THE ROLE OF DISPERSAL AND TRANSMISSION IN STRUCTURING MICROBIAL COMMUNITIES

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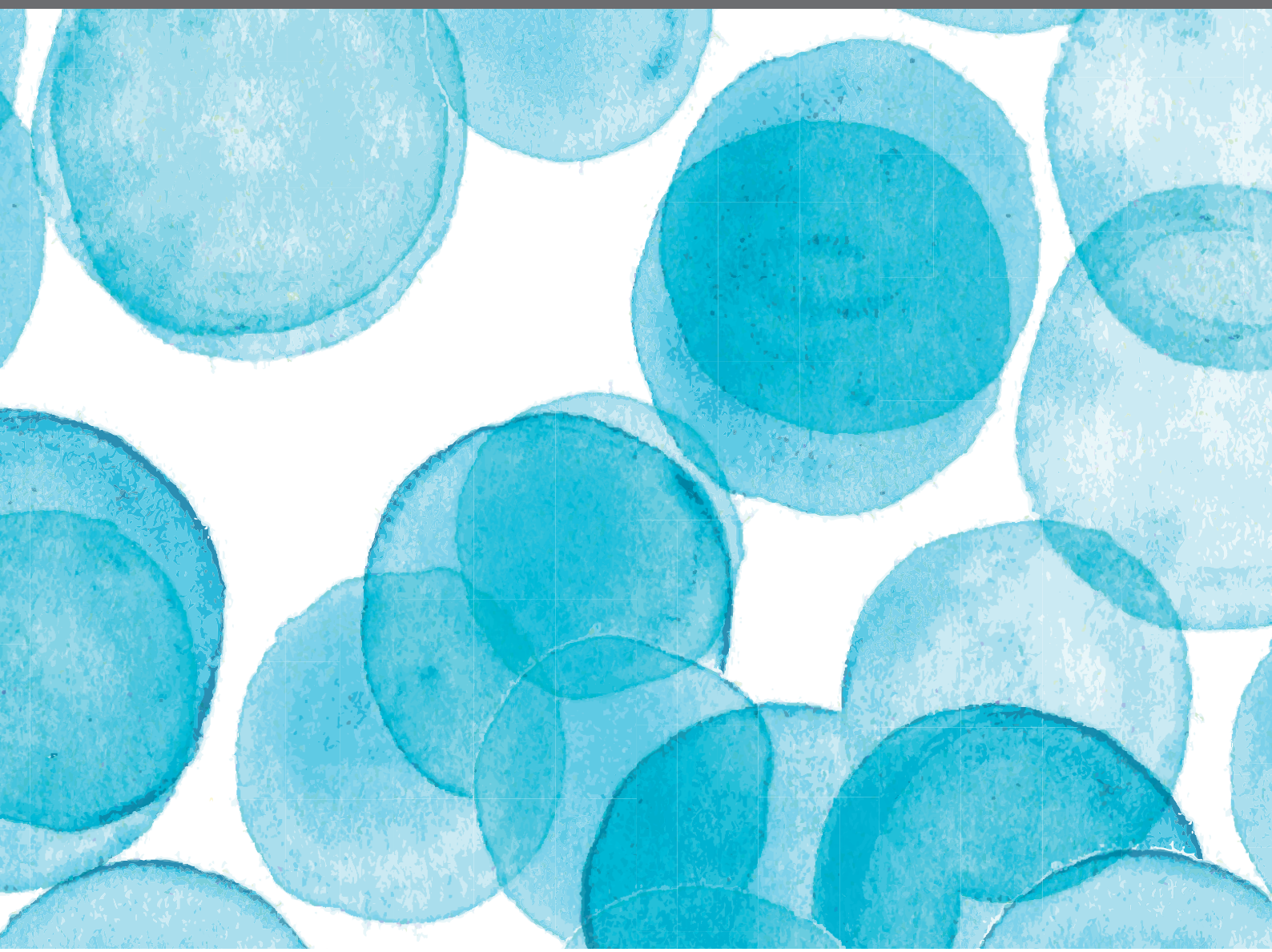

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THE ROLE OF DISPERSAL AND TRANSMISSION IN STRUCTURING MICROBIAL COMMUNITIES

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Editorial: The role of dispersal and transmission in structuring microbial communities

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microbiome, transmission, dispersal, symbiosis, microbial community diversity

Editorial on the Research Topic
The role of dispersal and transmission in structuring microbial communities

Microbial communities influence the systems they inhabit by driving ecosystem processes and promoting the health and fitness of plant and animals hosts. While an extensive body of work has documented variation in microbial community membership across hosts and systems, understanding the drivers of this variation remains a challenge. Much of the focus of these efforts has been on the characterization of host variation or the abiotic environment, and has overlooked the role of dispersal, i.e., the movement of organisms across space, and transmission, i.e., the movement of microbes among environments, hosts and between hosts and their environment. While quantifying or controlling the dispersal/transmission of microbial communities remains a technical and theoretical challenge, efforts to do so have led to insights into the assembly and function of microbial communities, highlighting the promising potential of a new wave of research. Our intention with this Research Topic is to draw attention to the important role that dispersal and transmission can play on the assembly, dynamics, and function of microbial communities and to highlight the unique ways that researchers are studying and conceptualizing microbial dispersal.

The study of dispersal and its implications for ecology and evolution have historically received more attention in macro-organismal communities (i.e., plants and animals) than microbial communities. Microbial ecologists can therefore take advantage of insights gained from plant and animal dispersal studies to examine similarities

and idiosyncrasies among macro- and microorganisms. Custer et al. provide a foundational review of the topic of microbial dispersal and its implications for microbial ecology and evolution. This review also highlights where microbial and macro-organismal communities may differ in regards to their dispersal abilities, e.g., through spore formation or exceptionally long dormancy periods, and how microbial communities may exhibit forms of community-wide dispersal that are less common in macro-organismal communities, such as community coalescence.

One important avenue that plant and animal hosts acquire members of their microbiome is through vertical transmission from parent to offspring. While this form of transmission is known to occur, the mechanisms by which it takes place are rarely characterized, including whether one or both parents contribute members. Baldassarre et al. experimentally induce spawning in the estuarine sea anemone *Nematostella vectensis* to track maternal and paternal contributions to offspring microbiome assembly. They present evidence for distinct bacterial contributions from mothers and fathers that are consistent across replicate families, suggesting both parents play an important role in vertical transmission of microbiome members. In Bathia et al. researchers using the freshwater polyp *Hydra viridissima* demonstrate that the animal's vertically-transmitted algal photobiont *Chlorella* alters the outcome of microbiome assembly. By rearing aposymbiotic (algae free) polyps in co-culture with algae-containing polyps, they demonstrate that the animals assemble distinct microbiomes, suggesting a role of the vertical photobiont in microbiome assembly. Together these works suggest both direct and indirect effects of vertical transmission on the microbiomes of invertebrate animals.

While vertical transmission is one important route for host colonization, many microbiome members arrive through horizontal transmission, i.e., from other hosts or the environment. Bergmann and Leveau provide a comprehensive review of how both vertical and horizontal acquisition modes shape the plant microbiome. They suggest a re-framing of plant microbiome assembly through the lens of metacommunity theory, which integrates principles of ecological filtering, species interactions, and dispersal across multiple scales. Weinhold also suggests a re-framing of microbiome acquisition by taking into account the spatial extent of animal movement, e.g., territory size, foraging range, and clustering behavior. Here it is argued that differences in these behaviors will directly impact microbiome acquisition through differential exposure to microbial diversity, and indirectly impact acquisition by altering the effects of local environment on the host. In line with these suggestions, Tipton et al. tracked the dispersal of arbuscular mycorrhizal fungi (AMF) that were experimentally inoculated onto host plants. They demonstrate that non-inoculated nearby plant neighbors can act as host "bridges" thereby aiding the dispersal of AMF, which may help guide ecological restoration attempts. Lastly, Bongrand et al. demonstrate that the horizontally acquired Hawaiian bobtail squid (*Euprymna scolopes*) symbiont *Vibrio fischeri* can undergo significant genomic diversification during its residence in the animal host and thereby influence *V. fischeri* population genetic structure in the water column between transmission events to other *E. scolopes* hosts.

Microbial transmission dynamics may also influence the onset of host disease, either directly through pathogen transmission or indirectly through the transmission of microbial taxa that interact with pathogens. Using tall fescue grass, O'Keefe et al. tested whether *Epichloë coenophiala*, a vertically transmitted fungal endophyte, protects the host against the pathogen *Rhizoctonia solani*. Contrary to expectations, they demonstrate that experimental inoculation of *Epichloë* actually increased the prevalence of host disease despite its reported benefits on host growth. In a similar realm, Ishaq et al. review the literature on how interactions between microbial transmission and environmental change may impact the health, disease prevalence, and future population sizes of the American lobster (*Homarus americanus*). In this study, authors suggests that changes to the American lobster's habitat range due to ocean warming may relate to the recent increased incidence of epizootic shell disease, either through heightened stress of the host, or through increased exposure to the causative pathogen in the water column. Together, these two studies highlight a gap in our understanding of the interplay between pathogen transmission and microbiome assembly, suggesting that a closer examination of these two processes may help shed light on the variability of disease outcome, especially under changing environmental conditions.

Beyond host-associated microbial systems, the study of microbial dispersal can shed light on the variation of microbial communities across different environments. Hariharan and Buckley examine the spatial structuring and dispersal limitation of soil *Streptomyces* along two elevational gradients, one steep gradient (>1,000 m) and one relatively less steep (<100 m). They conclude that the site with the steeper elevational gradient exhibits much higher beta diversity and suggest that this is likely driven by stronger species sorting due to sharper environmental changes. Maltz et al. also evaluate dispersal limitation using dust-associated microbial communities. Their study reveals effects of topography and elevation on microbial diversity and suggests that drought conditions may impose additional effects on dust microbial membership. Lastly, Hawkins and Zeglin examine how microbial inputs to the soil from bison dung may impact soil microbial community variation. They demonstrate that bison dung acts to increase local microbial diversity of the soil, but also drives increased community similarity over space, i.e. spatial

homogenization. Taken together, these studies highlight that dispersal limitation and environmental selection are two crucial factors to better understand environmental microbial community assembly.

To summarize, these 12 papers highlight the importance of microbial dispersal and transmission for understanding microbial community assembly. Hosts have evolved numerous mechanisms to transmit members of their microbiome to their offspring and a better understanding of these mechanisms will undoubtedly help researchers make sense of the enormous variation that exists in microbiome membership. Insights gained from the spatial structuring of microbial communities both in hosts and the environment will also shed light on the diversity of microbial dispersal strategies and environmental preferences. Finally, as demonstrated by several contributions to this special issue, dispersal and transmission may have important implications for microbiome functionality, including disease progression, microbe-microbe interactions, or host health. Thus by bringing together this diverse body of work we hope to illustrate the multifaceted role that dispersal and transmission play in microbiome dynamics.

Author contributions

KM wrote the editorial with input from all co-authors. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Contribution of Maternal and Paternal Transmission to Bacterial Colonization in *Nematostella vectensis*

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Microbial communities confer multiple beneficial effects to their multicellular hosts. To evaluate the evolutionary and ecological implications of the animal-microbe interactions, it is essential to understand how bacterial colonization is secured and maintained during the transition from one generation to the next. However, the mechanisms of symbiont transmission are poorly studied for many species, especially in marine environments, where the surrounding water constitutes an additional source of microbes. *Nematostella vectensis*, an estuarine cnidarian, has recently emerged as model organism for studies on host-microbes interactions. Here, we use this model organism to study the transmission of bacterial colonizers, evaluating the contribution of parental and environmental transmission to the establishment of bacterial communities of the offspring. We induced spawning in adult male and female polyps of *N. vectensis* and used their gametes for five individual fertilization experiments. While embryos developed into primary polyps, we sampled each developmental stage and its corresponding medium samples. By analyzing the microbial community compositions of all samples through 16S rRNA gene amplicon sequencing, we showed that all host tissues harbor microbiota significantly different from the surrounding medium. Interestingly, oocytes and sperms are associated with distinct bacterial communities, indicating the specific vertical transmission of bacterial colonizers by the gametes. These differences were consistent among all the five families analyzed. By overlapping the identified bacterial ASVs associated with gametes, offspring and parents, we identified specific bacterial ASVs that are well supported candidates for vertical transmission via mothers and fathers. This is the first study investigating bacteria transmission in *N. vectensis,* and among few on marine spawners that do not brood larvae. Our results shed light on the consistent yet distinct maternal and paternal transfer of bacterial symbionts along the different life stages and generations of an aquatic invertebrate.

Keywords: vertical transmission, horizontal transmission, marine symbioses, cnidaria, anthozoa

INTRODUCTION

Multicellular organisms originated in a world dominated by unicellular organisms. Thus, the current-day relationships of animals and microbes, from parasitism to mutualism, evolved most likely from ancient unicellular eukaryote–bacterial interactions (McFall-Ngai et al., 2013; Bosch and McFall-Ngai, 2021). In aquatic environments these relationships are essential components of animal health and physiology, influencing the nutrient cycling (Wegley et al., 2007; Raina et al., 2009; Lema et al., 2012; Santos et al., 2014), gut development (Rawls et al., 2004), resistance against pathogen colonization (Jung et al., 2009; Krediet et al., 2013; Fraune et al., 2015), osmoregulation and oxidative stress responses (Lesser, 1996; Bourne et al., 2016; Peixoto et al., 2017; Rosado et al., 2019), as well as larvae settlement and metamorphosis (Dobretsov and Qian, 2004; Hadfield, 2010; Tran and Hadfield, 2011; Huang et al., 2012).

Given the importance of these relationships, it is essential to understand how bacterial colonization is secured and maintained during the transition from one generation to the next (Bosch and McFall-Ngai, 2021). There are two ways animals acquire their bacterial symbionts, horizontal transmission, in which the bacterial symbionts are acquired from the environment, and vertical transmission in which the bacterial symbionts are transferred via the gametes or by direct contact with the parents. In most animals a combination of both mechanisms (mixed mode transmission) contributes to the establishment of early life bacterial colonization (Bright and Bulgheresi, 2010). While vertical transmission of bacterial symbionts facilitates the evolution and maintenance of mutualistic relationships (Koga et al., 2012; Bosch and McFall-Ngai, 2021), horizontal acquisition requires efficient host selection mechanisms to ensure appropriate bacterial colonization (Nyholm and McFall-Ngai, 2004; Franzenburg et al., 2013). For microbes that are transmitted horizontally, symbiotic life is facultative and free-living populations serve as reservoirs for colonization (Bright and Bulgheresi, 2010). In the marine environment, such free-living populations occur both in shallow and deep waters (Gros et al., 2003; Aida et al., 2008; Harmer et al., 2008) and, in some cases, are replenished by the release of symbionts from the host itself (Salerno et al., 2005). These bacteria provide a diverse pool of potential colonizers for horizontal acquisition and could confer advantages under changing environmental conditions (Hartmann et al., 2017).

As marine invertebrates have great diversity of life history, reproductive and developmental modes, they exhibit diverse modes of bacterial transmission (Russell, 2019). An accredited idea among authors was that species that brood their larvae, transmit symbionts to the next generations through direct contact of the parents with the offspring (Bright and Bulgheresi, 2010; Di Camillo et al., 2012; Bernasconi et al., 2019), while transmission in broadcast spawners, is dominated by horizontal transfer of bacteria from the surrounding water (van Oppen and Blackall, 2019). As more studies are being conducted, there are more evidences that do not necessarily support this idea (Nussbaumer et al., 2006; Apprill et al., 2012; Leite et al., 2017; Björk et al., 2019; Oliveira et al., 2020). Many broadcast spawners pass their symbionts to their offspring by incorporating them into the mucus that envelops oocyte and sperm bundles (Ceh et al., 2012; Ricardo et al., 2016; Leite et al., 2017) and a wide spectrum of mixed-mode transmission in brooders and in free and broadcast spawners is revealed (Sipkema et al., 2015; Fieth et al., 2016; Bernasconi et al., 2019; Damjanovic et al., 2020b).

Nematostella vectensis is an anthozoan cnidarian that lives burrowed in sediments of estuarine areas and is widely used as model organism in eco-evo-devo studies (Hand and Uhlinger, 1994; Fraune et al., 2016). *N. vectensis* reproduces both sexually and asexually and its full life cycle can be maintained under laboratory conditions. It is gonochoric and the sexual reproduction is triggered by changes in light exposure, food intake and increased temperature. Adult females release several hundreds of oocytes embedded in gelatinous sacks, while adult males release sperms directly into the surrounding water (Hand and Uhlinger, 1992; Fritzenwanker and Technau, 2002; Stefanik et al., 2013). The embryos develop inside the gelatinous sack and, within 1 to 2 days, ciliated planula larvae hatch from the oocytes and is released into the water where they remain freely swimming until settlement. After settlement, the planulae metamorphose into primary polyps. Under optimal conditions, the sexual maturity is reached within 3–4 months (Hand and Uhlinger, 1992).

Previous studies showed that adult *N. vectensis* harbors a specific microbiota whose composition changes in response to different environmental conditions and among geographic locations (Mortzfeld et al., 2016). In addition, sampling of different body regions (physa, mesenteries and capitulum) of the adult revealed a specific microbiota for each region, with specific dominance of spirochaetes bacteria within the capitulum (Bonacolta et al., 2021). Also changes in the diet lighting cycle induced differences in composition and relative abundance in *N. vectensis* microbiome (Leach et al., 2019). It has also been observed that different life stages (larva, juvenile, and adult) host specific microbiota (Mortzfeld et al., 2016; Domin et al., 2018).

In this study, we aimed at understanding how microbial symbionts are transmitted through generations and established in early life stages. Through metabarcoding of the 16S rRNA gene, we analyzed the microbial community composition in separated pairs of adult female and male polyps, and their corresponding newly released gametes, planula larvae and primary polyps. The comparisons to the corresponding medium microbiota allowed us to identify bacterial species that are specifically host associated and putatively maternally and paternally transmitted to the offspring.

MATERIALS AND METHODS

Animal Culture

Nematostella vectensis anemones were F1 offspring of CH2 × CH6 individuals collected from the Rhode River in Maryland, United States (Hand and Uhlinger, 1992; Fritzenwanker and Technau, 2002). Adult polyps were cultured in *N. vectensis* medium (NM) composed of 12.5 ppt artificial sea water (Red Sea) and maintained at 18°C in the dark. They were

fed five times a week with freshly hatched *Artemia* brine shrimps (Ocean Nutrition Sep-Art Artemia Cysts). Embryos were raised at 21°C in the dark and planulae or polyps were collected.

Spawning Induction and Fertilization

Five adult female polyps (labeled with numbers) and five adult male polyps (labeled with letters) were starved for 4 days prior to fertilization at standard conditions (18°C, in the dark), to avoid contamination from the food. A day before induction the animals were washed three times with sterile NM (autoclaved and filtered on 0.22 μm membrane) and separated into sterile six well plates. The induction was performed by exposing both males and females to increased temperature (25°C) and light for 13 h. After spawning, the adults were washed three times with sterile NM, snap-frozen in liquid nitrogen and stored at −80°C until DNA extraction. As control, the media where each female was kept before and during induction was filtered on a 0.22 μm membrane and stored at −80°C for DNA extraction. Sperms and oocyte sacks were individually collected into 1.5 ml tubes. Half of the sperms from each male polyp was collected for DNA extraction and the other half was used for fertilization of the oocytes. The sperms for DNA extraction were washed three times in sterile NM and stored at −80°C until processing. Each oocyte sack (here referred as oocytes) was washed three times in sterile NM and cut in two halves using a sterile scalpel; one half was collected for DNA extraction and the other half was fertilized. Four days post-fertilization (dpf), fertilized oocytes developed into planulae. Half of the planulae from each oocyte sack was washed three times with sterile NM and collected for DNA extraction, the other half was kept in the incubator for further development. Ten dpf, planulae developed into primary polyps that were washed three times with NM and collected for DNA extraction (**Figure 1**).

DNA Extraction and 16S rRNA Sequencing

The gDNA was extracted from adult animals, oocyte sacks, sperms, planulae, and primary polyps, with the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) as described in the manufacturer's protocol. DNA was eluted in 100 μL elution buffer. The eluate was kept frozen at −20°C until sequencing. For each sample the hypervariable regions V1 and V2 of bacterial 16S rRNA genes were amplified. The forward primer (5′- **AATGATACGGCGACCACCGAGATCTACAC** XXXXXXXX TATGGTAATTGT AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′- **CAAGCAGAAGACGGCATACGAGAT** XXXXXXXX AGTCAGTCAGCC TGCTGCCTCCCGTAGGA GT -3′) contained the Illumina Adaptor (in bold) p5 (forward) and p7 (reverse) (Fadrosh et al., 2014). Both primers contain a unique 8 base index (index; designated as XXXXXXXX) to tag each PCR product. For the PCR, 100 ng of template DNA (measured with Qubit) were added to 25 μl PCR reactions, which were performed using Phusion® Hot Start II DNA Polymerase (Finnzymes, Espoo, Finland). All dilutions were carried out using certified DNA-free PCR water (JT Baker). PCRs were conducted with the following cycling conditions [98°C—30 s, 30 × (98°C—9 s, 55°C—60 s, 72°C—90 s), 72°C—10 min] and checked on a 1.5% agarose gel. The concentration of the amplicons was estimated using a Gel Doc TM XR + System coupled with Image Lab TM Software (BioRad, Hercules, CA, United States) with 3 μl of O'GeneRulerTM 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA, United States) as the internal standard for band intensity measurement. The samples of individual gels were pooled into approximately equimolar sub-pools as indicated by band intensity and measured with the Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany). Sub-pools were mixed in an equimolar fashion and stored at −20°C until sequencing. Sequencing was performed on the Illumina MiSeq platform with v3 chemistry (Rausch et al., 2016). The raw data are deposited at the Sequence Read Archive (SRA) and available under the project ID PRJNA737505.

Analyses of Bacterial Communities

A total of 35 samples belonging to five separated families (five mothers, five fathers, five sperm batches, five oocyte sacks, five planulae batches, five primary polyps batches, and five medium controls) were submitted for 16S RNA gene sequencing. The 16S rRNA gene amplicon sequence analysis was conducted through the QIIME2 2021.4 platform (Bolyen et al., 2019). Sequences with 100% identity were grouped into ASVs and clustered against the SILVA RNA reference database (Quast et al., 2013; Yilmaz et al., 2014). Denoising and quality filtering were performed through the DADA2 plugin implemented in QIIME2 (Callahan et al., 2016, p. 2). A sample with less than 7000 reads was removed from the dataset, being considered as outlier. For the successive analysis, the number of ASVs per sample was normalized to 7000 reads.

Alpha-diversity was calculated using the Faith's PD, evenness, dominance and the total number of observed ASVs metrics implemented in QIIME2. Statistical significance was tested through the non-parametric Kruskal-Wallis test through QIIME2 and JASP 0.14.1 (JASP Team, 2020).

Beta-diversity was calculated in QIIME1 (Caporaso et al., 2010) and QIIME2 according with the different β-diversity metrics available (Binary-Pearson, Bray-Curtis, Jaccard, Weighted-Unifrac, and Unweighted-Unifrac). Statistical values of clustering were calculated using the non-parametric comparing categories methods PERMANOVA and Anosim implemented in QIIME2.

Bacterial ASVs associated with specific conditions were identified through LEfSe (Segata et al., 2011). LEfSe uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance, with respect to the biological conditions of interest; subsequently LEfSe uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature. To identify vertically transmitted bacteria ASVs, we performed pairwise comparisons of the surrounding medium microbiota with the microbiota of the *N. vectensis* samples. We were then able to infer ASVs associated with each animal life stage and therefore putative bacterial candidates for vertical transmission. The results obtained from LEfSe analyses were cross-checked against the outcomes of a logical test based on presence/absence

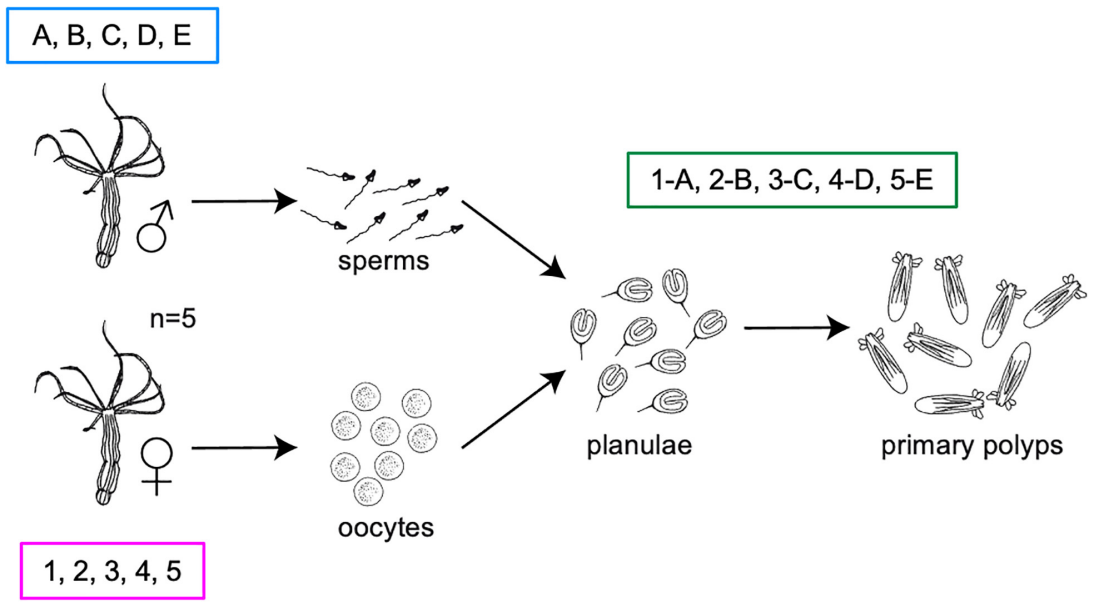

FIGURE 1 | Experimental design. Five pairs of parent polyps were induced for spawning. Their gametes and offspring were collected separately and used for further development and 16S rRNA gene sequencing.

data, performed directly on the ASV table generated for each sampled family. We assumed that a maternally transmitted bacterium should be present simultaneously in the microbiota of mother polyps, oocytes and planulae but absent from the medium and a paternally transmitted bacterium should be present in father polyps, sperms and planulae but absent from the medium. Bacterial ASVs that might be acquired horizontally from the medium by the early life stages were filtered by their concurrent presence in planula or primary polyps and medium and absence in the gametes.

RESULTS

Microbiota Associated With *N. vectensis* Tissues Is Distinct From Surrounding Medium

The experimental setup allowed us to assign gametes and offspring to their parent polyps in order to identify bacterial colonizers that are likely transmitted vertically among separated animal families and to differentiate them from the surrounding medium (**Figure 1**).

The sequencing was successful for 34 samples. After filtering and denoising, 2325 different ASVs were detected, with the number of reads per sample ranging between 678748 and 7026 (**Supplementary Table 1**). Beta-diversity analyses revealed that bacterial communities from the surrounding medium were distinct from those associated with host tissue (**Figure 2A** and **Table 1**), indicating a specific bacterial colonization in all life stages of *N. vectensis*. In contrast, α-diversity analyses revealed no significant differences between the bacterial communities of the medium to the bacterial communities associated with host tissues (**Figure 2B** and **Supplementary Table 2**). Although the medium showed a similar species richness compared to the animal tissues (**Figure 2B**), bacterial species in the medium showed lower diversity at the phylum level compared to those associated with the host (**Figure 2C**), e.g., Spirochaetota and Firmicutes are absent in the medium. In comparison, the host bacterial communities showed a higher evenness and lower dominance (**Figure 2C** and **Supplementary Table 2**) and harbored uniquely more bacterial species than the medium, with an overlap of 161 ASVs shared between both medium and host (**Supplementary Figure 1**). Given that the life stages of *N. vectensis* associate with specific bacterial communities distinct from the environment, a portion of these is likely to be non-random.

Specific Bacterial Communities Colonize Oocytes and Sperms

Analyzing the associated bacterial communities of *N. vectensis* according with their life stages revealed a clear clustering (**Figure 2D** and **Table 1**). Interestingly, the bacterial communities of sperms and oocytes were distinct from those of all the other life stages and from each other, indicating distinct mechanisms shaping the bacterial colonization of gametes (ANOSIM: $R = 0.444$, $p = 0.003$, **Figure 2D**), and a specific transmission from male and female parent polyps. Both planulae and primary polyps harbored similar bacterial communities (**Figure 2D**) that clustered between the sperms and oocytes samples, suggesting the contribution of maternal and paternal transmitted bacteria to early life stages colonization. While no significant differences in the bacterial α-diversity could be detected, sperms harbored bacterial communities with a slightly

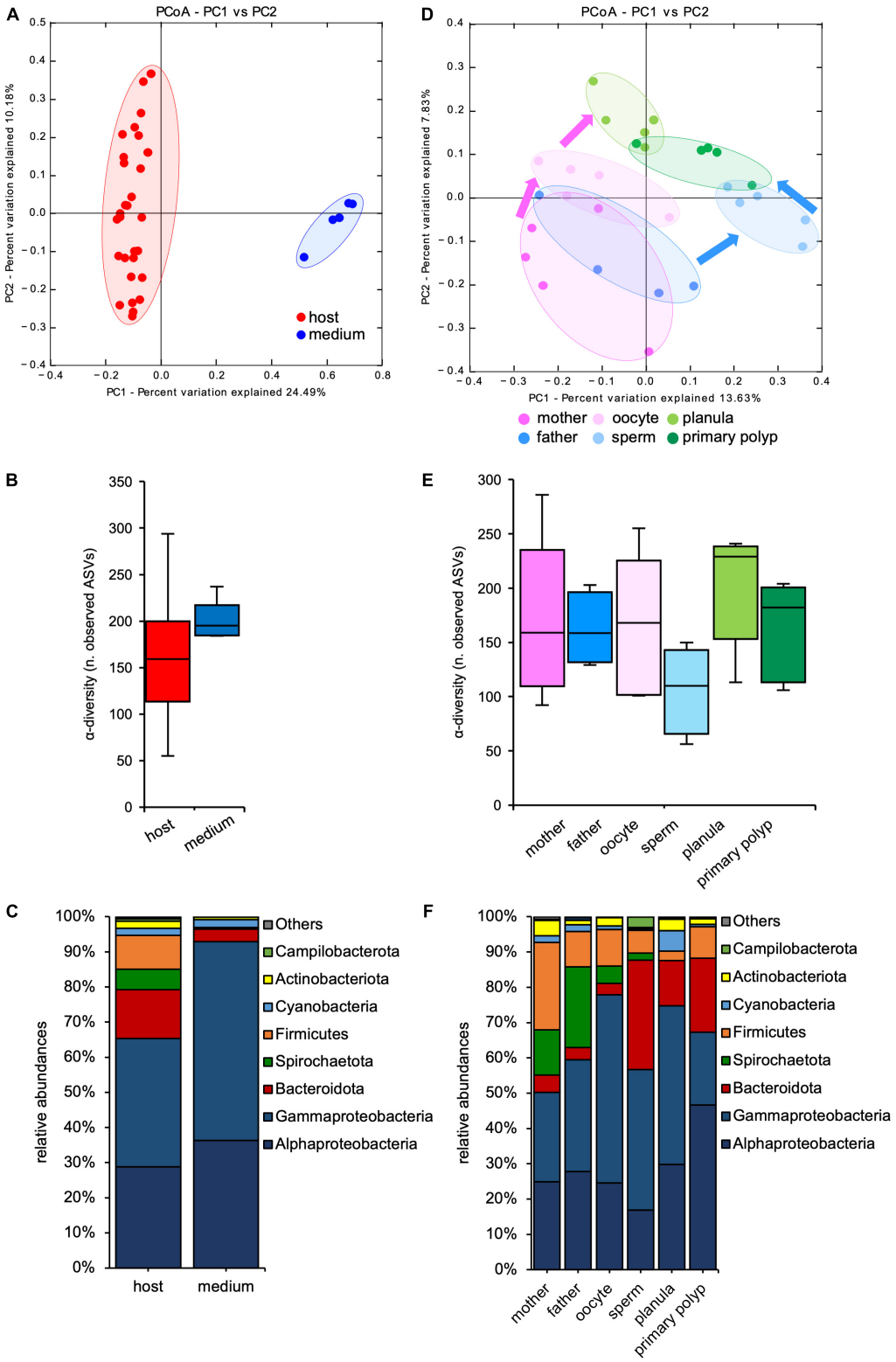

FIGURE 2 | Microbiota diversity analyses among sample source and life stage. **(A)** PCoA (based on Binary-Pearson metric, sampling depth = 7000) illustrating similarity of bacterial communities based on sample source; **(B)** α-diversity (observed ASVs) comparison of medium and animal tissue samples (max rarefaction depth = 7000, num. steps = 10); **(C)** relative abundance of main bacterial groups between host and medium samples; **(D)** PCoA (based on Binary-Pearson metric, sampling depth = 7000) illustrating similarity of bacterial communities based on developmental stage; **(E)** α-diversity (observed ASVs) comparison by developmental stage (max rarefaction depth = 7000, num. steps = 10); **(F)** relative abundance of main bacterial groups among different life stages.

TABLE 1 | Beta-diversity statistical tests comparing the different sample sources, the developmental stages and the families.

		ANOSIM		PERMANOVA	
Parameter	**β-diversity metric**	***R***	***p-value***	***pseudo-F***	***p-value***
host vs. medium	Binary-Pearson	0.987	0.001	10.130	0.001
	Bray-Curtis	0.940	0.001	9.757	0.001
	Jaccard	0.978	0.001	6.048	0.001
	Weighted-Unifrac	0.198	0.069	5.800	0.001
	Unweighted-Unifrac	0.865	0.001	6.559	0.001
life stage	Binary-Pearson	0.408	0.001	1.926	0.001
	Bray-Curtis	0.488	0.001	2.682	0.001
	Jaccard	0.458	0.001	1.544	0.001
	Weighted-Unifrac	0.468	0.001	3.553	0.001
	Unweighted-Unifrac	0.339	0.001	1.614	0.001
Family	Binary-Pearson	0.004	0.420	1.041	0.347
	Bray-Curtis	0.000	0.465	0.984	0.470
	Jaccard	−0.030	0.682	1.020	0.353
	Weighted-Unifrac	−0.031	0.698	0.978	0.467
	Unweighted-Unifrac	−0.031	0.683	0.975	0.573

Statistical analyses were performed (methods ANOSIM and PERMANOVA, number of permutations = 999) on each of the pairwise comparison distance matrices generated.

lower bacterial α-diversity compared to all the other samples (**Figure 2E** and **Supplementary Table 3**).

In contrast, no clustering (**Supplementary Figure 2A** and **Table 1**) and no differences in α-diversity (**Supplementary Figure 2B** and **Supplementary Table 4**) were evident according to family status.

Looking at the different bacterial groups, greater abundances of Spirochaetota (between 23.2 ± 17.2% and 5.2 ± 5.9%) and Firmicutes (between 27.3 ± 13.6% and 10.4 ± 7.8%) were evident in the adults and in the oocytes, while Bacteroidota were relatively more abundant in the sperms and in the offspring (between 27.6 ± 29.2% and 12.4 ± 12.4%). The abundance of Alphaproteobacteria increased from the adults (23.4 ± 10.9% and 27.2 ± 13.9%, respectively) through the primary polyps, in which they reached the maximum abundance (46.4 ± 9.3%) (**Figure 2F**). These differences among life stages and sexes suggest a differential transmission of specific bacterial groups through the gametes.

Offspring Bacterial Colonizers Originate From Oocytes, Sperms, and Surrounding Medium

By using the LEfSe algorithm (Segata et al., 2011) and the pairwise comparisons between host tissues and surrounding medium, we identified 15 ASVs that were significantly more abundant in mother polyps, oocytes, and planulae (**Figure 3A** and **Supplementary Table 5**) and 5 ASVs that were significantly more abundant in father polyps, sperms and planulae (**Figure 3B** and **Supplementary Table 5**), than in the surrounding medium. By overlapping these results with those obtained from the presence-absence calculations (**Supplementary Table 1**), we were further able to filter the LEfSe results to those candidates that were completely absent from the medium in any of the animal families (**Supplementary Table 6**). We ended up with 13 ASVs potentially transmitted by the mother and 5 ASVs potentially transmitted by the father (**Figure 3C**). The ASVs potentially transmitted by both mother and father polyps belong to the classes Alpha- and Gammaproteobacteria (**Supplementary Table 6**). In addition, one ASV potentially transmitted by mother polyps is a member of the Firmicutes phylum, while father polyps potentially transmit one member of the Bacteroidota phylum (**Figure 3C**). The high resolution of the presence-absence analysis, allowed us to also point out a slight variability in bacterial transmission that occur between different families (**Supplementary Table 6**).

In a final step we aimed at detecting bacterial ASVs that are present in the offspring and in the medium but not in the gametes, and thus, likely acquired horizontally from the medium. For planulae and primary polyps, we detected 24 and 25 ASVs, respectively, with an overlap of 10 ASVs shared between both developmental stages (**Supplementary Table 6**). In both planulae and primary polyps, these bacteria belonged to the classes Alpha- and Gammaproteobacteria, and the phyla Bacteroidota and Actinobacteriota. In addition, the primary polyps shared with the medium also four members of the Firmicutes (**Figure 3D**). These results suggest that each developmental stage is able to associate with different and specific environmental symbionts.

DISCUSSION

Maternal and Paternal Transmission of Bacterial Symbionts

A reliable transfer of specific symbionts is required to maintain the bacterial associations across generations. Thereby, vertically transmitted bacteria may represent beneficial symbionts,

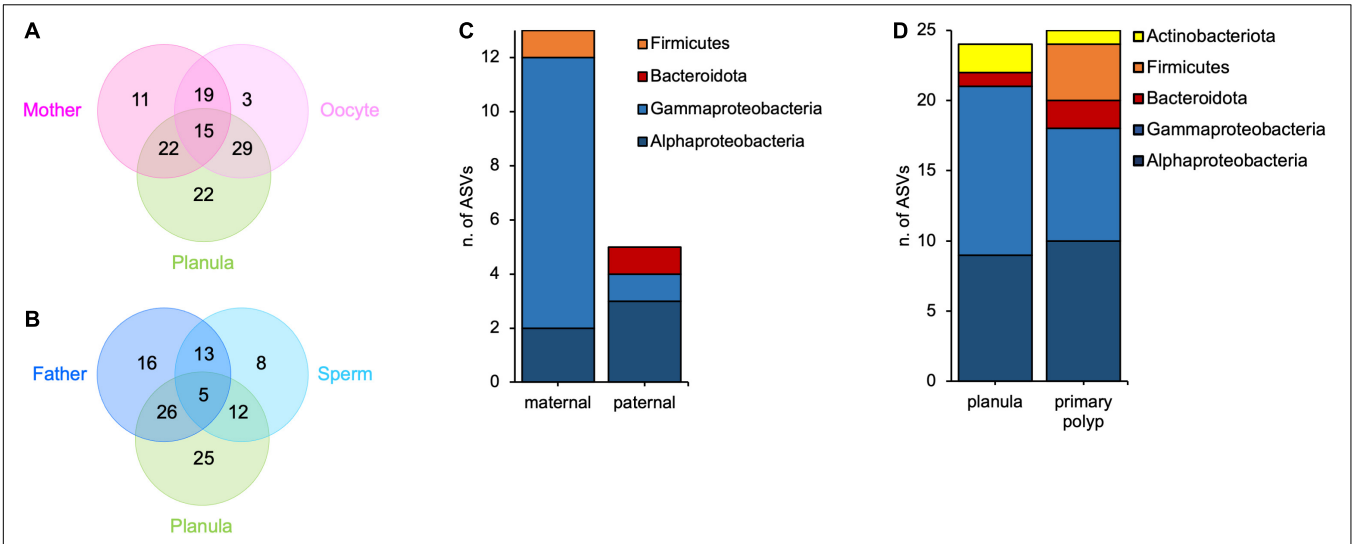

FIGURE 3 | Vertical and horizontal contributions to offspring microbiota. **(A)** Venn diagram showing the number of ASVs shared between mothers, oocytes and planulae; **(B)** Venn diagram showing the number of ASVs shared between fathers, sperms and planulae; **(C)** Bar-chart of parentally transmitted bacterial ASVs divided by major groups; **(D)** Bar-chart of environmentally transmitted bacterial ASVs divided by major groups.

necessary for animals' development and physiology, bacteria that lack a free-living stage, or more simply, bacteria that are present in the open water in too low abundance to be recruited (Salerno et al., 2005; Bright and Bulgheresi, 2010). So far, few studies have undertaken a detailed comparison of microbial communities of parents, gametes and progeny in marine organisms (Sipe et al., 2000; Bright and Bulgheresi, 2010; Sharp et al., 2012; Leite et al., 2017; Quigley et al., 2017, 2018, 2019; Bernasconi et al., 2019; Damjanovic et al., 2020a; Oliveira et al., 2020; Igawa-Ueda et al., 2021).

Our results, consistent with results obtained in former studies on broadcast spawning corals (Sharp et al., 2010; Quigley et al., 2017), indicate that *N. vectensis* transmits microbial symbionts to its offspring mainly maternally. Current knowledge is limited on the contribution of male parents to the progeny microbiota (Damiani et al., 2008; Watanabe et al., 2014; De Vooght et al., 2015); nevertheless, some examples exist regarding marine invertebrates (Usher et al., 2005; Sharp et al., 2010; Padilla-Gamiño et al., 2012; Kirk et al., 2013; Leite et al., 2017). In our study it seems that also male polyps transmit specific bacteria to the next generation.

As suggested from previous studies on corals, we hypothesize that in *N. vectensis* mother polyps incorporate bacterial colonizers into the mucus bundles that surround the oocytes (Ceh et al., 2012; Ricardo et al., 2016; Leite et al., 2017), while the sperms may acquire the bacteria before fertilization by horizontal transmission through water, which contains bacteria released by the parents (van Oppen and Blackall, 2019). This strategy is less specific than strict vertical transfer, however, not as non-specific as random horizontal acquisition of seawater communities (Ceh et al., 2013).

Our results are supported by fluorescence *in situ* hybridization (FISH) approaches applied on coral larvae and gametes, indicating the presence of bacteria in the ectoderm of brooded larvae (Sharp et al., 2012), but not inside gametes, embryos, and larvae of several broadcast spawners (Sharp et al., 2010).

Offspring Microbiota Results From Mixed-Mode Bacterial Transmission

Nematostella vectensis male and female polyps transmit different bacterial species through their gametes, with the oocytes contributing the most to the bacterial assemblage of the planulae, thus indicating distinct selecting forces. For instance, through the oocyte bundles, the mothers might provide the developing embryos with specific antimicrobial peptides (Fraune et al., 2010) and, in the case of *N. vectensis* also with nematosomes, multicellular motile bodies with putative defense function (Babonis et al., 2016). In vertebrates, like birds, fishes, and reptiles, passive immunity is transmitted through the deposition of antibodies in eggs (Grindstaff et al., 2003) and the fertilization envelope of fish eggs has demonstrated bactericidal activity (Kudo and Inoue, 1989).

Although not yet demonstrated, the bigger size of oocytes and the presence of a cytoplasm, in comparison to the sperm, may offer more room for carrying symbionts on the surface and/or intracellularly; sperms on the other hand, may carry strict symbionts in the nucleus (Usher et al., 2005; Watanabe et al., 2014). Additionally, the offspring can be partly colonized post-spawning through the uptake of microbial associates released by the parents into the surrounding seawater, as previously described (Apprill et al., 2009; Sharp et al., 2010; Ceh et al., 2012, 2013).

As already observed, the early life stages and adult microbiota in *N. vectensis* differ significantly (Mortzfeld et al., 2016; Domin et al., 2018). Our results suggest that a portion of the early life stages microbiota is the result of a parental transmission while another part is horizontally acquired from the environment

during ontogeny. This hypothesis is supported also by the tendency of higher bacterial species richness associated with the early developmental stages in comparison to that of the adults. Higher α-diversity in the early life stages has been described also in other studies on marine animals (Nyholm and McFall-Ngai, 2004; Littman et al., 2009; Fieth et al., 2016; Mortzfeld et al., 2016; Epstein et al., 2019) indicating an host filtering window during which the microbiota is shaped to a more stable community. This can relate with the different ecological (e.g., pelagic vs. benthonic, motile vs. sessile, preying vs. filter-feeding) and/or developmental requirements across the life stages of animals that have complex life cycles (Mortzfeld et al., 2016; Bosch and McFall-Ngai, 2021; Putnam, 2021). *N. vectensis*' life cycle includes a pelagic, freely swimming, not feeding planula larva and benthonic, sessile, preying primary polyp and adult stages. Therefore, it is easy to imagine that the symbiotic microbial community is impacted by the deep ecological and morphological changes during host development and that specific bacterial species may provide different benefits to the different life stages. As already pointed out (Fraune et al., 2010), organisms in which embryos develop outside the mother's body, in a potentially hostile environment, use a "be prepared" strategy involving species-specific, maternally produced antimicrobial peptides for protection. These antimicrobial peptides not only have bactericidal activity but also actively shape and select the colonizing bacterial community (Fraune et al., 2010; Fieth et al., 2016). It is likely that also *N. vectensis* is able to employ different mechanisms to shape and control its symbiotic microbiota in a life stage-specific manner.

Consistently with previous studies (Gilbert et al., 2012; Deines et al., 2017; Hernandez-Agreda et al., 2017; Goldsmith et al., 2018; Sullam et al., 2018; Glasl et al., 2019; Berg et al., 2020), our results showed that between different parents a slight variability of vertically and environmentally transmitted bacteria exists, highlighting the potential impact of host genotype and stochastic events on symbiotic community establishment of offspring.

The hologenome theory of evolution (Zilber-Rosenberg and Rosenberg, 2008) proposed that microbiome mediated plasticity of the host phenotype can be under selection pressure and may contribute to animal adaptation. Vertical transmission of the microbiota could therefore facilitate transgenerational adaptation of animals to changing conditions (Webster and Reusch, 2017), while horizontal transmission may mitigate some of the deleterious consequences of obligate host-association/strict vertical transmission (e.g., genome degradation and reduction of effective population size) (Russell, 2019). Concertedly, vertical transmission may secure the maintenance of coevolved beneficial bacteria, while horizontal acquirement of new bacterial partners increases the flexibility of beneficial effects under changing environmental conditions. Future studies should compare the function of vertically transmitted and horizontally acquired bacterial associates, providing important insights into the potential of microbial communities to promote animal adaptation to changing environmental conditions.

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 in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

LB, SL, and RB-S performed the experiment. LB performed the statistical analysis. LB and SF wrote the first draft of the manuscript. All authors contributed to conception and design of the study, manuscript revision, read, 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/fmicb.2021.726795/full#supplementary-material

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Bowel Movement: Integrating Host Mobility and Microbial Transmission Across Host Taxa

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The gut microbiota of animals displays a high degree of plasticity with respect to environmental or dietary adaptations and is shaped by factors like social interactions, diet diversity or the local environment. But the contribution of these drivers varies across host taxa and our ability to explain microbiome variability within wild populations remains limited. Terrestrial animals have divergent mobility ranges and can either crawl, walk or fly, from a couple of centimeters toward thousands of kilometers. Animal movement has been little regarded in host microbiota frameworks, though it can directly influence major drivers of the host microbiota: (1) Aggregation movement can enhance social transmissions, (2) foraging movement can extend range of diet diversity, and (3) dispersal movement determines the local environment of a host. Here, I would like to outline how movement behaviors of different host taxa matter for microbial acquisition across mammals, birds as well as insects. Host movement can have contrasting effects and either reduce or enlarge spatial scale. Increased dispersal movement could dissolve local effects of sampling location, while aggregation could enhance inter-host transmissions and uniformity among social groups. Host movement can also extend the boundaries of microbial dispersal limitations and connect habitat patches across plant-pollinator networks, while the microbiota of wild populations could converge toward a uniform pattern when mobility is interrupted in captivity or laboratory settings. Hence, the implementation of host movement would be a valuable addition to the metacommunity concept, to comprehend microbial dispersal within and across trophic levels.

Keywords: gut microbiota, movement ecology, microbial dispersal, host movement, migration, environmental acquisition, community assembly, social microbiome

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INTRODUCTION

Microbial associations with animal hosts are ubiquitous and increasingly recognized as important factor for the understanding of host ecology and evolution (Shapira, 2016; Foster et al., 2017; Kolodny et al., 2020; Moeller and Sanders, 2020). The gut microbiota of host animals provides various important roles regarding digestion, provision or nutrients or immune stimulating function within insects as well as vertebrate clades (Moran et al., 2019; Schmidt and Engel, 2021). There is a huge interest in advancing our understanding on how host-associations are formed and how much

inter-host transmission, environmental acquisition or host genetic factors play a role (Robinson et al., 2019; Mallott and Amato, 2021). While major focus is clearly on functional aspects for the host, reliable transmission routes and acquisition fidelity would sustain microbial associations even in the absence of mutualistic interactions (Leftwich et al., 2020; Sieber et al., 2021). For successful colonization and establishment as resident gut member, microbes must first encounter the host, which is increasingly more likely when one or both of the partners is mobile (Obeng et al., 2021). While microbial motility can be essential for the establishment of symbioses in aquatic and marine environments (Raina et al., 2019), terrestrial animals could directly alter the probability of encountering microbes by relocating themself. Host mobility range and frequency of movements differ across animal taxa, from highly mobile to more resident species.

Various studies applied a metacommunity concept for an eco-evolutionary understanding of host microbiome associations, including environmental microbes as a regional species pool (Adair and Douglas, 2017; Carrier and Reitzel, 2017; Miller et al., 2018). Host movement can extend these theoretical applications from a static toward a more dynamic acquisition process (Mihaljevic, 2012). Host aggregations reduces spatial scale and directly influence probability of inter-host transmissions, while host foraging movements could extend microbial acquisition range or increase microbial dispersal across different habitats.

While insects have (beside vertically transmitted obligate endosymbionts) relatively simple and species poor gut microbiotas (Colman et al., 2012; Engel and Moran, 2013; Jones et al., 2013), most mammals are known for a highly diverse and host taxon specific gut microbiota, that often fits to the phylogenetic distance of the host (the so called "phylosymbiosis" pattern) (Groussin et al., 2017; Groussin et al., 2020; Kohl, 2020). Though this is believed to be the result of an adaptive evolution of mammalian clades for dietary specialization, such a framework does not fit well to the microbiota of birds or bats, which show high inter individual dissimilarity and little influence of host phylogeny (Davenport et al., 2017; Lutz et al., 2019; Song et al., 2020).

With this manuscript I would like to highlight the role of host "movement" and mobility range as a neglected parameter explaining patterns in the microbiota of mammals, birds and insects and the potential transfer across host taxa.

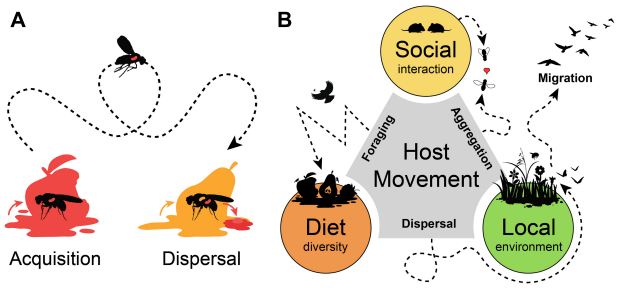

FIGURE 1 | Host movement promotes microbial acquisition and dispersal. **(A)** Host movement influences microbial acquisition range of a host and promotes microbial dispersal across larger distances. **(B)** Host movement and mobility range influence major drivers of host microbiota. Host aggregation movement promotes social interactions and microbial transmission among hosts. Foraging movement range determines diet diversity of a host. Dispersal movement, nomadism or migration relocate a host into another location from where environmental microbes can be acquired.

HOW HOST MOVEMENT INCREASES MICROBIAL DISPERSAL

By moving from one into another place animal hosts can vector microbes over a broad distance and increase their geographic distribution and dispersal rates. At the same time, a host can acquire different microbes from the new location, so that both effects of microbial *acquisition* and *dispersal* are often combined and hard to disentangle (**Figure 1A**). The vinegar fly *Drosophila melanogaster* is highly attracted by microbial volatiles and use this to find suitable oviposition sites within decaying and rotting fruits (Becher et al., 2012; Markow, 2015; Qiao et al., 2019). The highly mobile adults can disperse and vector microbes that accelerate the decaying process and support the development of the low-mobile larvae, which is a substantial aspect in the ecology of drosophilid and tephritid fruit flies (Wertheim et al., 2005; Wong et al., 2015; Pais et al., 2018). Under axenic conditions, adult flies respond with a restless behavior and increased locomotion activity (Schretter et al., 2018).

In pollination ecology, the high mobility and movement range of flying insects is a key factor to understand the dispersal abilities of the floral microbiota (Vannette and Fukami, 2017; Morris et al., 2019; Vannette, 2020). Pollinator foraging include repeated visitations of flowers by several different insects species, so that flowers can serve as hubs for microbial exchange within plant pollinator networks (Francis et al., 2021; Keller et al., 2021; Zemenick et al., 2021). Microbes can even directly influence pollinator behavior and preferences, altering floral visitation and nectar removal rates (Schaeffer et al., 2017; Rering et al., 2020; Jacquemyn et al., 2021). Similar, the distribution of multiple pollinator species drives parasite prevalence dynamics over the course of a season (Graystock et al., 2020). Like the common drone fly (*Eristalis tenax*), which promotes the dispersal of a hymenopteran gut parasite by contaminations of flower tissue with copious defecations (Figueroa et al., 2019; Davis et al., 2021).

HOW HOST MOVEMENT SHAPES THE HOST MICROBIOTA

A mobile host is not only a spreader, but also a receiver of microbes. Host animals can occupy various environmental niches, so that they can be associated with a diverse set of microbes (Carrier and Reitzel, 2017). The ecology and behavior of the host is an important aspect for host-microbial associations and their movement range would influence probabilities for microbial acquisition from different habitats or during social interaction (Ezenwa et al., 2012; Archie and Tung, 2015; Miller et al., 2018). Animals perform different kinds of movements,

like non-directional "station-keeping movements" when foraging for food in a restricted area, or "dispersal movement" and "nomadism" as an erratic translocation across different habitats, until "migration" as a highly directional long-distance form of movement (Schlägel et al., 2020). All these movement behaviors could be drivers of the host microbiota, as they allow "social" interactions of a host with conspecifics, influence foraging range and "diet" diversity and determine if a host stays within a single "local" environment or frequently moves across diverse habitats (**Figure 1B**). Predictions for the outcome could be divergent and case specific, as host mobility could enhance similarities among social group structures within geographic isolated populations. While for other hosts, a high dispersal rate and foraging range could blur local influences and result in high dissimilarities among individuals from a single habitat. Thus, the application of a unified framework for all animal hosts would be challenging, as the influence of stochastic and deterministic factors change with the ecological context of the host and its mobility range (Sieber et al., 2019; Mallott and Amato, 2021).

How Host Movement Defines the Microbial Acquisition Range

Animals with limited movement abilities depend largely on the regional pool of microbes in their vicinity, which could enhance the correlation of host microbiota with sampling location (**Figure 2A**, pale yellow). This category includes mainly very small animals or insect larvae. A prominent member would be probably *Caenorhabditis elegans,* which is literally dwelling in microbial rich habitats as it can be found in nature on decomposing plant material and rotting fruits (Frézal and Félix, 2015). *C. elegans* is mainly relocated by vectoring animals and its gut microbiota composition is largely determined by the local substrate conditions, which can be distinct from that under laboratory conditions (Zhang et al., 2017). Even larger insect larvae (i.e., caterpillars) are mainly influenced by the foliar microbiota of their local environment, when remaining on a single host plant. Caterpillars are not known to depend on microbial associations nor enrichment of host-specific microbiota and interindividual variability can be explained by different host plants or collection sites (Hammer et al., 2019; Jones et al., 2019; Mason et al., 2020).

FIGURE 2 | Host movement and mobility ranges across different host taxa. **(A)** Animals have diverse mobility ranges which could influence the probability for microbial acquisition and dispersal. Small animals or insect larvae (pale yellow) have limited movement abilities, while walking animals (yellow) can move into different environments and interact with other hosts. Flying animals (orange) can extend their foraging range to larger areas, while migrating species (red) can perform long-distance movements. **(B)** Potential extension of the microbial acquisition range across trophic levels. Insectivorous animals (i.e., bats or shrews) could acquire microbes from their prey (i.e., nocturnal moth) so that their microbiota would resemble more that of flying or migrating species.

The second category contains animals that can perform terrestrial locomotion or "walking" (**Figure 2A**, yellow). This includes most mammals (including humans), flightless birds or ground-dwelling insects. Such movement abilities increase the mobility range and enable selective foraging for host-specific diets or allows social interactions, which would both increase microbiota similarity among conspecifics. In the wood mouse (*Apodemus sylvaticus*), the tracking of individual movement patterns revealed, that social connectivity and encounters with conspecifics explains microbiota similarity better than spatial distances or genetic relatedness (Raulo et al., 2021). In humans and primates, social group structures or "co-housing" within a shared living environment have a stronger influence on microbiome similarity among individuals than host-genetic factors or kinship (Tung et al., 2015; Rothschild et al., 2018; Brito et al., 2019; Robinson et al., 2019). Over larger geographic distances dispersal limitations and prey preferences explain gut microbiota dissimilarities among carnivorous mammals in allopatric populations (Moeller et al., 2017). While at a smaller geographic distance (< 25 km), the microbiota of wild mammals does not cluster by location, but mainly by host species identity and dietary preference (Knowles et al., 2019). Movement allows a selective foraging for specific diets within the same habitat, so that herbivorous rodents (mice and voles) are dominated by Bacteroidetes and Firmicutes, while their sympatric insectivorous neighbors (shrews) show increased abundances of Proteobacteria (Knowles et al., 2019).

The third movement category includes all "flying" animals (**Figure 2A**, orange), which contains most birds, bats as well as several flying insects. Active flight enlarges the range for microbial acquisition further and might dissolve signatures of local habitat boundaries. Characteristic for birds and bats is the low correlation of their microbiota with host phylogeny and a minor influences of sampling locality or habitat type (Hird et al., 2015; Lutz et al., 2019; San Juan et al., 2020; Bodawatta et al., 2021). Studies that have combined individual flight behavior of birds with host microbiota analysis are extremely rare, but for the barn owl (*Tyto alba*) the medium foraging range indicated a positive correlation with microbial diversity (Corl et al., 2020). Also insects showed typically high dissimilarities among individuals, low biogeographic patterns and only a weak influence of host phylogeny (Colman et al., 2012; Jones et al., 2013; Yun et al., 2014; Bahrndorff et al., 2017; Wang et al., 2020). But flying insects do not necessarily cover larger absolute geographic distances than terrestrial animals, but mobility *per se* with a high frequency of movements within a smaller range could expose a host to heterogeneous microenvironments. This is particularly important for microbe–plant–pollinator interactions, since the dispersal of nectar microbes is directly linked to the foraging behavior of pollinators (Cullen et al., 2021; Francis et al., 2021). Wild bee species vary in their foraging ranges from a few hundred meters to several kilometers, while nocturnal

moth can easily exceed movement ranges to several hundred kilometers (Greenleaf et al., 2007; Satterfield et al., 2020).

The fourth movement category (**Figure 2A**, red) includes species that "migrate," which differs from the previous movement categories, as it is a seasonal directional movement over larger geographic distance. Migration has been primarily investigated in birds and butterflies regarding the long-distance dispersal of parasites or as a strategy to avoid pathogen infections (Altizer et al., 2011; Bartel et al., 2011; Boulinier et al., 2016; Viana et al., 2016). But a few studies have investigated if host migration influences gut microbial diversity. While migrating passerine birds showed a change of their microbiota following a dietary shift (Lewis et al., 2017; Skeen et al., 2021), other studies pointed mainly at physiological adaptations and little environmental acquisition of microbes (Risely et al., 2017, 2018; Wu et al., 2018; Turjeman et al., 2020). The influence of migration on the microbiota of birds seems rather marginal and could be mainly attributed to dietary shifts between geographic distant locations. With insects, the influence of migration is less clear. Though, insect migration is often associated with the spectacular mass migration events of the monarch butterfly (*Danaus plexippus*) or the painted lady (*Vanessa cardui*), migration abilities are not uncommon for other butterflies or nocturnal moth, but remain often unnoticed as they fly in lower numbers or at night (Chowdhury et al., 2021). But even seasonal mass migration events are easily overlooked, when insects are small and fly at high altitudes like the marmalade hoverfly (*Episyrphus balteatus*) (Hu et al., 2016; Wotton et al., 2019; Satterfield et al., 2020). But to what extent insect migration would influence microbiota acquisition or dispersal has not been fully elucidated yet.

Does 'Flight' or the Consumption of Mobile Insect Prey Shape the Host Microbiota?

A recent comparative study concludes that a convergent physiological adaption to "flight" (reduced dependence on microbes to reduce weight of the digestive system) shapes the bird as well as the bat microbiota (Song et al., 2020). Particular Proteobacteria seem somehow associated with "flight," as they are commonly found in birds, bats and insects, while most mammals are dominated by Bacteroidetes and Firmicutes (Brooks et al., 2016; Song et al., 2020). But there are notable exceptions. In the analysis of Song et al. (2020) the order of Insectivora (here mainly shrews of the genus *Crocidura* spp.) showed the second highest proportions of Proteobacteria and a less mammal-specific microbiota than Chiroptera (bats), directly followed by Pholidota (ant-eating Pangolins). If the observed pattern would be explained by a physiological adaptation to flight, this raises the question why terrestrial shrews have the most "bird-like" microbiota of all mammals.

What is intriguing about this observation is the possibility that insectivorous mammals might obtain their microbiota directly from their prey. In such a scenario, the consumption of insects would expand their microbial acquisition range, which resembles more that of a flying insect (**Figure 2B**). A transfer of microbes across predator-prey networks has been suggested for insectivorous birds and predatory insects (Tiede et al., 2017; Suenami et al., 2019; Dion-Phénix et al., 2021). The mammalian microbiota is strongly influenced by species identity and type of diet, but an increase of invertebrate prey (i.e., insects) within the diet correlates with a decrease in bacterial alpha diversity compared to mammals with a primarily herbivorous lifestyle (Knowles et al., 2019; Harrison et al., 2021). Tough "insectivory" includes the consumption of non-flying insects (i.e., ants or termites) as well as other invertebrates, such a dietary preference seems to result in a convergent adaptation in the microbiota of phylogenetically distant mammalian clades (Pilosa, Cingulata, Tubulidentata, and Carnivora) (Delsuc et al., 2014). Particular bats might be able to further expand their microbial acquisition range beyond their own flight range, as they consume a diverse variety of highly mobile insect species within the orders Lepidoptera, Diptera, and Coleoptera (Tiede et al., 2020). The Brazilian free-tailed bat (*Tadarida brasiliensis*) preys on high altitude flying insects, which includes several migrating species, and noctuid moth make on average 77% of their diet (Krauel et al., 2018). There is clearly more work needed to clarify to what extend the microbiota of insects influences the microbiota of insectivorous animals across tropic levels (**Figure 2B**), and what patterns would be predicted for the microbiota of highly mobile hosts.

Interruption of Host Movement Behavior in Captivity or Laboratory Settings

Captivity and laboratory settings can alter the outcome of microbiome studies tremendously and should be taken with caution when implying evolutionary context (Hird, 2017). As long as the insectivorous bat *Mops condylurus* preys on flying insects, they show higher interindividual variability with relative low alpha diversity in their fecal microbiota, but converge toward a more uniform community composition with increased alpha diversity when kept in captivity for 6 weeks (Edenborough et al., 2020). When brought into captivity, primates tend to shift toward a more "human-like" microbiota (Nishida and Ochman, 2021), which was attributed to a reduced diversity of food plants with lower fiber content compared to the naturally foraged diet of wild relatives (Clayton et al., 2016). Captivity has a significant effect on the microbiota of several mammal species (Kohl et al., 2014; McKenzie et al., 2017), and there is a lot potential to further explore how restrictions in foraging movement and range size shape the microbiome. Captivity alters also the microbiota of migratory as well as terrestrial birds, such as the crane and the brown kiwi (Xie et al., 2016; San Juan et al., 2021). Any restriction of animal movement behavior could disrupt natural host-microbial dispersal routes leading to more "uniform" results that confound the outcome obtained in laboratory settings or under captivity conditions.

CONCLUSION AND FUTURE PERSPECTIVES

A major challenge in the investigation of the microbiota of wild animals is the lack of a clear framework of what can be expected

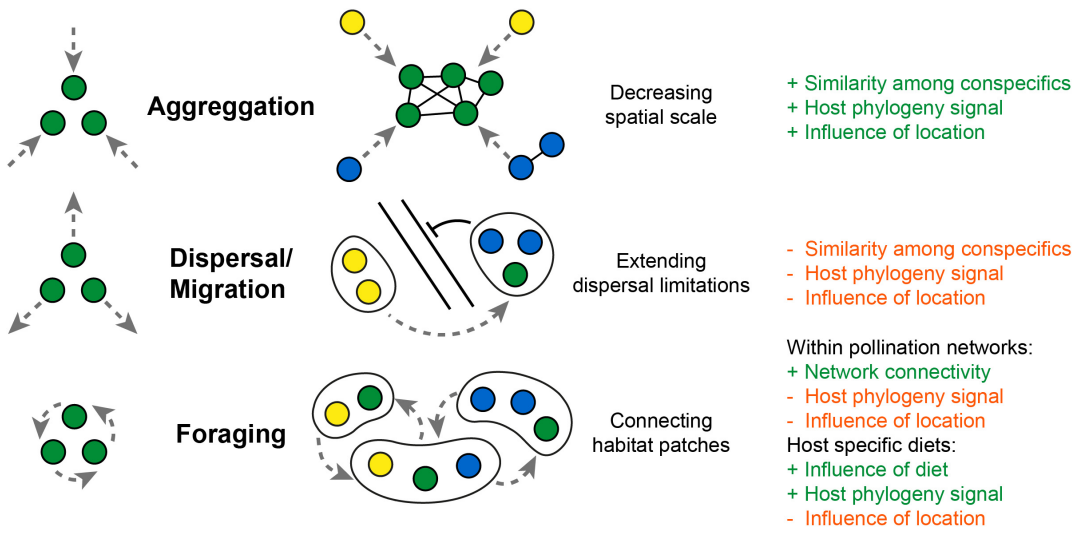

FIGURE 3 | Potential framework how moving hosts (as "floating islands") influence parameter of microbiota composition. Aggregation movement decreases spatial scale and promotes social exchange among conspecifics, which could increase correlation by host phylogeny. Dispersal movement could extend dispersal limitations of microbes but weakens the influence of local landscape parameters or host phylogeny. Foraging movement could connect habitat patches by distributing microbes across plant-pollinator networks and weakens influences of host phylogeny or the local environment. While a selective foraging for host specific diets (e.g., mammalian feeding guilds) would increase correlations with host diet and phylogeny (+ and – refers to an increase or decrease in correlation).

when host species identity or geographic location are failing short to predict the observed variability within wild populations. By an integration of flight behavior, the study by Song et al. (2020) marks a transition that elevates from a pure host phylogenetic perspective toward the integration of host ecology. There is an exciting potential for future research to combine hypothesis about animal behavior and movement decisions with the host microbiota (Davidson et al., 2020; Bo and Kohl, 2021).

