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INVERTEBRATE MICROBIOLOGY

Floral and Foliar Source Affect the Bee Nest Microbial Community

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Abstract

Managed pollinators such as the alfalfa leafcutting bee, *Megachile rotundata*, are essential to the production of a wide variety of agricultural crops. These pollinators encounter a diverse array of microbes when foraging for food and nest-building materials on various plants. To test the hypothesis that food and nest-building source affects the composition of the bee-nest microbiome, we exposed *M. rotundata* adults to treatments that varied both floral and foliar source in a 2 × 2 factorial design. We used 16S rRNA gene and internal transcribed spacer (ITS) sequencing to capture the bacterial and fungal diversity of the bee nests. We found that nest microbial communities were significantly different between treatments, indicating that bee nests become inoculated with environmentally derived microbes. We did not find evidence of interactions between the fungi and bacteria within our samples. Furthermore, both the bacterial and fungal communities were quite diverse and contained numerous exact sequence variants (ESVs) of known plant and bee pathogens that differed based on treatment. Our research indicates that bees deposit plant-associated microbes into their nests, including multiple plant pathogens such as smut fungi and bacteria that cause blight and wilt. The presence of plant pathogens in larval pollen provisions highlights the potential for bee nests to act as disease reservoirs across seasons. We therefore suggest that future research should investigate the ability of bees to transmit pathogens from nest to host plant.

Keywords Solitary bees · Microbial communities · ITS sequencing · 16S rRNA gene sequencing

Introduction

Pollinating insects are vital to agricultural plant productivity and increase the yield of approximately 75% of food crops worldwide [1]. While honey bees (*Apis mellifera*) are the main managed agricultural pollinator in the USA, there are several non-*Apis* commercial pollinators including bumble bees (*Bombus* spp., Family: Apidae), leafcutting bees (*Megachile* spp., Family: Megachilidae), and mason bees (*Osmia* spp., Family: Megachilidae) that are commercially used to pollinate various crops such as tomatoes, alfalfa seeds, and blueberries [2]. The lifestyles of managed bees vary considerably between individual species ranging from solitary to eusocial. For example, the solitary bee *Megachile rotundata*, an important pollinator of alfalfa for seed production in North America [3], constructs a linear series of brood cells made from cut leaves. Each brood cell is provisioned with a mass of pollen and nectar upon which a single egg is deposited. Development is completed in the brood cells, and adults have limited interaction with each other. Conversely, *A. mellifera*, a eusocial bee, builds complex perennial colonies and has a distinct caste system with strict control over reproduction [3, 4].

Honey bees and bumble bees harbor distinct gut microbial communities that are mainly transmitted by social interactions between colony-mates and, to a lesser degree, are inoculated by contact with hive surfaces [5, 6]. Due to the ancient symbiosis with their bacterial flora, the gut communities of individual species of social apid bees are fairly consistent worldwide [7]. In contrast with social apid bees, solitary bee species

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do not generally harbor a consistent microbiome, and bacteria and fungi that are found associated with these bees tend to be of environmental origin [8–13]. For example, McFrederick et al. [9] found that flower and wild megachilid bee samples can share the same taxon of *Lactobacillus*, and that this bacterium can dominate the microbial community of all life stages of the bee and its pollen provision. Likewise, in 2015, Lozo et al. [14] found that pollen collected by the mason bee *Osmia cornuta* contains mostly the environmentally acquired cosmopolitan bacterium *Pantoea agglomerans*. Recently, it has been established that providing honey bees with different sources of floral forage can subtly alter the gut microbiome [15, 16], and that bees may alter the composition of floral microbial diversity [17, 18]. However, no research has been conducted to determine the effects of a varied diet on either the bacterial or fungal communities of a model solitary bee species, *M. rotundata* [19], or the pollen provisions that it produces for its offspring.

Commercial and managed bees can vector insect diseases to wild populations of both solitary and social bees, such as pathogen spillover from managed to wild bumble bees and Deformed Wing Virus infections in *Osmia* spp. [20, 21]. Shared floral resources can serve as reservoirs of pathogen transmission between diseased and naïve bees [22]. As *M. rotundata* provides nourishment for its brood in the form of pollen provisions that it makes from floral resources, disease agents such as *Ascosphaera* (the fungi that causes chalkbrood [3]) and *Aspergillus* (the fungi that causes stonebrood [23]) can be obtained from floral surfaces and transmitted to the larvae via the pollen provision [24–26]. Notably, aside from insect disease, bees incidentally vector plant pathogenic fungi [27] and viruses [28] through visiting infected flowers and dispersing the disease through normal pollination behavior [29]. In addition, even if nonpathogenic microbes are deposited onto flowers through pollination, these exotic nectar-dwelling microbes can alter the chemistry and attractiveness of flowers to pollinators, which may ultimately affect plant fitness [30, 31].

Our study investigates several aspects of the microbial communities associated with *M. rotundata:* First, we determine if differing flower (nutritive material) or leaf sources (*M. rotundata* builds its nest from cut leaves) alter the bacteria and fungi found in pollen provisions. We did this by limiting the available pollen forage either to *Medicago sativa* (alfalfa) or *Phacelia tanacetifolia* and either to *Fagopyrum esculentum* (buckwheat) or *Tropaeolum majus* (nasturtium) as a leaf source. Each treatment was chosen based on the following criteria: *M. rotundata* is used heavily in alfalfa pollination [2], the bees demonstrate a strong preference in using buckwheat leaves for nesting [32], *Phacelia tanacetifolia* is known to produce copious amounts of nectar and pollen and is visited by a wide variety of bees [33], and nasturtium is known to produce antimicrobial compounds [34, 35]. The resulting pollen provisions were removed from cells and separated from the bee eggs. We performed amplicon sequencing of both the 16S rRNA genes for bacterial diversity and the internally transcribed spacer (ITS) region of the fungal rRNA gene to analyze fungal diversity. By analyzing the taxonomic origin of the sequences, we then determined the ability for *M. rotundata* to deposit potentially pathogenic or environmentally derived bacteria and fungi in their pollen provisions and correlated this to the plant sources on which the bees forage. Lastly, we compare the exact sequence variants (ESVs) of bacteria and fungi to establish if there are inter-kingdom interactions within the brood cell environment of *M. rotundata*.

