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Authors
Rothman, Jason A
Carroll, Mark J
Meikle, William G
et al.

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INVERTEBRATE MICROBIOLOGY

Longitudinal Effects of Supplemental Forage on the Honey Bee (*Apis mellifera*) Microbiota and Inter- and Intra-Colony Variability

Jason A. Rothman[1,2] · Mark J. Carroll[3] · William G. Meikle[3] · Kirk E. Anderson[3,4] · Quinn S. McFrederick[1]

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Abstract

Honey bees (*Apis mellifera*) provide vital pollination services for a variety of agricultural crops around the world and are known to host a consistent core bacterial microbiome. This symbiotic microbial community is essential to many facets of bee health, including likely nutrient acquisition, disease prevention and optimal physiological function. Being that the bee microbiome is likely involved in the digestion of nutrients, we either provided or excluded honey bee colonies from supplemental floral forage before being used for almond pollination. We then used 16S rRNA gene sequencing to examine the effects of forage treatment on the bees' microbial gut communities over four months. In agreement with previous studies, we found that the honey bee gut microbiota is quite stable over time. Similarly, we compared the gut communities of bees from separate colonies and sisters sampled from within the same hive over four months. Surprisingly, we found that the gut microbial communities of individual sisters from the same colony can exhibit as much variation as bees from different colonies. Supplemental floral forage had a subtle effect on the composition of the microbiome during the month of March only, with strains of *Gilliamella apicola*, *Lactobacillus*, and *Bartonella* being less proportionally abundant in bees exposed to forage in the winter. Collectively, our findings show that there is unexpected longitudinal variation within the gut microbial communities of sister honey bees and that supplemental floral forage can subtly alter the microbiome of managed honey bees.

Keywords honeybees · microbiome · supplemental forage · symbiosis

Introduction

Honey bees (*Apis mellifera*) provide vital pollination services for a variety of agricultural crops around the world [1]. In the United States alone, bee pollination provides over $11 billion in annual agricultural crop production [2]. Honey bees host a distinctive gut microbiome that is consistent across colonies worldwide [3, 4]. This stable microbial community is the result of an ancient and long-lasting symbiotic relationship between corbiculate apid bees and several bacterial taxa [5]. Both social interactions and contact with hive surfaces contribute to the colonization of the early adult gut community and stable maintenance of the microbial associations [6, 7]. These microbes have functional characteristics that are beneficial to bee health, such as the ability to degrade pectin [8], digest toxic sugars [9], stimulate the immune system [10], and protect against parasitism [11, 12]. Additionally, the core honey bee microbiome is known to directly affect host physiology by enhancing weight gain, maintaining an oxygen gradient in the gut, and producing short chain fatty acids that are likely absorbed and utilized by the host [13].

Honey bee colonies experience stress during the nutrient dearth surrounding almond (*Prunus dulcis*) pollination due to the lack of forage and their subsistence on supplemental feeds. Bees suffer nutritional deficiencies when they are limited to supplemental feedings of high fructose corn syrup (HFCS) [14] or when foraging in a monoculture system, such as

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✉ Quinn S. McFrederick
quinnmc@ucr.edu

[1] Department of Entomology, University of California, 139 Entomology Building, Riverside, CA 92521, USA

[2] Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521, USA

[3] Carl Hayden Bee Research Center, USDA-ARS, Tucson, AZ 85719, USA

[4] Department of Entomology, University of Arizona, Tucson, AZ 85721, USA

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almonds [15]. Notably, bees that are fed HFCS instead of honey show lower immune potential, hampered detoxification abilities, and changes in protein metabolism [14, 16].

In addition to affecting honey bee nutrition and health, supplemental feeds have the potential to directly or indirectly affect the gut microbial community of honey bees. Sucrose and HFCS lack the natural phytochemicals found in honey [17], and bacteria that form the honey bee gut microbiome vary in the different carbon sources that they utilize and in their enzymatic abilities [9, 18–20]. Diet may indirectly affect honey bee microbiota through host immune gene regulation. Diet affects the composition of the honey bee and human gut microbiomes, which can lead to microbial dysbiosis and disease manifestation [21–23]. If feeding bees sucrose syrup or HFCS in times of dearth lowers the abundance of microbes that play important roles in honey bee biology, nutrition and colony vigor later in the year may suffer.

As the effects of diet on the microbiome may ripple across a season, longitudinal analyses of experimental treatments on microbial communities are needed. Longitudinal analyses of microbiota can uncover hidden variation or alternative stable states that may be overlooked in time-static experiments [24, 25]. For example, longitudinal studies of the human microbiome have shown that there is a dynamic change in bacterial diversity [26] and human habitation can affect the microbes of our environments [27]. The honey bee is an excellent organism in which to study the evolution and ecology of microbial symbioses [28], yet there is a little information about the specific changes that occur in bee microbial communities across a season. There is a critical need to understand the temporal and seasonal variation of the honey bee microbiome.

