential plasticity of the existing host-symbiont(s) combination. If environmental conditions detrimentally affect even one component of this symbiotic consortium, it may lead to a cascade effect and the collapse of the entire symbiosis. Symbiosis is at the heart of the coral reef ecosystem, its existence, and its high biodiversity. Climate change may cause the demise of some of the cnidarian symbioses, leading to subsequent reduction in biodiversity on coral reefs.

Keywords: symbiosis, mutualism, coral, octocoral, sea anemone, anemonefish, coral reef fish, jellyfish

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INTRODUCTION

In 1878, de Bary defined symbiosis as the "living together of differently named organisms" (translated in Oulhen et al., 2016). "Living together" can lead to organisms existing in habitats where they may not survive if not for the symbiosis, increasing the biodiversity in that habitat. "Living together" can also create novel biological entities, a consortium of organisms. When symbionts are within (endosymbionts) or on (ectosymbionts) the tissue of the host, the host-symbiont(s) entity, the holobiont (Margulis, 1991), may exhibit novel physiological and ecological attributes. Although some debate the evolutionary utility of this term (Skillings, 2016), numerous holobiont characteristics differ from the properties of the organisms in isolation (reviewed in Goulet et al., 2020). Furthermore, host-symbiont genotypic combinations generate diverse holobionts that differ physiologically and ecologically from one another (Goulet et al., 2005). Therefore, different symbioses and holobionts can lead to increased variability within and between host species, both contributing to overall biodiversity in an ecosystem.

Symbioses are ubiquitous in terrestrial and aquatic habitats, from the tube worm-sulfur oxidizing bacteria in deep sea hydrothermal vents (Cavanaugh et al., 1981), to the fungal-blue green algal symbiosis in arctic lichens (Crittenden and Kershaw, 1978). In some habitats, symbioses are key to the existence of an entire ecosystem, as occurs on coral reefs. In this review, we focus on the symbioses between members of the phylum cnidaria (e.g., scleractinian corals, octocorals, sea anemones, jellyfish) and their symbionts. These symbioses shape biodiversity on coral reefs, but with climate change, these pivotal symbioses may falter, leading to a loss of biodiversity. The structural complexity of coral reefs may then be reduced (Rossi et al., 2019), detrimentally affecting other organisms in the ecosystem. The degree of reliance on the partners within cnidarian symbioses, and their fidelity, may potentially lead to their demise.

The Symbiotic Continuum and Its Influences on Biodiversity

de Bary's definition of symbiosis described a phenomenon, not its ramifications. Subsequently, attributes were assigned to symbioses based on benefits and costs, from mutualism (both partners benefit), commensalism (one partner benefits while the other is neither harmed nor gains a benefit), parasitism (one partner benefits while the other is harmed) (Saffo, 1993), to the recently added amensalism (one partner is harmed while the other is neither harmed nor gains a benefit) (Apprill, 2020). We adhere to the subdivisions of symbiosis (Saffo, 1993), as opposed to treating symbiosis as a synonym for mutualism (Douglas, 2010). Although the symbiotic states may appear as discrete boxes (**Figure 1A**), or a sliding continuum (**Figure 1B**; Lesser et al., 2013), neither portrayal captures the complexities of symbioses, since the former assumes that a symbiosis is constrained into one category while the latter implies that a mutualism needs to transition into a commensalism or amensalism before it may morph into a parasitism, and vice versa. Alternatively, symbiotic states may oscillate from one to another (**Figure 1C**) based on the context. Changing environmental conditions, which often occur in conjunction with climate change, may be an impetus for such symbiotic shifts. Conversely, if the symbiotic partner(s) rely on a certain symbiotic state, environmental changes may lead to symbiosis breakdown and even partner death (**Figure 1C**).

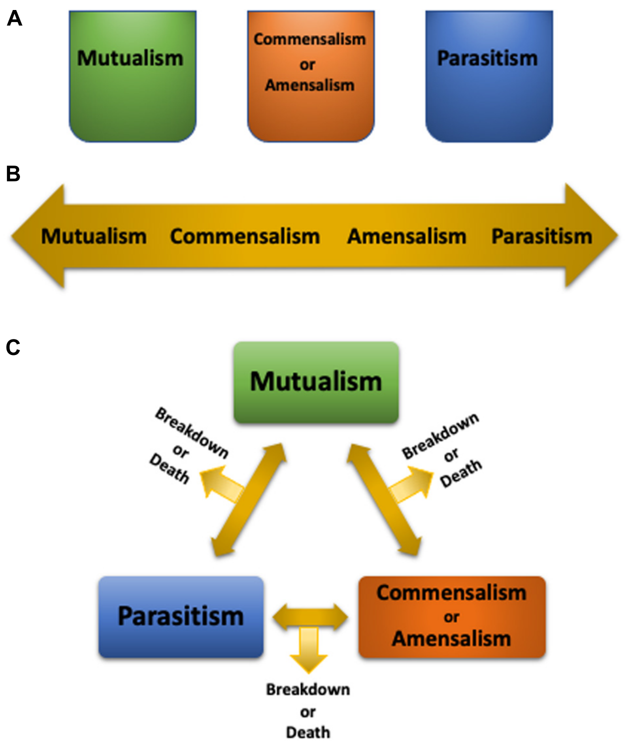

FIGURE 1 | Depiction of symbiosis states as discrete separate entities **(A)**, a continuum **(B)**, or as oscillating between one state to another based on context **(C)**.

The Benefits Brought About by Cnidarian Symbioses on Coral Reefs

The core of the coral reef ecosystem is the mutualism between members of the phylum cnidaria and unicellular dinoflagellate algae (family Symbiodiniaceae). Symbiodiniaceae transfer some of their photosynthetically fixed carbohydrates to the cnidarian host (Muscatine and Porter, 1977), contributing as much as 143% of the coral's maintenance respiration (Muscatine et al., 1984). But, Symbiodiniaceae differ in their metabolic contribution to their hosts, with some Symbiodiniaceae even acting as parasitic at some stage in host ontogeny (Banaszak et al., 2013) or under different environmental conditions (Lesser et al., 2013), reiterating symbiotic state oscillation (**Figure 1C**). The cnidarian host, through their pigments, may enhance Symbiodiniaceae photosynthesis (Schlichter et al., 1994), even in light limited mesophotic depths (Schlichter et al., 1986). Scleractinian corals' calcification is increased by Symbiodiniaceae, thereby enhancing coral growth which then affects the overall coral reef structure (Goreau and Goreau, 1959; Pearse and Muscatine, 1971), influencing the organisms that rely on the reef for habitat and/or food. From the host's nitrogenous wastes, Symbiodiniaceae gain a steady nitrogen source in an oligotrophic habitat (Muscatine and D'Elia, 1978). As endosymbionts, the Symbiodiniaceae exposure to environmental parameters such as ultraviolet radiation and thermal stress is attenuated (Kawaguti, 1944; Shibata, 1969; Goulet et al., 2005).

In addition to Symbiodiniacece, cnidarians contain bacteria and Archaea (reviewed in Knowlton and Rohwer, 2003; Bourne et al., 2016; Peixoto et al., 2017). Bacteria may provide a source of sulfur, nitrogen and carbon, and protect cnidarians via antibiotics, inhibitors, and out competing other microbes (Shashar et al., 1994; reviewed in Knowlton and Rohwer, 2003; McDevitt-Irwin et al., 2017; Peixoto et al., 2017). From cnidarian mucus, bacteria can obtain wax ester and triglycerides (Johannes, 1967; Benson and Muscatine, 1974). Cnidarians may also obtain benefits from Archaea aiding in nitrogen cycling, gene transfer from viruses, and fungal anti-microbial activity and contribution to the carbon and nitrogen cycles (reviewed in Knowlton and Rohwer, 2003 and Peixoto et al., 2017). On the other hand, viruses, bacteria, and fungi may harm the symbioses and are associated with cnidarian diseases (Richardson, 1998; Mera and Bourne, 2018). The data on non-Symbiodinaceae microbiome members are predominantly limited to identifying the entities involved, with a paucity of data on their role and function (Bourne et al., 2016). Furthermore, despite the tight coupling of scleractinian coral-bacteria phylogenies (Pollock et al., 2018), some argue that the relationship with bacteria should not be put in symbiotic terms (Mushegian and Ebert, 2016). Hence, although we recognize that bacteria, Archaea and other entities exist in cnidarians, given the current limited knowledge of their relationships, we do not discuss them in the subsequent sections.

Cnidarians also form symbioses with macro organisms such as bivalves (Mokady et al., 1998), crabs, shrimp (Glynn, 1980), and fish (Collingwood, 1868; Mariscal, 1970; Liberman et al., 1995; Howell et al., 2016) that continuously dwell within and between cnidarian branches/tentacles. Macro organisms can provide protection to their cnidarians; crabs and shrimp nip at the tube feet of the coral predator star fish *Acanthaster planci* (Glynn, 1980), and resident fishes chase away predators (Mariscal, 1970; Chase et al., 2014). Cnidarian dwelling fish remove sedimentation and debris from their hosts (Mariscal, 1970; Liberman et al., 1995). In addition, boring bivalves (Mokady et al., 1998), shrimp (Spotte, 1996), and fish (Roopin and Chadwick, 2009) provide their nitrogenous waste to the symbiosis. Coral dwelling fish's movements within their host contribute to coral oxygenation as proposed in Liberman et al. (1995) and demonstrated in Goldshmid et al. (2004). Many cnidarian dwelling macro organisms rely on their host cnidarian for their habitat and protection (Mariscal, 1970) in addition to utilizing the coral mucus, and occasionally coral tissue for nutrition.

Obligate vs. Facultative Cnidarian Symbioses

In most of the symbioses with coral reef cnidarians, at least one of the partners obligatorily engages in the symbiosis in order to survive. For example, many cnidarians obligatorily host Symbiodiniaceae, and some jellyfish even require Symbiodiniaceae infection to enter a stage in their life cycle (Ohdera et al., 2018; Djeghri et al., 2019). Conversely, Symbiodiniaceae genetic signatures were detected from open water samples (Decelle et al., 2018), and Symbiodiniaceae in feces of fish and a nudibranch that fed on anemones were viable (Muller Parker, 1984), indicating that the mutualism may not be reciprocally obligative. Perturbations can lead to a reduction in algal numbers and/or chlorophyll content per Symbiodiniaceae cell within cnidarian hosts, termed coral bleaching, which may lead to cnidarian death (Glynn, 1996). The same environmental perturbations, however, affect cnidarian symbioses differently, resulting in within (Berkelmans and van Oppen, 2006; Goulet et al., 2008; Sampayo et al., 2008) and between (Marshall and Baird, 2000; Goulet et al., 2008) species differences in susceptibility and survival. Coral species that can offset the loss of nutrients, brought about from the reduction in Symbiodiniaceae, with heterotrophic input via predation, often withstand perturbations better than coral species that are more autotrophic (Grottoli et al., 2006).

Many fish obligatorily associate with corals (Liberman et al., 1995) or sea anemones (Collingwood, 1868, reviewed in Fautin, 1991). Conversely, not all corals or anemones, even of the same species, at the same depth and habitat, host fish (Liberman et al., 1995; Chadwick and Arvedlund, 2005) or shrimp (Spotte, 1996), demonstrating the facultative nature of the relationships for the cnidarians. Furthermore, even though mutualisms are often referred to as a partnership between two organisms, most often mutualisms occur in networks, with a nested hierarchy between the members. Cnidarians epitomize the consortium concept (reviewed in Goulet et al., 2020) and the multi-level effects. Although macro symbionts associate with the cnidarian hosts, their nitrogen excretions provide nitrogen for the Symbiodiniaceae (Spotte, 1996; Mokady et al., 1998). The complexity of the interactions in the symbioses, along with their obligative vs. facultative nature, may further compound the effects of climate changes on them.

DISCUSSION

When symbioses face changing environmental conditions, several scenarios are plausible (Kiers et al., 2010). An existing mutualistic symbiosis may either thrive, or it may shift from beneficial to antagonistic (**Figure 1C**). Conversely, the current symbiosis may be dissolved and a new partnership formed, or the symbiosis may breakdown (**Figure 1C**; Kiers et al., 2010), potentially leading to the death of one if not all of the symbiotic partners (Kiers et al., 2010, 2015). Since holobiont adaptability may occur at a faster rate than evolutionary change of the hosts, cnidarian symbioses may survive climate change (Apprill, 2020). On the other hand, if the vast majority of cnidarian symbioses on coral reefs are obligatory with high fidelity, the range of potential scenarios for cnidarian symbioses faced with climate change may be limited, potentially leading to an overall reduction in cnidarian symbioses and biodiversity.

The Consequences of Obligate vs. Facultative Cnidarian Symbioses on Biodiversity on Coral Reefs Under Climate Change

A symbiosis can be obligate and resilient at the same time. If the symbiosis is physiologically plastic and robust, it will survive a perturbation. Alternatively, a stressor can adversely affect a symbiosis. The more reliant a partner is on the symbiosis, the more vulnerable it is if the symbiosis falters (Chomicki et al., 2019). When a coral bleaches, residual Symbiodiniaceae remain (Hayes and Bush, 1990), and if they repopulate the host, the symbiosis survives. Conversely, if a cnidarian host cannot repopulate or switch its Symbiodiniaceae, coral bleaching may lead to coral death.

If the obligate cnidarian-algal symbiosis is detrimentally affected by abiotic or biotic factors, other obligate symbionts will also be adversely affected. For example, the metabolic rate of juvenile clownfish *Amphiprion chrysopterus* inhabiting bleached *Heteractis magnifica* sea anemones was higher than in anemonefish inhabiting non-bleached anemones (Norin et al., 2018). Egg production in female *A. polymnus* in bleached *Stichodactyla hadonni* and *H. crispa* anemones was reduced by 38% compared to egg production in non-bleached anemones (Saenz-Agudelo et al., 2011). The effects on individual anemones and their symbionts in turn affects the populations of both. On a reef in the Gulf of Eilat (Aqaba) Red Sea, from 1997 to 2015 the number of the sea anemones, *H. crispa* and *Entacmaea quadricolor,* declined by 86% (Howell et al., 2016). Concurrently, a 74% reduction in the anemonefish *A. bicinctus* population occurred. In the original censuses, about 50% of sea anemones were inhabited by *A. bicinctus.* By 2015, all 25 sea anemones in the population were occupied by anemonefish, potentially limiting subsequent anemonefish recruitment (Howell et al., 2016). Hence, if the sea anemone population continues to decline on this reef, the reef biodiversity will be lowered with the loss of both the sea anemones and their resident anemonefish. Such a consequence occurred in the Keppel Islands, Australia, where a decade after a fishing moratorium not only did the sea anemone *E. quadricolor* and the anemonefish *A. melanopus* populations not bounce back to historic densities, but the anemone *H. crispa* and anemonefish *A. clarkii* were not seen in the surveys, demonstrating the dramatic decline in that symbiosis (Frisch et al., 2019).

Likewise, *Trapezia cymodoce*, a crab that obligatorily associates with scleractinian corals, was detrimentally affected when its host *Pocillopora damicornis* bleached (Stella et al., 2011). *Trapezia* egg clutch sizes were lower in bleached than in healthy corals (Stella et al., 2011). In bleached corals, either both members of the *Trapezia* breeding pair were lost, or only one *Trapezia* remained. A laboratory experiment demonstrated that *Trapezia* abandoned a bleached coral in search of a healthy one, with larger *Trapezia* usurping the resident smaller *Trapezia* (Stella et al., 2011). Hence, the bleaching in *P. damicornis* led to a reduction of the resident *Trapezia* fecundity and population and, through emigration, also affected *Trapezia* in healthy corals (Stella et al., 2011).

The Consequences of Cnidarian Symbiotic Partner(s) Fidelity vs. Flexibility on Coral Reef Biodiversity Under Climate Change

In both obligatory and facultative cnidarian symbioses, the symbionts may exhibit high fidelity, whereby a cnidarian species associates with a subset of symbionts, and vice versa. Alternatively, a host and/or symbiont may be flexible, forming symbioses indiscriminately. On coral reefs, many cnidarian symbioses demonstrate fidelity. Even if multiple entities can enter the host cnidarian, such as in initial Symbiodiniaceae or bacterial acquisition, winnowing occurs and the adult cnidarians exhibit symbiont affinity (Coffroth et al., 2001; Abrego et al., 2009; Epstein et al., 2019). Similarly, when a coral species hosts two or more Symbiodiniaceae genera, specific Symbiodiniaceae species within these genera associate with that given species (Goulet, 2006, 2007; Hume et al., 2020). Bacteria exhibit fidelity to cnidarian host species (Ainsworth et al., 2015; Shirur et al., 2016; van de Water et al., 2017, 2018; Huggett and Apprill, 2019; McCauley et al., 2020). Likewise, resident crabs, shrimp and fish associate with specific cnidarian species (Fishelson et al., 1974).

Is Fidelity Absolute or Context Dependent?

If fidelity occurs in a symbiosis, even for one of the partners, this may limit the potential range of responses of the holobiont to changing environmental conditions. Faced with climate change, with high fidelity, an existing host-symbiont(s) genotypic combination may be limited to the two outcomes of either surviving or dying. Alternatively, what appears as fidelity may be a context dependent consequence, i.e., under certain environmental conditions, a specific host-symbiont pairing may form since that is the best combination for those conditions. If the conditions change, then that host-symbiont combination may not be optimal, which may lead to a different host-symbiont symbiosis forming either during the establishment of the symbiosis (Baird et al., 2007) or in an existing symbiosis (Baker, 2003).

To tease apart if the fidelity exhibited in a certain symbiosis is absolute or context-dependent, one can pursue several investigative directions. Comparing cnidarian gene and metabolite expression profiles with homologous vs. heterologous symbionts may shed light on the cnidarian reaction to heterologous symbionts and if novel host-symbiont genotypic combinations are feasible (Matthews et al., 2017). Furthermore, if fidelity appears to exist because of the symbiosis' stability under certain environmental conditions, then if environmental parameters are pushed beyond a potential threshold, a different symbiosis partnership may form. Thus far, changing environmental conditions such as sea water temperatures, ultraviolet radiation, nutrient enrichment, reduction in pH and transplantation to different habitats on the same and different reefs have not resulted in the establishment of novel, persistent symbioses (reviewed in Goulet, 2006). In scleractinian corals, novel Symbiodiniaceae were detected following environmental change, but these Symbiodiniaceae were transient (Thornhill et al., 2006; Sampayo et al., 2008; Lee et al., 2016). If the perturbation did not lead to the demise of the symbiosis, the specific cnidarian-Symbiodiniaceae combination re-established itself. Nevertheless, one could argue that the symbioses have not been pushed to the point that would lead to flexibility (Baird et al., 2007). Thus far, experimental attempts at creating hardier artificial holobionts in the sea anemone *Exaiptasia*, by inoculating *Exaiptasia* with a more thermally tolerant Symbiodiniaceae, have not succeeded, with *Exaiptasia* displaying selectivity to its specific symbiont (Gabay et al., 2019; Herrera et al., 2020), countering the idea that these symbioses can change.

How Do the Effects of Climate Change on a Member of a Symbiotic Consortium Impact the Consortium as a Whole? Are There Cascade Effects?