Materials and Methods

Bee Treatments and Collections

We allowed the bees to forage on one of four possible treatments representing an exposure to either of two pollen sources (alfalfa or *Phacelia*) and to either of two leaf sources (buckwheat or nasturtium): alfalfa with buckwheat (AB), alfalfa with nasturtium (AN), *Phacelia* with buckwheat (PB), and *Phacelia* with nasturtium (PN). Four adjacent plots were planted with *Medicago sativa* (alfalfa), *Phacelia tanacetifolia*, buckwheat (*F. esculentum*), or nasturtium (*T. majus*) in North Logan, Utah. The timing of planting and irrigation was such that the bloom of alfalfa and *Phacelia* was coordinated, as well as leaf production by the buckwheat and nasturtium. The arrangement of the plots allowed for four screened field cages ($6.2 \times 6.2 \times 2$ m^3) to be positioned, so that half of the area was either alfalfa or *Phacelia* in combination with the other half being either buckwheat or nasturtium. A small plywood box ($35 \times 32 \times 28$ cm^3) was affixed to a pole 1.5 m above the ground in the center of each cage to serve as a shelter that held a polystyrene bee board ($7 \times 7 \times 9.5$ cm^3) with 49 cavities lined with paper straws. Overwintered prepupae cocooned in leaf-wrapped cells were incubated at 29 °C to complete bee development to adulthood, and emerged adults were released in cages approximately 3 weeks later. For each cage, we released 40 males and 20 females, and as females have been observed to nest in up to four cavities, it is unlikely that any one female dominated the available nesting space [36]. After 2 weeks of nesting, completed cells were removed.

The cells were cut out of the paper tubes and x-rayed to confirm that the eggs had not yet hatched. The cells were confirmed by visual observation that they consisted of the appropriate leaf and pollen sources. The sample sizes of the cells were as follows: AB ($N = 29$), AN ($N = 6$), PB ($N = 29$), and PN ($N = 30$). Alfalfa can serve as both a pollen and leaf source, so cells were confirmed to have leaf pieces of either buckwheat or nasturtium. Nasturtium was not used often in

the cages having both alfalfa and nasturtium, resulting in a lower number of cells.

DNA Extraction and Amplicon Sequencing Library Preparation

We used a modified DNA extraction protocol based on Engel et al. 2013 [37], Pennington et al. 2017 [38], and Pennington et al. 2018 [39]. We used sterile technique to remove the entire pollen provision of a *M. rotundata* brood cell and transferred it into 96-well tissue lysis plates (Qiagen, Valencia, CA). We then added 50–100 μL of 0.1 mm glass beads, one 3.4 mm steel-chrome bead (Biospec, Bartlesville, OK), and 180 μL of buffer ATL, and homogenized the samples with a Qiagen Tissuelyser at 30 Hz for 6 min. Lastly, we followed the remainder of the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) protocol for tissue samples to finish the DNA extraction. We also included three blanks to control for reagent contamination, which were subjected to the same preparation and sequencing procedures as the samples.

We prepared 16S rRNA gene libraries for paired-end Illumina MiSeq sequencing using the protocol from McFrederick and Rehan, 2016 [40] and Pennington et al. 2017 [41], while we followed the protocol from Smith and Peay, 2014 [42] and McFrederick and Rehan, 2018 [43] to prepare ITS libraries for fungi. We incorporated the 16S rRNA gene primer sequence, unique barcode sequence, and Illumina adapter sequence as in [44]. We used the primers 799F-mod3 (CMGGATTAGATACCCKGG) [45] and 1115R (AGGGTTGCGCTCGTTG) [44] to amplify the V5-V6 region of the 16S rRNA gene. Similarly, we used the primers ITS1f and ITS2 [43] along with a unique barcode sequence and Illumina adapter sequence to amplify the ITS region of interest. We used the following reaction conditions for PCR: 4 μL of template DNA, 0.5 μL of 10 μM forward primer, 0.5 μL of 10 μM reverse primer, 10 μL sterile water and 10 μL 2× Pfusion DNA polymerase (New England Biolabs, Ipswich, MA), an annealing temperature of 52 °C, and 30 cycles in a C1000 Touch thermal cycler (BioRad, Hercules, CA). We then used the MoBio UltraClean 96 PCR Clean-Up Kit (Invitrogen, Carlsbad, CA) to remove unincorporated primers and dNTPs. We performed a second PCR reaction using 1 μL of the cleaned PCR amplicons as a template with the primers PCR2F and PCR2R to complete the Illumina adapter construct [15, 40]. We performed this PCR with the following reaction conditions: 0.5 μL of 10 μM PCR2F, 0.5 μL of 10 μM PCR2R, 1 μL of template, 13 μL of water, and 10 μL of 2× Pfusion DNA polymerase for 15 cycles at an annealing temperature of 58 °C. We normalized the resulting amplicons with the SequalPrep Normalization kit following the supplied protocol (ThermoFisher Scientific, Waltham, MA). We pooled 5 μL of each normalized library and performed a final clean-up with a MoBio UltraClean PCR Clean-Up Kit. We then checked the amplicons on a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and sequenced the multiplexed libraries using a V3 Reagent Kit at 2 × 300 cycles on an Illumina MiSeq Sequencer (Illumina, San Diego, CA) at the UC Riverside Genomics Core Facility. Raw sequencing data are available on the NCBI Sequence Read Archive (SRA) under accession number SRP157103.

