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INVERTEBRATE MICROBIOLOGY

Flowers and Wild Megachilid Bees Share Microbes

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Abstract Transmission pathways have fundamental influence on microbial symbiont persistence and evolution. For example, the core gut microbiome of honey bees is transmitted socially and via hive surfaces, but some non-core bacteria associated with honey bees are also found on flowers, and these bacteria may therefore be transmitted indirectly between bees via flowers. Here, we test whether multiple flower and wild megachilid bee species share microbes, which would suggest that flowers may act as hubs of microbial transmission. We sampled the microbiomes of flowers (either bagged to exclude bees or open to allow bee visitation), adults, and larvae of seven megachilid bee species and their pollen provisions. We found a *Lactobacillus* operational taxonomic unit (OTU) in all samples but in the highest relative and absolute abundances in adult and larval bee guts and pollen provisions. The presence of the same bacterial types in open and bagged flowers, pollen provisions, and bees supports the hypothesis that flowers act as hubs of transmission of these bacteria between bees. The presence of bee-associated bacteria in flowers that have not been visited by bees suggests that these bacteria may also be transmitted to flowers via plant surfaces, the air, or minute insect vectors such as thrips. Phylogenetic analyses of nearly full-length 16S rRNA gene sequences indicated that the *Lactobacillus* OTU dominating in flower- and megachilid-associated microbiomes is monophyletic, and we propose the name *Lactobacillus micheneri* sp. nov. for this bacterium.

Keywords *Lactobacillus micheneri* · *Gilliamella* · *Arsenophonus* · Flower microbes · Floral transmission · Wild bees

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Introduction

How microbes assemble into microbiomes is a fundamental question in microbial ecology. For mammals and plants, environmental factors appear to be the most important, although there is gathering evidence that host genetics can influence microbiome structure [1]. In hominids, for example, *Bifidobactertium adolescentis* and several Bacteroidaceae species cospeciated with their hosts, indicating an ancient, coevolving relationship between host and at least some members of the gut microbiome [2]. Microbiome composition for many hosts may be most influenced by environmental factors such as diet or geography, yet heritability and vertical transmission may still play a role [1].

Insects and other invertebrates serve as important models for microbiome and symbiosis research [3, 4]. Insect microbiomes usually exhibit lower diversity than do vertebrate microbiomes, yet they exhibit a continuum of transmission modes [3]. Beneficial microbes are often host-specific and maternally inherited, as maternal inheritance typically couples the evolutionary interests of host and symbiont,

facilitating the evolution of cooperative (beneficial) host-microbiome interactions [5]. *Wolbachia* and other sex ratio distorters are fascinating exceptions, in that they are vertically transmitted but are reproductive manipulators instead of mutualists [6]. Another form of transmission that is akin to maternal inheritance is social transmission, which occurs between colony mates [3] and sexual partners [7] and can similarly couple the fates of the host and symbiont. A third mode of transmission is environmental acquisition of pathogenic, commensal, and beneficial microbes through contact with environmental microbes or via food. The broad-headed bug *Riptortus clavatus*, for example, acquires its beneficial *Burkholderia* symbionts from the soil [8].

Honey bees and bumble bees harbor distinct microbes that are shared via social transmission [9, 10] and, in the case of honey bees, contact with hive surfaces [11]. The "core" gut microbiota of honey bees and bumble bees appears to be mostly host-specific, while other social and solitary bee species appear to harbor mostly environmental bacteria [12]. In our previous work, we found that halictid bees, a solitary apid, and a megachilid bee associate with bacteria that have also been isolated from flowers or have close bacterial relatives that have been isolated from flowers [13–16]. For example, in wild bees, we have found a bacterium that is most closely related to *Lactobacillus kunkeei. Lactobacillus kunkeei* is found in the hive materials and larval guts of honey bees and has been isolated from flowers visited by honey bees [17]. However, *Lactobacillus kunkeei* strains isolated from flowers do not grow well in royal jelly, whereas strains isolated from honey bee larvae do, suggesting that some strains may be adapted to the honey bee niche [18]. Through deep sequencing of 16S rRNA gene amplicons, we have found considerable strain diversity in relatives of *L. kunkeei* that associate with wild bees [13, 15, 19] but the functional differences within this cryptic diversity is not understood.

Here, we address several unanswered questions regarding microbes found in association with wild megachilid bees and the flowers that they visit. First, we determine whether flowers and megachilid bees harbor the same microbes by sampling multiple megachilid bee species and multiple flowering plant species from two widely separated communities in central Texas. To determine whether the dominant *Lactobacillus* OTU from these samples is abundant in flowers, bee-associated samples, or both, we used quantitative PCR to measure absolute abundance. To determine if flowers that have not been visited by bees harbor the same microbes as those that have been visited by bees, we characterized the microbiomes of both bagged flowers and flowers that we had observed bees visiting. We further investigated strain diversity in three genera of megachilid bee- and flower-associated bacteria by sequencing nearly full-length 16S rRNA gene sequences and reconstructing the phylogenetic history of these bacteria.

Materials and Methods

Sample Collection

During April–June of 2013, we sampled bees, pollen provisions, and flowers from two sites separated by approximately 112 km in central Texas: a privately owned fallow field straddling Bexar and Guadalupe counties (29° 27′35.3″N 98° 07′ 33.2″W) and the Center for Environmental Research at Hornsby Bend (30° 14′08.6″N 97° 38′48.2″W, https://www.austintexas.gov/cer). To collect pollen provisions and larval bees, we placed three Logan Beemail shelters [20] at each site and filled the shelters with 16 by 37 by 127 mm untreated pine wood into which we drilled holes of 6.4, 6.7, or 7.1 mm in diameter. We checked these nests weekly or biweekly and transported completed nests (i.e., nests with the entrance sealed by the mother bee) back to the lab. We also netted adult bees on flowers within a 300-m radius of the nest boxes and immediately placed the adults in sterile tubes and then on ice for transport back to the laboratory. We collected adults and nests of seven species from three genera of megachilid bees: *Lithurgus gibbosus* and *Lithurgus littoralis*; *Megachile brevis*, *Megachile parallela* and *Megachile policaris*; and *Osmia chalybea* and *Osmia subfasciata* (see Table S1 for metadata).

To avoid contamination with airborne microbes, we opened the nests in a non-flow hood (a plastic tub turned on its side, sanitized with a 0.03 % sodium hypochlorite solution followed by 95 % ethanol) with a flame burning in front of the hood. We collected pollen provisions directly into tubes that had been sterilized by exposure to UV light (254 nm/>40 μW per square centimeter) for 10 min. To examine gut microbes in adult and larval bees, we surface-sterilized each bee with a 1-min wash in a 0.03 % sodium hypochlorite solution followed by three washes in sterilized, nano-pure water. Using flame-sterilized tools, we then dissected out the guts of the bees in paraffin wax dissecting trays that had been exposed to UV light as described above. After dissection, we immediately stored the guts at −80 °C until DNA extractions.