Our study addresses three aspects of the honey bee microbiome. First, we investigate the similarities and variation of the honey bee microbiome between foragers of separate colonies and sisters sampled from within the same hive in migratory bees used for almond pollination services. Second, we seek to understand the complex interplay between nutrition and the bacterial community of the honey bee gut through 16S rRNA gene sequencing surveys and by experimentally manipulating floral forage availability over winter. Third, we examine longitudinal variation in the honey bee microbiome in colonies used in migratory pollination.

Materials and Methods

Bee Husbandry and Experimental Setup

So that we may follow the practice of migratory beekeeping for almond pollination, we studied 40 honey bee colonies that occupied one to two, 10-frame deep Langstroth bee boxes with marked queens from source colonies at the University of Arizona Campus Agricultural Center. We placed eight colonies each in four sites, two sites near Red Rock Arizona, hereafter "RR", and two sites at the University of Arizona's Maricopa Agricultural Center in Maricopa, AZ, hereafter "MAC", for a total of 32 hives (see Supplemental Table S1 for geographic coordinates). To manipulate forage availability, colonies at the RR sites had access to ample forage provided in plots of *Brassica rapa*, while MAC sites had limited forage availability. To measure the effectiveness of our forage treatments, we used eight colonies as "pollen sentinels" (two per site) with pollen traps to determine whether bees had access to forage or not through visual inspection. Forage-supplemented bees at the RR sites were also found to have collected *Encelia farinosa*, *Larrea tridentata*, *Searsia lancea*, and *Erodium spp.*, while colonies at the MAC sites had access to about three times less forage [29]. We collected returning foragers from each colony and moved all colonies to Blackwell's Corner, CA where they were placed into groves of almonds that had not bloomed yet and had very little natural forage (the almond bloom was February 10 – March 1). We then collected incoming foragers post-bloom. All colonies were moved to a nearly forage-free holding yard at Keck's Corner, CA where they remained until the final sample of incoming foragers were collected. Incoming foragers were collected December 9–10, 2015, January 26–29, February 2–3 and April 2–5, 2016. All samples were immediately stored on dry ice, followed by long-term storage at −80 °C. In total, we analyzed three individual bee guts per time point from these 32 colonies (N = 289). Due to colony die-off, however, we were unable to sample bees from all 32 colonies at each time point (see Supplemental File F1 for the date that each sample was taken). All bee colonies received the same care as indicated in Table S1.

DNA Extraction and Next-Generation Sequencing of the Bacterial 16S rRNA Gene

We used the DNA extraction protocol from Engel et al. [30] in order to extract bacterial DNA from individual bee guts. We surface sterilized bees in a 0.1% sodium hypochlorite solution for 2 min, rinsed each bee in ultrapure water 3 times for 1 min each, then using sterile tools, aseptically dissected the entire gut from each bee and placed the gut into a sterile 2 mL screwcap tube. For reagent controls, we included three blank samples, which we included in all subsequent library preparation steps and sequence processing. We extracted total DNA from each sample with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) by adding 180 μL of buffer ATL and one 3.2 mm steel-chrome bead and 100 μL of 0.1 mm glass beads (BioSpec, Bartlesville, OK). We then bead-beat the samples for 6 min at 30 Hz. Next, we added 20 μL of

DNeasy kit-supplied Proteinase K to each sample, incubated the samples overnight at 57 °C, and subsequently followed the standard DNeasy extraction protocol.

We prepared libraries for Illumina MiSeq sequencing through a protocol using paired-end barcoding as in McFrederick and Rehan 2016 [31]. We used primers that incorporated a unique eight-mer barcode, the forward or reverse genomic DNA sequence and the forward or reverse Illumina adapter sequencing primer as in [32]. We used the primers 799-mod3 (CMGGATTAGATACCCKGG) [33] and modified 1115R (AGGGTTGCGCTCGTTG) [32] to amplify the hypervariable V5-V6 region of the 16S rRNA gene [34]. We performed PCR reactions with 4 μL of template DNA, 0.5 μL of 10 μM barcoded 799F primer, 0.5 μL of 10 μM barcoded 1115R primer, 10 μL sterile ultrapure water and 10 μL of 2× Pfusion High-Fidelity DNA polymerase (final concentration of 0.4 units) (New England Biolabs, Ipswich, MA) with an annealing temperature of 52 °C for 35 cycles in a C1000 Touch thermal cycler (BioRad, Hercules, CA). To remove unincorporated primers and dNTPs from the PCR reactions, we used the MoBio UltraClean PCR cleanup kit (MoBio Laboratories, Carlsbad, CA). Next, we used 1 μL of the cleaned PCR product as a template for a second PCR reaction using the primers PCR2F (CAAGCAGAAGACGGCATACG AGATCGGTCTCGGCATTCCTGC) and PCR2R (AATGATACGGCGACCACCGAGATCTACACTCT TTCCCTACACGACG) to generate the entire Illumina adapter sequence [32] under the following reaction conditions: 0.5 μL of 10 μM PCR2F, 0.5 μL of 10 μM PCR2R, 1 μL of cleaned and barcoded PCR amplicon, 13 μL of sterile ultrapure water and 10 μL of 2× Pfusion High-Fidelity DNA polymerase (final concentration of 0.4 units) for 15 cycles at an annealing temperature of 58 °C. In order to normalize the amount of DNA from each reaction, we used 18 μL of the library PCR amplicon and followed the protocol for SequalPrep Normalization plates (ThermoFisher Scientific, Waltham, MA). We pooled 5 μL of each of the normalized PCR products and cleaned up the pooled samples with a MoBio UltraClean PCR cleanup kit. We then analyzed the size and abundance of our amplicons on a 2100 Bioanalyzer (Agilent, Santa Clara, CA). We then sequenced the libraries using a MiSeq V3 Reagent Kit at 2 X 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 numbers SRR5532840-SRR5533128.