Bioinformatics and Statistics

We used QIIME2-2017.12 [46] to process the amplicon sequence libraries. First, we trimmed the low-quality ends off the reads with QIIME2. Then, we used DADA2 [47] to cluster our sequences into ESVs (sequences that are identical), identify chimeras, and remove reads with more than two expected errors. For 16S rRNA gene identification, we assigned taxonomy to the ESVs using the q2-feature-classifer [48] trained to the 799–1115 region of the 16S rRNA gene with the SILVA database [49], while we used the UNITE database [50] to classify ITS reads. We conducted confirmatory BLASTn searches against the NCBI nt/nr database (accessed May 2018) and manually removed ESVs from the resulting feature tables matching reagent contaminant DNA sequences as identified in our blank samples as in Salter et al. 2014 [51] along with ESVs that matched to chloroplast or mitochondria. Subsequently, we used MAFFT to align the representative sequences [52] and FastTree v2.1.3 to generate a phylogenetic tree of 16S rRNA gene sequences [53]. We used this tree and the filtered feature table to analyze alpha diversity, create rarefaction curves, and generate weighted and unweighted UniFrac distance matrices [54]. We additionally created a Bray-Curtis distance matrix with the 16S feature table. As the fungal ITS region is not amenable to phylogenetic analysis, we generated a Bray-Curtis distance matrix only for our analyses of ITS diversity. We then visualized the distance matrices through two-dimensional Principal Coordinates Analysis (PCoA). We analyzed the alpha diversity of our samples through the Shannon diversity index and assessed statistical significance through the Kruskal-Wallis test in QIIME2. We tested our beta diversity metrics for statistical significance in R v3.4.1 [55] with the packages "vegan" [56] and "DESeq2" through the Bioconductor package [57] along with "ggplot2" for graphing [58]. We built co-occurrence networks using CoNet [59] to test for interactions between fungal and bacterial ESVs, and ran Mantel tests with 999 permutations between the 16S and ITS Bray-Curtis distance matrices to test correlations between the overall fungal and bacterial diversity in our samples.

Results

Alpha Diversity and Library Statistics

We obtained a total of 343,960 quality-filtered bacterial 16S rRNA gene reads with an average of 3583 reads per sample ($N = 94$) that were clustered into 2016 ESVs, while we obtained 278,400 fungal reads with an average of 3026 reads per sample ($N = 92$) that were clustered into 379 ESVs (see Supplemental File SF1 for our ESV table). Through rarefaction analyses, we determined that we had representative ESV coverage and an acceptable remaining number of samples at a sequencing depth of 1566 ($N = 81$) and 792 reads ($N = 82$) for bacteria and fungi, respectively (Fig. SF1). There was a significant difference in alpha diversity (as measured by Shannon's H and Observed ESVs) across all treatments for bacteria (Shannon's $H = 42.0$, $P < 0.001$; Observed ESVs $H = 45.9$, $P < 0.001$), with pairwise comparisons showing significant differences across all sample pairings except PB versus PN (Benjamini-Hochberg corrected for multiple comparisons $P_{adj} < 0.05$; Fig. 1, Supplemental Table ST1). Conversely, alpha diversity was not significantly different across treatments for fungi (Shannon's $H = 4.38$, $P = 0.22$; Observed ESVs $H = 5.55$, $P = 0.14$) with no sample pairings showing significant differences ($P_{adj} > 0.05$, Supplemental Table ST1).

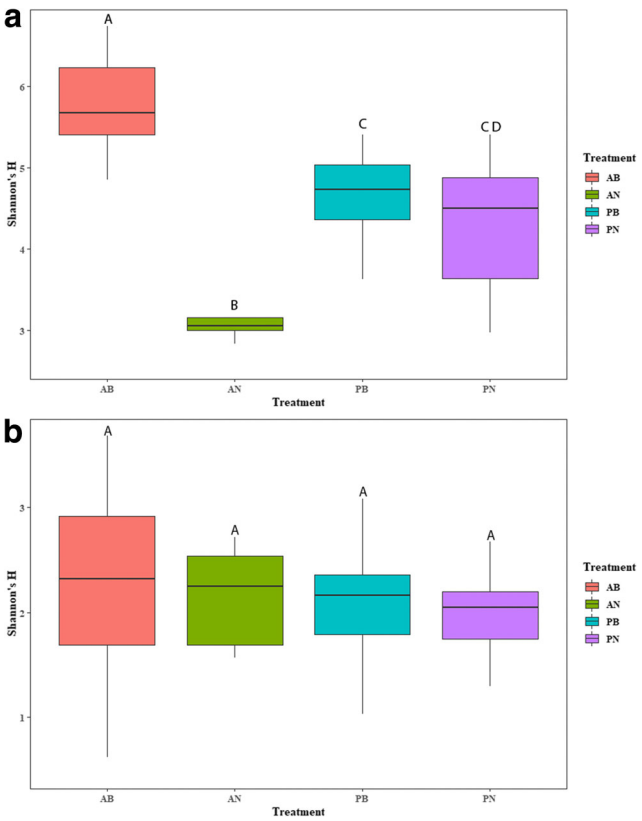

Fig. 1 Boxplots of alpha diversity for each treatment in the **a** bacterial and **b** fungal communities, of the sample treatments as measured by the Shannon diversity index. Bacterial alpha diversity was significantly different between treatments ($H = 42.0$, $P < 0.001$), with all treatment pairings except PB versus PN showing significant pairwise differences ($P_{adj} < 0.05$), while fungal alpha diversity was not significantly different between any treatment pairings or overall ($H = 4.38$, $P = 0.22$). Unique letters indicate significant pairwise differences and error bars denote 1.5× the interquartile range