In the past, the analysis of host microbiomes has been mainly performed from a "host-centric" viewpoint, following the tradition of genetic model systems in the search of a host-genetic basis that explains microbiome composition. The inclusion of the "microbes perspective" has challenged this view and brings community ecological principles and stochastic processes into host microbiota analysis (Obeng et al., 2021; Sieber et al., 2021). The host microbiota is not a constant trait, but shows context dependent plasticity and interindividual variability in time and space (Carrier and Reitzel, 2017). Especially the metacommunity concept has become very useful in describing host-associated microbiota as it provides a framework that integrates interhost transmission and environmental acquisition of microbes from an external pool (Miller et al., 2018). As a reference to island biogeography, hosts are often depicted as passive "microbial habitats" that become colonized by microbes, similar as islands become colonized by other macrobiota, while social connectivity is illustrated by clustering single host "islands" into "archipelagoes" as used in the analogy by Sarkar et al. (2020). Though this mainly refers to stable social group structures, it misses to depict the dynamic and transient nature of these interactions. Moving hosts would be more comparable to "floating islands" which constantly change their spatial distribution relative to each other, actively connect in social interactions or relocate themselves into different environments (**Figure 3**).

Similar as microbial movement (motility) turned out to be an important aspect of host microbiota associations within aquatic systems (Raina et al., 2019), host movement (mobility) could become a vital addition to the metacommunity concept explaining the acquisition and dispersal of microbes among terrestrial animals. Movement changes several parameters that influence microbiome composition and could strength similarities among conspecifics via selective foraging or social exchange (**Figure 3**). Social transmission would become merely a consequence of aggregation movement, which decreases spatial scale and enhances the probability of microbial transmission among conspecifics. But movement could also blur correlations with host phylogeny and lead to higher dissimilarity among conspecifics from the same location. The mobility range and foraging pattern of a host could be a key factor to fully comprehend the composition and diversity of the host microbiota. Though a direct tracking of host movement in the wild is indeed a challenge, the outstanding work by Raulo et al. (2021) and Skeen et al. (2021) demonstrates the value of repeated sampling and the integration of movement patterns as a new dimension into microbiota analysis of birds and mammals. But even for insects, where a direct tracking of individuals is less feasible, the investigation of transmission routes within plant pollinator networks becomes a promising step to better understand microbial dispersal among different host taxa and across the animal and plant kingdom (Keller et al., 2021; Zemenick et al., 2021). Still, a framework for predictions of microbiota composition of highly mobile flying hosts is missing, as increasing complexity of interactions makes it difficult to directly correlate host mobility range with gut microbial

diversity. Here, more work is needed to further explore if the same drivers can explain microbiota composition of flying hosts, resolving the patterns observed from insects, birds and bats.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AW conceived, designed, and wrote the manuscript.

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Evidence of Genomic Diversification in a Natural Symbiotic Population Within Its Host

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Planktonic cells of the luminous marine bacterium *Vibrio fischeri* establish themselves in the light-emitting organ of each generation of newly hatched *Euprymna scolopes* bobtail squid. A symbiont population is maintained within the 6 separated crypts of the organ for the ∼9-month life of the host. In the wild, the initial colonization step is typically accomplished by a handful of planktonic *V. fischeri* cells, leading to a species-specific, but often multi-strain, symbiont population. Within a few hours, the inoculating cells proliferate within the organ's individual crypts, after which there is evidently no supernumerary colonization. Nevertheless, every day at dawn, the majority of the symbionts is expelled, and the regrowth of the remaining ∼5% of cells provides a daily opportunity for the population to evolve and diverge, thereby increasing its genomic diversity. To begin to understand the extent of this diversification, we characterized the light-organ population of an adult animal. First, we used 16S sequencing to determine that species in the *V. fischeri* clade were essentially the only ones detectable within a field-caught *E. scolopes*. Efforts to colonize the host with a minor species that appeared to be identified, *V. litoralis*, revealed that, although some cells could be imaged within the organ, they were <0.1% of the typical *V. fischeri* population, and did not persist. Next, we determined the genome sequences of seventy-two isolates from one side of the organ. While all these isolates were associated with one of three clusters of *V. fischeri* strains, there was considerable genomic diversity within this natural symbiotic population. Comparative analyses revealed a significant difference in both the number and the presence/absence of genes within each cluster; in contrast, there was little accumulation of single-nucleotide polymorphisms. These data suggest that, in nature, the light organ is colonized by a small number of *V. fischeri* strains that can undergo significant genetic diversification, including by horizontal-gene transfer, over the course of ∼1500 generations of growth in the organ. When the resulting population of symbionts is expelled into seawater, its genomic mix provides the genetic basis for selection during the subsequent environmental dispersal, and transmission to the next host.

Keywords: *Vibrio fischeri*, comparative genomic, *Euprymna scolopes*, symbiosis, population biology

INTRODUCTION

Vibrio (Aliivibrio) fischeri is a marine gram-negative bacterium that can establish a symbiosis within the light-emitting organ of the Hawaiian bobtail squid *Euprymna scolopes*, providing bioluminescence to its partner each night in exchange for nutrients. When the aposymbiotic (i.e., symbiont-free) juvenile squid hatches from its egg, it specifically harvests *V. fischeri* cells from the ambient seawater (Nawroth et al., 2017; Visick et al., 2021). These bacteria enter and migrate through different tissue environments to finally reach and colonize the epithelium-lined crypts of the host's nascent light organ. Each lobe of the bilobed organ bears three pores, each leading to a separate crypt (McFall-Ngai, 2014), increasing the chance for a multi-strain inoculation, and reducing the opportunity for strain-strain competition. Every day at dawn, ∼95% of the symbiont population is expelled, and the remaining ∼5% regrow, repopulating the crypts within hours (Lee and Ruby, 1994). Therefore, the diversity of strains present in the light organ can arise from (i) the diversity of inoculating *V. fischeri* strains present in the local environment (Wollenberg and Ruby, 2009), (ii) the stochasticity of the initial colonization events (Bongrand and Ruby, 2018), and (iii) the subsequent evolution of those strains as they go through many daily cycles of population depletion and regrowth within the crypts. The genomic divergence resulting from this latter dynamic makes it of interest to determine the number of genomically distinct strains present in the light organ of a fully grown (i.e., >3 month-old) squid.

The presence of multiple, co-occurring strains of a symbiont species within a host, as well as the nature of their strain-level genetic differences, have become of increasing interest as a means to better understand the dynamics of host health and metabolism (Ansorge et al., 2019; Hinzke et al., 2021). The monospecificity of the *E. scolopes*/*V. fischeri* system simplifies the investigation of genomic differences between co-occurring strains in a natural association (Bongrand et al., 2016, 2020), as well as of the potential for behavioral interactions between these strains (Speare et al., 2018). In particular, because the differences between genomes are small due to the short periods of diversification, they may be difficult to determine using a purely metagenomic approach.

Previously, we have shown that the genome synteny of *V. fischeri* is surprisingly well conserved among *E. scolopes* light-organ symbionts collected across different geographic areas, and over a span of more than a decade (Bongrand et al., 2016). In addition, two colonization behaviors have been described during experimental inoculations of juvenile squid: (i) a dominant (D-type) behavior, in which one strain eclipses another during a co-infection of the host, and (ii) a sharing (S-type) behavior, in which both strains persist after a co-inoculation (Bongrand et al., 2016, 2020). One characteristic of D-type behavior is that such strains appear to reach and colonize the light-organ crypts more quickly than S-type strains (Bongrand and Ruby, 2018). Interestingly, both D-type and S-type strains can be found in field-caught animals (Wollenberg and Ruby, 2009), an unexpected outcome that may result from a sequential exposure of the newly hatched host to different *V. fischeri* strains in the environment (Bongrand and Ruby, 2018). Taken together, these characteristics of the squid-vibrio system make it a good model with which to study both colonization dynamics, and intra-species diversification within the symbiotic population of a host (Yawata et al., 2014; Duar et al., 2017; McLean et al., 2018; Bongrand and Ruby, 2019).

A previous rep-PCR study estimated that at least 6 to 8 strains were present in the light organ of a field-caught adult squid (Wollenberg and Ruby, 2009). However, under laboratory inoculation procedures, a single light organ can reportedly be colonized by as many as a hundred distinct mutant derivatives of the same strain (Brooks et al., 2014), indicating the potential for multi-strain populations in the symbiosis. In our study, we used culture-dependent and independent approaches to confirm that *V. fischeri* is essentially the only species detectable in the light organ of *E. scolopes*. In addition, we estimated the number of co-occurring strains of this species by whole-genome sequencing, using Illumina platform MiSeq (2*300bp), of around 80 isolates, obtained as colony-forming units (CFU) from one lobe (i.e., 3 crypts) of the light organ of a field-caught adult squid, collected off the island of Oahu, Hawaii, United States. All of the individual strains analyzed were identified as *V. fischeri*, and were closely associated with one of three genomic clusters of strains. While there was a substantial level of diversity in the total number and presence/absence of individual genes between and among these clusters, we found little evidence of significant evolutionary drift, as indicated by single nucleotide polymorphisms (SNP).

RESULTS AND DISCUSSION

Analysis of the Specificity of Light-Organ Occupancy

Vibrio fischeri has been described as the only bacterial species present in the light organ of the squid *E. scolopes*, based on the phenotypic identification of hundreds of CFU isolated from dozens of animals (Boettcher and Ruby, 1990; Ruby and Lee, 1998). The only reported exception to this specificity is that some isolates of the closely related (i.e., 97% 16S sequence identity) species *Vibrio logei* (Bang et al., 1978), subsequently proposed for renaming (Ast et al., 2009), can stably colonize *E. scolopes* juveniles, and are found co-occurring with *V. fischeri* as light-organ symbionts in other species of sepiolid squids (Fidopiastis et al., 1998). Here, a metagenomic approach using an Illumina platform MiSeq (2*300bp) intended to identify the symbionts present in the light organs of field-caught specimens of *E. scolopes*. Specifically, we sequenced the V3-V4 region of the 16S RNA genes of members of the bacterial population present in one of the two symbiont-containing core tissues, representing half of the bilaterally symmetrical light organ. Two adult squids from Maunalua Bay, Oahu, HI were sampled; one was collected in 2005 (Wollenberg and Ruby, 2009) and the other was obtained in 2019. Analyses of both produced the same outcome. Limiting the analysis to bacterial families identified in more than 1000 of the 176,118 total reads (i.e., >0.5%), only two categories were detected: "*Vibrionaceae*" (99%) and "unclassified at a family level" (1%) (**Figure 1**). *V. fischeri* strain ES114

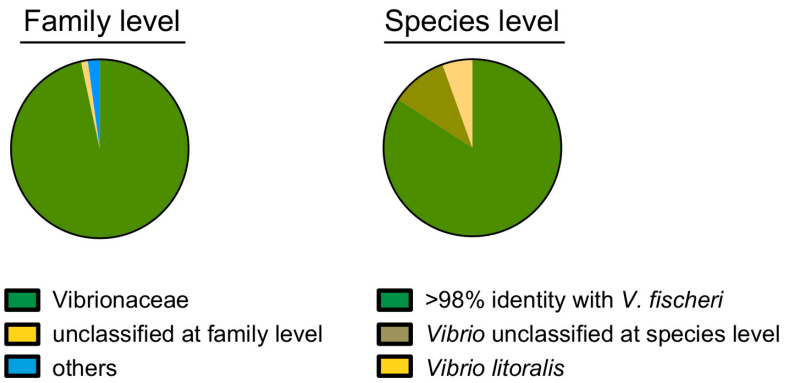

FIGURE 1 | Identification of the bacterial population within a symbiotic light organ at the family **(left)** and species **(right)** levels, based on the sequence of the V3-V4 regions of the 16S ribosomal RNA gene. Of the 176,118 total reads, 173,915 (99%) and 152,745 (87%) could be assigned to a family or species, respectively.

(Boettcher and Ruby, 1990) has been used as a genome reference for the species because of its complete, closed (Ruby et al., 2005), and subsequently updated (Mandel et al., 2008) sequence. This strain encodes twelve copies of the 16S DNA, comprising two distinguishable sequences that are 98% identical. In the available genomes of another 42 *V. fischeri* strains, including those draft genomes providing only one 16S sequence, there were a total of 4 versions of the typically analyzed hypervariable V3-V4 region (Wang and Qian, 2009), all of which share >98% identity. Therefore, we considered that, conservatively, strains with 98% or more identity with these *V. fischeri* sequences over this region belonged to this species. This assumption is consistent with the sequence similarity threshold for species differentiation described in the literature (Kim et al., 2014).

Within this context, 79%, 13%, and 5% of the 16S rRNA sequencing reads corresponded to, respectively: (i) *V. fischeri*, (ii) "unclassified at species level" and (iii) *V. litoralis* (**Figure 1**). *V. litoralis* is currently represented by two draft genome sequences in the database. The V3-V4 region for these two are identical to each other, and 96% identical to *V. fischeri*. Only two isolates of *V. litoralis* have been described (Nam et al., 2007), and essentially nothing else has been published about this species. It is difficult to distinguish with certainty two species with V3-V4 region sequences with >96% identity (Mysara et al., 2017), especially if the read-quality for accurate taxonomy assignment is taken into account (Bokulich et al., 2013). It has also been reported that there can be a limitation in the use of 16S-gene sequence comparisons for species-level taxonomy; for example, sequences belonging to the same species can be as little as 94% identical, while sequences from different species can be up to 97% identical (Lan et al., 2016). Thus, it is not unlikely that the 5% of 16S reads, indicated as *V. litoralis* (**Figure 1**), recovered from the light organ might, in fact, represent strains of *V. fischeri*. This result is consistent with an absence of *V. litoralis* among the 80 symbiont CFU sequenced in this study (see below), although this species is easily cultured (Nam et al., 2007). In addition, *V. litoralis* does not encode bacterial luciferase, and produces no luminescence (data not shown), and luminescence has been shown to be a strict requirement for persistence of *V. fischeri* in the light organ (Koch et al., 2014). Taken together, these data support the view that it is unlikely that members of this species are a resident population in the light organ. Overall these results confirm the specificity of the *E. scolopes*/*V. fischeri* association, consistent with the reported selective pressure in this niche for the symbiont (Nyholm et al., 2000).

To determine whether other bacterial species can enter the light organ transiently, especially when the juvenile is being initially colonized, we inoculated aposymbiotic squids with *V. fischeri* and one of three environmental marine isolates (**Figure 2A**). Because of the possibility that this species might be present in an adult light organ (**Figure 1**), we chose a strain of *V. litoralis*, as well as another Gram-negative bacterium that associates with marine larvae, *Pseudoalteromonas luteoviolacea*, and a Gram-positive marine bacterium *Bacillus aquimaris* (**Figure 2A**). Twenty-four hours after exposure to the inoculum, each animal was rinsed and homogenized, and the homogenate plated for CFU. While *B. aquimaris* was not detected, CFU of the other species were often found; however, in those squids inoculated with these species, there were only 0.01–0.5% as many CFU as in a colonization by *V. fischeri*. Nyholm et al. described a 2-h "permissive window" post initial exposure, during which < 2 μm-diameter beads or bacteria that were not *V. fischeri* could be found present in the newly hatched juvenile's light organ (Nyholm et al., 2002). Here we describe the presence of such non-symbiotic bacteria in the crypts after 24 h of initial exposure. Both results suggest that other bacterial species, while present for a short period of time during the juvenile stage, don't go on to colonize the squids; i.e., they are not capable of symbiosis, or of competing with *V. fischeri*. Our results further suggest that such non-symbiotic strains can remain longer than a few hours in the light organ. To determine where *V. litoralis* cells were within the light organ, we GFP-labeled a strain of this species and visualized co-inoculated squid by confocal microscopy (**Figures 2B,C**), and confirmed that a few dozen GFP-labeled *V. litoralis* cells were dispersed sporadically within the crypts of the light organ (**Supplementary Video 1**). To ask whether the presence of the natural symbiont had an impact on colonization by these strains, we performed both single- and

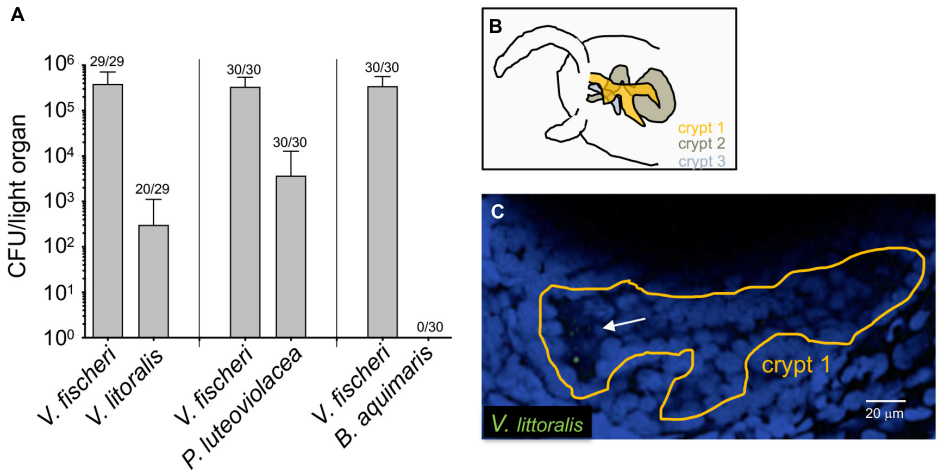

FIGURE 2 | Colonization of newly hatched *E. scolopes* by environmental bacteria. Juvenile squid were inoculated with pairs of strains consisting of *V. fischeri* and either *V. litoralis* (*n* = 29), *P. luteoviolacea* (*n* = 30) or *B. aquimaris* (*n* = 30) in three replicates (*n* = total number of animals in each condition). **(A)** The average number of colony-forming units (CFU) present in co-colonized light organs after 24 h. The number above each bar indicates the proportion of animals in which each strain was found; except for the *B. aquimaris* experiment, in which all the squids were only colonized with ES114; only animals in which both strains were detected were used to calculate the average. **(B)** Schematic of the interior of one side of a juvenile light organ, indicating the position of the three crypts. **(C)** One slice of a confocal microscope image of a crypt (tissue nuclei stained blue with TOPRO) in which 8-10 GFP-labeled *V. litoralis* cells (green) can be seen (arrow). A z-stack movie of the entire crypt can be found in **Supplementary Video 1**.

co-inoculations of juvenile squid with *V. fischeri* and one of the three other strains, and analyzed their success after 24 and 48 h (**Supplementary Figure 1**). Generally, the quantity of each strain, with or without the presence of *V. fischeri,* was similar, suggesting there was no evidence of either a strong competition or complementation by the presence of the natural symbiont on these environmental strains. However, a few squid harbored a very low number of *B. aquimaris* when *V. fischeri* was absent, compared to none when *V. fischeri* was present. This finding may suggest that newly hatched squid are more permissive to other bacteria when they don't encounter their symbiont. Indeed, the symbiont aggregates and concentrates at the pores of the light organ, giving itself an advantage compare to other species present in the environment (Nyholm et al., 2000), being absent will give a stochastic opportunity for other bacteria to reach and enter the pores of the light organ. In addition, we realize the inoculum used in these assays are also much higher than the one found in the environment which can increase the number of CFU present in the light organ for the non-symbiotic strains. We also wondered whether the non-*V. fischeri* species persisted in the light organ of the squid, and found that there were fewer of these bacteria at 48 h than at 24 h (**Supplementary Figure 1B**), suggesting that while they can enter, non-symbiotic environmental strains are just transient in the light organ of the squid. Nevertheless, they may indicate that other species can transiently provide new genetic material for the *V. fischeri* population that can take up and incorporate exogenous DNA by natural transformation (Pollack-Berti et al., 2010).

Overall, one aim in this study was to determine whether there is evidence for a significant number of an unrecognized species in the squid light organ, one that had been overlooked because it was not culturable by the methods used for isolating *V. fischeri*. However, no species other than *V. fischeri* appeared to be persistently present among the symbiont population.

Genomic Diversification Within the Symbiont Population

In nature, a juvenile *E. scolopes* typically becomes colonized by only a few cells of *V. fischeri* (Wollenberg and Ruby, 2012), drawn from an environmental pool of genomicly distinct strains (Bongrand et al., 2016). For the subsequent ∼9 months that the host lives, the symbiont population undergoes a daily cycle of expulsion and proliferate each morning (Schwartzman and Ruby, 2016). Thus, we predicted that an analysis of the population of a field-caught adult host light organ would reveal: (i) how many strains had initiated the population, (ii) what was their relative success, and (iii) whether there was evidence that the progeny had diverged from their ancestral genome. While studies selecting for better colonization have been performed in experimentally colonized animals, to date they have been initiated by inoculation with a single strain (Schuster et al., 2010; Soto and Nishiguchi, 2014; Pankey et al., 2017). Our interest was in examining the outcome of a multiple-strain colonization; however, arranging such a colonization experimentally can be challenging, even with as few as three strains (Bongrand and Ruby, 2018). Therefore, we decided to examine a snapshot in time of a naturally colonized adult light organ (i.e., >3 months after colonization) by genome-sequencing 80 CFU obtained from one side of the bilobed light organ of a 23-mm mantle-length, field-caught female (Wollenberg and Ruby, 2009). Of the initial 80 sequenced strains, 8 yielded DNA sequences that were of poor quality, so we excluded them from further analysis.

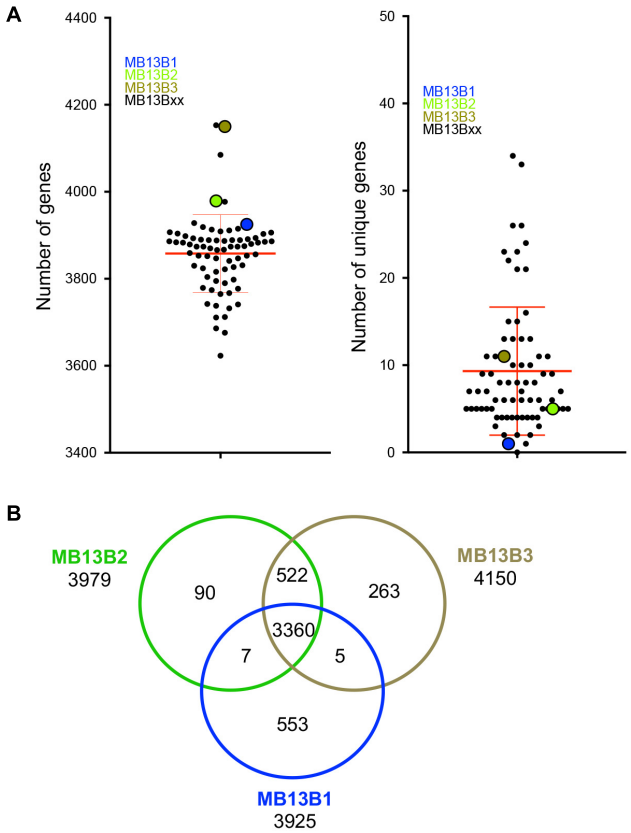

FIGURE 3 | (A) Total number of genes (left) and number of unique genes (right) for each MB13B isolate compared to all other 74 isolates. **(B)** Venn diagram indicating the number of total, shared and unique genes in a comparison between strains MB13B1, MB13B2 and MB13B3.

The number of predicted genes in each isolate's genome spanned from 3623 to 4153, with a mean of 3858 (**Figure 3A**), which is within the published range for *V. fischeri* (Bongrand et al., 2016). The number of unique genes in a given genome was calculated to be between 0 and 34 among the 72 isolates; however, because the data are from draft genomes, the actual number of unique genes may be different. Thus, the relatively low numbers of unique encoded proteins may be due to (i) limitations in the process of sequence assembly, and/or (ii) horizontal gene transfer (HGT) events experienced by members of the population, as well as gene loss amongst these strains during symbiosis. Indeed, the possibility of extensive HGT in the dense symbiont population is supported by the facts that (i) *V. fischeri* is naturally competent under conditions found in the symbiosis (Pollack-Berti et al., 2010) and (ii) HGT, such as plasmid transfer, has been reported between strains in the host (Dunn et al., 2005). The three previously characterized strains (Wollenberg and Ruby, 2009) isolated from this same light-organ lobe – MB13B1, MB13B2 and MB13B3 – encode 3915, 3979 and 4150 proteins, including 553, 90 and 263 unique proteins, respectively, when compared to each other (**Figure 3B**); strains MB13B2 and MB13B3 are more closely related to each other than either is to MB13B1 (Bongrand et al., 2016). Interestingly, the number of unique proteins decreased to 34 when the additional 72 available sequenced *V. fischeri* genomes were considered, a result that is consistent with a small number of ancestral/parent strains in the inoculum.

The core genome of *V. fischeri* consists of 2308 genes and, on a phylogenetic tree based on these core genes, all the sequenced isolates grouped with one of the three previously sequenced strains (**Figure 4**). This finding is confirmed by a pairwise gene-distance analysis of the genes belonging to the core genome. Interestingly only 30 of the 2308 core genes showed a pairwise distance between 0.1 and 0.5, suggesting that there was a low rate of evolutionary divergence in the light organ over the several months that the symbiosis had replicated in the crypts (estimated at approximately 500 generations). Because the intrinsic mutation rate of *V. fischeri* is reported to be low (Dillon et al., 2017), we predicted that there would be a low level of divergence among the progeny of the initial colonizers of this portion of the light-organ, unless these symbionts also experienced a significant degree of selection pressure.

The groups represented by strains MB13B1, MB13B2, and MB13B3 account for 93%, 4%, and 3% (respectively) of the total isolates sequenced, suggesting that strain MB13B1 is more highly represented in this half of the organ. One possible explanation for this disproportional representation is the available space and, thus, potential for growth, a strain has when proliferating within a crypt (Essock-Burns et al., 2020). A recent study showed that

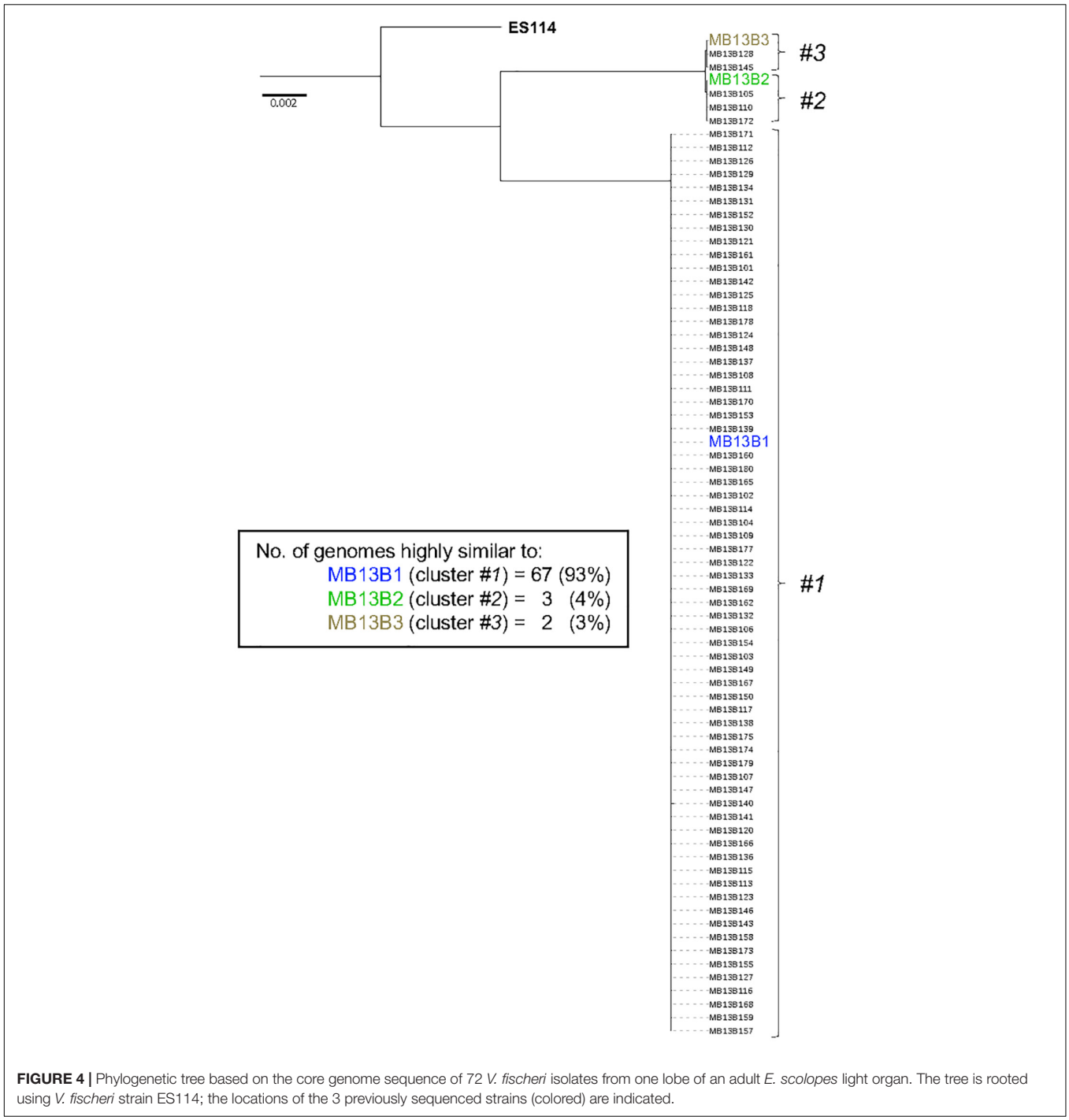

FIGURE 4 | Phylogenetic tree based on the core genome sequence of 72 *V. fischeri* isolates from one lobe of an adult *E. scolopes* light organ. The tree is rooted using *V. fischeri* strain ES114; the locations of the 3 previously sequenced strains (colored) are indicated.

in a polyclonal population present in the tubeworm symbiosis, the relative proportion of strains was more a result of their growth in the host (Polzin et al., 2019), a hypothesis that may hold here as well. Such a scenario would also help explain strain MB13B1's numerical advantage when strains MB13B2 and MB13B3 have been described as more effective at initiating a colonization (Bongrand and Ruby, 2018). This proportion could also be unintentionally biased during the picking of the colonies from the plate. A more accurate identification of the proportion of each strain may well be obtained by using a metagenomic approach, rather than one based on CFU selection.

Finally, an examination of the presence and absence of genes among the different isolates (**Figure 5**) suggested that considerable divergence within the initial inoculating strains had occurred post-colonization. The core genome among the 72 sequenced isolates consists of 2308 genes, which is low when compared to the 3170 genes found for 14 strains that were significantly more biologically and ecologically diverse

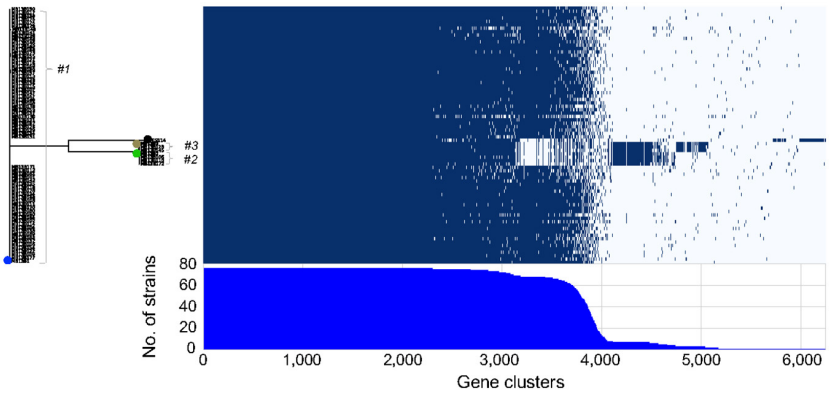

FIGURE 5 | Roary matrix indicating the presence or absence of genes in 6246 clusters among 75 light-organ isolates from lobe MB13B within clusters *#1-3*. Strain ES114, isolated from another light organ, is included for comparison (black dot). The positions of previously sequenced strains are indicated by blue (MB13B1), green (MB13B2), and brown (MB13B3) dots.

(Bongrand et al., 2016). This result will be an underestimate since some genes will be considered missing because of the technology used (short read) and the quality of the assembly. However, this could also result from frequent HGT occurring between the bacteria residing, or transiently occurring, in the light organ, and is consistent with the reported large number of gene loss and gain events that *Vibrio* spp. typically have during their evolution (Lin et al., 2018). Taken together, the data here support the conclusion that 3 strains initially colonized one lobe of squid MB13, and that their progeny have evolved into pseudo-clones harboring highly related, yet unique, genes sets. In a study of *V. cholerae* strains, the population in an individual patient was reported to experience more HGT than point mutations, which led the authors to conclude that strain divergence is due primarily to gene exchange within the *V. cholerae* population (Levade et al., 2017). The work presented here suggests that *V. cholerae* and *V. fischeri* may evolve in a similar manner within their respective hosts; however, while the cholera infection occupies the gut (a single communicating lumen) and is often clonal (Levade et al., 2017) the *V. fischeri* light-organ population typically derives from a few strains, likely corresponding to the several distinct crypt spaces it occupies (McFall-Ngai, 2014; Essock-Burns et al., 2020). It has also been shown in the gut microbiota that strains recovered from the same individual were more similar to each other than to strains from other individuals, and that the diversity within an individual host came principally from changes in gene content rather than from SNPs (Vatanen et al., 2018).

In our study, we confirmed that one side of the squid's bilobed light organ can become colonized by three strains. Because the frequency of co-colonization of a crypt by two different strains can be as low as ∼1% (Bongrand and Ruby, 2018), it seems likely that, in the ocean, the light organs of newly hatched squid are generally colonized by around 6 strains (Wollenberg and Ruby, 2009). Over the subsequent several months, the progeny of each strain within a crypt can diverge into many pseudo-clones characterized by a number of unique genes, but a low level of SNPs. Overall this genomic analysis suggests that *V. fischeri* may begin to go through an elimination of non-functional sequences after associating with its host (Bobay and Ochman, 2017).

Overall, this study aimed to begin to understand the mechanisms underlying the population diversity that can result from the association between a host and its horizontally transmitted symbiont. We used an amplicon-sequencing approach to confirm the single-species specificity of this symbiosis, and then used an Illumina platform MiSeq (2*300bp) to sequence cultured isolates to infer the genomic diversity of this population. Using these conclusions as a foundation, future studies will first examine additional symbiont populations in multiple adult light organs to establish hypotheses that describe the dynamics of population diversity in nature. Second, analyses of both the genomes of bacterial isolates and population metagenomes should determine the proportion of the different strains present in the light organs. Finally, studies should use the non-intrusive sampling afforded by the daily expulsion event to track the nature of the symbiont population of individual light organs over time, to determine whether there are age-specific dynamics of population diversification in a natural symbiotic relationship.

MATERIALS AND METHODS

Bacterial Strains

We performed this study on bacteria isolated from the crypt-containing central-core tissue of a single light-organ lobe of an adult specimen of *E. scolopes* (MB13) caught in Maunalua Bay, Oahu, HI and frozen in glycerol in 2005 (Wollenberg and Ruby, 2009). The internal tissue sample was dissected out, rinsed with sterile seawater, and frozen at −80°C. This sample was chosen because draft genomes were already available for three strains (MB13B1, MB13B2, and MB13B3) isolated from it, and included two phenotypically distinct groups (Bongrand et al., 2016). Three isolates of other

species of marine bacteria, identified as *Vibrio litoralis* strain DSM17657 (Nam et al., 2007), *Pseudoalteromonas luteoviolacea* strain HI1 (Huang et al., 2012) and *Bacillus aquimaris* strain TF-12 (Yoon et al., 2003) were also used in the study. Colonies of the latter two species could be easily distinguished from the *Vibrio* species by their purple and yellow-orange pigmentation, respectively.

16S RNA Gene Analyses

The symbiont-containing light-organ lobe tissue designated MB13B (Wollenberg and Ruby, 2009) was shipped to Omega Bioservices (Norcross, GA, United States) in ethanol, and processed to identify 16S gene sequences (Bioproject ID: PRJNA796326; BioSample accession number SAMN24847873). DNA was extracted using the Mag-Bind Universal Pathogen DNA kit (Omega), the library prepared with the KAPA HIFI PCR for 16S kit (KAPABIOSYSTEMS), and the 16S V3-V4 regions were sequenced using the Illumina primers (IlluminaF: CCTACGGGNGGCWGCAG; IlluminaR: GACTACHVGGGTATCTAATCC) on a Illumina MiSeq. The coverage per sample was ∼50K reads, with a paired-end read format of 2*300. Omega Bioservices also performed sequence classification using the Illumina's BaseSpace 16S rRNA application module, Illumina-curated version (May 2013) using the Greengenes taxonomic database in parallel with the Ribosomal Database Project (RDP) (Wang et al., 2007). The accuracy for species classification was estimated to be 98%.

Next Generation Sequencing

An aliquot of the −80°C frozen glycerol stock containing an homogenate of the right-side central core of the light organ of squid MB13 was spread onto Luria-Bertani Salt (LBS) agar medium containing (per liter) 20 g NaCl, 50 ml of 1 M Tris–HCl (pH 7.5), 10 g Bacto-Tryptone, 5 g yeast extract, and 12 g agar, and left overnight at 28°C. The following day, 80 colonies were picked at random, and cultured in LBS broth. After shaking overnight at 28°C, 500 μL of culture ($OD_{600\,nm}$ between 1 and 2) was centrifuged, and the pellet resuspended in 400 μL lysis buffer containing 40 mM EDTA, 50 mM Tris–HCl (pH 8.3) and 0.75 M sucrose, and frozen at −80°C. The suspension was thawed, and incubated with 1 mg lysozyme (Sigma Aldrich, St. Louis, MO, United States) per ml for 30 min at 37°C. Then, proteinase K (Roche, Basel, Switzerland) and SDS were added to 0.8 mg/ml and 1% final concentrations, respectively, and incubated for 2 h at 55°C. The released DNA was purified with a chemagic Magnetic Separation Module I (Perkin Elmer, Waltham, MA, United States), and quantified with the Quant-iT Picogreen dsDAN kit (Invitrogen). These genomic DNA samples were normalized, and libraries were prepared with the Nextera XT 96 DNA Library preparation kit (Illumina, San Diego, CA, United States). The sequencing was performed with a MiSeq V3 (Illumina) for 600 cycles, providing 22–25 million paired-end reads (2*300 bp) per run (sequence information can be found at Bioproject ID: PRJNA796703; BioSample accession numbers can be found in **Supplementary Table 1**).

Bioinformatic Analysis of the Genomic DNA

Genome assemblies were performed with SPAdes (Nurk et al., 2013), and annotated with prokka (Seemann, 2014). CheckM (Parks et al., 2015) was used to evaluate the completeness and potential contamination of the genomes, and genomes below 90% completeness were excluded. All genomes showed contamination estimates below 5%. High quality assemblies as well as publicly available genomes of *V. fischeri* strains MB13B1, MB13B2, MB13B3, and ES114 were used as input for a Roary pangenome analysis (Page et al., 2015). Roary was run using default parameters. FastTree2 (Price et al., 2010) was used to calculate a core genome tree using the concatenated alignment provided by Roary.

Colonization of the Squid Light Organ

The GFP-encoding plasmid pVSV102 (Dunn et al., 2006) was inserted into *V. litoralis* strain DSM17657 by conjugation as previously described (Stabb and Ruby, 2002). Overnight cultures of *V. litoralis* and *V. fischeri* were started in LBS medium, supplemented with kanamycin (50 μg per ml) when necessary to maintain pVSV102. Otherwise, bacteria were grown in seawater tryptone (SWT) media (Boettcher and Ruby, 1990). Bacteria were grown with shaking at 28°C until mid-exponential phase (∼0.5 $OD_{600\,nm}$), and diluted to a targeted concentration of 5000 cells per ml in 50 ml of filter-sterilized ocean water (FSOW) into which newly hatched juvenile squids were transferred. Squid were inoculated at room temperature with either one or both bacterial species, and maintained for 24 or 48 h. When the experiment lasted 48 h, the water was changed with fresh FSOW after 24 h. At the end of the experiment, the squid were rinsed individually twice for 1 min, and once for 5 min, in vials containing 4 ml FSOW, before being either frozen at −80°C, or anesthetized in 2% ethanol and fixed in 4% paraformaldehyde in mPBS (50 mM sodium phosphate buffer with 0.45 M NaCl, pH 7.4). The frozen animals were homogenized and aliquots plated on SWT agar plates, and the number of CFU that arose was used to calculate the symbiont population size in the light organ. Colonies of the GFP-labeled *V. litoralis* were differentiated from *V. fischeri* under a fluorescence dissecting scope, and colonies of *P. luteoviolacea* and *B. aquimaris* were identified by their distinct pigmentation. The fixed animals were rinsed four times for 30 min each in mPBS, and dissected. They were counterstained with TOPRO-3 (1:1000) in 1% TRITON-X100 mPBS, then rinsed four times for 15 min in mPBS. Light organs were mounted on a slide using Vectashield mounting medium, and imaged on a Zeiss LSM710 laser-scanning confocal microscope.

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://www.ncbi.nlm.nih.gov/, SAMN24847873; https://www.ncbi.nlm.nih.gov/, SAMN24907221–SAMN24907300.

ETHICS STATEMENT

The University of Hawaii Institutional and Animal Care and Use Committee (IACUC) is only allowed to review research using vertebrate animals. The communicating author has a letter from the University veterinarian that states the use of cephalopods in the research conducted in this study would pass IACUC standards if they were allowed to formally review it.

AUTHOR CONTRIBUTIONS

CB, EK, ER, and MM-N contributed to conception and design of the study. DM performed the bioinformatic analysis. CB and SL performed the animal colonizations. AR and CB performed the experimental sequencing. AR and ED provided training and facilities. CB wrote the first draft of the manuscript. CB, DM, and ER wrote sections of the manuscript. All authors contributed to manuscript revision, read, 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/fmicb.2022.854355/full#supplementary-material

Supplementary Video 1 | Colonization of newly hatched *E. scolopes* by *V. fischeri* and *V. litoralis* (GFP-labeled). A z-stack of the left side of a juvenile light organ (tissue nuclei stained blue with TOPRO) in which many GFP-labeled *V. litoralis* cells can be seen. No such fluorescent structures were observed in videos of light organs that were either uncolonized or exposed to bacteria without GFP labels.

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Microbial Dispersal, Including Bison Dung Vectored Dispersal, Increases Soil Microbial Diversity in a Grassland Ecosystem

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Microbial communities display biogeographical patterns that are driven by local environmental conditions and dispersal limitation, but the relative importance of underlying dispersal mechanisms and their consequences on community structure are not well described. High dispersal rates can cause soil microbial communities to become more homogenous across space and therefore it is important to identify factors that promote dispersal. This study experimentally manipulated microbial dispersal within different land management treatments at a native tallgrass prairie site, by changing the relative openness of soil to dispersal and by simulating vector dispersal *via* bison dung addition. We deployed experimental soil bags with mesh open or closed to dispersal, and placed bison dung over a subset of these bags, to areas with three different land managements: active bison grazing and annual fire, annual fire but no bison grazing, and no bison grazing with infrequent fire. We expected microbial dispersal to be highest in grazed and burned environments, and that the addition of dung would consistently increase overall microbial richness and lead to homogenization of communities over time. Results show that dispersal rates, as the accumulation of taxa over the course of the 3-month experiment, increase taxonomic richness similarly in all land management treatments. Additionally, bison dung seems to be serving as a dispersal and homogenization vector, based on the consistently higher taxon richness and increased community similarity across contrasting grazing and fire treatments when dung is added. This finding also points to microbial dispersal as an important function that herbivores perform in grassland ecosystems, and in turn, as a function that was lost at a continental scale following bison extermination across the Great Plains of North America in the nineteenth century. This study is the first to detect that dispersal and vector dispersal by grazing mammals promote grassland soil microbial diversity and affect microbial community composition.

Keywords: soil microbiology, microbial biogeography, grassland management, grazing (rangelands), fire

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INTRODUCTION

Microorganisms are the most diverse group of life on the planet (Locey and Lennon, 2016) and are integral to ecosystem functions such as nutrient cycling, biomass production, and carbon storage (Schimel and Schaeffer, 2012; Colman and Schimel, 2013; Glassman et al., 2018; Kuypers et al., 2018). Yet, a mechanistic understanding of the biogeography of microbial taxa lags behind

the extensive research for other organisms, such as plants and animals (Hanson et al., 2012). Accumulated evidence that microbial taxa can be dispersal limited and subject to the legacy of community assembly under historical environmental conditions, as opposed to being globally dispersed and filtered for survival by current local environmental conditions (Martiny et al., 2006; van der Gast, 2015), has created new questions about microbial metacommunity dynamics and the resulting biogeographical patterns that emerge.

Like macro-organismal communities, microbial communities assemble through a combination of dispersal from a regional taxon pool and successful growth in local conditions (Leibold et al., 2004; Hanson et al., 2012; Lindström and Langenheder, 2012; Nemergut et al., 2013). In some cases, the patterns that emerge from these mechanisms are similar to macro-organisms, while in other contexts they are different. For example, microbial taxa often display the same broad spatial scaling patterns that are found among plants and animals (Green and Bohannan, 2006; Locey and Lennon, 2016), but the strength of these patterns can be weaker for microbial life due to biological and methodological differences, such as dormancy and sampling extent (Locey, 2010; Meyer et al., 2018). One biogeographical pattern that exists across macrobial and microbial communities is that of distance-dissimilarity, or the decrease in community similarity with geographical distance (Soininen et al., 2007). The strength of the distance-dissimilarity relationship (i.e., the slope of regression line of community dissimilarity against geographic distance) depends on the balance between two main mechanisms of community assembly—environmental filtering and dispersal limitation (Nekola and White, 1999; Soininen et al., 2007; Hanson et al., 2012). Although environmental filtering has been more thoroughly investigated in microbial communities (Hanson et al., 2012), dispersal limitation can alter metacommunity dynamics by increasing similarity at close locations, while higher dispersal rates can increase similarity at farther locations through mass effects, which weakens the relationship between distance and dissimilarity. It is therefore critical to understand how different environmental attributes affect the interplay of underlying metacommunity assembly mechanisms.

Grasslands are diverse and globally important biomes that provide critical ecosystem services (Bengtsson et al., 2019) and are subject to environmental change due to shifting management practices, such as varying fire and grazing intensities (Bond et al., 2004; Briggs et al., 2005; Borer et al., 2014). How grassland fire and grazing management affects the relative importance of environmental filtering and dispersal limitation in structuring soil microbial communities is still unresolved. In northern China, Cao et al. (2016) determined that environmental filtering, mainly through soil pH and climatic factors, was the main process shaping microbial community distribution, but Richter-Heitmann et al. (2020) found the opposite, that purely deterministic assembly processes could not explain soil microbial diversity in temperate grasslands in Germany and that dispersal was important to both dominant and rare taxon dynamics. Overall, even if the mechanisms are unknown, it is clear that grassland soil microbial communities respond to large ungulate grazing *via* shifts in activity (Esch et al., 2013; Cline et al., 2017; Eldridge et al., 2017) and composition (Patra et al., 2005; Cline et al., 2017). Fire also alters the soil environment *via* direct heat, removal of organic matter, and subsequent changes to soil nutrient availability (Docherty et al., 2012). Although responses vary by grassland site, frequent fire can lead to increased soil microbial activity and shifts in composition (Pérez-Valera et al., 2017; Carson and Zeglin, 2018; Yang et al., 2020). These and many more studies demonstrate the importance of environmental filtering on grassland soil microbial communities, but dispersal could also have important consequences for microbial community assembly.

Dispersal is the least understood microbial community assembly mechanism in most ecosystems (Albright and Martiny, 2018), but could be affected by fire and grazing. In all grasslands, grazing is the critical ecological and evolutionary interaction between large herbivores and dominant plants (Stebbins, 1981). Before European settlement and "systematic slaughter" (in the words of Hornaday, 1889) of bison across the Great Plains, reducing the population to an estimated hundreds of animals by the end of the nineteenth century, bison numbered an estimated 25–30 million, and their range spanned more than a third of the continent (Lueck, 2002). Bison are particularly integral for North American tallgrass prairies as they hold a keystone role historically and contemporarily, increasing plant diversity, soil fertility, and forage quality in their zones of influence (Knapp et al., 1999). At sites across the Great Plains, bison grazing tends to decrease the strength of the soil microbial distance-dissimilarity relationship (Allenbrand, 2020), and bison reintroduction to Tallgrass prairie can cause convergence of soil microbial communities with varied management backgrounds, with their dung implicated as an important mechanism for this homogenization (Chantos, 2017). Also, North American bison have a distinct gut microbiome (Bergmann et al., 2015), as do most megaherbivores in more ancient grasslands (Kartzinel et al., 2019). Therefore, as large herbivores like bison move around the landscape, they may serve as vectors to disperse microbial cells *via* dung deposition. Concurrently, dispersal of microbial cells *via* aerial deposition and through water films in soil pores is also likely (Finlay and Clarke, 1999; Bottos et al., 2014; Yang and van Elsas, 2018; Elliott et al., 2019), and fire and grazing could influence how readily airborne cells reach the soil, since both create bare soil patches open to aerial inputs (Bakker et al., 2003; Henry et al., 2006). Further, fire can promote aerial dispersal of microbes, by aerosolizing viable soil microbial cells and spores (Kobziar et al., 2018; Moore et al., 2020). In sum, both bison grazing and fire could increase dispersal of soil microorganisms: Bison as a vector of dispersal, and fire as a direct vector, or non-vector mechanism that increases soil exposure to aerial dispersal.

To measure the degree to which non-vector and vector dispersal mechanisms are operating, it is necessary to experimentally alter the dominant factors predicted to be responsible for the pattern (Green and Bohannan, 2006). Two main research approaches, excluding modeling, can be taken for experimental evaluation of assembly mechanisms: environmental manipulation or altering dispersal rates (Hanson et al., 2012). Studies that manipulated microbial dispersal have been successful in altering rates and composition of dispersed

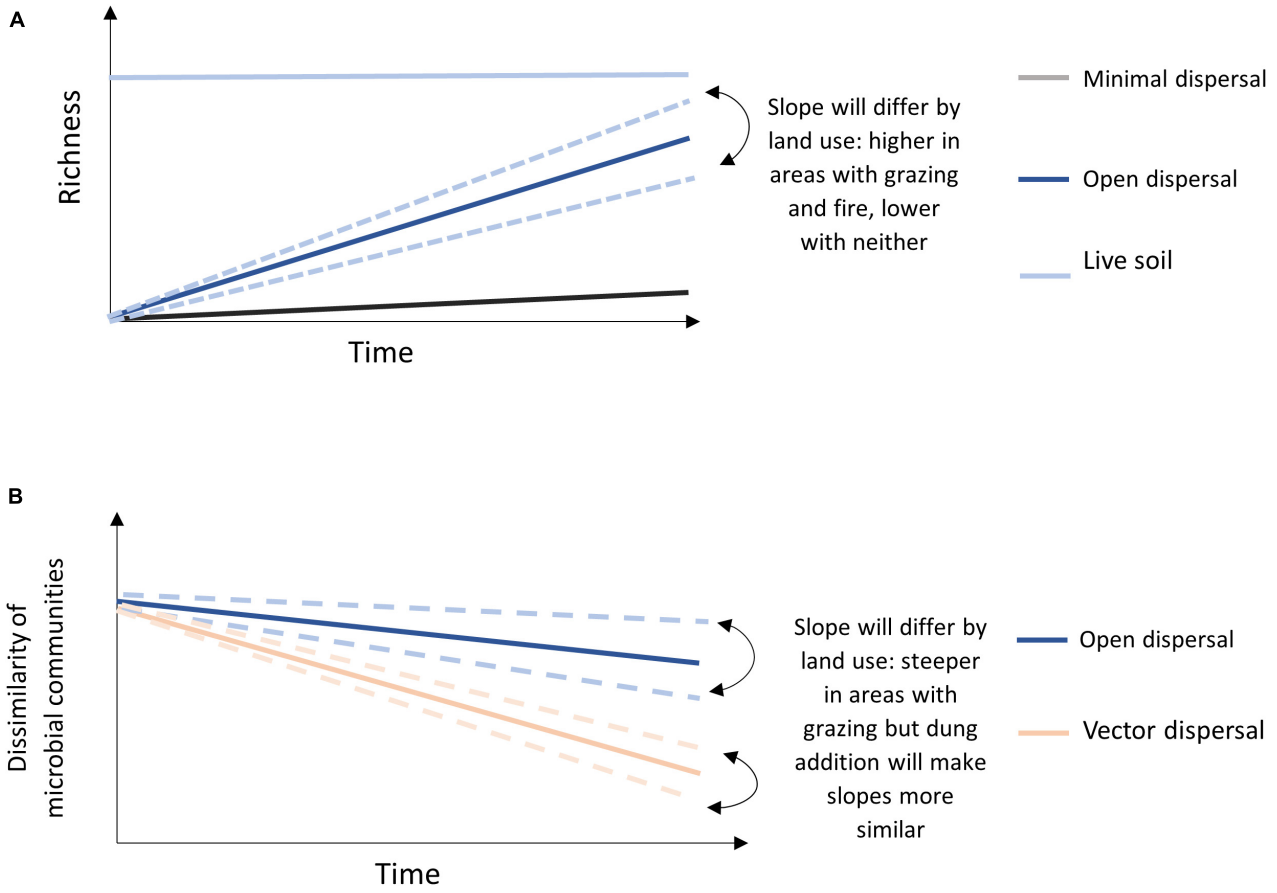

FIGURE 1 | Conceptual model of **(A)** predicted OTU accumulation over time for each treatment with indication of land management effect for the open sterile slope, and **(B)** predicted microbial community dissimilarity of open dispersal treatment and vector dispersal treatment (dung amended sterile soil) across land management treatments over time.

taxa, and showed that altered dispersal has a significant effect on community dynamics (Bell, 2010; Berga et al., 2015; Albright and Martiny, 2018). Therefore, we designed an experiment to learn how environmental manipulation (*via* differences in fire and grazing management), and alteration of dispersal rates (*via* manipulation of soil openness to aerial dispersal, and active addition of bison dung as a dispersal vector) influence dispersal limitation and subsequent assembly dynamics of soil microbial communities in a grassland ecosystem.

We hypothesized that dispersal of microbial taxa would be higher in burned and grazed areas than in unburned and ungrazed areas, but that burned area (open canopy) communities would display more heterogeneous assembly from aerial dispersal, while communities in grazed areas would converge in community composition due to bison-vectored dispersal (through bison dung). To test the hypotheses, we manipulated the potential rate of passive dispersal using soil bags with open or closed mesh (Albright and Martiny, 2018), and manipulated active dispersal using addition of fresh bison dung to sterilized and non-sterilized ("live") soil, and deployed these experiments in replicates across grazed, burned, and neither grazed nor burned watersheds at the Konza Prairie Biological Station (KPBS, Manhattan, KS, United States). Specific predictions included: (1) dispersal rates, or accumulation of new microbial taxa over time in sterilized soil open to dispersal, will occur in all conditions but will be highest in burned areas and lowest in unburned and ungrazed areas, and (2) vector dispersal, *via* the addition of bison dung, will increase the number of new microbial taxa in all environments, and also lead to microbial community convergence regardless of fire or grazing conditions (**Figure 1**).

MATERIALS AND METHODS

Study Location

This experiment was performed at Konza Prairie Biological Station (KPBS), located in northeastern Kansas, United States (39°05′N, 96°35′W), part of the Flint Hills region of KS and OK, one of the few, and largest, remaining native tracts of tallgrass prairie. KPBS was established as a research station in 1971, and became host to a Long-Term Ecological Research (LTER) project in 1980. Watershed scale treatments of differing fire intervals have been in place since the 1970s, and bison were

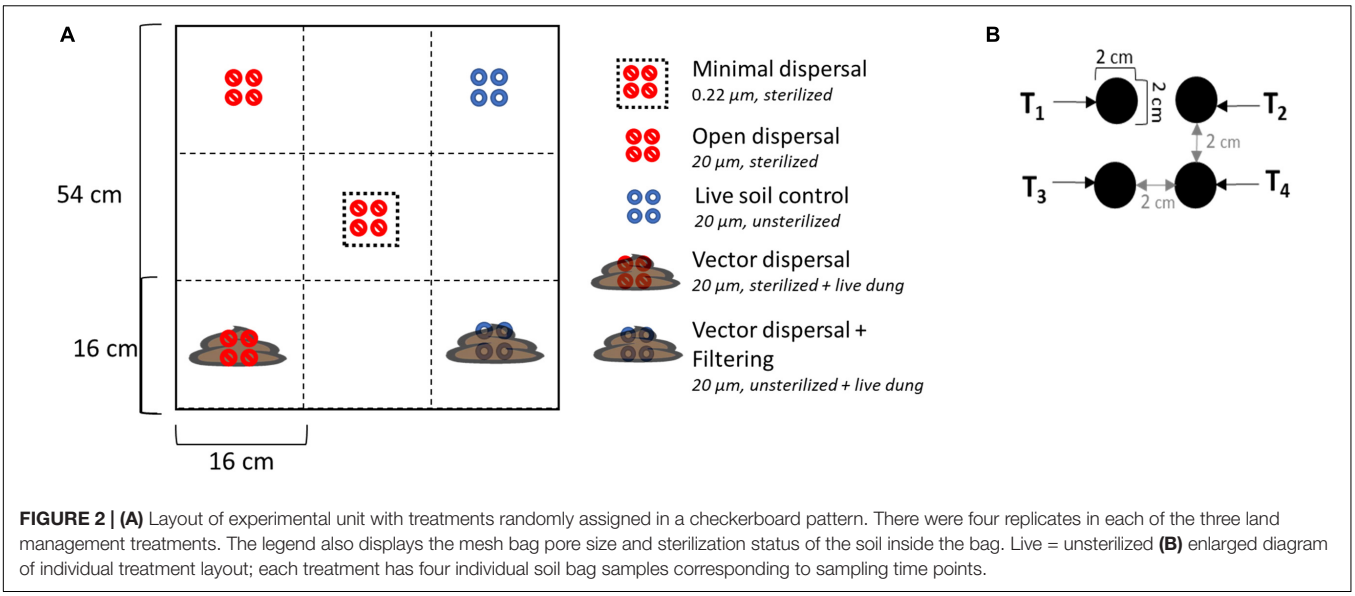

FIGURE 2 | (A) Layout of experimental unit with treatments randomly assigned in a checkerboard pattern. There were four replicates in each of the three land management treatments. The legend also displays the mesh bag pore size and sterilization status of the soil inside the bag. Live = unsterilized **(B)** enlarged diagram of individual treatment layout; each treatment has four individual soil bag samples corresponding to sampling time points.

reintroduced to a subset of these watersheds in the late 1980s—early 1990s, thus large areas with contrasting land management treatments have been maintained for decades. For this study, experimental research was restricted to upland soils (Florence series, Udic Argiustolls) in three of the environmental treatments: ungrazed and infrequently burned (20 year fire interval), bison-grazed and infrequently burned, and ungrazed and frequently burned (annual fire interval). No infrequently burned watersheds experienced fire in the study year, so are referred to as "unburned" treatments hereafter.

Experimental Design

Dispersal manipulations were installed across the experimental landscape, with four field replicates in each of the three different land use treatments. Each experimental unit contained five different dispersal treatments randomly assigned in a checkerboard pattern: sterilized soil closed to dispersal (minimal dispersal), sterilized soil open to dispersal (open dispersal), live soil open to dispersal (live soil control), sterilized soil open to dispersal from bison dung (vector dispersal), and live soil open to dispersal from bison dung (vector dispersal + filtering) (**Figure 2**). The open vs. closed dispersal contrast was achieved using nylon mesh (Tisch Scientific, North Bend, OH, United States) bags with pore sizes of 20 and 0.22 μm, respectively. These mesh sizes have been shown to successfully manipulate bacterial and archaeal migration rate (Albright and Martiny, 2018). The soil inside the bag was either live (unsterilized) or sterilized (*via* autoclaving at 121°C degrees for 20 min), and bags were always deployed to the same land use from which the soil was collected, to remove any confounding soil physicochemical environmental effects. In the vector dispersal treatments, recently collected fresh bison dung was deposited on top of the live and sterile soil bags after the bags were placed into the ground. Each treatment included four bags that were collected 1 day (T1), 1 week (T2), 1 month (T3), and 3 months (T4) post deployment, plus subsampling of the overlying bison dung at each time point. With 6 sample types (5 treatments + subsampling of dung), 3 land use treatments, 4 time points, and 4 replicates, 288 samples were collected in total.

Treatment Preparation and Installment

Fresh bison fecal samples were collected into gallon Ziploc bags using aseptic technique on 4 June 2019. Areas of the dung touching soil or vegetation was avoided, and dung was only collected from bison 2 years and older to ensure they had weaned and were eating a representative diet. The samples were kept on ice until transported back to the lab where they were stored at −20°C until further analysis. A subsample of approximately 50 mL was retained for reference data collection, and the remaining dung was divided in half to process for treatments.

Experimental unit locations were established and soil for the dispersal bags was collected from each sampling point within the unit using a 2 cm diameter soil auger to a depth of 2 cm. Soils were homogenized into one composite sample for each land use treatment, and plant material was removed, by sieving through 4 mm mesh using aseptic technique. A subsample for live soil and sterilized soil from each of the land use treatments was collected and stored at −20°C for characterization of initial soil microbial communities as a reference.

Open and closed dispersal soil bags were made with two different materials: nylon mesh with a pore size of 20 μm and a nylon membrane mesh with a pore size of 0.22 μm, respectively. Each bag had a dimension of 2 cm × 2 cm, but open and closed bags were constructed with two different methods. Using aseptic technique, the open bags were sewn using weather resistant nylon thread stitched along three edges with a folded edge to decrease the amount of stitching. Using aseptic technique, the closed bags were glued using Gorilla Glue Clear Grip along three edges with a folded edge. A small opening was left in each bag for filling, which was then closed with the corresponding method and bags were further processed according to dispersal

treatment. All bags used for live soil treatments were sterilized by autoclaving prior to filling and closed using aseptic technique and placed in UV-sterilized 1-L Nalgene bottles according to land use history. All bags used for sterile soil treatments were sterilized after filling by placing in UV-sterilized 1-L Nalgene bottles according to land use history and autoclaved with the lids loosely on. This allowed for aseptic transport to the field site for installation.

Soil bags were deployed back into sampling locations according to land use treatment. Live bison dung was deposited in equal amounts on top of soil bags according to treatment assignment. All soil bags and dung were placed underneath any surface litter that was present. At each sampling time point, the appropriate soil bag was extracted, transported to the lab, soil was transferred from the nylon bags to pre-labeled gamma-sterilized centrifuge tubes for storage at −20°C until further processing.

DNA Extraction and Polymerase Chain Reaction

Total genomic DNA (gDNA) was extracted from approximately 0.5 g of homogenized soil or dung per sample using the Qiagen DNeasy PowerSoil kit (Qiagen Sciences, Germantown, MD, United States) following manufacturer's instructions but with the following modifications: PowerBead Tubes were disrupted by bead beating for 20 s using a MP Biomedicals (Santa Ana, CA, United States) sample disruptor set at 4 m/s velocity, supernatant was transferred using the recommended minimum volume, and for final DNA elution step 50 μL of solution C6 was added and incubated for 5 min at room temperature before spinning down and repeated using the flow-through. In addition, since dung collected at later time points was markedly more desiccated and thus absorbed water, 500 μL of extraction buffer was added to the PowerBead column and then filled to capacity with dung even if dung mass was below 0.5 g. Genomic DNA (gDNA) was stored at −20°C until further analysis. Yield of gDNA was measured using a ThermoFisher Quant-iT PicoGreen dsDNA Assay Kit and quantified gram^{-1} dry soil (Thermo Fisher Scientific Inc., Waltham, MA, United States).

From the gDNA extracts, the bacterial and archaeal 16S rRNA gene was targeted for Illumina sequencing using universal primers (515F/926R) following established protocols (Caporaso et al., 2012; Parada et al., 2016) with one modification: Polymerase Chain Reaction (PCR) was run for 25 cycles instead of 35. Three technical replicates were run for each barcoded sample and reaction success was confirmed with 1% agarose gel electrophoresis. Upon successful PCR, technical replicates were pooled, cleaned using Exo-SapIT (Applied BioSystems, Foster City, CA, United States), and amplicon pools quantified using the Quant-iT PicoGreen assay kit (Life Technologies, Grand Island, NY, United States). Amplicon amounts were then normalized to 75 ng per barcoded sample, combined into one library and cleaned using a QIAquick Gel Extraction Kit (Qiagen, Germantown, MD, United States). The library was sequenced on a 2 × 250 paired-end read Illumina MiSeq run with 15% PhiX at the Kansas State University Integrated Genomics Facility.

Bioinformatics

Raw Illumina sequence data was processed using the QIIME2 software package (Bolyen et al., 2019). Sequences were demultiplexed and joined. Proceeding with only the forward reads, sequences were quality controlled and chimeras removed using Dada2 with default parameters, where reads truncated at the first instance of a quality score less than or equal to two (Callahan et al., 2016). The remaining sequences were clustered to 97% sequence similarity and assigned to operational taxonomic units (OTUs) using the open-reference workflow. OTUs were aligned to the GreenGenes (DeSantis et al., 2006) v 13.18 16S rRNA gene reference database and taxonomy assigned using a Naïve-Bayes classifier (Pedregosa et al., 2011) trained at 97% similarity. Singletons and doubletons (as per the rare feature cutoff threshold recommended in Bokulich et al., 2013), chloroplast sequences, and mitochondrial sequences were removed using filter functions before further analysis.

The remaining pre-processing, statistical analysis and visualizations were performed in R version 3.6.2 (R Core Team, 2019). The sequence library was further processed using phyloseq version 3.10 (McMurdie and Holmes, 2013) by creating a phyloseq object and removing samples that did not have at least 3,000 reads, resulting in a dataset with 272 samples and 6,500,692 total sequences with 15,326 unique OTUs. From this, two separate datasets were created: a rarefied dataset with all samples trimmed to 3,000 sequences by random sampling resulting in 272 samples and 816,000 total sequences with 11,664 unique OTUs, and a normalized data set by proportional transformation of each sample using total sequence counts resulting in 272 samples and 2,720,000 total sequences with 15,326 unique taxa. The low sequence count for the rarefied dataset was selected as the best approach to retain as many low-diversity samples as possible from the early experimental time points (**Supplementary Figure 1**).

Dispersal Analysis

All alpha diversity metrics were calculated using "phyloseq" and the statistical testing was done with base R and "vegan" (Oksanen et al., 2019). The alpha diversity metric of observed OTUs was calculated using the rarefied dataset with the estimate_richness function from "phyloseq" (McMurdie and Holmes, 2013). To test the effect of dispersal treatment, fire and grazing treatment, and the interaction between the two, on DNA yield and microbial richness for all time points, we used two-way analysis of variance (ANOVA) models and lsmeans function for *post hoc* pairwise comparison of groups. To test the effect of dispersal and land use treatment on DNA yield and microbial richness as a function of time, we used general linear models to perform analysis of covariance (ANCOVA) with the lm function (R Core Team, 2019) and Anova function (Fox and Weisberg, 2019). Response variables were assessed for normality, and only DNA yield needed to be log transformed to normalize data distribution prior to parametric statistical analysis. The models tested time, treatment, land use history and the pairwise interactions between all three (time by dispersal treatment, time by land use, dispersal treatment by land use, and the three-way

interaction). Pairwise ANCOVA models were performed on different treatment subsets to evaluate specific predictions regarding the effect of dispersal treatment and land use treatment on dispersal and richness (**Figure 1A**). For significant effects, least square means for all pairwise comparisons were used as the *post-hoc* test for identification of significantly different levels using the lsmeans function ("lsmeans," Length, 2016) and cld function ("multcompView," Graves et al., 2019).

Community Composition

A Bray-Curtis dissimilarity matrix was calculated from the normalized dataset to evaluate beta diversity, and community differences were visualized with non-metric multidimensional scaling (NMDS). A 3-way permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the effect on community composition of dispersal treatment, fire and grazing treatment, time, and their interaction using the adonis function in vegan with 999 permutations (Oksanen et al., 2019). To qualitatively evaluate community dispersion of dispersal treatments, the betadisper function in vegan was used to calculate the sample distance to group centroid of the Bray-Curtis dissimilarity matrix, with groups defined as dispersal treatments at each time point. The average distances were then plotted as function of time in days using ggplot, and used as the response variable to run ANCOVA to test the effects of dispersal treatment and time using the lm and anova functions. Least square means for all pairwise comparisons were used as the *post-hoc* test for identification of significantly different levels using the lsmeans and cld functions.