Since cnidarian symbioses can contain a multitude of symbiotic interactions, changes in environmental conditions may affect only one, some, or all of the partners, and the degree and the directionality of the effect may vary (Goulet, 2015). Furthermore, even if the environmental change directly affects only one of the partners, since the symbiotic partners are interconnected, the entire symbiotic consortium may be affected. For example, elevated seawater temperatures can detrimentally affect Symbiodiniaceae photosynthesis which may in turn detrimentally affect the cnidarian host and hence the entire symbiosis (Warner et al., 1999). Even if the Symbiodiniaceae are not directly influenced by a stressor, the cnidarian host may be affected (Baird et al., 2009). Since the Symbiodiniaceae reside within their cnidarian host, death of the host will lead to the demise of the entire symbiosis. Conversely, the interplay of the potential negative, neutral, and positive effects may, in essence, cancel each other out, resulting in the symbiotic cnidarian consortium surviving and potentially thriving under the new environmental conditions. In order to potentially predict the effects of a stressor on a cnidarian species, and hence coral reef biodiversity, one needs to assess the effects of the stressor on the consortium.

Selective symbiosis survival may lead to a reduction in biodiversity both within and between species. For example, on the Great Barrier Reef, the scleractinian coral *Stylophora pistillata* hosts multiple Symbiodiniaceae types within the genus *Cladocopium*. In a thermal event, *S. pistillata* colonies hosting *Cladocopium* C79 and C35/a died at a higher proportion than those hosting C78 and C8/a. As a result, although *S. pistillata* still existed on the reef, the symbiosis diversity within *S. pistillata* was reduced (Sampayo et al., 2008). Likewise, in the coral *Montipora digitata* genotypic variability in both the host coral and Symbiodiniaceae led to differential stress susceptibility between coral holobionts (Kavousi et al., 2020).

CONCLUSION

Living together in symbioses may enable organisms to inhabit environments where they may not exist in isolation. Obligate and/or high fidelity in symbioses may lead to increased biodiversity in an ecosystem because of the many intra and interspecific host-symbiont genotypic combinations. Such is the case on coral reefs where cnidarian symbioses drive the ecosystem. When environmental conditions change, mutualism may transition to parasitism, the symbiosis may be abandoned, the existing host-symbiont(s) genotypic combination may withstand the perturbation, or the entire symbiosis will collapse. The obligate and fidelity of many cnidarian symbioses will make symbiotic transitions probably unlikely. The strength of a specific obligate symbiosis may be its downfall, leading to a reduction in intra and interspecific biodiversity. Understanding the nature of cnidarian symbioses (obligative to facultative, fidelity vs. flexibility), the symbiotic consortium, and the effects of the environment on these symbioses, will advance our knowledge of the current and future biodiversity of cnidarian symbioses on coral reefs.

AUTHOR CONTRIBUTIONS

TLG and DG contributed to the conception of the review, manuscript revision, and read and approved the submitted version. TLG wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Quantifying Nutrient Trade in the Arbuscular Mycorrhizal Symbiosis Under Extreme Weather Events Using Quantum-Dot Tagged Phosphorus

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Given the current trends in climate change, extreme weather events are expected to increase in strength and frequency. Such events can impact species survival and species interactions. One of the most ubiquitous symbioses on earth is the nutrient exchange partnership between arbuscular mycorrhizal fungi and their host plants. While past work has shown that mycorrhizal fungi can help alleviate stress, it is unknown how phosphorus uptake by plants to fungi is affected by extreme weather events, such as flooding and heat waves. To test this response, we grew *Medicago truncatula* host plants with or without mycorrhizal fungi and then exposed them to extreme weather treatments: increasing soil temperature by 12°C, or by flooding the plant roots for 7 days. We measured plant and fungal performance, and quantified phosphorus (P) uptake before and after extreme weather treatments using a technique in which we tagged apatite, a form of rock phosphorus, with fluorescing quantum-dots (QDs) nanoparticles. We then measured fluorescence in root and shoot tissue at harvest. We found that plants and arbuscular mycorrhizal fungi were affected by soil flooding, with plant survival, fungal colonization and QD-apatite uptake decreasing under flooded conditions. We did not see these negative effects in the heat treatment. While the presence of arbuscular mycorrhizal fungi affected plant biomass allocation, leading to an increase in shoot biomass, the symbiosis did not increase plant survival, total biomass or QD uptake in either treatment. More generally, we found host tissue contained roughly 80% more QD-apatite from the pre-treatment compared to the post-treatment nutrient injection. Future studies should focus on various plant-fungal combinations to create databases on which predictive models to extreme weather events can be constructed.

Keywords: extreme weather, climate change, arbuscular mycorrhizal fungi, quantum-dots, symbiosis

INTRODUCTION

As the climate continues to warm, global ecosystems are experiencing an increase in the frequency and intensity of extreme weather events, such as sudden heat waves, droughts, torrential rains, and floods (IPCC, 2014; Allen et al., 2018). Extreme weather events can have dramatic impacts on the survival, abundance and distribution of species, and can even lead to the local extinction of species (Tinsley et al., 2015; Ray et al., 2016; Zylstra et al., 2019). While progress is being made in our ability to predict the effects of extreme weather events on single species, it is less understood how these events affect the interactions among species, such as in mutualism and parasitism (Harrison, 2000; Edwards and Richardson, 2004; Bronstein, 2015; Millar and Bennett, 2016; Gardner et al., 2017). Sequential extreme weather events can drive symbiotic interactions between species to break down (Rosenzweig et al., 2015), for example by disrupting partner services (Zhou et al., 2013). Likewise, partner abundance can affect the stability of mutualisms with mutualism losses occurring where symbionts are scarce (Chomicki and Renner, 2017). Changes in these species interactions can, in turn, affect species richness and ecosystem resilience, but this is not well understood (Chomicki et al., 2019). A key goal of global change research is to understand how changes in species interactions can be magnified at the ecosystem level (Dakos and Bascompte, 2014; Jordano, 2016).

One of the most ubiquitous species interactions on earth is the symbiosis formed between ∼70% of all terrestrial plants and arbuscular mycorrhizal fungi of the Glomeromycotina, a subphylum of the Mucoromycota (Spatafora et al., 2016; Brundrett and Tedersoo, 2018; Tedersoo et al., 2020). Arbuscular mycorrhizal fungi play a key role in nutrient cycling by forming an underground link between plants roots and soil bound nutrients. The fungi forage the soil for mineral nutrients and exchange these nutrients with host plants for sugars and fatty acids (Smith et al., 2011; Berruti et al., 2016; Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). In addition to this role in nutrient provisioning, there is a growing body of research demonstrating how arbuscular mycorrhizal fungi can protect host plants from biotic and abiotic stress (Mohan et al., 2014; Wu, 2017), including increased pathogen resistance (Martinez-Medina et al., 2016; Chialva et al., 2018), chemical tolerance (Meier et al., 2015), heavy metal protection (Husna et al., 2016), and mediation of salinity (Wu et al., 2010).

Given this important role in stress protection, a major question in the field is whether arbuscular mycorrhizal fungi can help mitigate the effects of extreme weather events, or if extreme weather events could drive a breakdown of the interaction. Our aim was to mimic a heat wave and a flooding event in mycorrhizal and non-mycorrhizal plants and to determine the effect of extreme weather events on the phosphorus (P) uptake, plant growth, and fungal success (as measured by copy number). To mimic a flood, we submerged replicates of the model species *Medicago truncatula* with water levels rising 0.5–1.0 cm above the soil for 7 days. To mimic a heat wave, we increased soil temperature from 28 to 40°C for 7 days using heating mats regulated with a digital thermostat.

We employed a new technique to study nutrient transfer in both mycorrhizal and non-mycorrhizal plants in which we tagged apatite, a natural form of rock P, with highly fluorescent quantum-dots (QDs) to create fluorescing QD-apatite (Whiteside et al., 2019; van't Padje et al., 2020a). QDs are nanoparticles that fluoresce in bright and pure colors when excited with UV light. We used a class of QDs in which a carboxyl polymer creates a protective coat to prevent organisms from being exposed to the toxicity of the heavy metal core, and allows us to conjugate the apatite to QDs. Past work has demonstrated that QD-apatite can be taken up by the fungal networks of *in vitro* root organ cultures, and transferred to host roots (**Figure 1**; Whiteside et al., 2019; van't Padje et al., 2020a,b). Similarly, QD-apatite can be taken up by the roots of whole-plants, but colonization by mycorrhizal fungi seem to increase this uptake (Whiteside et al., 2019). The exact uptake mechanism of QD-apatite by the fungus is still not known. However, various controls performed in past experiments have confirmed that fungi show no uptake affinity for unbound (i.e., unconjugated) QDs or for bare metal QD cores (i.e., "naked quantum dots"), meaning that the fungus will not take up QDs if they are not conjugated to a nutrient source like apatite (Whiteside et al., 2019). Further validations have shown that there are no differences in uptake and transfer affinity among different colors of QD-apatite, and that whole plants grown on QD-apatite show no signs of toxicity compared to plants growing on apatite lacking QD-cores (i.e., not conjugated to any QDs) (Whiteside et al., 2019).

Here, we injected sterile sand with QD-apatite of two distinct colors, adding one color to the sand as a pre-treatment (red, λ = 663 nm) and one color as a post-treatment (yellow, λ = 572 nm). This allowed us to determine QD-apatite uptake of plants before and after an extreme weather event. We expected that the QD-apatite uptake would decrease in plants exposed to extreme weather treatments, but that this effect would be reduced in plants colonized by arbuscular mycorrhizal fungi.

MATERIALS AND METHODS

Germination, Fungal Inoculation and Growing Conditions

We sterilized and scarified seeds of *M. truncatula* (Institut National de la Recherche Agronomique, Montpellier, France) by submerging the seeds in 95% H_2SO_4 for 6.5 min. We removed the acid by rinsing the seeds with dH_2O six times. We stored the seeds at 4°C in the dark for 4 days (Garcia et al., 2006). After 4 days, we placed four germination seeds per pot (75 mL; *d* = 6 cm), containing autoclaved RHP Agra-vermiculite (M3). We watered them with 25 mL of an 50% P modified Hoagland's solution (6.5 mM KNO_3, 3.25 mM K_2SO_4, 4 mM $Ca(NO_3)_2{\cdot}4H_2O$), 4 mM $CaCl_2{\cdot}2H_2O$, 4 mM $CaSO_4{\cdot}2H_2O$, 0.5 mM NH_4NO_3, 1 mM $NH_4H_2PO_4$, 1 mM KH_2PO_4, 1 mM $MgSO_4{\cdot}6H_2O$, 50 μM KCl, 35 μM H_3BO_3, 25 μM, 2 μM $MnSO_4{\cdot}4H_2O$, 2 μM $ZnSO_4{\cdot}7H_2O$, 0.5 μM $CuSO_4{\cdot}5H_2O$, 5 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 20 mM Fe(Na)EDTA ($C_{10}H_{12}N_2O_8FeNa$) (Hoagland and Arnon, 1950; Werner et al., 2018). We placed the pots in a climate room, under

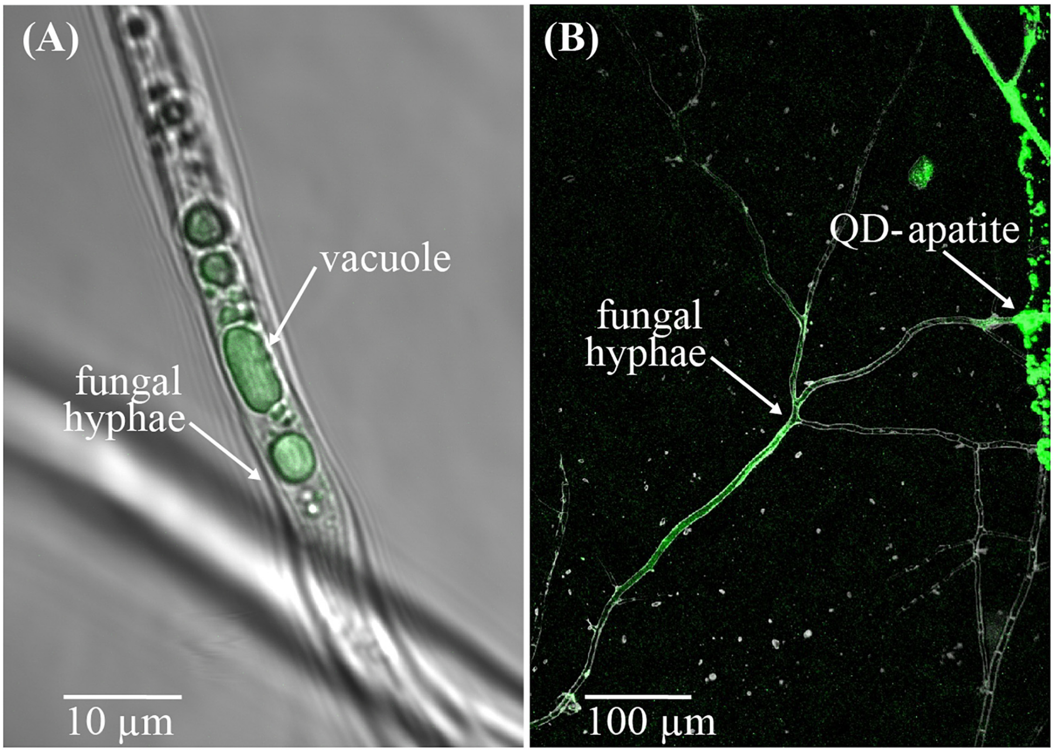

FIGURE 1 | QD-apatite uptake by arbuscular mycorrhizal fungus *Rhizophagus irregularis*. **(A)** Image of a fungal hypha from an *in vitro* culture with QD-apatite in its vacuoles. **(B)** Image of the arbuscular mycorrhiza fungus *Rhizophagus irregularis* near a patch of QD-apatite. Courtesy of M. D. Whiteside.

a light intensity of 170 μmol·m^{-2}·s^{-1}. We covered the seeds for the first 3 days and then shaded the seedlings for the following 7 days. After 10 days, we selected healthy seedlings, rinsed the roots with dH_2O and transferred them to plastic pots (320 mL) containing ∼270 g of quartz sand (>99.5% SiO2).

We prepared fungal inoculum by homogenizing mature *in vitro* root organ cultures of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (strain A5 Sanders Lab) grown on Ri T-DNA L-transformed carrot roots (*Daucus carota*) (Declerck et al., 2005). We inoculated half of the seedlings with 1.70 mL inoculum (∼1000 spores), and the other half with 1.70 mL dH_2O. We then fertilized all seedlings with 5 mL 50% P modified Hoagland's solution. We covered the sand with autoclaved white plastic beads, and placed each pot in a transparent plastic beaker (550 mL) to allow for flooding. We grew plants in a controlled climate room with a day-night cycle of 16–8 h, a day temperature of 22°C and a night temperature of 17°C. We kept the humidity at 75% and provided the plants with full spectrum day-light gas-discharge lamps with an intensity of 550 μmol·m^{-2}·s^{-1} at plant height (Barker et al., 2006). We watered plants twice a week with sterile dH_2O (Barker et al., 2006) to maintain a soil water content of 18% by weighting until 6 weeks, after which we increased the soil water content to 24% of the water holding capacity. Once per 2 weeks, we fertilized plants with 25 mL 50% P modified Hoagland's solution per pot. Mycorrhizal and non-mycorrhizal plants were randomly distributed in the climate room, and assigned to one of the three treatments: control, flooding, or heat. We grew a total of 72 plants, with 12 replicates per mycorrhizal × extreme weather treatment.

Nutrient Injections and Extreme Weather Treatments

We prepared two solutions of red (λ = 663 nm) and yellow (λ = 572 nm) QD-apatite to determine pre- and post-treatment nutrient uptake. To conjugate the QDs with hydroxyapatite, we added 150 mg Carboxyl CdSeS/ZnS Nanocrystals (CrystalPlex, Pittsburgh, PA, United States) of each color to 1 L 50% modified simulated body fluid–50% simulated body fluid solution (11.992 g NaCl; 1.966 g $NaHCO_3$; 0.447 g KCl; 0.457 g $MgCl_2 \cdot 6H_20$; 0.261 g K_2HPO_4; 0.416 g $CaCl_2$; 0.106 g Na_2SO_4) (Tang et al., 2010; Kawashita et al., 2012) and performed two separate reactions in the dark at 37°C. In the first reaction, small (∼8 nm) QD crystals were formed during 24 h. In the second reaction, the smaller crystals conjugated to bigger crystals (∼200 nm) during an exposure for 60 h, creating 15.64 g apatite per L (Kawashita et al., 2012), closely mimicking natural apatite (Sun et al., 2014). In between crystal formation, we placed the solutions on a shaker (100 oscillations/minute) for 24 h at room temperature. We replaced 80% of the supernatant with nanopure H_2O twice to remove unbound reagents, shaking the solutions by hand to re-precipitate between the washing steps. Our past analyses suggest that each nmol of QD-apatite contains ∼700 nmols of P (nmol

P:QD = 708:1)–for a complete description of the hydroxyapatite conjugation steps and validation tests, see Whiteside et al. (2019).

Five weeks after seedling transfer, we injected 5.7 mL red QD-apatite in the soil (0.33 g apatite/1000 g soil) for pre-treatment. This injection allowed us to determine baseline nutrient transfer before the plants were exposed to the treatments. After injection, we reduced fertilization to 12.5 mL 50% P modified Hoagland's solution per pot once per 2 weeks to stimulate QD-apatite uptake. We then allow plants to grow for 4 weeks before we started the treatments.

Treatments were initiated on adult plants, 9 weeks after seedling transfer. To mimic a flood, we submerged individual plants by adding dH_2O into the transparent beakers of 12 mycorrhizal and 12 non-mycorrhizal plants. We submerged the plants with water level rising 0.5–1 cm above the soil for 7 days. To mimic a heat wave, we placed 12 mycorrhizal and 12 non-mycorrhizal plants randomly on two heating mats (Bio Green GmbH & Co. KG, Bischoffen-Oberweidbach, Germany). The heating mats were regulated by a digital thermostat, which measured the soil temperature in the pot. We increased soil temperature from 28°C until 40°C for 7 days, keeping the original watering regime. After 1 week of the extreme weather treatments, we removed the water from the beakers of the flooded plants and placed the heat-treated plants randomly in the climate chamber. 12 mycorrhizal and 12 non-mycorrhizal plants were randomly assigned to a control treatment in which no extreme weather event was initiated. These controls plants were exposed to the same nutrient and green house conditions as the extreme weather plants, but were not exposed to flooding or heat treatments. Directly (same day) after the extreme weather treatments (or no-treatment in the case of the controls), we injected 2.5 mL yellow QD-apatite per pot (0.166 mg apatite/1000 g soil) to study if and how the nutrient transfer was influenced by the treatments as measure by the post-treatment QD-apatite injection.

Harvest

We harvested plants 11 weeks after seedling transfer, 7 days after the post-treatment QD-apatite injection. We washed roots in dH_2O and separated the root and shoot at the rosette. We placed plant material in paper bags to dry at 50°C for 48 h. We measured the dry mass of roots and shoot on an analytical balance, and subsampled root material for qPCR analysis of intraradical fungal colonization (∼20 mg) and root and shoot material for fluorescent analysis (∼6 mg) to determine the amount of QD-apatite in the tissue. We pulverized subsamples of root and shoot material using glass beads and a bead-beater speeding on 4 m/s for 40 s (Thermo Savant FastPrep Fp120 Cell homogenizer).