Beta Diversity and Differential Abundance of Bacteria and Fungi

Overall, we found that the top ten most abundant bacterial families present in our samples were as follows: *Enterobacteriaceae* (48.32%), *Lactobacillaceae* (5.59%), *Micrococcaceae* (3.13%), *Sphingomonadaceae* (2.76%), *Lachnospiraceae* (2.59%), *Methylobacteriaceae* (1.99%), *Comamonadaceae* (1.84%), *Microbacteriaceae* (1.65%), *Oxalobacteraceae* (1.58%), and *Burkholderiaceae* (1.57%) (Fig. 2a). Specifically, ESVs belonging to the genus *Pantoea* were the most abundant and comprised 35.26% of the total bacterial counts across all samples, with *Rosenbergiella* ESVs accounting for 8.81% and *Lactobacillus* ESVs representing 5.56% of total bacteria present.

We also analyzed the fungal composition of our samples and found that the ten most proportionally abundant families were as follows: *Pleosporaceae* (26.92%), *Erysiphaceae* (10.49%), *Phaeococcomycetaceae* (7.72%), *Rutstroemiaceae* (7.60%), *Hypocreales* (insertiae sedis; 6.93%), *Aspergillaceae* (5.56%), *Aureobasidiaceae* (5.04%), *Mortierellaceae* (3.25%), *Ophiostomataceae* (2.58%), and *Ustilaginaceae* (2.49%) (Fig. 2b). Specifically, ESVs belonging to the genus *Alternaria* were the most abundant in our samples, accounting for 17.75% of the total fungal reads, followed by *Golovinomyces* ESVs (7.90%) and *Phaeococcomyces* ESVs (7.16%).

We established that the beta diversity of bacterial communities associated with *M. rotundata* was significantly different between treatments as tested by Adonis (permutational ANOVA with 999 permutations) on both weighted ($F = 15.03$, $R^2 = 0.37$ and $P < 0.001$) and unweighted UniFrac distance matrices ($F = 4.36$, $R^2 = 0.15$, $P < 0.001$), and not between nests within treatments on both weighted ($F = 1.09$, $R^2 = 0.30$, $P = 0.33$) and unweighted UniFrac matrices ($F = 0.94$, $R^2 = 0.37$, $P = 0.82$). As there were few samples treated with both alfalfa and nasturtium (treatment AN, $N = 5$ after rarefaction), we removed those samples from the distance matrices and reanalyzed our resulting distance matrices. We found that our interpretation of the data is still valid for both weighted ($F = 16.63$, $R^2 = 0.31$, $P < 0.001$) and unweighted Unifrac ($F = 4.91$, $R^2 = 0.12$, $P < 0.001$) matrices without AN so we continued to analyze our data with treatment AN still included. To test for variability between nests, we also ran pairwise Adonis tests for each treatment's corresponding distance matrix and found that the beta diversity of the brood cell bacterial communities significantly differed for each sample

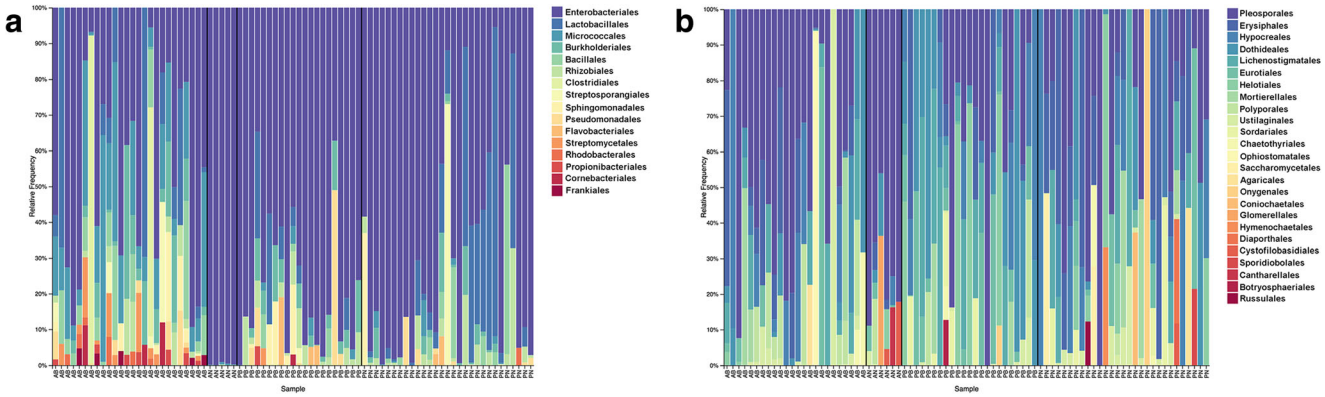

Fig. 2 Taxonomic order-level stacked bar plots of the **a** bacterial and **b** fungal communities of the samples grouped by treatment. Orders found at greater than 0.01% proportional abundance are included in this figure

pairing by weighted ($P < 0.02$, except PB versus PN [$P = 0.14$], Supplemental Table ST1) and unweighted UniFrac ($P < 0.04$, Supplemental Table ST1). To test for heterogeneous dispersion of the data in our PCoA ordination (Fig. 3), we analyzed the UniFrac distance matrices with the function "betadisper" in the R package "vegan." We found that there was significant dispersion between our samples for both unweighted ($F_{(3,77)} = 14.16$, $P < 0.001$) and weighted ($F_{(3,77)} = 9.81$, $P < 0.001$); however because Adonis PERMANOVA testing is robust to heterogeneous data [60] and the PCoA ordination exhibits clustering by treatment, the significant differences between treatments appear valid.