RESULTS

DNA Yield

In all sterile soil treatments, DNA yield was undetectable at time zero, and increased significantly over the incubation period (**Figure 3A** and **Table 1**). Throughout the experimental time-series, DNA yield remained lower in the minimal dispersal (0.2 μM mesh) treatment relative to the open dispersal (20 μM mesh) treatment, higher in vector-dispersal soil treatments than the open dispersal soil treatment, and higher in the live dung than the vector-dispersal soil treatments (**Table 2**). DNA yield did not change significantly through the sampling times for the live (unsterilized) soil treatments, but did increase significantly over time in live bison dung (**Tables 1**, **2** and **Figure 3A**). Land-use treatment effects were not significant (**Table 2** and **Supplementary Figure 2A**).

Richness

Microbial richness increased over time in all experimental dispersal treatments but was not affected by grazing or fire land-use treatment (**Figure 3B**, **Supplementary Figure 2B**, and **Tables 1**, **2**). While all slopes of OTU accumulation over time were similar, suggesting a dispersal rate of approximately 2–5 OTUs per day in all experimental treatments, there were significant differences in the intercepts of each model, indicating dispersal treatment effects on total richness that manifested early in the experimental time series (**Table 1**). The live soil control bags had lowest richness initially, and only reached the level of richness measured in intact field soil reference samples at the final, 3-month, sampling time (**Figure 3B**). In contrast, the sterilized soil treatments exposed to open dispersal accumulated higher richness in the first week of the experiment, and reached initial field soil reference levels after approximately 3 weeks (**Figure 3B** and **Table 1**). The vectored dispersal of microbes in live bison dung increased richness by hundreds of OTUs immediately, an effect that persisted for the duration of the experiment, maintained the highest richness overall, and weakened the slope of OTU accumulation over time (**Figure 3B** and **Tables 1**, **2**). Also, pure live dung had fewer observed OTUs than the soils with dung added (**Figure 3B** and **Tables 1**, **2**).

Community Composition

Soil microbial community composition was affected significantly by all treatments and their interactions (PERMANOVA, **Table 3**), with the highest amounts of variation explained by dispersal treatment (22.2%), time (10.1%), and the time by dispersal interaction (12.3%). In the NMDS ordination of all data, community composition shifted temporally, and soil and dung effects were also clearly separated (**Figure 4**). The community composition of vector dispersal treatments (soils with dung added) converged with the soil reference communities rapidly (after 1 day for the live soils, and after 1 month for the sterilized soils), while the open dispersal treatment communities became more similar to the reference soil communities over time, but did not converge after 3 months. The dispersion around the group centroid of vector dispersal microbial communities was consistently smaller than that of the open dispersal treatment communities without dung addition, and this difference was still apparent at 3 months ($F = 5.3$, $P = 0.00011$; *post-hoc* $P < 0.05$; **Figure 5**). There was no consistent trend in the compositional variance of the open treatment communities, while there was a steady decline in the minimal dispersal treatments over time ($F = 4.6$, $P = 0.034$; **Figure 5**).

DISCUSSION

This study shows that dispersal generally, including bison-dung vectored dispersal specifically, has a significant influence on soil microbial richness and composition. The experiment revealed that taxon accumulation over time *via* dispersal from aerial or proximate soil sources occurred under all treatment conditions, though fire and grazing management did not have the impact on dispersal rates that we predicted (**Figure 3B**, **Supplementary Figure 2**, and **Table 2**). Beyond the accumulation of taxa through non-vector dispersal, the dispersal through bison dung to both sterile and live soil resulted in an additive effect of hundreds of taxa, which may have contributed to community convergence over time (**Figure 5**).

It proved difficult to cut off microbial dispersal completely, as DNA and microbial taxa accumulated even in the "closed" (0.2 μM mesh) bags, despite DNA and richness levels below detection at the beginning of the experiment in that treatment,

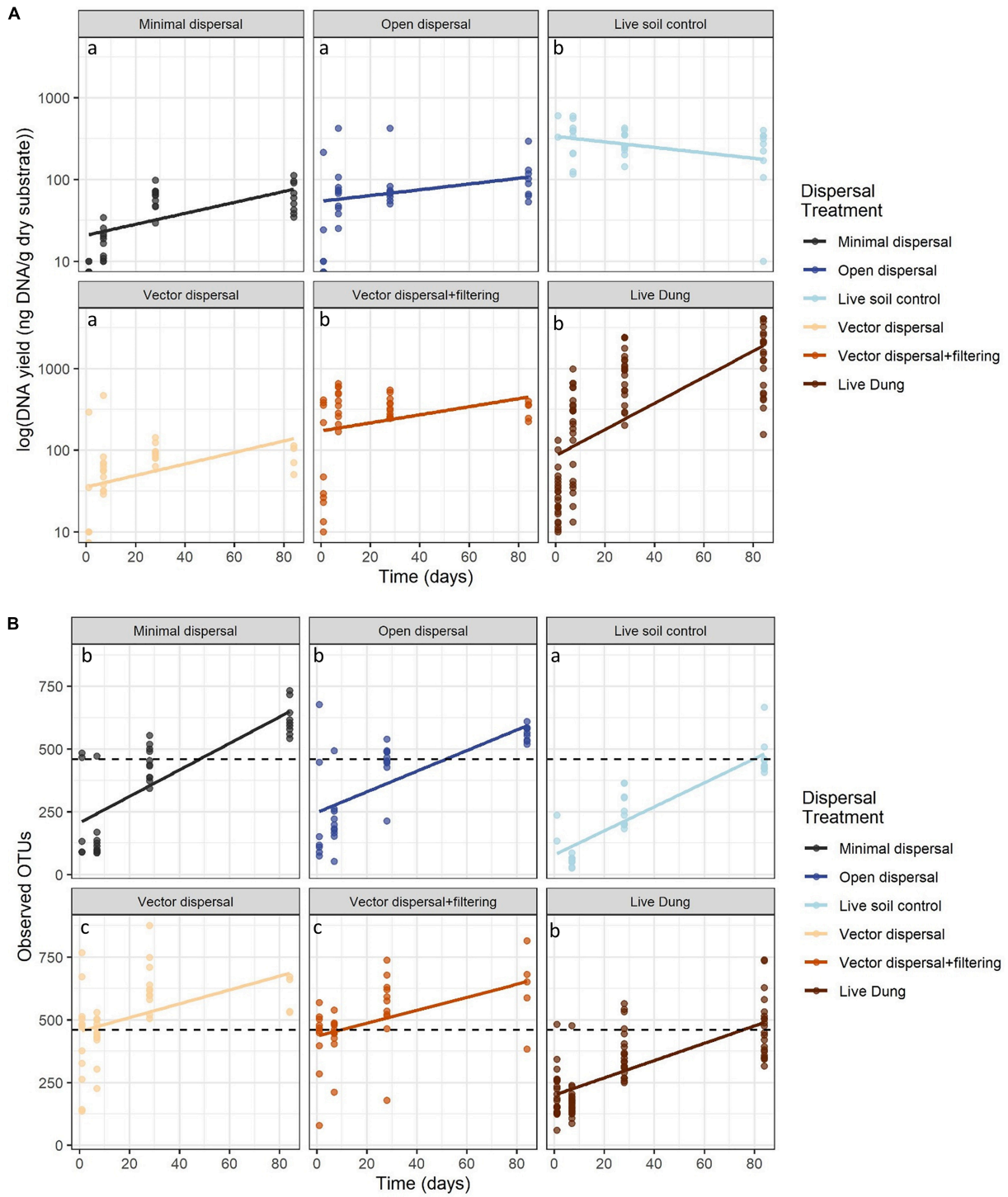

FIGURE 3 | (A) DNA yield (ng g-1 dry substrate) across time and **(B)** microbial richness (observed OTUs) across time with reference soil richness indicated by black dashed line. Ordinary least squares regression lines displayed and colored by treatment, and *post hoc* groupings for the intercept are indicated by lower-case letters.

which indicate the pre-experimental sterilization was successful. While the minimal dispersal treatment did not prevent microbial colonization, it did appear to successfully decrease the load of cells that were able to colonize and grow, since DNA levels remained lower through the experiment than the sterilized but "open" dispersal (20 μM mesh) bags (**Table 1**), which could in part be due to the smaller mesh size restricting dispersal to taxa with cell sizes smaller than 0.2 μM. Colonizers

TABLE 1 | Slopes and intercepts for full linear models for each experimental dispersal treatment pooled across land management types; model = log (DNA yield + 1) or OTU richness ~ Time in days *Treatment.

	DNA yield			OTU richness		
Dispersal treatment	**Slope, *p*-value**	**Intercept, *p*-value**	***Post hoc* groups for intercept**	**Slope, *p*-value**	**Intercept, *p*-value**	***Post hoc* groups for intercept**
Minimal dispersal	**0.02**	**11.9**	a	**5.3**	**205.5**	b
	0.0002	**<0.0001**		**<0.0001**	**<0.0001**	
Open dispersal	**0.02**	**28.7**	a	**4.1**	**247.3**	b
	0.018	**<0.0001**		**<0.0001**	**<0.0001**	
Live soil control	−0.01	**335.2**	b	**4.8**	**78.3**	a
	0.062	**<0.0001**		**<0.0001**	**0.0007**	
Vector dispersal	**0.02**	**26.7**	a	**2.8**	**453.3**	c
	0.01	**<0.0001**		**0.007**	**<0.0001**	
Vector dispersal + filtering	0.01	**173.6**	b	**2.6**	**434.9**	c
	0.092	**<0.0001**		**0.003**	**<0.0001**	
Live dung	**4.48**	**86.8**	b	**3.4**	**199.9**	b
	<0.0001	**<0.0001**		**<0.0001**	**<0.0001**	

Bolded values are significant. Post hoc groups for y-intercepts were defined using P < 0.05 significance threshold for least-squared means among group comparisons.

TABLE 2 | ANCOVA results for DNA yield (g g^{-1} dry substrate) and microbial richness (observed OTUs) for models comparing the treatment levels A: minimal dispersal, open dispersal; B: open dispersal, live soil control, vector dispersal, and vector dispersal + filtering; C: vector dispersal, vector dispersal + filtering, and live dung.

	A: Minimal vs. open dispersal		B: Open vs. vector dispersal		C: Live dung vs. vector dispersal	
Factors	**DNA yield F, P**	**Richness F, P**	**DNA yield F, P**	**Richness F, P**	**DNA yield F, P**	**Richness F, P**
Time	**6.04, 0.017**	**28.4, < 0.0001**	0.996, 0.32	**19.44, < 0.0001**	3.225, 0.075	**3.958, 0.049**
Dispersal treatment (Trt)	**4.4, 0.04**	0.0723, 0.800	**6.23, 0.0006**	**11.704, < 0.0001**	**4.92, 0.009**	**15.136, < 0.0001**
Land Mgt	0.925, 0.402	2.29, 0.11	0.322, 0.756	2.159, 0.12	1.238, 0.293	1.110, 0.332
Time*Trt	0.875, 0.353	0.143, 0.707	1.605, 0.192	1.3, 0.278	1.716, 0.183	0.212, 0.81
Time*Land Mgt	0.002, 0.998	1.04, 0.360	0.642, 0.528	0.697, 0.5	0.188, 0.829	0.44, 0.645
Trt*Land Mgt	0.657, 0.522	2.59, 0.083	0.737, 0.620	1.254, 0.284	1.183, 0.321	1.597, 0.178
Time*Trt*Land Mgt	0.286, 0.752	1.076, 0.348	0.314, 0.929	0.337, 0.916	0.53, 0.713	0.018, 0.999

Statistical results with P < 0.05 are shown in bold.

within or after 24 h of exposure could establish if carbon and nutrient sources left after the sterilization death of pre-existing microbial populations provided better environmental conditions for activity and growth. Additionally, dormant taxa in the form of spores or cysts may have survived sterilization and left the dormant state due to more favorable conditions (Locey, 2010), or traces of relict DNA may remain after sterilization (Carini et al., 2016). The slope of richness over time was significantly positive for all dispersal treatments (**Figure 3B**) and the trajectory of community change over time was similar (**Figure 4**), so while the DNA load in minimal dispersal bags tended to be lower than that of the open dispersal treatments (**Figure 3A**), the diversity of the source pool of colonizers appeared similar across all treatments and experimental units, which could point to the dispersal of the same small taxa or growth of the same dormant taxa across all of the sterilized treatments regardless of experimental bag mesh size.

TABLE 3 | PERMANOVA results across all dispersal treatments (Trt), time points (Time), and land management types (Land Mgt) for soil microbial community composition.

Factor	Sum of squares	*F*	R^2	*P*
Time	11.0	16.7	0.101	0.001*
Trt	24.2	15.7	0.222	0.001*
Land Mgt	2.3	5.2	0.021	0.001*
Time*Trt	13.4	4.1	0.123	0.001*
Time*Land Mgt	1.8	1.4	0.017	0.007*
Trt*Land Mgt	5.9	2.2	0.054	0.001*
Time*Trt*Land Mgt	7.3	1.1	0.067	0.027*

Statistical results with P < 0.05 are shown with an asterisk (*).

We found non-vectored dispersal to be important across all land use types and evident within 24 h, showing that dispersal is an important contributor to soil microbial richness and composition, and partly supporting our first prediction. Dispersal routes include aerial movement from wind and rain (Bottos et al., 2014) or active movement through the soil matrix (Yang and van Elsas, 2018), with both likely happening in our system. Microbial cells can be transported *via* wind-blown dust at local and regional scales, with distance traveled dependent on wind direction, speed,

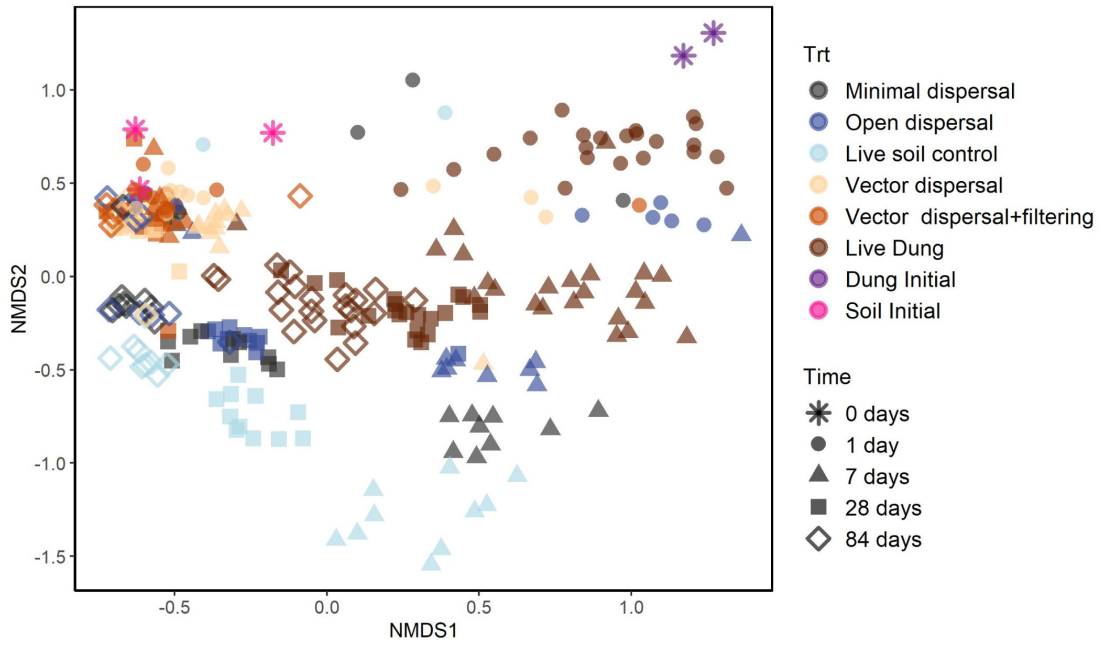

FIGURE 4 | NMDS ordination models of 16S rRNA gene community composition for all samples with colors representing experimental dispersal treatment (including reference soil and dung samples) and symbols representing sampling time.

and soil type (Sabacká et al., 2012; Acosta-Martínez et al., 2015; Elliott et al., 2019). The Great Plains are persistently windy, so it would not be unexpected for aeolian deposition to move microbes around the landscape. Secondly, microbial cells can move within the soil through water-filled pore spaces, which could result in dispersal to neighboring soil locations when water content is sufficiently high (Carson et al., 2010; Kravchenko et al., 2013; Yang and van Elsas, 2018). Movement of microbes within soil can also be driven by biotic interactions, as bacterial cells have been shown to use fungal hyphae as "highways" to navigate the soil matrix (Furuno et al., 2010; Warmink et al., 2011). More experimentation would be needed to parse contributions from these different mechanisms.

Contrary to our predictions, however, landscape-scale bison grazing and fire management treatments did not mediate microbial dispersal effects (**Figure 4** and **Table 3**). The lack of dispersal differences could be because very local scale effects, such as soil openness to dispersal (Albright et al., 2019) and influx of microbial populations from neighboring (sub-centimeters) soil and dung, might matter more than watershed scale environmental factors for overall dispersal rates. Alternatively, the effects of fire and grazing on dispersal might shift with time and our 3-month experiment may not have been long enough to capture this temporal variation. For example, transiently high aerial dispersal rates may have occurred immediately after spring burning, when soil was most exposed and more aerosolized cells were mobile (Kobziar et al., 2018), combined with higher dispersal impact in spring when soil had lower microbial biomass (Wang et al., 2012) and lower plant canopy cover. Our experiment was installed in early June, about 6 weeks after the annual fire; by this time, the peak influence of aerial dispersal might have passed. Also, partially burying the soil bags means that proximate soil communities may have been the dominant source of dispersal, rather than the aerial modes that underlaid our mechanistic predictions about fire and grazing effects. A follow-up dispersal experiment would need to be extended in time, and more explicitly measure aerial inputs of cells, to better evaluate the mechanisms of fire and grazing management on wind or rain driven dispersal.

Bison dung addition, mimicking vector dispersal by grazing ungulates, consistently and substantially increased soil microbial richness and changed the community composition (**Figures 3B**, **4** and **Table 1**). Thousands of bacterial and hundreds of archaeal taxa have been identified in bison fecal samples (Bergmann et al., 2015), so it is no surprise that dung may be an important vector of microbial dispersal. Further, a field bison dung incubation experiment conducted at a different tallgrass prairie site observed increased similarity among soil microbial communities after 3 weeks of exposure to the dung (Chantos, 2017). Following dung deposition, microbial dispersal to surface soil could also result from increased activity of dung-affiliated invertebrates, such as dung beetles (Slade et al., 2016), which may move their own host-associated microbiomes in and around the bison dung. Dung beetle abundance and diversity increases with bison presence and recent fire (Barber et al., 2017), and we anecdotally observed dung beetle activity, though we did not measure it. We also did not measure soil nutrient changes during this experiment; however, it is likely that dung quickly leaches labile nutrients and particulate organic matter, enhancing the fertility of the soil below and around it (Johnson and Matchett, 2001; Sitters and Olde Venterink, 2015), before it desiccates and hardens over the weeks of incubation.

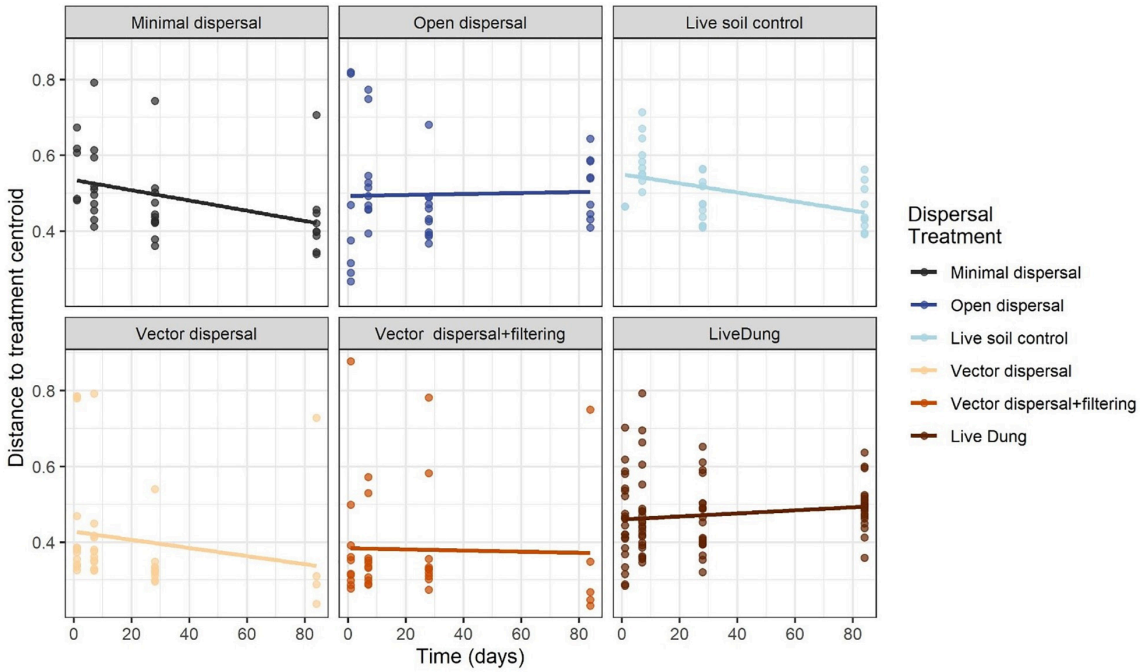

FIGURE 5 | Average distance to group centroids in multivariate space using the Bray-Curtis distance matrix across time for each dispersal experimental treatment. Values closer to 0 indicate less compositional variance across samples within that group, and therefore less dispersion.

However, another study saw no effect of bison dung addition on the C:N status of adjacent soil (Chantos, 2017), and therefore the role of environmental filtering through the fertilization effects of dung remains unclear. In our experiment, the dung addition clearly increased the number of microbial taxa in both sterilized and live soil relative to the corresponding treatments with no dung, but dung addition did not shift the live soil community composition toward that of pure dung. Rather, dung-sourced communities became rapidly more similar to soil (**Figure 3A** and **Table 1**). Also, as predicted, soil microbial communities converged more strongly and quickly with bison dung addition than with no dung (**Figure 5**). Thus, while the evidence that dung drives direct dispersal of microorganisms is clear, and it seems likely that the soil's distinct physicochemical habitat acts as an environmental filter for dung-sourced microorganisms, we still cannot infer the extent to which carbon and nutrients added through dung (Sitters et al., 2014) promoted the colonization and growth of certain taxa.

Unexpectedly, the live soil control experimental treatments had the lowest richness throughout the experiment, substantially less than the intact soil reference samples or the sterilized dispersal treatments (**Figure 3B** and **Table 1**). This suggests that removal of the soil from the field for experimental bag construction changed the microbial community, and that the 20 μM mesh barrier prevented the experimentally manipulated soils from recovering to a reference state (**Figure 4**). Laboratory processing of the soil to set up experimental soil bags could have killed certain taxa, providing the taxa remaining in the live soil control a competitive advantage over dispersers in the field due to earlier access to remaining nutrients, thus establishing communities with lower richness (Mouquet and Loreau, 2002; Svoboda et al., 2018). Such a priority effect is further supported by the combined observations of the live soil control treatments having the greatest DNA yield at the beginning of the experiment, but the lowest richness, indicating dominance of specific taxa that may have changed the community trajectory of these samples (Debray et al., 2022). The absence of this pattern in the initially live soil with dung addition could be because dung also serves as a nutrient source that alleviates resource scarcity, allowing dispersers a better chance of survival. Furthermore, biotic interactions are likely important for microbial community assembly, such that modification or suppression of interactions limits microbial richness in our experiment. For example, predation, which has been shown to increase microbial richness by reducing the survival of dominant taxa and allowing more rare or subordinate taxa to survive (Saleem et al., 2012; Jiang et al., 2017), would be minimal even in the "open" mesh experimental bags. Additionally, competition and cooperation with plant roots (Berg and Smalla, 2009; Haichar et al., 2014), fungi (Deveau et al., 2018), and invertebrates (Wardle, 2006; Bray et al., 2019) are well known biotic factors structuring microbial communities. Roots, invertebrates, and any organisms larger than 20 μm would have been unable to disperse into treatment bags, thus removing important multi-trophic interactions. The artificial conditions imposed by the dispersal experiment, in combination with the low rarefaction threshold required to fairly compare richness numbers among all samples (**Supplementary Figure 1**), emphasize that this work cannot be used to quantitatively predict *in situ* soil microbial richness levels.

The dual role of long-distance aerial and short-range within soil dispersal makes identifying a regional vs. local signal challenging, but nonetheless, a constant rate of passive dispersal could maintain higher soil diversity across the landscape in a relatively stochastic manner. In metacommunity theory, mass effects—the constant immigration of individuals because of high dispersal rates—can spatially homogenize communities and maintain the presence of rare taxa in communities (Leibold et al., 2004; Lindström and Langenheder, 2012). In microbial communities this effect might be stronger because of microorganisms' ability to enter dormancy and effectively serve as a "seed bank" if dispersed into initially unfavorable conditions (Locey et al., 2020; Wisnoski et al., 2020). In the context of grassland soil microbial community assembly, successful microbial passage through the ungulate digestive tract, either through dormancy or through facultatively anaerobic growth, serves as a strong filter antecedent to dispersal in dung. Before European colonization of the continent, bison migrated thousands of kilometers in mind-bogglingly high numbers across the North American Great Plains (Knapp et al., 1999). The global decline and extirpation of herbivore populations has detrimental consequences on many ecosystem attributes (Young et al., 2016). The extermination of bison from North America may have removed an important consumer-driven nutrient recycling function (Sitters and Olde Venterink, 2015) across Great Plains grasslands, and our results also suggest the likely loss of an important microbial dispersal mechanism that could impact soil microbial structure and function at both regional and local scales.

Overall, this experiment provides strong evidence that soil microbial dispersal is happening throughout the growing season in both grazed and burned land management environments in tallgrass prairie. Furthermore, vector dispersal through bison dung increases soil microbial community richness and homogenizes composition. Microbial dispersal has real and important consequences on community composition (Albright and Martiny, 2018) and function (Mallon et al., 2015; Evans et al., 2020), knowledge of which could be used to improve ecosystem management, conversation, and restoration. While the same mechanisms drive community assembly for all organisms, different biogeographical patterns may manifest due to the different scales at which these mechanisms act on microorganisms, making prediction of microbial structure and function less reliable when these different scales of influence are not taken into consideration. In this case study, bison's massively important historical role in grassland soil microbial community assembly, *via* dung-vectored dispersal, could be categorized as regional-scale deterministic mass effects, a category of influence that is not usually considered in metacommunity conceptual frameworks. A large number of contemporary studies on grassland soil microbial ecology are missing this factor, due to a lack of ungulate grazers within the study system. Our results contribute to both an increased understanding of grassland soil microbial community dynamics, and to a growing body of literature on soil microbial biogeography.

DATA AVAILABILITY STATEMENT

The data presented in this study can be found at the NCBI SRA database under Study PRJNA808890.

AUTHOR CONTRIBUTIONS

LZ conceived the initial study idea and edited the final manuscript. JH and LZ designed the study, performed bioinformatics, and contributed to data interpretation. JH carried out field and laboratory work, performed statistical analysis, and drafted the manuscript. 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/fmicb.2022.825193/full#supplementary-material

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REVIEW
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Ecological and Evolutionary Implications of Microbial Dispersal

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Dispersal is simply defined as the movement of species across space and time. Despite this terse definition, dispersal is an essential process with direct ecological and evolutionary implications that modulate community assembly and turnover. Seminal ecological studies have shown that environmental context (e.g., local edaphic properties, resident community), dispersal timing and frequency, and species traits, collectively account for patterns of species distribution resulting in either their persistence or unsuccessful establishment within local communities. Despite the key importance of this process, relatively little is known about how dispersal operates in microbiomes across divergent systems and community types. Here, we discuss parallels of macro- and micro-organismal ecology with a focus on idiosyncrasies that may lead to novel mechanisms by which dispersal affects the structure and function of microbiomes. Within the context of ecological implications, we revise the importance of short- and long-distance microbial dispersal through active and passive mechanisms, species traits, and community coalescence, and how these align with recent advances in metacommunity theory. Conversely, we enumerate how microbial dispersal can affect diversification rates of species by promoting gene influxes within local communities and/or shifting genes and allele frequencies *via* migration or *de novo* changes (e.g., horizontal gene transfer). Finally, we synthesize how observed microbial assemblages are the dynamic outcome of both successful and unsuccessful dispersal events of taxa and discuss these concepts in line with the literature, thus enabling a richer appreciation of this process in microbiome research.

Keywords: coalescence, community assembly, invasion, metacommunity ecology, microbiome

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INTRODUCTION

Biogeographic patterns of microbial communities and community responses to biotic/abiotic stressors have been studied primarily by considering environmental filtering or variance partitioning (i.e., deterministic selection). This is because microbes are presumed to have high dispersal rates, large population sizes, fast growth rates, and a propensity for dormancy (Xu et al., 2020). As such, these aspects collectively corroborate the notion that "*Everything is everywhere, but the environment selects*" (Baas-Becking, 1934). Though the "everything is everywhere" hypothesis was widely accepted through much of the twentieth century, mounting evidence that integrates ecological theory with characterizations of microbial communities has provided support to the contrary and suggests that microbes are instead dispersal-limited (Hubbell, 2001; Vellend, 2016). As such, apart from environmental selection, other community assembly processes, like dispersal, are gaining traction as important drivers of microbial community assembly and turnover (Nemergut et al., 2013; Dini-Andreote et al., 2015; Burns et al., 2017). In this review, we discuss the

role of dispersal in micro-organismal ecology and focus on idiosyncrasies between micro and macro systems that may lead to differences in how this fundamental process affects the structure and function of microbiomes. Within the context of ecological implications, we discuss the importance of short- and long-distance microbial dispersal through active and passive mechanisms, the value and concepts associated with species traits that favor dispersal and align these with metacommunity theory. In addition, we conceptualize the generalities of community coalescence (i.e., dispersal of entire communities), a phenomenon largely common in microbial communities. Conversely, we further detail how microbial dispersal can lead to changes in diversification rates dynamically affecting microbial evolution and eco-evolutionary dynamics.

DEFINING MICROBIAL DISPERSAL

Dispersal can be simply defined as the movement of organisms across space and time (Vellend, 2010). However, the process and underlying factors associated with dispersal are much more complex than this definition implies. Dispersal along with drift, selection, and differentiation (i.e., speciation), have conceptually been coined as four fundamental ecological processes responsible for the generation and maintenance of community structure (Vellend, 2010). Dispersal is yet an understudied process that can dynamically affect microbial communities. This general lack of explicit consideration of dispersal as a fundamental mechanism modulating microbial systems is mostly due to challenges associated with quantitative assessments of dispersal in observational microbiome studies, albeit relatively easy to manipulate and study it experimentally. Besides, unlike the other assembly processes, dispersal is not entirely a deterministic or stochastic process (Nemergut et al., 2013; Zhou and Ning, 2017). For example, dispersal may be more of a deterministic process when traits, like spore formation and dormancy, are common to select groups of microbes making them better adept for dispersal. On the other hand, dispersal is more of a stochastic process when density dependence favors more abundant taxa to disperse, as may be the case *via* passive dispersal mechanisms (Nemergut et al., 2013; Zhou and Ning, 2017).

When considering the definition, it is worth noting that the term dispersal does not imply a condition of successful establishment. Terms like "effective dispersal" (Nathan, 2013) or "migration" (Nemergut et al., 2013) can be used when explicitly referring to successful establishment following the dispersal event. In addition, studying the importance of dispersal can be even more complicated by the fact that "unsuccessful" dispersal events (i.e., the movement of species from one location to another followed by short-term persistence) can result in ecologically meaningful outcomes. For example, unsuccessful microbial dispersal has been shown to result in permanent shifts in microbial assemblages with long-lasting impacts on niche structure (Mallon et al., 2018; Amor et al., 2020). Also, it has been shown that transient invaders (or dispersal with fleeting establishment) can induce shifts in microbial communities between alternative stable states (Amor et al., 2020).

Active and Passive Dispersal in Microbes

Dispersal can be conceptually divided into two different mechanisms by which organisms move in space: active and passive (**Figure 1**). Active dispersal is initiated by inner processes in the microbial cell to direct movement, taking on different forms including hyphal growth and flagellar or ciliary motility. This type of movement can occur in response to predatory pressure, resource availability, or environmental chemical signaling (e.g., chemotaxis) (Hedlund et al., 1991; Fomina et al., 2000; Matz and Jürgens, 2005; McGonigle, 2007). The active movement of microbes is generally assumed to be of greater importance in more diffusible systems (e.g., aquatic habitats). For example, in ocean waters, in the absence of a chemosensory motile trait, bacterial cells rely on Brownian motion to move through the water column. These non-motile bacteria are limited to exploring ∼80 nanoliters per day, while motile bacteria can explore up to 1 ml (Stocker, 2012). Motility has obvious advantages and can facilitate resource capture over patches much larger than that of their non-motile counterparts. As such, traits associated with motility are widespread throughout aquatic microbes and can occur in upwards of 80% of the total community (Blackburn et al., 1998; Fenchel, 2002; Stocker and Seymour, 2012). Worth mentioning, such a type of motility is also important in terrestrial systems. For instance, assisting the movement of species within diffusible hotspots in the soil [e.g., plant-root surfaces (Aroney et al., 2021), fungal hyphae (Pion et al., 2013), see below].

Conversely, passive dispersal occurs when microbial movement is mediated by outside forces, such as the movement of water, wind, soils, animals, or even other microbes (Griffin, 2007; Ingham et al., 2011). This type of dispersal, particularly in terrestrial ecosystems, is often responsible for long-distance movements of microbial cells. For example, it has been shown that pathogenic, prokaryotic microorganisms residing in soils are passively dispersed inter-continentally during dust storms (Griffin, 2007; Gonzalez-Martin et al., 2014). This implies that unlike inter-continental dispersal of macro-organisms, which can take hundreds or thousands of years, global dispersal of microbes through passive mechanisms can occur very rapidly, as recently demonstrated by the shift endemicity to global ubiquity of the SARS-COV-2 virus (COVID-19) over a period of a few months.

Propagule Pressure

The concept of propagule pressure depicts the total effort of an organismal introduction in a system (**Figure 2**). This has been shown to have major importance in explaining the time-dependent success of invasion or dispersal (Lockwood et al., 2005; Simberloff, 2009; Jeschke and Starzer, 2018). Consisting of both frequency (number of dispersal events over time) and size (number of individuals per dispersal event), propagule pressure can vary temporally and have effects on community dynamics. Recently, Albright et al. (2021) provided a synthesis of strategies to prospectively engineer microbiomes based on dispersal dose (i.e., propagule size) and frequency. Within their framework, propagule size is conceptualized to interact with environmental stochastic extinction and density-dependent competitiveness,

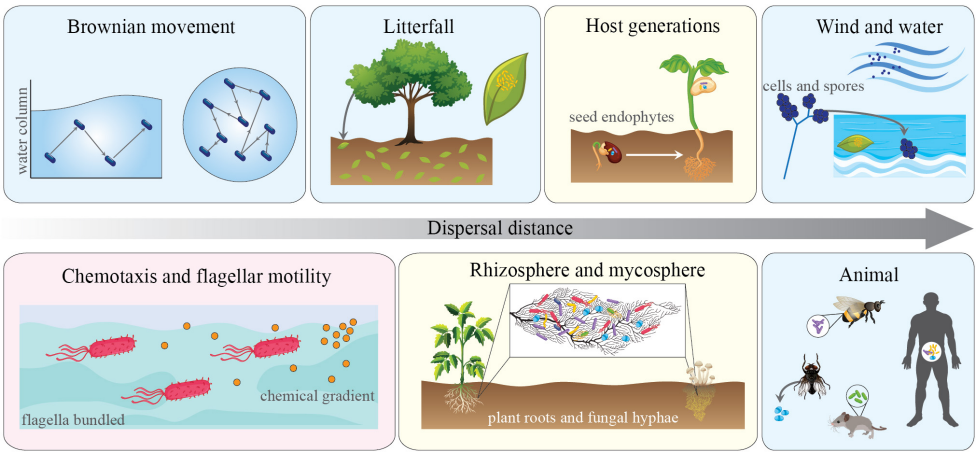

FIGURE 1 | Types and mechanisms of microbial dispersal and the modes by which they operate. Panels depict multiple instances of passive (light blue), active (light red) dispersal. Light yellow panels indicated that both modes of action operate simultaneously. Dispersal range of μM to upwards of hundreds of kilometers is indicated by the horizontal arrow. The figure includes different habitat types and local hotspots that align with the type and selection of traits associated with dispersal in different contexts (see main text).

while dispersal frequency acts to make niche pressure ephemeral and even promote biotic disturbance when taxa are added at different time points. As a general rule, high propagule pressure (i.e., more individuals introduced during a single event and/or more frequent introductions) typically results in a higher probability of establishment (Colautti et al., 2006). However, the importance of the individual components of propagule pressure may be dependent upon the identity of the invader and the composition of the recipient community. In freshwater bacterial mesocosms, competition has been shown to increase the importance of propagule size, while propagule frequency was shown to be most important in communities with lower growth rates (Jones et al., 2017). Other examples with implication for remediation success in soils show that increased propagule pressure results in higher efficiency of removal of environmental contaminants (e.g., petroleum pollution) (Avdalović et al., 2016; Li et al., 2016). Given that diverse microbes can produce an enormous number of propagules and often disperse inter-continentally, some have even gone as far as to describe microbial dispersal as a "microbial conveyor belt" (Mestre and Höfer, 2021). This concept suggests that microbes move around the globe through recurrent and cyclic dispersion, allowing selection to act upon specific traits associated with dispersal. Although this is the case for some microbial taxa and/or in particular systems, the explicit consideration of dispersal and propagule pressures at local and regional scales is likely to better inform on the importance of these processes modulating community assembly.

MICROBIAL TRAITS AND DISPERSAL

Evolutionary trade-offs mediated by differences in species traits are important for understanding the balance of fundamental processes giving rise to unique ecological assemblages (de Oliveira et al., 2020; Thompson et al., 2020). That is, species traits help to explain the nature of species interactions with the biotic and abiotic edaphic components. Hence, it is possible to assume that depicting specific microbial traits in line with habitat context can lead to a better understanding of how dispersal operates and can be "selected" for across divergent systems. In this section, we consider a set of specific microbial traits associated with microbial dispersal and link these with their relative importance across different environmental contexts. Additionally, we present a short summary of a recent effort made toward the integration of an ecological framework in microbial systems, with a particular focus on trade-offs in traits affecting organismal fitness (**Box 1**).

Motility and Chemotaxis

Short-distance active movement by microbes is determined by the presence of traits that allow the organism to sense environmental stimuli (e.g., molecular sensing of chemical gradients or predator pressure) and move toward more favorable conditions (Jarrell and McBride, 2008). These traits include those associated with chemotaxis, flagellar/ciliate motility, and even the "walking legs" of *Mycoplasma* (Miyata, 2008). The importance of motility traits, and thus their prevalence, likely relies upon the diffusibility of the system. For instance, drier soil systems may be less diffuse than wet soils or aquatic systems and thus favor other traits (e.g., antibiotic production/resistance) over the ability to actively move across space (Dini-Andreote et al., 2018). While evolutionary trade-offs likely decreased the prevalence of motility traits in soil, roots or fungal hyphae can act as highways to favor such strategies, thus facilitating the active microbial movement though a heterogenous soil matrix (Pion et al., 2013). On the other hand, in diffuse systems like aquatic habitats or wet soils, motility allows organisms to move across a larger area of the continuous landscape, with direct implications for their ability to exploit nutrient-rich patches (see the "active dispersal" section above for additional detail).

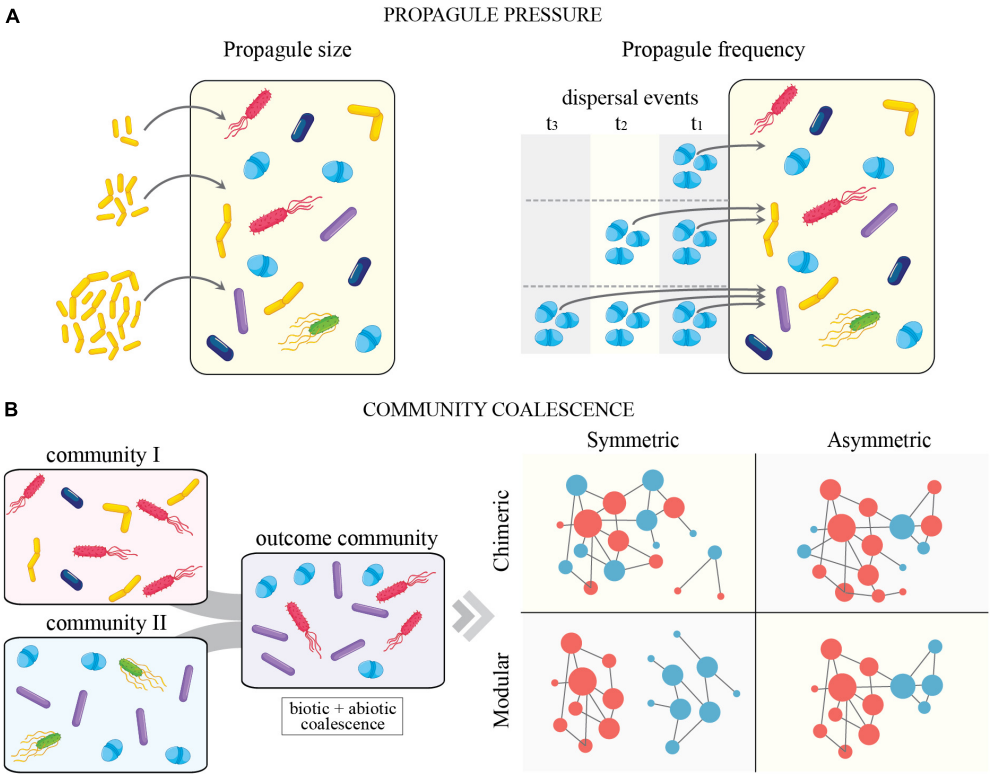

FIGURE 2 | (A) Conceptual depiction of propagule pressure (propagule size (left) and frequency (right) in microbial systems. **(B)** Conceptual diagram of community coalescence. Community coalescence is defined as the mixing of entire communities and their environments (*sensu* Rillig et al., 2015, 2016). **(B)** (Left) illustrates community coalescence in microbial systems. **(B)** (Right) depicts a modified version of possible coalescence outcomes from Castledine et al. (2020). In brief, symmetric and asymmetric coalescence indicates the degree to which one input community dominates over another after coalescence. Modular and chimeric coalescence relates to the degree of pre-existing (in previous communities) and novel species interactions (after coalescence), respectively. See Castledine et al. (2020) for additional details.

Spore Formation

While motility allows for short-distance movement, the ability to form spores facilitates the passive dispersal of microbes throughout space and time. Sporulation allows organisms to essentially "pack-up and move-on" to potentially more hospitable conditions. Such a trait can even allow an organism to persist over millions of years (Cano and Borucki, 1995), thus nicely connecting distinct evolutionary time-scales at local communities. The ability to form viable and long-lived spores make possible integral parts of the life cycle for many microbes and can provide resistance to environmental stressors like drought, heat, and even UV radiation (Huang and Hull, 2017). For example, in the pathogenic bacteria *Clostridium difficile*, endospore formation is required to reach the colon of their human hosts (Paredes-Sabja et al., 2014), while in fungi, both sexual and asexual spores can contribute to spatiotemporal dispersal through their often complex and dynamic life cycles (Wyatt et al., 2013).

Dormancy

Dormancy allows some microbes to enter a reversible state of low metabolic activity when exposed to unfavorable environmental conditions. This promotes the long-term persistence of microbial taxa within a local community (Lennon and Jones, 2011), and assists with long-distance dispersal of taxa *via* passive dispersal. Collectively, it has been shown that such traits are directly associated with patterns of population dynamics *via* temporal dispersal and the storage effect (Warner and Chesson, 1985). The strategy of dormancy can be found in several groups of microbes and encompasses a variety of phenotypes, ranging from latency in viral lifeforms to anhydrobiosis and cryobiosis in tardigrades (Lennon and Jones, 2011; Bertolani et al., 2019). Entering a state of dormancy allows an organism to bypass periods of suboptimal environmental conditions and reanimate when conditions become more favorable. Examples of dormancy in macro-organisms are also common (e.g., seasonal hibernation or extended diapause), though the temporal scale of dormancy in animals and plants is much shorter. It is worth noting that dormancy is also a metabolic costly trait. The mechanisms required for entering and exiting a state of dormancy demands a relatively large energy input, and during periods of dormancy, organisms must maintain cellular repair mechanisms or risk the chance of fatal damage to cellular machinery and loss of the ability to metabolic awaken upon appropriate environmental stimuli (Lennon and Jones, 2011).

BOX 1 | Extending Grime's competitor-stress tolerator-ruderal (C-S-R) framework to microbial traits.

Early work in macro-organismal systems focused on trade-offs in key fitness traits associated with resource acquisition and efficiency. This led to the development of the *r* vs. *k* framework, the colonization-competition trade-off, and the analogous copiotroph-oligotroph framework for microbes. Conversely, the Competitor-Stress Tolerant-Ruderal (C-S-R) triangle was developed to more holistically explain tradeoffs in life history traits of plants and incorporates the process dispersal (Grime, 1979; Koch, 2001). Recently, the C-S-R framework has been revisited and modified to categorize microbial life histories into three groups that are analogous to the original framework: high yield (Y), resource acquisition (A), and stress tolerance (S), forming the Y-A-S triangle. The Y-A-S framework has provided advances and insights into microbial life history strategies and may serve to further our understanding of traits that facilitate microbial persistence and survival (Wood et al., 2018; Malik et al., 2020). Within the context of the Y-A-S framework, dormancy may link characteristics of the "Y" and "S" lifestyles. Most apparent, dormancy facilitates the persistence of stress tolerant organisms, or "S" lifestyle adapted. The "S" individuals are either capable of dedicating resources to promote stress tolerance or enter a dormancy state depending on the type and intensity of environmental harshness. In this scenario, microbes capable of entering dormancy and disperse long distances may act as colonizers of new habitats. These organisms are likely to reanimate in an environment with excess resources, possibly at early successional stages. In this case, the "Y" life history would then be favored, thus aligning stress tolerators with high yield organisms. In fact, the "Y" and "S" lifestyles likely covary in a negative way with the type "A" organisms (resource acquisition) due to colonization-competition tradeoffs (Tilman, 1994).

Theory from macro-organismal ecology predicts that dispersal and dormancy will negatively covary due to life history trade-offs (Rees, 1993). That is, organisms capable of entering dormant states are not as reliant on changing physical locations, and vice-versa. However, negative covariation between dispersal and dormancy may not always be the case (Buoro and Carlson, 2014), and the opposite relationship may arise when there is a genetic link between traits that affect dormancy and dispersal (Peiman and Robinson, 2017; Wisnoski et al., 2019). In fact, it is plausible to assume that this is the norm rather than the exception for microbes, as dispersal across large spatial scales is largely mediated by passive events and requires dormancy and/or sporulation for long-term cell persistence. Depicting this relationship, Wisnoski et al. (2019) reviewed a set of studies that utilized aquatic zooplanktons as a model to understand positive dormancy-dispersal covariation. Collectively, these studies showed that organisms with durable propagules made a long-lasting contribution to the seed bank and were capable of dispersing longer distances due to their ability to survive the avian digestive tracts (i.e., suboptimal conditions) (Figuerola and Green, 2002; Viana et al., 2016). Thus, microbial reliance upon passive dispersal can result in positive dispersal-dormancy covariation, as traits that facilitate dormancy have an obvious mechanism to affect dispersal and vice-versa.

DISPERSAL OF ENTIRE COMMUNITIES AND COMMUNITY COALESCENCE

In classical ecology, dispersal refers to the movement of single species across space (Vellend, 2010). However, in striking contrast to macro-organismal systems, it is not uncommon for entire microbial communities to be dispersed simultaneously due to their small size and cohabitation of easily moveable units (e.g., soil particles, plant root segments, or animal intestinal tracts) (**Figure 2**). For an analogy, imagine an entire African savannah community, complete with herbivores, predators, and prey, simultaneously transported to a new location. Upon reaching a new destination, this community (termed as "donor"), begins to interact with the existing ecological system (termed as "resident") to produce a novel coalesced community. While we know this to be rare in macro-organismal systems, the movement of entire microbial assemblages is likely the norm rather than the exception (e.g., flooding events, soil tillage, animal excretion, shaking hands, etc.) (Rillig et al., 2015, 2016). Through the process known as "community coalescence," or mixing of complete communities and their environments, microbial assemblages with distinct ecologies and evolutionary histories are combined to produce a variety of outcomes that are dependent upon the type and strength of microbial interactions taking place within each assemblage (e.g., the complexity of trophic and ecological interactions) and their abiotic similarity (Rillig et al., 2015; Castledine et al., 2020).

Community coalescence has been broadly used to manage and manipulate microbiomes in agriculture and clinical settings, even though the concept was only recently formalized (Rillig et al., 2015, 2016). For example, fecal microbiome transplants have been implemented to treat several human diseases, including conditions like recurrent *Clostridium difficile* infection and Chron's disease (Gupta et al., 2016; Wilson et al., 2019; Sokol et al., 2020). However, desirable outcomes were not consistently achieved, and most of the variation in clinical outcome might relate to our yet inability to properly understand and predict the fundamental basis and outcomes of community coalescence. Likewise, coalescence has also been used to engineer soil microbial assemblages capable of steering restoration in ex-arable lands (Wubs et al., 2016), boosting crop production in organic agriculture (Ramos et al., 2019), or enhancing biomethane production in anaerobic digesters (Sierocinski et al., 2017), and has even been used to reduce nutrient flow-through and enhance recovery at wastewater treatment facilities (Wagner et al., 2002; Priya et al., 2021). Recently, the concept of *meta-gut* was proposed as a conceptual framework that utilizes coalescence to integrate the gut microbiome of excretion with the function these fecal microbiomes provide outside the host. To illustrate this new concept, a study investigated how the fecal microbiome of wild hippos dynamically alters the local biogeochemical processes of the pools these animals inhabit (Dutton et al., 2021).

ECOLOGICAL IMPLICATIONS OF MICROBIAL DISPERSAL

Dispersal is a central mechanism in ecology that can only be fully explored in the context of metacommunity theory, i.e., a set of local communities that are linked by dispersal of many potentially interacting species (Leibold et al., 2004). In line with that, all pre-conceptualized paradigms of metacommunities

[i.e., patch-dynamic (PD), species-sorting (SS), and mass-effects (ME); in addition to the neutral model (NM)], are actually four different perspectives that characterize how environmental heterogeneity (deterministic selection), and dispersal rates interplay to influence community diversity. In this section, we discuss recent advances in community ecology with a focus on the ecological implications of dispersal on structuring microbial community assemblages in line with metacommunity theory.

Metacommunity Theory for Microbes

A metacommunity is defined as a set of local communities with singular composition and functionality linked by dispersal events. Conceptually, theoretical and applied studies have based the metacommunity view through four perspectives: patch-dynamic, species-sorting, mass-effects, and neutral model (Leibold et al., 2004; **Figure 3**). In short, PD assumes that low levels of dispersal and species trade-off can lead to species coexistence at a regional scale. This implies the existence of distinct time scales between local and regional colonization-extinction dynamics. The SS perspective mostly focuses on the outcomes of species interactions occurring within local communities (habitat patches) that differ in their abiotic and biotic properties. This nicely aligns with the traditional view in microbial ecology (or the niche-based paradigm) and how microbiomes have mostly been studied in terms of variance partitioning. Conversely, ME aligns with SS but in a scenario where the rates of dispersal are too high and can lead to an overall homogenization of local communities over a regional scale. Lastly, NM assumes that species traits and environmental heterogeneity are equivalent across species and habitat types, and, as such, neutral for determining community composition and structure (Perkin et al., 2021).

These four metacommunity paradigms, driven by the movement of individuals among patches, combine ecological processes at local and regional spatial scales (Brown et al., 2017). Hence, metacommunity can be expressed through a set of continuous processes operating within and among interconnected communities. Local processes are mainly based on species interactions, environmental heterogeneity, and *in situ* perturbations, while regional processes are broadly driven by dispersal (Brown and Barney, 2021). Research efforts from macro-organismal ecology have provided examples and insights into the importance of dispersal and habitat heterogeneity for the generation and maintenance of species biodiversity across multiple spatio-temporal scales, including their interactive effects on community α-, β-, and γ-diversities. These can be extended to microbial systems by integrating metacommunity ecology with microbiome research, though these efforts may not be trivial.

Dispersal and Community Diversity

The rate and magnitude of dispersal have been shown to affect biodiversity at different scales. Predictive models show that intermediate rates of dispersal often result in higher levels of α-diversity (Mouquet and Loreau, 2002). While low rates of dispersal can lead to an overall dominance of highly competitive taxa and augment the importance of ecological drift (i.e., random birth and death events leading to rare species extinction). High levels of dispersal, however, can result in a homogenization of the local and regional species pools. This can lead to an overall short-term increase in stochasticity followed by an increase in deterministic selection at a regional scale that decreases species diversity (Mouquet and Loreau, 2003). As such, it is plausible that intermediate dispersal rates promote the continuous (re-) introduction of taxa and balance the effects of local selection by dispersal from the regional species pool. Additionally, different rates of dispersal across local communities often favor habitat heterogeneity and the differential performance of species across distinct local patches, which collectively account for species coexistence and promote biodiversity in heterogeneous landscapes (Thompson et al., 2020).

Experimental studies in macro- and micro-organism systems and meta-analyses have provided evidence to suggest idiosyncratic outcomes of the relationship between dispersal and local species diversity. Cadotte (2006) reported that while intermediate levels of dispersal can maximize α-diversity in animal communities, such effect does not hold for plant communities. These authors suggested an overall positive relationship, albeit the specific correlation may be non-linear and differ across organismal types. Conversely, Grainger and Gilbert (2016) further reported that approximately 50% of their surveyed experiments found a positive effect of dispersal on α-diversity, of which only 10% found this relationship to display a predicted hump-shaped pattern. In sum, these meta-analyses concluded that divergences between predicted theoretical outcomes and empirical studies are likely the result of three shortcomings in experimental design, including (*i*) failure to incorporate inter-specific differences in dispersal capabilities, (*ii*) heterogeneity in initial community structure and diversity across systems, and (*iii*) the lack of consideration of network structure among patches. All of which offers opportunities to further develop this synthesis in landscape microbial systems and/or *via* the design of prospective experimentation.

In microbial communities, manipulative experiments have provided valuable evidence for the influence of dispersal on local community diversity. Albright and Martiny (2018) examined the influence of dispersal rates in bacterial decomposer communities and showed that dispersal has a rate-dependent effect on community diversity affecting the decomposition rate. It was shown that selection in litter can decrease community diversity in the absence of dispersal due to competitive exclusion combined with ecological drift. Moreover, in another study, it was shown that increasing rates of dispersal result in a hump-shaped pattern of local species diversity (Evans et al., 2017). This pattern occurred because, after a certain level, high dispersal rates weaken local selection. As a follow-up, the authors suggested the strength of local environmental selection to play a role in the extent to which selection and dispersal interact in the system. For instance, by showing that weak selection imposed by low lignin:N ratio related to more labile litter chemistry (Evans et al., 2017).

The effect of dispersal rates on community divergences (i.e., β-diversity) is expected to negatively covary. That is, higher rates of dispersal will result in lower β-diversity by enhancing similarities across local communities (Loreau, 2000; Mouquet and Loreau, 2003; Cadotte, 2006; Grainger and Gilbert, 2016). While this relationship makes intuitive sense and has

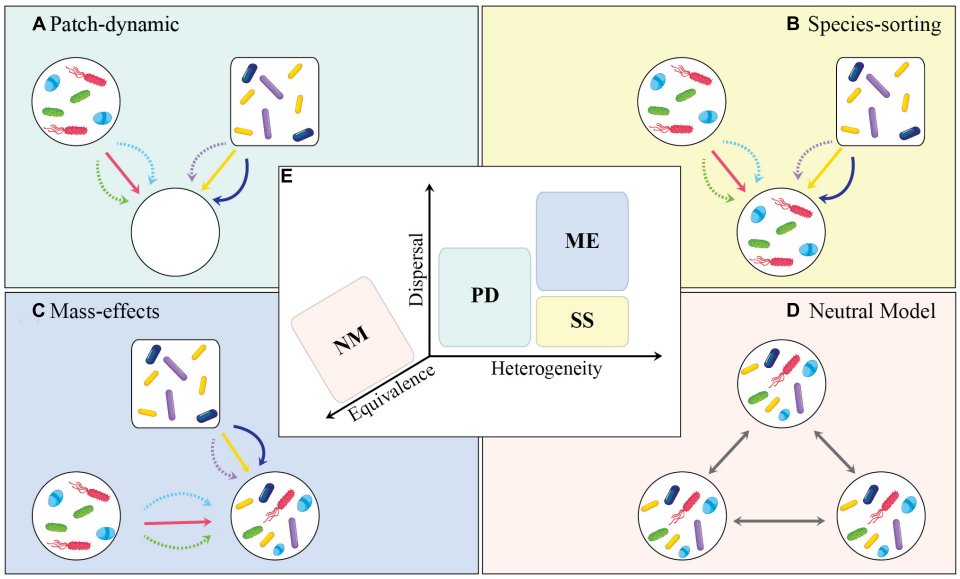

FIGURE 3 | Conceptual diagram depicting the importance of dispersal in metacommunity theory. Each panel shows one of the metacommunity paradigms: **(A)** Patch-dynamic (PD), **(B)** species-sorting (SS), **(C)** mass-effects (ME), and **(D)** neutral model (NM). **(E)** Metacommunity paradigms in relation to their place along the axes of dispersal, environmental heterogeneity, and equivalence. In **(A–D)**, the shapes of communities (i.e., circle and rectangle) indicate differences in abiotic conditions. Different microbial taxa are shown to disperse at different rates, i.e., solid lines indicate higher dispersal rates and dashed lines indicate lower dispersal rates. **(A)** Open patches offer new colonization opportunities as well as evolutionary tradeoffs in colonization and competition. **(B)** Local selection counterbalances the effect of dispersal in determining the community structure. **(C)** High levels of dispersal override the potential effect of local selection. **(D)** Neutral model assumes that all taxa are equally adept at dispersing and the environmental conditions are equivalent across patches. **(A–D)** Are adopted and modified from Leibold et al. (2004), and **(E)** was adopted from Logue et al. (2011).

been supported by literature (Grainger and Gilbert, 2016; Catano et al., 2017), recent experimentation using microbial communities has challenged this notion. Vannette and Fukami (2017) experimentally controlled dispersal vectors to study nectar microbial communities in *Mimulus aurantiacus* plants. These authors reported that nectar communities from the dispersal limited treatments resulted in the lowest measures of β-diversity from the treatment centroid; and the opposite pattern was found in treatments with the highest dispersal. The authors hypothesized that priority effects (i.e., the order of species arrival and their influence on the subsequent community outcome) and competitive interactions between introduced species can significantly alter the magnitude and directionality of the dispersal-diversity relationship. While additional evidence is required to support their findings across divergent systems, these results underscored the importance of incorporating the timing of dispersal as a potential mechanism responsible for divergent outcomes of diversity-dispersal relationships.

Dispersal and Community Assembly

Community assembly is highly influenced by the timing and frequency of species arrival *via* dispersal and subsequent changes in local abiotic conditions. At the initial phase of community assembly (or primary succession) higher stochasticity in species arrival can be expected (Dini-Andreote et al., 2015). For example, Jackson et al. (2001) observed that during the early stages of drinking water biofilm formation, communities were characterized by different populations that initially colonize the system. Over time, these initial colonizing species can change the local environment and progressively increase the level of environmental selection. As such, the order and timing of species immigration and changes to abiotic conditions, both early or later during community turnover, can affect species distributions and abundances. This concept, termed "priority effects" (Fukami, 2015), refers to the biotic component of historical contingencies and can be explained by two distinct mechanisms triggered by early arriving species: niche preemption and niche modification. Both of which exert continuous influence on community assembly and the subsequent patterns of community dynamics.

In microbial systems, studies examining priority effects have provided insights into the importance of timing of species arrival determining patterns of community assembly and functioning. For example, using wood-decaying fungi as a model system, immigration history was shown to result in large (ca. threefold) differences in fungal species richness and composition, both of which were associated with a similar magnitude in the rate of decomposition and carbon release from wood (Fukami et al., 2010). In another example, Cheong et al. (2021) examined priority effects in polymicrobial biofilms using a chronic human wound system and evaluated both bacterial and fungal communities, thus taking into account potential inter-kingdom species interactions. Their results provided evidence to support the notion that the balance between competitive and cooperative interactions in biofilms are largely mediated by the order of species arrival. Additionally, the importance of priority effects can also be considered within the context of community

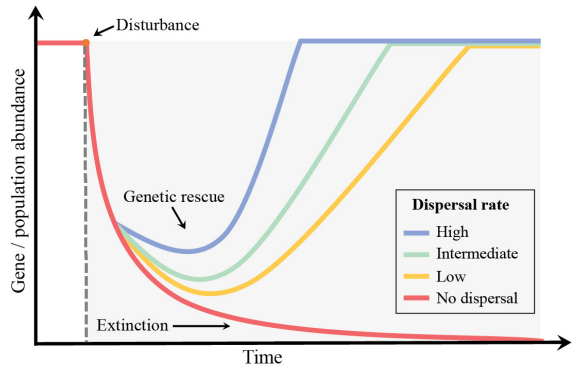

FIGURE 4 | Conceptual model displaying the potential decay in gene abundance due to population decline after disturbance. Dispersal contributes to genetic rescue and avoids loss of genetic variation. Different dispersal rates relate to the pace of genetic rescue over time.

coalescence (i.e., timing of arrival of entire communities). To illustrate this, Svoboda et al. (2018) experimentally evaluated the coalescence of fresh and brackish bacterioplankton communities. These authors inoculated sterile media with one of the donor communities and later the other community was inoculated at distinct time points (i.e., 0–96 h after the initial inoculation). The results revealed that the time after initial inoculation strengthens priority effects (i.e., time allowed for the establishment of the initial community), thus resulting in the lower success of taxa establishment from a later arriving community.

Dispersal and Ecosystem Function in Agroecosystems

Microbial inoculants in agriculture are often introduced to the soil environment to promote and/or enhance specific biological functions (e.g., plant nutrient acquisition, pathogen suppression, etc.). For example, by inoculating *Pseudomonas* sp. RU47 in the soil, Nassal et al. (2018) showed an increase in phosphatase activity associated with plant growth promotion *via* improved phosphorus uptake. In another example, the inoculation of *Acinetobacter calcoaceticus* strain CSY-P13 was shown to mitigate stress from ferulic and p-hydroxybenzoic acids in cucumber by activating antioxidant enzymatic activities and altering the overall bacterial community composition (Wu et al., 2018). In addition, their results showed that the inoculation with CSY-P13 increased the activities of phosphatase, catalase, urease, and sucrase enzymes in cucumber, representing the potential for deliberate inoculations of alien microbial species to elicit changes in nutrient cycling dynamics. While shifts in local community structure and function have been widely observed in inoculation-based studies, it is yet unclear whether a single inoculation or multiple introduction events are necessary to achieve desirable goals (i.e., the importance of dispersal frequency on ecosystem function and population establishment). For example, using long-term research plots inoculated with *Bradyrhizobium japonicum*, Narożna et al. (2015) showed persistent and viable populations of this N_2 fixing bacterium nearly 20-years after inoculation. Their results suggest that microbial inoculations with this particular species can remain viable in agroecosystems for extended periods of time, even in the absence of a suitable host. Most interestingly, in this particular case, only a single inoculation was proved to be sufficient for population establishment. In another study, however, Wang et al. (2021) examined the effects of multiple inoculation events with phosphate-solubilizing and N_2 fixing bacteria applied individually and in combination. Their results showed the resident soil microbiome to be resilient to inoculation, indicating the potential importance of repeated attempts. Last, they found subsequent inoculations to cause a sequential impact on the local community structure. This finding aligned with their initial hypothesis that successive inoculations can result in higher persistence and potential naturalization of inoculated taxa in the system.

EVOLUTIONARY IMPLICATIONS OF MICROBIAL DISPERSAL

Mostly due to challenges associated with properly defining the concept of species, and the fact that evolutionary changes can alter community dynamics even if new species are not created (Rainey and Travisano, 1998), the evolutionary implications of microbial dispersal can better be understood by considering how dispersal affects diversification (i.e., generation of genetic variation within populations or communities), rather than strictly focusing on speciation and species interactions. Differentiation in microbes occurs mostly by mutation and gene transfer, which in microbial systems also includes the uptake of environmental DNA (Hanson et al., 2012; Nemergut et al., 2013; Xu et al., 2020). Given their short generation times, fast growth rates, rapid genetic mutations, and gene transfer (Zhou et al., 2013, 2015; Zhou and Ning, 2017), the influence of evolutionary processes on microbial community dynamics are very important, albeit often neglected in landscape microbiome studies. These processes are–to some extent–mediated by dispersal events that determine the rate of gene flow across local and regional scales and have multifaceted eco-evolutionary implications.

Dispersal and Microbial Gene Flow

The movement of species in space and time fundamentally relates to the movement of genetic material and gene flow (i.e., the movement of genes into or out of a population). Changes in gene flow rates are responsible for population differentiation and speciation, and can modulate the genetic structure of ecological communities (Knowles, 2009; Kisel and Timothy, 2010; Hackel and Sanmartín, 2021). Like different dispersal rates influencing patterns of species diversity in metacommunities (see above), differences in the rates of gene flow mediated by dispersal also lead to variable levels of genetic variation across populations. For example, if the rate of gene flow is too high, two populations will have equivalent genetic variation and allele frequency leading to an overall genetic homogenization. On the contrary, isolated communities/populations (i.e., under low dispersal rates) are expected to experience accelerated genetic drift and speciation

from intense intraspecific differentiation (Puritz et al., 2012; Waters et al., 2020). This can result in a decrease in species genetic variation and likely enhance the susceptibility of a given population to environmental perturbation (Waters et al., 2020). As such, the consequences of differential gene flow rates can be interpreted analogously to the effects of dispersal on community diversity, functioning, and ecological properties.

Important to counterbalance the effect of genetic homogenization, microbes have different dispersal capabilities, all of which are driven by organismal intrinsic traits (motility and chemotaxis, spore formation, and dormancy) and the mechanisms of dispersal (passive and active dispersal) (see above). Some specific taxa (e.g., arbuscular mycorrhizal fungi) can reproduce and disperse *via* active and passive processes (i.e., spores, extraradical mycelium, dormancy). Such strategy reflects a broader spectrum of adaptation directly affecting the survival and genetic extension of a species (Paz et al., 2021). Besides, microbial traits linked to dispersal are also known to enhance gene flow through time scales (Jordano, 2017). For example, long-term dormancy of microbial cells can result in long-distance dispersal and/or long-term persistence, thus connecting genetically distinct communities and populations across evolutionary time scales.

Dispersal and Genetic Rescue

In theory, the success of genetic rescue is related to three key mechanisms. First, a population previously exposed to a low level of a stressor tends to increase the population size of resistant genotypes, thus favoring rapid adaptation when similar stress or disturbances are encountered (Gomulkiewicz and Holt, 1995; Bell and Gonzalez, 2009). Second, the initial population size is often positively correlated with the probability of species adaptation and survival in face of environmental disturbances (Lanfear et al., 2014), by reducing the risk of stochastic exclusion (Gomulkiewicz and Holt, 1995; Bell and Gonzalez, 2009; Gienapp et al., 2013). And third, the connectivity of populations across a landscape (similar to the metacommunity framework) enhances gene flow and genetic variation, which promote genetic rescue (Bell and Gonzalez, 2009; Bourne et al., 2014).