We used isohelix swabs (Cell Projects Ltd, UK) to sample all flower surfaces potentially contacted by bees. We swabbed the petals, nectaries, anthers, and stamens of each flower, placed the swabs on ice for transport back to the laboratory, and stored the swabs at −80 °C until we could perform DNA extractions. We used three approaches to sample and sequence the microbial communities on flowers. First, we targeted open flowers from which we collected adult bees: *Cirsium texanum* ($N = 2$), *Gaillardia pulchella* ($N = 1$), *Helenium amarum* ($N = 1$), *Helianthus annuus* ($N = 1$), *Monarda citriodora* ($N = 1$), *Opuntia engelmannii* ($N = 4$), and *Phacelia congesta* ($N = 3$). Second, to survey the microbiomes of flowers that have not been visited by bees, we covered unopened flowers with a 0.33-mm mesh bag, allowed the flower to open, and

then swabbed the flower (*C. texanum* $N = 4$, *G. pulchella* $N = 5$, *H. amarum* $N = 3$, *P. congesta* $N = 2$). The mesh size of the bags was chosen to exclude even the smallest bees and permit gas exchange of each flower but did not exclude small flower visitors such as thrips. Third, to survey bacteria generally found in flowering meadows, we randomly placed ten plots of 3-m diameter at Hornsby Bend and used a single swab to sample every flower in each plot. These plots were dominated by *Abutilon fruticosa*, *Callirhoe digitata*, *C. texanum*, *G. pulchella*, *H. amarum*, *O. engelmannii*, *P. congesta*, *Ratibida columnifera*, and *Solanum elaegnifolium*.

Illumina 16S rRNA Gene Amplicon Sequencing

Molecular Research (MR DNA, Shallowater, TX) performed DNA extractions and 16S amplicon sequencing on the Illumina MiSeq platform, using previously published protocols [13, 21]. Extractions were done using a Qiagen DNeasy blood and tissue extraction kit and a bead-beating step of 3 min at 30 Hz with a sterile 5-mm stainless steel bead and 0.1-mm glass beads was included to lyse gram-positive bacteria, as in previous bee-microbiome studies and as recommended in the Coloss BeeBook standard protocol [22–24]. To amplify the 16S rRNA gene, we used the 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) primer pair, again following previously published protocols and including negative controls [13, 21]. Two separate sequencing runs were performed. The first run comprised 40 samples, 8 (7 plant and 1 bee sample) of which contained a majority of reads originating from plant plastids. A second sequencing run was therefore performed with these 8 samples and 32 new samples where we first ran the PCR products out on a 1 % agarose gel and selected the larger bacterial band for gel purification and sequencing. For both runs, the sequencing reaction was performed on an Illumina MiSeq for 250 cycles for both paired ends of the amplicons; however, because the paired ends did not join, we used only the forward read in the subsequent analyses.

Bioinformatic and Statistical Analyses

To explore the alpha and beta diversity of flower and bee microbiomes, we used standard analyses as implemented in the QIIME pipeline [25]. We first analyzed our two datasets together but found that bias introduced by including or excluding the gel extraction step prevented comparative analyses across runs (Fig. S1). We therefore conducted diversity analyses within each sequencing run, and we present the results of the two runs separately. For both analyses, we used USEARCH for chimera checking [26] and Sumaclust for clustering of operational taxonomic units (OTUs) at 97 % sequence identity. Sumaclust performed consistently well in a recent test of OTU picking software [27]. We removed OTUs present at fewer than two reads per sequencing run. To assign taxonomic identity to the OTUs, we used the RDP naïve Bayesian classifier [28] with the Greengenes database [29], along with local BLAST searches using NCBI's 16S microbial database (downloaded December 3, 2015). We used this taxonomy to identify and remove plastid reads from our data. To align our quality-filtered FASTA file, we used the PyNAST aligner [30] along with the Greengenes database [29]. We visually inspected the alignment in Mesquite [31] and adjusted the alignment by eye. To create a phylogenetic tree as input for UniFrac analyses, we used FastTree [32] as implemented in QIIME.

After quality filtering, we calculated alpha and beta diversity in QIIME and used R [33] to graph the results. We created a heat map indicating the proportional abundance of the five most abundant OTUs within each sequencing run using the gplots package [34]. To visualize dissimilarity between samples based on proportional abundances of the top 10 OTUs, we used hierarchical clustering to create a dendrogram by which we organized the samples in our heat map. Before calculating alpha and beta diversity metrics, we first subsampled to 600 reads per sample for run 1 and 825 reads per sample for run 2. These subsampling depths allowed us to retain 33 samples in run 1 and 40 samples in run 2, but based on rarefaction curves (Fig. S2) and Good's coverage [35] from our alpha diversity analyses also captured the majority of the diversity found in our samples (see results). For beta diversity, we used QIIME to calculate unweighted and weighted UniFrac distances [36] and perform non-metric multidimensional scaling (NMDS). We also calculated generalized UniFrac distances using the GUniFrac library in R [37]. To test for differences between sample types, host species, and sites, we used Adonis analysis in the R Vegan package [38]. To test for differences in plant species and bee species only, we reduced the OTU tables to only plant or bee samples, then recalculated UniFrac distances and performed Adonis analysis. We also performed Kruskal-Wallis rank sum tests and Kendall rank correlation in R to analyze differences in the relative and absolute abundance of *Lactobacillus kunkeei* by sample type and correlate the qPCR and 16S amplicon data, respectively.

Quantitative PCR of *Lactobacillus kunkeei*-clade Bacteria

To quantify the abundance of flower- and bee-associated bacteria from the *Lactobacillus kunkeei*-clade, we developed a qPCR assay using the single-copy transcription elongation factor *greA* gene. We used *greA* sequence from an unpublished genome of a *Lactobacillus kunkeei* relative that was originally isolated from the sweat bee *Halictus ligatus* [13]. To develop primers specific to *Lactobacillus kunkeei*-clade

bacteria, we compared *greA* sequence from unpublished *Lactobacillus* genomes as well as publicly available *Lactobacillus* genomes. To check for specificity, we used NCBI's Primer-BLAST [39]. We selected a primer pair that produces a 71 base amplicon: greAF (5′-GAGA AGCATTTACTAAGCCAAC-3′) and greAR (5′-CATA TTGACCTTTACCACCAGAT-3′). We then cloned *greA* PCR products using the TopoTA cloning kit with One Shot Top10 component cells (Life Technologies, Carlsbad, CA), purified the plasmids, linearized the purified plasmids using the PstI-HF restriction enzyme (New England Biolabs, Ipswich, MA), and quantified the plasmids on a Qubit fluorometer (Life Technologies, Carlsbad, CA). We used a dilution series of these plasmids to create a standard curve for absolute quantification of our unknown samples on a CFX96 real-time thermal cycler (BioRad, Hercules, CA), using 10-μL reactions comprising 5 μL of Sso-Advanced mastermix (BioRad, Hercules, CA), 2-μL molecular grade water, 0.5 μL each of 2 μM primer, and 2 μL of 2× diluted DNA extraction. For absolute quantification, we ran each sample in triplicate with triplicate dilution series reactions for each plate. The efficiencies of these reactions ranged from 97 to 102 %. Our detection threshold was 10^3 copies, and we assigned a value of 10^3 to samples with copy numbers below this detection threshold, as in [9, 11].