DNA Isolation and Real Time qPCR

To quantify intraradical colonization, we extracted DNA from roots, using a modified protocol of the DNeasy Plant Mini Kit by Qiagen kit (Qiagen, Hombrechtikon, Switzerland), which included the addition of 10 μL internal standard, a plasmid containing cassava mosaic virus DNA (Engelmoer et al., 2014) after the lysis step to be able to correct for extraction efficiency (Kiers et al., 2011; Whiteside et al., 2019). We stored extracted DNA at −20°C for further analysis. We used Real Time qPCR to determine intraradical colonization of the host roots (Thonar et al., 2012). We prepared DNA samples by diluting the root samples 100 times to dilute PCR inhibiting proteins, and added 10 μL iTaq universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States), 0.32 μL forward primer, 0.32 μL reverse primer, 0.080 μL probe, and 5.28 μL nanopure water to each DNA sample of 4 μL sample (Kiers et al., 2011). We loaded the prepared DNA samples into white welled 96-well PCR plates (Bio-Rad, Hercules, CA, United States) and placed the samples in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). We measured the presence of *R. irregularis* and internal standard using two separate qPCR rounds for each sample. For the internal standard, we used the following cycle: denaturation at 95°C for 5 s, annealing at 50°C for 30 s and amplification at 72°C for 1 s. For *R. irregularis*, we used a cycle of denaturation at 95°C for 5 s, and at 50°C for 30 s, replicated 39 times. We exported Cq values with the CFX manager software, and set a baseline threshold of 500 relative fluorescent units. We converted Cq values to copy numbers (Kiers et al., 2011) and calculated extraction efficiency by dividing the Cq values of *R. irregularis* by the Cq value of the internal standard.

Fluorescent Analysis

We determined QD-apatite content of root and shoot material by measuring emission spectrum of plant tissue. We prepared the ground plant material by adding 150 μL 10 mM borate buffer per mg plant material. From each sample, we pipetted five replicates of 150 μL in a 96 wells plate with a glass bottom (Eppendorf AG, Hamburg, Germany). To reduce edge effects, we left the outmost wells empty. We measured the emission using a fluorescence a BioTek Synergy MX plate reader with Gen5^{TM} Data Analysis Software. Emission of root and shoot material was calculated from 450 to 800 nm, with steps of 2 nm by an excitation of 325 nm. We then translated the emission spectra to specific QD-apatite content in root and shoot using emission finger printing. This allowed us to separate the emission of the two QD colors, and the auto-fluorescence of the plant material (Zimmermann et al., 2003), using a custom script in Matlab Code (MathWorks, Natick, MA, United States) (Whiteside et al., 2019). We converged fluorescence intensities into the concentration of QD-apatite in root and shoot using calibration gradients of QDs of each color, composed of seven concentrations: 13.1 mM, 9.83 mM, 7.37 mM, 5.53 mM, 4.15 mM, 3.11 mM, and 2.33 mM (Whiteside et al., 2012a).

Statistical Analysis

All statistical analyses were performed in R version 3.3.4, with each treatment compared directly to the control. We first analyzed plant survival with a generalized linear model with a binomial error distribution, with the treatment (control, flood, or heat) and mycorrhizal status (mycorrhizal and non-mycorrhizal) as independent variables. This allowed us to produce ANOVA type III tables with a likelihood ratio as test statistic. We then removed dead plants from the dataset for further analysis. We analyzed the effect of the independent variables (treatment, mycorrhizal status and the treatment × mycorrhizal status interaction) on total biomass, root and shoot mass with an

ANOVA and an *F* test. We used an ANOVA with an *F* test on a generalized linear model with a gaussian error distribution to analyze intraradical colonization per mg of root. We used Wilcoxon rank sum tests to analyze the difference in nmol of pre-treatment QD-apatite between mycorrhizal and non-mycorrhizal plants per mg of root and shoot material (the difference in QD-apatite content of the root and shoot and the difference in uptake from the pre- and post-treatment), injection. We calculated the logarithm of the summed QD-apatite content of shoot and root as the total QD content and analyzed the effect of the independent variables with an ANOVA.

RESULTS

Plant Survival

We found that both heat and flooding treatments significantly influenced plant survival. At harvest, 11 weeks after seedling transfer, 63% of the flooded plants (15 of 24) and 21% of the heated plants (5 of 24) had died. In contrast, 100% (all 24) of the control plants survived. We found no evidence that mycorrhizal status of the plants significantly influenced overall plant survival (**Table 1**). Of the surviving flooded plants, four were mycorrhizal, and five non-mycorrhizal. In the heat treatment, eight mycorrhizal plants survived to harvest, and eleven non-mycorrhizal plants.

Plant Growth

We determined the effect of extreme weather treatments on plant growth by measuring plant biomass at harvest, removing the plant replicates that had died. We first compared the control to the heat treatment, with and without mycorrhizal fungi. Total plant biomass was not significantly affected by the heat treatment, the mycorrhizal status or the treatment × mycorrhizal status interaction (**Table 1**). The root biomass was also not significantly affected by the treatment, mycorrhizal status or treatment × mycorrhizal status interaction (**Table 1** and **Figure 2A**). However, while shoot biomass was not significantly affected by treatment effect, we found a significant mycorrhizal effect, with mycorrhizal plants having bigger shoots, independent of treatment (no significant treatment × mycorrhizal status interaction, **Table 1** and **Figure 2B**).

We then analyzed the effect of the flooding treatment on the biomass by comparing the control to the flooding treatment. Total biomass was significantly affected by the treatment, with flooded plants having a lower total biomass. However, total biomass was not significantly affected by mycorrhizal status or the treatment × mycorrhizal interaction (**Table 1**). Root biomass was likewise significantly lower in flooded plants, but there was no significant effect of mycorrhizal status or the treatment × mycorrhizal status interaction (**Table 1** and **Figure 2C**). The shoot mass was not significantly influenced by treatment or mycorrhizal status, but was significantly affected by the treatment × mycorrhizal status interaction: shoots of flooded mycorrhizal plants were larger than shoots of non-mycorrhizal flooded plants (**Table 1** and **Figure 2D**).

Fungal Colonization

We examined how extreme weather treatments affected intraradical fungal colonization on host roots. We compared the heat-treated plants with the control plants, and found no

TABLE 1 | Analysis of variance on the biomass of plants.

			Extreme weather treatment				
		Heat			Flood		
Plant survival			X^2	***p*-value**		X^2	***p*-value**
Extreme weather treatment			6.3506	0.0117		15.276	**0.001***
Mycorrhizal status			0.000	1.000		0.000	1.000
Treatment × mycorrhizal status interaction			0.000	1.000		0.000	1.000
Total plant biomass	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	39	0.011	0.917	29	11.611	**0.002***
Mycorrhizal status	1	39	0.427	0.517	29	0.292	0.593
Treatment × mycorrhizal status interaction	1	39	0.109	0.743	29	0.088	0.769
Root biomass	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	39	0.323	0.573	29	10.852	**0.003***
Mycorrhizal status	1	39	0.003	0.958	29	0.073	0.789
Treatment × mycorrhizal status interaction	1	39	0.038	0.846	29	0.420	0.522
Shoot biomass	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	39	1.933	0.172	29	1.727	0.199
Mycorrhizal status	1	39	5.417	**0.025***	29	3.792	0.061
Treatment × mycorrhizal status interaction	1	39	2.862	0.099	29	4.682	**0.039***

*Degrees of freedom (Df), residuals (res), and F- or X^2–values and p-values are given for the effect of the variables (treatment, mycorrhizal status, and the treatment × mycorrhizal status interaction). P-values in bold with an * have a significant effect of the variable (p-value < 0.05).*

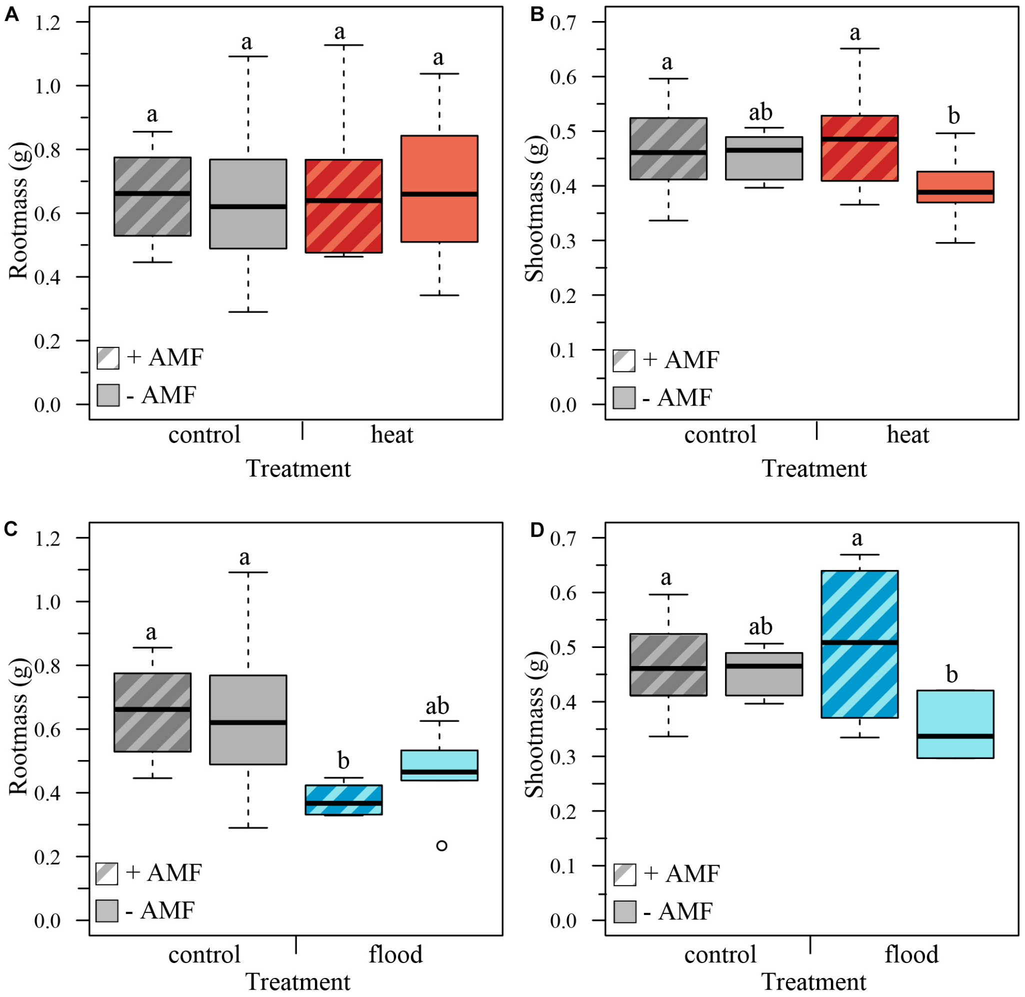

FIGURE 2 | Boxplots representing the plant biomass per treatment for mycorrhizal (+AMF) and non-mycorrhizal plants (–AMF). **(A)** Root biomass was not significantly influenced by heat treatment, mycorrhizal status or the treatment × mycorrhizal status interaction. **(B)** Shoot mass was not significantly influenced by the heat treatment nor the treatment × mycorrhizal status interaction, but was affected by the mycorrhizal status, with mycorrhizal plants having higher shoot biomass. **(C)** Root biomass was significantly affected by the flooding treatment, with flooded plants showing lower root biomass, but not by mycorrhizal status or treatment × mycorrhizal interaction. **(D)** Shoot biomass was not significantly affected by flooding treatment or mycorrhizal status but was significantly affected by the treatment × mycorrhizal status interaction: shoots of flooded mycorrhizal plants were significantly larger than shoots of flooded non-mycorrhizal plants. $n_{\text{contol},+AMF} = 12$, $n_{contol,\ \text{-AMF}} = 12$, $n_{heat,+AMF} = 8$, $n_{heat,\ -AMF} = 11$, $n_{\text{flood},+AMF} = 4$, $n_{\text{flood},\ \text{-AMF}} = 5$. Top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. Different letters indicate significant difference between the means (p-value < 0.05).

significant effect of treatment on the intraradical colonization per mg of root, or per total root (**Table 2** and **Figures 3A,B**). In contrast, when we compared the flooded plants with the control plants, we found a significant treatment effect, with flooding associated with a 58% drop in colonization per root and 94% drop in colonization per mg root (**Table 2** and **Figures 3C,D**).

QD-Apatite Uptake Before Exposure to Treatment (Pre-treatment)

We determined the QD status of plants before exposure to the extreme weather treatments by quantifying the red $\lambda = 666$ nm nmol QD-apatite per total root and total shoot tissue. Based on this pre-treatment injection, we found that mycorrhizal status did not significantly influence nmol of QD-apatite per total root (Wilcoxon rank sum test, $W = 314$, $p = 0.696$) or shoot (Wilcoxon rank sum test, $W = 234$, $p = 0.062$). In terms of allocation of QD-apatite across the plant, we found that total shoot contained on average three times more QD-apatite ($\lambda = 2.603$, SE < 0.187 nmol/total shoot) than total root ($\lambda = 0.889$, SE < 0.059 nmol/total root, paired Wilcoxon rank sum test: $W = 317$, $p \leq 0.0001$, **Figures 4A,B**).

QD-Apatite Uptake After Exposure to Treatment (Post-treatment)

We then quantified the post-treatment uptake by measuring the yellow, $\lambda = 572$ nm, QD-apatite in the host plants. We found that plants contained, on average, 80% less post-treatment QD-apatite compared to pre-treatment injection, independent of treatment.

TABLE 2 | Analysis of variance on the intraradical colonization and QD-apatite content per total root and total shoot.

		Extreme weather treatment					
		Heat			**Flood**		
Intraradical colonization per mg root	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	15	1.603	0.225	13	7.852	**0.015***
Intraradical colonization per total root	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	15	1.375	0.259	13	11.502	**0.005***
QD-apatite per total root	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	39	0.923	0.343	29	9.880	**0.004***
Mycorrhizal status	1	39	0.169	0.684	29	1.704	0.202
Treatment × mycorrhizal status	1	39	1.540	0.222	29	0.024	0.879
QD-apatite per total shoot	**Df**	**res**	***F*-value**	***p*-value**	**res**	***F*-value**	***p*-value**
Extreme weather treatment	1	39	0.056	0.814	29	2.046	0.163
Mycorrhizal status	1	39	0.820	0.371	29	0.194	0.663
Treatment × mycorrhizal status	1	39	0.954	0.335	29	0.559	0.461

*Degrees of freedom (Df), residuals (res), F- and p-values are given for the effect of the variables (treatment, mycorrhizal status, and the treatment × mycorrhizal status interaction). P-values in bold with an * have a significant effect of the variable (p-value < 0.05).*

We tested the effect of the heat treatment and mycorrhizal status on the nmol of QD-apatite per total root and total shoot by comparing the control plants to the heat-treated plants. We found that nmol of QD-apatite per total root was not significantly affected by treatment, mycorrhizal status or the treatment × mycorrhizal interaction (**Table 2** and **Figure 5A**). Likewise, we found that nmol QD-apatite per total of shoot was not significantly affected by treatment, mycorrhizal status or the interaction (**Table 2** and **Figure 5B**).

We quantified the effect of the flooding treatment by comparing the flooded plants with the control plants. We found that the nmol QD-apatite per total root was significantly lower in the flooded treatment, compared to control plants, but was not significantly affected by the mycorrhizal status or the treatment × mycorrhizal interaction (**Table 2** and **Figure 5C**). We found that nmol of QD-apatite per total shoot was not significantly affected by treatment, mycorrhizal status or the treatment × mycorrhizal status interaction (**Table 2** and **Figure 5D**).

DISCUSSION

Our aim was to determine how extreme weather events, specifically soil heating and flooding, modified the P uptake of plants and plants colonized by arbuscular mycorrhizal fungi. We found a strong effect of the flooding treatment on plant survival, with over 60% of the plants dying when exposed to flooding. We found a less strong effect of the soil heating treatment, with ∼20% of the plants dying. We found no evidence that mycorrhizal colonization of *R. irregularis* had an effect on the survival rates of plants (**Table 1**), but this effect is known to be highly species specific (Camprubi et al., 2012).

It is well established that flooding drives a stress response in plants, including metabolic modifications, such as inhibition of mitochondrial respiration and photosynthesis, leading to dramatic decline in plant growth and development and concurrent dysfunctions (Parent et al., 2008). We found that flooding was linked to a decrease in plant biomass (**Figures 2C,D**), intraradical colonization (**Figures 3C,D**), and nutrient uptake (**Figures 5C,D**).

Heat is also known to disrupt many physiological and biophysical processes, including photosynthesis and chlorophyll synthesis (Zhu et al., 2017). However, we did not see strong treatment effects on plant performance under our soil heating regime (**Figures 2A,B**). The stronger effects of the flooding treatment in our study could potentially be linked to the biology of *M. truncatula*, a Mediterranean herb known to tolerate high temperatures, but to be very sensitive to flooding (Küster et al., 2006). Additionally, our heating regime was potentially less intense than expected in nature because we only increased the soil temperature, without decreasing the water availability. This has the potential to reduce the stress experienced by plants and fungi in the heating treatment.

Mycorrhizal Fungi Affect Plant Biomass Allocation Under Extreme Weather Events

We expected to find a positive effect of mycorrhizal colonization on plant biomass. While mycorrhizal status had no effect on total plant biomass, we found that mycorrhizal status influenced the biomass allocation to above and below ground plant parts. Mycorrhizal plants had a higher shoot biomass than non-mycorrhizal plants (**Table 1** and **Figure 2**). This supports previous studies that have shown how arbuscular mycorrhizal fungi can modify biomass allocation of their host plants,

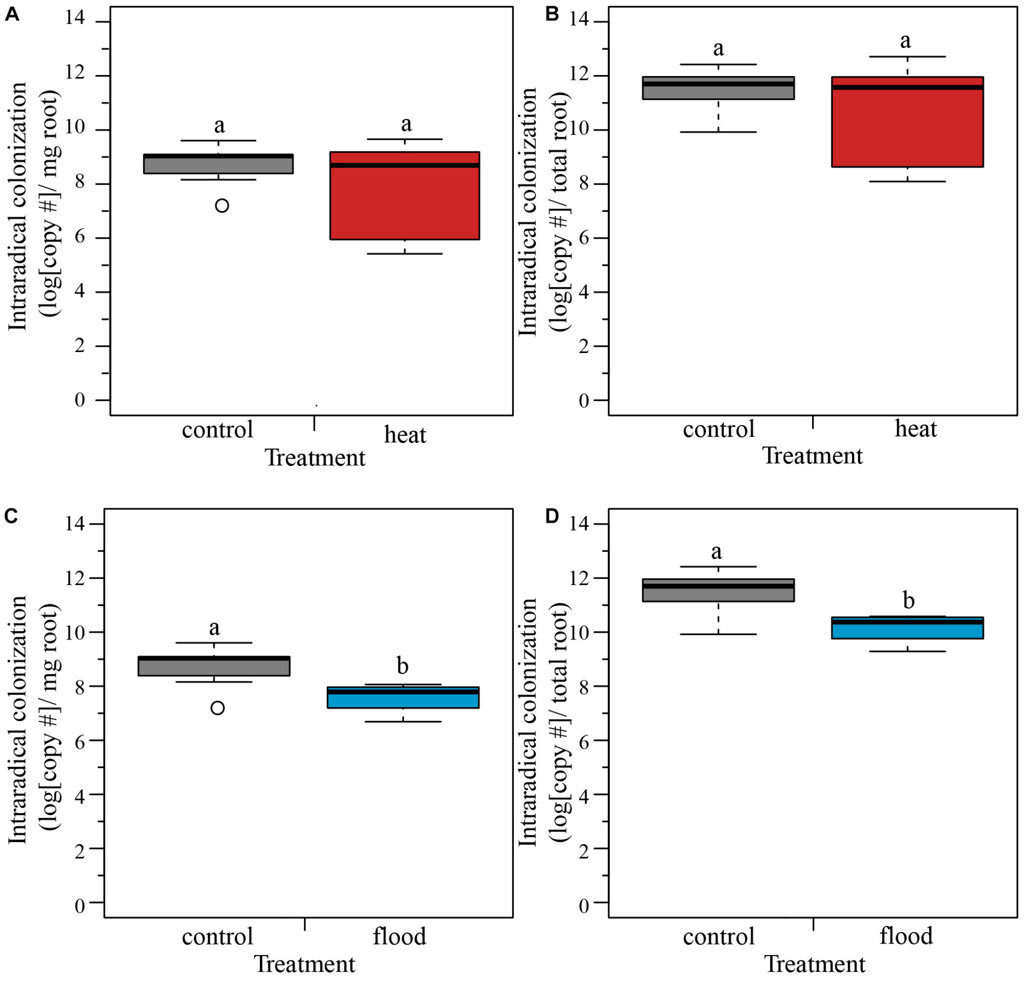

FIGURE 3 | Boxplots representing the intraradical colonization per mg and per total root. **(A,B)** The heat treatment did not significantly influence the fungal colonization of the host roots per mg or per total root. **(C,D)** The flooding treatment caused a significant lower intraradical colonization of the host roots, both per mg of root and per total root. $n_{\text{contol}} = 12$, $n_{\text{heat}} = 8$, $n_{\text{flood}} = 4$. Top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. Different letters indicate significant difference between the means (p-value < 0.05).

with mycorrhizal plants having higher shoot mass than non-mycorrhizal plants (Johnson et al., 2008; Zaller et al., 2011; Zhang et al., 2011). Non-mycorrhizal plants, similar to plants under low water or low nutrient conditions, may allocate less biomass to the shoot and more the roots to increase the uptake of limiting resource (Zhang et al., 2011). Many studies have shown that plant stressors, such as water availability, temperature, and heavy metals can also influence the biomass allocation patterns because these stressors alter the carbon allocation of plants [reviewed in Andersen and Rygiewicz (1991)]. Likewise, we found the lowest shoot mass was found in non-mycorrhizal plants exposed to the flooded treatment (**Table 1** and **Figure 2**).