Bioinformatics

We used macQIIME v1.91 [35] to process the 16S rRNA gene sequences. We used USEARCH v6.1 [36] to identify and remove chimeric sequences, and SUMACLUST [37] to cluster de novo Operational Taxonomic Units (OTUs) at 99% sequence identity and to remove OTUs with fewer than two sequences. We chose to use the 99% sequence identity threshold so that we may be able to see subtle differences in the bees' microbial communities [38, 39]. We assigned taxonomy to the OTUs using the Greengenes 16S rRNA gene database [40] and the RDP Naïve Bayesian Classifier [41] at 80% confidence. To verify taxonomic assignments, we conducted additional BLASTN searches [42] against the NCBI Nucleotide Collection (nr/nt) (accessed December, 2016). We then used the resulting taxonomy to remove mitochondria, chloroplast, and reagent contaminants (Supplemental File F1, identified from blank control samples and (Salter et al. 2014)) from the OTU table. After filtering, we used pynast [43] to align the sequences against the Greengenes core reference alignment [44] and generated a phylogeny using FastTree v2.1.3 [45]. We used this phylogeny, and an OTU Table [46] that we generated with QIIME to calculate alpha diversity, rarefaction sampling depth, Good's coverage, and to generate UniFrac and Bray-Curtis distance matrices [47]. Subsequently, we analyzed the resulting UniFrac matrices via Principal Coordinates Analysis, (PCoA) and Non-metric Multidimensional Scaling (NMDS), and used R [48] to plot the ordinations. We also used the R packages "vegan" [49] for significance testing on both weighted and unweighted UniFrac distance matrices, "ggplot2" to graph the change in weighted UniFrac distance of samples over time [50], and "metagenomeSeq_fitZIG" to analyze differentially abundant OTUs across time points and treatments [51], using the Bonferroni correction method to adjust for multiple comparisons. We analyzed homogeneity of dispersion in QIIME with PERMDISP (nonparametric Levene's test with 999 permutations). Lastly, we compared alpha diversities and weighted UniFrac distances with the QIIME script "make_distance_boxplots.py." This script performs nonparametric (999 permutations) two-way Student's t-tests in QIIME, and we used this test to compare the average UniFrac dissimilarity distances between individual workers' gut microbiota between and within colonies over time.

Results

Alpha Diversity and Library Coverage

We determined through rarefaction analysis that we could get representative coverage of sample species diversity with 10,000 reads per sample (Fig. S1). The average Good's coverage of the alpha diversity of our non-rarefied sample reads was 0.961 (minimum 0.676, maximum 0.996, standard deviation 0.033), and the average Good's coverage of the rarefied

reads was 0.950 (minimum 0.922, maximum 0.994, standard deviation 0.011). There were a total of 7,300,191 quality-filtered reads with an average of 25,260 reads per sample ($n = 289$) that were clustered into a total of 10,070 non-singleton, quality-filtered OTUs at 99% sequence identity. Based on two-tailed pairwise t-tests (999 Monte Carlo permutations) between the rarefied OTU tables of each sample, there was no significant difference ($P > 0.05$) of the effects of forage treatment, sampling date, or colony of origin on the Chao 1 alpha diversity index of the bee colonies. Across all samples, seven bacterial genera composed an average of 96.3% of the total OTU abundance: *Lactobacillus* (34.1%), *Gilliamella* (29.9%), *Snodgrassella* (17.6%), *Commensalibacter* (8.3%), *Bifidobacterium* (3.3%), *Bartonella* (1.7%) and *Erwinia* (1.4%). An OTU table with both RDP taxonomy and the top BLAST hit for each OTU and its abundance in the reagent control blank samples can be found in Supplementary File F1.