Phylogenetic Analyses

To further explore strain diversity, we sequenced most of the full length of the 16S rRNA gene from representative strains of three flower- and bee-associated bacteria: a bacterium closely related to *Lactobacillus apinorum* and *Lactobacillus kunkeei*, a bacterium related to *Gilliamella apicola*, and a bacterium related to *Arsenophonus nasoniae*. First, we generated 27F-1492R [40, 41] amplicons from megachilid bee samples from this study that were dominated by one of these bacteria: (1) for *Lactobacillus* OTU 0, we used Me.178.BG.P, a pollen provision from a *Megachile policaris* nest; (2) for *Gilliamella apicola*, we used ME.173.BG.G, the gut of a *Megachile policaris* larva; (3) for *Arsenophonus*, we used OS.40.HB.P, pollen from the scopa of an *O. chalybea* adult female. We used standard PCRs with 35 cycles and an annealing temperature of 52 °C. We then conducted TopoTA cloning with One Shot Top10 component cells (Life Technologies, Carlsbad, CA) and plasmid purification. Next, we used Sanger sequencing to obtain three to four unique DNA sequences for each bacteria, sequencing from each end with 27F or 1492R, respectively.

To verify that the *Lactobacillus kunkeei* relative is abundant on flowers and in wild bees, we cultured this bacterium directly from flowers and wild halictid bees found on the campus of UC Riverside in September 2015. To isolate this bacterium from flowers, we pipetted 50 μL of sterile physiologic saline solution into the base and nectaries of Indian mallow (*Abutilon* sp.) flowers ($N = 11$) that we had observed wild halictid bees visiting ($N = 12$: 4 *Agapostemon* sp., 4 *Augochlorella pomoniella*, 2 *Dialictus* sp., and 2 *Halictus tripartitrus*). To dislodge microbes, we mixed by pipetting a standard number of times, then aspirated the physiologic saline out of the flowers and transferred it to a sterile microcentrifuge tube. We placed the tubes on ice for transport back to the lab and then plated 20 μL of the suspension onto de Man, Rogosae, Sharpe agar plates [42] supplemented with 20 % fructose (MRS + F). For bees, we first observed them visiting flowers, then collected them into sterile tubes and stored them on ice for transport back to the lab. We dissected their guts under sterile conditions and homogenized the guts in 50-μL physiologic saline. We again plated 20 μL of the suspension on MRS + F plates. To isolate pure cultures, we picked single colonies and subcultured each culture three times before moving them into liquid MRS + F medium. We allowed these cultures to grow at room temperature until the culture was cloudy, then used the cells for DNA extraction. We performed PCR and Sanger sequencing on these flower and halictid bee isolates using the same conditions as for the clones described above.

To place these sequences into an evolutionary context, we created separate sequence alignments for each bacterium. For the *Lactobacillus* phylogeny, we added the newly obtained sequences to the alignment from McFrederick et al. [15], along with recently described honey-bee associated bacteria [43] and unpublished *Lactobacillus* sequences isolated from halictid and megachilid bees. For *Gilliamella* and *Arsenophonus*, we generated new alignments by searching GenBank [44] for 16S rRNA gene sequences from each genus and downloading all available 16S sequences. We also added *Gilliamella* sequences that were not retrieved with our searches but were included in Koch et al. [45] and Martinson et al. [9]. To simplify the resulting datasets, we clustered related sequences using CD-HIT [46] and included only one representative sequence per 97 % cluster. For outgroups, we used the same outgroup sequences as in Novakova et al. [47] and Koch et al. [45]. We then refined the alignments by eye in Mesquite [31] and performed maximum likelihood phylogenetic analysis using the GTRGAMMA model of nucleotide evolution and 1000 bootstrap pseudoreplicates with RAxML-HPV v 8.1.24 [48] on the CIPRES portal [49].

To further explore host associations of these bacteria, we searched NCBI's nucleotide database and Sequence Read Archive (SRA) with the newly sequenced 27F-1492R 16S rRNA gene sequences from *Lactobacillus* OTU 0, *Arsenophonus* OTU 5, and *Gilliamella* OTU 3 (see results) as queries. We recorded host associations for hits sharing 97 % or greater sequence identity when available. For the SRA, we searched datasets generated from honey bee microbiome

studies (PRJDB49 [24], SRA046735 [23], PRJNA225925 [11], PRJNA259199 [50], and PRJNA234448 [51]).

Data Availability

Raw Illumina sequencing data are available at the NCBI's SRA (accession number SRP058784). The complete OTU tables and sample metadata are presented in Table S1. Sanger sequencing reads of the 27F-1492R 16S rRNA gene sequences are available on NCBI (accession numbers KT833114–KT833126 and KX656646–KX656668).

Results

16S rRNA Gene Amplicon Data

Rarefaction analyses indicated that we were able to characterize most of the diversity found in our samples with as few as 600 reads per sample in run 1 and 825 reads per sample in run 2; most of the rarefaction curves leveled off or nearly leveled off at these sequencing depths (Fig. S2). Good's coverage estimates averaged 0.96 (minimum 0.925 and maximum 0.995) for run 1 and 0.96 (minimum 0.94 and maximum 0.98) for run 2. Run 1 resulted in 113,991 quality-filtered sequences while run 2 resulted in 99,793 quality-filtered sequences. While many OTUs were shared across both sequencing runs, each run identified OTUs that were not found in the other run. For example, OTU 5 (*Arsenophonus*) in sequencing run 1 was missing in run 2, and vice-versa for OTU 3 (*Gilliamella*) from sequencing run 2 (Figs. 1 and S3; Table S1). While we are unable to pinpoint exactly how the different methodologies resulted in the inclusion or exclusion of these OTUs, a likely explanation is that *Arsenophonus* was excluded in the gel extraction protocol, as its 16S amplicon was 31 bases shorter than that of the dominant OTU.

Across both sequencing runs, a *Lactobacillus* (Lactobacillaceae) OTU (OTU 0 in both sequencing runs) was the most abundant bacterium across all samples and dominated most communities regardless of whether the community originated from a flower or a bee (Fig. 1, Table S1). This OTU shared 99 % sequence identity with a member of the *Lactobacillus kunkeei*-clade first identified in association with the sweat bee *Augochlora pura* (Table S1, [13]) and 94 % sequence identity to *Lactobacillus kunkeei* NR_026404 [52]. Although abundant in many flower samples, OTU 0 dominated the microbiomes of adult bees, larval bees, and pollen provision samples in both sequencing runs (Fig. 1).