Dispersal can contributes to genetic rescue by increasing population size and consequently the rate of mutation and diversification (**Figure 4**) (Gomulkiewicz and Holt, 1995; Lenormand, 2002). For example, O'Connor et al. (2020) examined contrasting modes of dispersal and antibiotic selection history in *P. fluorescens* SBW25 using a gradient of antibiotic stress. This study showed that even though the previous exposure to antibiotics influenced genetic rescue in a metacommunity context, dispersal across community types and locally (i.e., dispersal through the antibiotic gradient) accelerated diversification rates. In another example, Low-Décarie et al. (2015) experimentally evaluated the importance of connectivity in soil microbial metacommunities for genetic rescue under herbicide stress. They showed that genetic rescue in a community context is dependent on the dispersal of both rare existing resistance lineages and new genotypes that arise through mutation following severe environmental degradation across the landscape.

Understanding the conditions and ecological processes that underpin the importance of dispersal affecting genetic rescue has implications for conservation biology and for the management of resistant pathogens (Alexander et al., 2014). For example, Cheptou et al. (2017) showed that highly fragmented systems result in negative effects on population rescue, even when sufficient adaptation is present, by driving the species to extinction and endangering the metapopulation dynamics. Furthermore, the beneficial association of microbes with hosts has also been shown to enhance host species rescue by increasing host fitness and enhancing survival chances (Mueller et al., 2020). In the case of pathogens, it was argued that globalization greatly contributed to the increased rate of evolution in resistant strains directly affecting their dispersal potential (Baquero et al., 2021). Several studies have shown the emergence of microbial resistant strains to pesticides and antibiotics, collectively highlighting an increased risk for agricultural systems and human health (Thanner et al., 2016; Hudson et al., 2017; Hernando-Amado et al., 2020). For example, antibiotic resistant microbes and their infections have been shown to affect access to healthcare and incur high costs of treatment (Hernando-Amado et al., 2020). In the United States alone, additional costs associated antibiotic resistance are estimated to increase $30 billion annually (van Duin and Paterson, 2016). Ecological studies can promote further understanding of microbial resistance pathways and strategies to improve control of antibiotic resistance. For instance, metapopulation theory can be used to understand emergence of resistant pathogens by incorporating dispersal barriers and population rescue decline (Koch et al., 2017).

Worth mentioning and different from macro-organisms, microbes are capable of uptaking genetic materials from the environment (i.e., dead cells or environmental DNA fragments). This illustrates a type of genetic rescue unique to microbes that is associated with the persistence and maintenance of genetic variation (or genes) rather than population rescue. For example, following exposure to treated wastewater containing free extracellular antibiotic resistance genes (eARGs), *Staphylococcus aureus* strains were found to be newly resistance to methicillin (Naquin et al., 2015). This study showed a successful natural extracellular transformation during chemical stress and provided evidence of resistance gene assimilation (most likely *mec*A—a methicillin resistance gene). In addition, Olwal et al. (2018) showed an increase in eDNA release by *S. epidermidis* biofilms in response to sub-lethal heat and oxidative stress. As such, the authors suggested that eDNA release could act as a potential mechanism underlying species resistance under physicochemical stress. Overall, this implies that the ability to incorporate extracellular eDNA can improve species survival and boost genetic rescue by upregulating stress resistant genes in stressful environments.

Dispersal in an Eco-Evolutionary Context

Dispersal is directly associated with the ecology and evolution of species interactions. A classic example comes from one of the most widespread and ecologically important instances of mutualism: the association of terrestrial plants with fungal mycorrhizal symbionts. This association allowed early land plants

to undergo the ecological transition to terrestrial life (Delwiche and Cooper, 2015; Morris et al., 2018), leading to both the dispersal and evolutionary radiation of the mycorrhizal fungi and terrestrial plants. Such examples of assisted dispersal by beneficial interaction occur more often than expected. Ingham et al. (2011) experimentally demonstrated beneficial co-dispersal between the non-mobile fungus *Aspergillus fumigatus* and the swarming bacterium *Paenibacillus vortex*. They showed *P. vortex* to rescue *A. fumigatus* from inhospitable environments and the transport of *A. fumigatus* conidia to areas containing a relatively higher concentration of antibiotics, which creates an environment more favorable to the fungal germination and lower bacterial competition. Furthermore, the authors hypothesized that *A. fumigatus* hyphae can facilitate bacterial movement through an otherwise impassable heterogeneous soil matrix, thus resulting in a relationship beneficial to both species. Other studies have provided support for this hypothesis by showing that the fungal mycelia can act as dispersal channels (or "fungal highways"), facilitating bacterial dispersal even under unfavorable conditions and environmental heterogeneity (Kohlmeier et al., 2005; Wick et al., 2007). To illustrate the concept, the hyphae of *Phomopsis liquidambaris* was shown to assist the migration of rhizobia from bulk soil to the rhizosphere of peanut (*Arachis hypogaea*) (Zhang et al., 2020). In another eco-evolutionary perspective, these fungal highways were investigated with respect to the movement of species associated with predator-prey interactions. Otto et al. (2017) experimentally evaluated the ability of the gram-negative bacterial predator *Bdellovibrio bacteriovorus* to utilize fungal mycelia as dispersal channels to locate suitable prey, in this case, *Pseudomonas fluorescens*. Although they found no predator dispersal through the fungal mycelia in the absence of prey, the predatory bacterium utilized the fungal mycelial highway when the prey was available. Last, these authors suggested that dispersal of predators *via* fungal hyphae is "activated" by the presence of the prey serving as a source of carbon and energy supply.

Dispersal can also mediate the evolutionary dynamics of competition-colonization trade-offs (Urban et al., 2020). Dispersal traits are expected to evolve as a consequence of species escaping competition or evading disturbance events, thus enhancing species survival and persistence at regional scales (Duputié and Massol, 2013). Conversely, it could be expected that the benefit of dispersal abilities decreases as species locally adapt and face an increased cost of dispersal, such as in the case with increased landscape fragmentation (Cenzer and M'Gonigle, 2019). To illustrate this, Dini-Andreote et al. (2018) investigated microbial adaptative traits along a gradient of saltmarsh formation. These authors revealed an overrepresentation of dispersal-related traits at a community level (i.e., chemotaxis and flagellar motility) in earlier stages of land formation, reflecting community dynamics in environments with frequent environmental change (i.e., flooding events). On the other hand, the gradual transition to a less diffusible habitat (i.e., the soil matrix) reflected in a progressive increase in the abundance of ARGs (i.e., competitive traits), in addition to a more versatile metabolism of carbohydrate-active enzymes. In sum, this study links a set of specific microbial traits directly associated with dispersal-competition trade-off during the eco-evolutionary transition of land colonization.

CONCLUDING REMARKS

Dispersal has long been recognized as one of the fundamental processes structuring ecological communities. Foundational studies have demonstrated its importance for species movement and how dispersal interacts with other assembly processes, such as selection (Ron et al., 2018). However, its importance in microbial systems has–to some extent–been neglected, mostly due to technical limitations, but also given the seminal paradigm of "everything is everywhere." Together, these factors have accounted for our yet inability to properly appreciate the importance of dispersal in structuring microbial communities; albeit seminal work has been done in explicitly studying dispersal in microbial systems. On the other hand, while large-scale experimental manipulations focusing on dispersal in macro-organismal systems remain unrealistic, the nature of microbial systems provides an easily manipulatable system, apt for experimentation that can undoubtedly further our understanding of dispersal on both the micro- and macro-ecological scales. With this, we argue that the field of ecology will benefit from future research that considers dispersal and its influence on taxonomic and functional diversity, as well as the influence of historical contingencies and community coalescence in microbial systems. Additionally, the importance of dispersal affecting eco-evolutionary outcomes deserves renewed attention due to expected increases in exotic invasion and gene flow across systems as a result of intensifying climate change and globalization.

AUTHOR CONTRIBUTIONS

GC, LB, and FD-A wrote, discussed, and reviewed the entire manuscript. All authors contributed to the article and approved the submitted version.

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A Microbial Mutualist Within Host Individuals Increases Parasite Transmission Between Host Individuals: Evidence From a Field Mesocosm Experiment

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The interactions among host-associated microbes and parasites can have clear consequences for disease susceptibility and progression within host individuals. Yet, empirical evidence for how these interactions impact parasite transmission between host individuals remains scarce. We address this scarcity by using a field mesocosm experiment to investigate the interaction between a systemic fungal endophyte, *Epichloë coenophiala*, and a fungal parasite, *Rhizoctonia solani*, in leaves of a grass host, tall fescue (*Lolium arundinaceum*). Specifically, we investigated how this interaction impacted transmission of the parasite under field conditions in replicated experimental host populations. *Epichloë*-inoculated populations tended to have greater disease prevalence over time, though this difference had weak statistical support. More clearly, *Epichloë*-inoculated populations experienced higher peak parasite prevalences than *Epichloë*-free populations. *Epichloë* conferred a benefit in growth; *Epichloë*-inoculated populations had greater aboveground biomass than *Epichloë*-free populations. Using biomass as a proxy, host density was correlated with peak parasite prevalence, but *Epichloë* still increased peak parasite prevalence after controlling for the effect of biomass. Together, these results suggest that within-host microbial interactions can impact disease at the population level. Further, while *Epichloë* is clearly a mutualist of tall fescue, it may not be a defensive mutualist in relation to *Rhizoctonia solani*.

Keywords: within-host microbial interactions, species interactions, co-infection, mesocosm experiment, transmission, disease ecology, plant parasites

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INTRODUCTION

Host organisms are commonly infected by defensive symbionts, which can interact with parasites to protect their hosts (reviewed in Clay, 2014; Hopkins et al., 2017). These defensive symbionts can have impacts on disease at the individual and population levels (Hopkins et al., 2017; O'Keeffe et al., 2017). As the ubiquity of diverse within-host microbial communities has come to be realized,

a challenge has been to link within- and between-host levels of disease dynamics (Clay et al., 2020). Here, we address this challenge by investigating the within- and between-host impacts of a defensive symbiont of a grass host on the severity and spread of a fungal parasite under field conditions.

Defensive symbionts can dramatically impact the survivorship, growth, and reproduction of parasites infecting the same host individual (Arnold et al., 2003; Costello et al., 2012; Clay, 2014; Santhanam et al., 2015; Hopkins et al., 2017). Within a host, defensive symbionts may prime a host immune response to parasites (Selosse et al., 2014) or directly interfere with an invading parasite (Gerardo and Parker, 2014). For example, systemic fungal endophytes of grasses can produce antimicrobial compounds that may reduce severity of disease caused by a parasite on a plant individual (Clay et al., 1989). Through such mechanisms, defensive symbionts can affect host susceptibility, parasite growth and replication, and subsequent parasite disease severity (Arnold et al., 2003; Oliver et al., 2005; Hussain et al., 2013).

Interactions among coinfecting symbionts within host individuals can have implications that scale up to populations (Cattadori et al., 2008; Telfer et al., 2010). Defensive symbionts can impact the growth and reproduction of parasites within a host, and within-host accumulation is often directly or indirectly linked to between-host transmission (Wintermantel et al., 2008; Tollenaere et al., 2016). Defensive symbionts may therefore be an important driver of epidemiological dynamics, which can have impacts on ecosystem function (Paseka et al., 2020). Yet, how within-host dynamics of defensive symbionts and parasites scale to impacts across host individuals remains an important frontier in disease ecology (Viney and Graham, 2013; Ezenwa and Jolles, 2015; Johnson et al., 2015).

There is a growing literature on how the defensive symbionts and other fungal species that grow within grass hosts interact with foliar fungal parasites to impact disease. Systemic fungal endophytes of grass hosts are considered defensive mutualists under most ecological conditions (Saikkonen et al., 2016). By modulating host defenses and/or producing alkaloids and other diverse toxins, these endophytes can suppress a wide range of fungal parasites under laboratory conditions (Tian et al., 2008; Pańka et al., 2013; Saikkonen et al., 2013; Panaccione et al., 2014; Card et al., 2021). Yet, a series of inoculation experiments under controlled settings found that while the systemic fungal endophyte, *Epichloë coenophiala*, did not directly impact within-host growth of fungal parasite, *Rhizoctonia solani*, it did so indirectly when the two species were coinfected with another fungal parasite, *Colletotrichum cereale* (O'Keeffe et al., 2021b). Further, within-host interactions among *R. solani* and coinfecting fungal parasites have been shown to scale up to impact transmission of parasites to host individuals (Halliday et al., 2017).

To investigate the impacts of a defensive symbiont on a parasite across levels of ecological organization, we conducted a field mesocosm experiment on a vertically transmitted fungal endophyte, *E. coenophiala*, the facultative fungal parasite *R. solani,* and a host grass, tall fescue (*Lolium arundinaceum*). We established experimental populations of *Epichloë*-inoculated and *Epichloë*-free plants in field mesocosms, inoculated them with the parasite, then performed repeated surveys of parasite damage on leaves. We provide evidence that while *Epichloë* clearly confers a benefit to its host at the host-individual level by increasing host aboveground biomass production, this endophyte can have a contrasting impact on parasite transmission at the host-population level.

MATERIALS AND METHODS

Study System

Within a grass host, tall fescue (*Lolium arundinaceum*), we investigated the interaction between two fungal symbionts: the parasite *Rhizoctonia solani* and the vertically transmitted systemic endophyte *Epichloë coenophiala*. *R. solani* is a facultative parasite, as it can persist in the soil as a saprobe. As a necrotrophic parasite, it extracts resources by killing host cells. *Rhizoctonia solani* is transmitted primarily by hyphae and sclerotia, and symptoms of *R. solani* on tall fescue can be observed as light brown lesions, of irregular shape, surrounded by dark brown borders. In contrast, *E. coenophiala* is considered a mutualist under most ecological conditions (Saikkonen et al., 2016) and is vertically transmitted by seed. While *E. coenophiala* consistently acts as a defensive mutualist with regard to herbivory, *E. coenophiala* can vary in its impact on parasites (Potter, 1980, 1982; Liu et al., 2006; Saikkonen et al., 2013). Empirical evidence for the direction of the interaction between *Epichloë* endophytes and *Rhizoctonia* parasites varies (Pańka et al., 2013; Halliday et al., 2017; O'Keeffe et al., 2021b).

Experimental Design and Setup

We investigated how within-host microbial interactions impact parasite transmission by conducting two field mesocosm experiments. This experiment was conducted at Widener Farm, an old field of the Duke Forest Teaching and Research Laboratory (Orange County, NC, United States), during the summer of 2018. This old field produced row crops until 1996 and has since been mowed to produce hay. During the 2013–2017 growing seasons, surveys of the tall fescue population at this site showed that symptoms from parasite, *R. solani*, began appearing on leaves in June or July, peaked in prevalence in August and September, and decreased in prevalence over the fall months (Halliday et al., 2017). Because the peak of this parasite epidemic at this site occurred in August in previous years (Halliday et al., 2017), we conducted each experiment during that time period in subsequent years, 2017 and 2018. While the overall design of each experiment was similar in 2017 and 2018, there were a few key differences and notably, parasite transmission was more successful in the 2018 experiment (**Supplementary Figure S1**). Owing to the relative lack of transmission in 2017 (for example, the peak parasite prevalence in 2018, averaged across all populations, was 739.6% higher than the peak parasite prevalence in 2017), we report the 2017 transmission methods and results in the supplement (**Supplementary Methods** and **Supplementary Table S1**), and here in the main text, we report the 2018 methods and results.

To test how the endophyte affects parasite spread across a host population, we manipulated the presence of *E. coenophiala* at the level of the host population. We planted a total of 26 populations, and each population was contained within a 45-inch (1.14

meter)-diameter plastic wading pool (Summer Escapes) to limit *R. solani* inoculum coming from the environment. Each population was randomly assigned one of two treatments: *E. coenophiala*-inoculated or *E. coenophiala*-free (herein referred to as *Epichloë*-inoculated and *Epichloë*-free). In total, there were 15 *Epichloë*-inoculated and 11 *Epichloë*-free populations. Two randomly selected populations in each *Epichloë* treatment (four total) were not inoculated with the parasite and served as experimental controls.

Each population consisted of 13 plants: one plant in the center of the population (which would ultimately be inoculated with the parasite), and 12 plants surrounding the central plant at distances of 12, 24, and 36 cm away (four plants at each distance; **Supplementary Figure S2**). The 338 plants within the experiment were propagated from *Epichloë*-free or *Epichloë*-inoculated seed produced by Tim Phillips at the University of Kentucky and the Noble Research Institute in Ardmore Oklahoma, respectively. All seed was from variety KY-31. Seed was germinated on 25 June 2018 and grown in a greenhouse for 6 weeks. The greenhouse temperature was kept between 19.7°C and 22.2°C, and light was supplemented between 9 AM and 7 PM if natural light fell below 350 W/m^2. All plants except for the central plants were transplanted into the contained field mesocosm experiment on Monday, 6 August 2018, 6 weeks after germination. Each population consisted of plants belonging to the same endophyte category (all *Epichloë*-inoculated or *Epichloë*-free) except all central plants were *Epichloë*-free. Plants were randomly assigned to one of the populations, and locations of the plants within the populations were also randomized. The populations were fully randomized in a 2 × 13 layout, with narrow paths separating populations (**Supplementary Figure S2**). The plants were given 4 days to acclimate to the field prior to the introduction of the parasite.

The plants that would ultimately be planted in the central position of the populations were transferred to growth chambers on 8 August 2018 and inoculated with an isolate of the parasite that was cultured in 2015 from a leaf lesion on a tall fescue plant in the same field as this experiment. Once in axenic culture, plugs of the leading edge of the culture were stored in mineral oil and potato dextrose broth in a −80°C freezer. We plated these plugs on potato dextrose agar and the resulting growth served as the source of inoculum for this experiment. Inoculum consisted of a 6-mm-diameter plug of potato dextrose agar with the leading edge of the parasite culture placed directly at the base of a leaf. Parasite infection success depends on a humid environment. To maintain moisture at the site of inoculation, a cotton ball wet with sterile water was placed over the inoculum, secured with tin foil and parafilm. The inoculated plants were placed in a growth chamber [Percival PGC-6L (Perry, Iowa)] for 2 days with a 12-h light/12-h dark cycle set at 28°C, and humidity was maintained at approximately 95% with humidifiers (Vicks V5100-N Ultrasonic Humidifier) on each shelf of the growth chambers. In addition to parasite-inoculated plants, four plants were mock-inoculated with plugs of potato dextrose agar without *R. solani* mycelia. After 2 days, all plants inoculated with *R. solani* exhibited parasite symptoms and were transplanted into the field mesocosm experiment on 10 August 2018. One mock-inoculated plant was transplanted into each of the four experimental control populations.

Data Collection

Following the placement of the central inoculated plant, twice a week, for 4 weeks, seven random leaves per plant were nondestructively selected and observed for the presence or absence of damage caused by the parasite, as well as any other parasite damage. Specifically, leaves were surveyed 4, 8, 11, 14, 18, 22, 25, and 28 days after parasite inoculation (eight surveys total). Each leaf was surveyed for the presence of any damage caused by parasites, herbivores, or abiotic sources. The symptoms of other fungal parasites of tall fescue in this location caused symptoms that are easily distinguished from those caused by *R. solani* (O'Keeffe et al., 2021a).

To measure disease severity over time, percent leaf area damaged by the parasite was quantified on individual leaves on one tagged tiller per plant once a week. On each leaf, the initial date of symptomatic infection by the parasite was recorded, and severity was estimated by visually comparing leaves to reference images of leaves of known infection severity (Mitchell et al., 2002, 2003; Halliday et al., 2017). Over the course of the experiment, three severity surveys were conducted (8, 14, and 22 days after parasite inoculation).

At the conclusion of the experiment, we collected and frozen (−20°C freezer) inch-long cross-sections of two tillers per plant to confirm endophyte presence. Endophyte infection was tested *via* immunoblot (Agrinostics Ltd., Co, Watkinsville, GA, United States). Additionally, aboveground biomass was harvested, dried, and weighed.

Data Analysis

Epichloë-inoculated seed did not always result in endophyte detection in the tillers tested at the end of the experiment. Overall, we detected the endophyte in aboveground tissue of 42% of *Epichloë*-inoculated plants. This resulted in variation in endophyte prevalence among the *Epichloë*-inoculated populations (minimum: 15.4%; maximum: 69.2%). We therefore analyzed our data in two separate ways: with endophyte treatment (two levels: *Epichloë*-free or *Epichloë*-inoculated) as a predictor, or with endophyte prevalence (continuous variable from 0 to 1) as a predictor.

Control populations (in which the central plant was mock-inoculated) did not exhibit symptoms of the parasite, confirming that mesocosm populations were not infected from environmental sources of inoculum. These control populations were therefore excluded from analyses.

The models based on parasite prevalence reported in the main text only include surveys until each population's peak parasite prevalence because we were interested in how endophyte presence impacts epidemic spread, which is no longer happening after the peak. We report the results of models based on parasite prevalence with all surveys in the supplement (**Supplementary Table S2**; **Supplementary Figure S3**). All analyses were performed in R, version 3.6.1.

Leaves were analyzed as host individuals because each parasite infection is restricted to a single leaf (as done by Halliday et al., 2017; O'Keeffe et al., 2021b). As described in more detail above, we surveyed leaves in two ways; in weekly severity surveys, we estimated the leaf area damaged on a sample of leaves, and

in twice weekly prevalence surveys, we sampled leaves for the presence/absence of disease symptoms. To summarize disease progression over time, area under the disease progress stairs (AUDPS) was calculated for each population using the audps function within the agricolae package (version 1.3; de Mendiburu and Yaseen, 2020). AUDPS estimated the integration of the development of disease progress experienced by each population by adding together polygon steps between each time point (Simko and Piepho, 2011). Using data from each of the two sets of surveys, we calculated AUDPS based on two different measures of disease. Specifically, we calculated AUDPS with the average leaf area damaged across all leaves surveyed within a population at each time point, and we separately calculated AUDPS with the prevalence of the parasite at each time point. When calculated with prevalence data, AUDPS was log-transformed to achieve homoscedasticity and normality of residuals. We investigated whether and how endophyte treatment affected these estimates of disease progression over time with a linear model.

To further evaluate the magnitude of epidemics, we investigated measurements of parasite prevalence repeated over time using linear mixed effects models. Data on proportion of leaves infected with the parasite were log-transformed to achieve homoscedasticity and normality of residuals. Using the nlme package (version 3.1) for linear mixed effects models, we modeled parasite prevalence within a population at a given time with a linear mixed effects model that included *Epichloë* inoculation treatment and a third-order polynomial of days after infection, as well as their interaction, as predictors (Pinheiro et al., 2013). We determined the appropriate polynomial to utilize using AIC. We included random slopes to account for repeated measures of the populations.

We additionally evaluated whether *Epichloë* inoculation treatment affected peak parasite prevalence. We quantified peak parasite prevalence as the highest proportion of leaves infected with the parasite at a given time point at the population level. We tested whether the *Epichloë* inoculation treatment affected peak parasite prevalence with a linear model that included *Epichloë* inoculation treatment as the predictor.

Under density-dependent transmission, the contact rate between susceptible and infected individuals depends on the host population density; transmission rate therefore increases with density. While host population density (here, the number of leaves in a population) was not measured in the 2018 experiment, aboveground biomass was measured at the conclusion of the experiment. To investigate whether host population density significantly correlated with biomass, we used data from the 2017 field mesocosm experiment in which host density was measured explicitly. We used a ranged major axis regression model (implemented in lmodel2, version 1.7; Legendre and Oksanen, 2018) to investigate whether there was a correlation between total aboveground biomass of plants and the total number of leaves in a given population at the end of the experiment. Both biomass and total leaves were log-transformed. As noted, there was relatively low transmission in the 2017 experiment, but this correlative analysis only considers biomass and number of leaves; it does not incorporate *Epichloë* treatment or disease data. Based on that correlation, we then used aboveground biomass as a proxy for host density of each population. Specifically, to test whether the effect of the *Epichloë* inoculation treatment on parasite peak prevalence was due to variation in host density, we added total aboveground biomass to the model as a covariate.

RESULTS

Epichloë-inoculated populations tended to experience more disease than *Epichloë*-free populations. *Epichloë*-inoculated populations experienced 8.3% higher disease integrated over time as measured by AUDPS calculated with disease prevalence (**Figure 1**; **Table 1**; $p = 0.051$), with much greater variation evident among *Epichloë*-free populations. Results when AUDPS was calculated with disease severity were generally consistent, although statistical support was weaker (**Supplementary Results**; **Supplementary Table S3**; **Supplementary Figures S4, S5**). A mixed model of prevalence over time complemented these results, as the *Epichloë* inoculation treatment tended to increase parasite prevalence over time, though this finding had weak statistical support (**Figure 2A**; **Table 2**; $p = 0.07$). The *Epichloë* inoculation treatment did clearly increase the peak parasite prevalence. Specifically, the *Epichloë*-inoculated populations had a 27% higher peak prevalence than *Epichloë*-free populations, with mean peak parasite prevalences of 0.43 and 0.34, respectively (**Figure 2B**; **Table 3**; $p = 0.007$). Together, these results suggest that while this endophyte may impact spread of a parasite across a

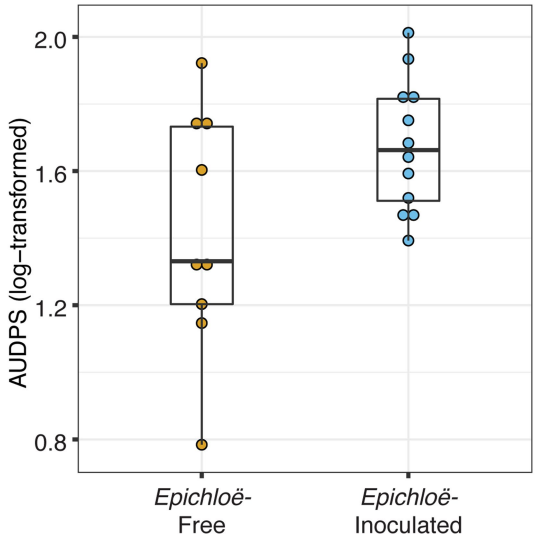

FIGURE 1 | *Epichloë*-inoculated populations had greater disease prevalence integrated over time. Disease prevalence was integrated over time for each population as log-transformed AUDPS (area under the disease progress stairs). AUDPS was estimated using disease prevalence over the eight surveys until each population's peak. Boxplots show median, 25th, and 75th percentile, with dots showing data for each population replicate. Whiskers extend to the lowest and highest values no further than ± 1.5 times the inter-quartile range.

population over time, it most clearly leads to higher parasite prevalence at the peak of an epidemic.

In addition to impacting parasite prevalence and disease severity, this endophyte also impacted host population aboveground biomass. Specifically, populations with higher prevalence of *Epichloë* also had higher aboveground biomass (**Figure 3**; **Table 4**; $p = 0.047$). Motivated by the expectation that transmission of the parasite is density dependent, we investigated whether higher biomass correlated with higher numbers of leaves in a population (i.e., host population density). As counting all leaves was not feasible in the 2018 experiment, we used data from the 2017 experiment, in which we counted all leaves in each population. In the data from 2017, we investigated whether there was a correlation between the aboveground biomass of the plant population measured at the end of the experiment and the total number of leaves surveyed at the end of the experiment. Population-level aboveground biomass and the number of leaves in a population were clearly positively correlated in 2017 (**Figure 4**; **Table 5**; Marginal $R^2 = 0.145$; $p = 0.045$). If we assume that the correlation between population biomass and leaf number from 2017 also held in 2018, then that suggests that in analyzing the 2018 experiment, host population aboveground biomass can be used a proxy for host population density.

TABLE 1 | *Epichloë*-inoculated populations tended to have heavier disease prevalence integrated over time as log-transformed AUDPS, though this difference had weak statistical support.

	AUDPS: parasite prevalence			
	F	**numDF**	**denDF**	***p***
Epichloë inoculation	4.33	1	20	0.051

TABLE 2 | *Epichloë*-inoculated populations tended to have greater disease prevalence tracked over time in a linear mixed model, though this difference had weak statistical support.

	numDF	**denDF**	***F***	***p***
Fixed effects				
Days after inoculation (third order polynomial)	3	120	67.6732	<0.0001
Epichloë status	1	20	0.9752	0.3358
Days after inoculation: *Epichloë* status	3	120	1.4091	0.0687

TABLE 3 | *Epichloë* inoculation clearly increased peak parasite prevalence experienced by a population.

	numDF	**denDF**	***F***	***p***
Epichloë inoculation	1	20	9.218	0.006795

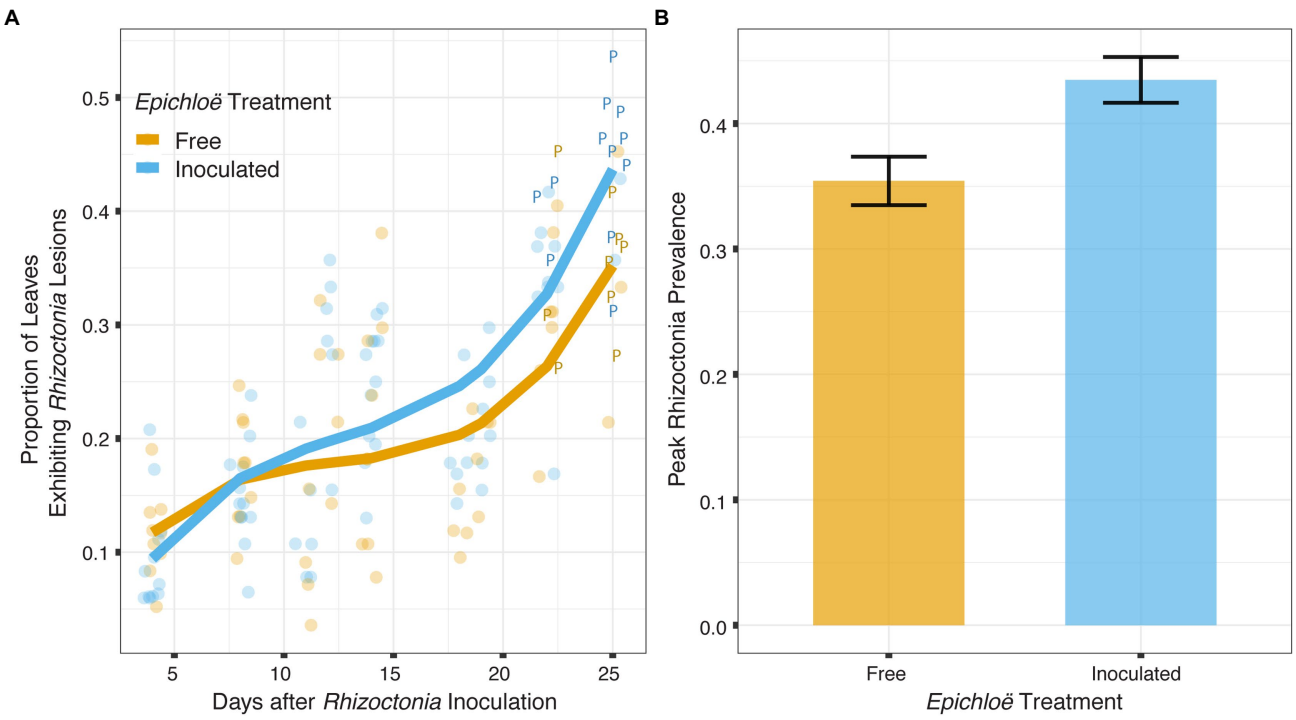

FIGURE 2 | *Epichloë*-inoculated populations tended to have greater disease prevalence over time, though this difference had weak statistical support in a linear mixed model. More clearly, *Epichloë* inoculation increased peak parasite prevalence experienced by a population. **(A)** Bold lines are model-predicted means of parasite prevalence over the course of 4 weeks post-inoculation with the parasite for populations in which *Epichloë* was inoculated (blue) and populations in which *Epichloë* was absent (orange). The peak parasite prevalence of each population is indicated by the letter "P." **(B)** *Epichloë*-inoculated populations had 8.6% higher peak parasite prevalence than *Epichloë*-free populations. Bars indicate mean peak parasite prevalence and error bars are ± 1 SE.

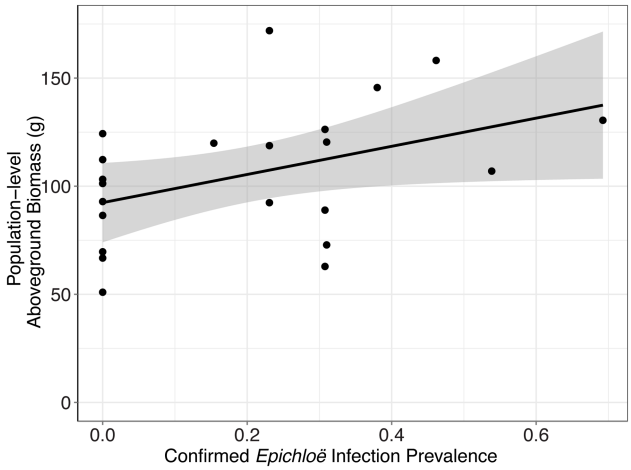

FIGURE 3 | *Epichloë* infection prevalence was associated with greater host aboveground biomass at the population level. Each point represents a host population, and the bold line represents the best fit linear model. At the population level, confirmed *Epichloë* prevalence was clearly positively associated with population-level host aboveground biomass at the end of the experiment ($p = 0.047$).

TABLE 4 | *Epichloë* prevalence was associated with greater final aboveground biomass of the host population.

	numDF	denDF	*F*	*p*
Epichloë prevalence	2	14	4.465	0.047

TABLE 5 | Aboveground biomass was correlated with number of leaves at the population level (permutation test of ranged major axis regression).

	Intercept	Slope	Angle	*p*
Biomass	−1.05	1.33	53.2	0.045

We then investigated whether higher levels of disease experienced by *Epichloë*-inoculated populations were driven by higher host densities. Based on the analysis of data from 2017, we used population-level aboveground biomass as a proxy for host population density and tested whether biomass accounted for the effect of *Epichloë* treatment on peak prevalence. Biomass and *Epichloë* treatment explained approximately 56% of variance in peak parasite prevalence. Biomass was positively correlated with peak parasite prevalence ($p = 0.006$), and independent of this association (interaction $p = 0.87$), *Epichloë* inoculation increased peak prevalence ($p = 0.01$; **Figure 5**; **Table 6**). While biomass did contribute to peak parasite prevalence, it did not account for the effect of the *Epichloë* treatment.

DISCUSSION

Our study provides experimental evidence that population-level disease dynamics can be impacted by the presence of a mutualist. Specifically, we investigated how parasite spread through a host population under field conditions responds to the presence of a mutualistic systemic endophyte under field conditions. We found that populations of tall fescue inoculated with the endophyte, *E. coenophiala*, counterintuitively experienced a higher peak prevalence of parasite, *R. solani*, over the course of the experimental epidemic.

The mutualistic relationship between cool-season grasses and vertically transmitted systemic fungal endophytes related to *Epichloë* has been studied extensively. While *Epichloë* endophyte infection has been shown to benefit host plants by increasing resistance to herbivores and seed predators, as well as providing protection against abiotic stressors (Saikkonen et al., 1998; Kauppinen et al., 2016), evidence for defending against infectious disease is less consistent (Potter, 1980, 1982; Liu et al., 2006; Saikkonen et al., 2013). In this experiment, we found that infection with *E. coenophiala* resulted in an increase in aboveground biomass, but there was no support for *E. coenophiala* limiting disease progression. *Epichloë*-inoculated populations actually experienced *R. solani* epidemics with higher peak prevalences. Our results are consistent with studies reporting no effect (Burpee and Bouton, 1993; Halliday et al., 2017; O'Keeffe et al., 2021b) or a facilitative effect of the endophyte on a parasite (Wäli et al., 2006; Halliday et al., 2017). Vertically transmitted fungal endophytes can impact fungal parasites via resource competition and changes in host immunity, which depends on parasite-feeding strategies (Saikkonen et al., 2013). While we expected that *R. solani* would be inhibited by *E. coenophiala* given previous findings that *E. coenophiala* most often suppressed disease caused by a relative of *R. solani* (Pańka et al., 2013), the direction of the effect of these endophytes on parasites likely depends on host genotype and environmental conditions (Krauss et al., 2007; Pańka et al., 2013). Further experimentation is needed to determine the mechanism underlying the potential facilitation of *R. solani* by *E. coenophiala*.

Epichloë-inoculated populations tended to have greater disease prevalence over time, though this difference had weak statistical support. More clearly, *Epichloë* inoculation increased peak parasite prevalence experienced by a population. We hypothesized that under density-dependent transmission, the benefit to the host

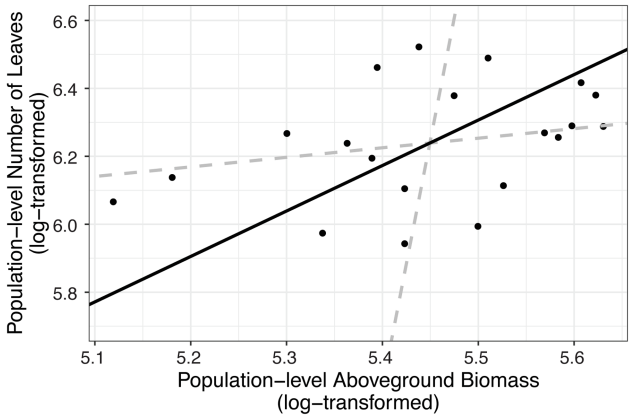

FIGURE 4 | Number of leaves in a population was correlated with dry aboveground biomass of a population. We summed the total number of leaves and the biomass (in grams) of each population (the level at which other analyses were performed). Each point represents a host population in the 2017 experiment. The black solid line indicates ranged major axis fit, and the gray dashed lines indicate 95% CIs. The total number of leaves (log-transformed) at the end of the experiment was correlated with log-transformed dry aboveground biomass ($R^2 = 0.145$, permutation test, $p = 0.045$).

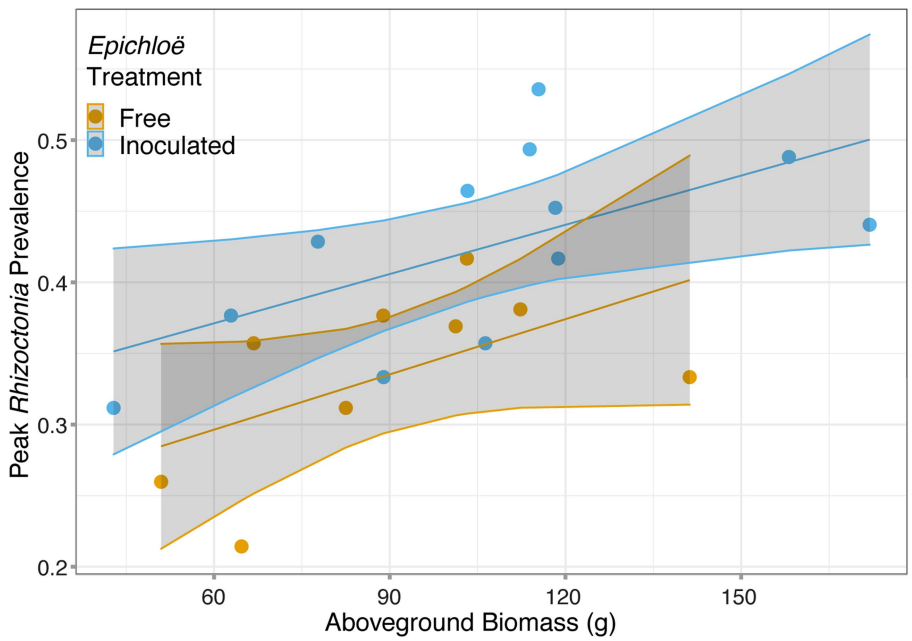

FIGURE 5 | Peak *Rhizoctonia* prevalence was correlated with dry aboveground biomass. Each population (represented with a point) was either inoculated with *Epichloë* (blue) or free of *Epichloë* (orange). Biomass predicted peak parasite prevalence ($p = 0.006$), and independent of this effect, *Epichloë* inoculation increased peak prevalence by 0.07 ($p = 0.01$).

individual of increased aboveground biomass, which in this case, correlated with host density (here, number of leaves), may result in a higher contact rate between hosts and consequently higher parasite peak prevalence. While biomass and peak parasite prevalence were significantly positively correlated, consistent with density dependent transmission, our results suggest that *Epichloë* impacted peak parasite prevalence beyond effects of biomass. One possible explanation is that *Epichloë* infection may have altered the growth of *Rhizoctonia* or other processes within host individuals that scaled up to the observed effect on peak prevalence in the host population. Alternatively, given that there is evidence that parasites can impact their host's biomass (Cordovez et al., 2017; Preston and Sauer, 2020), directly accounting for host density in models (rather than with biomass as a proxy) may more completely account for the impact of *Epichloë* on peak parasite prevalence.

There is growing evidence that the direction and magnitude of the consequences of within-host interactions are strongly affected by environmental context (Leung et al., 2018; Tracy et al., 2018). Our study provides a contribution to this understanding. By interrogating the impacts of an interaction

TABLE 6 | There was a clear effect of *Epichloë* inoculation on peak prevalence, even after accounting for the association of biomass with prevalence.

	numDF	denDF	*F*	*p*
Epichloë inoculation	1	20	12.6881	0.01
Biomass	1	20	9.126	0.006
Epichloë: biomass	1	20	0.02	0.87

between a systemic endophyte and parasite on parasite transmission at the population level, we expanded upon previous work which found no support for this hypothesized mutualist limiting parasite growth under controlled settings (O'Keeffe et al., 2021b). Foliar fungal parasites have been studied extensively and can serve as a suitable model system to investigate microbiome/parasite interactions under field settings. Here, we show that field mesocosm experiments offer the ability to investigate the effect of within-host microbial interactions on parasite spread.

Within-host microbial interactions can influence natural epidemics in complex ways (Mordecai et al., 2015; Clay et al., 2019). Across hosts, we found that populations inoculated with a mutualist counterintuitively experienced higher peak prevalence of this parasite. These results suggest that within-host interactions among parasites and non-pathogenic microbes can impact epidemic dynamics, and we propose that field mesocosm experiments can yield important insight into disease dynamics across populations under field settings.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://github.com/krokeeffe12/OKeeffe_fieldmesocosm.

AUTHOR CONTRIBUTIONS

KO, BW, and CM contributed to the conception and design of the study. KO and BW collected data. KO analyzed the data and wrote the first draft of the manuscript. 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/fmicb.2022.824211/full#supplementary-material

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Elevational Gradients Impose Dispersal Limitation on *Streptomyces*

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Dispersal governs microbial biogeography, but the rates and mechanisms of dispersal remain poorly characterized for most microbial taxa. Dispersal limitation is driven by limits on dissemination and establishment, respectively. Elevation gradients create striking patterns of biogeography because they produce steep environmental gradients at small spatial scales, and these gradients offer a powerful tool to examine mechanisms of dispersal limitation. We focus on *Streptomyces*, a bacterial genus common to soil, by using a taxon-specific phylogenetic marker, the RNA polymerase-encoding *rpoB* gene. By targeting *Streptomyces*, we assess dispersal limitation at finer phylogenetic resolution than is possible using whole community analyses. We characterized *Streptomyces* diversity at local spatial scales (100 to 3,000 m) in two temperate forest sites located in the Adirondacks region of New York State: Woods Lake (<100 m elevation change), and Whiteface Mountain (>1,000 m elevation change). Beta diversity varied considerably at both locations, indicative of dispersal limitation acting at local spatial scales, but beta diversity was significantly higher at Whiteface Mountain. Beta diversity varied across elevation at Whiteface Mountain, being lowest at the mountain's base. We show that *Streptomyces* taxa exhibit elevational preferences, and these preferences are phylogenetically conserved. These results indicate that habitat preferences influence *Streptomyces* biogeography and suggest that barriers to establishment structure *Streptomyces* communities at higher elevations. These data illustrate that *Streptomyces* biogeography is governed by dispersal limitation resulting from a complex mixture of stochastic and deterministic processes.

Keywords: biogeography, microbial, bacterial, diversity, soil, assembly, community

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INTRODUCTION

For more than a century, elevational gradients have yielded unique insights into the ecological and evolutionary mechanisms that generate patterns of biogeography (Grinnell, 1917). Steep elevation gradients generate rapid shifts in habitat characteristics over short spatial distances, a property that is useful in determining the degree to which dispersal is driven by spatial distance or variation in habitat characteristics (Sundqvist et al., 2013). Elevation gradients have a strong influence on the biodiversity of plants and animals (Peters et al., 2016) with many taxa exhibiting mid-elevation peaks or "hump-shaped curves" in alpha diversity (Rahbek, 2004; Moradi et al., 2020), and similar patterns have been observed for microbes (Fierer et al., 2011; Singh et al., 2012; Liu et al., 2016; Siles and Margesin, 2016). Elevation can affect biodiversity by a range of mechanisms including ecological filtering by habitat preference (Fierer et al., 2011; Wang et al., 2012; Shen et al., 2014;

Cho et al., 2018), variation in carrying capacity, and historical processes linked to patterns of climate change (Badgley and Fox, 2000; Rickart, 2001; de la Giroday et al., 2011; Schai-Braun et al., 2020). In addition, taxa that occupy mountain habitats are uniquely affected by historical climate change as warming climates tend to push species distributions toward higher elevations (Flesch, 2019; Marshall et al., 2020; Neate-Clegg et al., 2021), minimizing dispersal opportunities for species of plants and animals found at high elevations (Sekercioglu et al., 2008).

Biogeographical patterns can be driven by ecological mechanisms (*e.g.,* assembly processes driven by ecological filtering and ecological drift), evolutionary mechanisms (*e.g.,* speciation due to selection and drift), and historical contingency (*e.g.,* neutral processes linked to variation in geology and climate over time) (Hanson et al., 2012). Microbial biogeography is often thought to be constrained by ecological filtering, under the assumption that dispersal is largely unlimited and environmental gradients impose spatial structure on communities due to selection (Navarrete et al., 2015; Liu et al., 2018; Malard and Pearce, 2018; Malard et al., 2019). However, most evidence for unlimited microbial dispersal is obtained using highly conserved taxonomic markers (*e.g.*, rRNA genes) that have low taxonomic resolution and are insensitive to evolutionary processes that drive diversification (Hanson et al., 2012). Studies that use higher resolution taxonomic markers often find evidence for dispersal limitation with evidence that neutral processes can play a role in shaping patterns of microbial biogeography (Whitaker et al., 2003; Polz et al., 2013; Andam et al., 2016b; Choudoir et al., 2016; Choudoir and Buckley, 2018).

To explain the mechanisms that give rise to microbial biogeography we must first understand the forces that govern microbial dispersal. Dispersal is a two-part process comprised of dissemination, the movement from one place to another, and establishment, the successful colonization of a site characterized by the ongoing production of viable offspring (Martiny et al., 2006). Dissemination can be either passive (as driven by wind, erosion, currents, and organismal vectors) or active (as driven by motility or hyphal growth) (Yang and van Elsas, 2018). It is likely that capability for dissemination varies considerably between microbial taxa. For example, windborne dissemination is likely to vary in relation to cell size (Wilkinson et al., 2012), and cell shape likely influences microbial dissemination and establishment (Young, 2006). In addition, dissemination is influenced by environmental states. For example, soil texture and temperature influence spore transport in *Phytophthora* fungi (MacDonald and Duniway, 1978), and weather patterns can affect aerial dissemination (de Groot et al., 2021). Successful dissemination, however, is insufficient for successful dispersal, as microbes must still establish a sustainable population in the new site. Establishment requires that the habitat be suitable for growth, and that competitive interactions (*e.g.*, antagonism, or density-dependent blocking) do not prevent ongoing reproduction (Woody et al., 2007; Cheong et al., 2021).

We performed analysis of *rpoB* amplicons to investigate community assembly in *Streptomyces* along an elevational and spatial gradient in the Adirondacks region in New York State. The use of *rpoB* as a taxonomic marker for this genus improves taxonomic resolution significantly (Rong and Huang, 2012; Higgins et al., 2021) as compared to analyses made using 16S rRNA genes. The use of high-resolution taxonomic markers is essential for investigating the mechanisms that govern microbial biogeography (Hanson et al., 2012; Chase et al., 2017).

Streptomyces are bacteria that form aerial hyphae and arthrospores (Flärdh, 2003), which facilitate dissemination. They are common in soil habitats worldwide where they degrade a variety of common substrates derived from plant biomass (Yeager et al., 2017) and produce diverse antibiotics and secondary metabolites (Watve et al., 2001). Despite their high capacity for dissemination, and broad habitat tolerance, *Streptomyces* have been shown to exhibit endemism at regional scales, indicative of dispersal limitation (Andam et al., 2016a). Their wide distribution, ecological significance, theoretical capability for high dispersal, and their observed limited ranges make the *Streptomyces* genus an ideal group to understand dispersal and biogeography patterns in the soil.

We hypothesized that dispersal limitation would also occur at local scales, with dispersal limitation driven by barriers to establishment (*i.e.*, ecological filtering) rather than barriers to dissemination. To evaluate this hypothesis, we examined *Streptomyces* communities at two locations in the Adirondacks region of New York State. Sites at Whiteface Mountain varied greatly in elevation, while sites at Woods Lake varied little in elevation. All sites were broadly similar in habitat characteristics other than those linked to elevation. We predicted that high rates of local dissemination, coupled with ecological filtering as driven by elevation, would produce a strong gradient of beta diversity at Whiteface Mountain, and little variation in beta diversity at Woods Lake. We also predicted that ecological filtering would cause *Streptomyces* taxa to exhibit phylogenetic conservation with respect to elevation preference.

MATERIALS AND METHODS

Soil Sampling for Whiteface Mountain and Woods Lake

Soil samples were collected from nine locations on Whiteface Mountain (WM) in New York State (**Figure 1**). The average elevation change and horizontal distance between sites at WM was 347 and 1,361 m, respectively (metadata described in **Table 1**). The top of the mountain consists of shallow and well-drained loamy soil (Lythic Cryofolist) with moderately deep, well-drained Wallface-Skylight soils on gneiss bedrock at 1,200 m. The soil type changes to frigid Lithic Haplohumods characteristic of glaciated uplands below 800 m, and the base of Whiteface Mountain consists of deep, excessively drained sandy loam (Typic Haplorthod). To contrast the elevational gradient found at WM with the effects of spatial distance, we also collected samples from ten locations spanning two watersheds of Woods Lake (WL), which is situated in the Adirondacks region, 190 km from WM (**Figure 1**). Details of soil collection from Woods Lake are described elsewhere (Melvin et al., 2013). The average elevation change and horizontal distance between sites at WL was

19 and 353 m, respectively. WL contains sandy glacial till soils on top of hornblende granitic gneiss bedrock (Orthod Spodosols). Only non-limed soils from the Woods Lake watershed were included in this study.

At each sampling site, soil cores were collected in triplicate using a soil probe (2.5 cm diameter, 5 cm depth). Soil temperature was measured at the time of sampling. Samples used to test soil properties were air dried for 24 h and then sieved using a 2 mm mesh to remove plant debris and rocks, while soil used for DNA extraction was continuously stored at −20°C. Soil pH was measured using the 1:1 soil:water method described elsewhere (Kalra, 1995), and soil organic matter (SOM) content was measured by the loss-on-ignition method described in the Kellogg Soil Survey Laboratory Methods Manual (Burt, 2014).

DNA Extraction and Sequencing

DNA was extracted using the MoBio PowerSoil® DNA Isolation kit (Qiagen, Germantown, MD, United States) and quantified using the PicoGreen fluorometric assay (Thermo Fisher Scientific, Waltham, MA, United States). A 406 bp region of the RNA polymerase gene (*rpoB*) was amplified by PCR (∼25 ng DNA in a 25 μl reaction) using *Streptomyces*-specific primers Smyces_rpoB1563F and Smyces_rpoB1968R as described elsewhere (Higgins et al., 2021). The PCR reactions consisted of 25 ng DNA, 12.5 μl of Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, United States), 0.625 μl of 4X Quant-iT PicoGreen dsDNA assay reagent (Thermo Fisher Scientific, Waltham, MA, United States), and 1.25 μl each of 10 μM dual-barcoded forward and reverse primers modified for Illumina sequencing as described in Kozich et al. (2013). PCR products from triplicate reactions were pooled and normalized using the SequelPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA, United States). Fragments of 450 bp in length were size selected with a 1% agarose gel and subsequently extracted and purified from the gel band. Pooled samples were concentrated to 2 ng/μl using a vacuum concentrator and sequenced on an Illumina MiSeq instrument (2 × 300 bp) at the Biotechnology Resource Center, Cornell University.

Additional Datasets

In addition to the data generated from WM and WL, we also looked for evidence of elevational gradients in a larger dataset generated from soil samples obtained as part of the North American Soil Geochemical Landscapes Project (United States Geological Survey [USGS], 2012). This dataset consists of *Streptomyces rpoB* amplicons generated from 1,108 soil samples derived from sites across the United States and Mexico, spanning 7–3,483 m in elevation (average elevation 674 m). These *rpoB* amplicons were generated using the same primers and protocols described above (Steven Higgins, unpublished).

Data Analysis

Paired-end reads were joined using bbmerge (Bushnell et al., 2017) and trimmed using Trimmomatic-0.38 (Bolger et al., 2014). Sequences were dereplicated and size-sorted prior to OTU clustering at 99% identity using USEARCH (Edgar, 2010). The 0.99 similarity threshold corresponds to the species cut-off for *Streptomyces*, and provides better resolution for classifying *Streptomyces* at the species level than the 16S rRNA gene (Rong and Huang, 2012; Andam et al., 2016b; Higgins et al., 2021). OTUs were classified with SINTAX (Edgar, 2016). Sequences were aligned using MAFFT v7.475 (Katoh and Standley, 2013) and phylogenetic trees were constructed using the maximum likelihood method with RAxML 8.2.12 (Stamatakis, 2014).

Samples that had fewer than 18 sequences (first quartile value) were discarded from further analyses. All other samples were normalized using the Cumulative Sum Scaling method (Paulson et al., 2013), wherein OTU relative abundances within each sample are divided by the sample's library size (total number of reads in the sample). Downstream analyses for beta diversity estimates, phylogenetic signal, and phylogenetic clustering were performed using the phyloseq and picante R packages (Kembel et al., 2010; McMurdie and Holmes, 2013). Distance-decay relationships were quantified using the mgram function in the ecodist package (Goslee and Urban, 2007). The relative contributions of ecological processes like drift, selection and dispersal to community assembly were assessed using methods and code described elsewhere (Anderson et al., 2011; Stegen et al., 2013). The indicspecies R package (Cáceres and Legendre, 2009) was used for indicator species analyses, and RAxML (Stamatakis, 2014) was used to reconstruct phylogenetic relationships. Phylogenetic trees were visualized using iTol (Letunic and Bork, 2021). All analyses were performed in R version 3.6.1.

RESULTS

Streptomyces Diversity at Whiteface Mountain and Woods Lake

Streptomyces exhibited greater richness at WM (74 ± 69 OTUs, average and standard deviation) than at WL (6 ± 3, ave. and s.d.), and this result was significant (Mann Whitney *U*-test, $p = 0.004$). *Streptomyces* richness at WM was maximal at mid elevations (500–900 m, **Figure 2**), consistent with the classic hump-backed pattern of alpha diversity seen in previous studies (Singh et al., 2012; Liu et al., 2016; Kou et al., 2021). In contrast, sites at WL varied little in richness (**Figure 2**). Although WL had fewer *Streptomyces* OTUs than WM, rarefaction curves indicate that both sites were adequately sampled (**Supplementary Figure 1**).

Streptomyces exhibited greater beta diversity at WM (0.76 ± 0.28, unweighted UniFrac distance, ave. and s.d.) than at WL (0.35 ± 0.21, unweighted UniFrac distance, ave. and s.d.), and this difference was significant (Mann Whitney *U*-test, $p < 0.0001$). In addition, *Streptomyces* communities at WM and WL were highly dissimilar (0.84 ± 0.164, unweighted UniFrac distance, ave. and s.d.). Analysis of similarities (ANOSIM) indicates that elevation is the strongest predictor of beta diversity at WM ($R = 0.5284$, $p < 0.001$, 9,999 permutations). Additionally, beta diversity was partitioned into turnover and nestedness (Baselga, 2010). Species turnover underlies most of the beta diversity at both sites (90.91% at WM, 59.5% at WL), and turnover was significantly higher than nestedness at WM (paired

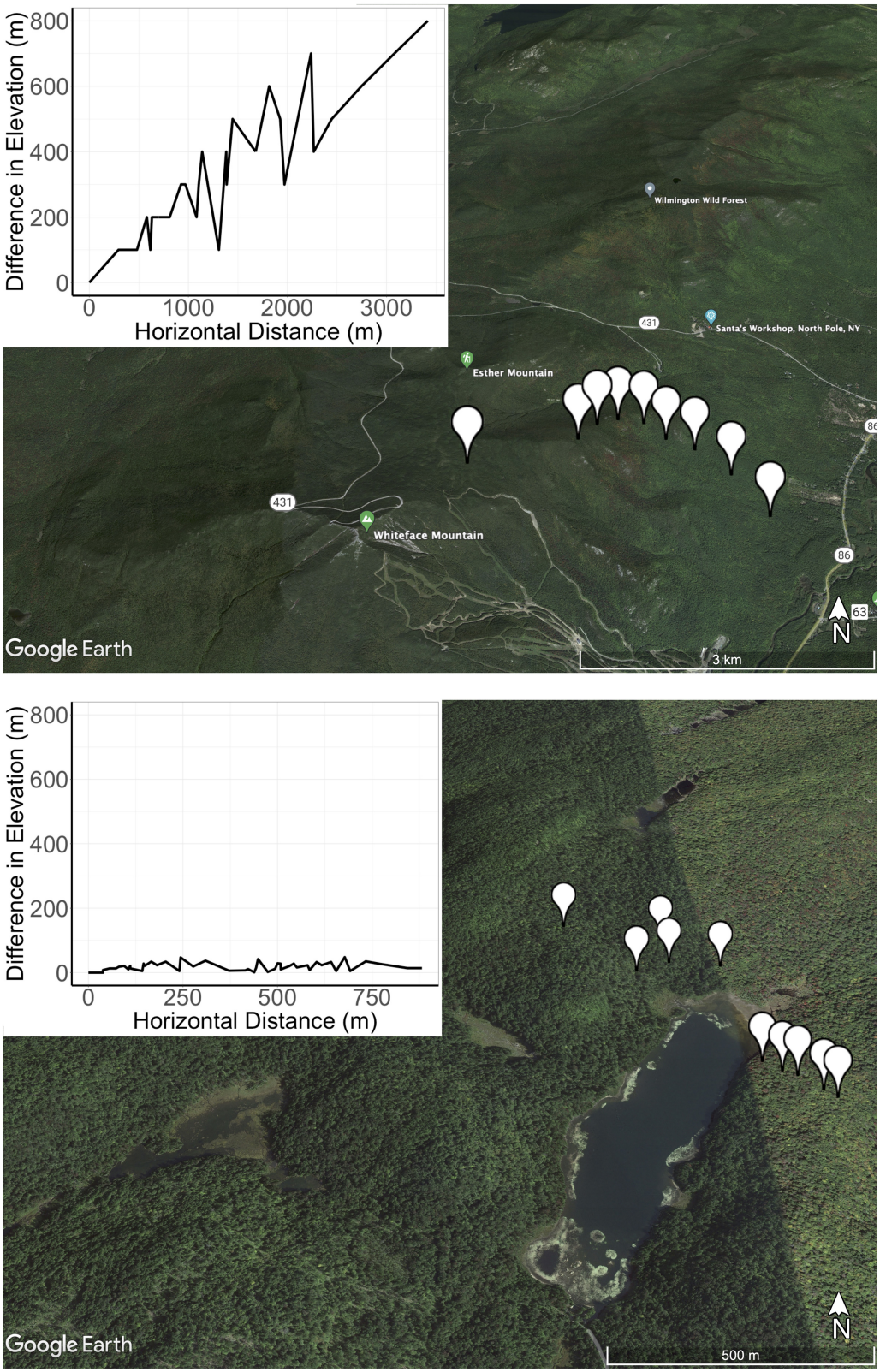

FIGURE 1 | The sites at Whiteface Mountain (WM; **top**) span more than 1,000 m elevation while those at Woods Lake (**bottom**) span less than 100 m elevation. The topographical profile is provided in the inset. Horizontal distances (X-axis) are measured as geodesic distance or the shortest distance between the GPS coordinates of each site while the Y-axis represents difference in elevation relevant to the base elevation at WM.

TABLE 1 | Spatial and environmental metadata for each sample at Whiteface Mountain and Woods Lake.

Sample	Elevation (m)	pH	Temperature	SOM	Watershed
S01_R01	1,200	3.93	15	16.6288192	NA
S01_R02	1,200	4.1	15	31.9330525	NA
S01_R03	1,200	3.74	15	29.1874668	NA
S02_R01	1,100	3.86	16	22.1387585	NA
S02_R02	1,100	3.61	17	53.9605634	NA
S02_R03	1,100	3.86	16	35.9873995	NA
S03_R02	1,000	3.67	16	26.3294986	NA
S03_R03	1,000	4.05	17	27.4130845	NA
S04_R02	900	3.93	17	33.735336	NA
S04_R03	900	4.69	17	27.6732296	NA
S05_R01	800	4.42	19	15.7430731	NA
S05_R02	800	4.52	19	14.0397492	NA
S05_R03	800	4.45	18	14.61	NA
S06_R01	700	4.96	20	17.9205852	NA
S06_R02	700	4.54	19	22.4786858	NA
S06_R03	700	4.95	18	13.2271829	NA
S07_R02	600	5.09	20	7.89907312	NA
S07_R03	600	4.7	19	8.87326813	NA
S08_R01	500	5.08	20	5.65392979	NA
S08_R02	500	4.81	20	5.04150015	NA
S08_R03	500	4.95	20	6.75924035	NA
S09_R01	400	4.81	19	11.4845938	NA
S09_R02	400	5.07	19	28.7365177	NA
S09_R03	400	5.11	19	9.08149529	NA
C1A1	610	4.14	NA	NA	C1
C1B1	615	4.1	NA	NA	C1
C1C1	657	4.28	NA	NA	C1
C1D1	620	4.16	NA	NA	C1
C1E1	638	4.59	NA	NA	C1
C2A1	609	4.45	NA	NA	C2
C2B1	622	4.62	NA	NA	C2
C2C1	630	4.28	NA	NA	C2
C2D1	643	4.21	NA	NA	C2
C2E1	643	4.88	NA	NA	C2

Sites that start with C (e.g., C1A1) are in WL, and sites that start with S (e.g., S01_R01) are in WM.

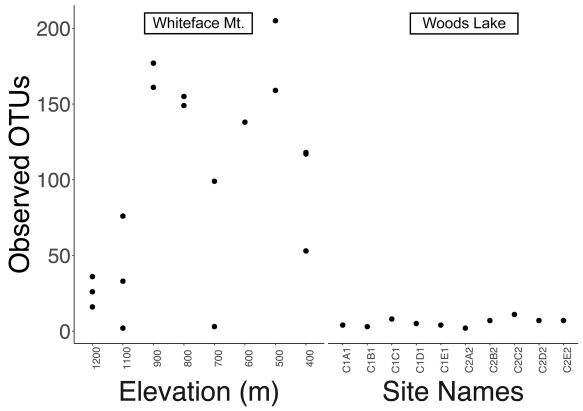

FIGURE 2 | Alpha diversity is represented as number of observed OTUs in each sample. *Streptomyces* at Whiteface Mountain (**left**) exhibited maximal richness at mid elevations with decreasing alpha diversity at the highest and lowest sites. *Streptomyces* at Woods Lake (WL; **right**) exhibited little change in alpha diversity with respect to elevation change. Sample names are provided instead of elevations in the right panel because elevation does not vary across the WL watershed (largest elevation difference is 33 m, **Table 1**).

t-test, $p < 0.0001$; **Supplementary Table 1**). Taken together, these results indicate that dispersal is limited between WM and WL, and it is limited across elevation at WM.

A high amount of species turnover indicates that species are replaced from local pools rather than from the regional meta-community. Only 12 OTUs were shared between WM and WL. Ten of these shared OTUs have high relative abundance as compared to random expectations based on a random draw from the regional meta-community (paired *t*-test, 1000 permutations, $p = 0.002$), indicating that high-abundance OTUs are more likely to be shared at regional scales than we would expect due to chance.

Elevation Drives Community Structure on Whiteface Mountain

If elevation causes phylogenetic clustering, beta diversity should correlate with elevation change. Of the five variables tested, elevation was the only variable that was significantly correlated with beta diversity (**Figure 3** and **Supplementary Figure 2**). The correlation coefficients indicate an intermediate-strength relationship, consistent with previous findings of dispersal limitation in other bacterial communities (Bell, 2010; Angermeyer et al., 2016). Beta diversity at WL was not significantly correlated with elevation, horizontal distance, pH, or any other measured variables.

Community assembly was subdivided into selection, dispersal, and ecological drift [as described by Stegen et al. (2012, 2013)]. Ecological drift was the dominant assembly process within WL. At WM, variable selection was the dominant assembly process at high elevation (above 1,000 m), as expected if ecological filtering is driven by elevation (**Figure 4A**). However, at WM, the importance of homogenizing dispersal increases at elevations below 1,000 m (**Figure 4A**). This result suggests that dissemination at WM is driven by downward movement of soil and water from high to low elevation, with ecological filtering due to variable selection limiting the establishment of "high elevation clades" at mid and low elevation sites, while homogenizing dispersal is more common between mid and low elevation sites (**Figure 4B**).