Beta Diversity

To discern patterns of clustering by either forage treatment or sampling date, we performed Principal Coordinates Analysis (PCoA) on weighted UniFrac distances (Fig. S2 and S3) and Non-metric MultiDimensional Scaling (NMDS, Fig. 1) on both weighted and unweighted UniFrac distance matrices, which did not show any obvious visual clustering by forage treatment or sampling date. Two notable exceptions are the slight clustering in the NMDS ordination of weighted UniFrac distance by treatment during the

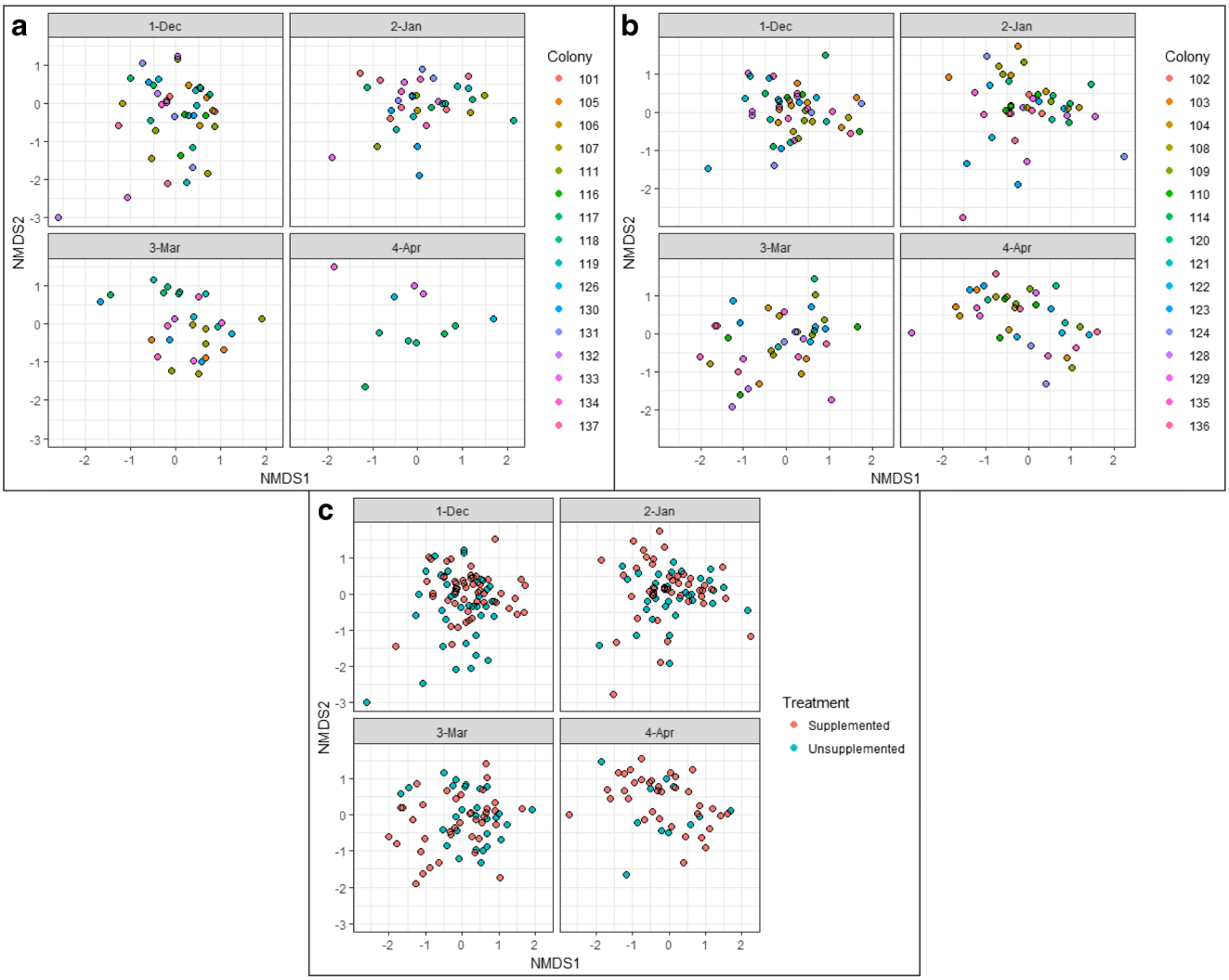

Fig. 1 Non-metric Multidimensional Scaling (NMDS) plots of the weighted UniFrac distance of a) Foragers that did not receive supplemental forage separated by timepoint and grouped by colony of origin, b) Foragers that received supplemental foragers separated by timepoint and grouped by colony of origin and c) Individual foragers grouped by forage treatment by timepoint. The only significant differences between the microbiota of supplemented and non-supplemented bees occurred during the March timepoint ($P = 0.042$). The only significant variation of within colony versus between colony bee microbial communities also occurred during the March timepoint