NMDS ordination of weighted, unweighted, and generalized UniFrac distance matrices exhibited no obvious clustering in either dataset (Fig. 2 and Fig. S4). Some patterns, however, did emerge and were verified by Adonis analysis. For example, the microbiomes of different species differed significantly in run 2 (weighted UniFrac distance matrix, Adonis $F = 2.18$, $R^2 = 0.52$, $P = 0.006$) and approached significance in run 1 ($F = 1.74$, $R^2 = 0.40$, $P = 0.061$). Much of this appears to be driven by differences between bee-associated and flower-associated samples; within-flowers species did not differ (run 1 $F = 0.65$, $R^2 = 0.19$, $P = 0.81$ but note that ten flower samples in run 1 were multispecies plots, run 2 $F = 0.75$, $R^2 = 0.27$, $P = 0.60$) and within-bees species differed significantly only in run 2 (run 1 $F = 1.23$, $R^2 = 0.29$, $P = 0.337$; run 2 $F = 2.56$, $R^2 = 0.52$, $P = 0.041$). *Osmia* and *Megachile* adult samples mostly overlapped in run 1 but showed greater dispersion in run 2. Although we included only two *Lithurgus* adult samples, these clustered away from both *Osmia* and *Megachile* adult samples. While some bee and pollen provision samples overlapped (adults, larvae, and pollen provisions in run 2), other bee and pollen provision samples were dissimilar and widely dispersed in the ordination. Run 1 included only one site, but run 2 microbiomes significantly differed by site ($F = 7.65$, $R^2 = 0.168$ $P = 0.002$). Microbiomes from bagged flowers and bee-visited flowers overlapped in both runs and were not significantly different (Run 1 $F = 0.75$, $R^2 = 0.05$, $P = 0.55$; run 2 $F = 1.50$, $R^2 = 0.08$, $P = 0.13$).

Quantitative PCR

OTU 0 (the *Lactobacillus kunkeei* relative) occurred in high absolute abundance across nearly half of the bee-associated samples (Fig. 3). Although this bacterium was present in every sample in our 16S amplicon survey, many of these same samples fell below the detection threshold in our qPCR analysis (Fig. 3). For example, only one flower sample was above our detection threshold. As our qPCR detection threshold was 1000 copies, following [9, 11], and the flower swab DNA extractions had low quantities of DNA (average 1.01 ng/μL, s.d. = 0.5), this does not suggest that OTU 0 was absent from these samples but instead that it occurred at levels below the detection threshold. The highest *Lactobacillus kunkeei* abundance across all samples was found in the gut of an adult female *M. policaris* bee (Me.198.Bg.G). Only one flower sample, taken from a flower plot comprising *C. texanum*, *Brassica rapa*, and unidentified Asteraceae flowers, had detectable levels of OTU 0 via qPCR.

Our qPCR measures of absolute abundance were correlated with the relative abundances of OTU 0 in our 16S-amplicon survey (Kendall $\tau = 0.544$, $P < 0.001$). We found no significant differences, however, in the relative abundance of OTU 0 as determined in our 16S-amplicon survey (Kruskal-Wallis $X^2 = 10.17$, $df = 5$, $P = 0.07$), but absolute abundance as measured with our qPCR assay was significantly different among sample types (Fig. 3, Kruskal-Wallis $X^2 = 23.68$, $df = 3$, $P < 0.001$).

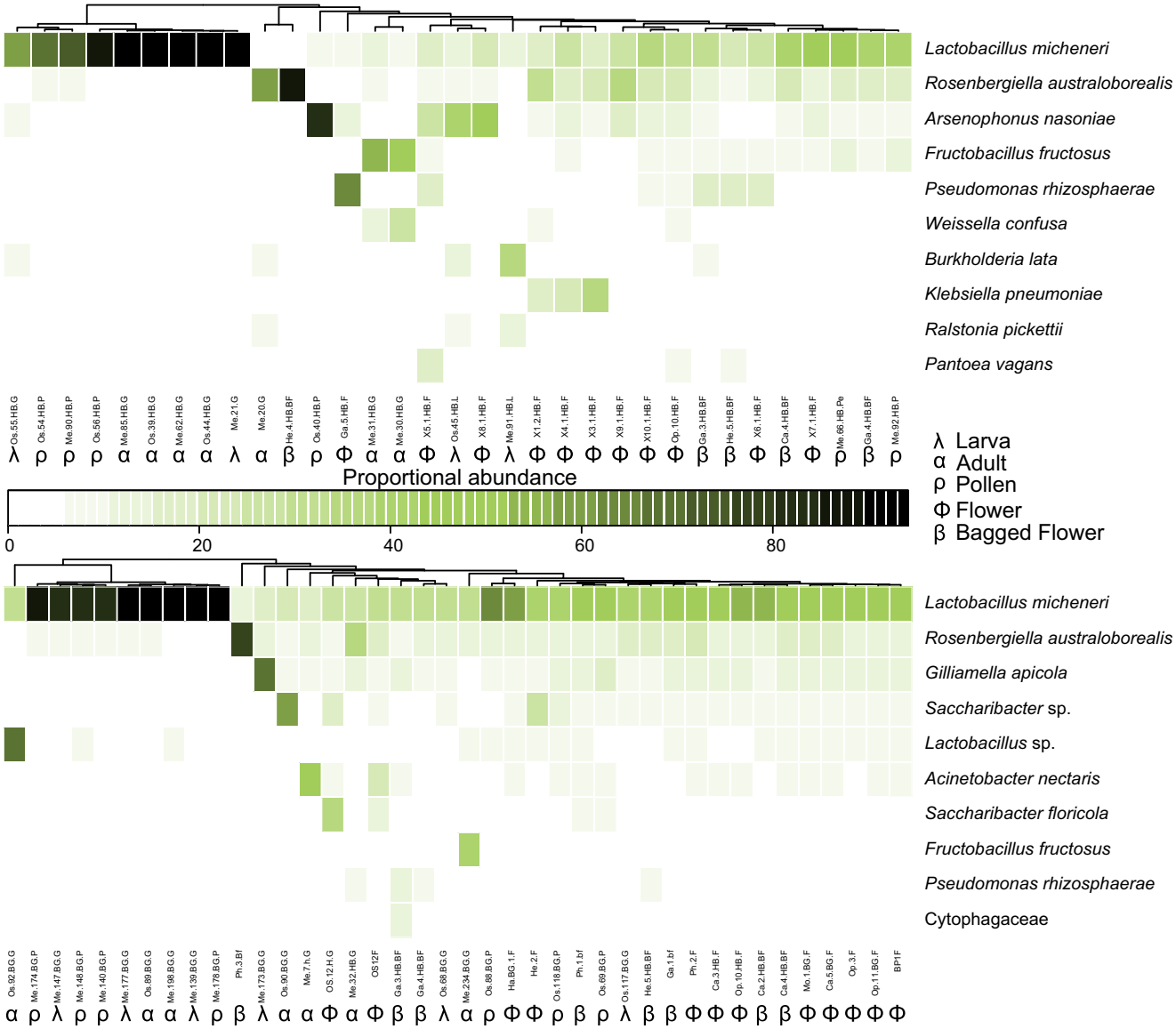


Fig. 1 Heat map of the 10 OTUs with the highest relative abundance per sequencing run, in descending order of relative abundance. Each *column* represents a sample as indicated in the legend, each *row* represents an OTU, and *shading* of the cell represents relative abundance as indicated in the legend. Samples are arranged by hierarchical clustering as shown by the *dendrogram* in the column header. The *Lactobacillus micheneri* OTU (OTU 0) was detected in every sample but had the highest relative abundance in clusters of samples in each sequencing run comprising adult bees, pollen provisions, and larvae

Phylogenetic Analyses and Proposal of *Lactobacillus micheneri* sp. nov.