Flooding, but Not Heat, Decreases Mycorrhizal Colonization

We then tested the effects of the extreme weather treatments on intraradical colonization rates as measured by qPCR. In flooded plants, we found that arbuscular mycorrhizal fungi showed a surprising resilience against the flooding treatment, still colonizing roots after a 7-day exposure to flooded conditions (**Figures 3C,D**). Previous research has demonstrated species-specific effects of colonization by arbuscular mycorrhizal fungi, with some plant-fungal combinations experiencing increased colonization associated with flooding, while others show a reduction, or no effect (Hartmond et al., 1987; Wu et al., 2013). There may also be an important time component: in rice, colonization of arbuscular mycorrhizal fungi gradually decreases over time during flooding condition due to changed root morphology (Vallino et al., 2014). Our study suggests that colonization is negatively affected by the flooding treatment, but did not lead to the total loss of mycorrhizal colonization.

In contrast to flooding, we found that the heat treatment did not influence the fungal colonization rates significantly (**Figures 3A,B**). One explanation is that our heat treatment was neither strong, nor long enough to trigger a negative effect on the fungal symbiont. When the soil temperature increases, the carbon exchange rate and the absorption of nutrients as P increases which could negate the negative effects of increased temperatures (Andersen and Rygiewicz, 1991). We aimed to induce only a heat stress, however the heat-treated plants might have experienced drought related effects as well. While the majority of work has found positive effects of AM fungi on drought tolerance (Bárzana et al., 2015; Quiroga et al., 2017; Li et al., 2019; Fracasso et al., 2020), a transcriptomic analysis in sorghum suggested that drought can negatively impact the functionality of the symbiosis (Varoquaux et al., 2019). Similar negative effects have been found in barley under higher AMF richness (Sendek et al., 2019). However, the vast majority of studies suggest that AMF can improve tolerance to temperature stress. AMF can enhance water and nutrient uptake, and induce plant production of ROS-scavenging anti-oxidant compounds, reduce oxidative stress, improve photosynthesis, and increase accumulation of osmolytes (Zhu et al., 2017). However, because these effects differ depending on host species, fungal species/fungal diversity, and soil type, there are many open questions as to how plant-soil biotic interactions will respond under climate change. New research has shown that even the presence versus absence of intrahyphal endobacteria in AM fungi such as *Gigaspora margarita* plays an important role in modulating stress (Chialva et al., 2020). More broadly, while it is known that plants can actively recruit microorganisms to buffer the environmental stress of drought (Naylor and Coleman-derr, 2018; Andreo-jimenez et al., 2019; Vigani et al., 2019; Veach et al., 2020), it is unknown how these microorganisms interact with the functionality of mycorrhizal roots.

Mycorrhizal Status Did Not Increase Nutrient Transfer

The use of QD-apatite to study P transfer from fungi to host plants is an emerging technique (Whiteside et al., 2019; van't Padje et al., 2020a,b), and many open questions remain. Using emission finger printing, we were able to quantify florescence in root and shoot tissue of QD-apatite injections of two different colors in whole plants, representing nutrient uptake from pre- and post-extreme weather treatments. Data on pre-treatment uptake (i.e., red QDs, λ = 666 nm) suggested that tagged apatite was taken up by plants, and then successfully transferred to growing leaves: shoots contained on average three times more QD-apatite than roots (**Figure 4**). This is important because it further validates our QD-apatite method (Whiteside et al., 2019), showing that tagged nutrients accumulated in the growing host shoots tissues as expected.

After the pre-treatment, we next quantified QD-apatite uptake post-extreme weather treatments. The most important result was that plants contained, on average, 80% less QD-apatite compared to QD-apatite from the pre-treatment injection, independent of treatment. This suggests that either the plants were saturated with P from the first injection, or that not enough time had passed since the injection for the QD-apatite to be incorporated into host tissue (7 days). We found no significant effect of mycorrhizal status of the plants on QD uptake in the heat treatment (**Table 2**). This result is not surprising given that the heat treatment did not induce a change in plant biomass or mycorrhizal status. We did find that there was a significant effect of the flooding treatment on QD uptake in post-treatment QD injections: roots of flooded plants contained significantly less nmol QD-apatite than roots of control plants (**Table 2** and **Figure 5D**). While research has shown that flooding can increase the solubility of P in soils by dissolution of P apatite (Chien, 1977), the lower root biomass and lower colonization of fungi in roots of flooded plants suggests that these host roots and fungal symbionts were stressed, and were not able to take up or transfer as much QD-apatite as control plants.

Our work provides further evidence that plants and arbuscular mycorrhizal fungi are able to take up QD-apatite, and incorporate

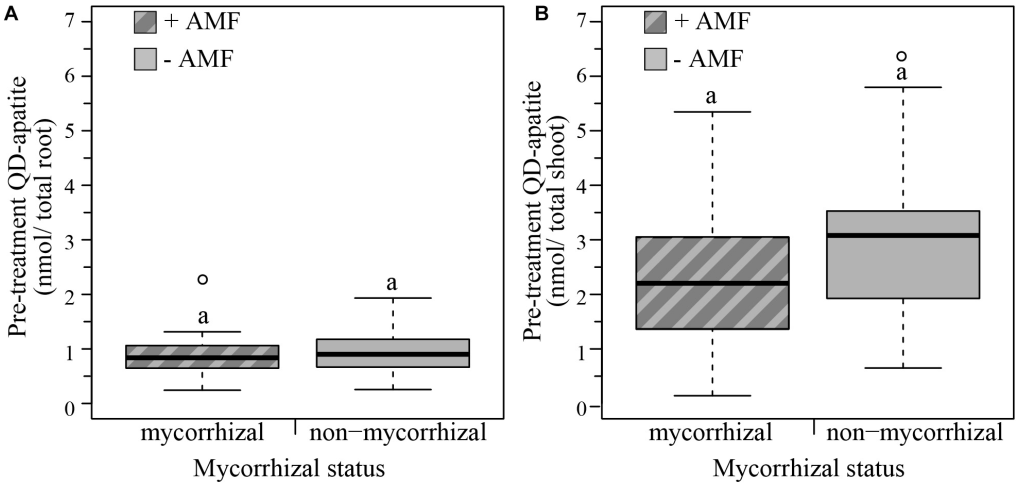

FIGURE 4 | Boxplots representing pre-treatment QD-apatite content per total root and total shoot for mycorrhizal (+AMF) and non-mycorrhizal plants (–AMF). **(A)** The amount QD-apatite in total roots was not significantly different between mycorrhizal and non-mycorrhizal plants. **(B)** The amount of QD-apatite in total shoots was also not significantly different between mycorrhizal and non-mycorrhizal plants. n_{+AMF} = 24, n_{-AMF} = 28. Top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. Different letters indicate significant difference between the means (p-value < 0.05).

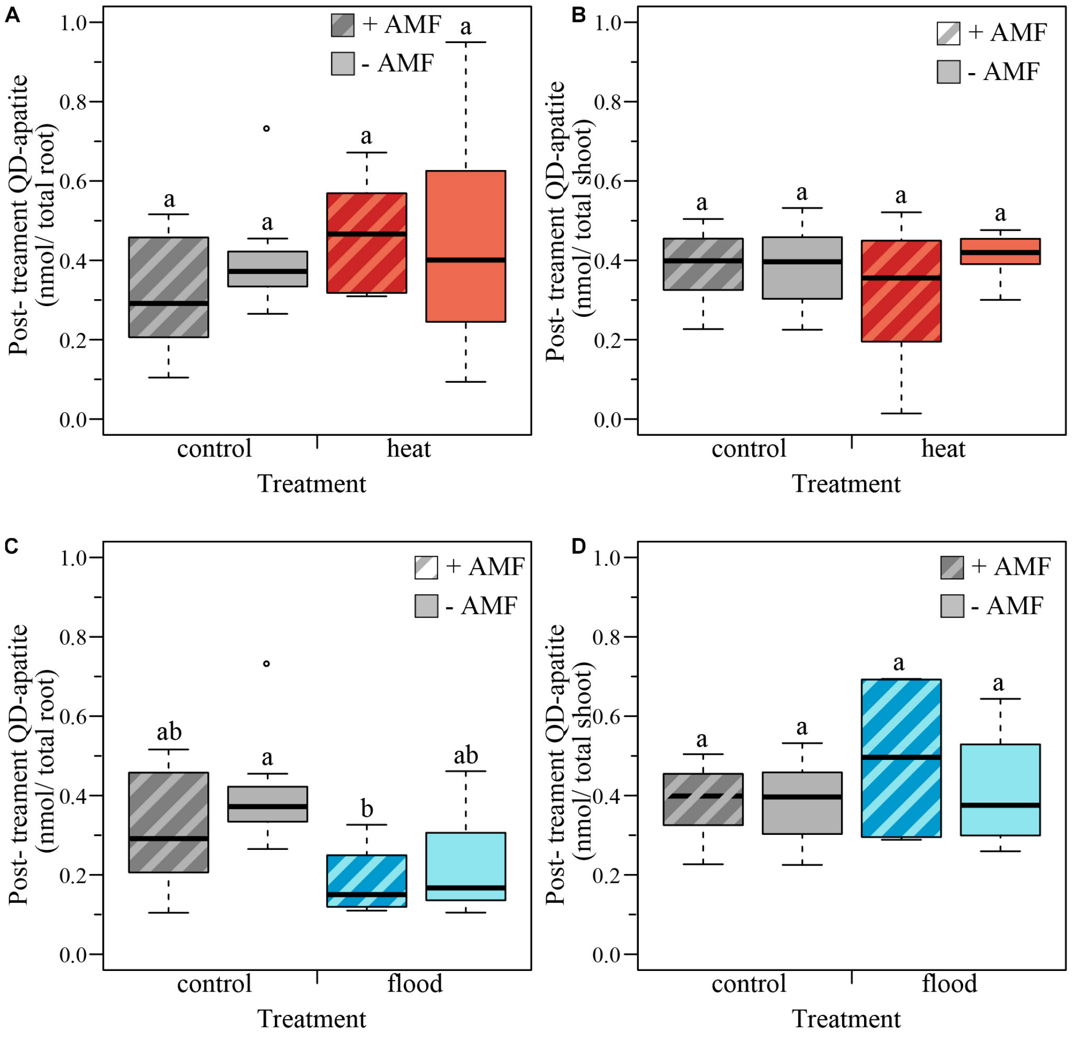

FIGURE 5 | Boxplots representing the post-treatment QD-apatite content per total root and total shoot for mycorrhizal (+AMF) and non-mycorrhizal plants (–AMF). **(A)** The amount QD-apatite in total root was not significantly affected by heat treatment, mycorrhizal status or the treatment × mycorrhizal status interaction. **(B)** The amount of QD-apatite was in total shoot was not significantly affected by the heat treatment, mycorrhizal status nor the treatment × mycorrhizal status interaction. **(C)** The amount of QD-apatite in total roots was significantly affected by the flooding treatment: flooded roots contained less QD-apatite. However, QD content was not significantly affected by mycorrhizal status or the treatment × mycorrhizal status interaction. **(D)** The amount of QD-apatite in shoots was not significantly affected by the flooded treatment, mycorrhizal status or the treatment × mycorrhizal status interaction. $n_{contol,+AMF} = 12$, $n_{contol,\ -AMF} = 12$, $n_{heat,+AMF} = 8$, $n_{heat,\ -AMF} = 11$, $n_{flood,+AMF} = 4$, $n_{flood,\ -AMF} = 5$. Top and bottom of the box indicate the first and third quartile, and the whiskers indicate the minimum and maximum values. Different letters indicate significant difference between the means (p-value < 0.05).

the fluorescing QD-apatite into their tissue (**Figure 1**; Whiteside et al., 2019; van't Padje et al., 2020a), as has been shown previously using QD-tagged amino acids (Whiteside et al., 2009, 2012a,b). However, a major limitation in our current approach is the inability to quantify the rate at which P is dissociated from the QD core across different biological tissue. Additionally, while the specific pathways of QD-apatite uptake in plants is still unknown, there has been work describing the mechanisms of nanoparticles by plants roots [extensively reviewed by Schwab et al. (2016)]. Uptake of larger QD particles is likely via endocytosis, i.e., invagination of the cell membrane. The most common endocytosis pathway in plant roots is clathrin dependent, enabling the uptake of particles of ∼70 to 120 nm diameter (Šamaj, 2012), but nanoparticles can also be taken up via clathrin-independent endocytosis (Etxeberria et al., 2006). Once inside the plant root cell, the nanoparticles can cross the cells simplistically via cell wall pores. Previous studies have measured cell wall pores diameter, and found that the diameter varies between 5 and 20 nm (McCann et al., 1990). Transport of nanoparticles can also occur apoplastically and via the vascular system of the plants (reviewed in Schwab et al., 2016). Our data confirm past research in QDs showing that once inside the plant roots, the QD are transported to the shoots and eventually into the mesophyll cells and chloroplasts (Whiteside et al., 2009, 2019).

Less is known about the fungal uptake of nutrients tagged with nanoparticles, especially in arbuscular mycorrhizal fungi. In yeast, QD tagged glutathione has been shown to be taken up using ADP1-encoded transporters (Gustafsson et al., 2015). However, for larger particles, such as apatite crystals used here, endocytosis is the most likely mechanism. This idea is supported by recent bright-field imaging videos of nutrient flows that showed large vacuoles inside hyphae when the fungus was given access to QD-tagged apatite. These large vacuoles were conspicuously absent in flows when the fungus has no access to QD-tagged apatite (van't Padje et al., 2020a). Endocytosis has been found to be important for particle uptake in filamentous fungal hyphae (Fischer-Parton et al., 2000; Read and Kalkman, 2003). Likewise, the budding yeasts *Saccharomyces cerevisiae* (Lu et al., 2016), and *Candida albicans* have been shown to use clathrin mediated endocytosis. In the case of *C. albicans*, endocytosis can result in invagination of vacuoles with diameters of ∼100 nm (Epp et al., 2013). Arbuscular mycorrhizal fungi likely rely on endocytosis for the uptake of QD-apatite crystals, which can be as large as ∼200 nm directly after chemical synthesis (Whiteside et al., 2019). However, as the fungi dissolute the apatite (Pel et al., 2018), the particle size will decrease prior to uptake (∼8–20 nm).

We did not find a significant effect of mycorrhizal status on the nmol of QD-apatite per total root or shoot. Past work has shown that can arbuscular mycorrhizal fungi facilitate the uptake of apatite in plants (Pel et al., 2018), likely through dissolution of the apatite into smaller crystals before uptake. Likewise, Whiteside et al., 2019 found that colonization by mycorrhizal fungi significantly increased QD-apatite in plant tissue after ∼7 weeks. Given this past evidence, we had expected to see a positive effect of colonization on QD-apatite uptake for the host. However, given that we also did not see a positive effect of fungal colonization on total plant biomass, this is further evidence that not all plant-fungal combinations result in P benefits for host plants (Hoeksema et al., 2010) and that this is highly context dependent (Li et al., 2008; Chialva et al., 2020).

While some progress is being made in our ability to predict the effects of extreme weather events on single species, an open research question is how these events affect the interactions among species, and thus biodiversity more generally. Our work suggests that both plants and arbuscular mycorrhizal fungi were negatively affected by soil flooding, with plant survival, fungal colonization and QD-apatite uptake decreasing under flooded conditions. These effects were less severe in the heat treatment. While we found no evidence that the plant-fungal combination tested affected P uptake by plants exposed to extreme weather events, future studies should develop a standardized protocol to test these and other weather-related stress effects, with the aim of developing databases on which predictive models can be constructed. As we test more species combinations to extreme weather, we can better understand how these events will shape biodiversity through symbiosis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://github.com/anoukvantpadje/Extreme_weather.

AUTHOR CONTRIBUTIONS

AP and LC designed and performed the experiments. AP performed the statistical analysis and wrote the main text. PB was involved with revision of the main text. EK was involved in the experimental design and revision of the main text. All authors contributed to the article and approved the submitted version.

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REVIEW
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Environmental Stress Selects for Innovations That Drive Vibrio Symbiont Diversity

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Symbiotic bacteria in the Vibrionaceae are a dynamic group of γ-Proteobacteria that are commonly found throughout the world. Although they primarily are free-living in the environment, they can be commonly found associated with various Eukarya, either as beneficial or pathogenic symbionts. Interestingly, this dual lifestyle (free-living or in symbiosis) enables the bacteria to have enormous ecological breadth, where they can accommodate a variety of stresses in both stages. Here, we discuss some of the most common stressors that *Vibrio* bacteria encounter when in their free-living state or associated with an animal host, and how some of the mechanisms that are used to cope with these stressors can be used as an evolutionary advantage that increases their diversity both in the environment and within their specific hosts.

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INTRODUCTION

The Vibrionaceae are a cosmopolitan family of gram-negative bacteria that form special associations with metazoan organisms (Thompson et al., 2006). These associations can be beneficial, commensal, and even pathogenic (Gomez-Gil et al., 2014). Close study of associations between the Vibrionaceae and their metazoan hosts have led to many important discoveries about the evolution of such unique partnerships, the molecular cross-talk that is established between different organisms, and how the partnership is more resilient to changes in the surrounding environment (Bourne et al., 2009). Examining how the entire "holobiome" (host + symbionts) responds to changes in the surrounding milieu can provide a barometer not only for the maintenance and selection of host-microbe relationships, but communities of other nested organisms that rely on each other for ecosystem health (McFall-Ngai, 2007; McFall-Ngai et al., 2013). This in turn produces a wide variety of host-symbiont assemblages, that would not exist without both partners being in association with one another. The holobiome thus is an independent "super organism" that can survive under conditions where neither symbiont or host cannot-thereby increasing the diversity and breadth of their habitat.