March sampling point and by colony throughout all timepoints. Although there was no obvious visual clustering by either treatment or sampling date, Adonis analyses (PERMANOVA with 999 permutations) indicated that there were significant effects of: (a) treatment on the beta diversity of the bee gut bacterial communities by both unweighted ($F = 1.59$, $R^2 = 0.006$, $P = 0.011$) and weighted UniFrac distances ($F = 2.87$, $R^2 = 0.01$, $P = 0.02$), (b) sampling date by both unweighted ($F = 1.41$, $R^2 = 0.02$, $P = 0.008$) and weighted UniFrac distances ($F = 2.12$, $R^2 = 0.02$, $P = 0.017$), and the (c) interaction of sampling date and forage treatment by weighted UniFrac only ($F = 2.23$, $R^2 = 0.02$, $P = 0.008$), but not unweighted UniFrac ($F = 1.09$, $R^2 = 0.01$, $P = 0.194$). It is worth noting the small R^2 values of these analyses, which indicates that there are other, major sources of variation that are likely more important than sampling date or forage treatment. We therefore conducted further analyses to elucidate the sources of some of this variation. We found that the two sites within each treatment did not yield significantly different gut diversity ($F = 1.52$, $R^2 = 0.02$, $P = 0.092$) from each other, as measured by Adonis. We analyzed the beta diversity of (a) the bee colony of origin, which showed a significant difference in both the unweighted ($F = 1.29$, $R^2 = 0.14$, $P = 0.001$) and weighted ($F = 1.62$, $R^2 = 0.16$, $P = 0.001$) UniFrac distances and (b) the interaction of colony of origin and sampling date in both unweighted ($F = 1.11$, $R^2 = 0.23$, $P = 0.001$) and weighted ($F = 1.21$, $R^2 = 0.23$, $P = 0.035$). To rule out the effect of unequal dispersion causing a Type I error in our Adonis analyses, we evaluated the dispersion of the sample groupings of forage, treatment and sampling date through PERMDISP (permutational dispersion of beta diversity with 999 permutations) on weighted UniFrac distances and found that treatment ($F = 0.15$, $P = 0.71$), sampling date ($F = 0.58$, $P = 0.63$) and colony of origin ($F = 1.98$, $P = 0.053$) did not have heterogeneous dispersion.

Differential Abundance of Bacterial Taxa

There were significant differences of relative bacterial abundance based on both time and forage treatments. Seven OTUs showed a significant decrease in proportional abundance in the March sampling date regardless of forage treatment as compared to all other sample dates: A strain of unknown *Acetobacteraceae* ("denovo39," $Padj = 0.006$), one strain of *Snodgrassella alvi* ("denovo29," $Padj = 0.011$), two separate strains of *Commensalibacter* sp. ("denovo4" and "denovo42," $Padj = 0.034$ and 0.041 respectively), a strain of *Frischella perrara* ("denovo12," $Padj = 0.034$), and one *Gilliamella apicola* strain ("denovo23," $Padj = 0.034$). There were no differentially abundant OTUs throughout the rest of the sampling dates. There were seven proportionally less abundant OTUs and one proportionally more abundant OTU present in the March sampling date within the forage-supplemented bees compared to unsupplemented bees: Two separate strains of *Gilliamella apicola* ("denovo26" "denovo40," and $Padj < 0.001$ and 0.017 respectively), three separate strains of *Lactobacillus* ("denovo22," "denovo 28" and "denovo45," $Padj < 0.001$, 0.017 and 0.040 respectively), two separate strains of *Bartonella* sp. ("denovo 34" and "denovo15," $Padj = 0.040$ for each) and one *Morganella* sp. ("denovo37," $Padj = 0.019$) (Fig. 2). All of these bacterial taxa were less abundant within the forage-supplemented bees, with the exception of the *Morganella* OTU.

Time Series Analyses

To further explore the effect of the interaction between forage and time, we used a violin plot to visualize the weighted UniFrac distances of each treatment over time (Fig. 3). The overall distance between treatments per timepoint is generally low regardless of treatment exposure. There are significant differences between the distances within forage-supplemented and unsupplemented bees, with bees that did not receive a forage treatment differing between treatments most during the March time point. This can be illustrated by Adonis analyses on both unweighted and weighted UniFrac distances matrices for each sampling date. There was a significant, albeit small effect of forage treatment on the weighted UniFrac distances from within the January time point ($F = 2.28$, $R^2 = 0.03$, $P = 0.041$), and both unweighted ($F = 1.40$, $R^2 = 0.02$, $P = 0.033$) and weighted ($F = 2.39$, $R^2 = 0.04$, $P = 0.042$) UniFrac distances from within the March sampling date. The rest of the time series did not show any significant effect of treatment. Additionally, there was a significant difference between the weighted UniFrac distances of colonies at the March time point (Adonis: $F = 1.71$, $R^2 = 0.45$, $P < 0.001$). Similarly, there was a significant effect of the interaction between the colony of origin and sampling date regardless of treatment, so we generated several plots to visualize how these interactions may be contributing to the overall difference in microbial diversity between colonies (Fig. S3). A nonparametric two-tailed Student's t-test failed to detect difference in the worker gut UniFrac distances between sisters from within the same colonies and between workers of different colonies of origin over time ($t = -1.80$, $P = 0.075$, statistical power = 0.90). However, when sampling dates are considered separately, we found that the microbiome of sisters within colony was significantly different from workers between colonies during the March timepoint (Student's nonparametric two-tailed t-test: $t = -2.63$, $P = 0.011$), while there was no significant difference between these groups at any