The *Lactobacillus* 16S rRNA gene phylogeny resolved OTU 0 as reciprocally monophyletic to the clade comprising *Lactobacillus apinorum* and *Lactobacillus kunkeei* (Fig. 4, also Fig. S5 for complete *Lactobacillus* phylogeny). The OTU 0 clade included bacteria that were isolated from diverse wild bees including *Cauplicana yarrowi*, *Diadasia opuntiae*, *Megachile* spp., *Osmia* spp., *Augochlorella pomoniella*, *Agapostemon* spp., *Dialictus* sp., *Halictus tripartitus*, and *Halictus ligatus*. BLAST searches against NCBI's nucleotide database and honey bee microbiome projects on the SRA database revealed that OTU 0 shared 97 % or greater sequence identity mainly with bacteria associated with wild bees [12, 13]. This OTU was also abundant in honey bee food (corbicular pollen [53] and honey bee bread and corbicular pollen [50]) but rare in the honey bee gut (8 sequences were present in the honey bee crop [53], 1 sequence in honey bee guts from Massachusetts [24], and 59 sequences from a larger survey of honey bee guts [23]). Due to monophyly of this lineage and its association with wild bees from several families, we propose the species epithet *micheneri* for OTU 0, to honor the contributions of the late Charles D. Michener to the field of melittology (the study of wild bees). GenBank accession KT833121 is a representative 16S rRNA gene sequence

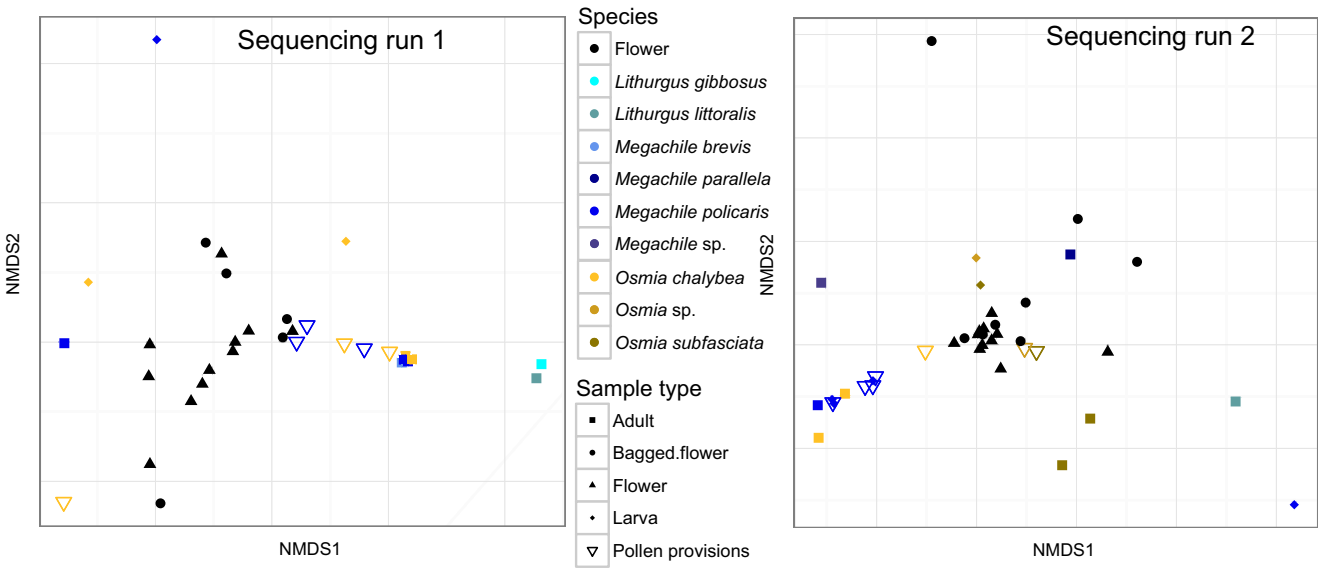


Fig. 2 Ordination of weighted UniFrac matrices with non-metric multidimensional scaling. Each *point* represents a sample, and sample type is indicated as in the legend. No sample types exhibited exclusive clustering. Stress for the NMDS analysis of run 1 samples = 0.08 while stress for the weighted NMDS analysis of run 2 samples = 0.11

of a *Lactobacillus micheneri* strain isolated from the halictid bee *Halictus ligatus*. This *Lactobacillus micheneri* strain shares 97 % sequence identity with the reference sequence of *Lactobacillus apinorum*, NR_126247. As the 16S rRNA gene sequence of *Lactobacillus apinorum* NR_126247 [43] shares 99 % sequence identity with the reference sequence for *Lactobacillus kunkeei*, NR_026404, *Lactobacillus micheneri* is clearly differentiated from these two honey-bee associated bacteria. *Lactobacillus micheneri* grows in aerobic conditions at 25–30 °C on de Man, Rogosa, Sharpe [42] plus 2–20 % fructose agar.

The *Gilliamella* phylogeny was poorly resolved overall (Fig. 5), but the OTU 3 sequences formed a monophyletic group that included sequences isolated from a single honey bee colony from Arizona [12]. BLAST searches against honey bee microbiome studies on the SRA revealed that strains with 99 to 100 % sequence identity to *Gilliamella* OTU 3 occur in corbicular pollen and bee bread [50] as well as honey bee worker guts [11, 23, 24, 51].

As in previous studies [47], the *Arsenophonus* phylogeny was poorly resolved with 16S rRNA gene sequence (Fig. S6). OTU 5 sequences, however, formed a monophyletic group closely related to *Arsenophonus nasoniae*. BLAST searches revealed that OTU 5 was found in honey bee corbicular pollen, bee bread, honey bee crops, and honey bee hindguts [11, 50, 53].

Discussion

Our data show that flowers harbor bacteria that are shared across many wild megachilid bee species, and flowers therefore may act as hubs of transmission. For example, OTU 0 (*Lactobacillus micheneri*) was present in every sample, regardless of whether the sample was from flowers, pollen provisions, or adult or larval megachilid bees. Adult bees may both obtain and deposit microbes at flowers, so that flowers may serve as connected hubs in a network of transmission [54]. Our data, however, suggest that bee visitation may not be necessary for the presence of these bacteria on flowers. We found the same OTUs in flowers whether the flower was bagged to exclude bees or had been visited by bees. Flowers may therefore provide habitat for metapopulations of easily dispersed microbes, as has been found for microbial eukaryotes in aquatic systems [55]. Bee visitation may increase the populations of these microbes on flowers but does not appear to be necessary for them to be present.