We also established that there was a significant difference in the fungal communities of our samples by analyzing the ITS Bray-Curtis distance matrix with Adonis PERMANOVA ($F = 1.81$, $R^2 = 0.07$, $P < 0.001$) with pairwise tests showing significant differences between all sample pairings ($P_{adj} < 0.03$, except AB versus AN [$P = 0.12$], Supplemental Table ST1) and no significant effect of nest straw within treatment ($F = 0.92$, $R^2 = 0.40$, $P = 0.97$). We found that there was slight clustering by treatment by performing a PCoA ordination on the distance matrix (Fig. 3), and we did not find that our ITS data was heterogeneously dispersed ($F_{(3,78)} = 1.73$, $P = 0.170$).

We used "DESeq2" to observe the fold difference between treatments of ESVs constituting 1% or greater proportional abundance in our samples. We compared these ESVs between each sample pairing (pairwise between AN, AB, PN, and PB) along with samples grouped entirely by pollen source (all *Phacelia* compared to all alfalfa) or leaf source (all buckwheat compared to all nasturtium). For bacteria, we found four *Pantoea* ESVs that were more proportionally abundant in provisions with phacelia versus alfalfa pollens, and seven ESVs (five *Pantoea* and two *Rosenbergiella*) that were less proportionally abundant in pollen provisions collected from cells having nasturtium versus buckwheat leaf pieces (Benjamini-Hochberg corrected $P_{adj} < 0.05$, Fig. 4). Each of the analyses of sampling pairings and the corresponding ESVs can be found in Supplemental Table ST2, and in every case, the only differentially abundant ESVs were of the genera *Pantoea* or *Rosenbergiella*. Likewise, the pollen provisions had a diverse bacterial community with few ESVs present in 50% or greater of our samples (three ESVs of *Pantoea* and one ESV of *Rosenbergiella*) and no fungi common across 50% or more samples.

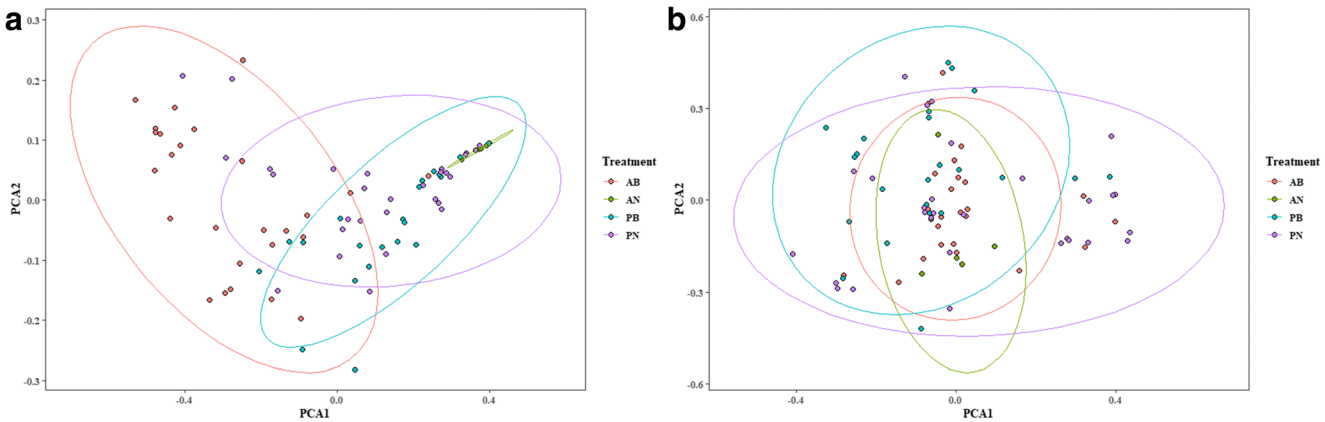

Fig. 3 Principal Coordinates Analysis (PCoA) of the *M. rotundata* pollen provision bacterial communities grouped by treatment of **a** weighted UniFrac distance matrix and **b** the fungal community Bray-Curtis distance matrix. Ellipses denote 95% confidence intervals