When not associated with a host, free-living, planktonic vibrios are subjected to multiple abiotic and biotic factors that select against various traits involved in colonization, infection, and eventually persistence (**Figure 1**; Piculell et al., 2008; Bright and Bulgheresi, 2010; Medina and Sachs, 2010). The distribution of vibrios that associate with animal hosts has been shown to be independent of host availability, while ecological factors such as temperature and salinity have been shown to impact *Vibrio* proliferation in the water column (Takemura et al., 2014). Recent reviews of the impact that environmental factors have on *Vibrio* biodiversity have focused primarily on human disease, aquaculture, or the population dynamics between the benign and pathogenic states of environmental vibrios (Takemura et al., 2014; Vezzulli et al., 2015). For example, a recent, long term evaluation of pathogenic *Vibrio* populations demonstrated that rising sea surface temperatures (SST) correlate with increased numbers of human infections (Vezzulli et al., 2012). Thus, temperature is just one abiotic factor that has tremendous effects on ecosystem health (Pantos et al., 2015). How *Vibrio* bacteria respond to such changes, and whether stress increases bacterial fitness and their ability to diversify among different associations with various eukaryotes, has yet to be determined. Most recently, the definition of "stress" in *Vibrio* was previously defined in an earlier review (Soto et al., 2010), focusing on environmental vibrios when not associated with a host.

The importance of studying the dynamics of these *Vibrio*-animal associations cannot be overstated. Impacting human health and economy, *Vibrio* bacteria are being probed for answers to some of the most pressing questions about how environmental factors influence bacterial fitness and biodiversity. Studies using models where both *Vibrio* bacteria and animal host can be studied independently or in symbioses, researchers have examined the effects of various abiotic and biotic factors that eventually shape these relationships (Chavez-Dozal and Nishiguchi, 2011; Chavez-Dozal et al., 2015). Evaluating not only both abiotic and biotic factors that facilitate *Vibrio*-animal relationships, but how various stressors influence genotype, physiology, environment, and geography, may help determine the basis for diversity among these unique symbiotic relationships (Piculell et al., 2008; Bright and Bulgheresi, 2010; Medina and Sachs, 2010; Pantos et al., 2015). Herein, the most common stressors that *Vibrio* bacteria encounter is discussed, including the free-living environment and animal hosts. Moreover, how these stressors can drive increased bacterial diversity in the free-living state and hosts is addressed. Three interesting empirical case studies have been completed with temperature and pH quite recently, which readers are encouraged to read (Cohen et al., 2019, 2020; Nourabadi and Nishiguchi, 2021).

TEMPERATURE DRIVES BACTERIAL SPECIATION

Vibrio bacteria have been observed in a wide range of habitats that vary in temperature, creating conditions that select for specific phenotypes that can survive and proliferate, both in their free-living mode as well as in their symbiotic lifestyle (Kimbell et al., 2002; Soto et al., 2009). Most notably, populations can vary, particularly when there are specific temperature gradients commonly found in nature (Jones et al., 2006; Coryell et al., 2018). For example, when sampling populations of *Euprymna tasmanica*, the sepiolid host squid for *Vibrio fischeri* in Australia, a distinct population break occurs within the symbionts where the habitat changes, particularly due to currents from the south (colder) and those from the north (warmer; Jones et al., 2006). This can also be observed in environments that have seasonal changes in water temperature, or those driven by currents (Coryell et al., 2018). Interestingly, when vibrio bacteria are "forced" into specific temperature regimes, they can rapidly adapt to survival outside the host, which oftentimes promotes the coevolution between hosts and their symbionts (Cohen et al., 2019). This temperature switch can subsequently facilitate gene expression of a wide variety of mechanisms that allow the bacterium to become more virulent to hosts (Kimes et al., 2002) as well as invade new host populations by relocating and surviving at a new environmental temperature (González-Escalona, 2015). Gene expression of such proteins such as Hsps (heat shock proteins), which are molecular chaperones that are upregulated in bacteria during heat stress (Madigan et al., 2018) are found to have an important role in the symbioses between nitrogen-fixing rhizobia (e.g., *Rhizobium* and *Bradyrhizobium*) and leguminous plants (Alexandre and Oliveira, 2016), but are not well studied in the Vibrionaceae. Albeit most stress comes in the form of higher temperatures (inciting stress in the host), drops in temperature can also elicit a change in gene expression (Liu et al., 2016), creating new ecological niches for those populations of *Vibrio* bacteria (**Figure 2**). This creates a situation where *Vibrio* bacteria must either adapt to these conditions or migrate to better environments (Richardson et al., 2012; Sunday et al., 2012).

OSMOTIC STRESS CHANGES PHENOTYPIC VARIATION

The Vibrionaceae have evolved mechanisms to cope with hypertonic and hypotonic stress. During hypotonic stress, cells must regulate to maintain the proper cytoplasmic concentrations of metabolites and ions, prevent lysis, and preserve ionic strength and pH (Bartlett, 2006; Nourabadi and Nishiguchi, 2021). Under hypotonicity, some vibrios may increase putrescine content to compensate for decreased K^+ that are necessary to stabilize the phosphate backbones of nucleic acids (Soto et al., 2010). Hypertonicity, however, promotes dehydration and shriveling of cells. Microorganisms must be able to import or synthesize counterbalancing solutes that are compatible with metabolic and physiological functions. K^+ uptake is a common mechanism to compensate for increased external osmolarity. Additionally, negative counter-ions (e.g., glutamate) must also be concurrently imported into the cell or synthesized *de novo* to sustain the same intracellular net charge (Sleator and Hill, 2001). Alternatively, cells can forgo K^+ uptake and import or synthesize neutral compatible solutes, as they carry no charge. Ectoine is such an example and its biosynthesis may be unique to the genus *Vibrio* (Bartlett, 2006). Interestingly,

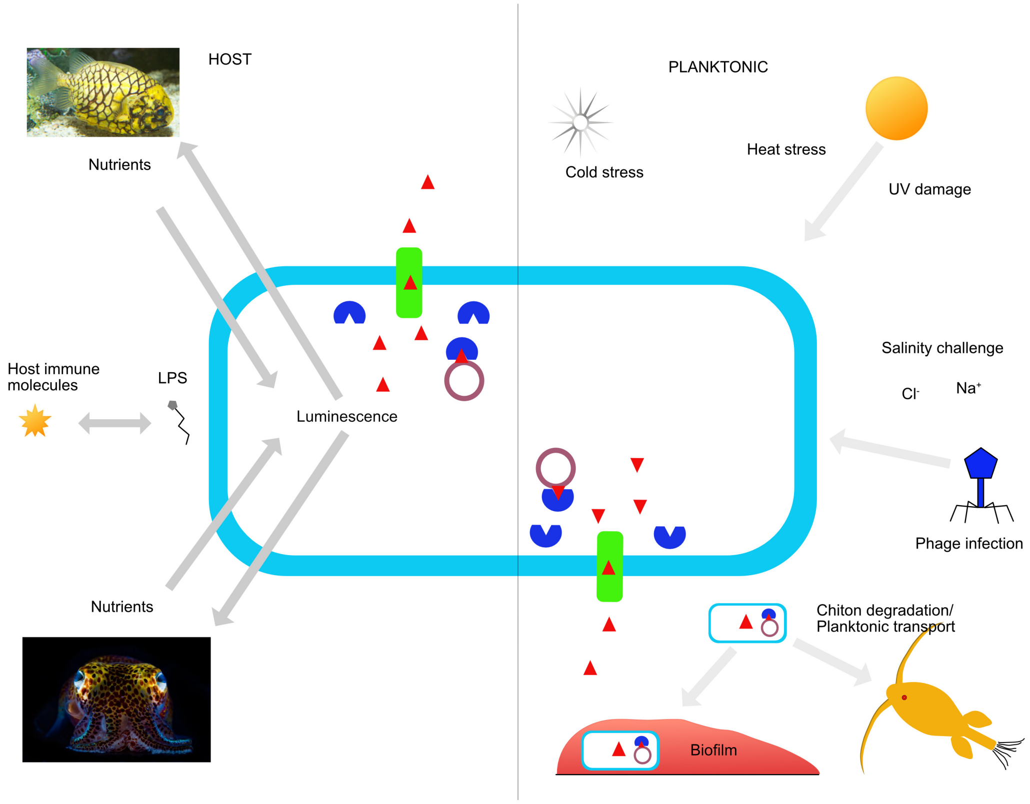

FIGURE 1 | Response to environmental cues inside and outside the host by *V. fischeri*. A diagrammatic representation of the various impacts and responses that *V. fischeri* endures as a result of planktonic or host associated factors. Some mechanisms are universal, but induce different responses according to the environmental context. One example is quorum sensing, used for light generation and host immune response suppression inside a host, while these same signaling and response pathways are employed planktonically to ensure survival and persistence when nutrients are limiting. Host factors drive specificity among certain ecotypes of *V. fischeri*, thus creating a hierarchy of diverse strains that range in host compatibility. See **Figure 2** for key.

Vibrio fischeri can synthesize disaccharide trehalose, which is also a neutral compatible solute. Vibrios can also incorporate polyunsaturated fatty acids in the cell membrane. This allows excess Na^+ to exit the cell during hypertonic stress (Valentine and Valentine, 2004). Unsurprisingly, more avenues are available to bacteria to neutralize high osmolarity than hypotonicity. As a result, there are few vibrios that tolerate freshwater, *Vibrio cholerae* and *Vibrio mimicus* are two exceptions (Soto and Nishiguchi, 2014). Evidence exists that osmolar stress can affect host-microbe interactions—including pathogenesis, mutualisms, and commensalisms.

Generally, microbial diversity peaks at optimal environments and decreases at extreme ones (Ruhl et al., 2018). However, this conclusion has not always held true for environments where microbial diversity was evaluated with respect to osmotic conditions. For example, habitat salinity has been found to be linearly and inversely related to diversity for all three domains of life (Ruhl et al., 2018). In fact, salinity explained 44% of the variation in numerous diversity metrics (OTUs, Shannon index, and Phylogenetic Diversity) within the domain Bacteria. In the domain Archaea, diversity has even been found to increase at higher salinities (Ruhl et al., 2018). Since a diversity of compatible solutes can be used as osmolytes (e.g., ions, sugars, polyols, amino acids, and their respective derivatives such as ectoines and betaines) within the domains Bacteria and Archaea, there are myriad ways microbial diversification can proceed at extreme salinities (Da Costa et al., 2006; Empadinhas and Da Costa, 2006).

ULTRAVIOLET LIGHT AND DNA DAMAGE INDUCE PHENOTYPIC CHANGE

In bacteria, the SOS response is the change in gene expression that occurs when there is extensive DNA damage (Fry et al., 2005). Consequently, many genes involved in DNA repair are upregulated, including DNA polymerases IV (*dinB*) and V (*umuCD*). In the Vibrionaceae, the SOS response involves the expression of at least twenty genes (Sanchez-Alberola et al., 2012). For microorganisms that routinely engage in host-microbe interactions, the microbial chromosome can be a consistent target for inflicting substantial DNA damage by host immune cells via a respiratory burst (see oxidative stress) (O'Rourke et al., 2003). In the Vibrionaceae, induction of the SOS response is frequently associated with increased virulence and pathogenicity. However, the SOS response is not necessary for toxin production and intestinal colonization in suckling mice (Quinones et al., 2006),

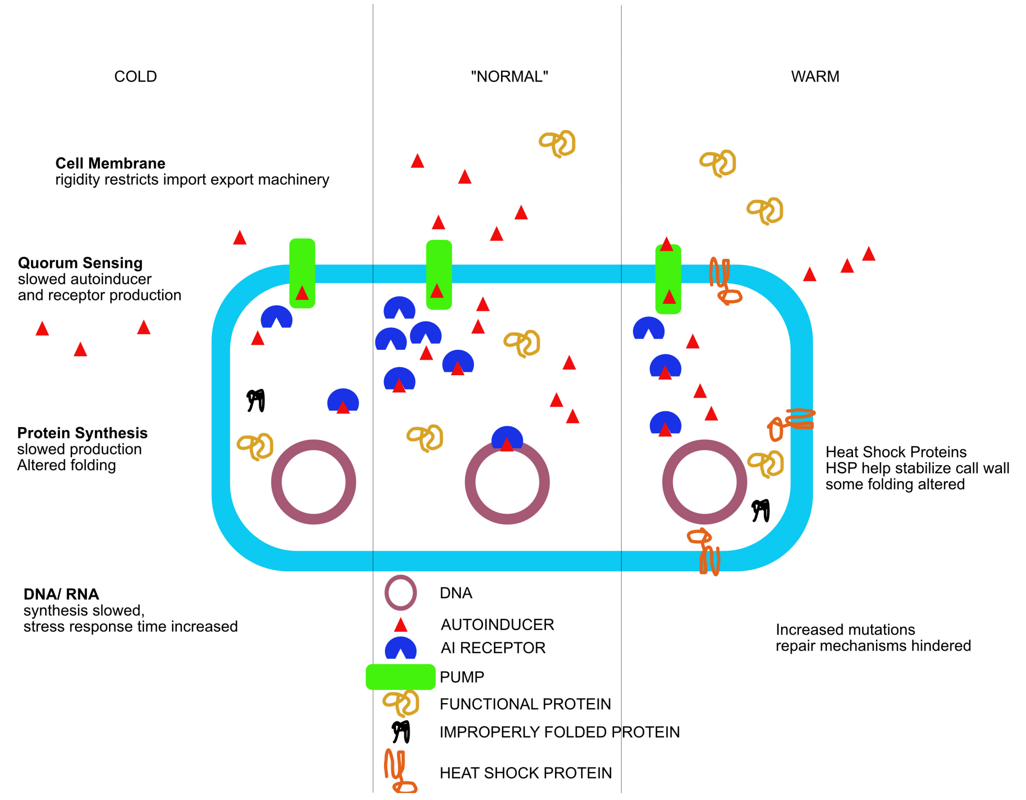

FIGURE 2 | The response of environmentally transmitted *Vibrio fischeri* to temperature stress as a driver for diversification. Cell features and processes are affected differently when presented with a cold (left side of figure), or hot (right side of figure) environmental challenge. Metabolic and signaling processes are altered in response to these temperature changes, effecting both genotype and phenotype. Subsequently, these adapted strains diversify and become a novel ecotype, possibly leading to speciation.

which is perplexing given the multitude of genes governed by the SOS system in *Vibrio cholerae* (Krin et al., 2018). Perhaps *Vibrio cholerae* utilizes other chromosomal repair systems such as base excision repair and mismatch repair, which can function independently of SOS (Davies et al., 2011). There is also the photolyase reaction, which is capable of DNA repair and requires light for activation (Thiagarajan et al., 2011; Yamamoto et al., 2017). *Vibrio cholerae* strains exist that are bioluminescent (Zo et al., 2009), and the visible light produced from bioluminescence can conceivably stimulate photolyase DNA repair. Photolyase DNA repair has also been proposed as an additional mechanism that is available to bioluminescent bacteria at night (Czyz et al., 2000). Thus, bioluminescent bacteria can continue to ameliorate DNA damage in the dark. Although light can be used as an activator for DNA repair, the roles of the SOS response and most bacterial DNA repair systems in the squid-*Vibrio* mutualism have not yet been rigorously investigated. Nonetheless, the photolyase reaction does not appear to have a major contribution to for combating stress in vibrio bacteria (Walker et al., 2006).

DNA damage caused by ultraviolet light can also lead to mutations that generate transient diversity via various mechanisms, which ultimately enhances a microbial population's chance of survival (Aertsen and Michiels, 2005). These mechanisms include transient and constitutive mutators and the activation of contingency loci. These processes can facilitate the targeting of mutations in the genome that are customized to the specific needs of a certain time and particular environment (Massey and Buckling, 2002). Additionally, mutations induced by ultraviolet light can also reveal genetic variation within a microbial population that was previously latent due to "genetic buffering" mechanisms (Aertsen and Michiels, 2005). Consequently, the revelation of cryptic polygenic variation becomes possible in a microbial population as a result of ultraviolet light as an environmental stressor. Thus, even within a clonal population, intrapopulation diversity can be manufactured to ensure survival after prolonged exposure to ultraviolet light (Booth, 2002).

OXIDATIVE STRESS

Reactive oxygen species (ROS) include superoxide anion, peroxides, hydroxyl radical, and other toxic chemical agents containing oxygen (Imlay, 2019). ROS are highly unstable and cause deleterious effects by oxidizing cellular components. Bacteria are constantly exposed to ROS due to endogenous biochemical reactions (oxidative phosphorylation) and extrinsic processes external to the cell. Hydrogen peroxide produced from the photooxidation of water, via UV light from the sun, is a common cause of exogenous ROS (Imlay, 2019). Bacteria have evolved a diverse array of tools to neutralize ROS. Two regulons that combat oxidative stress include *oxy*R and *sox*RS, which have been well characterized is *Escherichia coli* (Chiang and Schellhorn, 2012). The regulons *oxy*R and *sox*RS control at least 30 and 100 genes, respectively. *Oxy*R is mainly activated by the presence of hydrogen peroxide, while *sox*RS is stimulated by multiple ROS and other oxidizing agents. Catalase gene expression is also governed by *oxyR*. *Sox*RS is hypothesized to combat superoxide anion, including the upregulation of superoxide dismutase (Schellhorn et al., 2016). Both *oxy*R and *sox*RS are present within the Vibrionaceae (Vattanaviboon et al., 2003; Chiang and Schellhorn, 2012). Since superoxide anion and hydrogen peroxide are two ROS that regularly arise in living cells, bacteria have evolved regulons that are highly sensitive, including *oxy*R and *sox*RS. In *Vibrio vulnificus*, the *sox*RS regulon encompasses *cad*BA, a locus that encodes for a lysine-cadaverine antiporter and lysine decarboxylase, respectively (Kim et al., 2006). *cadBA* is involved in superoxide detoxification. The physiological response to oxidative stress at least partially overlaps with other bacterial stress responses, including heat shock (Storz et al., 1990). Several genes involved in the oxidative stress response are also controlled by *rpo*S, which is a stationary phase and general stress response regulator (Chiang and Schellhorn, 2012). Neutralizing the ROS produced by the respiratory burst of host immune cells is necessary for microorganisms involved in host-microbe interactions, including *Vibrio fischeri* during sepiolid squid colonization (Visick and Ruby, 1998). Additionally, bioluminescence, a key symbiotic phenotype in the squid-*Vibrio* mutualism is controlled by the redox-responsive regulator ArcA (Bose et al., 2007), where the ArcAB system represses the *lux* operon by ArcA binding and regulating the transcription of the *luxCDABEG* promoter. Although the deletion of *arcA* in *V. fischeri* increases luminescence *in vitro*, it does not repress symbiotic luminescence *in vivo* but rather causes a decrease in colonization competitiveness (Bose et al., 2007). Given that there may be alternative metabolic pathways that ArcAB regulates, as well as other genes responsible for reducing stress to oxygen and nitric oxide exposure (Aox; Dunn, 2018), the mechanisms of combating oxidative stress are complex. Oxidative stress also causes DNA damage that produces diverse subpopulations that can extend the range of conditions in which communities can thrive (Boles and Singh, 2008). These genetic variants are created due to double-stranded DNA breaks which are repaired by recombinatorial genes. In fact, this process is known to occur in biofilms, where endogenous oxidative stress is responsible for increasing the mutation rate as much as 10-fold (Boles and Singh, 2008; Steenackers et al., 2016). Little is known about the role of oxidative stress in *Vibrio* biofilms, but given the number of genetic regulators that modulate how the cell responds to oxygen and other respiratory stressors, they are more likely to have a major role in modulating the viability of biofilms in the environment and in possibly the development of the host light organs during symbiosis (Patelunas and Nishiguchi, 2018).