Bacteria associated with flowers and adult bees also occurred in larvae, suggesting that adult bees transmit these bacteria to their offspring via pollen provisions and the shared nest environment. In the current work, we did not study pupae or newly emerged adults, but previous work has shown that these stages lack gut microbes [9, 19]. As solitary bees, megachilids have no opportunity for social transmission between cohabiting nestmates, suggesting that environmental transmission is important for inoculation of the newly emerged adult gut. The ubiquity of bacteria such as *Lactobacillus micheneri* across flower and bee species further suggests that interspecies transmission is also rampant. We lack direct experimental evidence, but our data support the hypothesis that this transmission occurs on flowers, although we cannot exclude other possible sources such as inoculation of newly emerged adults in their brood cell or from leaf surfaces.

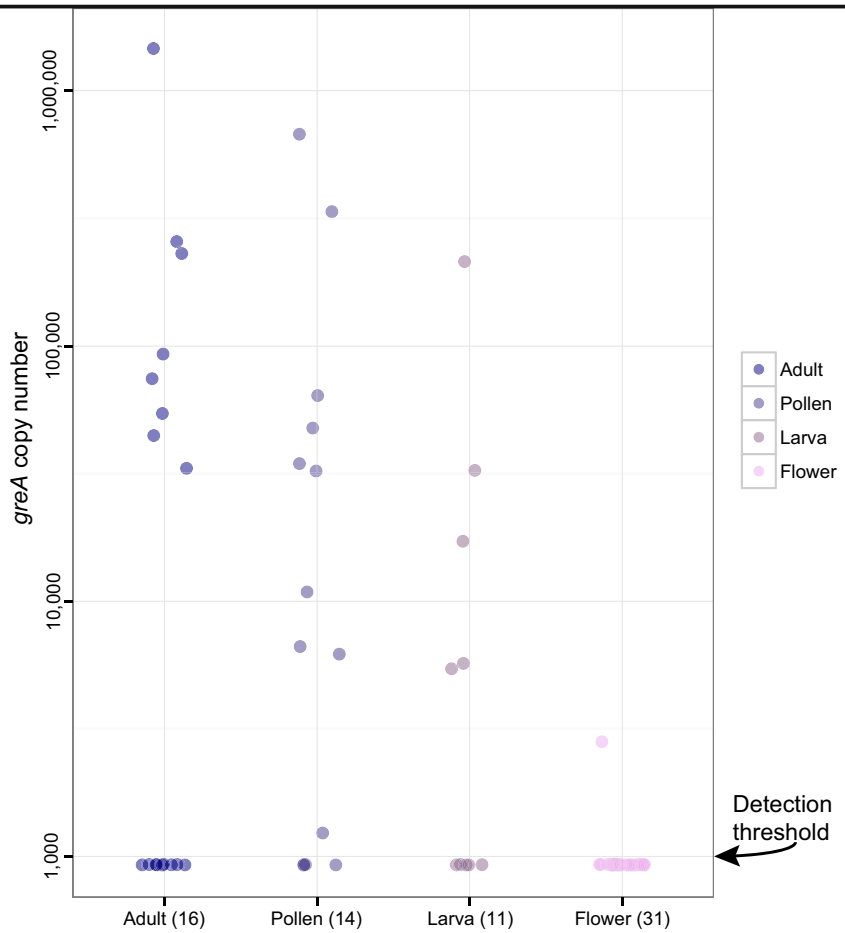


Fig. 3 Absolute quantification of *Lactobacillus micheneri* with quantitative PCR of the single copy of *greA* gene. Copy number is presented on a log scale, and the number of samples from each sample type is given in parentheses on the x-axis. Our 16S rRNA survey indicated that *Lactobacillus micheneri* was present in all samples, but many samples had quantities of this bacterium below our qPCR detection threshold (1000 cells per reaction). *Lactobacillus micheneri* was abundant in some adult, pollen, and larva samples but detectable in only one flower sample

Several possible explanations for the presence of these bacteria on unvisited flowers exist. Plants have long been known to be sources of airborne bacteria [56], and one possible explanation is that bacteria in the air or on leaf surfaces colonize flowers as they open. The flower bags may also have allowed bacteria to enter flowers via feces from nearby foraging bees. Another possibility is that these bacteria were transmitted by thrips or other flower visitors that were small enough [57] to fit through the mesh of our flower bags.

Other researchers have previously investigated transmission of bacteria and unicellular eukaryotic pathogens between bees on flowers. Durrer and Schmid-Hempel [58] showed that flowers are hubs of transmission for the bumble bee parasite *Crithidia bombi*. Graystock et al. [54] showed that unicellular eukaryotic pathogens can be florally transmitted between honey and bumble bees. Anderson et al. [17] found that the honey bee crop, honey bee hive materials, and floral nectar from four plant species can harbor the same bacterial types. Flower visitation by carpenter bees, but not honey or bumble bees, increased microbial abundance on flowers [59]. Aizenberg-Gershtein et al. [60] compared bagged and open flower microbiomes to the exoskeletal microbiomes of honey bees and found that two open-flower samples clustered with one bee sample while one open-flower sample clustered with three bagged-flower samples, suggesting that honey bee visitation may change the floral microbiome.

Our study differs from these previous studies in several ways. First, we investigated the microbiome of multiple wild flower and megachilid bee species at two distant sites. Second, while recent microbiome analyses compared flower and surface (exoskeletal) microbiomes of bees [59, 60], we compare flower microbiomes to microbiomes from pollen provisions and the guts of larval and adult bees, and we show that the same OTUs are found in flowers and bee-associated samples. We also provide the first evidence that *Lactobacillus micheneri*, *Gilliamella*, and *Arsenophonus* occur on flowers.