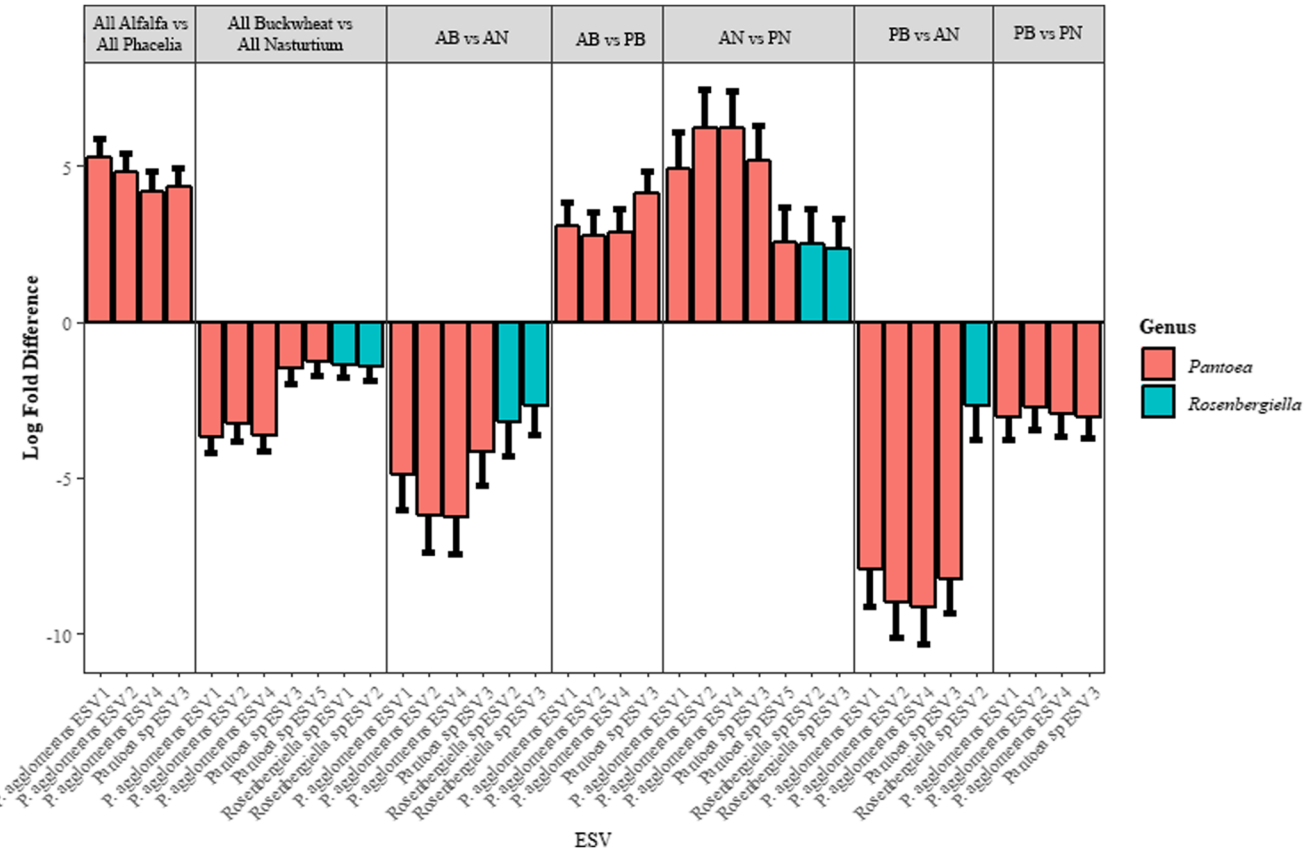

Fig. 4 Bar plot of the $\log_2$ fold difference in significantly differentially abundant ($P_{adj} < 0.05$) exact sequence variants (ESVs) of bacteria within the pollen provisions of *M. rotundata* binned by treatment pairings and colored by genus: All buckwheat (B) versus all nasturtium (N) treatments and all alfalfa (A) versus all *Phacelia* (P) treatments along with permutations of each treatment. Error bars denote standard error

We analyzed our ITS data in the same fashion as our bacterial reads and compared the log fold difference of ESVs between treatments at greater than 1% abundance. We found that there were eight differentially abundant ESVs in *Phacelia* versus alfalfa treatments and four differentially abundant ESVs when comparing nasturtium to buckwheat treatments (Fig. 5). Similar to the results of our bacterial analyses, each of the sample pairings yielded differentially abundant ESVs ($P_{adj} < 0.05$, Supplemental Table ST3). In all cases, the only significantly different ESVs were identified in the fungal genera *Pezicula*, *Ganoderma*, *Aspergillus*, *Stemphylium*, *Ustilago*, *Trichothecium*, *Golovinomyces*, *Aureobasidium*, *Phaeococcomyces*, and *Alternatria*. Specifically, when comparing the fungi found in alfalfa versus *Phacelia* treatments, we found differentially abundant ESVs including *Alternaria*, which are saprophytic fungi that are commonly found in soil and on decaying plants and can act as major plant pathogens causing blight in multiple crops [61] and have been previously identified in *M. rotundata* larval guts [62]. Abundant fungal reads corresponding to *Golovinomyces* (one of the causative agents of powdery mildew) [63] were found in our samples, as well as *Phaeococcomyces* (a genus of potentially pathogenic black yeast) [64], the mild plant pathogen *Pezicula heterochroma* [65], an ESV of *Ustilago* smut fungus [66], of which this taxon has been previously found in *M. rotundata* [62], an ESV of the leaf spot disease agent *Stemphylium eturmiunum* [67], the pink rot fungus *Trichothecium roseum* [68], and the widespread putative opportunistic bee pathogen *Aspergillus niger* [23, 62]. We found many other genera and unique ESVs in lesser abundance including the chalkbrood-causing fungus *Ascosphaera aggregata* [3], *Debaryomyces hansenii*, which has been previously isolated from stingless bees [69], and *Colletotrichum trifolii*, an important pathogen of alfalfa. We also found that our samples harbored a wide diversity of fungi and that no ESV was present in greater than 50% of the samples. We compared the Bray-Curtis distance matrices of the bacterial and fungal abundances for each sample and found that differences between their complete bacterial composition are independent from the differences between the complete fungal composition ($\rho = -0.04$, $P = 0.333$). Lastly, we built co-occurrence networks with CoNet and did not find any significant interactions (Benjamini-Hochberg corrected for multiple comparisons $P_{adj} < 0.05$)