GRAZING AND PREDATION SELECT FOR MORE DIVERSE PHENOTYPES

Bacteria are prone to grazing and predation from numerous organisms. "Grazing" generally refers to consuming numerous prey individuals at once, whereas "predation" is used more commonly when the prey item is one individual (Atlas and Bartha, 1998). Vibrio bacteria have evolved numerous strategies to hinder being the prey of other organisms, especially against eukaryotic microbes such as ciliates and amoebas (Matz and Kjelleberg, 2005). After being ingested by protists, some vibrio species produce toxins which can kill grazers or predators. Other strains can block digestion and grow within the grazer or predator trying to feed on them. The grazer or predator is then killed or made ill by a bacterial infection (Matz and Kjelleberg, 2005). Additionally, oversizing and microcolony formation are other phenotypes that are selected to resist being preyed upon by microbial eukaryotes (Matz and Kjelleberg, 2005). Vibrios can also utilize their ability to develop complex biofilms that enables bacteria to escape grazing or predation, especially in aquatic habitats (Matz et al., 2005; Chavez-Dozal et al., 2013). Although vibrio bacterial biofilms can still be devoured, biofilms are generally less susceptible to bacterivory than their planktonic counterparts (cells in the water column) and the capability to produce strong biofilm varies from strain to strain. Recent data supports that predation selects for the hearty biofilm phenotype, and vibrio bacteria can use this strategy to their advantage to settle on various substrates or within their hosts (Chavez-Dozal et al., 2013; Chavez-Dozal et al., 2021).

BIOFILM REGULATION AND VARIABILITY IN *VIBRIO*

Vibrio biofilms may be regulated by various quorum sensing mechanisms. This entails the use of a secreted autoinducer (AI), namely a homoserine lactone (HSL) derivative that is produced by each individual cell and is secreted to the exterior environment. Small concentrations of autoinducer do not have any type of effect on the *Vibrio* bacteria themselves, but once they are in high enough concentration (e.g., a quorum), the HSLs are able to induce or inhibit gene regulatory pathways by their interaction with the regulatory gene for a specific operon(s). Thus, this chemical communication amongst same or similar species of *Vibrio* bacteria allow them to regulate specific genetic mechanisms that are important for the entire population of *Vibrio* bacteria to thrive and outcompete other bacteria for resources. Biofilms are particularly important for bacterial survival in the environment, since they provide a refuge for exposure to antibiotics, phage predation, and protozoan grazing than their planktonic counterparts. Many of the components that define *Vibrio* biofilms such as their extracellular matrix (Faruque et al., 2006; Smith et al., 2015), polysaccharides, and attachment pili are regulated by AIs, as well as their dispersal once the biofilm community is no longer viable. Since biofilms are especially important for *Vibrio* bacteria to colonize and persist in a specific host species or for their subsequent dispersal, the molecular signals which induce the production and continuity of these biofilms are tightly regulated and specific. Biofilm communities are also varied, thus contributing to the diversity of species and species complexes that are represented in both mono and multi-type biofilms.

The most well studied type of regulation that is linked to biofilm production is that found in *Vibrio cholerae*. Determining how cholerae is able to transition between the environment (where no virulence factors are expressed) to the human gut (where virulence is expressed) has been the forefront of most cholera research. Studies focusing on temperature acclimation as a cue as well as other abiotic changes have shown that the molecular mechanisms that transition the bacterium to a free-living, non-pathogenic state to its ability to inflict damage in the host gastrointestinal tract are intricately related to complex signals that determine the behavior of the bacterium. For example, the temperature "sensor" that is capable of regulating pili and other virulence related loci is important in how *V. cholerae* can be innocuous in the water column, yet benign until it is ingested by the proper host where conditions are ripe for growth and exploitation of host resources (Townsley et al., 2016). *V. cholerae* bacteria are also able to detect important sources of energy, such as chitin in the surrounding environment (Meibom et al., 2004; Markov et al., 2015). *V. cholerae* can metabolize chitin as an carbon source, and at the same time, produce high levels of ammonia and antiprotozoan compounds that reduce grazing pressure on biofilm structures (Sun et al., 2015). Moreover, recent studies investigating filamentous *V. cholerae* strains have found that these morphotypes are capable of producing biofilms without the presence of matrix proteins, and instead use filaments to attach to chitin when in their free-living (environmental) state (Wucher et al., 2019). Additionally, changes in osmotic pressure between the external environment and the *V. cholerae* biofilm matrix promotes expansion on certain surfaces by increasing the colony size and subsequently enhances nutrient uptake and expanding on areas where other bacteria may be growing (Yan et al., 2017). This change in osmotic pressure in the matrix allows the cells that produce the matrix proteins to outcompete the non-matrix competing strains (cheaters) through physical exclusion (Yan et al., 2017). Thus, *V. cholerae* biofilms are capable of adapting to various abiotic or biotic factors to establish and maintain biofilm structure and maintain a colony that is more resistant to planktonic cells trying to invade the biofilm. Given that not all *V. cholerae* strains are capable of expressing these behaviors, selection of these given morphotypes demonstrates the breadth and diversity that *Vibrio* bacteria and in particular, *V. cholerae* has obtained for its bimodal life-style.

Interestingly, marine mutualistic bacteria such as *V. fischeri*, form biofilms inside their host squids during colonization, and are maintained until the squid "vents" the bacteria from the light organ with the cue of dawn. The bioluminescent bacterium *V. fischeri*, which is in association with sepiolid squids (Cephalopoda: Sepiolidae) is beneficial to the host by providing light for a behavior termed counterillumination (Jones and Nishiguchi, 2004). Sepiolid squids use the bioluminescence to match downwelling moonlight at night when they are out hunting or finding mates. In return, the squid host provides nutrients and increases the growth rate by as much as four times compared to when the bacteria are in the environment (Soto et al., 2009). This mutualism has been a model to study beneficial symbiosis for over 30 years, and the mechanisms of biofilm formation and light production have been well studied (Visick, 2009). Biofilm formation in *V. fischeri* has been closely tied to the success of colonization and proliferation in squid light organs; bacteria first form a biofilm on the outside of the pores that eventually lead into the crypt spaces that lie within the light organ of the squid (Nyholm and Nishiguchi, 2008). Differences exist between symbiotic *V. fischeri*, and those strains that are free-living and cannot colonize host squids (Nishiguchi et al., 1998; Nishiguchi, 2002; Chavez-Dozal and Nishiguchi, 2011), which may be due to the fact that these free-living strains lack specific regulators for biofilm production that are responsible for colonization and persistence (Chavez-Dozal et al., 2021; Mandel et al., 2009; Thompson et al., 2018). Additionally, biotic factors outside of the squid, such as protozoan grazing, have impacted how *V. fischeri* (both symbiotic and non-symbiotic) is susceptible to selective pressures that influence biofilm production and are thought to have driven the diversity of various biofilm phenotypes observed in nature (Chavez-Dozal et al., 2013).

BACTERIOPHAGES

Phages definitely impose a tremendous burden on bacterial populations as ecological parasites or even predators (Atlas and Bartha, 1998). Phages can impose negative frequency-dependent selection on their hosts, which can in turn maintain high levels of diversity in bacterial communities within various ecosystems. However, phages can also benefit bacterial populations in numerous ways (Taylor et al., 2018). Some virologists even hypothesize that phage can even serve as mutualists for their bacterial hosts under certain circumstances. As prophages inserted into bacterial chromosomes, host cells can gain antibiotic resistance, immunity to other phages, beneficial alterations to motility and chemotaxis, and enhanced quorum sensing properties (Taylor et al., 2018). Moreover, prophages might enable lysogenized bacteria to utilize metabolites or nutrients that were previously inaccessible. Prophages can even permit lysogens to colonize novel eukaryotic hosts or to acquire new virulence factors (e.g., CTXΦ phage for *V. cholerae*). Prophages as lysogen symbionts might be especially relevant for prokaryotes colonizing eukaryotic hosts, including animals (Taylor et al., 2018). For instance, prophages sometimes enable bacteria and archaea to evade or suppress the immune systems of multicellular hosts (Mirzaei and Maurice, 2017). The role of phages in the mutualism between sepiolid squid and *Vibrio fischeri* is largely unexplored. Since seawater is continuous with the fluid in the squid light organ, phages in the ocean could conceivably enter the light organ crypts to influence the squid-*Vibrio* symbiosis (Soto and Nishiguchi, 2014).

Phage introduce genetic diversity in bacteria by promoting horizontal gene transfer via transduction (Jiang and Paul, 1998). Transduction can facilitate the dissemination of genes for antibiotic resistance, the catabolism of novel carbon substrates, detoxification of deleterious substances, and virulence factors (Qiu et al., 2009; Von Wintersdorff et al., 2016; Bhandari and Karn, 2019). Conceivably, the spread of loci involved in stress response and elevated tolerance to drastic environmental fluctuations can also occur through transduction. However, the exact role of phage transduction in the lateral transfer of stress response genes is unclear. Since stress response in bacteria may involve operons if not regulons, generalized transduction would perhaps be a better candidate than specialized transduction, since more genetic material can be passed onto new host cells by phage (Popa et al., 2017). If transduction is able to promote the horizontal transfer of stress genetic networks, phage and environmental stressors will have an even larger role in impacting prokaryotic diversity than previously considered. Interestingly, one mechanism involved in managing cell membrane stress is the phage shock response (Joly et al., 2010). Consequently, stress regulons might even be able to suppress their own transduction by phage to some extent. For instance, phage infection in *Vibrio alginolyticus* leads to upregulation of molecular chaperones that bind to nucleic acid (RNA and DNA), which could inhibit phage genome replication (Luo et al., 2018).

CONCLUSION

There is a need to ascertain how quickly organisms can adapt to different environments; with the noticeable change in global climate, it is to our benefit to determine if these changes will increase or decrease biodiversity (Duarte, 2014). Using model symbiotic associations to examine how microbial diversity responds to environmental change can potentially offer insights into the organisms, communities, and ecosystems that these microbes interact with. Understanding the interactions between genes, function, and the factors which select for specific *Vibrio* genotypes will help determine whether microbial associations with other organisms will survive in response to stress from the environment.

AUTHOR CONTRIBUTIONS

WS and MN designed and wrote the manuscript equally. Both authors contributed to the article and approved the submitted version.

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Spheres of Influence: Host Tree Proximity and Soil Chemistry Shape rRNA, but Not DNA, Communities of Symbiotic and Free-Living Soil Fungi in a Mixed Hardwood-Conifer Forest

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Host and symbiont diversity are inextricably linked across partnerships and ecosystems, with degree of partner reliance governing the strength of this correlation. In many forest soils, symbiotic ectomycorrhizal fungi coexist and compete with free-living saprotrophic fungi, with the outcomes of these interactions shaping resource availability and competitive outcomes for the trees aboveground. Traditional approaches to characterizing these communities rely on DNA sequencing of a ribosomal precursor RNA gene (the internal transcribed spacer region), but directly sequencing the precursor rRNA may provide a more functionally relevant perspective on the potentially active fungal communities. Here, we map ectomycorrhizal and saprotrophic soil fungal communities through a mixed hardwood-conifer forest to assess how above- and belowground diversity linkages compare across these differently adapted guilds. Using highly spatially resolved transects (sampled every 2 m) and well-mapped stands of varying host tree diversity, we sought to understand the relative influence of symbiosis versus environment in predicting fungal diversity measures. Canopy species in this forest included two oaks (*Quercus agrifolia* and *Quercus douglasii*) and one pine (*Pinus sabiniana*). At the scale of our study, spatial turnover in rRNA-based communities was much more predictable from measurable environmental attributes than DNA-based communities. And while turnover of ectomycorrhizal fungi and saprotrophs were predictable by the presence and abundance of different canopy species, they both responded strongly to soil nutrient characteristics, namely pH and nitrogen availability, highlighting the niche overlap of these coexisting guilds and the strong influence of aboveground plants on belowground fungal communities.

Keywords: aboveground-belowground linkages, ectomycorrhizal fungi, fungal trophic mode, neighbor effects, rDNA, saprotrophic fungi, spatial community turnover

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INTRODUCTION

Forest communities are shaped by symbiosis. Plant roots engage with staggeringly diverse communities of soil organisms: many of these interactions are symbiotic, and their effects on plant health and resource movement through soils can have profound impacts on the functioning of ecosystems (Baldrian, 2017; Crowther et al., 2019). One key symbiosis structuring many forests is the ectomycorrhizal association, an essential nutritional mutualism for roughly 60% of tree stems on the planet (Steidinger et al., 2019). The diversity of ectomycorrhizal fungal mutualists available to plant roots belowground has a powerful impact on the composition and structure of the plant community aboveground (Dickie et al., 2010; Gao et al., 2013; Koide et al., 2014). This partnership between plants and fungi also influences the structure and function of soil communities: Ectomycorrhizal fungi, with their reliable source of host carbon, can inhibit decomposition and mediate soil carbon storage by competing directly with saprotrophic fungi for soil resources (Gadgil and Gadgil, 1975; Averill and Hawkes, 2016), although this effect varies substantially based on the traits of the fungi and the environment in which they interact (Fernandez et al., 2020).

When sampled at a landscape scale, plant and fungal diversity are clearly linked. In forested systems, host specificity of the ectomycorrhizal community means that turnover in aboveground host tree community membership is mirrored belowground (Tedersoo et al., 2012; Moeller et al., 2015) and ectomycorrhizal fungal species richness is known to track the genus richness of available host plants (Gao et al., 2013). This allows for some predictive linkages between aboveground plant communities and belowground function: pine forests, for example, support a distinctive set of ectomycorrhizal fungi with conserved enzymatic capabilities (Talbot et al., 2013, 2014), and exhibit distinct soil chemistry and decomposition rates that are likely driven by these fungal associations. Yet aboveground-belowground linkages are complicated by environmental context and stochastic patterns of community assembly. Across the range of a single host tree species, the fungal community may turn over in response to climatological factors (Moeller et al., 2014; Bui et al., 2020) as well as dispersal limitation (Peay et al., 2010).

This context dependency becomes especially important when examining these above-belowground linkages at a fine spatial scale. Each tree influences and responds to a belowground rooting zone on the scale of meters, and is subject to habitat heterogeneity at that scale: it does not experience the habitat of an entire forest. The fungi, too, can extend from several centimeters to many meters belowground (Vincenot and Selosse, 2017), exposing them to substantial fine-scale variation in soil conditions. Competition among fungi for resource patches including decomposing organic matter (Fukami et al., 2010) and host root exudates (Smith et al., 2018) may also enhance community turnover at small scales (Pickles et al., 2012). Studies investigating the fine scale spatial turnover of fungal communities find that fungal community composition turns over on a scale ranging from centimeters to several meters (Lilleskov et al., 2004; Bahram et al., 2013), driven by factors such as host availability, host and fungal taxonomy, competition, and stochastic processes (Pickles et al., 2012; Bahram et al., 2013). Thus, communities of fungi and roots may be shaped strongly at the fine scale by factors that disappear when averaged across an entire landscape, obscuring the specific environmental conditions and species interactions that drive fungal diversity (Izzo et al., 2005).

Fine-scale coupling between belowground fungal communities and aboveground tree communities is shaped by how fungi interact with trees. Ectomycorrhizal fungi, reliant on host roots for carbon, might be expected to respond more strongly to host availability than saprotrophic fungi. Trees, however, have an enormous impact on the soil, in ways that go beyond carbon subsidies to their ectomycorrhizal partners. Litter inputs, for example, can vary both in quantity and in chemical quality across different host plants (Scott and Binkley, 1997). This variation could have profound effects on both ectomycorrhizal fungi and saprotrophic fungi, since both guilds must obtain nitrogen, phosphorus, and other resources from similar substrates; the outcomes of competition between these guilds may depend strongly on litter chemistry (Smith and Wan, 2019). Highlighting the powerful but divergent influences of tree identity on these guilds, Nguyen et al. (2016b) found that that host phylogenetic diversity can be an important influence on ectomycorrhizal fungal communities, while saprotrophs may be controlled more by chemical inputs such as plant leaf nitrogen and aboveground tree biomass. However, in other settings, ectomycorrhizal and saprotrophic fungi respond similarly to aboveground vegetation, revealing only subtle differentiation between the forces structuring these groups (Tedersoo et al., 2016; Hiiesalu et al., 2017). Both guilds of fungi should be influenced by aboveground plant communities, but in fundamentally different ways. It remains unclear when these distinct mechanisms should lead these guilds to respond similarly to aboveground plant communities, and when their responses should diverge on the basis of their different trophic strategies.

To understand how ectomycorrhizal and saprotrophic fungi respond to aboveground plant diversity, we must identify the fungi making up a given community. Fungal community characterization, and ectomycorrhizal characterization specifically, has traditionally relied on either sporocarp surveys or soil environmental DNA for the description of communities. As high-throughput sequencing technologies have become increasingly accessible, amplicon sequencing of the fungal internal transcribed spacer (ITS) region, a gene encoding a precursor component of mature ribosomal RNA, has become a standard for community characterization at the species level. Sequencing DNA, however, is an imperfect way to measure fungal communities: DNA can be preserved in soils for months to years (Nielsen et al., 2006), both as extracellular molecules sorbed to clays (Ogram et al., 1988; Morrissey et al., 2015), and within necromass and dormant propagules (Lennon and Jones, 2011; Carini et al., 2016). Fungal spores, too, can make it difficult to detect active community members using DNA, since fungi can disperse over great distances (Peay and Bruns, 2014; Horton, 2017), and their spores may persist in a dormant but intact state for many years (Bruns et al., 2009). To limit the influence of these forms of inconsistent data, researchers in the past have selected root tips from ectomycorrhizal plants and sequenced the individual fungi on each tip using Sanger sequencing (Moeller et al., 2014; Kumar and Satyanarayana, 2002; Janowski et al., 2019). This practice, though, is labor intensive and can be biased toward species with more conspicuous mycorrhizas.

Additionally, saprotrophic fungi cannot be assessed in a parallel way because they cannot be isolated by visual identification of host association.

Ribosomal RNA-based community profiling can produce a more functionally relevant snapshot of microbial community composition (Dlott et al., 2015; Sorensen and Shade, 2020), allowing researchers to characterize which members of a community have the greatest protein synthesis potential – that is, the greatest number of ribosomes (Blazewicz et al., 2013). Thus, rRNA-based community profiling can reflect the potentially active, functional community at a site at the time of sampling, while DNA-based profiling of rRNA genes, like ITS, reveals all the fungi whose DNA is present at the site, whether or not they are active members of the community (Baldrian et al., 2012; Barnard et al., 2013; Liao et al., 2014; Žifčáková et al., 2016; Wutkowska et al., 2019). The ITS region is particularly well suited to capturing the truly active members of a fungal community, because it is a precursor rRNA molecule absent even from dormant spores (van der Linde and Haller, 2013). The ITS region itself is spliced out before a ribosome becomes active (Schoch et al., 2012), so any fungi with measurable ITS rRNA molecules are in the process of producing new ribosomes. These fungi are more likely to be active than fungi present only as DNA, since they are actively building the protein synthesis enzymes that drive their ecological functions.

Here, we use both precursor rRNA and DNA-based ITS communities to examine the connections between the spatial turnover in aboveground plant communities and the turnover in soil fungal communities, comparing ectomycorrhizal (symbiotic) and saprotrophic (free-living) fungi across four transects with varied aboveground plant community composition. Using high resolution sampling and mapping of above-ground canopy trees, we created a spatially explicit set of influences on each sequenced soil community. Each sample had a unique set of soil chemical characteristics and continuous ectomycorrhizal host availability data. We extracted and sequenced amplicons from both RNA and DNA in each soil sample in order to compare turnover at the meter scale within both potentially active (precursor rRNA-based) and more broadly inclusive DNA-based community profiles. We anticipated that host plant diversity would have a more pronounced effect on the potentially active, RNA-based community than the longer-term, DNA-based community, and that the RNA-based community composition would be more variable across our samples. We also hypothesized that ectomycorrhizal fungal communities would be much more responsive to the availability of host trees than saprotrophs, but that both guilds of fungi would respond to soil resource and chemical properties.