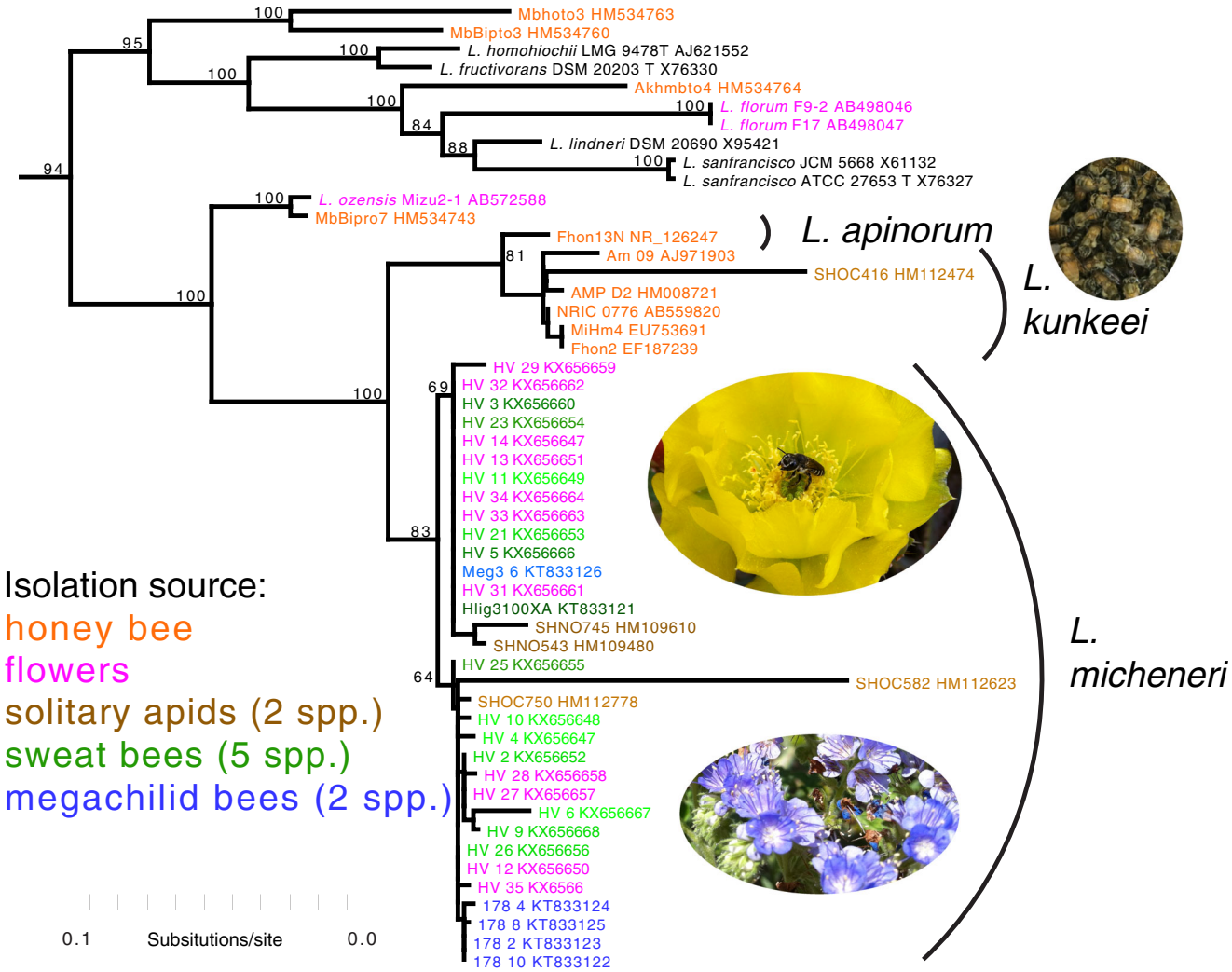
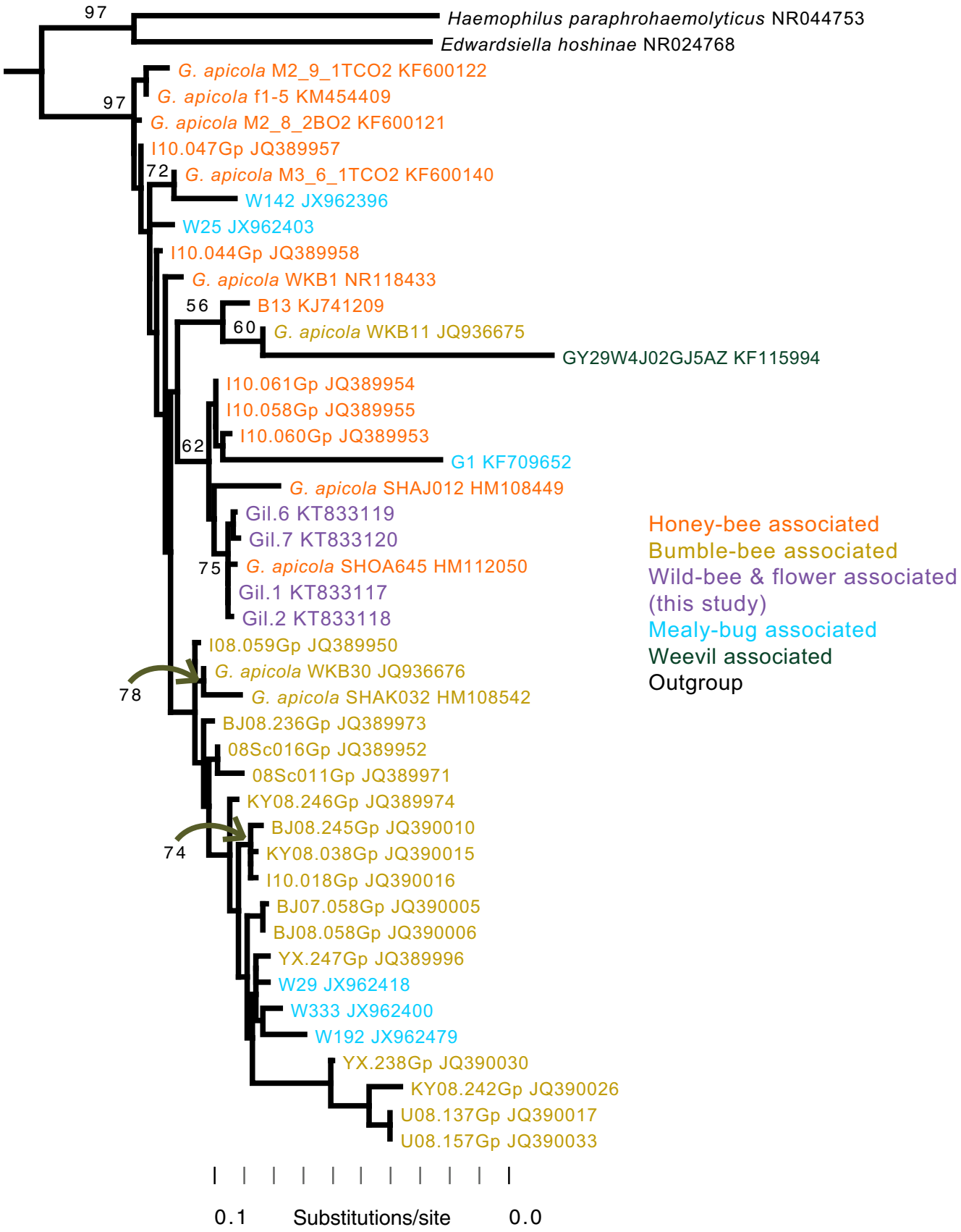


Fig. 4 Maximum likelihood phylogeny of lactobacilli, trimmed to highlight the *Lactobacillus kunkeei*/*Lactobacillus micheneri* clade. For full phylogeny including outgroups, refer to Fig. S5. *Numbers on nodes* are bootstrap support values from 1000 pseudoreplicates; values below 50 are not reported. Isolation source is coded by *color* as indicated in the legend, with different host species coded by *shade within color*. Flower isolates are from Indian mallow (*Abutilon* sp.) flowers. *Lactobacillus micheneri* was supported as being reciprocally monophyletic to the clade that includes *Lactobacillus kunkeei* and *Lactobacillus apinorum*

Our qPCR data show that *Lactobacillus micheneri* can reach high abundance in the adult gut, the larval gut, and pollen provisions. The resources sustaining bacterial growth in flowers are found in greater abundance in the pollen provisions and guts of wild megachilid bees than in flowers; for example, *Megachile rotundata* visits hundreds to thousands of flowers on multiple foraging trips to provision one brood cell [61]. The finding that *Lactobacillus micheneri* occurs at lower absolute abundance in flowers may simply reflect the lower amount of starting material in the flower samples compared to the bee and pollen provision samples. The bee gut, however, is a very different environment than flowers, but one that *Lactobacillus micheneri* nevertheless thrives in. *Lactobacillus micheneri* may therefore be adapted to the bee gut, but further work is needed to test this hypothesis.