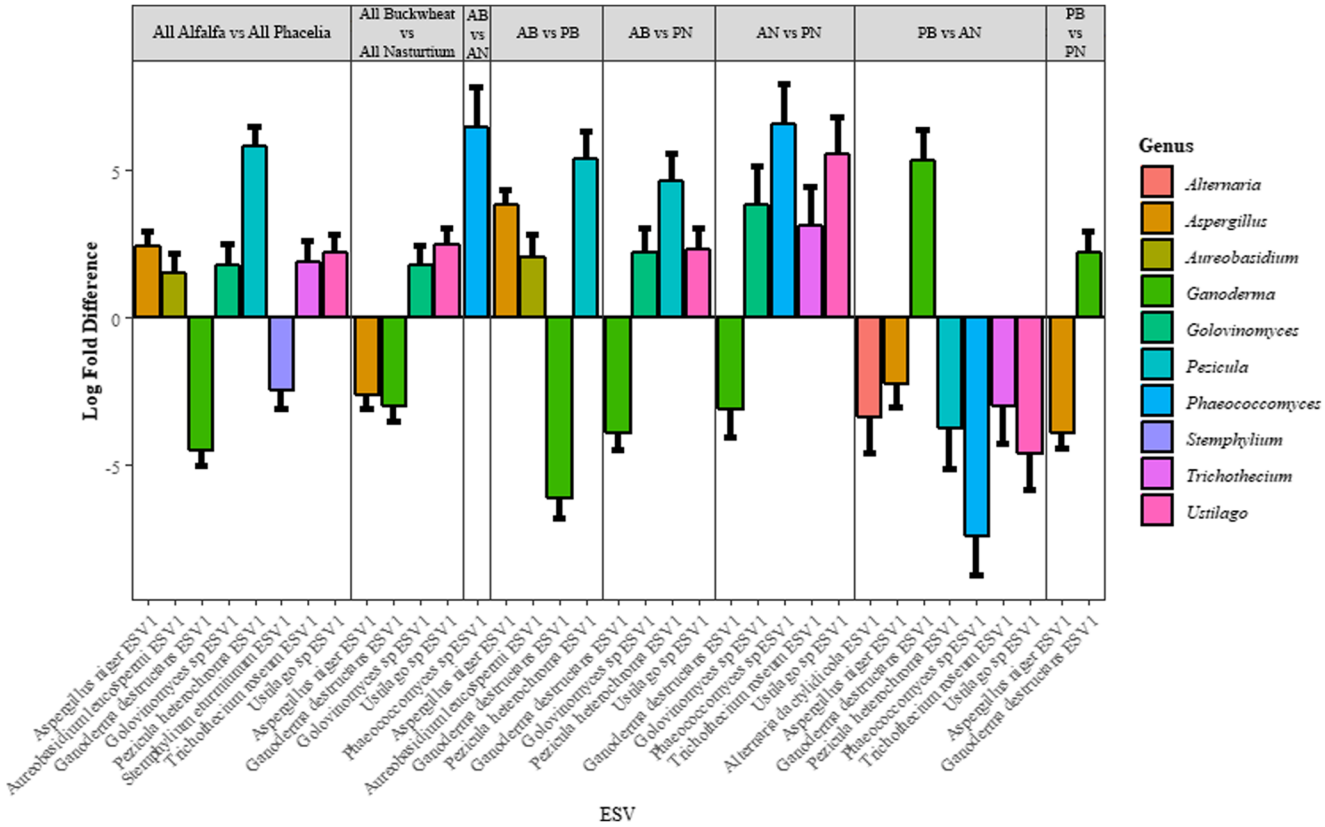

Fig. 5 Bar plot of the $\log_2$ fold difference between differentially abundant exact sequence variants (ESVs) of fungi within the pollen provisions of *M. rotundata* separated by treatment pairings and grouped by genus: All buckwheat (B) versus all nasturtium (N) treatments and all alfalfa (A) versus all *Phacelia* (P) treatments along with permutations of each treatment. Only significantly different ($P_{adj} < 0.05$) ESVs are shown; error bars signify standard error

between fungal and bacterial ESVs within any of our samples or treatments.

Discussion

The Microbial Diversity of *M. rotundata* Pollen Provisions

Overall, we found that varying both foliar and floral resources can affect the bacterial and fungal communities of *M. rotundata* pollen provisions. This result indicates that these resources harbor distinct microbes that are transmissible to bee pollen provisions and may be distributed back out to the environment upon emergence of the next bee generation. We also established that varying the source of pollen or leaves can affect the diversity of bacteria but interestingly not fungi. While it is currently unknown if bees inoculate flowers with many different microbes, this hypothesis was previously tested by Ushio et al. [18] and the authors of the study were able to show that some bacterial taxa were transferred to flowers upon pollination.

Our results support previous studies that have shown that solitary bees such as *Megachile* spp. and *Ceratina* spp. are largely exposed to microorganisms from their environment and can deposit those microbes into their pollen provisions [9, 40]. Similarly, Aizenberg-Gershtein et al. [17] suggested that honey bees may share bacteria with the flowers that they visit and may alter the floral microbial community. Our experiment extends these previous studies, as we analyzed both bacterial and fungal communities and showed that they differ based on pollen or nesting material source. By simultaneously sequencing bacteria and fungi, we were able to test for correlations between these two Kingdoms. We did not find any correlation between fungi and bacteria regardless of treatment, both at the community and individual ESV level. This suggests that inter-kingdom competition or other interactions do not appear to occur based on pollen or leaf sources within *M. rotundata* nests. This result somewhat agrees with past work that showed that pollen provision-associated bacteria do not affect fungi, while fungi may increase the diversity of the bacterial community through a currently unknown mechanism [62]. Conversely, DeGrandi-Hoffman et al. [70] found that honey bee exposure to fungicides did not affect their microbiomes. While our results are insightful, they do not

indicate if the bees are picking up live microbes, or if there is any effect on bee health or metabolism. Similarly, there does not seem to be consensus on the interactions between bee-associated bacteria and fungi, so future studies should focus on these plant- and bee-associated microbes, their interactions, and their potential functions in solitary bees.