Indicator Species Analysis

We conducted indicator species analysis to identify OTUs specific to elevation zones in WM (multipatt function, indicspecies R package). 16 OTUs were associated with elevations above 1,000 m and 14 OTUs with elevations below 500 m. We evaluated pairwise phylogenetic distance of the indicator species with respect to elevation (**Figure 5**). Low-elevation indicator species exhibited more phylogenetically similarity to each other than expected due to chance (*t*-test, $p = 0.006$), while high-elevation indicator OTUs were more

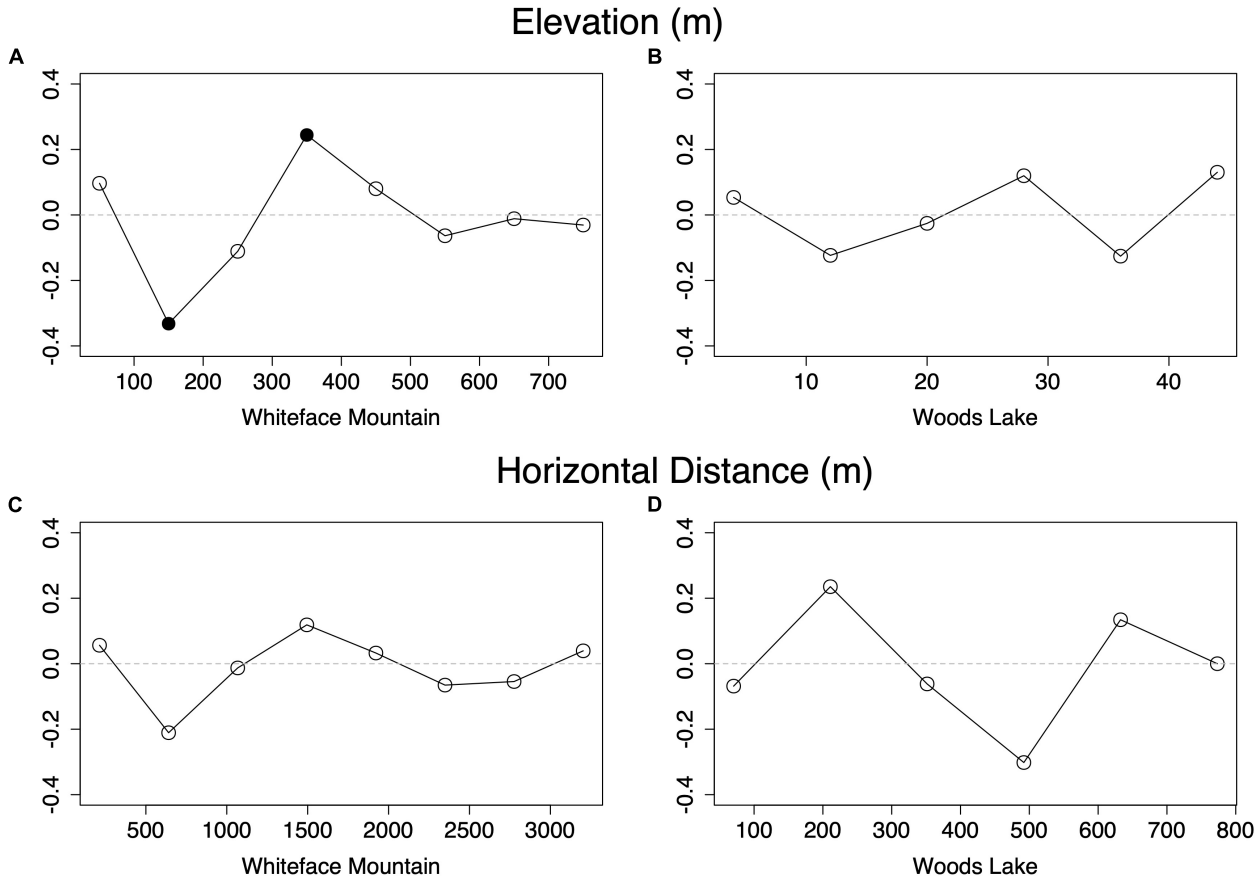

FIGURE 3 | Partial Mantel tests indicate that beta diversity varies significantly with respect to elevation at Whiteface Mountain **(A)** but not at Woods Lake **(B)**, and that beta diversity does not significantly correlate with horizontal distance at either site **(C,D)**. Filled points indicate significant correlation ($p < 0.01$).

diverse indicating the presence of multiple phylogenetic clusters (**Figure 5**).

Evaluating Indicator OTU Distribution in a Continental-Scale Dataset

We further evaluated the elevational preferences of indicator OTUs by examining their distribution in a continental-scale dataset of *Streptomyces* biogeography. The continental-scale dataset contains *rpoB* sequences from *Streptomyces* communities across North America (see "Materials and Methods" section). Briefly, indicator OTUs from WM were identified in the continental-scale dataset by clustering at 99% identity. The preferred elevation for these OTUs was calculated as the normalized abundance-weighted average of elevations for all sites at which the OTU was detected. Elevational preference at WM had a strong influence on elevational distribution for conspecific OTUs in the continental survey (Cohen's $d = 0.7$, **Figure 6**).

Phylogenetic reconstruction of WM indicator OTUs and their continental relatives (**Figure 7**) indicated that mixed clades (clades with both high- and low-indicator OTUs) were more evolutionarily divergent than clades with only one indicator type as quantified by mean pairwise phylogenetic distances within each clade (*t*-test with 1,000 permutations, Bonferroni corrected $p = 0.002$). Additionally, clades with only high-elevation OTUs contained less divergence than those with only low-elevation OTUs (*t*-test with 1,000 permutations, Bonferroni corrected $p = 0.006$).

Patristic distance calculations suggest that low-elevation clades in **Figure 7** are younger than the mixed or high-elevation clades, as they have shorter root-to-tip distances (0.09 ± 0.06 as compared to 0.16 ± 0.11 for high-elevation OTUs; Kruskal-Wallis test, $p = 0.004$). Hence, OTUs that are now localized above 1,000 m exhibit greater evolutionary divergence than those present at lower elevations.

DISCUSSION

We show that *Streptomyces* communities are dispersal limited at local spatial scales and that dispersal limitation is governed by limits to both dissemination and establishment. *Streptomyces* communities at Woods Lake had low rates of dispersal, resulting in ecological drift at spatial scales spanning hundreds of meters. Low dispersal could result from low rates of dissemination, but the ability of *Streptomyces* to make aerial hyphae and desiccation

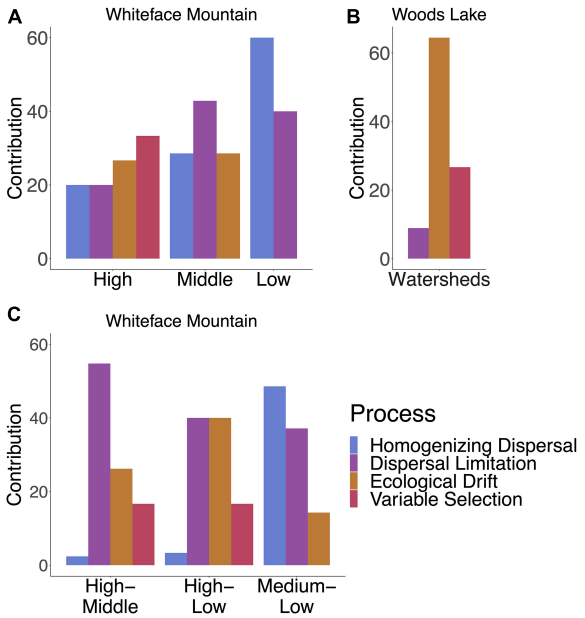

FIGURE 4 | Relative contributions of selection, dispersal, and ecological drift to community assembly vary between sites and within elevation zones in Whiteface Mountain. For WM, High indicates elevations above 1,000 m, Low indicates elevations below 500 m, and Middle indicates 500–1,000 m. **(A)** Variable selection drives beta diversity at sites above 1,000 m in WM while lower elevations have higher levels of homogenizing dispersal resulting in reduced beta diversity. **(B)** Dispersal limitation is highest between sites above 1,000 m and the rest of the mountain, while dispersal plays an important role in homogenizing *Streptomyces* communities between lower elevations. **(C)** Community assembly at WL is dominated by ecological drift with some variable selection.

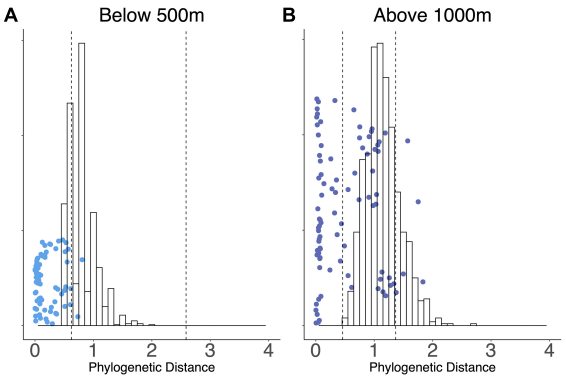

FIGURE 5 | Scatter plots show the distribution of pairwise phylogenetic distances **(A)** between indicator OTUs below 500 m, and **(B)** between indicator OTUs above 1,000 m. The histograms represent a bootstrapped distribution of 1,000 random draws from pairwise phylogenetic distances within the WM community, and 95% confidence intervals are indicated by vertical dashed lines. Each dot represents the phylogenetic distance between a pair of OTUs in that respective category.

resistant spores, and the fact that long range dispersal has been observed (Andam et al., 2016b; Choudoir et al., 2016; Higgins et al., 2021), suggest that limits to dissemination are unlikely to structure communities at local scales. Given the minimal habitat variation among sites at WL, and the broad habitat suitability expected for *Streptomyces*, it also seems unlikely that low dispersal at WL is driven by ecological filtering. Hence, we hypothesize that low dispersal and ecological drift at WL is likely driven by biotic interactions such as antagonism or density dependent blocking (Waters et al., 2013). *Streptomyces* are well known to produce diverse antimicrobial compounds, and other secondary metabolites, that alter biotic interactions (Kinkel et al., 2014; Schlatter and Kinkel, 2014; Vaz Jauri and Kinkel, 2014; Essarioui et al., 2016; Otto-Hanson and Kinkel, 2020). It seems likely that biotic interactions generate barriers to establishment that govern the structure of *Streptomyces* communities at WL.

We see strong evidence of *Streptomyces* dispersal limitation at Whiteface Mountain, with elevation having significant impacts on both dissemination and establishment. Certain clades preferentially occupy either high or low elevation habitats, indicating that barriers to establishment alter community structure across the mountain. The fact that these elevational habitat preferences are also observed in a continental-scale dataset suggests that ecological filtering by habitat preference constrains *Streptomyces* community structure across elevation. However, we also observe that homogenizing dispersal increases toward the base of the mountain (**Figure 4**), and this suggests that the elevation gradient favors dissemination, likely due to movement of material down the mountain. Both alpha and beta diversity are significantly higher at WM than WL, consistent with the expectation that strong environmental gradients amplify patterns of microbial diversity despite high rates of dissemination. We expect that competitive interactions influence *Streptomyces* community composition at both WL and WM, but that the effect of elevation on dissemination and establishment is the main driver of community structure at WM.

Previous studies offer conflicting evidence for the effect of elevation on microbial biogeography. While research across a montane elevational gradient in Peru showed no effect of elevation on bacterial communities in soil (Fierer et al., 2011), a similar analysis on soils from the Andes Mountains found that bacterial and fungal diversity both varied with respect to elevation (Nottingham et al., 2018). Other studies have documented variation in bacterial community structure across elevation, attributing such variation to a range of factors including soil pH (Cho et al., 2018), aspect (Wu et al., 2017), soil carbon (King et al., 2008), and seasonality (Lazzaro et al., 2015; Zhu et al., 2020).

Conflicting evidence on the relationship between bacterial biogeography and elevation could result from variation in spatial scales, habitat variability, and the phylogenetic resolution of taxonomic markers. Several environmental variables can co-vary with elevation (Sundqvist et al., 2013), making it difficult to disentangle the effect of elevation as opposed to other co-varying gradients. We also know that the spatial scale and taxonomic resolution at which diversity is measured can influence our ability to observe patterns of biogeography (Bent et al., 2003; Martiny et al., 2011; van de Guchte, 2017). Most prior studies of microbial diversity across elevation gradients have been performed using the 16S rRNA gene as a taxonomic marker. The

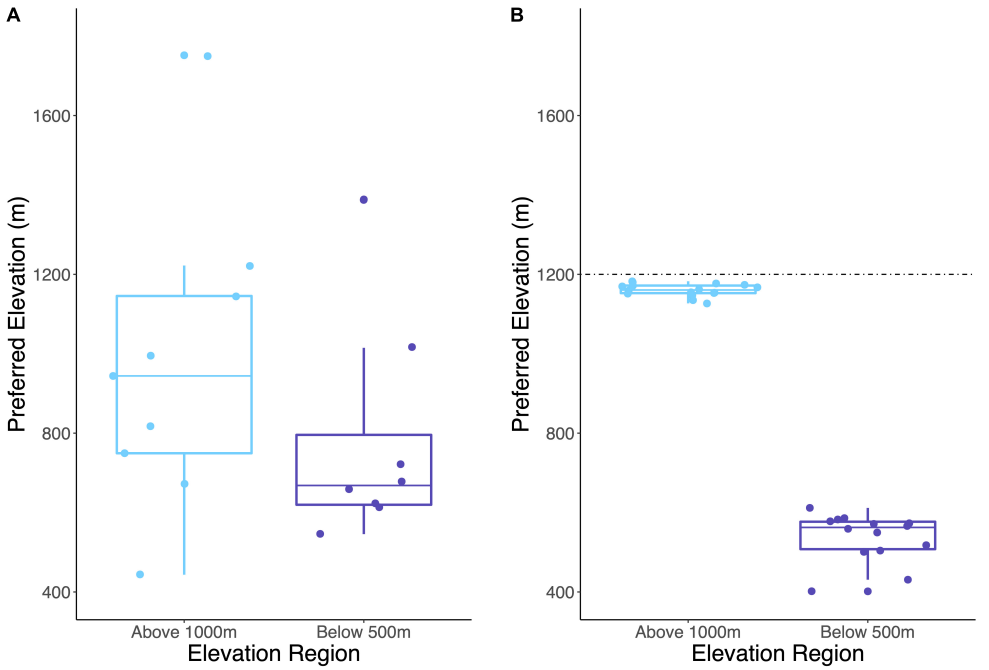

FIGURE 6 | Abundance-weighted average elevation of each indicator OTU or its conspecific representative is represented as preferred elevation for **(A)** continental-scale distribution of *Streptomyces* and **(B)** the distribution of *Streptomyces* at WM. The dashed line indicates maximum elevation sampled at WM. Preferred elevations are significantly different between High and Low indicator OTUs at WM (Welch's t-test, $p < 0.001$).

low phylogenetic resolution of this marker makes it unsuitable for assessing mechanisms of dispersal limitation (Choudoir et al., 2012). For example, common taxonomic units defined on the basis of the 16S rRNA gene encompass strains whose ancestors may have diverged 50–150 million years ago (Ochman et al., 1999), and such taxonomic units lack the resolution needed to resolve the mechanisms that underlie extant patterns of microbial biogeography (Hanson et al., 2012). Our ability to identify the effect of environmental gradients on species distributions improves in proportion to the phylogenetic resolution at which diversity is characterized (Ramirez et al., 2018). Many phenotypic traits are conserved among closely related strains (Martiny et al., 2015; Barnett et al., 2021), and so experiments that use taxon-specific, fine-scale phylogenetic markers are vital to illustrate the processes driving microbial biogeography. Several examples of non-16S gene markers already exist in the literature; *dsrA*, *nirK*, *nirS*, and other MLST-based schemes have been used to detect biogeographical patterns in environmental bacteria (Whitaker et al., 2003; Boucher et al., 2011; Angermeyer et al., 2016; Kou et al., 2021; Liao et al., 2021). In this study, the use of a *Streptomyces*-specific amplicon marker allows the exploration of phylogenetic patterns at a fine scale, sufficient for exploring the mechanisms that govern microbial dispersal.

Contemporary and historical climate variation is likely to influence patterns of *Streptomyces* biogeography. Elevation has a clear impact on temperature, as land temperatures decline 0.42°C for every 100 m of elevation, such that a 200 m change in elevation approximates the temperature shift associated with a 1° change in latitude (Montgomery, 2006). Phylogenetic conservation of thermal traits has been shown to influence *Streptomyces* dispersal across latitude (Choudoir and Buckley, 2018), and such thermal adaptation likely contributes to the latitudinal diversity gradient observed for North American *Streptomyces* (Andam et al., 2016b). Whiteface Mountain is one of the highest peaks in the Adirondacks (1,484 m above sea level). Geological evidence indicates that the mountain was glaciated along with the entire Adirondacks region, until glacial retreat about 10,000 years ago (Franzi et al., 2000). This geological timeline means that *Streptomyces* have arrived fairly recently to WM and WL and hence the time for local diversification was limited. Prior to the period of glacial retreat, about 12,000 years ago, the climate in the Adirondacks region would have been approximately 2°C cooler than current conditions (Kaufman et al., 2020). Over time, as the climate warmed, species adapted to warmer climates would have dispersed into the wider Adirondacks region while cold adapted species would have found their habitat restricted to higher and higher positions on the mountain. In this scenario, we hypothesize that ecosystem properties linked to climate variation influence microbial dispersal in soils by controlling the probability that species are able to establish at new sites. Changes in elevation influence a wide range of ecological variables both above and belowground (Sundqvist et al., 2013), and so it would be imprudent to conclude that temperature is the most important variable delimiting establishment, but it seems fair to conclude that ecological properties associated with climate variation can be expected to alter patterns of microbial establishment in soils.

Our findings indicate that a mixture of stochastic and deterministic processes govern *Streptomyces* dispersal.

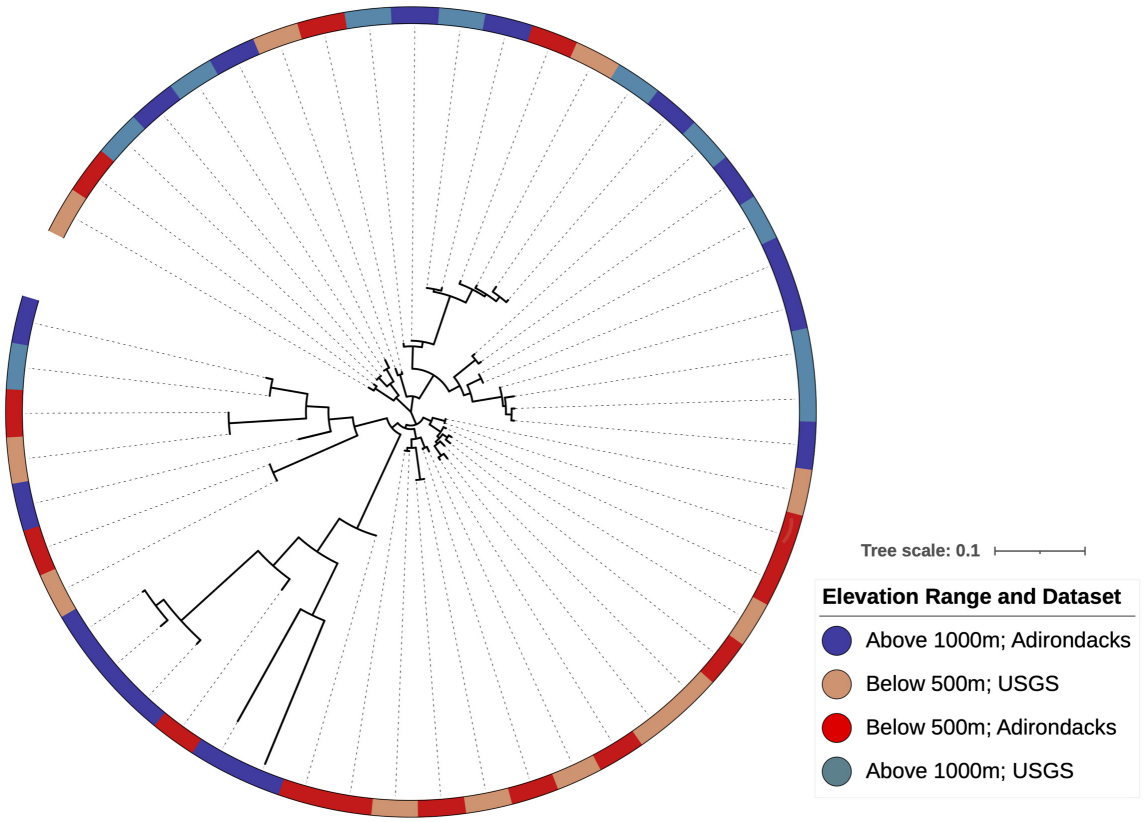

FIGURE 7 | Phylogenetic reconstruction of indicator OTU lineages shows clade-level conservation of elevational preferences within *Streptomyces* found at WM. Some clades show mixed elevation preferences with branch lengths suggesting that indicator OTUs above 1,000 m have greater evolutionary divergence relative to those found at lower elevations. Colors are indicative of elevation and dataset for each OTU as indicated in the legend.

Streptomyces form aerial hyphae that produce desiccation-resistant, hydrophobic spores and their physiological traits should support broad habitat tolerance. As a result, we would generally assume that *Streptomyces* have a greater dispersal capacity than most other soil bacteria. However, we found high dissimilarity in communities that occupied similar habitats and similar elevations (350–450 m elevation) at both WL and WM. This result suggests local limits on dispersal, likely driven by capacity for establishment as determined by competitive interactions between existing species and new immigrants. However, we did see evidence for homogenizing dispersal at the base of Whiteface Mountain suggesting that high rates of dissemination, likely driven by mass transport down the mountain, might overwhelm the ability of deterministic processes to constrain community assembly patterns. Our ability to identify dispersal limitation was enabled by the high phylogenetic resolution of the *rpoB* marker that we used, since 16S rRNA analyses provide little ability to resolve patterns of dispersal in *Streptomyces* (Higgins et al., 2021). The existence and impact of dispersal limitation has now been documented for several microbial taxa across a range of ecosystems (Staley and Gosink, 2002; Whitaker et al., 2003; Bell, 2010; Eisenlord et al., 2012; Albright and Martiny, 2017; Bottos et al., 2018; Evans et al., 2019). Hence, it seems likely that microbial dispersal is finite and subject to change over time based on contemporary processes and historical contingencies (Hewitt, 2000; Mennicken et al., 2020; Liao et al., 2021). In the case of *Streptomyces*, we hypothesize that contemporary climate variation is a major determinant of establishment. We also hypothesize that historical variation in climate has contributed significantly to extant patterns of microbial biogeography in North America because rates of dispersal are low relative to rates of climate variation during the Quaternary Period.

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://www.ncbi.nlm.nih.gov/, PRJNA790982.

AUTHOR CONTRIBUTIONS

JH performed the research, analysis, writing, and editing. DB supervised research and analysis and assisted with writing and editing. 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/fmicb.2022.856263/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves show that most sites within WM and all WL sites have been sampled to saturation.

Supplementary Figure 2 | Elevation and horizontal distance are the best predictors of beta diversity at WM as measured by unweighted UniFrac distance along all measured environmental and spatial gradients.

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PERSPECTIVE
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Many Questions Remain Unanswered About the Role of Microbial Transmission in Epizootic Shell Disease in American Lobsters (*Homarus americanus*)

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Despite decades of research on lobster species' biology, ecology, and microbiology, there are still unresolved questions about the microbial communities which associate in or on lobsters under healthy or diseased states, microbial acquisition, as well as microbial transmission between lobsters and between lobsters and their environment. There is an untapped opportunity for metagenomics, metatranscriptomics, and metabolomics to be added to the existing wealth of knowledge to more precisely track disease transmission, etiology, and host-microbe dynamics. Moreover, we need to gain this knowledge of wild lobster microbiomes before climate change alters environmental and host-microbial communities more than it likely already has, throwing a socioeconomically critical industry into disarray. As with so many animal species, the effects of climate change often manifest as changes in movement, and in this perspective piece, we consider the movement of the American lobster (*Homarus americanus*), Atlantic Ocean currents, and the microorganisms associated with either.

Keywords: microbial community assembly, environmental microbiome, epizootic shell disease, shell associated community, Atlantic Ocean microbiome

INTRODUCTION

The American lobster, *Homarus americanus* (family Nephropidae), is an iconic and delicious (authors, personal communications) marine crustacean found along coastal waters in the Northwestern Atlantic Ocean, from latitude ~56° N, present day Labrador Peninsula in Canada, down to latitude ~34° N, present day North Carolina, in the United States. Lobsters have a hard carapace, which they molt regularly to grow, and large but unequally sized front claws used for feeding and defense (Aiken, 1980). While not all lobster species have been extensively studied, generally, lobsters are mid-trophic level community members, who transfer energy from primary producers further up the food chain (Radhakrishnan et al., 2019). In addition

to exhibiting some grazing of algae and plants and predation on various bivalves and crustaceans, lobsters primarily scavenge detritus and turn over decaying material in the ecosystem (Radhakrishnan et al., 2019). In the past few decades, increasingly prevalent infectious disease, a slow northward migration, and stress from warming or acidifying waters have piqued research interests, and a significant amount of research on lobster biology and ecology has been accomplished.

Marine ecosystems are an untapped opportunity for metagenomics, metatranscriptomics, and metabolomics to increase our knowledge of ocean and lobster-associated microbiomes—the collective genomes of the bacteria, fungi, archaea, protozoa, and viruses in a community. For example, phages are known to infect and destroy large numbers of marine microorganisms in very short periods of time, effectively remodeling the microbial ecosystem (Breitbart et al., 2018). Further, horizontal gene transfer, whether mediated by phage infection or otherwise, is extremely common in hosts (Degnan, 2014) and marine systems (Nakamura, 2019). Metagenomics and whole-genome sequencing would allow for greater resolution of genomic data and the ability to better identify microbial individuals. Coupled with time-resolved sampling, this would allow for better tracking of individual microbiota over time and space. Changing environmental conditions are known to rapidly effect changes in microbial gene expression, and plasticity in biochemical substrates allows the same microbial individual to act very differently. Here, we consider the microbiological, biological, ecological, social, and economical impacts of the movement of lobsters, ocean currents, and the microorganisms associated with either.

Slowly but Steadily, Lobsters Are Migrating Northward

Perhaps the most apparent change in movement is that of animal habitat. Lobsters prefer colder waters and become physiologically stressed after extended periods in waters warmer than 22°C. In the past several decades, densities have dramatically declined in coastal waters which have warmed, as lobster populations slowly migrate north at a rate of ~43 miles per decade (Pinsky et al., 2013). While the Gulf of Maine has seen a recent lobster population boom, attributed to this range shift, Gulf waters are also warming quickly and populations are expected to continue to march northward. Meanwhile, warming temperatures are altering animal health, lobster-associated microbial communities, and ocean microbial communities, further mechanisms for destabilizing the population.

American lobsters have long been part of the diet and livelihood of coastal communities, beginning with historical accounts through present day, including Beothuk, Eastern Abenaki, Mi'kmaq, Passamaquoddy, and Wampanoag peoples [The Centre for Indigenous Peoples' Nutrition and Environment (CINE), 2017], as well as peoples in New England and Mid-Atlantic U.S., and the eastern maritime provinces of Canada. Currently, lobster catch, or landings, measures from ~10 million pounds per year in southern New England to ~140 million pounds per year from the Gulf of Maine, representing 79% of the total value caught by Maine fishermen (Atlantic States Marine Fisheries Commission, 2021; Department of Marine Resources, 2021) where the industry generates around half a billion dollars annually (Commercial Fishing Historical Landings Data, 2020). The lobster industry in Canada generates nearly 1 billion dollars annually (Government of Canada, Fisheries and Oceans Statistical Services, 2021). Intra-industry relations between indigenous and non-indigenous Canadian fishermen as well as negotiations between Maine fishermen and both marine mammal conservation groups and offshore energy producers have often turned contentious, illustrating how vital the lobster industry is along the eastern seaboard. The lucrative nature of the current market, combined with high barriers to entry, ongoing territorial disputes, and conflicts with state and federal policy makers have led to prolonged regulatory and social battles in many coastal areas (Levinson-King, 2020). In recent decades, a myriad of reasons, including overfishing of other lucrative species and climate change driven population shifts, have led to the siloing of Maine's lobster fishers, with many families and communities depending primarily on lobster for income (Steneck et al., 2011; Stoll et al., 2016). In Southern New England, where lobster abundance declined by around 70% in recent years, state managers suggested a complete closure of the fishery (Steneck et al., 2011). In Maine, where rural communities tie their cultural identity to the lobster fishery and have little flexibility to pursue other opportunities (Stoll et al., 2016), a similar collapse of the industry would have significant and widespread social and economic impacts, in addition to the ecological impacts.

Epizootic Shell Disease Is More Prevalent Over Time and With Increased Temperature

Considering emerging diseases, epizootic shell disease (ESD) has been particularly perplexing since it was first observed in *H. americanus* in the mid-1990s and has dramatically increased in prevalence along U.S. coastal waters (Castro et al., 2012). Shell diseases in crustaceans are common, generally cause softening or pitting of the shell, and depending on the disease, have microbial or environmental causes (Tlusty et al., 2007; Sweet and Bateman, 2015) In ESD, shell degradation can lead to lesions with poly-microbial communities present but it does not spread to internal tissues (Watson, 2005). Research studies have linked ESD to disease-associated mortality (Hoenig et al., 2017; Groner et al., 2018), but it remains to be determined if ESD microbial communities are the direct cause of mortality or if shell degradation increases the susceptibility of the animal to other infections, predation, and/or physical damage. Chitinolytic bacteria were originally suspected of degrading shells, and though present and active on shells, microbial chitinase enzymes are not as active on shells as microbial proteolytic and cellulolytic enzymes (Bell et al., 2012). Disease diagnosis is difficult prior to the development of physical signs of disease, and many potential bacterial pathogens have been identified ubiquitously on apparently healthy lobsters, (e.g., Meres, 2016; Bouchard, 2018; Ishaq et al., 2021a) or in marine environments (Watson, 2005). A study used amplicon sequencing and did not find putative ESD pathogens to also exist in tank biofilms and that putative pathogens may be associated with more

disease symptoms but not disease onset (Whitten et al., 2014). Rather than being associated with a different community, ESD is associated with having more bacterial growth present, as confirmed by scanning electron microscopy of shells (Tlusty et al., 2007).

In decades of research, the causative microbial or viral agent for ESD has not been identified, though many studies attempted to Castro et al. (2012). Previous research has used several regions of the 16S rRNA gene to identify bacterial species using denaturing gradient gels (e.g., Chistoserdov et al., 2012; Quinn et al., 2013) or communities using amplicon sequencing (e.g., Whitten et al., 2014; Meres, 2016; Reardon et al., 2018), and one study used amplicon sequencing on the 18S rRNA gene to identify eukaryotes associated with ESD (Quinn et al., 2009). One study using denaturing gradient gels also used infection models under experimental conditions to determine if putative infectious bacteria might be causative agents (Quinn et al., 2012). To our knowledge, no study has explored the microbial community using shotgun sequencing metagenomics techniques to identify microbial genomes of any bacteria, fungi, archaea, protozoan, or virus present or using shotgun sequencing metatranscriptomics to identify active gene transcriptions from microorganisms present, from healthy and diseased shells to investigate those functional changes.

Further, lobsters do not respond to ESD as they respond to typical bacterial infections, although there are some gene expression changes in ESD lobsters which indicate less growth and more innate immune activity (Tarrant et al., 2010). *In vitro* antimicrobial activity of hemolymph collected from lobsters was not higher in ESD lobsters compared to healthy controls (Bouchard, 2018) and was lower in both ESD and healthy lobster hemolymph compared to hemolymph spiked with the bacteria *Escherichia coli* as a positive control (Bouchard, 2018). Assuming that the lack of response is because the ESD-associated microbial community is recognized by the lobster as commensal, this lends weight to the theory that shell microbial community taxonomic structure is not changed, only microbial function is. The immune response dynamic was suppressed when hemolymph was collected from ESD lobsters that had been housed in warmer waters; *in vitro* antimicrobial activity was lowest in those kept in the warmest waters compared to medium or low temperatures (Dove et al., 2005; Bouchard, 2018). ESD progression is faster in lobsters housed under warmer temperatures for prolonged periods (Barris et al., 2018) and, regardless of when or where the study took place, ESD is more prevalent in ocean waters which are routinely above 22°C (Glenn and Pugh, 2006; Tanaka et al., 2017; Groner et al., 2018). Studies that use higher resolution of spatial patterns of ESD prevalence and environmental conditions support the importance of water temperature, as ESD can be found even in areas of otherwise optimal lobster habitat (Tanaka et al., 2017).

INTERACTIONS OF MOVEMENT, MOLTING BEHAVIOR, AND TEMPERATURE

Disease transmission dynamics of ESD have not been elucidated, and while the general consensus is that ESD is not transmitted from one animal to the next under laboratory conditions, this appears to be based on one note in a conference proceeding (Duboise and Moulton, 2005). This consensus is further tempered as most aquaculture-based lobsters are housed individually to prevent antagonism or cannibalism, which would preclude horizontal transmission of bacteria *via* direct contact, as well as *via* water if there is only one lobster per tank. Multiple lobsters may be housed per tank and separated by divider screens which allow water flow, which would theoretically spread the infection if it were simply a factor of localized exposure to certain microorganisms. There is also a general consensus that it is too difficult for tank systems to replicate the complex factors which would contribute to disease transmission in the wild. Further, because tank systems regularly filter water, possible causative agents could be removed from the tank system over time. In a study of wild-caught lobsters which were group housed but individually separated by mesh screens which allowed water flow, ESD signs did not worsen over time in most lobsters nor did they appear on apparently healthy lobsters after being co-housed with ESD-affected lobsters for over a year (Bouchard, 2018).

In wild populations along the United States coast, daily lobster movement has not been documented to be a factor in spread of disease (Watson, 2005), as even very mobile lobsters tend to have localized ranges (Watson, 2005; Scopel et al., 2009), and migration from deeper, cooler water to shallower, warmer water, and back was not associated with the spread of ESD (Watson, 2005). Those lobsters which migrate long distances appear to move further south and tend toward shallower waters on the move (Watson, 2005), contrary to the hypothesis that diseased lobsters would seek cooler waters in which to recuperate. However, movement of lobsters and disease transmission may be more of a problem in other locations: lobsters along the Canadian coast have been documented to move farther (Campbell, 1986; Morse et al., 2018), and American lobsters were released in the late 1990s and became invasive around Scandinavia and Britain where they pose various risks to European lobsters, *Homarus gammarus* (Whitten et al., 2014).

Lobsters molt regularly to shed their outer carapace and grow a new, larger shell. Molting frequency is somewhat seasonal based on food availability and ocean temperatures, and typically takes place during summer (June to August) and again in fall (September to October). The molt itself leaves lobsters vulnerable to environmental conditions and may result in subsequent disease signs (Aiken, 1980; Howell et al., 2005; Groner et al., 2018). If conditions are not favorable, or if a female is egg-bearing, the time between moltings will be prolonged. Taking longer to molt can increase ESD progression (Groner et al., 2018). Warmer temperature can induce molting earlier in spring (Groner et al., 2018), and molting out of sync with typical seasonal conditions can increase the risk of ESD if summer temperatures were hotter or prolonged (Groner et al., 2018). In wild and cultured lobsters, molting can reduce or remove signs of ESD entirely; however, if the outer carapace and deeper layers have been damaged, the new shell may show scarring or deformities (Stevens, 2009), which if severe enough can

prevent detachment of the old carapace. It is often in the process of molt that lobsters die from ESD-related issues.

There is another hypothetical mechanism for horizontal transmission which previous research has considered. Lobsters can be cannibalistic or in the process of molt, lobsters may consume shed carapaces (Aiken, 1980), including the associated microbial community, although uneaten molted shells are removed from aquaculture systems to prevent fouling tank water. Consuming a molted shell that contains potentially pathogenic microorganisms raises several questions. If ESD were caused by infectious or toxin-producing microorganisms, would consuming a carapace and associated microbial community be linked to morbidity and mortality in lobsters, as those hypothetical pathogens wreaked havoc internally? Various studies refute this possible mechanism, discussed in Tlusty et al. (2007). There is evidence that healthy Caribbean spiny lobsters (*Panulirus argus*) will avoid other spiny lobsters that are infected with a virus (Behringer et al., 2006), although *Homarus americanus* females do not avoid ESD-positive males in laboratory settings based on olfactory cues in the water (Rycroft et al., 2012).

WARMER WATER COULD ALTER HOW AND WHICH MICROORGANISMS ARE CARRIED BY OCEAN CURRENTS

Ocean microbial communities are diverse and dynamic, and coastal anthropogenic activities can alter the microorganisms present, nutrient cycling, and other ecological processes (Stewart et al., 2008; Nogales et al., 2011), as well as induce antimicrobial resistance (Chen et al., 2019). Ocean currents are known to curate marine microbial communities by geographic location (Cavicchioli, 2015) and depth (Zinger et al., 2011). Ocean currents were also implicated as driving the bacterial community assembled on the shell of topshell sea snails, *Phorcus sauciatus*, found in coastal Northeastern Atlantic Ocean waters around Europe and the Mediterranean (Sousa et al., 2021). Not only are currents transporting microbial communities, but the varying environmental conditions along the way may alter the microorganisms, including making them tolerant to higher temperatures (Doblin and van Sebille, 2016).

Air currents, too, transport bacterial and fungal communities which are assembled from land, water, or urban environments where the clouds were formed (Amato et al., 2017). Combined with local weather, aerosolized microorganisms contribute to disease outbreaks when environmental conditions cause animal and human hosts to be more susceptible to infection or to microbial transmission (e.g., coughing more during dust storms; Griffin, 2007). It is feasible that microbial transmission along air or water currents could contribute to removing, adding, or circulating disruptive microorganisms in coastal waters which affects lobsters. For example, land-sourced microbial communities which are transported in air (Mayol et al., 2017) or water runoff (Adyasari et al., 2020) change the microbial communities found in coastal ocean waters, and this could have a protracted effect in summer when water circulates more slowly. During the summer, the Gulf Stream slows along eastern North America as warming land temperatures alter winds and push them *across* coastal water currents on an east–west axis, rather than *with* water currents on a northward path (Roarty et al., 2020). Around Cape Cod, summer winds contribute to a recirculating eddy in Massachusetts Bay which does not mix with deeper water nearly as much as it does in winter (Robinson, 2005).

While the hypothesis of currents and ESD transmission was discussed in a regional conference panel (Robinson, 2005), and air and water currents have been extensively studied in coastal waters in this region, including for lobster larval dispersal, this has never been evaluated for currents and ESD *via* water or vectored by larvae. If climate change and/or disease affects lobster populations in warmer waters, will microbial dispersal along ocean currents bring disease from southern to northern lobster populations even before temperatures rise there? Ocean currents have been implicated in the rapid and far-reaching spread of infectious disease in marine animals (McCallum et al., 2003), including in corals (Dobbelaere et al., 2020), starfish (Aalto et al., 2020), and fish (Stene et al., 2014; Alaliyat et al., 2019), using agent-based, environmental-based, and combination modeling of empirical infection data. While not yet implicated in lobster shell diseases, there is a plausible scenario in which ocean currents flowing from southern New England, where disease is more prevalent (Glenn and Pugh, 2006; Tanaka et al., 2017) and warmer temperatures select hardier microorganisms (Mayers et al., 2016), bring taxonomically similar but functionally different microorganisms further north. Or, phage dispersal along currents could induce horizontal gene transfer and a change of activity in existing host-associated microorganisms (Degnan, 2014). Even if those microbial or viral travelers are not infectious, *per se*, those microorganisms would still be in a geographic position to take advantage of altered lobster homeostasis due to warming waters or thinner shells caused by increasing acidity.

Warming and acidifying ocean waters work independently, and conjunctively, to weaken lobster immune and physical defenses (e.g., shell quality) and make them more susceptible to infectious disease (Harrington et al., 2020). While host susceptibility does play a small role in ESD, published studies suggest it has more to do with structural capacity of the shell than a weakened immune response or other symptoms associated with heat stress in terrestrial animals, e.g., "leaky gut" (Tlusty et al., 2007).

Separate from environmental conditions and host susceptibility is the capacity for microbes to colonize hosts and transfer between them, which could temper disease transmission. In an elegantly simple microbial transmission model, a bacterium was inoculated into germ-free zebrafish (*Danio rerio*) to track immigration and emigration through an animal population. *Aeromonas veronii* was isolated from the gastrointestinal tract of conventional zebrafish with typical microbiota, and colony lines created in which the same bacteria were added to a new germ-free fish colony, re-isolated, and added to new fish for a total of 22 passages. As compared to the ancestral bacterial lines, these replicate lines were more "fish-associated" and were able to colonize more fish in a given period of time, as well

as cultivate larger cell counts in those fish they colonized (Robinson et al., 2018). Robinson et al. made use of a modified *A. veronii* with increased capacity for genetic mutation, to better study adaptation to a host. However, given enough time and the right environmental conditions, it is likely that other microorganisms could become better at colonizing animals in aquaculture systems.

In the case of ESD, in which it appears that the same bacteria are present but forming larger and more complex biofilms, the assembly, and succession of that shell biofilm community over time is likely to affect transmission dynamics. Further, water temperatures affect biofilm formation and dynamics, and so do pollution and nutrients in water runoff that finds its way into coastal waters—even if the pollution itself is not affecting lobsters directly. For example, *Aquimarina* and *Thalassobius* species of bacteria have long been posited to be involved with ESD lesions (Chistoserdov et al., 2012). Both were found to be part of biofilms associated with plastic waste after incubation in sea water for 40 days, though both were rare until after a further 94-day incubation (Jacquin et al., 2021), implying they might join biofilms after initial colonizations.

MARINE AND AQUACULTURE ENVIRONMENTS FOSTER DIFFERENT DYNAMICS AFFECTING MICROBIAL TRANSMISSION

Marine and aquaculture environments offer contrasts in their dynamics which likely generate distinct mechanisms of selection for host disease resistance or susceptibility, for microbial transmission, and for pathogen virulence or symbiosis. Marine environments have wildly diverse microbial communities which turn over quickly and dramatically with phage infection (Breitbart et al., 2018), weather patterns (Angly et al., 2016), or human settlement pollution (Nogales et al., 2011; Chen et al., 2019). Marine environments are likely to select for traits which favor microbial transmission between different animal hosts, as well as for long-term survival in the water when moving between hosts (Rebollar et al., 2016). Host-associated and free-living *Aquimarina* species were not demonstrated to have different biochemical capacities, although species varied widely in their abilities to be commensal or pathogenic (Silva et al., 2019). Silva et al. further noted that while many host-associated microbial strains have reduced genome sizes compared to their free-living, wild-type counterparts, *Aquimarina* did not differ in genome size by isolation source and that it had a larger genome than most known marine isolates. This could imply that *Aquimarina* are particularly adept at switching from host- to environmental-associated as needed.

Aquaculture facilities may select for host-species specificity in enclosed production systems, in which the only hosts available are from a single species and most likely at the same lifestage. Aquaculture production, like any selective practice, has the potential to increase or decrease microbial virulence, discussed in Kennedy et al. (2016). Aquaculture systems can be static and refreshed, flow through with no recirculation (one-time pass through), or recirculating with mechanical and biofiltration. All aquaculture facilities present surfaces on which microbial biofilms might accumulate (de Carvalho, 2018). Over time, even with regular draining and cleaning, microbial inhabitants may increase their ability to attach to surfaces, produce exopolysaccharides and other film materials, and remain in the system. Even if these biofilm-formers are not infectious, an abundance of microbial biofilms can still generate toxins or other compounds which affect host health. For example, microbial biofilms can alter the chemistry of surfaces and prevent shellfish larvae from settling and continuing their development (Qian et al., 2007). While lobster larvae do not attach upon settlement, biofilms on surfaces can affect the hospitality of their local environment. Biofilms also generate planktonic microbial cells which may be ingested or come into contact with animals. Poly-microbial biofilms can offer long-term protection and safe harbor for infectious microorganisms, which may then be released into the tank system to cause sporadic outbreaks (Levipan et al., 2020). However, previous studies which track bacterial communities in tanks and lobsters have not demonstrated much correlation between bacterial concentration and health in spiny lobster phyllosoma (larvae; Bourne et al., 2004), or American lobster adults (Bouchard, 2018).

DISCUSSION: WHERE CAN AQUACULTURE DRAW LESSONS FROM?

Aquaculture systems can both simplify and complicate marine host microbiomes, as they contain a fraction of the biological diversity—not to mention typically offering a single and nearly static ecosystem—which dramatically reduces microbial diversity in water and animals. For example, preliminary data indicate that bacterial communities on lobster shells have dramatically more bacterial diversity when coming from ocean waters versus having spent several months in filtered-and -recirculated-water tank systems, even with light exposure and water temperatures which mimic seasonal variation (Ishaq et al., 2021a,b). This effect can be beneficial for isolating and removing infectious disease from a population, especially if the causative agent is an obligate host-associated microorganism or virus.

However, this does not remove the possibility of infectious built-environment-associated microorganisms. Aquaculture systems are potentially open to similar problems faced by water management systems in human settlements: pipes and storage tanks can foster microbial communities (Borella et al., 2004; Douterelo et al., 2016; Ling et al., 2018), and surfaces could act as attachment points for biofilms—especially in pipes with valves or other connection points. No sterilization mechanism is completely effective forever, either, as evidenced by the selection of chlorine-tolerant microorganisms in disinfection systems for human settlement water treatment (Liu et al., 2018).

Nor does a depauperate tank microbial community allow animal hosts to accumulate a diverse host-associated community

which may protect them from infections. For example, laboratory mice in the same cage will transfer microorganisms through physical contact and grooming behaviors; transfer *via* air, cage surfaces, or other objects (i.e., fomites); and through feces as mice are coprophagic. If small groups of animals are left in the same cage under clean laboratory conditions, eventually their microbial communities will homogenize between individuals in each cage (Hildebrand et al., 2013). Over time, the cages act like islands, and without an influx of new microorganisms, the collective microbial community in the different cages will experience drift or random changes to the abundance of those remaining microorganisms (Nemergut et al., 2013). Yet, microbial transmission within a tank system does not appear to be spreading ESD among tank mates, even when lobsters showing ESD signs were grouped together which would theoretically increase negative microbial exposures (Bouchard, 2018).

Probiotics have been considered as a treatment or preventative for ESD (Underwood, 2018), as many aquatic microorganisms and viruses are antagonistic toward other species. Probiotics might be effective as topical treatments for tank or shell bacteria; however, while a topical probiotic could hypothetically be effective, it would not be practical at a scale needed to make an appreciable difference in wild populations. Not only are aquaculture facilities lacking to house the number of diseased lobsters which are caught annually, but application as a topical treatment to diseased lobsters which are captured and released would be extremely labor intensive and creates the potential for ecological ramifications of the probiotic in the wild. Further, probiotics may not be effective if ESD is mediated by altered microbial activity of the typical shell microbiota. In that hypothetical scenario, then, a probiotic or even bacteriophage-based topical treatment would need to disrupt or remove the biofilm on shells to give lobsters time to heal and perhaps molt before reassembly. If molting times were predicted by geographic region and seasonal temperature fluctuations, this strategy could be implemented to time it with molting. Given the sheer number of lobsters which are captured each year, this would be a sizable and costly undertaking, but priority could be given to ovigerous females to maximize impact. Probiotics could also be implemented fairly easily in aquaculture systems, or in other short-term lobster holding tanks, though it may take weeks or months to see any improvement using microbial treatment alone. Tank environmental conditions could be manipulated subsequently to induce molting after receiving anti-biofilm treatments, but this alters the molting pace by a scale of weeks, not days.

Despite economically driven interest and decades of research on lobster biology, ecology, and microbiology, there are still unresolved questions about lobster microbial communities in general, and regarding ESD in particular. There is an untapped opportunity for metagenomics, transcriptomics, and metabolomics to be added to the existing wealth of knowledge, to more precisely track disease transmission, etiology, and host-microbe dynamics.

AUTHOR CONTRIBUTIONS

SI conceptualized and wrote this perspective. SI, ST, MT, JM, HH, JK, GL, and DB contributed to refining the scope and ideas, writing, and reviewing. All authors contributed to the article and approved the submitted version.

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Symbiotic Algae of *Hydra viridissima* Play a Key Role in Maintaining Homeostatic Bacterial Colonization

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The freshwater polyp *Hydra viridissima* (*H. viridissima*) harbors endosymbiotic *Chlorella* algae in addition to a species-specific microbiome. The molecular basis of the symbiosis between *Hydra* and *Chlorella* has been characterized to be metabolic in nature. Here, we studied the interaction between the extracellularly located microbiota and the algal photobiont, which resides in *Hydra*'s endodermal epithelium, with main focus on *Legionella* bacterium. We aimed at evaluating the influence of the symbiotic algae on microbial colonization and in shaping the host microbiome. We report that the microbiome composition of symbiotic and aposymbiotic (algae free) *H. viridissima* is significantly different and dominated by *Legionella spp. Hvir* in aposymbiotic animals. Co-cultivation of these animals resulted in horizontal transmission of *Legionella spp. Hvir* bacteria from aposymbiotic to symbiotic animals. Acquisition of this bacterium increased the release of algae into ambient water. From there, algae could subsequently be taken up again by the aposymbiotic animals. The presence of algal symbionts had negative impact on *Legionella spp. Hvir* and resulted in a decrease of the relative abundance of this bacterium. Prolonged co-cultivation ultimately resulted in the disappearance of the *Legionella spp. Hvir* bacterium from the *Hydra* tissue. Our observations suggest an important role of the photobiont in controlling an invasive species in a metacommunity and, thereby, shaping the microbiome.

Keywords: symbiosis, microbiome, tripartite interactions, horizontal transmission, co-cultivation

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INTRODUCTION

For over half a century, *Hydra viridissima* (*H. viridissima*) has been a subject of study for symbiosis between the host polyp and the endosymbiotic *Chlorella* algae (Muscatine and Lenhoff, 1963, 1965; Muscatine et al., 1975; McAuley, 1981a,b; Thorington and Margulis, 1981; McAuley and Darrah, 1990; **Figure 1A**). We have shown previously that the mutual exchange of metabolites forms the basis of this symbiotic relationship (Hamada et al., 2018). The algae provide fixed carbon in the form of maltose to the host that provides a significant competitive advantage compared to the aposymbiotic animals during periods of starvation, promoting oogenesis, and allow a faster population growth rate (Habetha et al., 2003; Hamada et al., 2018). Symbiotic algae have also been shown to protect the host under temperature stress conditions (Ye et al., 2019a,b). A hallmark of

this strong interdependence is the loss of algal autonomy, as it must depend on the host for survival. Genomic analysis of *Chlorella A99* revealed degeneracy in the nitrate assimilation pathway that renders it to depend on host-derived glutamine as the source of nitrogen (Hamada et al., 2018). As a consequence, all the attempts to cultivate these symbiotic algae *in vitro* have failed so far (Hamada et al., 2018).

Apart from the symbiotic algae, the green *H. viridissima* also harbors a distinct bacterial community (Franzenburg et al., 2013b). The bacterial composition for any given *Hydra* species is specific and mirrors the phylogenetic relationship of their hosts (Fraune and Bosch, 2007; Franzenburg et al., 2013b), a pattern termed as phylosymbiosis (Brucker and Bordenstein, 2013). Species-specific antimicrobial peptides and stem cell transcription factor FoxO are involved in shaping *Hydra*'s microbiota, which is remarkably stable over time (Fraune and Bosch, 2007; Franzenburg et al., 2013a; Augustin et al., 2017; Mortzfeld et al., 2018; Bosch and Zasloff, 2021). *Hydra*-associated bacteria protect the host against fungal pathogen infection (Fraune et al., 2015) and influence the host behavior (Murillo-Rincon et al., 2017; Klimovich et al., 2020). In *Hydra*, the bacterial symbionts are known to be vertically transmitted through egg after sexual reproduction following a temporal pattern of colonization (Franzenburg et al., 2013a) or *via* budding from parent polyp. However, the role of horizontal transmission between individuals or through environment is understudied. Moreover, it is unclear yet how in *H. viridissima*, the host, endosymbiotic algae, and the extracellular bacteria impact each other and interact to maintain a tripartite relationship.

Here, we wondered if the alga had any influence on the microbial composition of the polyp. We used *H. viridissima* strain A99 associated with its native *Chlorella A99* symbiont and species-specific bacteria and compared its microbiome with that of the aposymbiotic animals. We tested the role of horizontal transmission of bacteria and other factors of the co-cultivated hosts in determining the microbiome composition. We also tested the role of algal symbionts in shaping the composition of the host-associated microbiome under different cultivation conditions. Our primary focus member of microbiome is *Legionella spp. Hvir*, as we observed clear dynamics in the proportion of this bacterium from being present in trace amounts to be the main colonizer in the symbiotic animals. We could observe the impact of horizontally transmitted bacteria, specifically *Legionella spp. Hvir*, on the host fitness and the effect of eventual spread of *Chlorella* photobiont on the control of *Legionella* bacterium in the metacommunity.

RESULTS

Microbiome Composition of Symbiotic and Aposymbiotic *Hydra viridissima* Is Distinctly Different

Symbiotic and aposymbiotic *H. viridissima* polyps maintain a profoundly different microbiome [Adonis, R^2 = 0.35679, *p*-value (Bonferroni corrected) = 0.005] (**Figure 1B**). While the microbiome of symbiotic animals is dominated by Betaproteobacteriales, aposymbiotic polyps are mostly colonized by bacteria belonging to the Legionellales group (**Figure 1B**). The prevalent operational taxonomic unit (OTU) in aposymbiotic polyps belongs to the genus *Legionella* (henceforth *Legionella spp. Hvir*). In the symbiotic animals, this microbe is present only in very small numbers (**Figure 1C** and **Supplementary Figure 1**). Since aposymbiotic animals were generated from symbiotic *H. viridissima* polyps (see Section "Materials and Methods"), they are genetically identical and we suspected that the absence of *Chlorella* algae in aposymbiotic polyps was responsible for the observed difference in the microbiome composition. This assumption is supported by the observation that the Chao1 index for alpha-diversity was higher for the symbiotic animals as compared to the aposymbiotic polyps, indicating a loss of low abundance taxa in the aposymbiotic animals (**Figure 1D**; Apo_mono_cultivation vs. Sym_mono_cultivation, *t*-test, p-value < 0.01).

Co-cultivation Affects the Microbiome Composition of Symbiotic and Aposymbiotic *Hydra viridissima*

While *Legionella spp. Hvir* is present in very low numbers (0.03%) in symbiotic *H. viridissima* polyps, it accounts for up to 47.8% of the microbiome in the aposymbiotic animals. When cultured separately, both the *H. viridissima* lines maintain these distinct microbiome signatures indefinitely. However, co-cultivating the two lines for 4 weeks (**Figure 1C**) resulted in a clear shift of the microbiota in the symbiotic animals and a significant increase of the average relative abundance of *Legionella spp. Hvir*. In symbiotic *H. viridissima* polyps co-cultivated with aposymbiotic polyps, *Legionella spp. Hvir* accounted for up to 50.9% of associated bacteria. This increase in the *Legionella spp. Hvir* abundance was consistent across all the samples of the co-cultivated symbiotic animals (**Supplementary Table 1**). We presume that this is due to co-housing and the horizontal transfer of *Legionella spp. Hvir* bacteria from aposymbiotic to symbiotic polyps. Despite this shift in microbial composition, it was noteworthy that the Chao1 alpha-diversity index remained higher for the symbiotic polyps as compared to the aposymbiotic animals (**Figure 1D**; Apo_co_cultivation vs. Sym_co _cultivation, *t*-test, p-value < 0.01; Apo_co_cultivation vs. Apo_mono_cultivation, *t*-test, p-value > 0.05). This portrays the role of *Chlorella* symbiont in maintaining the diversity of bacteria on host.

Legionella spp. Hvir Can Be Transmitted Through Water

To find out how, during the period of co-cultivation, the bacterium spread from aposymbiotic *H. viridissima* to the symbiotic polyps, we next explored whether we could transfer the bacteria simply by exchanging the culture medium and exposing monocultivated symbiotic polyps to water taken from the aposymbiotic culture. As shown in **Figures 2A,B**, culturing symbiotic animals in the non-filtered water from aposymbiotic animals lead to the presence of a considerable proportion

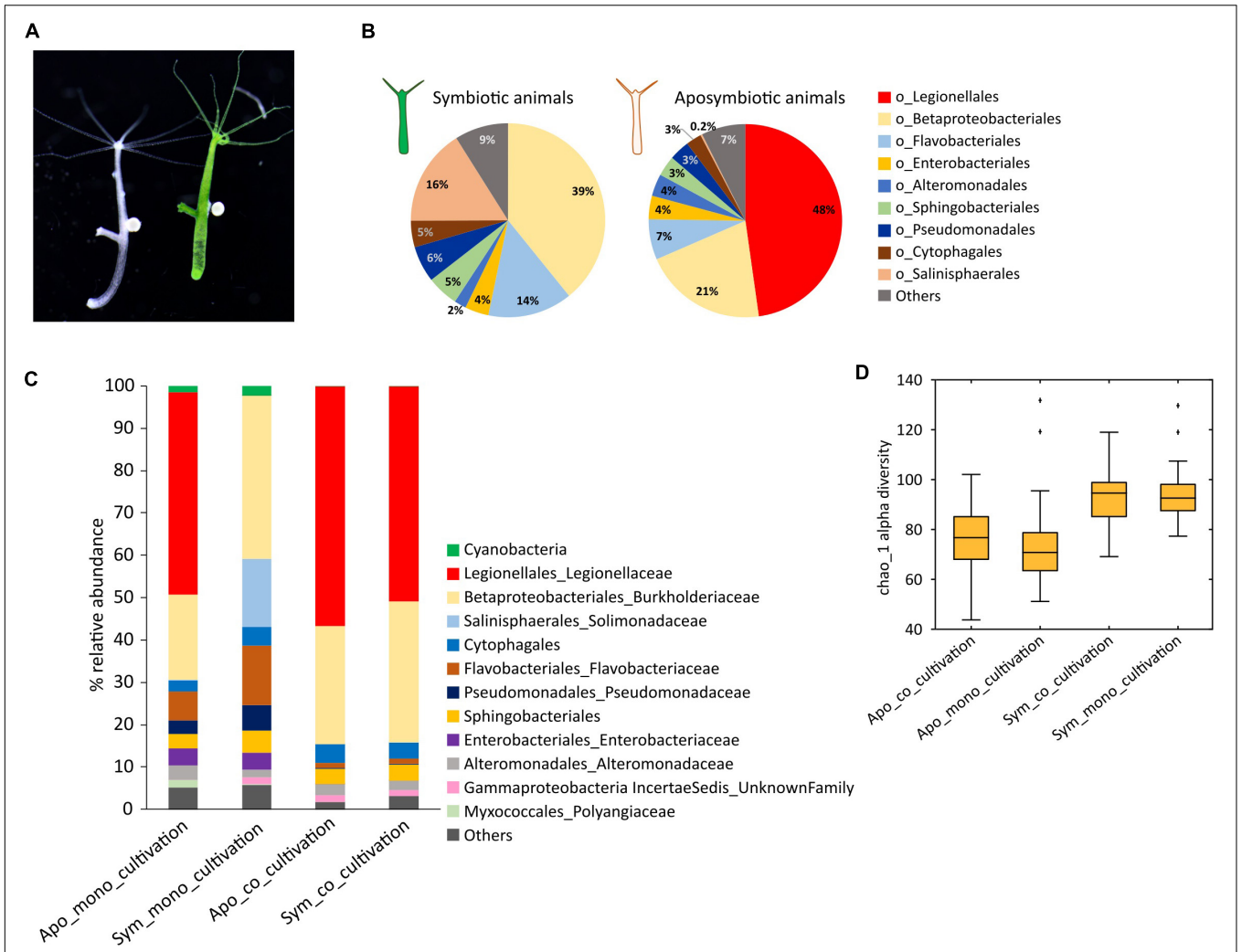

FIGURE 1 | Co-cultivation affects the microbiome composition of symbiotic and aposymbiotic *Hydra viridissima* (*H. viridissima*) polyps. **(A)** Symbiotic (green) and aposymbiotic (white) *Hydra viridissima* polyps. **(B)** Pie charts showing the mean relative abundances ($N = 58$) of bacteria at order level of symbiotic and aposymbiotic *H. viridissima*. **(C)** Taxonomic bar charts representing mean relative abundances of bacteria in co-cultivated and monocultivated symbiotic and aposymbiotic *H. viridissima*. Co-cultivation of symbiotic with aposymbiotic animals drastically affects the microbiome of symbiotic animals, in particular due to the acquisition of a single operational taxonomic unit (OTU), *Legionella spp. Hvir*. **(D)** Box plots showing the alpha-diversity (Chao1) of symbiotic and aposymbiotic polyps under mono- and co-cultivation.

(∼20%) of *Legionella spp. Hvir* in symbiotic *H. viridissima* polyps. Since the co-cultivated culture (4 weeks) also had both the strain of animals colonized with *Legionella spp. Hvir*, we decided to check if the transfer of bacterium can also occurs through the co-cultivation medium. Interestingly and also shown in **Figure 2B**, the transfer of bacterium only happened when culture medium was taken from an aposymbiotic culture, but not from co-cultivated culture. Consistently, the water from a co-cultivated culture also did not contain any *Legionella* bacteria (**Figure 2B**). We conclude that *Legionella* from aposymbiotic polyps is transmitted *via* water only in a purely aposymbiotic culture or immediately after the start of the co-cultivation experiment. Moreover, this migration of *Legionella spp. Hvir* is directional toward the symbiotic animals (**Supplementary Figure 2**). In a long-term co-cultivation experiment, *Legionella* bacteria do not seem to be able to leave the aposymbiotic polyps or the symbiotic polyps anymore.

Non-bacterial Fractions of Water Affect the Microbiome Composition

The filter-sterilized (with 0.2 μ filter) culture water of the aposymbiotic animals did not contain any bacteria; however, it still resulted in an alteration in the microbiome composition of the symbiotic animals (**Figure 2B**—Filtered_apo_water) as compared to the native composition of monocultivated symbiotic animals (**Figure 1C**—Sym_mono_cultivation). To identify the contributing factor responsible for this alteration, we divided the water into the fractions containing bacteria, viruses, and metabolites before treating the symbiotic animals (**Figure 2C** and

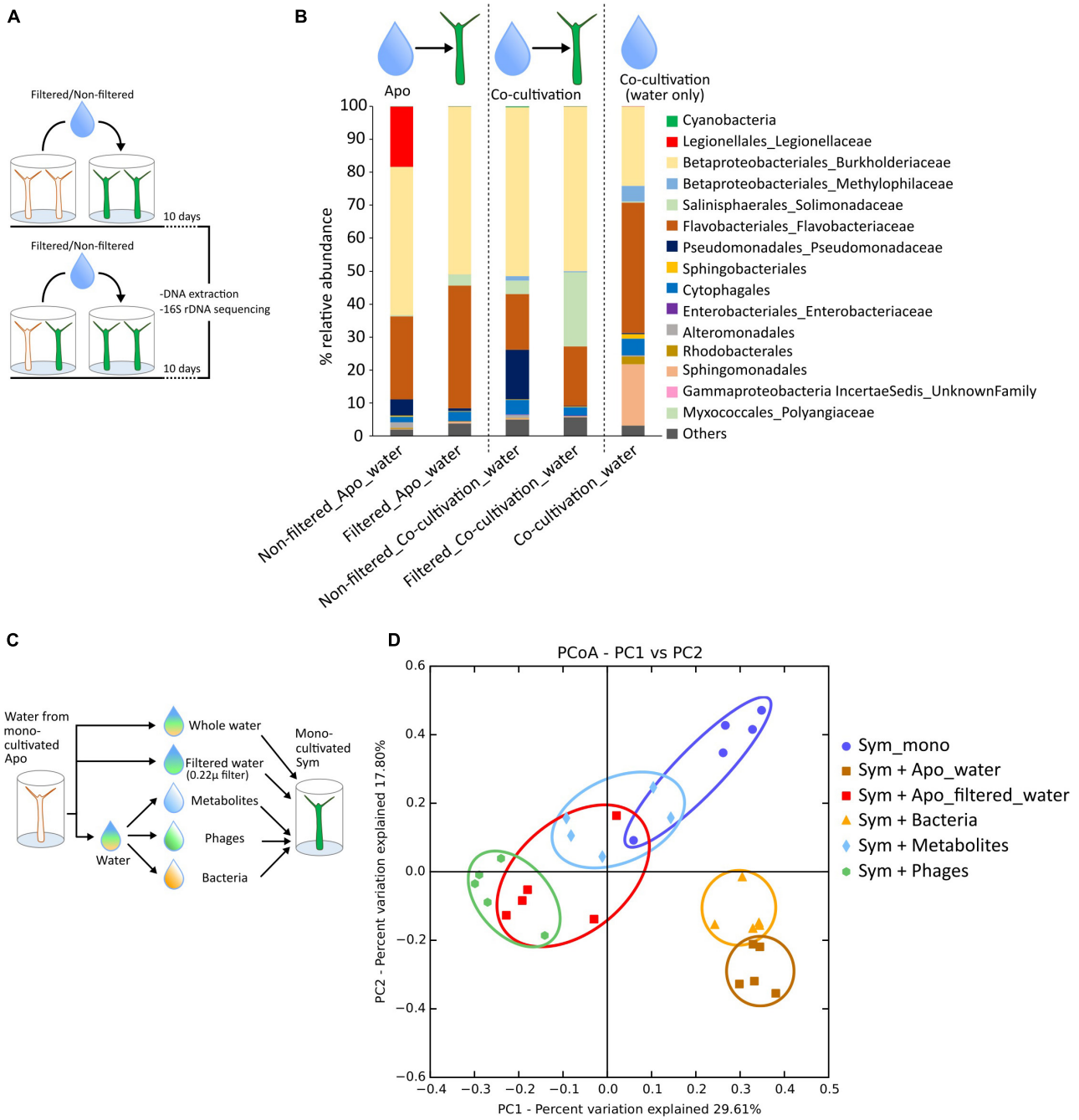

FIGURE 2 | *Legionella* is transferable through water. **(A)** Experimental setup to study the horizontal transfer of *Legionella spp. Hvir*. The mono-cultivated symbiotic animals were incubated in water from the aposymbiotic and co-cultivated animals for 10 days. The water filtered with 0.2 µ filter was used as control. **(B)** Taxa summary plots showing microbiome composition of the symbiotic animals treated with filtered/non-filtered water from aposymbiotic animals and co-cultivated animals along with microbiome composition of water of co-cultivated animals. **(C)** Experimental setup to study the effect of different fractions of water of aposymbiotic animals on the symbiotic animals. **(D)** Principal coordinate analysis (PCoA) plot constructed from Bray–Curtis dissimilarity matrix showing the microbiome composition of the symbiotic animals. The ellipses are manually drawn for visual representation.

Section "Materials and Methods"). It was noteworthy to observe that the virus fraction had a huge impact on the shift in the microbiome composition of the symbiotic animals (**Figure 2D**) probably through action of phages. A similar trend was also observed with the metabolite fraction. Expectedly, the bacterial fraction had a similar effect as the complete culture water from the aposymbiotic animals, with the main contributing factor being an increase in the *Legionella spp. Hvir* relative abundance

(**Supplementary Figure 3**; For detailed statistics, please refer **Supplementary Table 3**).

Transfer of Microbes Influences the Host Fitness

An altered microbiome composition often has an impact on the host (Tiffany and Bäumler, 2019). When assayed for fitness alteration, we observed that the acquisition of *Legionella spp. Hvir* reduced the population growth rates in the co-cultivated symbiotic animals (**Figure 3A**). Moreover, a similar effect is also observed in aposymbiotic animals, which upon co-cultivation displayed a reduced population growth rate. However, the reason for the observed growth rate in aposymbiotic animals can rather be correlated to the colonization by the algal symbionts than the altered bacterial composition (**Supplementary Figure 4**).

The symbiotic animals release a small number of algae in the surrounding water, but they tend to release an increased number of algal symbionts in response to stress (McAuley, 1981b), also observed during coral bleaching events. Since *Legionella spp. Hvir* is fitness costly for the host (**Figure 3A**), we investigated its effect on the algal release in the symbiotic animals by incubating an equal number of symbiotic polyps with aposymbiotic polyps in an equal amount of sterile culture water for 24 h (see Section "Materials and Methods" and **Supplementary Figure 5**). During the early phase of co-cultivation (4 days post co-cultivation), we observed an increased level of algal release per polyp as compared to monocultivated and long-term co-cultivated (8 weeks) animals (**Figure 3B**). We predict that the early colonization of host by *Legionella spp. Hvir* induces stress in the polyp triggering an increased release of algal symbionts in the ambient water.

Acquisition of *Chlorella* and Prolonged Co-cultivation Negatively Affect *Legionella spp. Hvir* Relative Abundance

The released algae by the symbiotic animals can also act as a means of horizontal transfer of the symbionts (Miyokawa et al., 2018). These cells can be taken up by the aposymbiotic animals and can be converted to symbiotic animals. To study the effect of these acquired symbiotic algae on the *Legionella spp. Hvir*, we fed the aposymbiotic animals with the freshly extracted symbiotic algae and observed the microbial composition of the newly formed symbiotic animals. After 8 weeks, the relative abundance of *Legionella spp. Hvir* reduced in the aposymbiotic animals fed with algae as compared to the controls (**Figure 3C**). Moreover, a long-term co-cultivation for over 6 months resulted in acquisition of algae by all the aposymbiotic animals rendering them to symbiotic state. It further leads to a reduction in the relative abundance of *Legionella spp. Hvir* in the population to undetectable levels in 16S rRNA gene sequencing (**Figure 3C**).