Lactobacillus, Gilliamella, and Arsenophonus Diversity in the Pollination Landscape

Our phylogenetic analyses revealed and/or resolved novel lineages of flower- and bee-associated bacteria. For example, *Lactobacillus micheneri* was reciprocally monophyletic to the honey bee-associated clade that includes *Lactobacillus apinorum* and *Lactobacillus kunkeei*. We previously reported *Lactobacillus micheneri* associated with the sweat bee *Augochlora pura* [13] but were unable to resolve its phylogenetic placement with the shorter 16S rRNA gene sequence used in that previous analysis. BLAST searches further showed that *Lactobacillus micheneri* is found on pollen entering honey bee colonies and in honey bee bread but is rarely found in the honey bee gut. Why diverse wild bee guts harbor *Lactobacillus*


97
Haemophilus paraphrohaemolyticus NR044753
Edwardsiella hoshinae NR024768
G. apicola M2_9_1TCO2 KF600122
97
G. apicola f1-5 KM454409
G. apicola M2_8_2BO2 KF600121
I10.047Gp JQ389957
72
G. apicola M3_6_1TCO2 KF600140
W142 JX962396
W25 JX962403
I10.044Gp JQ389958
G. apicola WKB1 NR118433
56
B13 KJ741209
60
G. apicola WKB11 JQ936675
GY29W4J02GJ5AZ KF115994
I10.061Gp JQ389954
I10.058Gp JQ389955
62
I10.060Gp JQ389953
G1 KF709652
G. apicola SHAJ012 HM108449
Gil.6 KT833119
Gil.7 KT833120
75
G. apicola SHOA645 HM112050
Gil.1 KT833117
Gil.2 KT833118
I08.059Gp JQ389950
G. apicola WKB30 JQ936676
78
G. apicola SHAK032 HM108542
BJ08.236Gp JQ389973
08Sc016Gp JQ389952
08Sc011Gp JQ389971
KY08.246Gp JQ389974
BJ08.245Gp JQ390010
74
KY08.038Gp JQ390015
I10.018Gp JQ390016
BJ07.058Gp JQ390005
BJ08.058Gp JQ390006
YX.247Gp JQ389996
W29 JX962418
W333 JX962400
W192 JX962479
YX.238Gp JQ390030
KY08.242Gp JQ390026
U08.137Gp JQ390017
U08.157Gp JQ390033
0.1
Substitutions/site
0.0
Honey-bee associated
Bumble-bee associated
Wild-bee & flower associated
(this study)
Mealy-bug associated
Weevil associated
Outgroup

◀ **Fig. 5** Maximum likelihood phylogeny of *Gilliamella*. *Numbers on nodes* are bootstrap support values from 1000 pseudoreplicates; values less than 50 are not reported. *Gilliamella* from wild bees and flowers was supported as being monophyletic and distinct from the core honey bee and bumble bee bacteria

micheneri whereas honey bee guts do not remains an open question. The reciprocal question, i.e., why *Lactobacillus kunkeei* occurs in honey bee samples but not in wild bee samples, is equally compelling. Our data agree with the previously posited hypothesis that strains of *Lactobacillus kunkeei* are adapted to the honey bee niche while other closely related strains may be adapted to the flower niche [18].

Gilliamella apicola is part of the honey bee core hindgut microbiome and is thought to be specific to the honey bee gut [62, 63]. We previously reported a close relative of *Gilliamella apicola* associated with sweat bees in the genus *Megalopta* [19], and here, we found a bacterial lineage in many of our flower, pollen provision, and bee gut samples that is 99 % identical in 16S rRNA gene sequence to a *Gilliamella apicola* strain from honey bees [12, 23]. Our phylogenetic analysis returned this lineage as a monophyletic group that is distinct from the majority of the honey bee- and bumble bee-associated *Gilliamella apicola* lineages. While previously found in honey bees, this *Gilliamella apicola* strain was detected in only one of four honey bee colonies from one apiary and appears to be a minor constituent of the honey bee microbiome [12, 23]. Additionally, this flower- and bee-associated *Gilliamella* strain shares 96 % (27F 250 base pair Illumina fragment) or 98 % (27F-1492R fragment) sequence identity to the *Gilliamella apicola* type strain [64]. This flower- and bee-associated strain may therefore represent a distinct lineage of *Gilliamella* that can form broad associations with bees (including sporadic association with honey bees) and flowers. Further studies of this bacterium are needed to determine whether *Gilliamella* found in flowers are active and can survive and replicate outside of the bee gut.

Arsenophonus has been previously reported from solitary and wild bees [14, 65] and honey bees [51, 60]. Here, we additionally report *Arsenophonus* from flowers. Our phylogenetic analysis returned these sequences as monophyletic, but embedded within a larger clade containing endosymbionts of *Nasonia*, ticks, whiteflies, and aphids. The occurrence of this *Arsenophonus* lineage on flowers suggests that it may not be an obligate endosymbiont but can be environmentally transmitted. How this bacterium is able to persist outside insect hosts and what role it plays in plant and/or bee health merits further investigation.

Conclusions and Future Directions

Our data suggest that flowers may act as hubs of transmission for bacteria that are found associated with multiple bee species. For example, we found a *Lactobacillus* OTU in all samples but in the highest relative and absolute abundances in adult and larval megachilid bee guts and pollen provisions. Future studies should therefore address the hypothesis that this bacterium is best adapted to the bee niche. Phylogenetic analyses of nearly full-length 16S rRNA gene sequences indicated that this *Lactobacillus* OTU is monophyletic, and we propose the name *Lactobacillus micheneri* sp. nov. for this bacterium.

Many unanswered questions remain regarding the microbiomes associated with wild and solitary bees. We found several abundant bacteria in bagged flowers, including *Lactobacillus micheneri*, *Gilliamella*, and *Arsenophonus*, suggesting that these bacteria may colonize flowers via the air, leaf surfaces, or smaller insect vectors such as thrips. Experiments controlling for possible environmental sources of flower inoculation could determine from which of these sources flowers recruit microbes into their microbiomes. Transcriptomic studies of bacteria in flowers, pollen provisions, and bee guts could reveal how active these bacteria are in their various niches, as well as the degree to which these bacteria are adapted to these varied environments. The main outstanding questions, however, are the functional roles that these bacteria play in bee fitness. Fitness experiments combined with metagenomic sequencing approaches will help unravel the effects these abundant bacteria have on their hosts.

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