Effects of Floral and Foliar Treatments on Bacterial Diversity

Pantoea ESVs were the most abundant bacteria present in our samples, regardless of treatment. Bacteria within this genus are cosmopolitan and are known to cause disease in some plant species (e.g., *Pantoea stewartii* causes Stewart's wilt in corn and *Pantoea agglomerans* can cause various disease symptoms in specific plant hosts), but remain commensal and epiphytic in many others [71]. *Pantoea* have been identified in honey bee colonies and corbiculate pollen [72], honey bee bread [73], flowers [9], in pollen collected by the megachilid bees *O. cornuta* [14] in *O. bicornis* [12], and in the apid bee *Ceratina calcarata* [40]. Our data shows that *Pantoea* ESVs are more proportionately abundant in the pollen provisions of bees foraging on treatments containing alfalfa, which indicates that these plants harbor differing amounts or ESVs of bacteria that can be transmitted to the provisions of *M. rotundata*. Likewise, treatments containing nasturtium also contained a higher proportional abundance of *Pantoea* and *Rosenbergiella* ESVs, again suggesting that bees can vector specific bacteria from plants to pollen provisions. We also found other taxa in high relative abundance, including the nectar-dwelling microbe *Rosenbergiella* [74] and taxa within the *Lactobacillus micheneri* clade of flower- and solitary bee-associated bacteria [11]. Lastly, we found other taxa in appreciable abundance, including the devastating plant pathogen *Ralstonia solanacearum* [75] and *Clostridium* that have been previously isolated from *M. rotundata* [62], although we did not find plant pathogens that are specifically vectored by bees.

Our data also suggest that *M. rotundata* might be selecting the floral and leaf sources it uses to build its nest to minimize pathogens. As reviewed by McArt et al. [76], there is an increasing body of literature that indicates that pollinators have a complex interaction with their floral hosts. Data from studies indicate that bumble bees and honey bees may select pollens from floral hosts to acquire plant secondary compounds that can reduce pathogen levels in the bees [77]. Here, nasturtium was in part chosen to ask if the leaves would be associated with an altered microbial community. Nasturtium leaves are a source of isothiocyanates that have been found to be antimicrobial when used to treat bacterial infections in humans [34, 35, 78]. Our data indicates that nasturtium may affect microbes, as the proportional differential abundance of microbes is lower for most treatments involving nasturtium. We are unable to determine if there is any antimicrobial activity of nasturtium as our data is compositional, and we cannot say for certain if the specific microbes are less proportionally abundant due to treatment, as we did not quantify the microbes in our study. Given the choice between alfalfa and nasturtium leaves, the bees tended to prefer alfalfa, resulting in many fewer cells being made using nasturtium leaves.

Collectively, the results of our experiment show that the bacterial community in a pollen provision differs based on the plant that a mother bee collects from. These bacteria will likely be ingested by the larva, as previous studies have found that the pollen provisions and larvae share at least some microbes [9]. Whether these bacteria colonize newly emerged adult bees and are transmitted back into the environment merits further study.

Effects of Floral and Foliar Treatments on Fungal Diversity

Through our ITS gene survey data, we show that *M. rotundata* can deposit potentially pathogenic fungi into their brood cells and may transmit these pathogens to other plants upon emergence of the next bee generation. We found that there were significantly differentially abundant fungal plant pathogen genera (*Pezicula*, *Ganoderma*, *Aspergillus*, *Stemphylium*, *Ustilago*, *Trichothecium*, *Golovinomyces*, *Aureobasidium*, *Phaeococcomyces*, and *Alternatria*) in our samples between treatments, and to the best of our knowledge, these genera have not been previously identified in any survey of bee pollen. Our data suggests that it is plausible that the bees transmit these microbes upon emergence or that the foraging bees already have the fungi on them and are spreading disease. Many plant diseases are known to be vectored by pollinators [27]. Previous studies have shown that the smut fungus, *Microbotryum violaceum*, is vectored from diseased to healthy flowers by visiting pollinators [79]. Likewise, the causative agent of mummyberry (*Monilinia vaccinii-corymbosi*) also uses bees as vectors by attracting pollinators to then carry the infective conidia to a naïve plant host [80].

Apart from plant pathogens, we also found the causative agent of chalkbrood disease in *M. rotundata*—the fungi *Ascosphaera aggregata*—present in pollen provisions. This disease is thought to be transmitted to larvae from the mother from either her natal nest or the environment [24], although we are unable to discern transmission mode from our data. Other potential bee fungal pathogens like *Aspergillus niger* were also found, indicating that bee larvae may be exposed in their nests.

Similar to our results obtained from bacterial sequencing, our fungal survey data suggest that bees contact plant pathogenic microbes in their environment and deposit them into their pollen provisions. *M. rotundata* may be a common fungal disease vector, and more controllable and manipulative transmission studies should be conducted in the future.

Conclusion

Our study shows that the solitary bee, *M. rotundata*, can deposit plant-associated bacteria and fungi into the pollen provisions of their brood cells. Furthermore, our data suggests that bees can transmit plant pathogens into their nests, highlighting their potential role as vectors of plant diseases. Through manipulating the plants that the bees foraged on for pollen and leaf material, we also demonstrated that bees pick up differing proportions of both disease-causing and commensal microorganisms depending on their source plant, including bacteria and fungi that cause plant wilt and blight. Our study reports important and agriculturally relevant findings that may assist others in controlling the spread of plant pathogens and understanding the intricate relationship between pollinators and plants. We suggest that future studies examine the ability of both solitary and social bees to vector diverse infections to plants via pollination or leaf-collecting behavior and the interactions between the microbes associated with bees and plants.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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