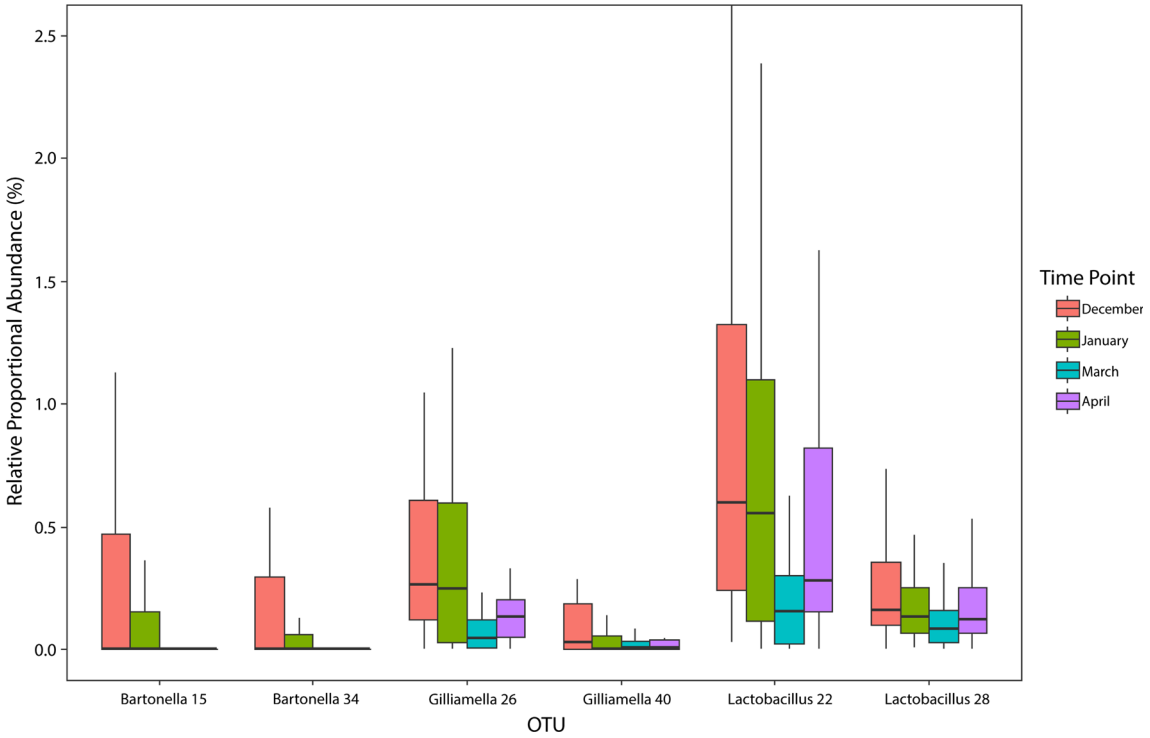

Fig. 2 Proportional abundance of differentially abundant bacterial taxa binned by the sampling time point within forage-treated bees. Only March had a significantly decreased proportional abundance of two Bartonella, Gilliamella and Lactobacillus OTUs as compared to the initial December timepoint. (*Padj.* <0.05). Only OTUs with greater than 0.1% proportional abundance in at least one time point are visualized. Error bars show 1.5× the interquartile range

other timepoint (both Adonis and Student's t-test, $P > 0.05$). Lastly, as reported in Carroll et al. 2017 [29], colonies experienced significant die-off with 81% of unsupplemented colonies failing versus 25% of forage-supplemented colonies, with the majority of the colony failure occurring in March and April. There was no