MATERIALS AND METHODS

Sampling Site and Sample Collection

We conducted our study at the Sedgwick Reserve, part of the University of California Natural Reserve System, in Santa Barbara County, California in January, 2020. This southern California site experiences a Mediterranean climate of hot, dry summers and cool, wet winters (Davis et al., 2011), leading us to sample in the winter wet season when fungi could be expected to be most active. We selected four 30 m transects with varied aboveground plant community composition within 0.2 km of one another (**Figure 1**). The canopy species on our transects were gray pine (*Pinus sabiniana*, abbreviated PISA hereafter), blue oak (*Quercus douglasii*, abbreviated QUDO), and coast live oak (*Quercus agrifolia*, abbreviated QUAG). These two oak species are more phylogenetically distinct from the pine than one another, though blue oaks are deciduous and belong to the white oak group while coast live oaks are evergreen and belong to the red oak group (Plumb and Gomez, 1983). Previous studies have described distinct differences in the ectomycorrhizal (but not saprotrophic) communities associated with such phylogenetically diverged oaks (Morris et al., 2008), as well as between oaks and co-occurring pine species (Smith et al., 2009; Suz et al., 2017; Rasmussen et al., 2018).

Soils across the four transects derived from the same alluvium parent materials and were Chamise shaly loams (transects one, three, and half of two: Clayey-skeletal, mixed, active, thermic Ultic Palexerolls), Elder loams (transect four: Coarse-loamy, mixed, superactive, thermic Cumulic Haploxerolls), and Positas fine sandy loams (half of transect two: fine, smectitic, thermic Mollic Palexeralfs) (Soil Survey Staff, Natural Resources Conservation Service, and United States Department of Agriculture, 2020). We sampled each 30 m transect every 2 m (n = 16 soil cores per transect, including 0 and 30 m end points) by removing surface vegetation and using a hand auger to a depth of 25 cm. We emptied auger contents into an ethanol-sterilized bin and homogenized them by passing through a 4 mm soil sieve and mixing before collecting a sample for nucleic acid extraction. These samples were weighed into sterile 15 mL tubes (1–2 g soil per sample) and flash frozen on dry ice; samples were placed in a −80°C freezer within 24 h of collection. A separate 1-quart homogenized soil sample from the same core was placed in a clean plastic bag for chemical analysis and stored in a cooler with ice until placement in a 4°C refrigerator within 24 h.

We quantified aboveground plant community composition for each soil core by surveying host trees of all three species whose canopies extended to within 10 m of the transect (Day et al., 2010). For each individual tree, we measured diameter at breast height (DBH) and position relative to the transect (by measuring the distance of the tree to the nearest location on the transect tape to the nearest centimeter). This allowed us to create a high-resolution map of aboveground plant diversity. To compute the host influence on each soil core, we calculated the angular size of each tree relative to each sampling point (Hein et al., 2018), expressing the potential influence of each tree species as the percentage of each core's 360° panoramic field of view that was occupied by that host species. If a host tree was too far from a given soil core to include that core in its rooting zone – that is, if the core was outside the tree's sphere of influence – we excluded that tree from the calculation. We

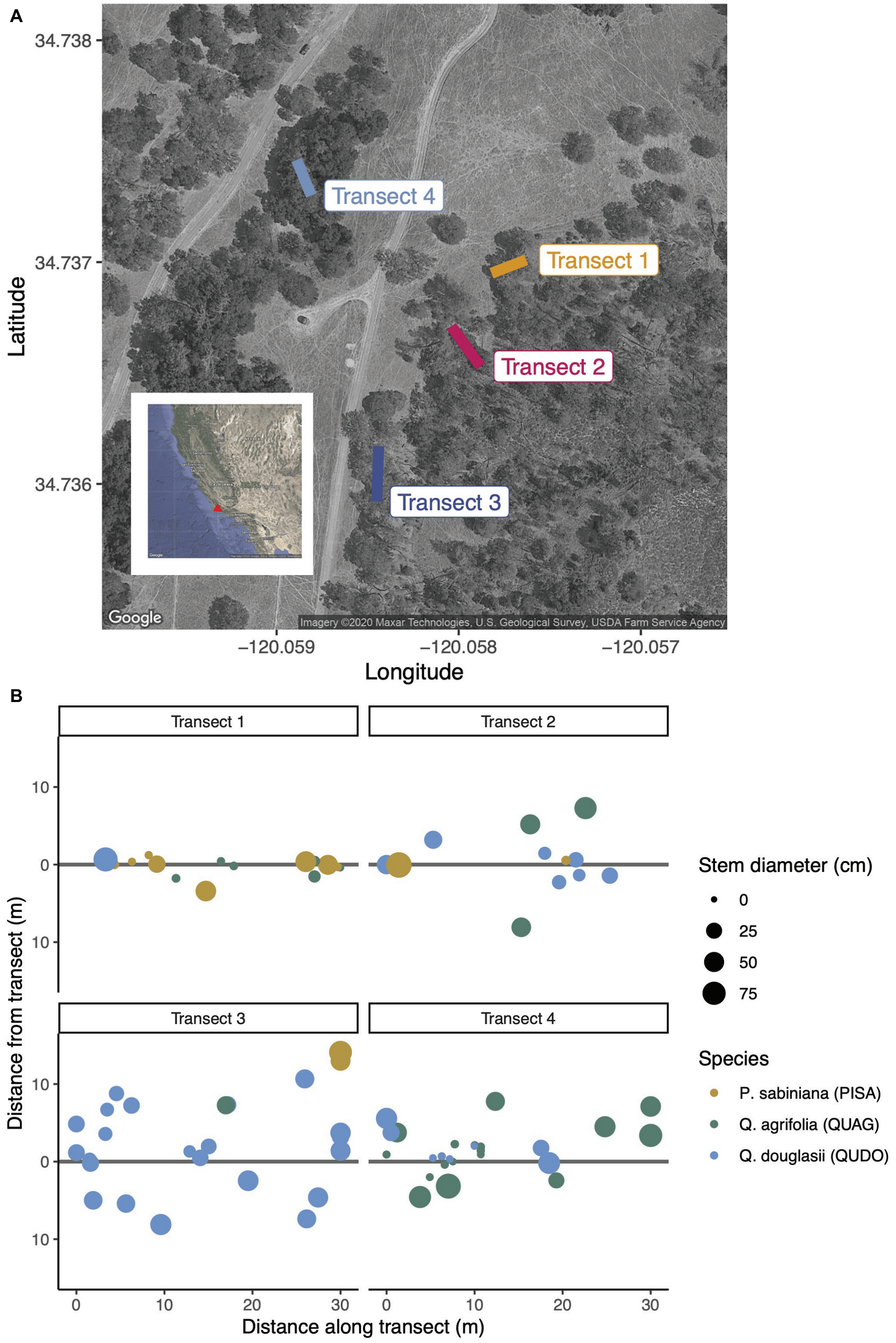

FIGURE 1 | (A) Four 30 m transects were selected to capture different canopy communities across the mixed conifer-hardwood forest at Sedgwick Reserve in Santa Barbara County, CA, United States. The maximum distance between two transect endpoints (Transect 3 and Transect 4) is approximately 175 m. **(B)** Canopy communities were inventoried based on their minimum distance to the transect. The species surveyed were *Pinus sabiniana* (PISA, brown circles), *Quercus agrifolia* (QUAG, green circles), and *Q. douglasii* (QUDO, blue circles). Circle size is proportionate to stem diameter (DBH), and distance from the transect represents physical location of each tree.

computed this distance as ½ of the tree's maximum rooting diameter, calculated using a formula from a meta-analysis linking maximum rooting radius (r) to DBH (Day et al., 2010):

$$r = 526.6 \times (1 - e^{-0.096 \times DBH}) \tag{1}$$

Soil Chemical Analysis

Within 1 week of sampling, we subsampled and weighed refrigerated soils, then dried them to constant mass in an oven at 45–60°C and reweighed them to determine percent moisture. The remainder of our soil analyses were performed at Brookside Laboratories Inc., on dried soil (OH, Untied States). Briefly, total carbon and total nitrogen were evaluated via dry combustion on an elementar vario EL cube and divided for site C:N ratios. Phosphorus was extracted via the Bray II method and other trace nutrients were extracted with Mehlich III extractions (data not shown). Soil pH was evaluated using a 1:1 soil to water slurry.

We compared soil properties between transects using analysis of variance (ANOVA) tests with a Tukey's honest significant difference post-hoc test to assign significance groups. We used linear regression to evaluate correlation between tree-driven parameters and soil chemical properties, selecting our models based on lowest AIC score when grouping all transects' data. From this point, we tested for Pearson's correlation coefficient between selected parameters to choose which would be included in later analyses.

Nucleic Acid Extraction and Sequencing

We co-extracted RNA and DNA from 1 to 2 g of soil using the Qiagen RNeasy Powersoil Total RNA Kit and RNeasy Powersoil DNA Elution Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. We reverse transcribed cDNA from the RNA extracts using the Qiagen OneStep RT-PCR kit and used Promega GoTaq Green mastermix for the DNA extracts. We amplified all samples with the ITS1 primer pair ITS1F-KYO1 and ITS2-KYO1 (Toju et al., 2012) and the following thermal cycler steps: 95°C for 3 min, 35 rounds of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s, then a final elongation step of 5 min at 72°C. We ligated Illumina indices with 3 min at 95°C, 35 cycles of 95°C for 30 s, 47°C for 30 s, 72°C for 30 s, then a final elongation step of 72°C for 5 min. Finally, we purified samples with AMPure XP beads and diluted them to 10 ng/uL before multiplexing all samples for sequencing in a single run. Amplicons were sequenced on the Illumina MiSeq platform with 250-bp paired-end reads at the California NanoSystems Institute (UC Santa Barbara).

Bioinformatics Pipeline

We processed amplicon sequences using the DADA2 pipeline (Callahan et al., 2016). To assess the broadest fungal community, we processed only forward sequences, as described in Pauvert et al. (2019). We removed adapter and primer sequences using the "cutadapt" package and removed chimeras before filtering (Martin, 2011). Our *DADA2::filterandtrim* settings removed any sequences with an "N" assignment and truncated reads at the first Q-score of 2 or lower, with a minimum read length of 50-bp. We chose not to rarefy our sequences due to the potential for false positives in differential abundance testing and our intention to use a consistent dataset through both richness and turnover (beta diversity) estimates (McMurdie and Holmes, 2014). We finally clustered our amplicon sequence variants (ASVs) at a 97-percent similarity threshold using the *DECIPHER* package before making taxon assignments on these operational taxonomic units (OTUs) with the UNITE database (Nilsson et al., 2019; Wright, 2020). We assigned fungal functional guilds using the FUNGuild database and retained OTUs called as either "probable" or "highly probable," further filtering for any OTU with "Ectomycorrhizal" or "Saprotroph" in its guild assignment to be in that guild for our analyses (Nguyen et al., 2016a). Finally, we removed any samples with fewer than 500 reads after guild assignment and also removed any OTU with only a single read to maintain the power of later analyses to infer differences between samples.

Statistical Analyses

We compared DNA and RNA amplicon communities from the whole library and as subsets assigned (through FUNGuild) to ectomycorrhizal or saprotrophic fungal guilds. To understand patterns of diversity between transects and nucleic acid extracts, we assessed the whole library for species richness as the total number of OTUs and the Shannon's diversity index using the *vegan* package in R. To quantify spatial community turnover (beta diversity), we also computed Bray–Curtis OTU community dissimilarities based on relative taxon abundance data with the *distance* function in the *Phyloseq* package (McMurdie and Holmes, 2013). We calculated differences across transects with linear regression and Tukey's honest significant difference tests. Comparisons of DNA to RNA diversity metrics within transects were assessed with two-sample *t*-tests.

To visualize patterns of fungal community turnover as they related to tree identity, we created non-metric multidimensional scaling (NMDS) plots of Bray–Curtis dissimilarity using the *metaMDS* function in the *vegan* package, looking specifically at the ectomycorrhizal and saprotroph guilds across RNA- and DNA-extracted community profiles. We used permutational analysis of variance tests (PERMANOVA; *adonis2* function in the *vegan* package) to quantify the influence of pine (PISA angular size) and oak (QUDO plus QUAG angular size) on community composition, grouping at the genus level to assess broader above-ground plant influence. In order to understand how much of the overall fungal community turnover could be attributed to forest canopy changes or soil core proximity, we employed Mantel tests on OTU and tree availability Bray–Curtis dissimilarity matrices using the *mantel* function in the *vegan* package.

To investigate how aboveground plant composition and soil chemistry interacted to drive fungal community turnover, we compared community dissimilarity matrices against environmental dissimilarity matrices including soil chemical data and canopy survey data, analyzing each of the same four groups as in the NMDS plots (ectomycorrhizal DNA, ectomycorrhizal RNA, saprotroph DNA, and saprotroph RNA).

For these analyses, we used the *bioenv* function to select the subset of environmental factors that were most strongly correlated with community turnover. In total, the list of factors selected for one or more groups included PISA angular size, QUAG angular size, QUDO angular size, pH, nitrogen percentage, soil phosphorus concentration, litter depth, and C:N ratio. From this set of predictors, we removed correlated predictors (C:N ratio, correlated with PISA, and litter depth, correlated with pH) and used all remaining predictors in a PERMANOVA test of each group to assess relative predictive power between canopy and environmental factors. For these tests, we included transect as a random predictor.

RESULTS

Site Properties and Aboveground Plant Diversity

Across our soil cores, we captured substantial diversity in tree species influence and soil chemistry (**Figures 1B**, **2**). On average, cores in Transects 1 and 2 had the greatest influence by gray pine (PISA), with very limited to zero pine influence in Transects 3 and 4, respectively. Cores in Transects 1, 2, and 4 (but not 3) exhibited considerable influence of coast live oak (QUAG), and all transects had large amounts of blue oak (QUDO), though substantial within-transect variation existed. Soil chemistry varied subtly but significantly in our study. We found significantly lower phosphorus levels in Transects 1 and 3 than in Transects 2 and 4. Nitrogen was variable across transects (**Figure 2E**), with Transect 1 having the lowest percent nitrogen and Transect 3 having the highest. We found small but consistent transect-specific differences in pH, though generally all sites were near neutral (**Figure 2F**).

Both pH and C:N ratio were strongly correlated with tree-driven factors. The influence of pine was correlated with C:N with a Pearson's r-value of 0.491 and a p-value of $p < 0.001$. Similarly, deeper litter depth was correlated with lower pH with a Pearson's r-value of 0.392 and a p-value of $p < 0.01$. To avoid including tightly correlated parameters in later analyses, we chose to drop C:N ratio and litter depth in favor of retaining PISA angular size and pH, respectively, in our PERMANOVA tests of community dissimilarity.

Belowground Fungal Diversity

We successfully extracted, amplified, and sequenced 61 of 64 DNA samples and 62 of 64 RNA samples. (The five unsequenced samples were randomly distributed among our study samples.) After filtering, our dataset contained just over 15.7 million reads, with an average of roughly 127,000 sequences per sample. We found 5,264 unique OTUs in 123 samples (see **Supplementary Figure 1** for species accumulation curves). Of these OTUs, 3,192 were observed in the RNA samples, 4,153 were observed in the DNA samples, and 2,450 were observed in both. Subsetting our data to ectomycorrhizal and saprotroph OTUs reduced our OTU count to 430 and 396 OTUs and 1.85 million and 1.58 million reads, respectively (see **Supplementary Figure 2** for a visualization of all fungi in dataset). RNA and DNA samples accounted for 46 and 54 percent of the total read counts, respectively. Species accumulation data suggest that read depth was closer to saturation in the RNA communities and that the subset of saprotrophic fungi were closer than either the ectomycorrhizal guild or the total community to saturating OTU richness (**Supplementary Figure 1**).

Operational taxonomic unit richness was stable across transects in the DNA communities, with no significant differences ($p < 0.05$), though Transect 3 had greater OTU abundance than the other transects in the RNA communities ($p < 0.001$, **Figure 3A**). DNA communities were generally more OTU-rich than their RNA counterparts, with significantly higher richness in Transects 1 and 4 ($p < 0.05$ and $p < 0.001$, respectively). Shannon diversity of DNA and RNA within transects was similar except for Transect 2 where the RNA community was significantly more diverse ($p < 0.01$) than in the DNA samples (**Figure 3B**). Richness and Shannon diversity trends were similar when rarefied data were analyzed with the most notable difference being a relative increase in richness in RNA samples versus DNA (**Supplementary Figure 3**). The most consistent difference in measured diversity was in fungal turnover (beta diversity). The DNA communities again were indistinguishable between transects but RNA varied widely (**Figure 3C**). Transect 1 had the highest spatial turnover (significantly higher than T2 and T3, $p < 0.05$ and $p < 0.001$, respectively) and Transect 3 the lowest (significantly lower than T1 and T4, $p < 0.001$ and $p < 0.05$, respectively). Across transects, each RNA community had significantly greater spatial turnover than the corresponding DNA community ($p < 0.001$ for all transects).

Drivers of Spatial Turnover in Belowground Communities

In our NMDS plots, aboveground plant diversity drove turnover in belowground diversity in fungal RNA but not DNA communities (**Figure 4** and **Supplementary Figure 2**). In the RNA samples, communities closer to a pine host separated from those closer to an oak host. Our host influence PERMANOVA revealed that, in the RNA communities, ectomycorrhizal fungal community turnover was significantly, but not strongly, predicted by both pine influence (pseudo-F = 2.794, $R^2 = 0.050$, $p < 0.001$) and oak influence (pseudo-F = 1.658, $R^2 = 0.030$, $p < 0.05$). The saprotrophic fungal community behaved similarly, although the influence of pine hosts was not statistically significant (pine: pseudo-F = 1.500, $R^2 = 0.027$, $p = 0.055$; oak: pseudo-F = 2.201, $R^2 = 0.040$, $p < 0.01$). Similar analysis of DNA-based communities did not reveal significant structuring by pine or oak host influence (**Supplementary Table 1**).

In RNA communities, the spatial turnover in fungal community composition was significantly predicted by spatial turnover in aboveground plant community composition. This relationship between above- and below-ground diversity was strongest when analyzing all fungi in the data set (Mantel $r = 0.25992$, $p < 0.01$), although it was also significantly predictive for ectomycorrhizal fungi ($r = 0.11021$, $p < 0.05$) and showed a weaker trend for saprotrophic fungi ($r = 0.1519$, $p = 0.056$).