Shifting the Co-cultivated Animals to Monocultivation Restores the Microbiome

If the shift observed in the co-cultivated symbiotic animals is only subjective to co-cultivation with the aposymbiotic animals, then removal from co-cultivation should result in the restoration of the microbial composition. Therefore, the 8-week co-cultivated symbiotic animals were transferred to monocultivation (**Figure 3D**) for 2 weeks and the microbiome composition was compared to the mono- and co-cultivated animals. As shown in **Figures 3E,F**, the weighted UniFrac distance between the monocultivated animals and the animals removed from the co-cultivation significantly reduced (**Figure 3F**, $^{***}p$-value < 0.001) as compared to the co-cultivated symbiotic animals. The detailed statistical results of pairwise comparison of all the treatments using Adonis test with weighted UniFrac distances as input are given in **Supplementary Table 4**. This indicated the restoration of the native/homeostatic symbiotic microbiome.

DISCUSSION

Symbiosis study traditionally focused on bipartite host–microbe interactions such as the plant root–*Rhizobium* symbiosis or algae–fungi symbiosis in lichen, while tripartite and multipartite associations received lesser attention. However, it is becoming evident that long-term symbiotic persistence is prevalent not only as two-party, but also as more complex multipartite systems. This study demonstrates that in the green *H. viridissima* species, the photosynthetic *Chlorella* symbiont plays an important role in stabilizing the host bacterial community. Culturing aposymbiotic and symbiotic polyps in a shared environment resulted in horizontal transmission of bacteria from aposymbiotic animals to symbiotic polyps and a dramatic alteration of the microbiome in symbiotic animals. The main driver of alteration in the microbial community of symbiotic animals was identified to be a single bacterial OTU, *Legionella spp. Hvir*. *Hydra* is a freshwater organism and *Legionella* is a common habitant in freshwater bodies. *Legionella* already colonized in very low abundance the host before the symbiotic animals was collected for laboratory culturing. The aposymbiotic animals were later generated from these same animals using [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (DCMU) treatment and were maintained as a separate culture for at least over 20 years (Habetha et al., 2003). On the aposymbiotic hosts, in absence of algae, *Legionella* bacterium became a dominant colonizer possessing invasive properties that also enables it to transiently colonize the symbiotic hosts through horizontal transmission.

Markedly, the long-term co-culturing study revealed that transfer of *Legionella spp. Hvir* from aposymbiotic to symbiotic polyps resulted in a decreased growth rate of symbiotic polyps and an increased algal release, indicating considerable fitness costs for symbiotic animals. Interestingly, over a longer period of time, the spread of algal symbionts among the co-cultured polyps and subsequent recolonization of aposymbiotic animals with *Chlorella* algae lead to a restoration of the microbiome to the native state of monocultivated symbiotic animals. Although the mechanism allowing horizontal transmission remains to be uncovered, it was intriguing to observe that *Legionella spp. Hvir* migrates actively toward the symbiotic hosts (**Supplementary Figure 2**). We speculate that this chemotactic movement of

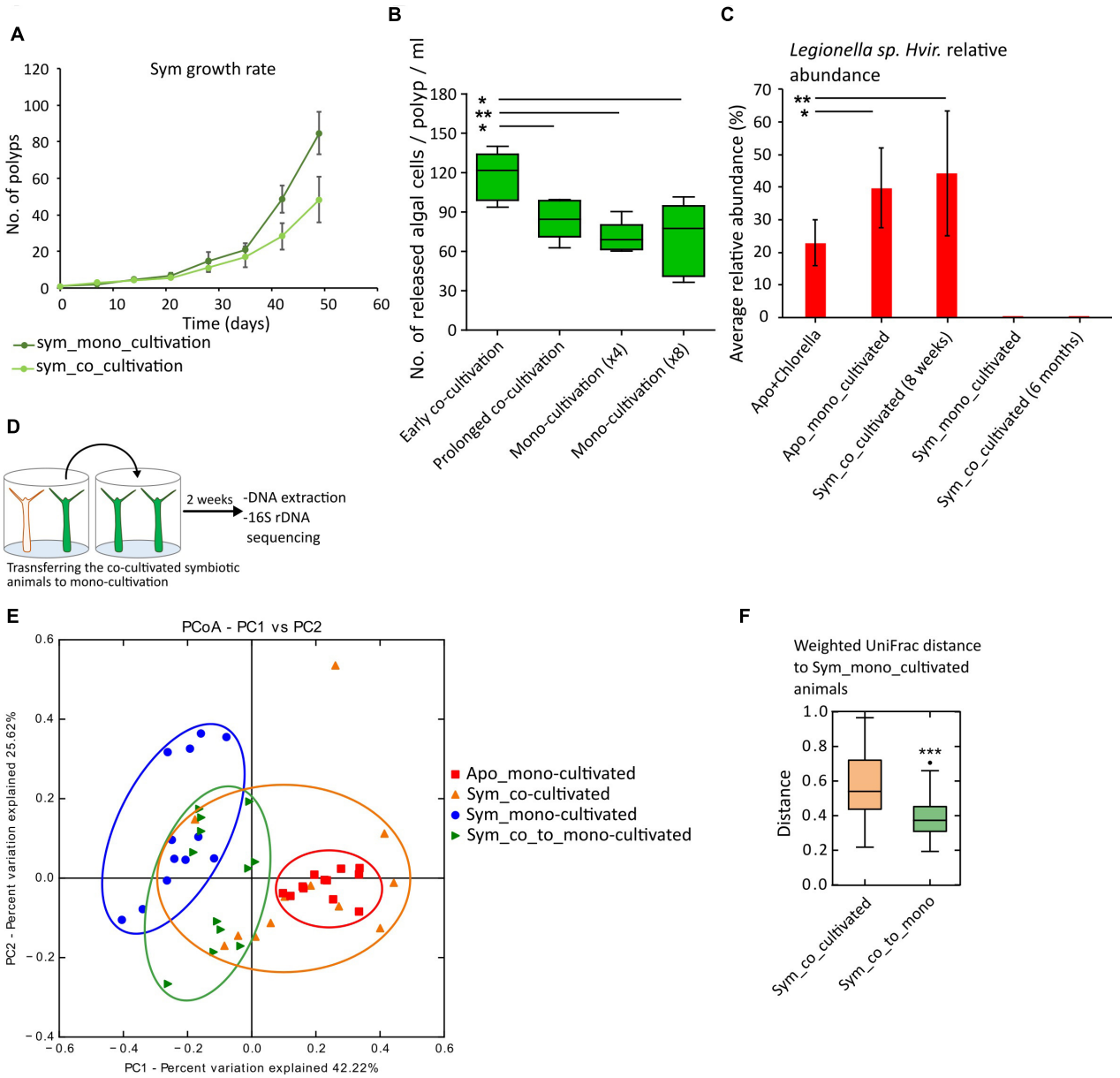

FIGURE 3 | Effect of co-cultivation on the host fitness and microbiome dynamics under co-/mono-cultivation. **(A)** Population growth rate, as a fitness measure of the mono- and co-cultivated symbiotic animals, was lower for co-cultivated animals. **(B)** Immediate early acquisition of *Legionella spp. Hvir* results in an increased expulsion of algae as compared to prolonged co-cultivation and monocultivation of symbiotic animals ($n = 5$, t-test, $^*p$-value < 0.05, $^{**}p < 0.01$). **(C)** Bar chart showing the effect of algal acquisition on *Legionella spp. Hvir* in aposymbiotic animals and prolonged co-cultivation for 6 months ($n = 6$, t-test, $^*p < 0.05$, $^{**}p < 0.01$). **(D–F)** Effect of transferring the co-cultivated animals to monocultivation. **(D)** Experimental setup. **(E)** Principal component analysis (PCA) plot showing the microbiome composition of the symbiotic animals under mono- and co-cultivation and 2 weeks post-transfer from co- to monocultivation and aposymbiotic animals. **(F)** Box plots comparing the weighted UniFrac distance between symbiotic animals under co-cultivation and post-transfer to monocultivation to monocultivated symbiotic animals.

a *Legionella* species toward the symbiotic *H. viridissima* is caused by secretory products of the algae. Supporting this view, earlier study has demonstrated that many *Legionella* species possess chemotaxis genes and also show swarming behavior (Appelt and Heuner, 2017). We conclude that our observations suggest complex interactions between the members of the *H. viridissima* metaorganism and that the *Chlorella* photobiont is involved in controlling invading bacteria and stabilizing the resident microbiota.

With the available experimental evidences, it is difficult to provide any mechanistic explanation underlying the observed phenomenon, a major limitation being not possessing *Legionella spp. Hvir* in the culture. There might be several factors at play that affect the observed trend in microbial composition, including

horizontal transmission, other bacterial symbionts, but we cannot rule out the involvement of the algal symbionts in *Legionella* mitigation. It is known for the renowned squid-vibrio symbiotic system that the *Vibrio* bacteria show chemotaxis in response to the host-derived chitin oligosaccharides (Mandel et al., 2012). Similar chemotactic behavior has been observed for several species of *Rhizobium* bacteria toward the plant roots (Aroney et al., 2021). Similarly, we hypothesize that algal metabolites in *Hydra* might be actively attracting the invasive species such as *Legionella* and altering the colonization niche to stop its further spread like "lure-and-kill" mechanism.

Legionella bacteria are generally perceived as pathogenic bacteria and are responsible for various human diseases, commonly an inhabitant of freshwater bodies. We have reported here an existence of a symbiotic strain of *Legionella* whose pathogenicity varies between the hosts. After colonizing the symbiotic animals, there is a transient pathogenic effect of bacterium on host fitness, but prolonged colonization reduces the bacterial virulence.

As shown in **Figure 4**, we present the dynamics of the bacterium in the population under the effect of horizontal transfer and the dominant symbiont, *Chlorella*. It is noteworthy that with the spread of algal symbionts in the population, the load of *Legionella spp*. *Hvir* decreases. Although the *Chlorella* photobiont and the bacterial microbiota colonize separate niches in the *H. viridissima* holobiont, the endosymbiotic algae can influence microenvironmental physicochemical parameters such as sugar, pH, and oxygen level and, thus, the properties of microbial habitat. Indeed, photosynthetic activity of *Chlorella* has been shown to involve transcriptional changes not only in the endoderm of *H. viridissima*, but also in the ectoderm that gives rise to the microbial habitat, the glycocalyx (Hamada et al., 2018). The fact that the spread of *Chlorella* symbiont correlates with *Legionella* mitigation in the community is consistent with an increased susceptibility in corals to white band disease post-bleaching event, resulting in the loss of immunity provided by the endosymbiotic algae (Muller et al., 2018). This transmission dynamics of symbionts would determine the outcome of the microbiome composition of different individuals in a population until it reaches the steady state. This modularity of the metaorganism, to alter the symbiont composition to adapt to the prevailing environment, makes the system resilient against adverse biotic or abiotic factors and improves the survival chances. This would also hold true for the natural conditions where the composition of a metacommunity is more complex. Collectively, these findings demonstrate the complexity of interactions in a tripartite symbiosis and the beneficial role of the *Chlorella* photobiont in maintaining a specific microbiome. This is of relevance because interhost dispersal of bacteria is not a rare phenomenon. Transmission of microbes from one individual to another one in a shared environment has been documented previously for aquatic systems, as exemplified in corals (Grupstra et al., 2021), zebrafish (Burns et al., 2017), microcosm (Shen et al., 2018), and zooplankton (Grossart et al., 2010). Moreover, co-housing of mice has a profound effect on their gut microbiome (Caruso et al., 2019; Robertson et al., 2019). It needs reductionistic model systems such as *Hydra* (Wittlieb et al., 2006; Augustin et al., 2012; Bosch, 2012; Murillo-Rincon et al., 2017; Hamada et al., 2018) for a functional understanding of the mechanisms involved.

MATERIALS AND METHODS

Animal Husbandry

Experiments were carried out with the Australian *H. viridissima* strain A99, which was obtained from Dr. Richard Campbell, Irvine. In this study, the symbiotic hosts refer to the hosts with algal symbionts and the aposymbiotic hosts refer to the same animals without algal symbionts. However, both the lines of animals contain bacterial symbionts. Aposymbiotic polyps were obtained by photobleaching using 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) as described before (Habetha et al., 2003) and subsequently cultivated for more than 1 year prior to the start of the experiments. All the animals were cultivated at constant temperature (18°C), light conditions (12 h/12 h light/dark rhythm), and culture medium (0.28 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.5 mM $NaHCO_3$, and 0.08 mM KCO_3) according to the standard procedure (Lenhoff and Brown, 1970). For the co-cultivation experiments, symbiotic and aposymbiotic animals were co-cultivated and fed three times per week with freshly hatched *Artemia salina* for 4 weeks before DNA isolation. Before guide DNA (gDNA) extraction, polyps were starved for 72 h.

Deoxyribonucleic Acid Extraction

For each biological replicate (n = 6), combined hydra and bacterial DNA were isolated from ten polyps. Animals were washed three times in sterile filtered (0.2 μm) culture medium and subsequently ruptured in buffer ATL (Qiagen, Germany) by vortexing. Algae were removed by centrifugation; suspensions were centrifuged three times at 350 *g* for 2 min. After each centrifugation step, supernatant was transferred to new tube. The gDNA was extracted from the third supernatant using the DNeasy Blood & Tissue Kit (Qiagen, Germany) with elution performed in 50 μl AE buffer. The extracted DNA was stored at −20°C until sequencing.

16S Ribosomal Ribonucleic Acid Gene Sequence Analysis

16S ribosomal RNA (rRNA) gene amplicon sequence analysis was conducted using the Quantitative Insights Into Microbial Ecology (QIIME) 1.8.0 package (Caporaso et al., 2010). Using the sequence FASTA file, a quality file, and a mapping file, which assigned the 10 nt barcodes to the corresponding sample as input, the sequences were analyzed using the following parameters: length between 300 and 400 bp, no ambiguous bases, and no mismatch to the primer sequence. Chimeric sequences were identified using ChimeraSlayer (Haas et al., 2011). We manually curated the chimeric sequences and as one of the criteria we

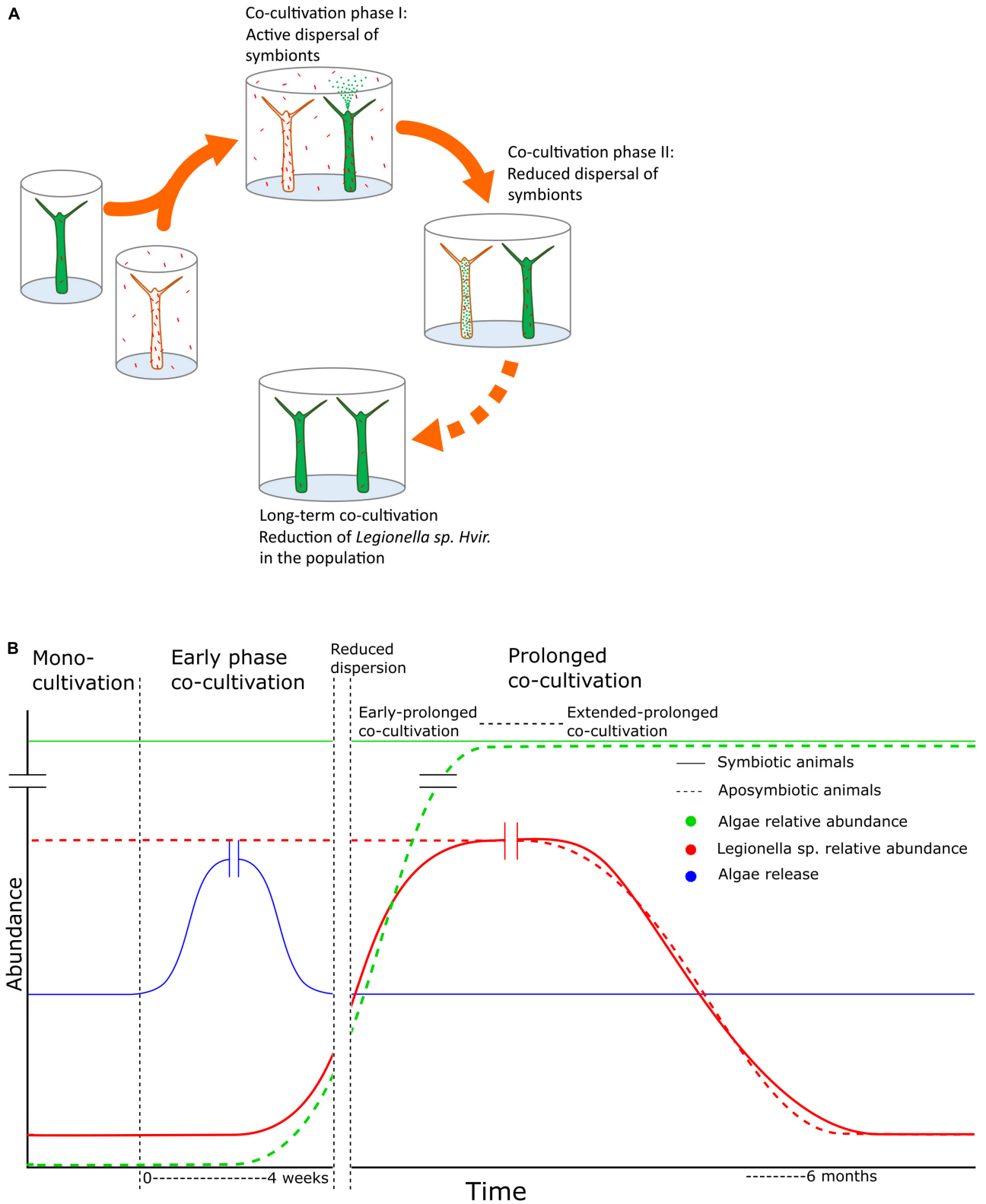

FIGURE 4 | Summary figure. Summarized graphically in panels **(A,B)**, we represent the temporal dynamics of *Legionella spp. Hvir* in the population of aposymbiotic and symbiotic animals over a prolonged co-cultivation. There is a phase of active dispersal of symbionts during early co-cultivation followed by reduced dispersal. During this phase, the microbiome of both the strains changes over the course of time till the system reaches the phase of equilibrium in respect of alteration of microbiome composition and the entire population has a uniform microbial composition.

considered those sequences that are present in at least two independent samples as false positive for being chimera. We considered it highly unlikely for an exact chimeric sequence to be present in two or more independent samples and, thus, decided to retain them. Sequences were rarified to the lowest number of reads in the dataset which for samples were just 2,300 reads. Such samples were accepted after manual curation through rarefaction plot and checking. Subsequently, sequences were grouped into operational taxonomic units (OTUs) at a $\geq$ 97% sequence identity threshold and mapped against SILVA_132 database (Quast et al., 2013).

Migration Assay

As shown in **Supplementary Figure 2A**, 1 ml syringes were filled with 1 ml of sterile culture water. In the treatment group, 10 symbiotic polyps were placed in each syringe and the control syringes were filled with only water without polyps. These syringes were placed vertically in 1 L of water from the aposymbiotic animals (source pool). This source pool contained aposymbiotic animals for 1 week before the start of experiment and was removed during the experiment. The experiment was performed with six biological replicates, in six different setups, but the water for the source pool came from the same culture vessel to maintain equal bacterial load as the starter source. The syringes were incubated for 48 h in contact with the source pool water. At the end of incubation, the water in the syringes was carefully collected and used for DNA extraction and quantification.

Legionella spp. Hvir Quantification Using Real-Time PCR

Legionella-specific primers were designed using the V1–V2 region sequences obtained from 16S rDNA sequencing: forward 5′-CTCTCAGACCAGCTACCGAT-3′ and reverse 5′-TACTAGATGGGTGGCGAGTG-3′. They were checked by aligning against the Ribosomal Database Project (RDP) database using the RDP probe (Cole et al., 2014) and against the 16S rRNA library of *H. viridissima* to avoid any non-specific amplification. For the migration assay, 1 ml of water was collected from the syringe or the source pool (**Supplementary Figure 2A**) and centrifuged at 20,000 rcf for an hour and a half. A total of 950 μl of the supernatant was removed away to avoid any disturbance in the pellet. DNA was isolated from the remaining 50 μl water + pellet using the DNeasy Blood & Tissue Kit (Qiagen, Germany) with elution performed in 25 μl AE buffer. Real-time PCR was performed on 1 μl of the template DNA using the GoTaq qPCR Master Mix (Promega, Madison, WI, United States) and ABI Prism 7300 (Applied Biosystems, Foster City, CA, United States). The qPCR was performed in duplicate with six biological replicates each.

Fractionation of Water From Aposymbiotic Animals

The aposymbiotic animals were incubated in sterile S-medium for 4 days without feeding. After 4 days, this medium was used to prepare three different fractions containing the bacteria/viruses/metabolites. The medium was centrifuged at 20,000 *g* for 30 min to collect bacteria in the pellet. The pellet was resuspended in a smaller volume of sterile S-medium. The supernatant was filtered through a sterile 0.2 u filter to remove bacterial particles. Half of the supernatant was used to collect viral particles and other half for metabolite fraction. One half of the supernatant was filtered through 0.02 u filter to remove viral particles and obtain metabolite fraction. Polyethylene glycol (PEG) was added to rest of the supernatant suspension to achieve the final concentration of 5% to precipitate viral particles. The mixture was centrifuged at 20,000 *g* for 75 min. The supernatant was carefully discarded and the pellet was resuspended in sterile S-medium. The animals were incubated in each of the water fraction for 48 h before DNA isolation for 16S rRNA sequencing. All the samples were treated using the same water source and collected simultaneously to avoid any additional variation and contamination.

Algal Release Quantification

For early co-cultivation, monocultivated symbiotic polyps were co-cultivated with monocultivated aposymbiotic polyps for 48 h prior to start of experiment. For prolonged co-cultivation, the animals were co-cultivated for 1 month. At 0 h before the start of experiment, four symbiotic polyps of monocultivated animals or four polyps each of symbiotic and aposymbiotic animals, each of early/prolonged co-cultivation, were incubated in 1 ml of sterile culture water (**Supplementary Figure 5**). As an additional animal-density control, 8 polyps of monocultivated symbiotic animals were used (**Supplementary Figure 5**) to match the co-cultivated animals. After 24 h, 900 μl of water was carefully collected and centrifuged at 14,000 rpm for 5 min to pellet the algae. A total of 850 μl of the supernatant was carefully removed away and the pellet was resuspended in the remaining 50 μl of water. It was immediately subjected to counting using flow cytometry on the BD FACSCalibur with CellQuestPro v5.2 (*Becton–Dickinson*) with a blue laser at 488 nm using forward scatter and FL3 filter (>670 nm). The data were further analyzed with FCSalyzer 0.9.13-alpha[1] and total number of algal cells expelled per polyp per ml of water was calculated.

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: Sequence Read Archive (SRA), accession no: PRJNA810523.

AUTHOR CONTRIBUTIONS

JB, KS, SF, TL, and TB planned the experiments. JB, KS, and TL executed the experiments. PR provided cell counting facility and logistics. JB analyzed the data. JB, SF, and TB wrote, reviewed, and

[1]https://sourceforge.net/projects/fcsalyzer/

edited the manuscript. 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/fmicb.2022.869666/full#supplementary-material

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Landscape Topography and Regional Drought Alters Dust Microbiomes in the Sierra Nevada of California

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Dust provides an ecologically significant input of nutrients, especially in slowly eroding ecosystems where chemical weathering intensity limits nutrient inputs from underlying bedrock. In addition to nutrient inputs, incoming dust is a vector for dispersing dust-associated microorganisms. While little is known about dust-microbial dispersal, dust deposits may have transformative effects on ecosystems far from where the dust was emitted. Using molecular analyses, we examined spatiotemporal variation in incoming dust microbiomes along an elevational gradient within the Sierra Nevada of California. We sampled throughout two dry seasons and found that dust microbiomes differed by elevation across two summer dry seasons (2014 and 2015), which corresponded to competing droughts in dust source areas. Dust microbial taxa richness decreased with elevation and was inversely proportional to dust heterogeneity. Likewise, dust phosphorus content increased with elevation. At lower elevations, early season dust microbiomes were more diverse than those found later in the year. The relative abundances of microbial groups shifted during the summer dry season. Furthermore, mutualistic fungal diversity increased with elevation, which may have corresponded with the biogeography of their plant hosts. Although dust fungal pathogen diversity was equivalent across elevations, elevation and sampling month interactions for the relative abundance, diversity, and richness of fungal pathogens suggest that these pathogens differed temporally across elevations, with potential implications for humans and wildlife. This study shows that landscape topography and droughts in source locations may alter the composition and diversity of ecologically relevant dust-associated microorganisms.

Keywords: aeolian processes, Asian desert, bacteria, biogeochemistry, dispersal, fungi, montane, provenance

INTRODUCTION

Each year, more than two billion metric tons of aerosolized soil particles (i.e., dust) are entrained into air currents and uplifted into the atmosphere of our Earth (Moulin et al., 1997; Perkins, 2001; Kellogg et al., 2004). Fine dust generation is expected to increase with droughts in source locations, leading to greater transport of material globally (Pu and Ginoux, 2017). One important—yet often overlooked—aspect of dust deposition is the functional role of far-traveled dust as a vector for microbial dispersal. Because microbial processes are important for regulating ecosystem functions (Bardgett et al., 2008; Mendes et al., 2015), in resource-limited montane systems, microorganisms and dust-associated nutrients have major effects on ecosystem functioning (Arvin et al., 2017). Therefore, it is essential to understand how abiotic factors like elevation or source-region drought affect dust-driven microbial dispersal and biogeography in montane systems.

Although air is often a vector of microbial dispersal (Bowers et al., 2009, 2011, 2012, 2013; Smith et al., 2013; Yooseph et al., 2013), the airborne environment can also be a true habitat for microorganisms capable of tolerating atmospheric conditions (Womack et al., 2010). Soil or root-associated fungal morphologies or life history traits, such as belowground mycorrhizal spore production, may determine the airborne dispersal ability and range of fungal taxa (Kivlin et al., 2014). For bacteria and archaea, motility and chemotaxis accelerate bacterial dispersal in porous environments, yet little is known about whether active cell dispersal confers similar advantages in airborne environments (Scheidweiler et al., 2020). Dispersal limitations or motility may ultimately dictate the functional capacity of resultant microbial communities in deposition zones.

Upon deposition, dust may reduce or eliminate nutrient limitations in aquatic ecosystems. For instance, dust promotes oceanic biological productivity by alleviating micronutrient deficiencies, such as iron (Fe), in the eastern equatorial Pacific (Winckler et al., 2016). Similarly, dust can serve as an exogenous subsidy to nutrient-poor terrestrial ecosystems such as slowly eroding ecosystems where slow rates of weathering limit nutrient supply from underlying bedrock. In a resource-limited montane ecosystem, Arvin et al. (2017) suggested that most of the growth requirement of native *Pinus jeffreyi* trees for the essential nutrient phosphorus (P) is dust derived. Additionally, microorganisms deposited with dust may support macronutrient bioavailability and plant uptake, and thus maintain vegetation community structure (Allen et al., 2003; Bever et al., 2010; Alguacil et al., 2011).

While exogenous inputs from dust can support biological productivity, they may also harm resident terrestrial biota. Fungal plant pathogens, such as those causing powdery mildew or sooty molds, infect a wide variety of plants, leading to both crop losses and forest blights (Peetz et al., 2007; Carisse et al., 2008). Dust storms and disturbance are linked to localized outbreaks of diseases caused by inhaling soil-dwelling fungal pathogens, such as *Coccidiodes* species, which causes valley fever (Gorris et al., 2018). Airborne chemicals, bacterial cells, and fungal spores within human lungs may produce damaging oxidative reactions (Samake et al., 2017). Metabolic and ecophysiological differences among microbial groups, including pathogenicity, underscore the importance of examining microbial diversity within airborne environments.

Environmental and temporal factors may differentially affect the composition and activity of airborne microbial communities (Griffin et al., 2002; Brodie et al., 2007). Airborne microbial interactions may expedite wet deposition and reduce particulate transit time. Shorter transit time may promote viability upon deposition, as it also limits airborne microorganisms' exposure to environmental stress (Schlichting, 1964; Tesson and Santl-Temkiv, 2018). Yet, airborne microbes vary in their tolerance of stressful environmental conditions (Ehresmann and Hatch, 1975). For instance, cyanobacteria can withstand a wide range of atmospheric humidity, while microalgae prefer high humidity. Because cyanobacteria are highly resilient to airborne stress and produce compounds toxic to humans or animals (Carmichael and Li, 2006; Nielsen and Jiang, 2020; Wu et al., 2021) recent studies have concentrated on dispersal trajectories of this microbial group.

Although the consequences of microbial dispersal are vast, we know little about global vectors of microbial dispersal. Temperature gradients may stratify the airborne environment into distinct biogeographic zones and influence dispersal trajectories (Womack et al., 2010). Discrete events, like dust storms and wildfires, entrain particulate-transported microorganisms and biological ice-nucleating particles into air currents through convective columns (Kobziar et al., 2018; Kobziar and Thompson, 2020; Moore et al., 2021). Additionally, climatic and topographic features of depositional areas may filter dust-driven microbial transport into distinct terrestrial biogeographic zones.

Considering the potential implications of dust inputs for ecosystem structure and function, we evaluated the microbial dynamics and biophysical composition of dust along an elevation gradient on the western slope of the Sierra Nevada of California. To examine the effects of clinal variation on dust-associated microorganisms, we analyze incoming dust deposited at four study sites along an elevation gradient. This allows us to evaluate the relative contributions of drought, distance, and spatial features on biophysical properties of dust. We characterized dust microbiomes across two unusually dry years (2014 and 2015) to examine interactions among dust provenance and nutrient balance on microbial communities associated with incoming dust to the Sierra Nevada of California.

Our previous research found shifts in dust-driven nutrient inputs to this montane ecosystem. We demonstrated that the relative abundance of global (Asian) dust vs. regional (California's San Joaquin Valley) sources of dust delivered to these study sites differed across elevations and varied between study years (Aarons et al., 2019). We also showed that sources of dust change throughout the dry season (Aciego et al., 2017), which is characterized by warm, dry summers with little to no precipitation from mid-May to October. Using isotope mixing models, we demonstrated that Asian dust inputs, combined with regional sources of dust, resulted in more heterogeneous dust materials.

In this complementary study, we aimed to determine whether the composition of dust-associated bacterial and fungal community structure and function also differ across elevations and in response to droughts in global and regional dust source areas. The observed patterns in dust heterogeneity may correspond with increasingly intricate dust microbiomes, such as more phylogenetically or functionally diverse microbial communities. Deposited dust mixtures containing substantial contributions from both Asian and Californian dust sources may not only be more chemically complex than dust originating from one source region, but they may also be more complex microbially. Our study coincided with droughts in both California (Asner et al., 2016) and Asia (Zhang and Zhou, 2015), which increase dust emissions (Pu and Ginoux, 2017) and microbial entrainment in emitted dust (Kellogg et al., 2004), thus contributing more fine particulates and biological materials to these complex dust mixtures. Interannual variation in the location and severity of drought events may drive compositional differences in microbial community structure and the distribution of bacterial and fungal functional groups within dust deposited along this elevation gradient in the Sierra Nevada of California (**Figure 1**, modified from Aciego et al. (2017), Aarons et al. (2019) and **Supplementary Table 1**). We hypothesized that microbial diversity will be greater at higher elevations rather than at lower elevations, owing to more complex dust from multiple sources being deposited at higher elevations. We further hypothesize that these diversity trends will be more pronounced early in the dry season, and attenuate over time because regional soils from California's San Joaquin Valley may be drier later in the dry season, and therefore aerosolize more fine dust from regional sources which will deposit across all elevations. Furthermore, we hypothesize that more dust-associated pathogens will be found at lower elevations, due to greater human-induced disturbance.

RESULTS

Influence of Dust Heterogeneity and Elevation on Microbial Communities

We found that dust deposition to the Sierra Nevada study sites varied in proportions of dust originating from Asian and San Joaquin Valley sources (Aciego et al., 2017; Aarons et al., 2019). Our metric of dust heterogeneity (related to the proportion of Asian dust), calculated from concentrations of strontium (Sr) and Sr isotopic compositions ($^{87}Sr/^{86}Sr$) in San JoaquinValley and Asian Desert dust endmembers (as per Ingram and Lin, 2002; Yokoo et al., 2004) peaked when contributions of Asian dust approached those of San Joaquin Valley dust. While we used Sr concentrations and Sr isotopic compositions to calculate the metric of ranked dust heterogeneity, we also calculated neodymium (Nd) isotopic concentrations for use in determining dust provenance. Comparing elemental concentrations and radiogenic Sr and Nd isotope compositions, we quantified the role of regionally vs. globally supplied dust on microbial community composition across the elevation gradient. Dust heterogeneity, as reported from 2015, includes isotopic data to pair with all dust samples collected that year. When we reported dust heterogeneity from both years, we used paired isotopic analyses for samples in both July and August 2014; however, we used September 2014 dust samples as a proxy for October 2014 samples to coincide with the subsequent molecular analyses of dust collected during that sampling month.

Using this metric, we found that dust heterogeneity positively correlated with elevation ($p_{adj} < 0.001$, $R^2_{adj} = 0.542$). The dust at the lowest elevation site, San Joaquin Experimental Range (SJER; 400 m), was significantly less heterogeneous than dust deposited to the other sites. In contrast, dust detected at the highest elevation site, Short Hair (2,700 m), was more heterogeneous than at any other site. As dust heterogeneity increased with elevation, both bacterial and archaeal (hereafter, bacterial; $p_{adj} = 0.010$, $R^2_{adj} = 0.178$; **Figure 2**) and fungal ($p_{adj} = 0.005$, $R^2_{adj} = 0.221$; **Figure 2**) taxa richness declined. More heterogeneous dust at higher elevations corresponded to lower 16S rRNA gene microbial and ITS1 fungal taxa richness; likewise, less heterogeneous dust at lower elevations harbored greater 16S rRNA gene microbial and ITS1 fungal taxa richness. No effects of dust weights (m * g) were found for microbial richness or diversity (fungal taxa richness: $p = 0.559$; fungal alpha diversity: $p = 0.650$; bacterial taxa richness: $p = 0.193$; bacterial alpha diversity: $p = 0.410$); likewise, dust weights were not correlated with sampling duration.

We calculated alpha diversity with the Shannon-Wiener index, using rarefied OTU counts and detected no interannual differences (**Supplementary Figure 1**); bacterial counts were rarefied to 34,559 counts and fungi were rarefied to 7,302 counts using the "rrarefy" function from the "vegan" package (Oksanen et al., 2012). Although fungal alpha diversity differed by elevation ($p < 0.001$), elevation had no effect on bacterial alpha diversity ($p = 0.412$; **Supplementary Figure 2**).

No differences in bacterial alpha diversity were found by either month ($p = 0.119$) or year ($p = 0.634$; **Supplementary Figure 1**). Moreover, we found no overall temporal effects of sampling year ($p = 0.413$) or sampling month ($p = 0.159$) on fungal alpha diversity (**Supplementary Figure 1**). Apart from Short Hair, bacterial diversity was equivalent between years within each site (SJER $p = 0.694$, Soaproot $p = 0.627$, Providence $p = 0.737$); bacterial diversity at Short Hair was higher in 2015 than in 2014 (**Supplementary Figure 2**; $p = 0.020$). For fungal diversity, no interannual variation was detected at SJER ($p = 0.732$), Soaproot ($p = 0.545$), or Short Hair ($p = 0.147$), but we found higher fungal diversity in 2014 than in 2015 at Providence ($p = 0.009$).

Using PERMANOVA, we characterized compositional shifts and found that both fungal ($p_{adj} \leq 0.001$) and bacterial ($p_{adj} = 0.005$) community composition varied along with the heterogeneity of deposited dust (**Figure 3**). Both bacterial community composition ($p_{adj} = 0.001$) and fungal composition shifted significantly by elevation ($p_{adj} < 0.001$). Elevation also significantly affected microbial communities at phylum and class levels, with significant effects on the composition of bacterial phyla ($p_{adj} = 0.003$) and fungal phyla ($p_{adj} = 0.002$), as well as both bacterial classes ($p_{adj} = 0.001$) and fungal classes ($p_{adj} = 0.001$). Additionally, when we restricted our analyses within specific

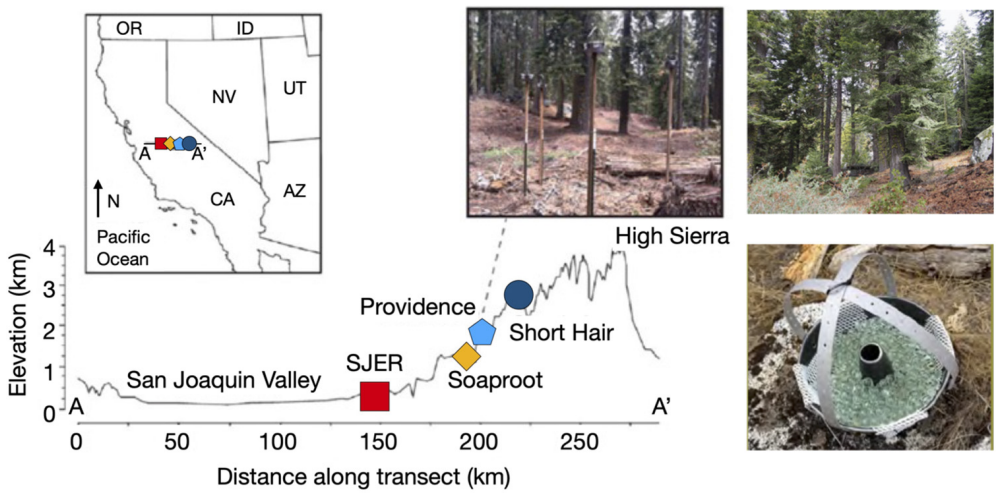

FIGURE 1 | Map of sampling transect, dust collectors, and sites along the elevation gradient. Map illustrating sites from the San Joaquin Valley (low elevation; red square indicating SJER, 400 m elevation) to the High Sierra (high elevation; dark blue circle symbolizing Short Hair, 2,700 m). Inset image on the map of the state of California, United States illustrates sampling transect from A-A' with sites (left to right, SJER, Soaproot 1,100 m, Providence 2,000 m, Short Hair) increasing with distance from the Pacific Ocean and rising in elevation. Dust collectors deployed at Providence, to the right of the inset map. Photograph of the highest elevation site at Short Hair is shown on the top right. Photograph of the dust collector, composed of a Teflon-lined Bundt pan filled with glass marbles and lined with Kevlar mesh shown at the bottom right (adapted from Aciego et al., 2017 and Aarons et al., 2019).

sites, both fungal and bacterial community composition shifted annually (Bacteria $p = 0.004$; Fungi $p < 0.001$) and monthly (Bacteria $p = 0.017$; Fungi $p = 0.001$). Using Canonical correspondence analyses (CCA), we also found that elevation was a significant driver of fungal community composition during both years, and of bacterial community composition during 2015 (**Figure 4**). The relative abundance of fungal and bacterial classes varied over the course of the study (**Supplementary Figure 3**).

Influence of Abiotic Factors and Dust Chemistry on Microbial Communities

Dust P concentrations were positively correlated with elevation during 2015 ($p < 0.001$, $R^2 = 0.61$), but not in 2014 ($p = 0.880$, $R^2 = 0.003$). In 2015, PERMANOVA analyses revealed that both fungal and bacterial community compositions were correlated with dust P (Bacteria $p = 0.004$; Fungi $p = 0.001$). Additionally, we found significant effects of dust sulfur (S) concentrations on fungal community composition ($p = 0.005$).

Fungal ($p \leq 0.001$) and bacterial ($p \leq 0.001$) community compositions were significantly related to dust chemistry (concentrations of elements or radiogenic isotopic compositions). Secondarily, of all the sampling months, the latest sampling date (October) in 2015 had a strong effect on structuring both fungal and bacterial communities (**Figure 4**).

PERMANOVA analyses revealed that isotope ratios used in determining dust provenance—Sr and Nd—were also correlated with both fungal ($^{143}Nd/^{144}Nd$, $p < 0.001$; $^{87}Sr/^{86}Sr$, $p = 0.001$) and bacterial ($^{143}Nd/^{144}Nd$, $p < 0.001$; $^{87}Sr/^{86}Sr$, $p = 0.002$) community composition. All Nd isotope compositions are reported hereafter as ε_{Nd}, which is defined as $\varepsilon_{Nd} = ([(^{143}Nd/^{144}Nd)_{sample}/(^{143}Nd/^{144}Nd)_{CHUR}]) - 1 \times 10^4$, where $^{143}Nd/^{144}Nd)_{CHUR}$ is the Nd isotopic composition of the Chondritic Uniform Reservoir (CHUR; Jacobsen and Wasserberg, 1980).

Early vs. Late Dry Season Dust

We examined presence-absence data to calculate bacterial and fungal taxa richness and found that at the lowest elevation site (SJER; 400 m), fungal taxa richness was higher earlier (July) than later (August and October) in the 2015 dry season ($p = 0.027$; **Figure 5**). At SJER, bacterial richness exhibited a similar, marginally significant, trend ($p = 0.065$). In contrast, at Providence (2,000 m)—the second-highest elevation site—bacterial taxa richness was lower earlier in the 2015 dry season and increased later ($p = 0.017$); fungal taxa richness was statistically equivalent during the same time period ($p = 0.720$; **Figure 5**). We compared dust microbial diversity at each site between early and late dry season sampling periods and found that bacterial diversity was higher earlier in the dry season at the two lowest elevation sites (SJER, $p = 0.03$; Soaproot, 1,100 m, $p = 0.03$). Although dust from Short Hair (2,700 m) was the most heterogeneous, dust microbial richness and diversity did not vary across the dry season at this site for either fungi or bacteria.

We evaluated microbial community composition in dust collected in the early and late dry season via PERMANOVA analyses and detected significant compositional shifts in fungal community structure later in the dry season ($p = 0.017$; **Supplementary Figure 3**). Additionally, we detected a marginally significant interaction among elevation and fungal classes ($p_{adj} = 0.008$). In 2015, we found that the relative abundance of Arthoniomycetes (composed of ascomycete cup fungi with bitunicate apothecia) was higher in early dry season dust than in later-season dust (**Supplementary Figure 4**). In contrast, the relative abundance of fungi from Cystobasidiomycetes

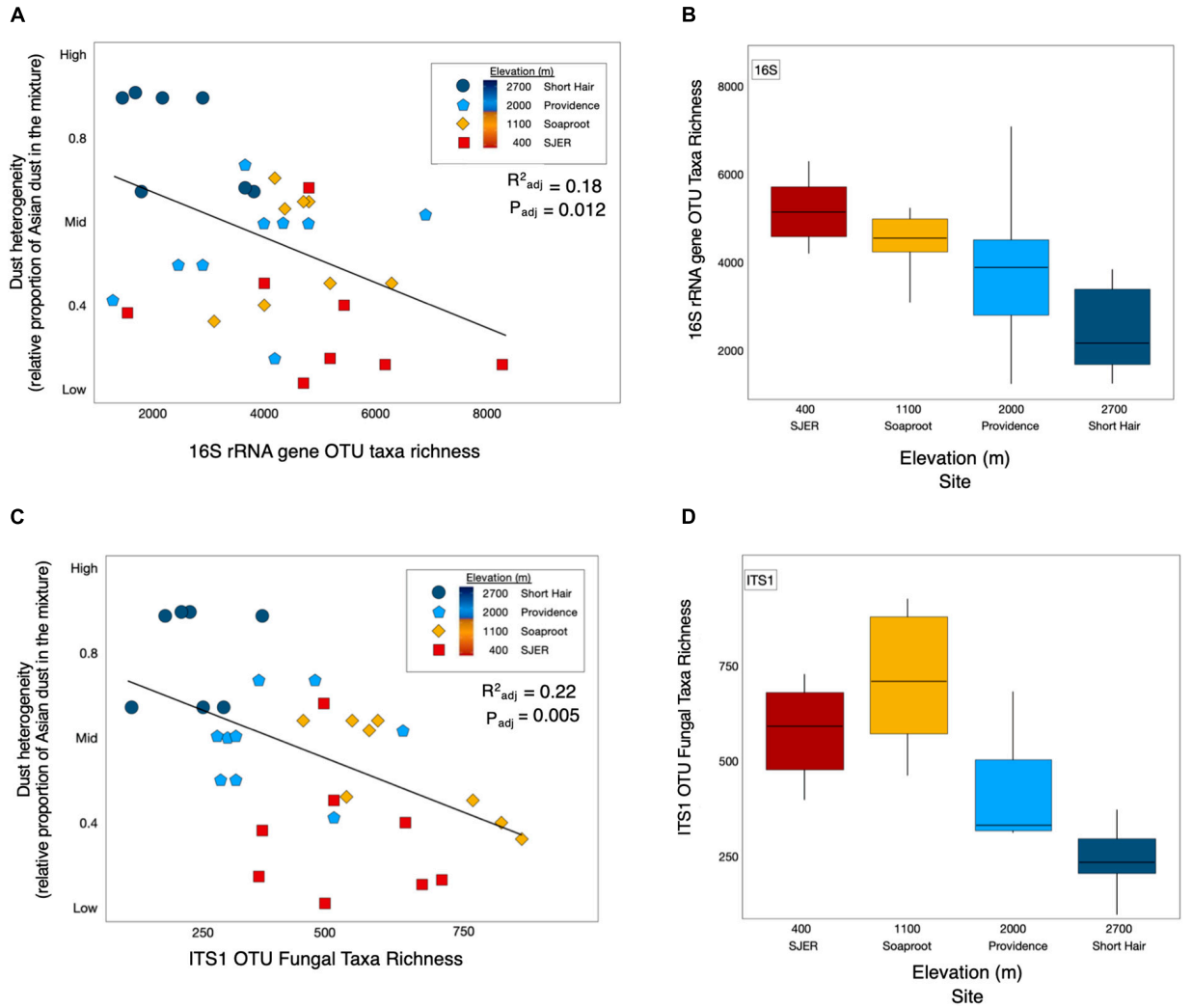

FIGURE 2 | Dust heterogeneity varies by elevation and 16S rRNA gene OTU bacterial and fungal ITS1 microbial taxa richness. Dust complexity and 16S rRNA gene OTU microbial taxa richness varied by site along the elevation gradient. Linear regression of bacterial and archaeal taxa richness from 16S rRNA marker gene targeted amplicon sequencing shows that microbial taxa richness corresponded with dust heterogeneity and elevation **(A)**. Microbial taxa richness varies by site along the elevation gradient **(B)**. ITS1 fungal OTU taxa richness **(C)** was correlated with dust heterogeneity **(C)** and elevation **(D)** across sites in the study.

(a group of simple-septate basidiomycete fungi from the Pucciniomycotina) was lower earlier in the 2015 dry season than later (**Supplementary Figure 4**). Although there was a significant interaction between sampling year and early or late dry season dust for the relative abundance of bacterial classes (p_{adj} = 0.022), no differences were detected for bacterial phyla (p_{adj} = 0.353). However, the relative abundance of Acidobacteria was higher earlier than later in the 2015 dry season (p = 0.031; **Supplementary Figure 4**). Likewise, the relative abundance of Cyanobacteria (p = 0.001) was also higher earlier than later in the dry season (p = 0.001; **Supplementary Figure 4**).

Variation in Fungal Functional Groups

We detected compositional shifts across elevation in fungal mutualists ($p < 0.001$, as in lichen-forming or mycorrhizal fungal mutualists), fungal pathogens ($p < 0.001$), and saprotrophic fungi (i.e., decomposers, $p < 0.001$). Across sampling years, we detected compositional shifts in fungal mutualists ($p < 0.001$) and decomposers ($p < 0.001$). Sampling month (p = 0.029) significantly shifted the community structure of fungal mutualists. Along with these observed compositional shifts, the diversity of fungal mutualists significantly increased incrementally with increasing elevation (i.e., clinally; **Figure 6** and **Supplementary Table 2**). The relative abundance of fungal mutualists increased with elevation (p = 0.002), with a greater abundance of mutualistic fungi at higher elevation than at lower elevation sites.

Interactions between elevation and sampling month were correlated with richness (p = 0.040), diversity (p = 0.050), and relative abundance of fungal pathogens (p = 0.042). The relative abundance of fungal pathogenic and decomposer taxa varied temporally, with interactions by sampling year and month

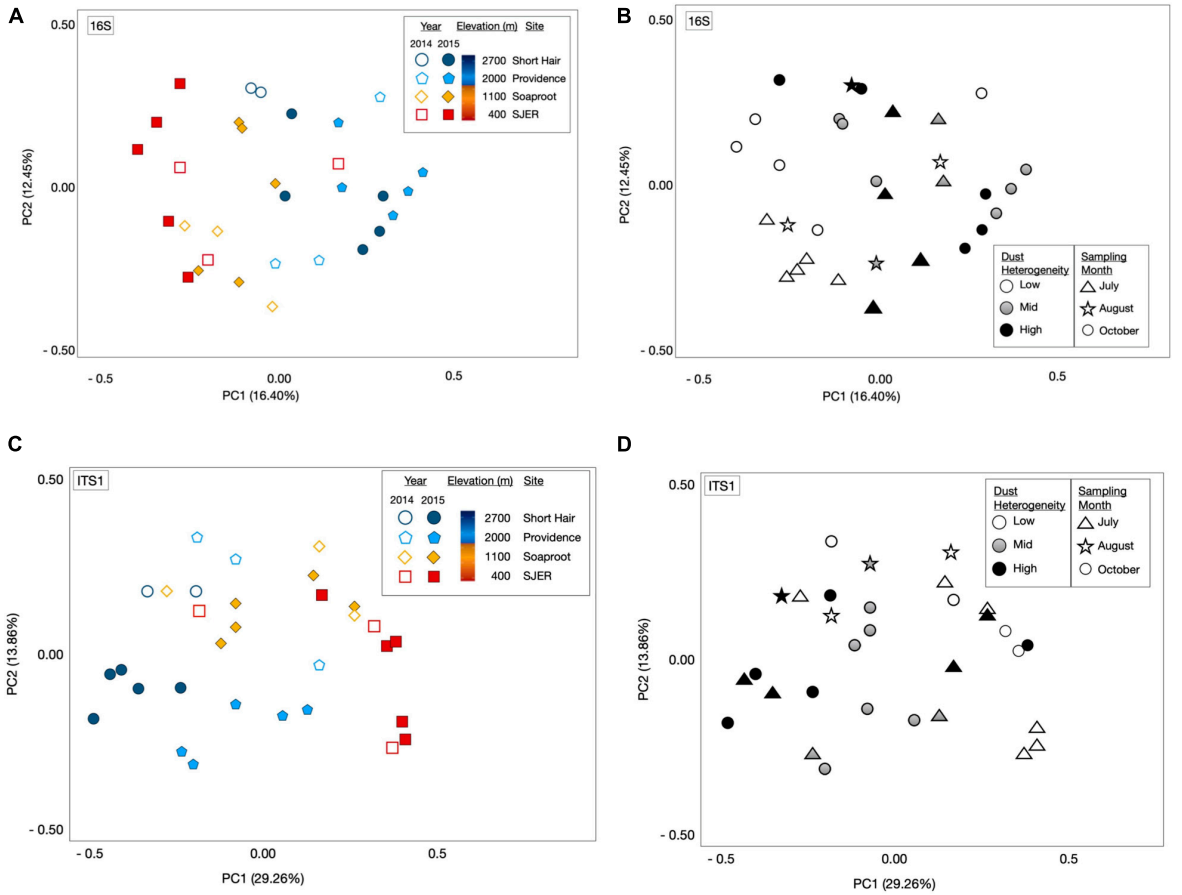

FIGURE 3 | Microbial community composition shifts by elevation and sampling date. Principal coordinate analyses (PCoA) of 16S rRNA gene bacterial OTU microbial community composition revealed from targeted amplicon sequencing of marker genes **(A)** and the amplicons of the fungal ITS1 **(C)** region illustrated structural shifts by elevation and sampling year, as well as dust heterogeneity and sampling month **(B,D)**. Microbial communities represented by either open or filled shapes, corresponding to dust sampling year; two California drought years (2014 and 2015) and one Asian drought year (2015). 2014 dust microbiomes **(A)**, shown as open shapes with outlines colored by site); 2015 color filled shapes **(A)** with black outlines; colors corresponded to sites (as per legend, and on **Figure 1**: Map). Bacterial [16S rRNA marker genes; **(A)**] and fungal communities [ITS1 region; **(C)**] varied across elevation and sampling year. Dust bacterial **(B)** and fungal **(D)** communities in greyscale, ranked by dust heterogeneity (Low = white; Mid = gray; High = black), which was related to the proportion of Asian dust in samples deposited in California's Sierra Nevada. Symbols in **(B)** (bacterial communities) and **(D)** (fungal communities) represented sampling months, with triangles for July, stars for August, and circles for dust samples collected in October.

(pathogens, $p = 0.039$; decomposer, $p = 0.026$). Indicator species analysis yielded a total of 22 significant taxa across all elevations, with a majority (15) of these found at Short Hair, the highest elevation site (**Supplementary Table 3**).

DISCUSSION

Our results suggest that dust-associated microbial communities in the Sierra Nevada of California are correlated with the chemical composition of dust and the environmental conditions in dust-source locations or recipient ecosystems. Overall, the differences in these dust-associated microbial communities appear to have corresponded to persistent drought conditions in California during our 2-year study, as well as the emergent drought in Asia in year two only, affecting the heterogeneity of dust deposited in both years. For the Asian drought year, dust heterogeneity (as related to the proportion of Asian dust in the mixtures) increased throughout the dry season. The competing droughts in Asia and California may have enhanced microbial diversity at lower elevations, while hindering dust-driven microbial inputs to highest elevations if dust-associated microorganisms were released during long-distance transport. These findings suggest that droughts in source locations may influence long-distance dispersal trajectories and the composition of both dust-bacterial and fungal communities in the Sierra Nevada.

Dust Heterogeneity and Microbial Structure

Despite the expected finding of more heterogeneous dust at higher elevations, surprisingly, these dust mixtures harbored

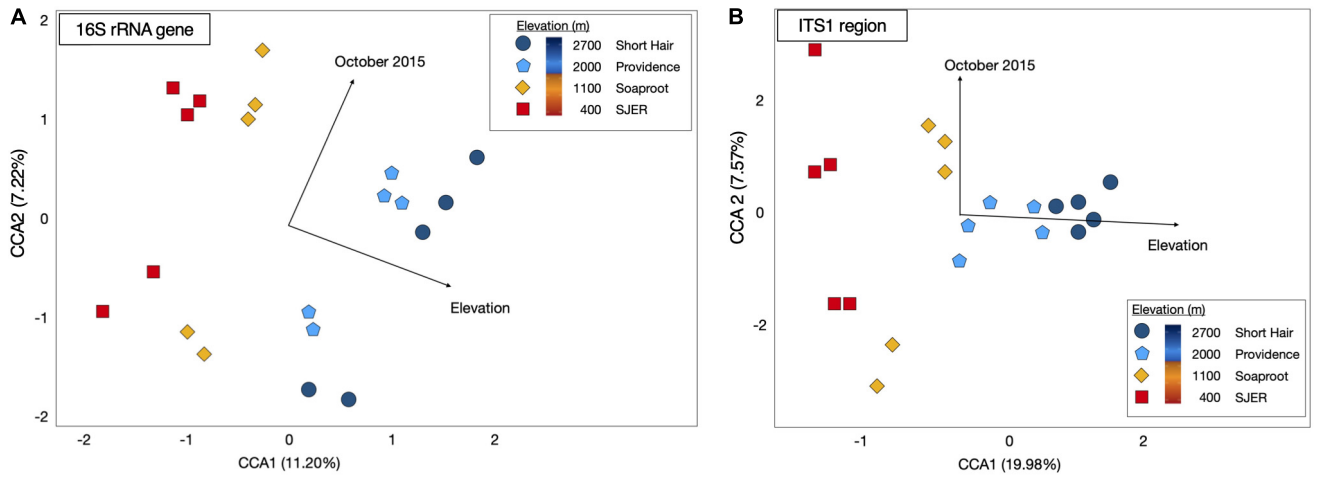

FIGURE 4 | Canonical Correspondence Analysis (CCA) for dust-bacterial 16S rRNA targeted amplicons from marker genes **(A)** and ITS1 region amplicons for dust-fungal **(B)** microbiomes. Elevation and October sampling date contributed to structuring microbial communities. Dust-associated bacterial (16S rRNA marker gene amplicons) communities from 2015 were colored by site [colored symbols, **(A)**]. Dust fungal communities (ITS1) from 2015 were shown in **(B)**. Both fungal and bacterial communities were structured by elevation and the October 2015 sampling date, as evidenced by arrows representing the direction of the effect; arrow length corresponded to the magnitude of the effect of these environmental or temporal variables. CCA1 for dust-associated bacteria represented 11.20% of the variation; CCA2 for these bacterial 16S rRNA OTUs represented 7.22% of the variation; CCA1 for dust-associated fungal ITS communities represented 19.98% of the variation; CCA2 for dust fungal amplicons represented 7.57% of the variation in the composition of these communities.

fewer microbial taxa than less-heterogeneous dust. Based on this finding, we rejected our hypothesis that more heterogeneous dust would harbor greater microbial taxa. Fewer detectable taxa in more heterogeneous dust suggests that microorganisms sorbed to dust particles traveling from Asia may have been jettisoned during the long-distance journey (Tesson and Santl-Temkiv, 2018). Although DNA from inviable organisms may still be detected by our molecular analyses, microbial viability may have been hindered in transit or prior to deposition, impacting the long-distance dispersal ability of a subset of viable dust microorganisms (Kellogg et al., 2004). Along with a reduction in San Joaquin Valley dust inputs, we found a corresponding decrease in the microbial richness within more heterogeneous dust mixtures.

Although we predicted that these diversity trends would attenuate over the dry season as higher elevation sites became progressively hotter and drier, we found varying richness and diversity patterns across elevations, years, and microbial groups. For instance, at the second highest elevation site, bacterial richness peaked late in the 2015 dry season, which may have resulted from more favorable site conditions. In contrast, fungal diversity peaked early in the dry season at the two lower elevation sites (SJER and Soaproot), with greater fungal taxa richness early in the 2015 dry season at SJER. Further, we found that dust microbial diversity and richness did not vary throughout the dry season at the highest elevation site (Short Hair). During this study, dust from Short Hair was characterized by low fungal and microbial diversity, which may have been related to cold temperatures or inhospitable conditions in this high elevation site. Thus, our hypothesis that dust microbial diversity trends would be more pronounced early in the dry season was partially supported, but only at our two lowest elevation sites that harbored the least heterogeneous dust.

Drought and Dry Season Impacts

Human activities, such as irrigation and farming, may alter seasonal edaphic properties and soil microbial communities. Although soil in natural systems would have gotten progressively drier by July 2015 at lower elevation sites, agricultural production, such as almond farming, at locations near these elevations may continue year-round and promote favorable microbial growth conditions. During these particular California drought years, productive farmland had been fallowed from lack of water resources (Swain, 2015). Differences in the extent and type of activities at low and high elevations may be related to these observed diversity patterns, as emissions from the San Joaquin Valley contributed to deposited dust in response to human-induced disturbance at low elevations. Our isotopic analyses showed that dust deposited at these two low-elevation sites was predominantly from the San Joaquin Valley (Aciego et al., 2017), suggesting that a diverse suite of dust microorganisms may have been emitted from putatively parched soils early in the dry season during this record-breaking drought in California (Swain et al., 2014; Swain, 2015).

Our findings on dust variation across months and between years underscore the importance of drought and soil disturbances in driving microbial dispersal in montane ecosystems, including droughts in source locations. The extreme drought in California during both years of our study likely reduced vegetative cover and decreased soil stability (Field et al., 2010), which led to greater regional dust promotion. Locations like the San Joaquin Valley of California, characterized by heightened human activities, agricultural production, and soil disturbance, are

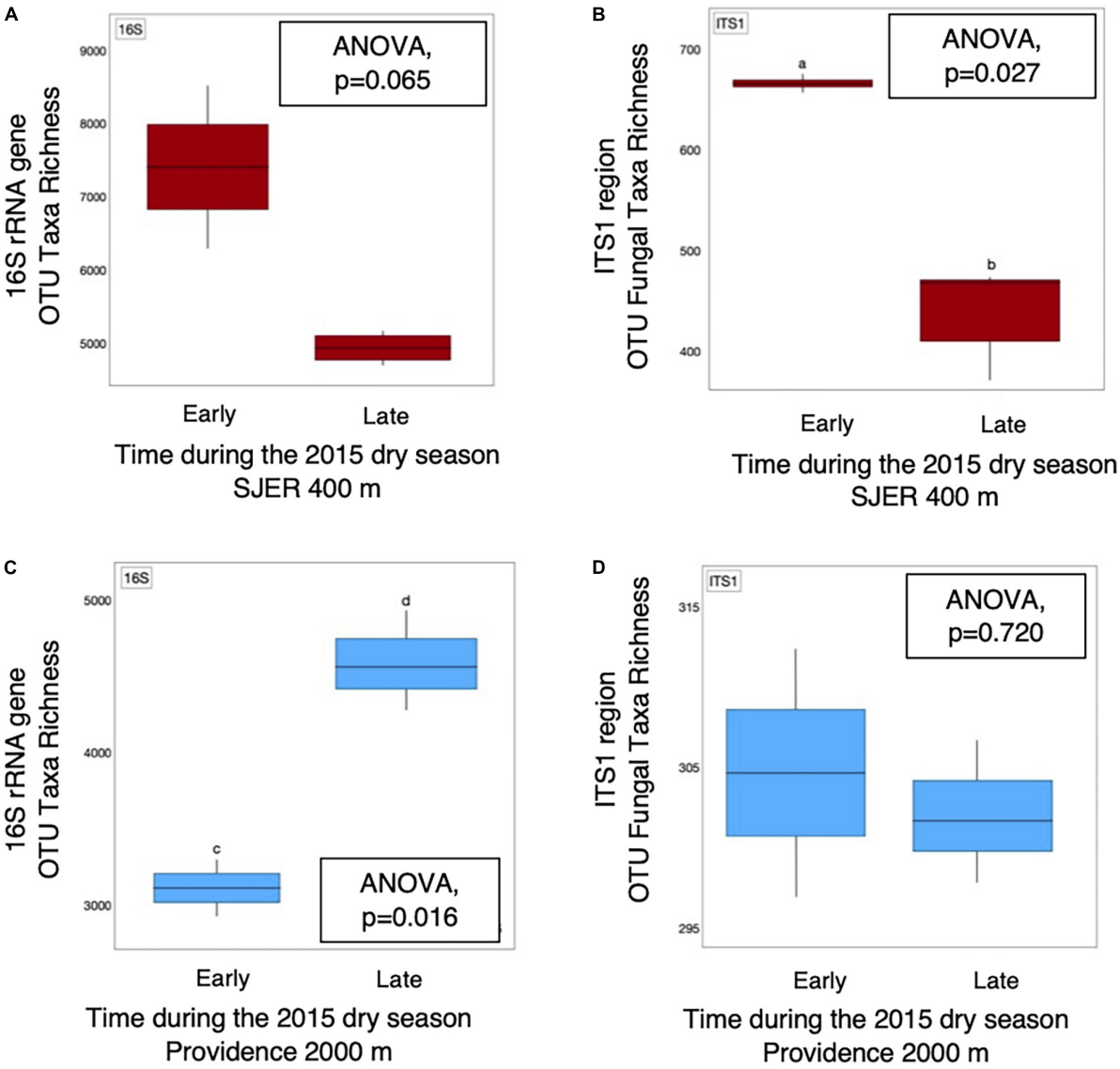

FIGURE 5 | OTU taxa richness of dust microbial communities detected from 16S rRNA marker genes and amplicons from the fungal ITS1 region varied throughout the 2015 dry season. Bacterial **(A)** taxa richness was equivalent, with marginal differences detected, at SJER (400 m), the lowest elevation site throughout the 2015 dry season. At the same site, fungal **(B)** taxa richness was significantly higher in July (early in the dry season), than late in the dry season (in October). In contrast, at Providence (2,000 m), the second highest site, dust bacterial **(C)** taxa richness was significantly lower early in the dry season, than later during that same dry season. Dust fungal richness **(D)** was equivalent at Providence throughout that same time period.

particularly vulnerable to erosion during drought conditions and can emit large quantities of dust. Dust contributions to the Sierra Nevada from the San Joaquin Valley grew substantially between summer and fall 2014 (Aciego et al., 2017), suggesting that the microorganisms and nutrients in San Joaquin Valley dust may have played an increasingly important role in these ecosystems throughout the dry season. Although dust-associated microbial diversity was unchanged during this period, both fungal and bacterial composition differed monthly across each sampling year, providing partial support for our hypothesis that dust-associated microbial communities would vary throughout the dry season. In addition, as the percentage of dust inputs from Asia increased throughout the 2015 dry season during an extreme drought in Asia (Aarons et al., 2019), microbial community composition shifted by sampling month as well as by dust provenance. In comparison to the emerging 2015 drought in Asia, these California landscapes were already burdened by heightened dust emissions, and exacerbated by a multi-year drought coupled with regional land use practices. Likewise, these introduced Asian inputs yielded more heterogeneous dust mixtures deposited in the Sierra Nevada as the season became progressively drier.

Given the variation in Asian dust inputs as the 2015 drought emerged, we observed interannual variation in dust fungi deposited to the Sierra Nevada during two California

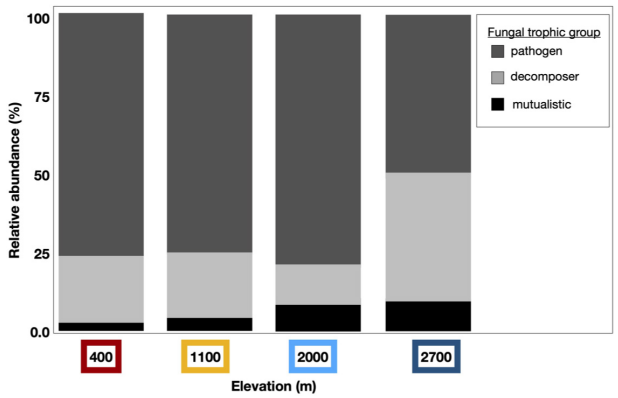

FIGURE 6 | Dust fungal ITS1 trophic groups vary by elevation. Dust-associated fungal OTUs were assigned to functional groups using the online application FUNGuild; Those fungal taxa with high confidence levels were curated and binned by trophic group to pathogens (dark gray), decomposers (light gray), or mutualistic fungi (black). Bar graphs represent the relative abundance of these trophic groups within assigned fungal taxa from each elevation. The relative abundance of mutualistic fungal taxa increased clinally by elevation and more fungal pathogens were found at lower elevations than at the highest elevation site.

drought years. In addition to compositional differences in fungal decomposers and mutualists, the relative abundance of dust-associated fungal pathogens differed across sampling years. Overall, these findings provided partial support for our hypothesis that source region droughts would drive interannual differences in dust microbial communities and fungal functional group abundance within this elevation gradient.

Landscape Topography and Microbial Processes

Our finding that clinal increases in dust P concentrations spiked during our second sampling year suggests that this dust may have exerted ecosystem-level influences at high elevations. Previous studies have illustrated greater airborne flux of bioavailable P delivered to high elevation sites, with implications for terrestrial ecosystem productivity (O'Day et al., 2020). In this slowly eroding, dust-reliant montane ecosystem, dust-associated mineral P contributions elicit transformative effects on vegetation communities, soil and ecosystem development, and biogeochemical cycling (Arvin et al., 2017; Gu et al., 2019). As Longo et al. (2014) demonstrated in Mediterranean aerosols, a portion of this soluble organic P may be microbially derived. While little is known about how dust elemental concentrations affect microbial activity or drive the structure of dust-associated microbiomes in airborne ecosystems, some microorganisms may use airborne dust as refugia and access labile carbon compounds to fuel their metabolic activities.