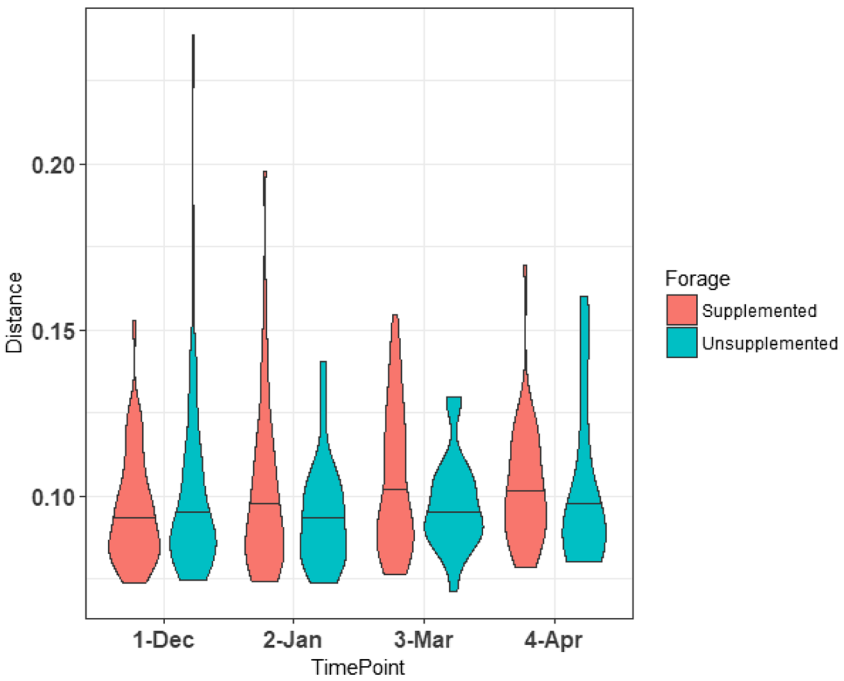

Fig. 3 Violin plot of the weighted UniFrac distances compared within treatment groups and between treatment groups of forage-supplemented and non-supplemented bees at all timepoints. The bee gut microbiome changes slightly over time, with forage-supplemented bees having significantly different microbiomes in both the January ($F = 2.28$, $R^2 = 0.03$, $P = 0.041$), and March ($F = 2.39$, $R^2 = 0.04$, $P = 0.042$) time points

significant difference in mortality between supplemented and unsupplemented colonies at the December or January timepoints.

Discussion

Microbial Diversity between and within Colonies

We found that the microbiome of migratory honey bees is quite stable over time, regardless of the availability of winter forage. This finding supports previous studies suggesting that the honey bee microbiome is stable, as transmission of the *A. mellifera* worker microbiome occurs mainly during social interactions within the colony and contact with colony surfaces [6, 52]. These findings are also in line with the general understanding that many insect species, such as American Cockroaches (*Periplaneta americana*) [53] and turtle ants (*Cephalotes* sp.) [54] have a consistent microbiome, and that maintaining this host/microbe relationship over time is important for the overall vitality of bees [55–58]. Despite this stability, our comparisons of the microbiota of adult foragers from the same colony show that within-colony variation exists. Likewise, there is variation between the microbiomes of different colonies as a whole. However, somewhat surprisingly, there can be more variability between the gut microbes in sisters of the same colony than between colonies. This can potentially confound the results of experiments and result in confusing or misleading interpretation of data, as many culture-independent 16S rRNA gene sequencing studies of the microbial community of honey bees also pool multiple colonial sister samples together [10, 52, 59–61]. Possible sources of this variation could include paternal diversity through queens laying eggs fathered by multiple males [62], foragers drifting between colonies or variation in the ages of bees sampled, as we could not always collect foragers with corbiculate pollen. The variation in forager ages over time is likely exacerbated during almond pollination as workers are foraging heavily and are under heavy stress. As the succession of the gut community has been shown to vary with age and stress, this could account for the decreased abundance of *G. apicola* in the foragers during almond pollination, which may be contributing to a dysbiotic gut community [63]. Within a colony, foragers interact with different plant species and individuals and may contact different floral chemicals that likely affect their gut microbiome [64]. Given the degree of variation in microbiome structure within a colony, we do not recommend that researchers pool honey bee guts for microbial analyses and instead separately sequence and analyze multiple individual bees from colony replicates.

Migratory Beekeeping

The process of migratory beekeeping transports honey bee colonies to multiple different environments over the course of a single year. These new environments expose migratory honey bee colonies to a variety of pathogens, pesticides, and lack of natural forage [65]. In our study, we followed migratory colonies from the desert near Tucson, Arizona to the almond orchards and bee-holding yards of Central California. We expected to find variation of the gut microbiome across this migration due to the availability of different sugars [9], novel pathogens [66], and seasonal variation [39]. Interestingly, we do not find that moving honey bee colonies long distances causes large shifts in the gut microbial community, and that only minor changes resulted from a combination of forage availability and migration. Future studies should investigate the underlying stability of the bee microbiome's response to migration solely, without the effects of forage supplementation. Also, as we analyzed the bees' microbial communities with 16S rDNA surveys instead of transcriptomics, we are unable to discern patterns of differential gene expression or strain-level genomic differences. These outstanding questions about the specific bacterial strains' genomic variation and the possible effects on migratory honey bees could also be specifically studied in future experiments.