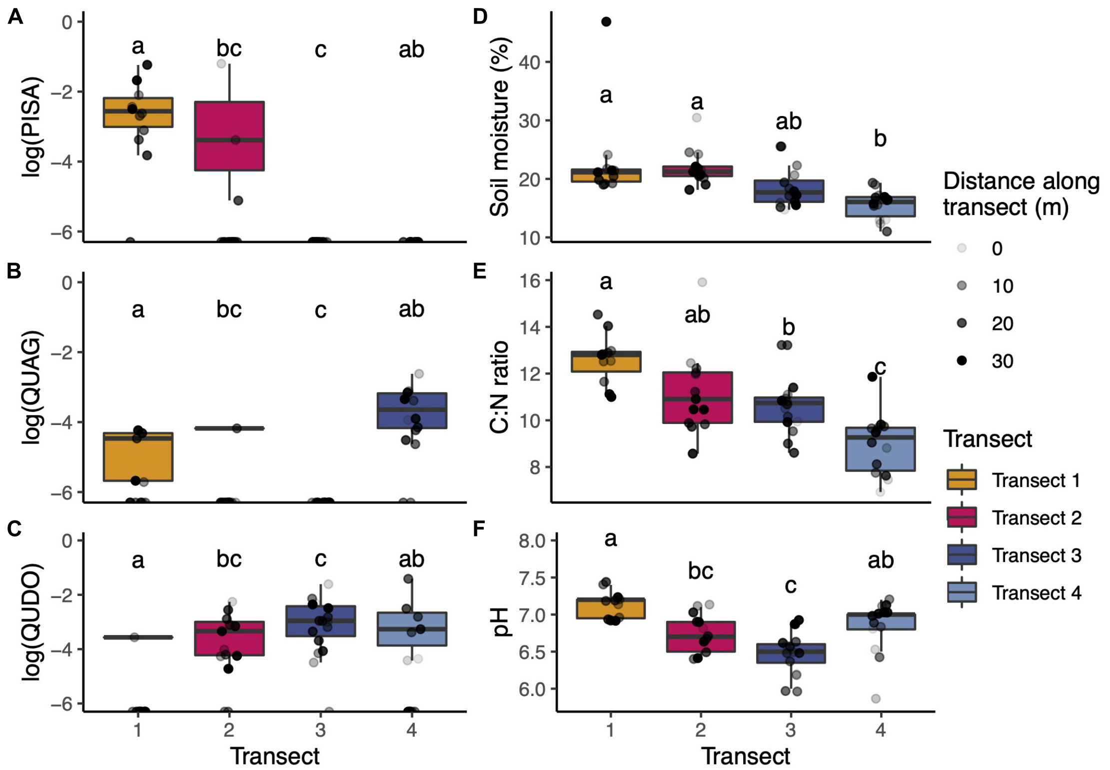

FIGURE 2 | (A–C) Aboveground plant and soil properties of transects. Transects varied in the composition of their aboveground tree communities, measured in proportion of each soil core's field-of-view represented by each host type (note log transformation of the *y*-axis). **(D–F)** Soil chemical properties were relatively consistent across transects with the greatest variation arising in C:N ratio (correlated with PISA). Boxplots span the 25th to 75th percentile with median values marked with the horizontal line. Whiskers depict at maximum ±1.5 times the IQR from the lower hinge. Data from individual soil cores are shown as points overlaid on each boxplot. Letters indicate statistically significant differences in transect means at the $p < 0.05$ level (ANOVA with Tukey's HSD correction for multiple hypothesis tests).

The DNA fungal communities did not respond significantly to aboveground plant turnover (**Supplementary Table 2**). RNA communities from samples taken closer to one another were not significantly correlated in Mantel tests of any community subset (**Supplementary Table 3**). DNA-based fungal communities did not exhibit any significant spatial autocorrelation (**Supplementary Table 3**).

Examining spatial turnover with the PERMANOVA allowed us to identify significant drivers of community turnover in the RNA samples, but not in the DNA samples (**Table 1**). None of our predictors explained more than 5% of the variation in spatial turnover for RNA-based fungal communities, however, and the entire model only accounted for 20 and 25% of the variation in saprotrophic and ectomycorrhizal communities, respectively. RNA community turnover was significantly predicted in both saprotroph and ectomycorrhizal communities by pH and soil nitrogen levels (**Table 1**). Additionally, saprotroph RNA communities were significantly predicted by the influence of QUDO hosts, while ectomycorrhizal communities were correlated with PISA influence and soil phosphorus. A similar model with transect as a predictor (instead of a random effect) yielded similar results, although transect identity explained approximately 8% of the variation in all communities when included explicitly as a predictor, and effects of soil chemistry and aboveground plant community were less strong than in the main analysis (**Supplementary Table 4**).

DISCUSSION

Understanding the ways in which aboveground diversity maps onto belowground patterns of spatial turnover in fungal communities is an important step toward predicting both the function and the vulnerabilities of forested ecosystems. We show here that both aboveground tree community composition and soil chemistry influence fungal communities regardless of fungal trophic mode, but that the chemical factors affecting symbiotic ectomycorrhizal fungi are not identical to those influencing free-living saprotrophs. These patterns were clearly discernible in the precursor rRNA communities, but were not significant for DNA communities.

Although both nucleic acid types allowed us to detect similar levels of fungal OTU richness, the precursor rRNA-based communities responded much more predictably to measured soil and plant factors than the DNA-based communities, which appeared to be mostly stochastic. This is likely due, at least in

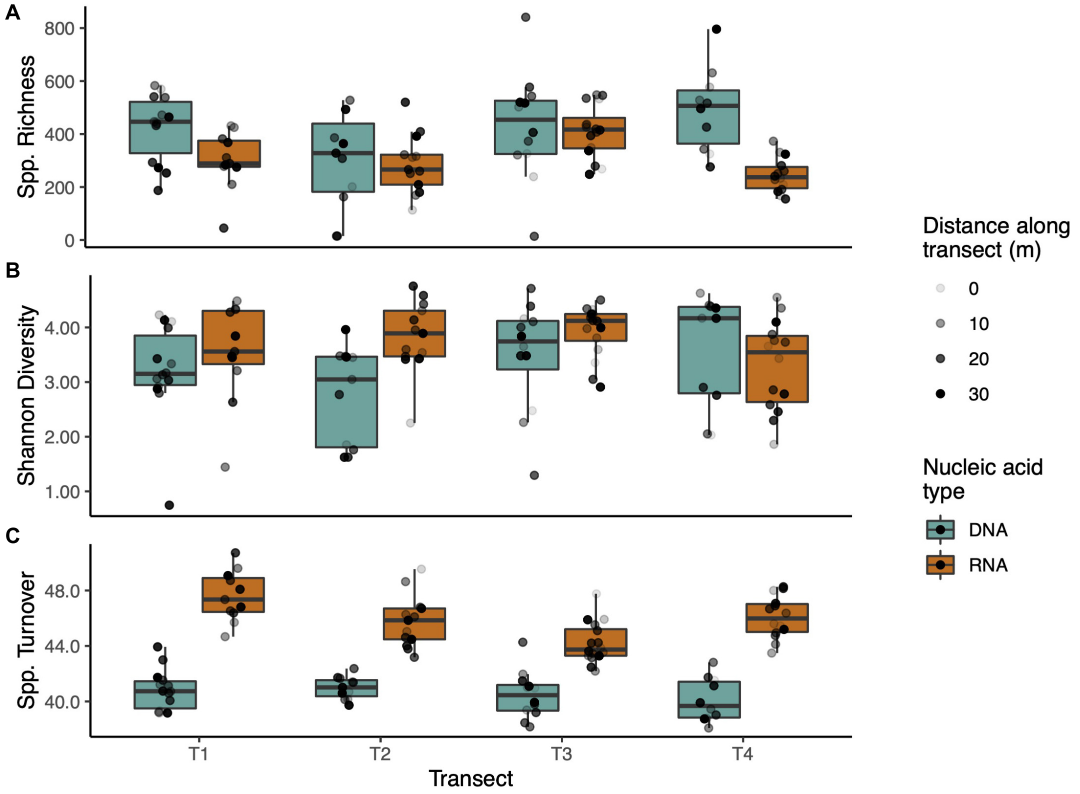

FIGURE 3 | Alpha and beta diversity of transects. **(A)** Transects did not differ significantly in species richness, though DNA communities generally captured greater richness. **(B)** Shannon diversity was consistent among transects and nucleic acid types. **(C)** Species turnover (beta diversity) was much higher in RNA communities than DNA communities. Boxplots span the 25th to 75th percentile with median values marked with the horizontal line. Whiskers depict at maximum ±1.5 times the IQR from the lower hinge. Points are scattered individual data points shaded by distance along the transect.

part, to the fact that precursor rRNA represents the metabolically active component of the fungal community (van der Linde and Haller, 2013; Wutkowska et al., 2019), and may therefore more closely mirror local environmental conditions. Supporting this possibility, other studies that have examined both nucleic acids in soils have also found that rRNA-based communities respond to a greater number of deterministic factors than DNA-based communities, or respond to those factors more strongly (Romanowicz et al., 2016; Lüneberg et al., 2018; Lasa et al., 2019; Meyer et al., 2019; Nawaz et al., 2019; Wutkowska et al., 2019). In some cases, like the present study, rRNA communities are the only ones in which deterministic effects on community structure are statistically detectable (Ragot et al., 2016; Gill et al., 2017). DNA- and precursor rRNA-based communities can exhibit more similar structures and environmental responses, but studies that find this tend to be conducted at a much larger spatial scale and to target substantially greater habitat diversity than ours did (Baldrian et al., 2012; Žifčáková et al., 2016). At the relatively fine spatial scale of our study, the potentially active fungal community (precursor rRNA-based) provided more useful ecological insight than the DNA-based community.

We found that soil pH, soil nutrients, and canopy species all had significant capacity to predict community turnover in the potentially active community for both saprotrophs and ectomycorrhizal fungi, but the variance explained by these factors was small (**Table 1**). This may be attributable to the fact that the levels of these factors did not, themselves, vary substantially at the relatively small spatial scale of this study. In this context, it is perhaps a testament to the importance of these soil factors that their influence was detectable even within this fairly limited range of values. Detecting these factors, which subtly but significantly influenced the structure of potentially active ectomycorrhizal and saprotrophic fungal communities, allowed us to disentangle how these distinct trophic guilds responded to their shared environment. We hypothesized that symbiotic ectomycorrhizal communities would track aboveground plant composition more closely than free-living saprotrophs, but this hypothesis was only partially supported by our precursor rRNA community data. In our study system, ectomycorrhizal community turnover was significantly predicted by pine influence, a result consistent with prior work on ectomycorrhizal root tips in mixed woodlands (Smith et al., 2009; Suz et al., 2017). The spatial turnover in saprotrophic fungi, by contrast, was significantly predicted by oaks (**Table 1**). Other belowground factors, such as nitrogen availability and pH, were significant predictors of both communities.

In part, effects on these two fungal guilds likely arise from the cascading effects of host trees on the soil community. While ectomycorrhizal host plants directly supply their belowground partners with carbon, they also indirectly influence the whole

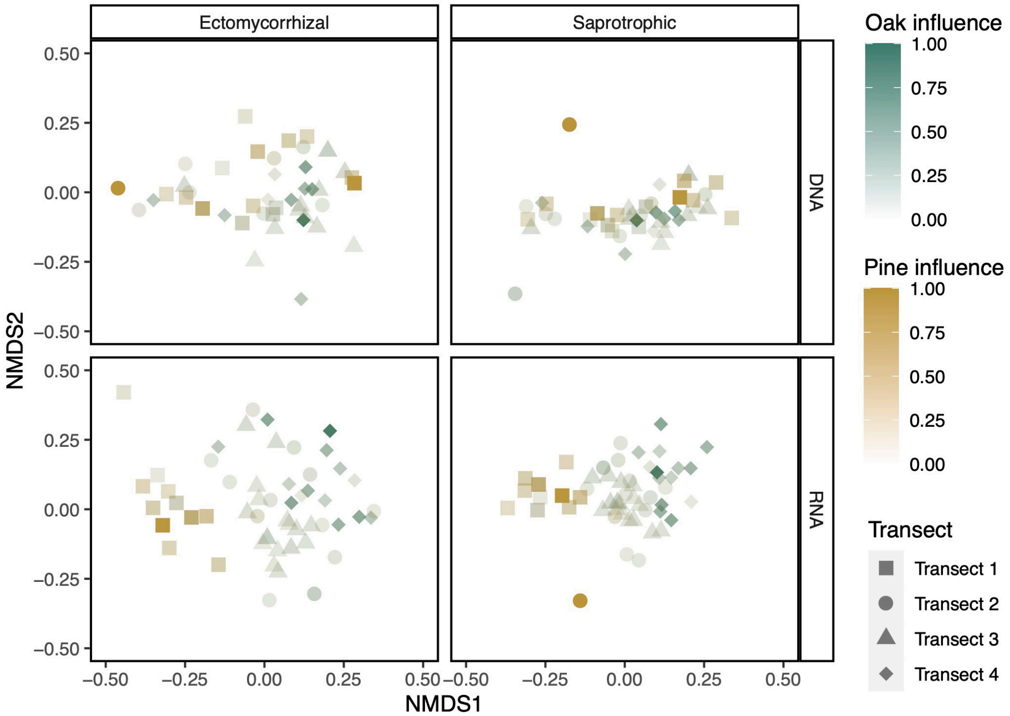

FIGURE 4 | NMDS plots show a separation by aboveground influence in both ectomycorrhizal and saprotrophic communities when displaying RNA but not DNA community data. Colors are scaled to the maximum influence of each canopy genus in any soil core. NMDS plots were constructed using Bray–Curtis dissimilarity.

TABLE 1 | PERMANOVA results show the overlap in predictive parameters between saprotrophic and ectomycorrhizal fungi.

	DNA				**RNA**			
	Saprotroph		**Ectomycorrhizal**		**Saprotroph**		**Ectomycorrhizal**	
	R^2	**Pr (>*F*)**	R^2	**Pr (>*F*)**	R^2	**Pr (>*F*)**	R^2	**Pr (>*F*)**
Gray pine (PISA)	0.0175	0.815	0.0095	0.988	0.0192	0.268	0.0269	**0.028**
Coast live oak (QUAG)	0.0168	0.859	0.0284	0.242	0.0202	0.235	0.0212	0.121
Blue oak (QUDO)	0.0191	0.754	0.027	0.278	0.0393	**0.004**	0.0223	0.095
pH	0.0136	0.964	0.0232	0.405	0.0329	**0.009**	0.0489	**0.001**
Phosphorus (mg/kg)	0.0169	0.877	0.0181	0.704	0.0186	0.348	0.0322	**0.01**
Percent nitrogen	0.02	0.674	0.0219	0.511	0.0434	**0.002**	0.0488	**0.001**
Residual	0.8867	NA	0.8684	NA	0.8048	NA	0.7576	NA
Total	1	NA	1	NA	1	NA	1	NA

Parameters were significantly predictive in both RNA communities but neither DNA community. Tests were performed on all transects together with transect identity integrated as a random effect. Pr(>F) values smaller than 0.05 were bolded in the table to help draw readers to significant values. P-values less than 0.05 are considered significant and are presented in bold.

fungal community through root exudates and leaf litter (Aponte et al., 2010; Hugoni et al., 2018). For example, blue oak litter shapes the seasonality of nutrient deposition (Callaway and Nadkarni, 1991) and understory productivity (Callaway et al., 1991) in California woodlands. Indeed, in our study, we found that the pine availability correlated with soil C:N ratios, and the depth of surface litter correlated with soil pH. Soil pH is a dominant factor predicting soil microbial community structure (van der Linde et al., 2018), and soil C:N ratios indicate the relative cost of decomposition of soil organic matter and, thus, its bioavailability to free-living saprotrophs (Manzoni et al., 2010; Smith and Wan, 2019). The overlap in these resource-availability predictors between ectomycorrhizal fungi and saprotrophs underscores the niche similarity of these fungal guilds (Bödeker et al., 2016), despite their different trophic strategies. Our results emphasize the profound influence that aboveground tree communities can have on belowground fungal assemblages, both directly, through symbiotic interactions, and indirectly, by mediating soil chemistry with litter inputs and root exudates. Ectomycorrhizal and saprotrophic fungi share an environment and are affected by similar environmental factors, but these guilds perform fundamentally distinct ecosystem functions (Talbot et al., 2013; Averill et al., 2014; Lindahl and Tunlid, 2015; Bödeker et al., 2016). Understanding how these guilds will be differentially affected by ecological stressors will be important for predicting their contributions to ecosystem function moving forward.

The strength of host identity effects in our ecosystem is consistent with results from prior studies in California woodlands, particularly studies showing the effects of oak host identity using DNA-based sampling of ectomycorrhizal root tips (Morris et al., 2008; Smith et al., 2009). Further, ectomycorrhizal communities diverged more strongly between oak and pine hosts, than between oak species, consistent with the phylogenetic distances of the host trees (Tedersoo et al., 2013). Host effects may be particularly strong in these woodlands, where the water-limited environment creates a patchwork of trees and grass cover (**Figures 1**, **2**; Borchert et al., 1991). This habitat mosaic results in greater spatial separation (and reduced rooting zone overlap) among host trees, potentially increasing our ability to detect differences in fungal community composition because of physical separation between hosts. In denser canopies, or more speciose systems, such signals might be more difficult to detect.

Despite the influences of these deterministic environmental factors on precursor rRNA-based communities, our analyses were unable to explain more than 25% of the variance in spatial turnover of potentially active fungal assemblages. The large amount of unaccounted-for variation likely stems from a combination of deterministic factors, such as unmeasured environmental variables and biotic interactions belowground, and the stochastic and partially stochastic processes well known to play important roles in community assembly, such as dispersal, ecological drift, and historical contingency (Peay and Bruns, 2014; Fukami, 2015; Vellend, 2016; Bogar and Peay, 2017). Although studies tend to agree that, for instance, pH and nitrogen are important in structuring fungal communities, it is not uncommon for much of the variance in fungal communities to remain unaccounted for even after modeling the influence of soil and plant traits, especially at relatively fine spatial scales (Romanowicz et al., 2016; Ren et al., 2018) or when focusing on functionally specific subsets of the total fungal community (Kivlin and Hawkes, 2016; Glassman et al., 2017; Daws et al., 2020). Because dispersal limitation likely played only a modest role in determining community membership at the fine spatial scale of our study (tens of meters) (Peay et al., 2012; Horton, 2017), it is especially striking that we found consistent, significant variation in the potentially active fungal communities predictable with soil and plant factors. Both tree identity and soil chemistry were significant factors structuring the active fungal communities in this study, even at a spatial scale where stochastic processes also likely exerted powerful influence.

CONCLUSION

In our study, active fungal communities, characterized with precursor rRNA (ITS), responded significantly to measured ecological factors, providing insight into the linkages between aboveground canopy composition and the community structure of symbiotic ectomycorrhizal and free-living saprotrophic fungi. DNA-based communities provided little ecological insight at this scale. The turnover of ectomycorrhizal and saprotrophic communities were differentially predicted by the availability of canopy species, though both were driven by similar soil properties. The overlap in predictive factors between the ectomycorrhizal and saprotrophic guilds reiterates their degree of niche overlap and the critical nature of symbiosis in structuring these communities. Our study suggests that, at relatively fine (meter) scales, precursor rRNA may allow researchers to identify ecological factors that affect fungal community function, while DNA would obscure these relationships. Future work should investigate whether precursor rRNA-based communities are, in fact, consistently more responsive to deterministic predictors than DNA-based communities, and at what spatial and temporal scales this is most true. In addition, manipulative experiments that isolate direct plant influences, such as symbiotic associations, from indirect plant effects on the soil environment, such as litter input, would provide valuable insight into the mechanisms driving differences between ectomycorrhizal and saprotrophic fungal community dynamics.

DATA AVAILABILITY STATEMENT

All analyses were performed in R [version 3.6.3 (R Core Team, 2020)] using the R Studio interface [version 1.2.5001 (RStudio Team, 2020)]. Scripts and data are available on Github (https://github.com/gaberunte/spheres_of_influence). Data files not stored on Github (due to size constraints) are available upon request from GR. Sequences are available through GenBank with Bioproject accession numbers PRJNA684619 and SRR13253516–SRR13253632.

AUTHOR CONTRIBUTIONS

LB, GR, and HM designed the study and led the sample collection effort. AS extracted all nucleic acids and prepared sequencing libraries with GR, and developed the host influence metric. LB and GR designed the analyses. GR conducted all data processing and statistical analyses. All authors contributed to manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.641732/full#supplementary-material

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