Previous research in soil and subalpine snow shows that bacterial communities may be structured by environmental changes that increase clinally or co-vary with elevation (Bowers et al., 2012; Carey et al., 2016). Likewise, our results indicate that low and high elevational extremes may serve as environmental filters (Meier et al., 2010; Bahram et al., 2012; Zimmerman and Vitousek, 2012; Coince et al., 2014). However, although dust-associated microbial diversity and community composition differed by elevation, these patterns did not exhibit a clinal trend (i.e., changes were not proportional along the elevation gradient). Dust compositional and diversity changes by elevation may have been driven by a mixture of incoming microorganisms and ambient microbial communities at each site. Landscape features and upper canopy plants, such as conifers that host endophytic and phyllosphere microbiomes (Kinkel, 1991; Osono, 2006; Sieber, 2007; Frank et al., 2017), may release dust and microorganisms that then intermix with the aeolian microbiome. Abiotic factors like temperature and precipitation vary clinally along our elevation gradient, and the combination of these parameters shapes the realized niche (Vetaas, 2002) and corresponds with transition zones for dominant plants in our study locations.

We found that the diversity of plant-associated mycorrhizal fungal taxa increased with elevation, which may have corresponded with the compositional, biogeographic, or phenological patterns associated with plant hosts and differing environmental conditions at the highest elevations. In particular, a greater proportion of the fungal community at our highest elevation sites was composed of mutualistic fungal symbionts, such as lichen-forming fungi *Buellia griseovirens* and *Fulgidea sierrae*. *Fulgidea sierrae* has previously been identified from coniferous forests in montane ecosystems in California (Bendiksby and Timdal, 2013) and may have therefore originated from local sources and subsequently been entrained in Sierra Nevada dust. Our previous study from soil in a tropical montane cloud forest (TMCF, Looby et al., 2016) shows that the relative abundance of arbuscular mycorrhizal fungi decreases with increasing elevation during the dry season, while ectomycorrhizal fungal diversity increases with elevation during the TMCF wet season. Likewise, mutualistic fungi increased with elevation in our study's temperate montane ecosystem, which may also correlate with increasing abundance or variety of ectomycorrhizal host trees at higher elevations. In contrast, *Trichophaea*, an ectomycorrhizal fungus from the Pyronemataceae family, was indicative of our lowest elevation site. This taxon belongs to a fungal group that emerges following disturbance, such as post-catastrophic wildfire (Glassman et al., 2016), so greater disturbances or warmer-drier conditions at this site may correspond to the abundance of this taxon.

Beyond mutualistic or host associations, indicators of higher elevation sites included several types of free-living basidiomycetous yeasts, including *Rhodotorula mucilaginosa, Vishniacozyma* spp., *Cryptococcus* spp., and *Naganishia* spp. Tremellomycetes were only indicators of the highest elevation sites; in particular, *Vishniacozyma* and *Naganishia* spp. contains taxa that are psychrophilic and have been isolated in extremely cold locations, such as from retreating glaciers in the Canadian High Arctic (Tsuji et al., 2019) and extreme high elevations in the Atacama region (Vimercati et al., 2016; Schmidt et al., 2017). Snowpack and colder temperatures at our highest elevation site likely promoted favorable conditions for cold-tolerant (i.e., psychrophilic) microbial taxa more than the

warmer-drier conditions at our lower elevation sites. Either these taxa were equipped with traits to survive harsh atmospheric conditions during long-distance transport or the presence of these psychrophilic taxa may be an indication of ambient microbial inputs from depositional sites. Another indicator of our highest elevation sites was *Phlebia centrifuga*, a wood-decaying basidiomycete fungus from the Polyporales order, which has previously been touted as an indicator of old-growth forests in Nordic countries (Makela et al., 2018). *Phlebia centrifuga* has an exposed spore-bearing surface (hymenophore) and crust-like corticoid growth on decomposing tree trunks, often found in more pristine or unmanaged forests, and this orientation may have contributed to the abundance of basidiospores entering the dust microbiome at the highest elevation site.

Intra-Seasonal Temporal Variation

We detected interannual temporal variation in both decomposer and mutualistic fungal communities. Only fungal mutualists exhibited monthly variation in community structure; these patterns persisted within years or for individual elevations. Fungi from the Arthoniales order also increased in abundance throughout the 2015 dry season. Members of this order are known to disperse long distances. Indeed, many dimorphic Arthonialean lichens reproduce both asexually, with soredia for airborne dispersal, and sexually, with ascospores (Tehler et al., 2009). Although this study did not sample microorganisms while in transit, tropical or subtropical Arthoniales taxa may have traveled along the jet stream to California early in the dry season but then potentially tapered off as the season progressed. In contrast, our analyses suggest that members of the Cystobasidiomycetes class were more abundant in the late 2015 dry season. Previous studies have shown that basidiomycete yeasts from Cystobasidiomycetes disperse together with lichen soredia as airborne contaminants traveling with lichen mycobionts (Cernajova and Skaloud, 2020). Although we did find an abundance of cold-adapted basidiomycete yeasts from Cystobasidiomycetes in higher-elevation dust samples, this did not correspond with abundance of lichen-forming fungi (Turchetti et al., 2013).

Consistent with our finding that dust from higher elevation sites harbored cold-adapted yeasts, the features of lower elevation sites may exert selective pressure to encourage taxa with traits facilitating their survival in warmer or drier conditions. For instance, a particularly abundant bacterial phylum at low-elevation sites, Firmicutes, consists of desiccation-resistant spore formers that may thrive in the hot-dry conditions found at these sites. Taxa from the phylum Acidobacteria were more abundant earlier than later in the 2015 dry season; this trend was likely driven by the abundance of Acidobacterial taxa early in the dry season at the Soaproot site. Previous studies have shown a remarkable rise in the relative abundance of Acidobacteria during dust events (Tang et al., 2018), specifically within the atmospheric environment of the Gobi Desert (Maki et al., 2017). We may have therefore expected rising Acidobacterial taxa richness or abundance as the 2015 dry season progressed, and more Asian dust entered our study system. Nevertheless, dust storm events in Asian deserts in 2015 likely contributed substantial Acidobacterial taxa to the atmosphere and subsequent deposition zones throughout the dry season.

Human and Ecosystem Implications

Our findings on both temporal and elevation-linked differences in fungal functional group composition have important implications for recipient ecosystems and human health. In particular, the abundance and type of fungal pathogens present could determine the extent to which host taxa are infected and detrimentally affected by pathogenesis. Some airborne plant pathogens, such as those causing powdery mildews and sooty molds, may lead to crop losses or forest blights (Peetz et al., 2007; Carisse et al., 2008). Previous studies have shown that air pollution, which may be more prevalent at lower elevations, interacts with fungal pathogens to exacerbate asthmatic responses and leads to chronic pulmonary disease or organic toxic dust syndrome in human hosts (Rask-Andersen and Malmberg, 1990; Tunnicliffe et al., 2001; Peng et al., 2019). Overall, the persistence of these fungal groups likely has important consequences for ecological and human health.

Although our sequencing approach and molecular analyses may impart biases that constrain our interpretation of the total abundance of pathogenic reads, the effects of month and elevation on fungal pathogens provides partial support for our hypothesis that more dust-associated pathogens would be found lower in elevation. In our study, we detected the greatest relative abundance of fungal pathogenic sequences at lower elevations, and this pathogenic signal diminished at the highest elevation. There may be a greater dominance of fungal pathogens at the lowest elevation sites corresponding with more human activities and soil disturbance, as is characteristic of the San Joaquin Valley of California. The impact of elevation and sampling month on fungal pathogen richness, diversity, and the relative abundance of fungal pathogens suggests that interacting spatial and temporal factors may determine the spread and distribution of ecologically relevant pathogens.

Interactions among soil microbial communities at our sites and the incoming aeolian microbiome may yield unique compositional and functional attributes of the resultant dust-associated microbiome. Incoming microorganisms traveling long distances may be either active in the air (Dimmick et al., 1979; Tang et al., 2018) or dormant such that they maintain viability upon deposition (Prospero et al., 2005). To stay viable, airborne microorganisms must be equipped with traits that allow them to withstand turbulent conditions and long-distance transport (Chagnon et al., 2013; Krause et al., 2014; Singh et al., 2019). Conditions at recipient sites may determine whether deposited microorganisms perish, proliferate, or associate with plant, animal, or human hosts (Schmidt et al., 2020).

The elevation-related shifts we observed in plant-associated mycorrhizal fungi likely have functional implications for resident biotic communities and contribute to patterns in ecosystem function across this elevational gradient. In general, the functional capacity of fungal communities can have transformative effects on ecosystems, as both decomposer and mutualistic fungi alter decomposition rates and are the engines of nutrient cycling, including carbon and macro-nutrient cycles

(Ehrenfeld et al., 2005), which may be particularly important in slowly eroding montane systems.

Study Limitations and Future Research

Although our study is one of the first to investigate how microbial communities and the chemical composition of dust change along an elevational gradient, it did have some limitations. For instance, our 16S rRNA gene analyses of bacterial communities yielded many sequences similar to eukaryotic photosynthetic taxa, which may have been better examined with sequencing technologies targeting longer genomic portions, such as single-molecule real-time sequencing using the PacBio Sequel Systems (Menlo Park, CA, United States) platform. Although previous studies have reported complex species-specific interactions between bacteria and microalgae, this phenomenon remains understudied (Schwenk et al., 2014). As microalgae can survive the harsh conditions of airborne transport, soil or dust-associated microorganisms have likewise evolved photoprotective pigments to survive environmental stress (Rehakova et al., 2019). However, the activity of airborne bacteria across varying temperatures and their taxon-specific interactions with photosynthetic micro-eukaryotes would both be promising avenues for future aeolian microbial research, especially throughout the dry season in California (Maki and Willoughby, 1978).

Because of the remoteness of our sampling locations and constraints associated with our sampling campaigns, dust collectors were deployed for varying durations of time prior to collection. Although the durations differed for dust collections across samples, deployment duration and dust mass was unrelated to microbial diversity or richness, ostensibly because dust flux is variable on annual timescales. After deposited dust accumulated, we subsequently extracted DNA from passive dust collectors by suspending this dust into sterile water and transporting it before performing DNA extraction and molecular analyses. While collectors were sterilized before placement each year, we subsequently replaced collectors after rinsing only with sterile water, such that relic DNA may have remained in our replaced collectors following rinsing. This may have confounded our interpretation of temporal variation in microbial community structure or chemical analyses of dust within a given sampling year, although it would not have impacted elevation-based findings. Our methods were also unable to discern between active microorganisms and dead microbial fragments or relic DNA that may have accumulated with our collected dust. However, we repeated all community composition and diversity analyses with binary data indicating presence/absence of individual taxa and taxa richness, which revealed that trends identified based on relative abundance were also identified in the presence/absence data. Therefore, we are confident in our reporting and interpretation of dust-associated microbial communities over time.

In addition, our molecular and bioinformatic techniques were not able to differentiate between viable DNA and extracellular DNA from dead microorganisms, which may persist for several years and obfuscate estimates of extant microbial diversity (Carini et al., 2017). Without quantifying relative ribosomal quantities (Placella et al., 2012), using stable isotope probing techniques to calculate excess atom fractions (Hungate et al., 2015), applying transcriptional and (Placella and Firestone, 2013) metatranscriptional techniques (Shakya et al., 2019), or measuring CO_2 pulses and microbial metabolism in dust (upon deposition or with exposure to favorable conditions), it would be challenging to discern the active portion from relic molecular products. Although our study was not designed to tease apart the relative contributions of global vs. local microbial inputs to the dust microbiome, higher intercontinental transport may have resulted in more exogenous microbial deposits. Yet, since both bacterial and fungal communities were correlated with the radiogenic nuclides used to determine dust provenance, microorganisms traveling long distances may have experienced selective forces during long distance transit.

Another avenue for future study would be to analyze dust and soil physicochemical properties, as well as soil and phyllosphere microbial communities at these study sites. Although no dust samples were analyzed for pH, we acknowledge that dust pH or ambient moisture measurements could have aided in the interpretation of our results, as certain microorganisms have specific tolerance ranges. Removing leaf cuticles or the use of specialized instrumentation, such as an ultrasonic bath, to separate surficial leaf associations from endophytic microorganisms could provide insight into ambient microbial inputs from local sites (Guzman et al., 2020). Analyzing microbial communities from transoceanic dust source locations, such as the Gobi or Taklamakan Deserts (Aciego et al., 2017), would provide valuable baseline data for bioinformatically teasing apart local vs. long-distance contributions to the dust-associated microbiome. Weather station instrumentation at source and recipient locations, coupled with soil biogeochemical measurements across locations, may also help quantify the relative impact of incoming dust on ecosystem function. Although soil biogeochemistry was beyond the scope of this study, our future research will examine both dust- and soil-based microbial communities and soil biogeochemical cycling across unusually dry years and an unusually wet year along this same elevation gradient.

CONCLUSION

Our results suggest that landscape topography and drought events in dust source locations may alter the composition and diversity of dust-associated microorganisms deposited into a temperate montane ecosystem. Each year of our study, we found that elevation determined dust-microbial community composition, suggesting elevation filtered the microbial inputs from dust and the ambient microorganisms at each site intermixed with incoming microorganisms to yield distinct structural and functional attributes. Dust-associated microorganisms play numerous functional roles in ecosystems, and dust biogeochemistry has implications for human health, biodiversity, and food security. Factors exacerbated by global change, including drought and development in dust source locations, may result in drier, unstable landscapes, increasing susceptibility to wind erosion and amplifying dust-promoting conditions in the future. Therefore, our findings

have crucial implications for how dust-associated microbial dispersal may respond to future climate scenarios, providing critical information on dust-driven microbial dynamics and biogeography in montane ecosystems. These data provide evidence that elevation and time since the last precipitation event may influence the structure and functioning of dust-associated microbial communities. Our study underscores the importance of drought events in dust source locations on long-distance microbial dispersal and the interactions among temporal factors and landscape topography on dust-associated microbial communities in montane ecosystems.

MATERIALS AND METHODS

Study Sites

Study sites were in California, United States, on the western side of the Sierra Nevada in the Southern Sierra Critical Zone Observatory (SSCZO). Sites ranged from oak savannah to subalpine forest, crossing the rain-snow transition zone (**Supplementary Figure 1**). These sites include the San Joaquin Experimental Range (SJER; 400 m above sea level), Soaproot (1,100 m), Providence (2,000 m), and Short Hair (2,700 m) (sites described in detail in O'Geen et al., 2018). The region is characterized by a Mediterranean climate with cool, wet winters and warm, dry summers with generally little to no precipitation from mid-May to October (i.e., dry season). From 2012 to 2015, the region experienced severe drought (Robeson, 2015).

The unprecedented magnitude of the drought in California during 2012–2015 (Griffin and Anchukaitis, 2014; Diffenbaugh et al., 2015; Robeson, 2015) resulted in low soil moisture, increasing the likelihood for wind erosion. This may be especially relevant in locations characterized by frequent soil disturbance from tillage, such as in California's San Joaquin Valley. During 2015, northern China—and large areas of northeastern Asia—experienced their most severe drought in the past 60 years (Wang and He, 2015). According to the Standardized Precipitation Evapotranspiration Index-(SPEI database), large areas of Mongolia, north China and northeastern Asia were under heightened drought conditions in July–October 2015 (Vicente-Serrano et al., 2010).

Dust contributions from the San Joaquin Valley grew significantly throughout the dry season in 2014, suggesting that a significant portion of dust was attributable to the extreme regional drought in California (Aciego et al., 2017). In contrast, the percentage of dust inputs from Asia increased throughout the 2015 dry season (Aarons et al., 2019). We determined that these changes were likely related to the prolonged drought in Asia during our second year of sampling. This remarkable interannual pattern highlights both the sensitivity of dust production and transport to drought and the teleconnections of dust transport in terrestrial ecosystems.

Field Dust Collection

We used passive dust collectors (Nordic Ware, St. Louis Park, MN, United States) to characterize dust-associated bacterial and fungal community composition during the 2014 and 2015 dry seasons along this ∼2,300 m elevational transect (**Figure 1**). Dust samples were collected between July and October 2014 and again between July and October 2015. Five replicate dust collectors were deployed per location. In 2014, we deployed dust collectors in response to worsening drought, with collectors deployed at SJER, Soaproot, and Providence sites between June 6–7, 2014, and at Short Hair on July 5, 2014. Later deployment at the Short Hair site was necessary due to snowpack, which prevented earlier access. Dust collectors were sampled from SJER, Soaproot, and Providence between July 5–6, 2014 and then redeployed. Collectors were then sampled from all four sites on August 6–7, Sept 6–7, and October 8–9, 2014, for deployment lengths of 29–31 days. Collectors were sampled for microbial communities in July, August, and October 2014, and were retained for isotopic and elemental analyses in July, August and September 2014 (see Aarons et al., 2019). In 2015, we maximized the duration of collection due to limited resources, deploying collectors at each site between April 2 and 4, 2015. We collected samples on July 15 and between October 7 and 10, 2015, resulting in a deployment length of 103–104 days for the first sample and 91–93 days for the second.

Constraints precluded us from obtaining isotopic analyses from October 2014; therefore, we used isotopic and other dust-analysis data from samples collected in September 2014, 29 days previously, as a proxy for October 2014, to complement our molecular analyses of dust collected during that sampling month. In July and August 2014, and July and October 2015, we paired molecular analyses with the results of our isotopic fingerprinting and trace elemental analyses (Aciego et al., 2017; Aarons et al., 2019) of collected dust.

Our passive collectors (as per Aciego et al., 2017), which consisted of Teflon-lined Bundt pans, secondarily lined with Kevlar mesh (Industrial Netting Inc., Maple Grove, MN, United States) and filled with glass marbles (12.7 mm diameter; Brooklyn, NY, United States; **Figure 1**), were used to collect dust at the four study sites. Prior to deployment in the field, all collection materials were acid washed in 2 M HCl, with rinses of 18.2 MΩ water between each reagent cleaning step, prior to deployment.

We deployed collectors 2 m above ground level atop wooden posts within open-canopy locations at each site. Collectors were deployed upon these posts to minimize the contribution of local particulate material from nearby canopy trees or surface soil saltation. To recover dust-microbial samples from the collectors in the field, we used 18.2 MΩ water to rinse the marbles within the collector and then transferred the water-and-dust suspension to acid-washed 1 L Nalgene bottles (low density polyethylene; **Figure 1**), which were subsequently processed back at the laboratory.

Sample Processing for Elemental and Isotopic Analyses

Samples were frozen and processed using techniques previously described in Aciego et al. (2009); Aarons et al. (2019), and using procedures outlined in Koornneef et al. (2015). Frozen samples were melted and immediately filtered to exclude the soluble

contribution with consecutive 30 and 0.2 μm polycarbonate filters. Following filtration, these samples were weighed for dust mass (weights, m * g) and then dust was dissolved directly off of the 0.2 μm filter using techniques previously described in Aciego et al. (2009). We calculated dust heterogeneity as detailed in Aarons et al. (2019), using the dust source location concentrations of Sr in San Joaquin Valley and Asian dust endmembers (Ingram and Lin, 2002; Yokoo et al., 2004) to calculate the proportion of San Joaquin Valley and Asian dust in each measured sample. We used endmember isotope-mixing models, as modified from White (2013), to determine the relative inputs of San Joaquin Valley-derived dust vs. Asian-derived dust in our dust mixtures: $^{87}Sr/^{86}Sr_M = [^{87}Sr/^{86}Sr_A * f * (Sr_A/Sr_M)] + [^{87}Sr/^{86}SrB * (1—f) * Sr_B/Sr_M)]$. Since our dust mixtures varied by the proportion of Asian dust in the samples (Aciego et al., 2017; Aarons et al., 2019), we used the proportion of Asian dust as the metric of dust heterogeneity.

Aliquots of each sample were measured for P or S concentrations on a Nu Instruments Attom high-resolution inductively coupled plasma mass spectrometer at the University of California Irvine. These aliquots were diluted in 2% HNO_3 and blanks, standards, and samples were spiked with a mixed internal standard prior to analyses, as per Aarons et al. (2019).

Laboratory Dust Filtration

We filtered dust suspensions into an acid-wash sterilized glass funnel through sterile 0.2 μm filters (47-mm diameter; Pall Supor 200 Sterile Grid filters, Pall Corporation, Port Washington, NY, United States) into a collecting flask below the acid-wash sterilized vacuum filtration unit. Filters were then stored in sterile Whirlpak bags at –20°C. To test for contamination, we filtered 1 L sterile Milli-Q water through a blank 0.2 μm filter using the same filtration apparatus as a negative control, which was processed alongside the dust samples. After samples were extracted and amplified, blank filters were free of DNA, indicating that contamination did not occur during processing.

Molecular Analyses

We extracted microbial DNA from frozen 0.2 μm filters (Pall, NY, United States) using a MO BIO PowerWater DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, United States; Qiagen Inc.), followed by cell lysis directly from filters, following the manufacturer's instructions. Our DNA extracts were then quantified using a NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). For prokaryotes, we amplified DNA extracts in duplicate using primers targeting the V3-V4 region of the 16S rRNA gene (S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21; Klindworth et al., 2013). We conducted polymerase chain reaction (PCR) by combining 2.5 μL DNA template, 5 μL each of 1 μM forward and reverse primers, and 12.5 μL KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, United States), totaling a 25 μL reaction. Thermocycler conditions were as follows: 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by an extension step for 5 min at 72°C. After amplification, we combined and purified the duplicate PCR products using Agencourt AMPure XP Beads (Beckman Coulter Genomics, Danvers, MA, United States). For purified bacterial amplicons, we conducted a second round of PCR to attach dual indices and sequencing adapters to each amplified sample using the Nextera XT Index Kit (Illumina, San Diego, CA, United States). For each of these reactions, 5 μL DNA, 5 μL each of 1 μM forward and reverse index primers, 25 μL KAPA HiFi HotStart ReadyMix, and 10 μL PCR grade water were combined to create a 50 μL reaction. Thermocycler conditions were programmed as follows: 95°C for 3 min, followed by 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by an extension step for 5 min at 72°C. We then conducted a second purification step using AMPure Beads (described above) on our indexed amplicons. Next, we quantified our purified PCR products using the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Grand Island, NY, United States). As a final step, we pooled samples in equimolar concentrations and sequenced them using an Illumina MiSeq instrument at the University of California Riverside (UCR) Genomics Core Facility.

To target fungal amplicons, we used modified versions of the universal fungal primers ITS1F and ITS2 (described in Smith and Peay, 2014), which were improved as part of the Earth Microbiome Project (Walters et al., 2016). This primer set accurately identifies fungi to the specific epithet level, but may have limited phylogenetic resolution (Schoch et al., 2012; Lindahl et al., 2013; Öpik et al., 2014; Yarza et al., 2017). We performed PCR amplification in 25 μL reactions including 1 μL of 10 μM for each primer (forward and reverse), 1 μL DNA, 12.5 μL of Taq 2X Master Mix (New England Biolabs, Ipswich, MA, United States) and 9.5 μL diH2O, as per Collins et al. (2016). Thermocyclers were programmed to 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, with a final extension step at 72°C for 10 min. As described in Walters et al. (2016), our forward primer sequences contained unique 12-base Golay barcodes as indexes for this single indexed reaction (Hamady et al., 2008). For fungal amplicons, we purified our PCR products using a NucleoSpin Gel-Extraction kit (Macherey-Nagel GmbH &Co. KG, Düren, Germany). Purified samples were quantified with a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, United States), prior to pooling in equimolar concentrations. We sequenced our library of fungal amplicons in a multiplexed run on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, United States) at UCR Genomics Core Facility. A total of 2,490,644 paired-end sequence reads were deposited to the Sequences Read Archive (SRA) database associated with BioProject accession number SUB10961273; with 7,548,435 paired end sequences submitted databases associated with BioSample submission accession SUB1096275 Southern Sierra Dust Targeted loci environmental.

Bioinformatics

Fungal ITS1 amplicon sequences were analyzed with AMPtk: the Amplicon Toolkit for NGS data (formerly UFITS; v1.2.4; Palmer et al., 2018), as per Pombubpa et al. (2020). Demultiplexed paired-end sequences were pre-processed, respectively, as both forward and reverse reads, trimmed to a maximum of 300 bp; any read with lengths below 100 bp in length was

discarded. Quantitative Insights into Microbial Ecology (QIIME; Kuczynski et al., 2012) was used to quality filter 16S amplicon sequences and determine taxonomic identity against the SILVA reference database (Quast et al., 2013), using 97% similarity for OTUs. Singletons were removed and analysis of similarity was performed in QIIME for 16S rRNA genes.

Functional or Microbial Group Analyses

We explored the significance of dust-associated phylogenetic taxa detected from libraries constructed for 16S rRNA or ITS1 targeted amplicon sequencing. To examine responses of the fungal community (ITS1), we assigned OTUs to functional groups using the online application FUNguild "http://www.stbates.org/guilds/app.php" 2019-02-12, Nguyen et al., 2016). After processing OTUs through FUNguild, our OTUs were assigned to Taxon, Trophic Mode, Guild, and Growth Morphology. FUNguild functional group assignments were as follows: pathotrophs, pathotroph-saprotrophs and pathotroph-symbiotrophs were assigned to the "pathogen group," saprotrophs and saprotroph-pathotrophs were assigned to the "decomposer group," and all saprotroph-symbiotrophs and symbiotrophs were assigned to the "mutualist group." We kept only FUNguild assignments at the confidence level of "highly probable (**Supplementary Table 3**)," removing all taxa that were at the confidence level of "possible" from the analyses. Finally, we compared the relative abundance of prokaryotic phyla present above a 1% threshold across conditions in the study.

Statistical Analyses

We used the "diversity" function in the "vegan" package to calculate Shannon's Diversity (i.e., alpha diversity) with the Shannon-Wiener index, using rarefied OTU counts. We used the "rrarefy" function from the "vegan" package, as per Oksanen et al. (2012), with bacterial counts rarefied to 34,559 or fungal counts rarefied to 7,302. After singleton sequences were removed, raw OTU counts were used to calculate taxa richness as to not lose rare taxa that could be present. We reported adjusted *p*-values that were calculated using the Bonferroni correction with the "adjust_*p*-value" function from the "rstatix" package in R to control the family-wise error rate. For linear regressions, adjusted R^2 values were calculated using the "stat_regline_equation" function within the "ggpubr" package in R (Kassambara, 2020).

We fit linear models using the "lm" function from the "stats" package in R, with bacterial alpha diversity, bacterial taxa richness, fungal alpha diversity, or fungal taxa richness as response variables and elevation, concentrations of radiogenic isotopic composition, concentrations of trace elements, month, or year as predictive variables. ANOVA and Tukey's Honest Significant Differences *post-hoc* tests were used to determine if there were significant differences between elevations or sampling timepoint as categorical variables. We used the "aov" function to compare the variation in these response variables within and among sites, sampling months and sampling years, or time in the dry season. To evaluate whether elevation or sampling timepoint affected microbial or functional group diversity, richness, or relative abundance, we used the "kruskal.test" function from the "stats" package; if significant, we tested the significance of pairwise comparisons. To control for the family-wise error rate, Bonferroni corrections were applied to all reported statistical tests using the "adjust *p*-value" function from the "rstatix" package (Kassambara, 2021). All statistical analyses were performed in R version 4.0.5 (R Core Team, 2018).

Beta diversity and community composition were calculated and visualized for each locus using principal coordinate analyses (PCoA) of the associated relativized feature table (McMurdie and Holmes, 2013; Borcard et al., 2018). We calculated distance matrices using the Bray–Curtis Dissimilarity Index from relativized OTU counts using the "decostand" (method = total) function from "vegan." We standardized varying sequence depths with the "total" option in the "decostand" function in the "vegan" package in R, transforming raw counts into proportions of each OTU per sample. With the "betadisper" function, we checked for between-group dispersion in fungal or bacterial composition between early or late timepoints within the dry season. For dust heterogeneity, we performed linear regressions using microbial alpha diversity (calculated from rarefied data) or taxa richness for fungi and bacteria. We also used dust heterogeneity to produce ordinations for visualizing compositional differences across microbial groups.

As dispersion in fungal and bacterial composition did not significantly differ by early vs. late dry season sampling timepoints, we decided to conduct permutational multivariate analysis of variance (PERMANOVA) with the "vegan" function "adonis" and "adonis2" in R (999 permutations; Oksanen et al., 2012) with relativized Bray–Curtis dissimilarity matrices to check between group differences and evaluate how composition varied across groups.

Using PERMANOVA, we assessed significant differences in composition between sampling years, sampling months, or abiotic parameters—such as the concentration of radiogenic isotopic composition, concentration of trace metals, or macronutrients—on compositional changes. Site was used as a blocking variable (strata) to restrict permutations within a particular site, as appropriate. Beta diversity and community composition were visualized for each locus using principal coordinate analyses (PCoA) of the associated relativized feature table. Additionally, we relativized by phyla and class, respectively, for bacteria and fungi and conducted PERMANOVAs to compare the composition of fungal and bacterial classes, phyla, as well as by fungal functional groups, by site, month, year, or time in the dry season.

For Canonical Correspondence Analysis (CCA), we ran a Detrended Correspondence Analysis (DCA) on the relativized feature tables for fungi and bacteria, respectively, using the "decorana" function from the "vegan" package to determine the homogeneity and respective variation in these data. Concentrations of trace elements and radiogenic isotopic composition were scaled prior to running the DCAs and CCAs using the "scale" function from "R-base" package. Based on the length of the first DCA axis, we ran CCAs using the relativized feature table and all associated metadata. We calculated adjusted R^2 value to obtain the overall variation explained by the CCA models. Then, ANOVAs were run on the CCA model to

determine which environmental variables contributed most to the variation within the composition data (using RsquareAdj and anova from the "vegan" package). Finally, we used the "ordistep" function from the "vegan" package to determine which environmental variables significantly constrained the variation in the composition data. Only significant environmental drivers were shown in CCA plots.

We used "decostand," to examine the relationship between the relative abundance of the most abundant bacterial or fungal classes by elevation and sampling month, across both years of the study (**Supplementary Figure 5**). We used non-parametric Kruskal-Wallis rank sum tests when the response variable was not normally distributed. Also using "decostand," we calculated the relative abundance of FUNGuild-assigned taxa (Nguyen et al., 2016), binned into fungal functional groups. We fit linear models using the "lm" function from the "stats" package in R to compare the richness or diversity of functional or taxonomic groups by elevation, month, or year, as well as to examine the relationship between the relative abundance of pathogenic, mutualistic, or decomposer fungi across the elevation gradient. We used dust heterogeneity, as the ranked proportion of Asian dust in the dust mixtures, to produce ordinations for visualizing compositional differences across microbial groups. Additionally, we constructed linear models to investigate interacting effects of elevation and sampling month, or month within year, on the richness, diversity, and relative abundance of these fungal groups. Adjusted R^2 values for these regressions were calculated using the "stat_regline_equation" function from the "ggpubr" package (Kassambara, 2020).

Indicator Species Analysis

We tested for indicator taxa associated with each elevation for the ITS1 region, with a particular interest in fungal taxa at the highest or lowest elevations. We calculated indicator values using the "multiplatt" function with 9,999 permutations in the "indicspecies" R package (De Caceres and Legendre, 2009). Indicator value indices are used for assessing the predictive value of taxa as indicators of conditions present in the different groups (De Caceres and Legendre, 2009). We only retained taxa with a p-value < 0.05 as significant (**Supplementary Table 3**).

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://ncbi.nlm.nih.gov/bioproject/PRJNA797677, NCBI Sequence Read Archive—PRJNA797677.

AUTHOR CONTRIBUTIONS

SH, EA, CC, SAa, SAc, and JB conceived of the study. SH, EA, CC, JB, ND, MBl, MM, NP, SAc, SAa, and MBa executed the study, collected the samples, processed samples, and generated data. EA, SH, JS, SAa, and SAc provided supplies, instrumentation, and funding to conduct analyses. HF, NP, and MM analyzed data and generated figures. MM wrote the manuscript. SH, HF, EA, CC, ND, MBl, MBa, MM, NP, SAa, and SAc edited the manuscript and commented on description of the study. 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/fmicb.2022.856454/full#supplementary-material

Supplementary Figure 1 | Bacterial 16S rRNA marker gene alpha diversity **(A)** and fungal ITS1 region alpha diversity **(B)** in dust collected during either 2014 or 2015 dry seasons in this study.

Supplementary Figure 2 | Bacterial 16S rRNA marker gene alpha diversity **(A)** and fungal ITS region alpha diversity **(B)** across elevations during both years of the study.

Supplementary Figure 3 | The relative abundance of 16S rRNA gene bacterial phyla in 2014 **(A)** and 2015 **(B)** and the relative abundance in ITS1 region of fungal classes in 2014 **(C)** and 2015 **(D)** across elevations.

Supplementary Figure 4 | The relative abundance of microbial groups detected through 16S rRNA marker gene amplification targeting bacterial 16S rRNA gene

and fungal ITS1 region amplicons across the dry season. The relative abundance of Acidobacteria **(A)** and Cyanobacteria **(B)** and Arthoniomycetes **(C)** were more abundant early in the dry season than later that season; the relative abundance of Cystobasidiomycetes **(D)** increased later in the dry season. Fungal ITS1 taxa summaries are shown by elevation **(E)**.

Supplementary Table 1 | Site and sampling characteristics. Coordinates, elevation, and deployment dates for each of four sites in the elevation gradient in the Sierra Nevada of California. The International Geo Sample Number (IGSN) is an alphanumeric code that uniquely identifies samples taken from the environment.

Supplementary Table 2 | Fungal taxa representing fungal tropic modes. Fungal taxa associated with each functional group, as assigned through FUNguild (as per Nguyen et al., 2016).

Supplementary Table 3 | Indicator species analyses for significant fungal taxa (to genus- or species-level). Only significant p-values ($p < 0.05$) reported.

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A metacommunity ecology approach to understanding microbial community assembly in developing plant seeds

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Microorganisms have the potential to affect plant seed germination and seedling fitness, ultimately impacting plant health and community dynamics. Because seed-associated microbiota are highly variable across individual plants, plant species, and environments, it is challenging to identify the dominant processes that underlie the assembly, composition, and influence of these communities. We propose here that metacommunity ecology provides a conceptually useful framework for studying the microbiota of developing seeds, by the application of metacommunity principles of filtering, species interactions, and dispersal at multiple scales. Many studies in seed microbial ecology already describe individual assembly processes in a pattern-based manner, such as correlating seed microbiome composition with genotype or tracking diversity metrics across treatments in dispersal limitation experiments. But we see a lot of opportunities to examine understudied aspects of seed microbiology, including trait-based research on mechanisms of filtering and dispersal at the micro-scale, the use of pollination exclusion experiments in macro-scale seed studies, and an in-depth evaluation of how these processes interact *via* priority effect experiments and joint species distribution modeling.

KEYWORDS

priority effects, selection, dispersal, metacommunities, epiphytes, endophytes, community assembly, seed microbiota

Introduction

The plant microbiota, defined here as the community of bacteria, fungi, archaea, viruses, and other microscopic organisms that live on (i.e., epiphytically) or in (i.e., endophytically) plant tissues (Hardoim et al., 2015), confer many services as well as disservices to their hosts, including disease development and defense (Busby et al., 2016), protection against herbivory (Shikano et al., 2017), tolerance of abiotic stress (Rodriguez et al., 2004), and aid in nutrient uptake (Christian et al., 2019). These microbial communities associate with all plant tissues (Hardoim et al., 2015), including seeds (Mundt and Hinkle, 1976; Ganley and Newcombe, 2006; U'Ren et al., 2009; Hodgson et al., 2014; Barret et al., 2015). Seeds play a major role in plant communities as agents of dispersal, genetic diversity,

and regeneration (Fenner and Thompson, 2005), and they have significant economic and social value through agriculture (Nabhan, 2012). Seeds also are a major bottleneck in natural plant populations, as they face heightened mortality from abiotic stressors, pests, pathogens, and predators (Bever et al., 2015). As the initial source of inoculum in a plant's life cycle, seed microbes are can be transmitted across plant generations and have lifelong impacts (Barret et al., 2015; Abdelfattah et al., 2021). Consequently, understanding how seeds acquire and interact with their microbiota, for example, *via* priority effects (Fukami, 2015; Ridout et al., 2019; Johnston-Monje et al., 2021) or according to the Primary Symbiont Hypothesis (Newcombe et al., 2018), has implications for improving seed health, seedling establishment, and plant community structure. Previous work on seed microbiota has primarily taken a pattern-based approach to studying assembly processes (e.g., Rezki et al., 2018). Such an approach uses culturing (Ganley and Newcombe, 2006; U'Ren et al., 2009; Heitmann et al., 2021) and/or next-generation sequencing (e.g., Barret et al., 2015; Rezki et al., 2018; Prado et al., 2020; Bergmann and Busby, 2021; Fort et al., 2021) to compare, contrast, and correlate patterns in microbial community composition, diversity, and species co-occurrences. Typically, however, these community data provide limited (i.e., mostly indirect) insights into processes such as dispersal, microbe-plant interactions, and microbe-microbe interactions. Given that seed microbial communities are highly variable across individual plants, plant species, and locations (Simonin et al., 2021), such pattern-based data cannot always be used to predict assembly outcomes. Moreover, such studies often consider how these assembly processes occur at a single spatial scale (e.g., between sites or plant populations; Klaedtke et al., 2016; Adam et al., 2018; Chartrel et al., 2021). We hypothesize that a mechanistic, multi-scale approach would provide a more complete understanding of how microbial communities assemble in seeds, with the field of metacommunity ecology providing a theoretical framework for such an approach.

Metacommunity theory accounts for the interaction between ecological processes and habitat heterogeneity across spatio-temporal scales to impact community patterns (Leibold and Chase, 2018). This emphasis on multiple scales and heterogeneity can help explain the main drivers of community assembly and patterns of biodiversity and co-occurrence (Leibold and Chase, 2018). Plant-associated microbial communities vary widely across environmental gradients (Barge et al., 2019) and host genetics (Wagner et al., 2016) from the levels of tissues (Cregger et al., 2018) to populations (Bergmann and Busby, 2021). As such, treating individual plants as heterogeneous habitats for microorganisms that are embedded in a larger, heterogeneous landscape of multiple plants representing different species provides a new approach to observing, testing, and modeling drivers of microbial community variation (Figure 1). However, the study of microbiota through a metacommunity lens is still relatively new, both for animals (Miller et al., 2018) and plants (Christian et al., 2015; Borer et al., 2016), and the plant seed represents a relatively understudied microbiome in this context.

In this review, we address how mechanisms of seed microbial community assembly have been studied at different spatial micro-, meso-, and macro-scales (Figure 1), and advocate for a metacommunity-based approach to seed microbiology in future work. For this review, we use the definition of community assembly from Fukami (2010): "the construction and maintenance of local communities through sequential, repeated immigration of species from the regional species pool." Additionally, most studies that we cover in our review will be focused on fungi and bacteria (Table 1). We acknowledge that archaea, viruses, and protists are frequent members of plant-associated microbial communities (Trivedi et al., 2020), many plant viruses are seed-transmitted (Sastry, 2013), and viruses can play a major role in the diversity and function of soil microbial communities (Albright et al., 2022). However, the ecological roles of these microbes in plant microbial communities, including those of seeds, are still largely unknown. As such, we cannot speak on their contributions to seed microbiota assembly here and recommend new research on these microbes in seeds. We will first summarize the modes of microbial acquisition into seeds, and how metacommunity ecology frames this assembly process. We then discuss studies of seed microbiome assembly which examine the processes of filtering, species interactions, dispersal, and ecological drift. We specifically highlight studies that address assembly processes during seed development and maturation, as these stages are understudied compared to seed dormancy and germination, and they are likely the source of microbes that persist between plant generations (Chesneau et al., 2020). Lastly, we suggest future lines of research to gain a more mechanistic, scale-explicit understanding of seed microbiome assembly.

Acquisition modes of seed microbiota

Plant seeds are generally composed of three tissues: a seed coat which provides physical protection (Belmonte et al., 2013), an embryo which is the precursor to the seedling and is made up of an immature root, a stem, and one or more embryonic leaves (Boesewinkel and Bouman, 1995; Bewley et al., 2012; Bewley and Nonogaki, 2017), and an endosperm which typically consists of carbohydrates and proteins and provides nutrition for the embryo during germination and growth before photosynthesis can occur (Berger, 1999). Seed development involves three stages (Belmonte et al., 2013; Bewley and Nonogaki, 2017). Following fertilization by pollen, the egg cells divide and differentiate into the embryo and endosperm tissues, in a process called histodifferentiation (Bewley and Nonogaki, 2017). Next, the cells expand and mature with reduced division, and seed mass increases during this filling stage, as nutrient reserves are deposited into the endosperm (Bewley and Nonogaki, 2017). After this, nutrient accumulation declines, and the seed goes into maturation drying and loses about 10%–15% moisture content before it is ready to be dispersed (Angelovici et al., 2010; Bewley and Nonogaki, 2017).

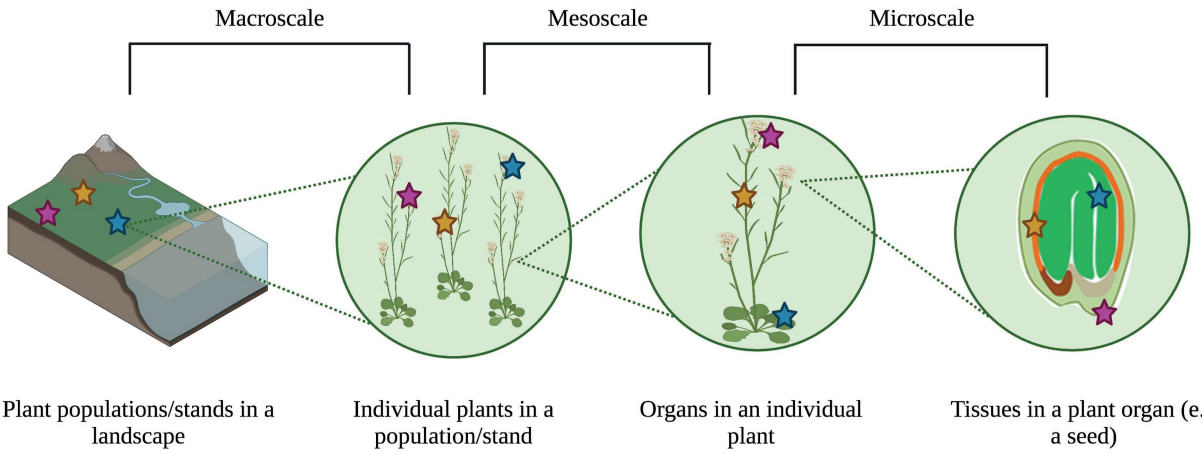

FIGURE 1
Variation in plant microbial communities can be observed at the macro-scale (i.e., meters to kilometers, or within and among sampling sites), meso-scale (i.e., centimeters to meters, or within and among plant organs to individuals), and micro-scale (i.e., nanometers to centimeters, or within and among plant organs) scales. The different colored stars represented distinct microbial communities at each scale. Image created through BioRender.com.

TABLE 1 Commonly found fungal and bacterial taxa in seeds across broad plant groups.

Plant group	Commonly observed[a] fungal taxa	Commonly observed[a] bacterial taxa	Fungal taxa with known phytopathogenic representatives[b]	Bacterial taxa with known phytopathogenic representatives[b]
Angiosperms (monocots; e.g., grasses)	*Alternaria, Aspergillus, Colletotrichum, Epichloë, Fusarium*	*Pantoea, Pseudomonas, Sphingomonas, Burkholderia, Enterobacter*	*Alternaria, Aspergillus, Colletotrichum, Fusarium*	*Pseudomonas*
Angiosperms (dicots; e.g., brassicas, the rose family)	*Alternaria, Cladosporium, Cryptococcus, Stemphylium, Aureobasidium*	*Pseudomonas, Pantoea, Bacillus, Acinetobacter, Erwinia*	*Alternaria, Cladosporium, Stemphylium*	*Pseudomonas, Erwinia*
Gymnosperms (e.g., pines, firs, cypresses)	*Cladosporium, Hormonema, Trichoderma, Alternaria, Caloscypha*	*Pseudomonas, Bacillus, Enterobacter, Erwinia, Lysinibacillus*	*Alternaria, Cladosporium, Hormonema*	*Pseudomonas, Erwinia*

[a]Commonly observed taxa are defined here as the top five most mentioned among the most abundant genera that were identified in a selection of representative papers (Supplementary Table 1). This table is not meant to be exhaustive or quantitative and only offers a qualitative sense for the types of fungal and bacterial taxa that these studies recognized as most likely to find associated with seeds. For more extensive reviews of bacteria and fungi found across plants, we recommend referring to Simonin et al. (2021) and Newcombe et al. (2022).
[b]Putative pathogens were defined as any of the commonly observed genera that have pathogenic strains reported in the UC Integrated Pest Management Plant Disease List (http://ipm.ucanr.edu/PMG/menu.disease.html).

During seed development, microbes may enter the seed tissues *via* three distinct routes of transmission: vertical, floral, and horizontal (Maude, 1996). Vertical transmission involves microbes traveling from other organs (e.g., stems, roots) of the mother plant to the developing embryo. Such transmission is cited as an ecologically important way for plants to inherit beneficial microbes across generations (Barret et al., 2015) and for seed-associated pathogens to disperse (Darsonval et al., 2008; Darrasse et al., 2010, 2018; Barret et al., 2016). Vertical transmission has long been observed in grasses, which are hosts to clavicipitaceous fungal endophytes (i.e., fungi in the family Clavicipitaceae; Porras-Alfaro and Bayman, 2011) such as *Epichloe* (Schardl et al., 2004; Truyens et al., 2015). Vertical seed transmission has also been observed for non-clavicipitaceous endophytes in *Setaria viridis* (Rodríguez et al., 2020), *Triticum* (Vujanovic et al., 2019), *Quercus* (Fort et al., 2021), and other plants (Newcombe et al., 2018). Floral transmission of microbes into seeds has been studied extensively for pathogens such as *Monilinia vaccinii-corymbosi* in blueberry (Ngugi and Scherm, 2004) and *Acidovorax citrulli* in watermelon (Walcott et al., 2003; Lessl et al., 2007; Dutta et al., 2012, 2015). However, flower-to-seed transmission has also been observed for commensal and beneficial bacteria, for example in *Brassica napus* (Prado et al., 2020). The microbial contributions of the vertical and floral transmission pathways are likely to vary based on a plant species' pollination mode (Chesneau et al., 2020). Horizontal transmission is the acquisition of seed microbes from the environment, either prior to or after the maturation of the seed as it is still attached to the mother plant (Deckert et al., 2019) or as

matured seed disperse and becomes colonized from sources such as air (suggested in Gandolfi et al., 2013), water (Crocker et al., 2016), animals (Correia et al., 2019; Lash et al., 2020), soil (Fort et al., 2021), and other seeds in storage (suggested in Bergmann and Busby, 2021). Seed dormancy and germination are likely to represent a very active period of such horizontal transmission, as soil microbes interact with seed exudates and pre-existing microorganisms on and within the seed (Nelson, 1990; Ofek et al., 2011).

The case for studying seed microbiome assembly through a metacommunity lens

The metacommunity concept was formally described by Leibold et al. (2004), who defined metacommunities as sets of local communities that are interconnected by dispersal. This definition arose out of a need to better account for spatio-temporal scales in ecological studies (Leibold et al., 2004), and also included the impacts of dispersal and habitat heterogeneity on community patterns (Leibold and Chase, 2018). Since it was first described, metacommunity theory has adopted Vellend (2010, 2016) synthesis that community assembly and composition are driven by four categories of processes: (1) abiotic and host filtering, (2) species interactions, (3) dispersal, and (4) ecological drift (Leibold and Chase, 2018). Categories 1 and 2 (filters and interactions) represent a deterministic or niche-based process of selection where differences in fitness between taxa, species, or guilds lead to differences in their abundances (Vellend, 2010). Dispersal is the stochastic (or neutral, chance-based) process by which taxa move between local communities (Vellend, 2010). Finally, drift is the stochastic fluctuation in species abundances, often due to chance birth, death, and migration events (i.e., demographic stochasticity; Leibold and Chase, 2018).

Framing plant microbiomes as metacommunities provides an integrated view of the drivers of their composition, function, and evolution, and of the impacts of these drivers on host health (Mihaljevic, 2012). Traditional metacommunity ecology states that filtering and species interactions occur at the local scale (i.e., less than one square meter to several square kilometers; Cornell and Lawton, 1992), while dispersal and drift occur at the regional scale (i.e., many square kilometers; Cornell and Lawton, 1992; Leibold and Chase, 2018). However, categorizing processes as "local" or "regional" is relative to the community that is being studied, and depends on the scales of interest and on defining the boundaries between a local community and a regional metacommunity. For plant microbiota, including those associated with seeds, the terms "local" and "regional" are contextual because microbes primarily behave at very small scales (i.e., the micrometer scale; Leveau et al., 2018; Leveau, 2019), although they can be affected by much larger scale factors (e.g., plant genotype or climate gradients across kilometers; Dini-Andreote et al., 2020). Furthermore, microbes can be ubiquitous across habitats at multiple scales, blurring the boundaries between patches of local communities in the landscape of interest (Mony et al., 2020). As we apply the first principles of metacommunity ecology to plant and seed microbiology below, we will therefore use three categories of spatial scale: macro- (i.e., meters to kilometers, or within and among sampling sites), meso- (i.e., centimeters to meters, or within and among plant organs to individuals), and micro- (i.e., nano-to centimeters, or within and among plant organs) scales. Integrating the study of assembly processes across these three scales should give a more complete picture of how microbial communities are assembled, and how emergent community patterns occur at individual scales (Ricklefs, 1987; Shade et al., 2018).

Deterministic processes: Abiotic filtering, host filtering, and species interactions

Abiotic filtering

Several studies have shown that seed microbial communities differ significantly across geographic locations, i.e., at the macro-scale, for example in *B. napus* (Morales Moreira et al., 2021), *Elymus nutans* (Guo et al., 2020), *Phelipanche ramosa* (Huet et al., 2020) and *Pseudotsuga menziesii* (Bergmann and Busby, 2021). For most of these studies, the abiotic factors that are important for structuring these seed microbial communities remain to be identified. However, we can assume that these factors are similar to the ones that drive macro-scale differences in the microbial communities on/in other parts of the plant. In communities associated with leaves, roots, and fruits, such factors include temperature (Zimmerman and Vitousek, 2012), precipitation (Zimmerman and Vitousek, 2012; Barge et al., 2019), humidity (Bokulich et al., 2014), and soil conditions (e.g., available cations, soil pH; Johnston-Monje et al., 2016). In a study of aboveground microbial communities in *Vitis vinifera,* Bokulich et al. (2014) found that fungal communities of seeded fruit were associated with net precipitation, relative humidity, and temperature. During dormancy in the soil, the bacterial communities of *Noccaea caerulescens* seeds were correlated with soil pH and cation composition (Durand et al., 2020).

Not much is known either about variation in seed microbial community as a function of abiotic factors at the meso-and micro-scales, although again, much can be learned from studies on other aboveground plant tissues. At the meso-scale of an individual plant, microbial communities can vary with tissue location such as canopy height in trees. Unterseher et al. (2007) cultured fungi from leaves at different canopy heights in several tree species. They found that species richness was greater in the lower canopy. Harrison et al. (2016) went on to use next-generation sequencing in a survey of the needle fungi of *Sequoia sempervirens* at different height positions, and found that there were distinct communities present at each height across trees. While they did not measure

microclimate variables within the trees sampled, they suggested that the observed variation could be attributed to the amount of sunlight (Harrison et al., 2016). At the micro-scale (i.e., across parts of a single plant organ), factors such as exposure to ultraviolet (UV) radiation and water availability can also be important. Hayes et al. (2021) described variation in the bacterial communities and UV radiation along individual flower petals in two sunflower species. They found that while there was no significant difference in community composition along petals, there was variation in UV tolerance in association with source petal position (Hayes et al., 2021). Another potentially important factor may be water availability, which has been shown to affect bacterial survival, growth, and movement on leaf surfaces (Doan et al., 2020).

For many macro-scale studies, a major limitation is the use of location as a proxy for environmental conditions, which precludes linking variation in microbial communities to specific environmental factors. Because site effects are impacted by environmental, spatial, and temporal factors, it can be difficult to parse out how location and environment influence seed microbiota (Bergmann and Busby, 2021). Also, most of these studies do not explore if and how environmental conditions actually select for microbial traits and taxa. *In vitro* experiments suggest that there is potential for environmental filtering, as demonstrated by thermotolerance in fungal endophytes of desert plants (Sangamesh et al., 2018), salt stress tolerance in fungal root endophytes (Gaber et al., 2020), water stress tolerance in bacterial endophytes (Yandigeri et al., 2012; Daranas et al., 2018), and oxidative stress tolerance in the fungal endophyte *Epichloë festucae* (Eaton et al., 2008). Similar characterization of seed microbial tolerance and survival when challenged with different environmental conditions could provide a more mechanistic understanding of abiotic filtering. Such studies would be particularly insightful at the micro-and meso-scales.

Host filtering

Variation in plant microbial communities is often studied and interpreted as a result of plant genetics, which represents filtering through host selection. Studies at the macro-and meso-scales have revealed that plant genetics can significantly impact microbial community composition in different parts of the plant, although seeds are clearly underrepresented in the body of literature on this topic. Microbial community variation has been associated with specific genes in leaves and roots of various plants (Horton et al., 2014; Wagner et al., 2016; Deng et al., 2021), an approach that has not yet been applied to seeds, as far as we know. Seed line (i.e., familial line of descent traced to an individual seed) has been weakly associated with microbial community variation in *Zea mays* (Yang et al., 2020) and *B. napus* (Morales Moreira et al., 2021). Seed accessions (i.e., populations) of *Oryza* were also associated with variation in bacterial and fungal community composition, with significant compositional shifts between wild and domesticated accessions (Kim et al., 2020). In a study of the bacterial and fungal communities associated with grapes, Singh et al. (2018) found that host genotype had an impact particularly within individual sites, whereas abiotic conditions better explained microbial community variation between sites. This is consistent with the notion that host effects are difficult to reveal without carefully controlling for environmental factors, which would suggest, by extension, that environmental factors may have a greater relative impact on seed microbiota than plant genotype. A recent study showed however that the fungal community composition of *Quercus petraea* internal seed tissue was largely influenced by the mother plant, with only weak significant environmental influences (Fort et al., 2021).

Studying the roles of plant functional traits in seed microbiome assembly and dynamics provides the mechanistic framework to understand host filtering. Some of the clearest examples of these mechanisms come from the field of plant pathology, where plant traits can be used to predict disease outcomes (Fahey et al., 2020). One obvious suite of traits to study are plant defenses. As agents of plant regeneration, seeds are one of the most defended plant organs, protected by both chemical and physical defenses (Zangerl and Bazzaz, 1992; Fuerst et al., 2014; Fricke and Wright, 2016; Wang et al., 2018). Some of these defenses come from the mother plant, such as through innate floral defenses in angiosperms (Rhoades and Cates, 1976). Many studies on plant defense traits are obviously focused on protection against pests and pathogens (Dalling et al., 2020), but can be extended to other members of the microbial community (Fort et al., 2021).

A number of studies have been conducted to test how microbes interact with seeds at the micro-scale. Using microscopy, the microbial communities within seeds of *Citrullus lanatus* (Dutta et al., 2012) and *Q. petraea* (Fort et al., 2021) were found to differ in abundance and composition depending on seed sub-structure. Since *Q. petraea* is a wind-pollinated species, the variation in seed sub-structure colonization observed by Fort et al. (2021) suggests physical filtering of microbes during vertical and horizontal transmission. Although few studies have explored the role of micromorphology of developing seeds in microbial community acquisition (e.g., Eyre et al., 2019), there are plenty examples of such micro-scale studies come from work on the floral microbiome. Spinelli et al. (2005) used microscopy and fluorescent tagging to study the growth and movement of the bacteria *Erwinia amylovora* and *Pantoea agglomerans* on flowers of apple (*Malus domestica*) and pear (*Pyrus communis*). They found that the bacteria migrate from the stigma to the nectaries along a stylar groove in both species, indicating topographical effects on survival, population growth, and dispersal (Spinelli et al., 2005). Similarly, Steven et al. (2018) characterized at the high spatial resolution the floral bacterial communities on apple (*M. domestica*) using next-generation sequencing and found that different flower parts were enriched with different bacterial families (Steven et al., 2018). It is intriguing to think that variation in microtopography on flowers and stigmas may contribute to

host filtering during the process of flower-to-seed horizontal transmission of microorganisms.

Species interactions

The role of species interactions in metacommunity dynamics is important, but often overlooked in metacommunity ecology studies (Leibold et al., 2020). In plant microbiota research in general, much focus has been on pathogen antagonism interactions, for example with an eye toward applications in disease control (Busby et al., 2016). However, there is much interest and opportunity to better understand interactions between and among non-pathogens in plant and also seed microbial communities. As with traditional ecology studies, much of the work on species interactions in seed microbial communities focuses on competition and antagonism. For example, Raghavendra et al. (2013) inoculated *Centaurea stoebe* flowers with pairs of fungi and then cultured those fungi out of mature seeds. They always isolated the same single fungus from each pairing out of seeds across parent genotypes, and proposed that competition was the primary driver of selection (Raghavendra et al., 2013). Fungi compete for space and resources in *Q. petraea* seeds (Fort et al., 2021), and have negative interactions with bacteria in *Populus trichocarpa* seeds (Heitmann et al., 2021). Similar competitive exclusion has been observed in floral stigma communities (Cui et al., 2020), and in dormant seeds within the soil (Fuerst et al., 2018). However, seed microbes can also coexist *via* niche partitioning and other interactions. For example, Torres-Cortés et al. (2019) looked at how transmission of several bacterial pathogens impacted the composition of *Raphanus sativus* seed microbiomes. They found that these pathogens did not alter the composition of the seed microbiome, suggesting that differences in resource usage (niche partitioning) lead to coexistence between taxa (Torres-Cortés et al., 2019). A more complete understanding of the types and outcomes of microbial species interactions prior to and during seed development is desirable.

Stochastic processes: Dispersal and ecological drift

Dispersal

As with filtering, microbial dispersal to seeds occurs at multiple nested spatial scales, with different mechanisms at play for each spatial scale. For example, at the micro-scale, dispersal from floral stigmas to seeds can be impacted by variation in the level of protection or nutrients that are available to microbial colonizers, which is closely tied to stigma surface topography. The presence of pollen may also be important, as it has been shown that germinating pollen can enhance the flower-to-seed transmission of pathogens (Walcott et al., 2003) and that some bacteria can even induce pollen germination (Christensen et al., 2021). While there are no studies looking at the connection between floral topography and seed microbial transmission, experiments with flowers (Spinelli et al., 2005) and leaves (Doan et al., 2020) have demonstrated that bacterial dispersal is influenced by plant surface topography and surface water distribution. Conducting similar micro-scale inoculation experiments like these in flower-to-seed systems will illuminate how microbes actually move.

Seeing that microbes can be florally transmitted to seed, we need to consider studies on the dispersal of floral microbes to understand seed microbial communities at the meso-and macro-scales. One major finding from floral studies is that microbes are dispersal limited at regional scales (Belisle et al., 2012). For example, Belisle et al., 2012 found that yeast frequency in nectar communities of *Mimulus aurantiacus* was correlated with flower proximity, and they inferred that dispersal limitation was controlled by pollinator behavior. In a study on the floral microbiome across wildflower species of California, Vannette et al. (2020) observed that fungi were more dispersal limited between individual flowers and plant species than bacteria. Another major finding has been that pollinators can vector microbes between flowers and influence microbial community patterns. For example, Vannette and Fukami (2017) explored the variable effects of dispersal limitation on beta diversity in the nectar microbiome. Using a pollinator exclusion experiment, they found that increased dispersal by pollinators raised beta diversity and hypothesized that this increase was due to the stochasticity of dispersal timing which strengthens priority effects (Vannette and Fukami, 2017). A pollinator exclusion experiment in *B. napus* demonstrated that pollinators can also vector bacteria to seeds through flowers, impacting the local and regional diversity (Prado et al., 2020). These experiments indicate that dispersal may have unique effects on diversity in flower microbiome metacommunities *via* arrival history. However, all of these studies were performed only on the macro-scale, and they did not characterize dispersal traits. Furthermore, the association between dispersal patterns in floral microbial communities and those in seed communities has yet to be studied. Future experiments should explore if dispersal traits and arrival history consistently enhance beta diversity in flower and seed microbial communities among spatial scales (Vannette and Fukami, 2017).

Ecological drift

Ecological drift is defined as random fluctuations in species abundances over time, and can be driven by random birth, death, and migration events (Leibold and Chase, 2018). Drift is particularly important when local communities are small (Fukami, 2004) and filtering is weak (Chase, 2010). This is key to note for seed microbial communities because they typically have low population sizes and low species richness (Newcombe et al., 2018; Bergmann and Busby, 2021). Random migration events may be particularly important for seed microbes, such as those vectored by rain or wind (Shade et al., 2017). However, while there

is a lot of interest in drift and stochasticity in seed microbe research (Shade et al., 2017), drift as a process is difficult to study because it is hard to manipulate. Metacommunity ecologists have also generally found it difficult to get direct evidence of drift, with limited examples from experiments testing the coexistence of ecologically equivalent taxa (Leibold and Chase, 2018). One alternative approach to direct observation used in plant microbiota studies is to fit community data to neutral models, where community members are assumed to be ecologically equivalent, and non-significant variation in community composition across samples is explained by neutral processes. Rezki et al. (2018) took this approach when studying the seed microbiota of *R. sativus* by fitting fungal and bacterial community data to a Sloan neutral model (Sloan et al., 2007). This model accounts for neutral birth, death, and immigration rates, and estimates immigration rates into communities based on species frequencies across samples (Sloan et al., 2007). Immigration rates within the confidence interval of the predicted values imply that drift is structuring the community (Sloan et al., 2007). Based on the model, they found that bacterial community assembly was driven primarily by drift, while fungal communities were driven more by dispersal (Rezki et al., 2018). This study indicated that drift is important for some seed microbes, and more model-fitting studies or coexistence experiments are needed.

Interactions between assembly processes across scales

At its core, metacommunity ecology emphasizes not only how the processes described above play out individually, but also how they interact with each other to produce emergent community patterns across scales. In plant microbiome research, the interaction between abiotic and host filters, also known as genotype-by-environment (GxE) interactions, has been of growing interest because it provides a more holistic explanation for microbiome variation (Wagner et al., 2016). Such an explanation can be applied to seed microbial communities, which may vary with seed nutrient profiles, osmotic stress, and water availability. However, as previously mentioned, GxE studies on plant microbiota face a scale problem where genotype and environment become synonymous at the micro-scale. Taking a plant trait-based approach to these studies may make the role of these effects more clear, and can connect micro-and macro-scales *via* host local adaptation.

While not emphasized as much as GxE interactions, the interaction between dispersal and filtering is also important during seed microbiome assembly. At the micro-scale, variation in the plant surface landscape (e.g., water availability and topography of the surface) can create differences in dispersal limitation between taxa. Doan et al. (2020) demonstrated this interaction on synthetic leaf surfaces, finding that surface water acted as a conduit for bacterial dispersal. This effect may also be present in floral stigmas, which are highly heterogeneous landscapes (Spinelli et al., 2005). Indeed, in their work on transmission of the pathogen *A. citrulli* from watermelon flowers and fruit to seeds, Dutta et al. (2012) found that inoculum from the flower dispersed more frequently and ended up in deeper seed tissues (e.g., endosperm, embryo) than inoculum from the fruit. While these examples suggest that heterogeneity in the plant landscape impacts dispersal limitation to seeds, more studies are needed.

Dispersal also intersects with species interactions, most clearly through historical contingency or priority effects (Fukami, 2015). In this phenomenon, the arrival order of community members dictates assembly outcomes, typically with an advantage to taxa that arrive first (Fukami, 2015). Priority effects can occur either through niche preemption, where the first colonizers fill all available niches, or by niche modification, where the first colonizers alter the environment and its resulting niches (Fukami, 2015). These effects are often cited as important in seed communities because they have few members (Bergmann and Busby, 2021). However, priority effect experiments in plant microbiota have typically been done in leaf (Leopold and Busby, 2020) and wood (Hiscox et al., 2015; Leopold et al., 2017) communities (Maignien et al., 2014). As such, there is a need to understand the role of priority effects in seed communities.

An exciting new approach for studying the multiple, interactive processes of dispersal, filtering, drift, and species interaction is with Joint Species Distribution Models (JSDMs), which extend single-species distributions to community-level dynamics (Ovaskainen et al., 2017). Leibold et al. (2020) used these models in tandem with variation partitioning to explain the internal structure of simulated metacommunities. They found that this approach was a promising way to connect metacommunity pattern data to multiple assembly processes (Leibold et al., 2020). In the seed microbiology literature, Fort et al. (2021) used JSDMs to infer how maternal filtering and abiotic filtering contributed to seed mycobiome (fungal microbiome) variation in *Q. petraea* seeds (i.e., acorns). They found that fungal guild (e.g., pathogen, saprotroph, etc.) influenced which taxa varied with abiotic filters, with elevation selecting saprotrophs and seed specialists, and all taxon co-occurrences were positive associations (Fort et al., 2021). While JSDMs were not used in a metacommunity context for this study, and they are limited in their omission of abundance data, these models provide an integrative approach for looking at seed microbiome assembly.

Future directions

Multiple tools exist for exploring and exposing the effects and interactions of filtering, species interactions, dispersal, and drift on microbial community assembly of individual seeds at multiple spatial scales. However, future work can do a better job of integrating and connecting metacommunity ecology models to traditional seed microbial ecology studies at micro-, meso-and macro-scales. One technical challenge of taking this approach

pertains to the interrogation of microbial communities in individual seeds. Culture-based studies of individual seeds report low isolation frequencies, with most seeds containing zero or one microbial taxon (Newcombe et al., 2018, 2022). Additionally, most sequence-based studies to date pool seeds by fields or other groupings (Rezki et al., 2018; Kim et al., 2020; Mascot-Gómez et al., 2021; Morales Moreira et al., 2021; Wassermann et al., 2022). As exceptions, Bergmann and Busby (2021) and Fort et al. (2021) sequenced fungi from individual tree seeds and found that sequencing depth was fairly high. However, the tree species in these studies produce large seeds; sequence-based detection of microbiota might be more difficult in small-seeded species (e.g., *Arabidopsis*). Additionally, it is often difficult or impossible to treat individual seeds as independent since experimental treatments or predictors are often applied at the fruit or plant level. To resolve these issues, future work could focus on species where seeds are fertilized independently (*Helianthus annuus, Quercus* sp.), or one seed per fruit/plant could be sampled for large-seeded species. Alternatively, seeds could be pooled at the fruit or plant levels, since these are the levels where treatments are often applied and they sufficiently capture the variation in seed microbiota (Bintarti et al., 2022) while still allowing for a metacommunity approach at the meso-and macro-scales. The appropriate level of pooling should be selected based on the transmission pathway of interest (i.e., fruit level for floral transmission, plant level for vertical transmission; Bintarti et al., 2022). Finally, seeds of large-fruited species could be pooled by parts of the fruit/pod for spatially explicit sampling at the meso-and micro-scales. These scale-explicit pooling approaches, along with the use of additional methods at the meso-and micro-scales (e.g., microscopy of individual seeds, inoculation experiments with synthetic communities), will allow for characterization of microbiota at or near the individual seed level while mitigating issues of low DNA amounts and cross-contamination.

At the micro-scale, there are many opportunities to take a traits-based approach to host filtering of seed microbiota. Experiments can go beyond studying if plant traits have an effect to testing what these effects are (e.g., changes in microbial colonization rates, fitness, dispersal, and species interactions). These experiments could also take a microbial trait-based approach to host filtering (and other processes) and identify the genes required for successful transmission, which are still largely unknown (Chesneau et al., 2020). This could provide valuable insights into the genes required for transmission across the different pathways (Chesneau et al., 2020). Furthermore, metagenomic analyses across plants, populations, species, etc., could determine if these transmission-associated genes are common across metacommunities. Such information could show if there is functional conservation across microbial communities, even if they are taxonomically variable. Finally, micro-scale experiments can also test how microbial community assembly is impacted by the interplay between deterministic and stochastic processes.