The Effects of Forage and Time on Microbiome Structure

The microbiota of bees and other insects can be altered by many external factors, including pesticides [67], foreign bacteria [68], diet [21, 69], pharmaceutical pollution [70, 71] and habitat [72]. Our data show that exposure to natural floral forage during the winter months has only subtle effects on the composition and possible function of the honey bee gut microbiota. The main source of variation in microbiome composition with respect to forage effects was the March sampling date, as this is the only time point where there were any differentially abundant bacterial taxa between the forage and no forage treatments, although this may also be an artifact of our choice of 99%-similar OTUs. To address the possibility of 99% OTU clustering introducing noise, we also clustered OTUs at 97% and found no difference in the community-level interpretation of our data. Being that the forage-supplemented bees were exposed to a diet of varied flowers, it is likely that they are consuming different types of sugars which can be metabolized by the bee gut microorganisms [9, 73], or that the bees acquired some rare strains of bacteria from the flowers themselves [74, 75]. Being that the bees may bring floral bacteria back to their colony and inoculate the hive surfaces, honey, pollen stores and bee bread with these microbes [76, 77], it is entirely plausible that future generations of bees will have altered microbiota as compared to

unsupplemented bees. Likewise, as the bees are consuming floral plant compounds, such as *p*-coumaric acid, there may be differential expression of immunity genes that alter the ability of the gut symbionts to persist [10, 14] or that these compounds are being deposited into food stores and hive surfaces. Interestingly, the majority of OTUs that were differentially abundant were *less* abundant in the forage-supplemented bees, which suggests that the added stress of almond pollination could affect the gut bacteria. However, being that our data are proportional abundancies rather than absolute, it is also plausible that the forage supplementation causes other bacteria to be more proportionally abundant without decreasing the absolute abundance of the core microbes. Additionally, the microbial communities between forage-supplemented and unsupplemented bees were not significantly different once the colonies were removed from the almonds and kept in a common environment, which likely emphasizes the importance of the mass almond pollination event on the bees' microbes.

Our study included samples at four time points from December to April, with most interesting and significant effects occurring in the March timepoint. This sample consisted of bees that foraged in the blooming almond orchards for most of February, and after receiving supplemental forage throughout the month of January. The combined effects of exposure to forage and almond pollination indicate that these factors synergistically affect the microbiome of honey bees by increasing the bacterial beta diversity of the gut. This result could originate from effects of forage on the quality of the hypopharyngeal gland secretions of nurse bees in the winter months [21], which could affect the phenotypes of foraging workers that we collected in March. Although the rates of turnover in microbiome structure varies over time, these differences leveled off by the end of the experiment, supporting the notion that despite transient changes to the microbiota, the gut community of honey bees is reasonably consistent.

Longitudinal variation of the gut microbiome has been studied in humans [26, 78, 79], wood mice [80], and to a limited extent, honey bees [39, 55, 58]. For example, Ludvigsen et al. [39] showed that microbial species richness of the midgut of foragers increases throughout the months of May–October, and attribute most of the change of the microbes' α-diversity to differing diets throughout their experiment. Hroncova et al. [55], using Denaturing Gel Gradient Electrophoresis (DGGE), found significant microbial variability between sisters of the same colony, as well as ontogenetic variation. However, DGGE does not allow for the fine-scale taxonomic ranking that next-generation sequencing does, which is important for observing subtle changes in microbial communities [81]. Hroncova et al. [55] also does not recommend pooling biological replicate samples together, and we concur with this recommendation. Corby-Harris et al. [58] found that the microbial gut communities of foragers collected in fall and spring were quite similar.

Our research differs from the above-mentioned studies in several ways. Ludvigsen et al. [39] studied the midgut, rather than the entire gut, as was done here. While investigating bacteria found in the midgut is insightful to understanding the bacterial community of the honey bee gut, the hindgut contains more than 95% of the total bacterial microbiota [52] so a study of the entire gut would be expected to capture more of the bacterial diversity in each bee. Hroncova et al. [55] examined developmental changes to the bee microbiome, whereas this study focused on variation in bee microbiomes across a season. Finally, Corby-Harris et al. [58] binned OTUs by 97% sequence similarity, while we used 99% to account for strain variability; they pooled 10 forager guts together, while we sequenced individual bees; and they sampled 14 colonies from within the same apiary while we sampled bees from 32 colonies across four different sites and two treatments. These major methodological differences make our studies complementary and simultaneously useful, while still investigating different hypothesis in the microbiomes of honey bees.

Our study shows that supplementing managed honey bees with floral forage before almond pollination can subtly change the composition of their bacterial gut communities. Importantly, we were also able to show that there is variation of the bee microbiome between individual sisters of the same colony. Lastly, we examined the plasticity of the honey bee microbiome over time in managed colonies used for pollination services. We suggest that future studies investigate the source of the within-colony bee microbiome variability and what specific microbial genomic features are involved in honey bees' response to a migratory lifestyle.

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