In addition to these tests of microbial and plant trait impacts, experiments testing the role of dispersal in seed microbial community assembly among spatial scales should be conducted. At the micro-scale, experiments using synthetic microbial communities on stigmas with varying chemistry and topography can demonstrate how dispersal and selection occur between flowers and seeds, and what the role is of plant genetics and microbial adaptations. At the macro-scale, pollinator exclusion experiments similar to those in Prado et al. (2020) could be conducted across sites in natural landscapes. By using sites at varying distances and connectivity levels from each other, and analyzing both within- and among-site seed microbial community variation, one may obtain new information about how pollinators and patch connectivity impact multi-scale dispersal ability. These proposed studies would elucidate how dispersal contributes to metacommunity assembly among spatial scales.

Along with these single-process studies, we envision studying the interactions between processes through both observational and experimental studies. As JSDMs continue to be refined to model nested and continuous metacommunities, they will provide a way to analyze seed microbiome patterns and their associated assembly processes that is more sophisticated than previous modeling approaches. Additionally, priority effect experiments conducted at multiple points in the seed life cycle (e.g., pollination, dispersal, germination) may reveal how historical contingencies impact seed microbiome assembly throughout the seed life cycle. Such experiments would also test the Primary Symbiont Hypothesis (Newcombe et al., 2018), which argues that seed communities are dominated by a single microbe with significant functional consequences for the plant.

Finally, questions will need to be asked about seed microbiome assembly that go beyond just testing for spatial mechanisms. Primary among these questions is: what fitness benefit does transmission into seeds provide to microbes and their host plants? Such a question gets at the eco-evolutionary dynamics in these microbial metacommunities, which can have long-term consequences for both microbes and plants. Because microbial communities behave and evolve at shorter time-scales than macro-organisms (Nemergut et al., 2013), it is feasible to design simple experiments testing how microbes evolve in response to plant defenses, nutrient availability, and micromorphology. Such eco-evolutionary studies may have applications in understanding microbial community shifts with crop domestication (Kim et al., 2020). Additionally, both microbes and seeds have dormant stages, which can impact metacommunity dynamics through tradeoffs with dispersal and delayed responses to environmental conditions (Wisnoski et al., 2019). The role of dormancy in seed and plant microbial metacommunity assembly has yet to be explored, so there is much room to study how dormancy impacts these systems over longer temporal scales. Finally, a hot topic in plant microbiome research is how to modify plant microbial communities for climate resilience and other beneficial traits (Busby et al., 2017; Mitter et al., 2017). However, the impacts of climate change-associated disturbances on plant microbiomes

have been limited to pattern-based studies in leaves and roots (e.g., Kivlin et al., 2013). As such, more work can be done on how disturbances alter seed microbiome assembly processes and outcomes.

Conclusion

Seed microbial community assembly, much like the assembly of microbial communities associated with other plant parts, is the result of complex interactions between multi-scale processes. Metacommunity ecology provides a conceptual framework for identifying these processes, and provides new statistical and theoretical tools for testing their interactions. We advocate for the spatially explicit, multi-scale study of seed microbial community assembly, with emphasis on the effects of plant topography and chemistry on micro-scale dispersal and persistence, the role of pollinators and seed dispersers on macro-scale dispersal, and the interactions between processes. These new avenues of study will provide a more generalizable understanding of seed microbiome assembly, with potential applications in plant conservation and sustainable agriculture.

Author contributions

GB wrote and revised the manuscript and created the manuscript figure and tables. JL revised the manuscript and associated tables. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.877519/full#supplementary-material

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Arbuscular Mycorrhizal Fungi Taxa Show Variable Patterns of Micro-Scale Dispersal in Prairie Restorations

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Human land use disturbance is a major contributor to the loss of natural plant communities, and this is particularly true in areas used for agriculture, such as the Midwestern tallgrass prairies of the United States. Previous work has shown that arbuscular mycorrhizal fungi (AMF) additions can increase native plant survival and success in plant community restorations, but the dispersal of AMF in these systems is poorly understood. In this study, we examined the dispersal of AMF taxa inoculated into four tallgrass prairie restorations. At each site, we inoculated native plant species with greenhouse-cultured native AMF taxa or whole soil collected from a nearby unplowed prairie. We monitored AMF dispersal, AMF biomass, plant growth, and plant community composition, at different distances from inoculation. In two sites, we assessed the role of plant hosts in dispersal, by placing known AMF hosts in a "bridge" and "island" pattern on either side of the inoculation points. We found that AMF taxa differ in their dispersal ability, with some taxa spreading to 2-m in the first year and others remaining closer to the inoculation point. We also found evidence that AMF spread altered non-inoculated neighboring plant growth and community composition in certain sites. These results represent the most comprehensive attempt to date to evaluate AMF spread.

Keywords: dispersal, restoration ecology, grassland, inocula, plant–microbial interactions, arbuscular mycorrhizal fungi

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INTRODUCTION

In recent decades, the role of plant–soil microbial interactions in structuring plant communities has gained attention in the field of ecology. Feedback between plants and the soil community can impact plant succession, invasion, community composition, and plant diversity (Van der Putten et al., 2013; Bever et al., 2015). The dynamics of plant–soil feedback can depend on the level of dispersal of both plant and soil organisms (Molofsky and Bever, 2002; Bever et al., 2012; Michaels et al., 2020). This is particularly true of mutualisms, in which local dispersal is associated with increased stability (Bever et al., 2009; Mack, 2012). Microbial dispersal limitation can influence biogeography (Delavaux et al., 2019, 2021) and succession and the speed of recovery

post-disturbance (Middleton and Bever, 2012; Kardol et al., 2014; Bauer et al., 2015; Wubs et al., 2016).

In many degraded ecosystems, restoring soil microbial communities and plant–microbial interactions have been proposed as essential to re-establishing complete and diverse native plant communities (Koziol et al., 2018). Specifically, in grasslands, arbuscular mycorrhizal fungi (AMF) may play an important role in structuring plant communities. Late successional plant species are more likely to rely on AMF and show AMF species-specific growth responses compared to early successional native or non-native plant species (Koziol and Bever, 2015, 2016, 2017; Cheeke et al., 2019). However, AMF community composition in grasslands with a history of disturbance remains different from those of remnant prairies, even in cases when remnant undisturbed prairies are nearby (House and Bever, 2018; Tipton et al., 2018). Some rare AMF taxa remain absent in disturbed sites altogether, suggesting that some AMF species do not readily re-establish disturbed sites on their own (House and Bever, 2018; Tipton et al., 2018). The absence or decreasing abundance of certain AMF taxa could explain why in sites with a history of large soil disturbances, late-successional or rare plant species often do not re-establish, even with abundant seed addition (Martin et al., 2005; Polley et al., 2005).

Although dispersal mechanisms are well known for higher-order organisms (Clobert et al., 2012), AMF dispersal mechanisms are still poorly understood (Paz et al., 2020). Studies suggest that some AMF spores can disperse *via* wind, but most wind-dispersed spores are small spore species belonging to Glomeraceae, and survival of aerially dispersed spores can be low (Warner et al., 1987; Allen et al., 1989; Egan et al., 2014; Chaudhary et al., 2020). A few species of *Glomeromycota* produce specialized subterranean sporocarps that are attractive to rodents, which act as dispersal agents (Gehring et al., 2002; Mangan and Adler, 2002). All AMF can also disperse *via* hyphal spread in the soil, and for many taxa, this may be the primary mechanism of dispersal. The rate of spread through hyphal growth is not well characterized (Paz et al., 2020), but is likely dependent on the local environment. As AMF are obligate mutualists, compatible plant hosts are necessary for their survival and the availability of quality host roots will likely influence the hyphal rate of spread. However, although still not well characterized, some studies suggest that AM hyphae can spread out into the soil to at least 1-m distances with few plant hosts (Chaudhary et al., 2014). The rate of spread will also likely vary between AMF taxa, as AMF display different growth strategies, with some producing more or less external hyphae than others (Abbott and Robson, 1985; Friese and Allen, 1991), and vary in their response to particular host species (Bever et al., 1996). Therefore, certain AMF taxa may be able to explore soil at greater distances than others.

When native plant species in restored prairie sites are inoculated with AMF collected from the remnant prairie, benefits can extend to non-inoculated plants at least 2-m away from the inocula source within the first growing season (Middleton and Bever, 2012; Middleton et al., 2015). This suggests that AMF hyphae can move meters through the soil in one growing season. However, the identity of the AMF that has spread and whether particular taxonomic groups are more likely to spread is not known. Moreover, it is unclear whether these hyphae move *via* infections of new hosts, moving from one plant to the next, or if individual hyphae can spread long distances without intermediate plant hosts. Should they be moved through intermediate hosts, then host quality in the plant community would modify the rates of the AMF hyphal spread. For example, we can hypothesize that certain AMF species may spread faster and farther with appropriate hosts along the way. Alternatively, some AMF species may be able to spread long distances independent of the plant community composition.

In this study, we integrate field inoculation of nurse plants with environmental sequencing to evaluate the rate of spread of native AMF from points of inoculation into the surrounding plant community during restoration. We placed native AMF inocula along with native nurse plants into sites once dominated by non-native grass species. Using environmental sequencing, we tracked the spread of AMF taxa present in the inocula in the plant community to determine how far and quickly AMF spread from inoculation points into the soil. We tracked the spread of AMF in areas with and without established "bridges" of native plant hosts, to determine the role of host quality in AMF movement through the soil. We also measured changes in the plant community and relative abundance of AMF using phospholipid fatty acid (PLFA) analysis to determine how the spread of inoculated AMF may alter above- and below-ground communities over time.

MATERIALS AND METHODS

Study System

We established prairie reconstruction experiments at three different field sites across the Midwest in both 2014 and 2015: Chanute Air Force Base near Rantoul, IL (Chanute, 40°28′76.96″N, −88°13′36.23″W, 2014), Ft. Riley Military Base in Ft. Riley, KS (Ft. Riley, 2014), and Tinker Air Force Base near Oklahoma City, OK (Tinker, 35°24′54.96″N 97°24′37.02″W, 2015). Chanute was dominated by C_3 *Schedonorus arundinaceus*. At Ft. Riley, we established two different sites with two unique grass dominants: C_3 *Bromus inermis* (Ft. Riley *B. inermis*) and C_4 *Bothriochloa bladhii* (Ft. Riley *B. bladhii*). Tinker was dominated by C_4 *Bothriochloa ischaemum*. Because of variation in dominant exotic invaders and other environmental variations between sites, we used different plant removal methods in each site before prairie planting (**Supplementary Table S1**), including disking, spraying 5% glyphosate, or installing black tarps (4.88 × 4.88 m) to solarize plots following the methods of Upadhyaya and Blackshaw (2007).

Inocula

Whole Soil Inocula

To compare the effects of pure AMF inocula to inocula containing the full suite of soil microbes, we collected and created a "whole soil" treatment for our experiment. We collected whole soil inocula at remnant prairies ranging from 0 to 25 km from each field site (Koziol et al., 2021). At remnant prairies, we collected 0.5 L for each of the five randomized samples of field soil

to a depth of approximately 10 cm. The soil was sieved through an 8 mm sieve and stored at 4°C before being used as inoculum. Both Ft. Riley sites received the same whole soil inocula. Sub-samples from each whole soil inocula were stored at−20°C for molecular identification.

AMF Inocula

AMF inocula used in this study contained AMF collected and cultured from remnant prairies. In 2012, we extracted spores from the field-collected remnant prairie soil from the same locations used for the whole soil treatments using the methods of Morton et al. (1993). AMF species were separated microscopically. We created single spore cultures using the methods of Koziol and Bever (2016) (See **Supplementary Material**). Before field inoculation, multiple spore cultures from each site were mixed to create three site-specific cultures for each of the restoration sites (Ft. Riley, Tinker, and Chanute). Sub-samples from each site-specific mixed culture were stored at −20°C for molecular identification.

Experimental Design

In May 2014 at Chanute and Ft. Riley and in May 2015 at Tinker, we assigned treatments to plots using a randomized block design. Chanute contained 9 blocks and 27 plots, while all other sites contained 7 blocks and 21 plots in total (69 plots total across all four sites). Each of the three plots in each spatially stratified block was randomly assigned one of the following treatments: nurse plants established with AMF inocula, nurse plants established with whole prairie soil inocula, or nurse plants established with sterilized control soil (hereafter referred to as non-inoculated). Because sites spread across a large rainfall and geographic gradient, different nurse plant species were used in various sites (**Supplementary Table S1**; **Supplementary Methods S2** for nurse plant growth and inoculation). We planted 16 nurse plants in a row down the center of each of the 16-m^2 plots (**Figure 1**). Each plot received four replicates of each nurse plant species of the corresponding treatment (16 nurse plants total). Replicates were repeated in the same order across each plot in each site. At Chanute and Tinker, we also planted grass test plants inoculated with sterilized control soil (non-inoculated) on either side of the nurse plant row (*Andropogon gerardii* in Tinker and *Schizachyrium scoparium* in Chanute). On one randomly selected side, we planted three test plants in three rows 0.5-m apart (bridge side, **Figure 1**). On the opposite side, we planted three test plants 2-m away from the nurse plants (island treatment).

Soil and Root Sampling

Each growing season, we collected soil and root samples for molecular and phospholipid fatty acid (PLFA)/neutral lipid fatty acid (NLFA) analysis (**Supplementary Table S2**). In sites without test plants, we collected at the nurse plant row, 0.5-m away from the nurse plant row, 1.0-m away, and 2-m away from the nurse plant row. For Chanute and Tinker, we collected at the nurse plant row, 0.5-m away on the bridge side to collect near test plants, and 2-m away on both the island and bridge side (both years at Chanute and the second year at Tinker, **Figure 1**). We collected four 2-cm diameter samples approximately 10 cm deep using a soil core along each sampling row. Sampling was concentrated away from the plot edges. Soil corers were cleaned with 80% ethanol between each sampling row. Soil corers for each row were mixed and split into sub-samples fro molecular and PLFA/NLFA analysis.

Molecular Analysis

Fresh roots from all 2014 samples and Chanute samples in 2015 were used for DNA extraction. For all other 2015 samples and inocula samples, the soil corers containing roots were frozen before extraction, and DNA was extracted from soil sample/root mixes. DNA was extracted from 0.025 g roots or 0.25 g of soil/roots using the Power Soil Kit (Qiagen, Carlsbad, CA) with a modified beat beating step. To amplify AMF-specific sequences of the large subunit (LSU), we conducted PCR with the primer pair LROR (Bunyard et al., 1994) and FLR2 (van Tuinen et al., 1998) amplifying an approximately 850-bp region. PCR amplification procedure was as follows: 94°C for 5 min; then 35 cycles of (1) 94°C for 30 s, (2) 48°C for 30 s, and (3) 72°C for 45 s; and ending with 72°C for 10 min. We purified PCR products with the AMPure XP bead system (Beckman Coulter, Indianapolis, IN). An equimolar amount from each sample was pooled and sequenced on the Illumina MiSeq platform to produce two non-overlapping 300 bp reads (Center for Genomics and Bioinformatics, Indiana University).

The resulting sequences were quality screened (quality score = 10) per read pair, and we removed chimeras using the –uchime_denovo function in VSEARCH (Rognes et al., 2016). Because individual AMF cells contain high levels of rDNA sequence variation (House et al., 2016) and we were interested in the movement of isolates rather than genetic variants within isolates, we analyzed OTUs rather than ASVs. We clustered the resulting sequences using AbundantOTU (Ye, 2010), using a 97% sequence similarity threshold. We then added and aligned the consensus OTU sequences to a reference alignment of AMF fungal sequences (House et al., 2016) using MAFFT (Katoh and Standley, 2013). This reference database consisted of sequences from the 350-bp D2 region of the nuclear-large subunit (LSU) rRNA gene in a previously published, Krüger et al. (2012) database with additional supplemental sequences from GenBank with confident species identification (House et al., 2016). We removed sequences that aligned poorly with the reference sequences and then created a rooted Maximum Likelihood (ML) phylogeny with the remaining consensus sequences and the reference sequences using RAxML (Stamatakis, 2014, RRID:SCR_006086), with *Mortierella elongata* as an outgroup. The rooted tree was used to remove any other OTU that did not cluster within the Glomeromycota database. All analyses were done for each site separately. Sequence counts were turned into proportions (total number of OTU sequences out of the total number of sequences in that sample) to account for variation in sequence number among samples.

To determine shared OTUs among sites, sequence data for each of the identified OTUs in all sites was compiled in alignment with the same in-house AMF database (House et al., 2016) to generate a maximum likelihood phylogenetic tree. Aligned sequences were uploaded to the CIPRES science gateway

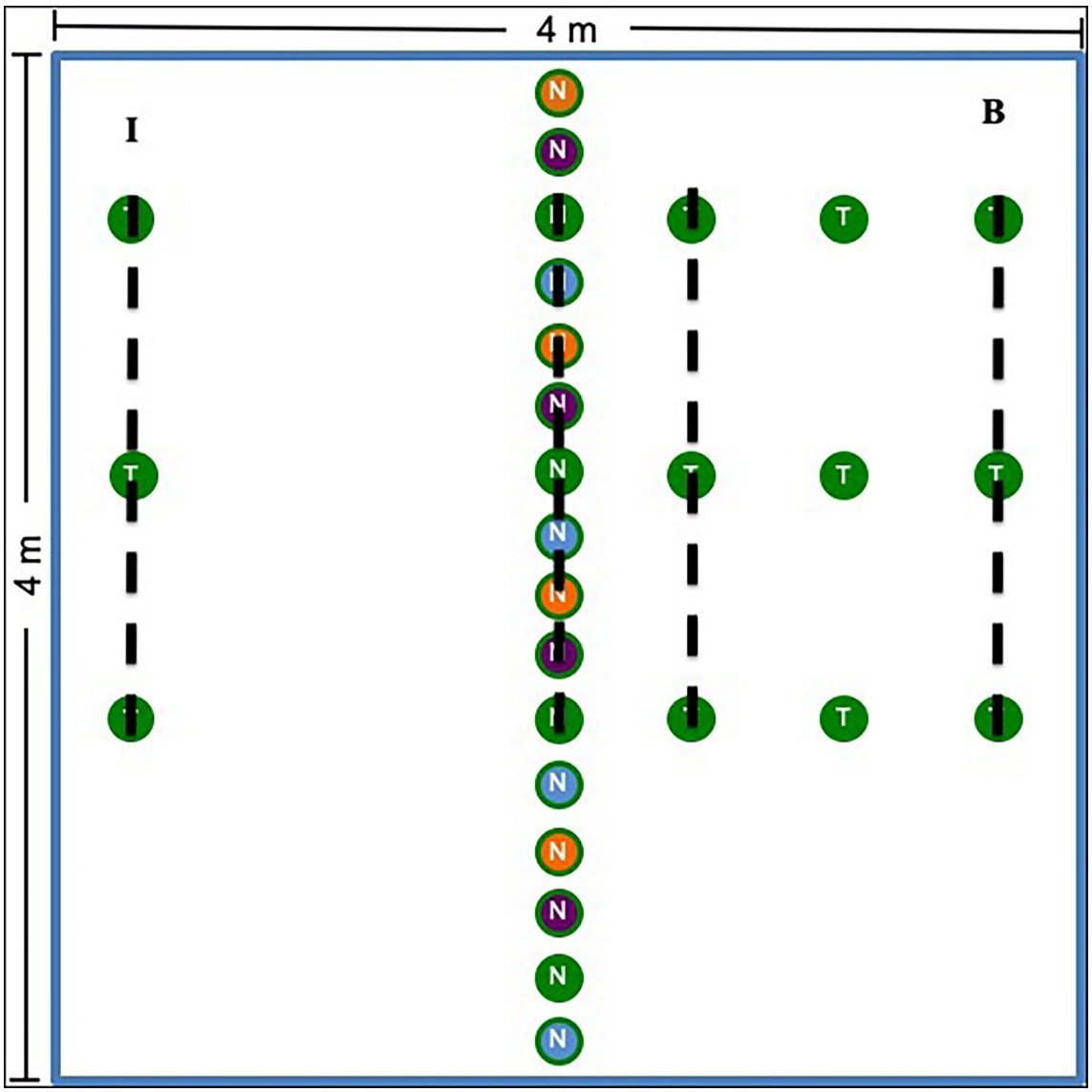

FIGURE 1 | Plot design for the experiment. The center row represents the nurse plant row, which was either inoculated with whole prairie soil, prairie AMF only, or sterilized control soil (non-inoculated) depending on the plot treatment. The colors represent species. Four species were used as nurse plants. In Chanute and Tinker, test plants (*S. scoparium* at Chanute and *A. gerardii* at Tinker) were planted in rows 0.5-, 1-, and 2-m away from the nurse plant row. On one side, only the 2-m plants were added (I), whereas on the other side (B), plants were placed at 0.5-, 1-, and 2-m away from the nurse plant row. The dotted lines represent where soil and root samples were taken for molecular analysis. Plant community data were collected 0.5-, 1-, and 2-m away from the nurse plant row, and the side on which these data were taken was selected randomly each year and varied by site.

(Miller et al., 2010, RRID:SCR_008439) for analysis *via* RaxML (Stamatakis, 2014, RRID:SCR_006086)) using default settings with the following changes: 1,000 bootstrap iterations and print bootstrap values. Criteria for determining identical OTUs from the phylogenetic tree were as follows: (1) starting from the terminal node, collapse branches if support values are <70, otherwise retain original branching pattern, (2) cannot collapse branches where branching pattern is unresolved/polyphyletic, and (3) collapse split branches with ≥ 70 if there is no genetic distance between OTUs. Because one species of AMF can contain multiple OTUs (House et al., 2016), these collapsed OTUs often contained multiple OTUs from the same site. We call these collapsed OTUs "virtual OTUs" as they are similar in purpose to the virtual taxa designated by Opik et al. in the MaarjAM database (Opik et al., 2010).

Other Metrics to Measure the Impact of Inoculation and Dispersal

Relative AMF Biomass

We also assessed how inocula dispersed and impacted the surrounding plant and soil community. Phospholipid fatty

acid (PLFA) and neutral lipid fatty acid (NLFA) biomarker analyses were conducted to determine the relative abundances of extra-radical AMF. Phospholipid fatty acids are constituents of biological membranes that can be used to estimate the biomass of fungi (Tunlid and White, 1992), while neutral lipid fatty acids act as storage products and serve as the primary energy reserve in fungi (Larsen and Bødker, 2001). Total lipids were extracted from freeze-dried soil samples using a modification of the Bligh and Dyer (1959) extraction method described in detail by Allison and Miller (2005). The fatty acids were then analyzed by gas chromatography and mass spectrometry detection using Agilent GC 7890A/MS 5975C. Biomarkers 16:1ω5c, 22:1ω13 (tightly correlated with *Glomus* spp.), and 20:1ω9 (tightly correlated with *Gigaspora* spp.) were used to assess extra-radical AMF biomass.

Plant Community

We also assessed plant community identity along the nurse plant row, and 0.5 m, 1.0-, 1.5-, and 2.0-m away from the nurse plant row in the growing season between 2014 and 2016 using the point-intersect method (Middleton and Bever, 2012). The plant community was always assessed on the island side of the plot for Chanute, but at Tinker, the side (B or I) was randomly chosen each year.

Test Plants

At Chanute and Tinker, test plant species at different distances from the nurse plant row on both the island and bridge side were measured each growing season. Although these two sites contained different test plant species (*S. scoparium* at Chanute and *A. gerardii* at Tinker), we recorded leaf count and height at each site.

Statistical Analysis

Dispersal of Inoculated OTUs

For each site, we first determined which AMF OTUs were present in the AMF and whole soil inocula used in each experiment. OTUs present in our inocula were often present in sites before inoculation or in sterile plots. Because of this, we could not use simple presence/absence at various distances and treatments to determine dispersal. We used separate Multivariate Analysis of Variance (MANOVA) tests to determine whether inoculated OTUs were more abundant in inoculated nurse plant rows compared to non-inoculated control nurse plant rows. We then used a separate MANOVA to determine whether inoculated OTUs decreased with distance from inoculated nurse plant rows, to determine the percentage of OTUs fitting into particular spread categories. Finally, for Chanute and Tinker, we used a separate MANOVA to determine whether inoculated OTUs were in greater abundance on the bridge compared to the island side at 2-m away. For all MANOVAs, we analyzed the site and year separately. All MANOVAs were performed in SAS (RRID:SCR_008567).

We categorized all present OTUs into the following spread categories, using contrasts of marginal means between each distance from the distance MANOVAs:

(1) No spread from the nurse plant row: OTU made up a significantly higher proportion of the AMF community in the nurse plant row compared to both 0.5- and 2-m away
(2) Spread 0.5-m away from the nurse plant row: OTU relative abundance in nurse plant row and 0.5-m away were not significantly different, but the OTU made up a significantly lower proportion of the AMF community 2-m away compared to other distances

We then have three categories involving varying levels of confidence in spread to 2-m away:

(3) Distance decay: the nurse plant row and 0.5-m away were not significantly different, 0.5-m and 2-m away were not significantly different, but the OTU made up a significantly higher proportion of the AMF community in the nurse plant row compared to 2-m away, or overall decrease with distance from the nurse plant row (although there was no statistically significant trend with distance, an overall distance effect was present, with the OTU decreasing in proportion to the rest of the community with distance)
(4) Spread to 2-m away: showed no significant differences across distance and inoculated nurse plant rows had higher relative abundance compared to non-inoculated nurse plant rows and
(5) Inconclusive distance effects (significant differences between different distances, but not displaying any clear distance effects or no differences across a distance or between inoculated and non-inoculated nurse plant rows).

At Chanute and Tinker, if there was a significantly higher proportion of the OTU on the bridge side compared to the island at 2-m away from the nurse plant row, and there was also no significant difference between the varying distances on the bridge side, we considered this spread to 2-m away. This information was assessed for Chanute in both years 1 and 2, and only the second year for Tinker. In the results, OTUs that spread to 2-m away include results from this analysis at these two sites.

We counted each OTU present in either the AMF or whole soil inocula as one trial. If an OTU was present in both, its spread category was assessed for both the AMF-inoculated plots and whole soil–inoculated plots. This resulted in 185 trials at Chanute (61 and 124 OTUs in the AMF and whole soil inocula, respectively), 133 trials for each Ft. Riley site (46 and 87 OTUs in the AMF and whole soil inocula, respectively), and 103 trials for Tinker (15 and 88 OTUs in the AMF and whole soil inocula, respectively). We used these trials to determine the proportion of OTUs in each spread category at each site and year.

Dispersal of OTUs Shared Among Sites

Using the virtual OTUs determined in the phylogenetic tree for all sites, we determined whether the same virtual OTU behaved similarly across sites. We assessed the proportion of trials for each virtual OTU that fit into one dominant spread category. As in other analyses, a trial is an OTU in a certain treatment, site, and year. We eliminated all trials that fit in the unknown spread category and then selected virtual OTUs that had at least 2 or more trials (19 total taxa) from at least two or

more different original OTUs. We then examined whether these virtual OTUs had more than half of their trials in one particular spread category.

Dispersal of Glomeromycota Taxonomic Families

We tested for consistent differences in patterns of spread with different phylogenetic groupings using two approaches. First, using our entire dataset, we tested whether OTUs whose spread could be determined (i.e., spread categories 1–4), differed consistently between phylogenetic groupings. In practice, our power was limited by the few OTUs that could be confirmed to have spread and we, therefore, grouped all OTUs that could be determined to have spread out from their nurse plant (spread categories 2–4) and contrasted the likelihood of spreading to that of the absence of spread (spread category 0). Differences in the number of OTUs that spread between AMF families were tested using Chi-square tests in proc genmod in SAS (RRID:SCR_00856). This approach treated each OTU as an independent. Our second approach focused on the virtual OTUs that we were able to link between years, which allowed assessment of whether individual virtual OTUs spread across years. For this analysis, we used a generalized mixed model that tested the fixed effects of the AMF family and its interaction with Site and Year, and we identified OTU within the AMF family and interactions with year and site as a random effect. We used binomial error and logit links Proc Glimmix in SAS (RRID:SCR_00856).

Relative AMF Biomass

To assess the effects of inoculation on relative AMF abundances (through PLFA and NLFA analyses), generalized linear models (GLMs) were employed with the site, inoculum, distance from nurse plant row, and year as the main effects. Because of the left-skewed, positive nature of the data, GLMs with a gamma error distribution and log link were used.

To assess how AMF biomass varied between island and bridge sides for both Tinker and Chanute, we ran a separate generalized linear model for each site. In each model, we included treatment and side (island vs. bridge). Non-inoculated control plots were included at Tinker but not in the Chanute analysis. Both analyses used PLFA and NLFA data and were performed in base R (version 4.1.0).

Plant Community

Because plant community composition data were collected across the distance from the nurse plant row for all three treatments, we were able to analyze plant community richness, diversity, evenness, and composition across the distance for sterile control, whole soil, and AM fungi inocula plots in the one analysis. We used permutational multivariate analysis of variance (PERMANOVA) to assess how the plant community changed across sites, years, soil treatment, distance from nurse plant rows, and all interactions. We also included an experimental block within each site as a control. Because site and year were important in predicting plant community composition (see Results section), we also used PERMANOVA to assess plant community composition across inoculation treatment and distance from nurse plant row in specific sites and years. All PERMANOVAs were conducted using the adonis function in R.

We also used the vegan package in R to calculate richness, Shannon diversity, and evenness. Then, we used mixed effects models to assess how treatment, distance from nurse plant row, and the interaction impacted richness, Shannon diversity, and evenness across all sites and years. All mixed effect models had plot and block nested within site. We ran separate mixed effects models for specific sites and years to assess variation seen in the larger model. All mixed effect models were conducted using the PROC MIXED function in SAS.

Test Plants

We used a mixed effects model to assess how the test plant size (height and leaf number) at Chanute and Tinker varied by treatment, distance from the nurse plant row, and between the island and bridge side using the PROC MIXED function in SAS. Both leaf number and height were log transformed to meet assumptions of normality if necessary. We used a generalized linear mixed effects model to assess survival by the treatment, distance from nurse plant row, and between island and bridge in Chanute and Tinker using the PROC GLIMMIX function in SAS. Survival was estimated as a proportion of plants at each distance and bridge/island location for each plot, and then logit transformed to meet assumptions of normality. We conducted a separate analysis for each year for both plant size and survival.

RESULTS

Molecular Analysis of Dispersal of Inoculated OTUs

Dispersal of Inoculated OTUs

The total number of observed AMF OTUs in our inocula treatments varied for each site: Chanute-185, Tinker-79, and Ft. Riley *B. inermis* and *B. bladhii*-133. The number of OTUs that were present in either one inocula treatment or that were common among both inocula treatments also varied between sites (**Table 1**). The ability to detect the presence of inocula OTUs and major spread patterns across inocula OTUs varied by site and year. For each site, OTUs that spread to either 0.5 or 2 m, failed to spread or were unable to be distinguished as having spread or not

TABLE 1 | The number of OTUs found in both AMF and Whole Soil inoculum treatment regimens at each site and the number of shared OTUs that showed different spread patterns in the two different treatments.

Study site	Number of OTUs found in AMF and whole soil treatments	Number of OTUs in different spread categories in AMF vs. whole soil
Ft. Riley *Bothriochloa bladhii*	42	16
Ft. Riley *Bromus inermis*	42	15
Tinker	11	2
Chanute	44	25

For a detailed assessment of OTUs, see ***Supplementary Table S3****.*

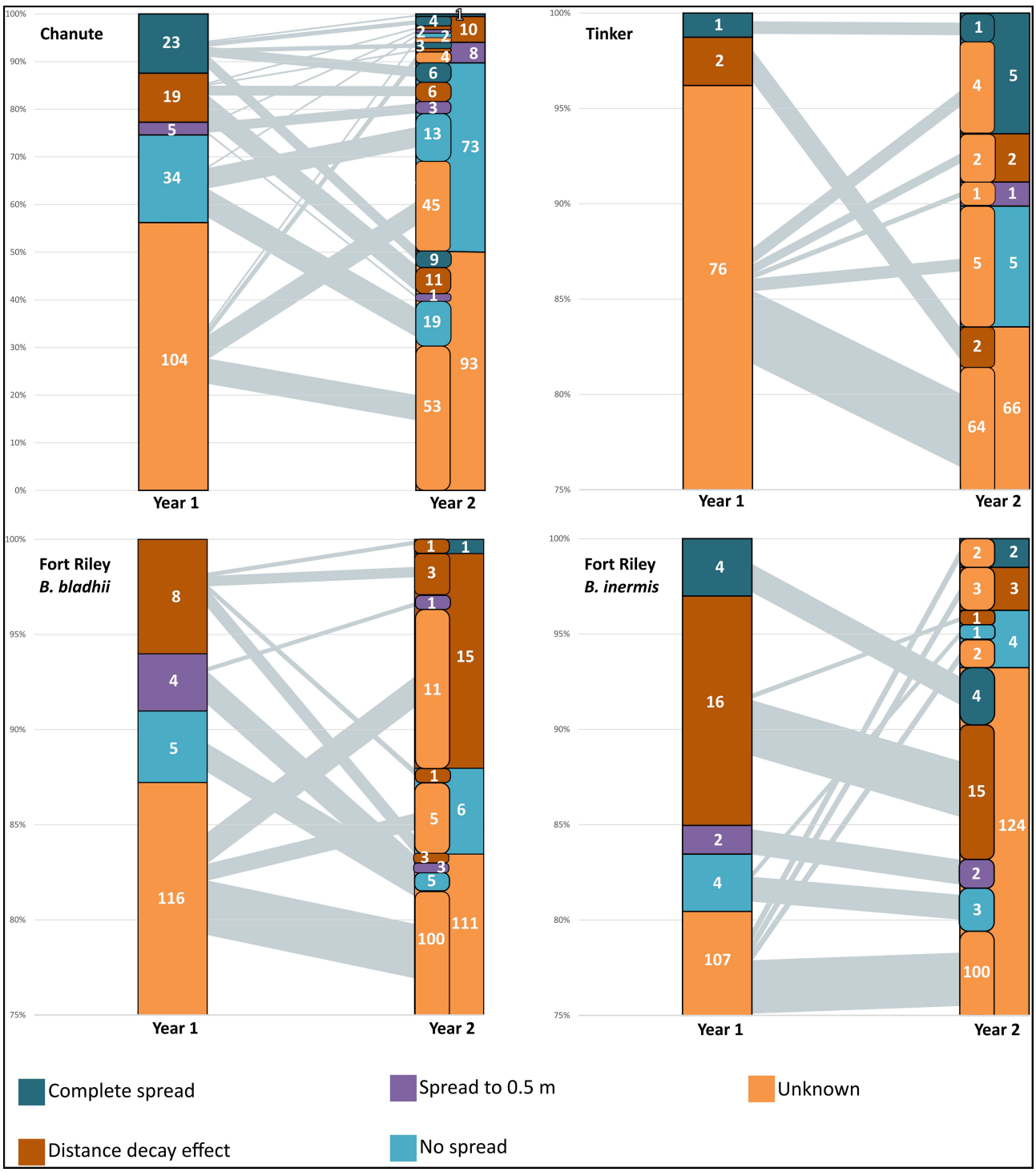

FIGURE 2 | Spread of AMF OTUs from nurse plant row by sites in years 1 and 2. The number of OTUs in each spread category is presented. The left-side column in year 2 represents the contribution of OTUs from year 1 categories in each year 2 category. Black lines from year 1 to year 2 represent the amount of OTUs from categories in the first year to the second—the thicker the line, the greater the amount of OTUs from year 1 category in the year 2 category. The largest category in all sites was unknown, and there was evidence of the complete spread of some AMF in all sites by the second year.

spread (unknown) in the first year often shifted into other spread categories in the second year (**Figure 2**). The largest category at every site was the unknown or undetermined spread category.

At Chanute in the first year, inocula OTUs overall made up a higher proportion of the AMF community in whole soil nurse plant rows compared to rows of non-inoculated nurse

plants ($F_{1,16} = 5.6$, $p < 0.05$). Additionally, this proportion decreased precipitously with increasing distance from the nurse plant row for inoculated plots overall ($F^5_{38} = 6.5$, $p < 0.001$). Although the unknown spread category had the largest number of OTUs (104 out of 185 total, **Figure 2**), 34 OTUs did not significantly spread away from the nurse plant row, 5 spread to 0.5 m, 19 showed distance decay (declined with distance), and 23 OTUs were confirmed to spread at least 2-m away from the nurse plant row (**Figure 2**). In the second year, inoculated nurse plant rows had a marginally significantly greater abundance of inoculated OTUs compared to non-inoculated nurse plant rows ($F^1_{16} = 3.9$, $p < 0.1$). More specifically, the AMF-only inoculation plots had a greater proportion of inocula OTUs compared to non-inoculated plots ($F^1_{16} = 4.5$, $p = 0.05$). The overall distance effect remained in the second year, with an abundance of inoculated OTUs decreasing with distance from the nurse plant row ($F^5_{37} = 30.8$, $p < 0.0001$). The unknown category was again the largest (93 OTUs out of 185 total), but 73 OTUs did not spread away from the nurse plant row, with 13 of those remaining in that category from the following year. Two OTUs spread further in the second year, while 22 OTUs moved backward *via* the spread category, being detected closer to the nurse plant row in the second year compared to the first year (**Figure 2**).

At Ft. Riley *B. inermis*, Ft. Riley *B. bladhii*, and Tinker, inoculated OTUs were not more abundant in inoculated nurse plant rows compared to non-inoculated nurse plant rows, although at Tinker they were marginally significantly more abundant in whole soil compared to non-inoculated control nurse plant rows in the second year ($F^1_{52} = 3.1$, $p = 0.08$). At Tinker in the second year, inocula OTUs did vary marginally across distance ($F^5_{30} = 2.48$, $p = 0.054$). This was driven by differences between the nurse plant row and 0.5 m distance in whole soil plots ($F^1_{30} = 4.79$, $p < 0.05$) and, overall, differences in inoculated plots between the nurse plant row and 0.5 m ($F^1_{30} = 3.2$, $p < 0.1$) and 0.5-m and 2.0-m ($F^1_{30} = 3.8$, $p < 0.1$).

In all three of these sites, just as in Chanute, OTUs were in different spread categories for each site and year (**Figure 2**), but OTUs with an unknown or undetectable spread pattern made up 80–95% of the OTUs in both years. For Ft. Riley *B. bladhii*, Ft. Riley *B inermis*, and Tinker, 89, 92, and 100%, respectively, of the OTUs in a detectable spread category in the first year were in the unknown category in the second year (**Figure 2**).

At each study site, some proportion of OTUs were found in both the whole soil and the AMF inoculum treatments. These OTUs were recorded and often showed different spread characteristics in whole vs. AMF-inoculated plots (**Supplementary Table S3**). Chanute had the largest proportion of OTUs in both whole and AMF inocula in different spread categories (25 of 44).

At Chanute, there was a marginally significantly higher proportion of inoculated OTUs at the Bridge side 2-m away from the nurse plant row compared to the island side ($F^1_{21} = 4.1$, $p = 0.06$) in the first year, but no significant differences overall in the second year. At Tinker, there was not a significant difference in the abundance of the inocula OTUs between the island and bridge overall.

Dispersal of Glomeromycota Taxonomic Families

Taxonomic placement of OTUs found in the different spread categories resulted in 105 OTUs identified to family in Chanute, 74 in Tinker, and 125 in Ft. Riley. These data were then used to determine spread patterns at each site in each AMF family (**Figure 3**). The greatest number of OTUs to spread through the soil in the three spread categories occurred in the Glomeraceae and Claroideoglomeraceae families at Chanute (18 and 9, respectively, year 1), and the Glomeraceae at the Ft. Riley *B. inermis* site (18 in year 1). OTUs assigned to families were used in a statistical analysis of the spread by the family across the sites each year. No strong relationship was found between any family and their ability to spread in the soil substrate. At Chanute, which had the most data, Paraglomerales tended to be more likely to spread from the nurse plant row than Glomerales ($\chi^2 = 3.48$, $p = 0.06$). Common OTUs identified across years within the Chanute site were more likely to spread from the nurse plant row in the second year ($F^1_{35} = 12.38$, $p = 0.001$, **Figure 4**; **Supplementary Table S4**) and Glomeraceae tended to be more likely to spread from the nurse plant row than Claroideoglomeraceae ($F^1_{35} = 3.09$, $p = 0.088$). PLFA biomarkers indicated no significant shift in biomass of genera *Glomus* and *Gigaspora*.

Spread of OTUs Shared Among Sites

Of those 15 OTUs containing two or more trials across more than one site, 13 of them showed more than 50% of their trials in one specific category, which is ∼87% of those OTUs shared among sites (**Table 2**). Of these, 11 OTUs were dominated by the no spread category, one was dominated by the spread to 0.5 category, and 2 were dominated by the distance decay category.

Other Metrics to Measure the Impact of Inoculation and Dispersal

Relative AMF Biomass

A significant three-way interaction between site, distance from nurse plant row, and the year was detected when utilizing both NLFA ($F^5_{61} = 2.80$, $p < 0.03$; **Supplementary Table S5**) and PLFA ($F^5_{54} = 38.5$, $p < 0.0001$; **Supplementary Table S6**), with general decreases from year 1 to 2, as well as with increasing distances from the nurse plant row (**Figure 5**; **Supplementary Figure S1**).

When comparing the bridge vs. the island side of plots at 1.5 m from the nurse plant row at Tinker through NLFA, no significant effects of side or treatment, or the interaction, were detected (**Supplementary Figure S2**). However, through PLFA analyses, there was a significant side to treatment interaction ($F^2_{54} = 3.1$, $p < 0.05$). In both the whole soil and sterile control plots, AMF biomass at 1.5-m away was higher on the island side compared to the bridge side. But, in the AMF plots, the bridge side had slightly higher AMF biomass than the island side (**Supplementary Figure S3**). At Chanute, there was a significant effect of side, with significantly greater relative AMF biomass on island sides ($F^2_{54} = 6.68$, $p = 0.01$), and this difference was more noticeable in the AMF treatment, relative to whole soil (**Supplementary Figure S4**). There was no significant difference in relative AMF biomass measured through PLFAs between the island and bridge side at Chanute (**Supplementary Figure S5**).

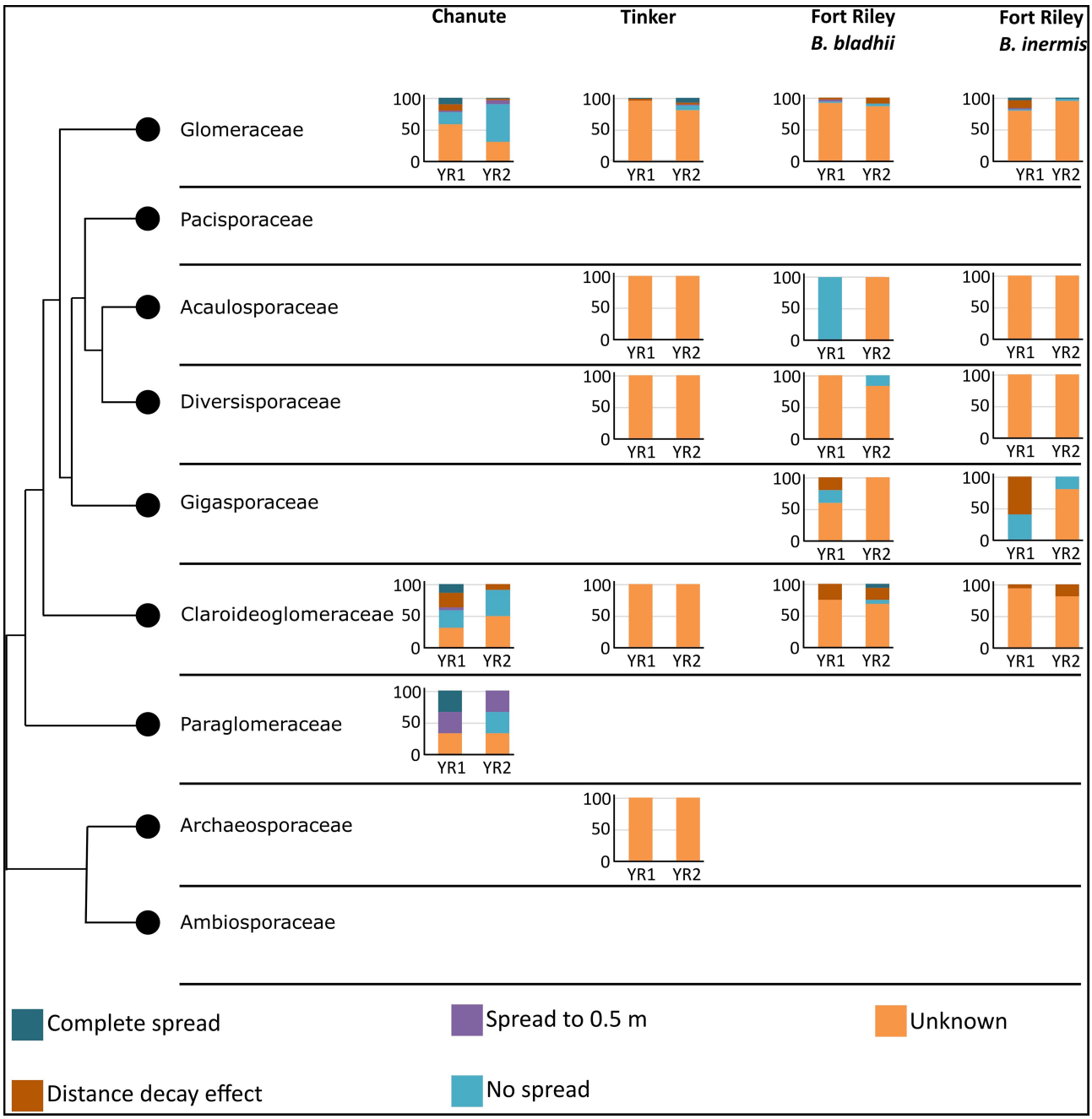

FIGURE 3 | Spread categories by taxonomy. Y-axis represents the percentage of trials in each category. Graphs are broken down into site and year. Colors represent the spread categories.

Plant Community

Overall, richness ($F^3_{78} = 9.7$, $p < 0.0001$), diversity ($F^3_{78} = 4.3$, $p < 0.01$), and plant community composition (NMDS1, $F^3_{78} = 18.1$, $p < 0.0001$; NMDS2, $F^3_{78} = 4.4$, $p < 0.01$) varied by site and year. In all sites except Ft. Riley *B. bladhii*, richness and diversity increased across time (**Supplementary Figure S6**). Within specific sites and years results varied, but when soil treatment was a significant predictor of plant diversity, richness, or evenness, inoculated plots (AMF and whole soil) or AMF plots alone had significantly higher plant diversity values. Diversity ($F^6_{52} = 2.3$, $p < 0.05$) and evenness ($F^6_{78} = 2.7$, $p = 0.02$) also differed by soil treatment, year, and site, which was driven by higher diversity and evenness in AMF compared to whole plots in the third year at Ft. Riley *B. inermis* (**Figure 6**; **Supplementary Figures S7, S8**).

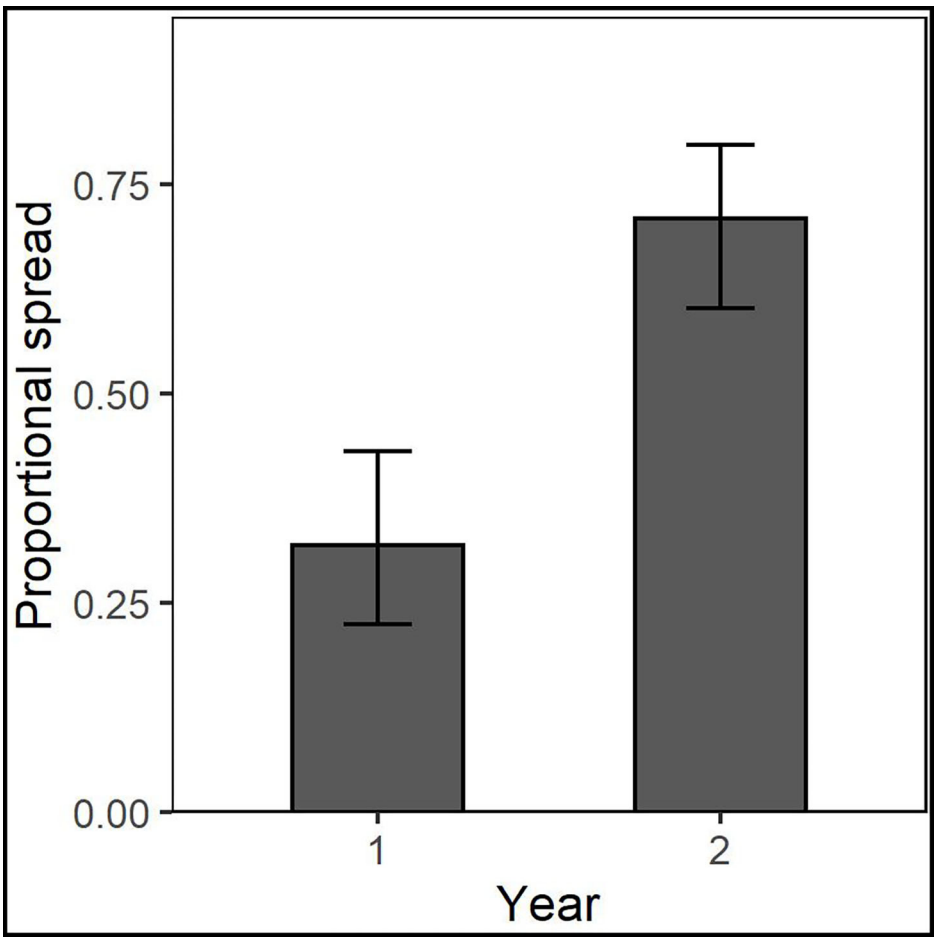

FIGURE 4 | Common OTUs identified across years within the Chanute site were more likely to spread from the nurse plant row in the second year.

Both sites with monocultures of *B. bladhii* before restoration efforts (Ft. Riley *B. bladhii* and Tinker) displayed significant differences in plant richness, diversity, and evenness across soil treatment and distance in certain years. At Tinker, inoculated plots (AMF and whole plots) had a marginally significantly higher plant richness than sterile plots overall ($F^1_{12} = 3.2$, $p < 0.1$, **Figure 6A**). At *Ft. Riley B. bladhii*, plant richness was significantly higher in AMF plots compared to both sterile and whole plots in the first year ($F^2_{12} = 4.5$, $p < 0.05$; **Figure 6B**), although there were no significant differences in the second or third year. Both these sites also showed some significant changes with distance from the nurse plant row. At Ft. Riley *B. bladhii*, plant richness in year 1 and plant evenness in year 2 decreased away from the nurse plant row in AMF plots but increased in whole soil plots with distance from the nurse plant row ($F^2_{39} = 4.1$, $p < 0.05$; $F^1_{39} = 3.8$, $p < 0.1$; **Supplementary Figures S9A,B**). In the third year, both AMF and whole soil plots had the highest plant richness ($F^1_{39} = 4.3$, $p < 0.05$) and diversity ($F^1_{39} = 4.4$, $p < 0.05$) near the nurse plant row compared to sterile plots, where richness and diversity increased with distance (**Supplementary Figures S9C,D**). At Tinker overall, plant community evenness decreased with distance in AMF plots and increased in whole plots ($F^1_{96} = 4.1$, $p < 0.05$, **Supplementary Figure S9E**).

Plant community composition (PERMANOVA) varied by soil treatment at both sites originally dominated by *B. bladhii*. At Tinker, the plant community was significantly different across the soil treatments, regardless of distance from the nurse plant row ($F^2_{112} = 2.4$, $p < 0.01$, **Supplementary Figure S10C**). At the Ft. Riley *B. bladhii* site, plant community composition was significantly different across soil treatments in the first year ($F^2_{55} = 1.9$, $p < 0.05$, **Supplementary Figure S10A**), and marginally significantly different across inoculation treatment in the third year ($F^2_{55} = 1.9$, $p < 0.1$, **Supplementary Figure S10B**). Soil treatment was also important for explaining community composition in the third year at Ft. Riley *B. inermis* ($F^2_{55} = 2.0$, $p < 0.05$, **Supplementary Figure S10D**).

Test Plants

At Tinker, test plants in inoculated plots were larger overall ($F^2_{12} = 5$, $p < 0.05$, **Supplementary Figure S11**). There was also a significant distance effect ($F^4_{66} = 7$, $p < 0.0001$), where test plant

TABLE 2 | Trials of virtual OTUs across sites.

Virtual OTUs	Number of trials	Number of non-unknown trials	Sites with non-unknown trials	No spread	Spread to 0.5-m	Distance decay effect	Complete spread to 2-m
1	10	2	Chanute, Ft. Riley *B. bladhii*	100%	0	0	0
2	20	7	Chanute, Ft. Riley *B. bladhii*, Ft. Riley *B. inermis*	86%	0	14%	0
3	42	18	Chanute, Ft. Riley *B. inermis*, Tinkr	78%	0	11%	11%
4	46	17	Chanute, Ft. Riley *B. bladhii*, Tinkr	53%	18%	29%	0%
7	12	2	Ft. Riley *B. bladhii*, Tinker	100%	0	0%	0
9	12	3	Chantue, Ft. Riley *B. inermis*	33%	0	0%	67%
10	6	2	Chanute	100%	0	0%	0%
14	14	4	Chanute	75%	0	25%	0%
16	14	2	Chanute	0%	0	50%	50%
17	12	4	Chanute	100%	0	0%	0%
18	12	4	Chanute	100%	0	0%	0%
19	6	2	Chanute, Ft. Riley *B. inermis*	0%	100%	0%	0%
20	10	3	Chanute, Ft. Riley *B. inermis*	67%	0	33%	0%
21	6	4	Chanute	75%	0	25%	0%
22	6	2	Tinker	0%	0	50%	50%

This table only includes the virtual OTUs that had more than one trial (one trial= an OTU in treatment in a year) when all trials that fit into an unknown spread category were removed. Once the trials that fit into an unknown category were removed, some virtual OTUs only had trials on one site.

species in the nurse plant row were larger compared to all other distances (**Supplementary Figure S11**), but the bridge and island sides did not vary significantly in size. Although the size of the plants varied by treatment and distance at Tinker, survival did not vary across any experimental variables. Survival of *S. scoparium* test plants was high across all distances, treatments, and years in Chanute, with 90% of the plants surviving to the third year. Test plant size and survival at Chanute varied with distance and side (island vs. bridge) in year 3 but did not follow any pattern moving toward or away from the nurse plant row.

DISCUSSION

This work evaluates AMF spread across four experiments distributed across three states and indicates that rates of AMF spread vary among taxa, are context dependent, and can impact neighboring plant communities. We found that many members of the AMF community were generally slow to spread from the site of inoculation, with most detectable OTUs (those not in the unknown category) either not spreading, spreading only to the 0.5 m, or spreading with a distance decay function. These results are supported by our findings that increases in relative AMF biomass were also slow, with increasing distances from the sites of inoculation. Together, these results indicate that AMF is both slow to spread and slow to increase in abundance from the site of colonization or inoculation—but that they can indeed spread. This result is consistent with previous studies showing variation in the mycorrhizal community closer to and away from mycorrhizal hosts (Chaudhary et al., 2014) and with evidence of the benefits of inoculation of native AMF spreading several meters from points of inoculation (Middleton and Bever, 2012; Middleton et al., 2015). We found the rate of the dispersal of AMF in the soil varies strongly among taxa, but that spread was not strongly predicted by the family. Generally, the proportion of AMF dispersing to 2 m is dependent on the presence of high-quality plant hosts and is affected by environmental context.

Environmental variation between sites likely influenced AMF spread from inoculation points, and thus our ability to detect this spread. For example, at Chanute, the AMF community was greatly reduced at a broad spatial scale through whole site disking and removal of existing vegetation. Because of these management procedures, we were able to better detect spread patterns for a high proportion of inoculated OTUs. However, at Tinker and Ft. Riley sites, where vegetation in areas outside of the plot tarp treatment was not removed, competition from other AMF or dispersal of AMF OTUs from outside the plot may explain the large abundance of OTUs displaying non-significant or unknown spread patterns in these sites. In support of this dispersal from the edge hypothesis, plots at Ft. Riley *B. inermis* generally showed an increase in AMF biomass moving away from the nurse plant row (calculated *via* PLFA) patterns in the second year of the experiment (**Supplementary Figure S1**). Sites also varied in dominant plant species before restoration. Previous studies have shown that land use history, soil nutrient levels, and dominant plant species can alter AMF communities (House and Bever, 2018; Tipton et al., 2018) and that mycorrhizal communities vary most at the site or regional scale (Chaudhary et al., 2014). This study suggests that site history may also alter either the spread of AMF from inoculation sites or the ability to detect this spread using molecular techniques in the field.

The use of island and bridge host plants on either side of the inoculation row allowed us to further distinguish spread rates among plant hosts, where hosts on the bridge side aided the spread of native inoculated AMF away from the inoculated

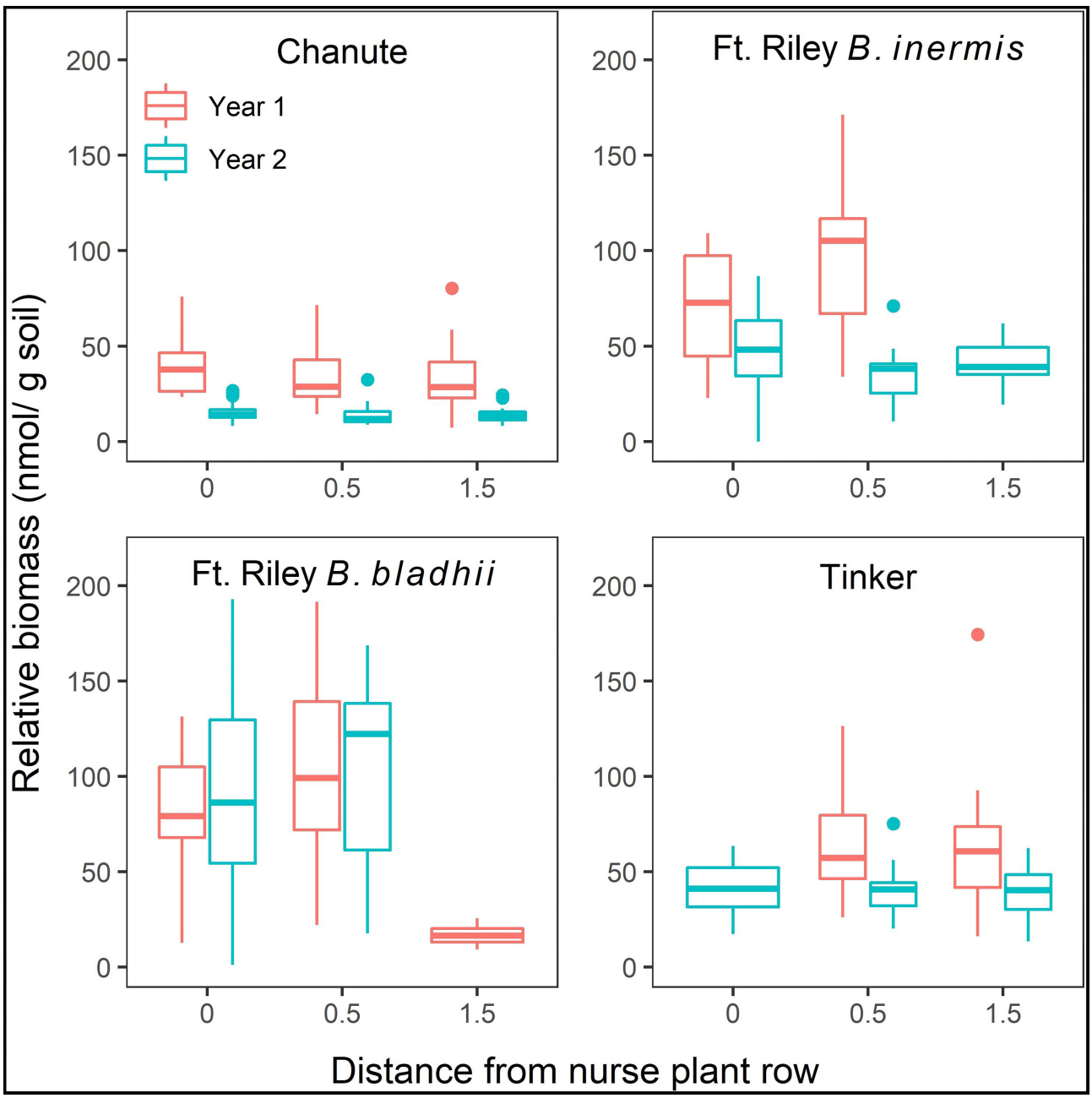

FIGURE 5 | The effects of site, distance from nurse plant row (X-axis), and year on relative abundances of extra-radical arbuscular mycorrhizal (AM) fungi (mean ± SE) measured through NLFA. Red bars represent measurements from year 1, and blue bars represent measurements from year 2.

nurse plants. Other studies have shown the benefits of the spread of inoculated native AMF to prairie plant growth up to 2 m in distance (Middleton and Bever, 2012; Middleton et al., 2015). Our results suggest that the rates of spread and benefit will depend in part on the establishment of mycotrophic host plants. In the prairie system, late-successional plant species are more responsive to native inocula than early-successional native and non-native plant species that establish quickly from seed and dominate the first several years of restoration (Cheeke et al., 2019). We showed that planting a row of non-inoculated late-successional host plant species at 0.5 m distances (the bridge) resulted in a greater proportion of inoculated OTUs spreading to 2.0 m and greater PLFA abundances compared to weedy host plants that dominated from seed (the island). Although we did not see significant differences in survival of these test plants with distance from inoculated nurse plant rows, *A. gerardii* plants were

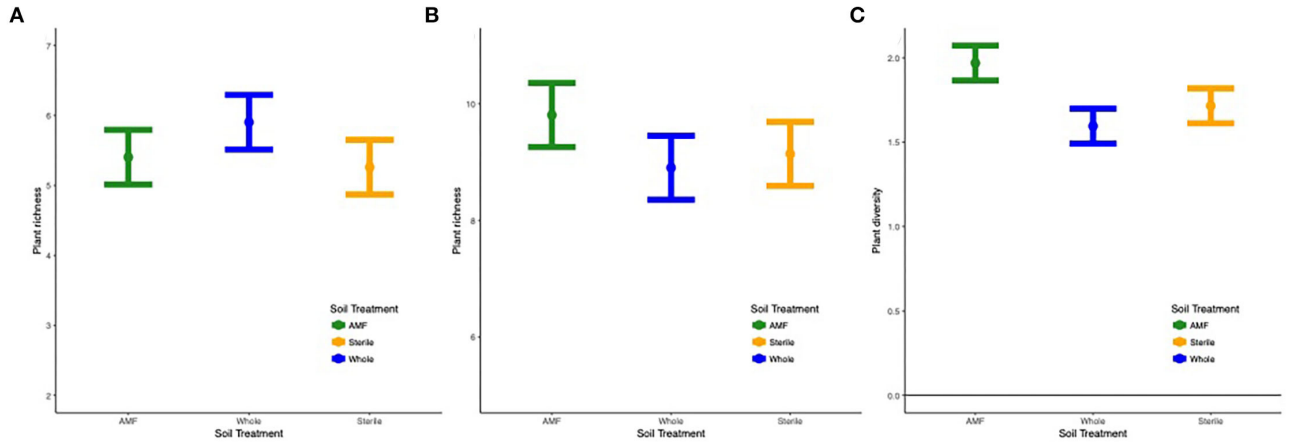

FIGURE 6 | (A) Plant richness across the three soil treatments in **(A)** Tinker site across all years and **(B)** Ft. Riley *B. bladhii* site in year 1 and **(C)** diversity in Ft. Riley *B. inermis* site in year 3.

larger in the nurse plant row compared to all other distances (**Supplementary Figure S11**), suggesting that inoculation did impact overall growth. We also know both *S. scoparium* and *A. gerardii* are hosts of native AMF (Anderson et al., 1994) and could facilitate AMF hypha through the soil. Chaudhary et al. (2014) found that spore abundance but not hyphal density varied between shrub understory and un-vegetated open space 1-m away, suggesting that hyphae may be able to spread at least 1 m from a host. However, these were established shrubs and the taxonomic identity of these hyphae was not tested. It could be that some AMF taxa rely more on the presence of a plant host for dispersal, compared to other taxa. In restoration, when new plant vegetation is added, presence of a host may be essential for dispersal of some AMF taxa. Although we did not examine how bridge and island sides varied in plant community diversity, future studies should examine how existing host plants could improve the spread of AMF inocula and thus increase abundance of late successional and AMF-dependent plant species away from inoculation points.

Our assessment of dispersal patterns as a function of AMF taxonomy yielded mixed results. Our data showed only weak patterns of OTUs in Paraglomerales tending to spread more than those in Glomerales and OTUs in Glomeraceae tending to spread more than those in Claroideoglomeraceae within the Chanute restoration site. However, when assessing the spread of the same OTUs at different sites, there appeared to be some support for identical spread patterns across varied environmental, host, and land use history parameters (**Supplementary Table S3**). Moreover, at the Chanute site, individual OTUs likelihood of spread increased from year 1 to year 2. Therefore, these data suggest that the spread of AMF through soil by hyphal growth is not solely dictated by the environment, but varies depending on the fungal taxon in question. This is in line with previous research on hyphal growth in soil for other fungal groups (Friese and Allen, 1991).

Given that so many native plant species are dependent on mycorrhizae (Koziol and Bever, 2015; Bauer et al., 2018; Cheeke et al., 2019) and that the presence of specific species of mycorrhizal fungi can strongly influence the growth of native plant species (Koziol and Bever, 2016; Cheeke et al., 2019), the rate of AMF spread from sites of colonization could have important impacts on the resulting plant community that occurs as AMF spread or does not spread. Our results support that slow AMF spread from inoculation sites limited plant community establishment. For instance, as we observed fungal OTUs spreading across distance and years, we observed increases in plant community richness and diversity across years with amendment with AMF or whole soil inoculum at some sites (**Supplementary Figures S4, S5**; **Figure 6**). Although whether or not these inocula had a significant impact on the plant community differed between year and site, when there was a significant effect, it included an increase in diversity and richness in AMF-inoculated plots (**Supplementary Figures S7, S8**; **Figure 6**), suggesting these soil amendments have the potential to alter the plant community at scales beyond the nurse plant level. Past work has also indicated that the presence of specific fungi in the field can increase seed recruitment, diversity, and native richness (Koziol and Bever, 2017) and that these benefits of AMF increase with the density of native AMF inocula (Koziol et al., 2020).

This work expands much-needed work on dispersal mechanisms of AMF, especially how dispersal *via* soil varies among taxa and environmental contexts. However, determining the dispersal of many AMF taxa was difficult using this approach in the field. Many of the AMF OTUs present in our inocula were also present at a background level in sites before the experiment, leaving us unable to determine dispersal patterns for some taxa. Different tools should be utilized in the future to expand our knowledge of dispersal, including controlled experiments involving multiple

taxonomic groups, to determine the propagule type and dispersal rate of multiple species. A better understanding of AMF dispersal can lead to more efficient plans for the reintroduction of these fungi, to the benefit of the plant species that depend on them, and to the improvement of ecological restoration.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession number PRJNA818716.

AUTHOR CONTRIBUTIONS

JB, PS, and GW conceived and designed the experiment. AT, GH, ED, and LK collected data. AT, DN, GH, LK, and JB analyzed the data. AT wrote the manuscript. All authors contributed toward the materials and edits to manuscript drafts. 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/fmicb.2022.827293/full#supplementary-material

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