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Exploring the bee microbiome: Distributions, interactions, and functions

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Exploring the bee microbiome: Distributions, interactions, and functions

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Table of contents

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Significant compositional and functional variation reveals the patterns of gut microbiota evolution among the widespread Asian honeybee populations

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The gut microbiome is a crucial element that facilitates a host's adaptation to a changing environment. Compared to the western honeybee *Apis mellifera,* the Asian honeybee, *Apis cerana* populations across its natural range remain mostly semi-feral and are less affected by bee management, which provides a good system to investigate how gut microbiota evolve under environmental heterogeneity on large geographic scales. We compared and analyzed the gut microbiomes of 99 Asian honeybees, from genetically diverged populations covering 13 provinces across China. Bacterial composition varied significantly across populations at phylotype, sequence-discrete population (SDP), and strain levels, but with extensive overlaps, indicating that the diversity of microbial community among *A. cerana* populations is driven by nestedness. Pollen diets were significantly correlated with both the composition and function of the gut microbiome. Core bacteria, *Gilliamella* and *Lactobacillus* Firm-5, showed antagonistic turnovers and contributed to the enrichment in carbohydrate transport and metabolism. By feeding and inoculation bioassays, we confirmed that the variations in pollen polysaccharide composition contributed to the trade-off of these core bacteria. Progressive change, i.e., nestedness, is the foundation of gut microbiome evolution among the Asian honeybee. Such a transition during the co-diversification of gut microbiomes is affected by environmental factors, diets in general, and pollen polysaccharides in particular.

KEYWORDS

gut microbiota, Asian honeybee, population variation, pollen, nectar

Introduction

The gut microbiome often serves as a critical component in the host's adaptation to a changing environment (Suzuki and Ley, 2020). Gut microbiota can benefit host animals in nutrition provision, pathogen resistance, and modulations of development and behavior (Cryan and Dinan, 2012; Engel and Moran, 2013; Kamada et al., 2013; Sommer and Bäckhed, 2013). On the other hand, gut microbiota may be shaped by the host's adjustments to changing environments, such as range expansion accompanied by diet shifts (Baldo et al., 2015; Michel et al., 2018). In particular, for widespread species found in a large geographic range, environmental heterogeneity is expected to influence their gut microbiota (Yatsunenko et al., 2012; Henderson et al., 2015). This is because the geographic location of animal populations is linked with varied host genetics, local vegetation, and environmental microbe sources.

Studies based on *Apis mellifera* have established the framework for honeybee gut microbiota, revealing their essential role in the biology of the honeybee, such as facilitating pollen digestion (Engel et al., 2012; Zheng et al., 2019), host development (Zheng et al., 2017), and pathogen resistance (Kwong et al., 2017a; Wu et al., 2020). The species of honeybees each maintain a relatively simple but stable gut microbiota, comprising 5–9 core bacteria (>95% of total abundance) from phyla Proteobacteria, Firmicutes, and Actinobacteria (Kwong and Moran, 2016; Kwong et al., 2017b). Ancestry reconstruction of these core microbes suggested that they have probably become part of the symbiont system in the common ancestry of all extent corbiculate bees (Kwong et al., 2017b). Interestingly, although the honeybees share much of the core microbes at the phylotype level, each host species possesses a species-specific microbial community (Kwong et al., 2017b), with most core microbes showing distinct strain diversities among hosts, e.g., between *Apis mellifera* and *Apis cerana* (Ellegaard et al., 2020). However, little is known about how these gut symbionts have evolved within their hosts.

Among the different honeybee species, both the western (*A. mellifera*) and eastern honeybees (*A. cerana*) are widely distributed across tropical and temperate climates, each with endemic populations adapted to local habitats (Wallberg et al., 2014; Ji et al., 2020). Compared to *A. mellifera, A. cerana* populations across its natural range (much of eastern, southern, and southeastern Asia) (Radloff et al., 2010) remain mostly semi-feral and are less affected by bee management, which provides a good system to investigate how gut microbiota evolve under environmental heterogeneity on large geographic scales. However, investigations based on 16S rRNA did not provide sufficient resolution to differentiate tropical *A. cerana* populations from those of the temperate zones (Kwong et al., 2017b). There is still a knowledge gap on the biogeographical variation of gut microbes among *A. cerana* populations.

Our recent work on the evolution of mainland *A. cerana* revealed that multiple peripheral subspecies had radiated from a common central ancestral population and adapted independently to diverse habitats (Ji et al., 2020). During the most recent radiation period (~100 ka), selective pressures imposed by diverse habitats, especially those of the changing floras, led to the convergent adaptation of the honeybee, where genes associated with sucrose sensitivity and foraging labor division had been repeatedly selected (Ji et al., 2020). We hypothesized that the gut microbiota of *A. cerana* had also evolved along with host range expansion, subspecies differentiation, and habitat adaptation. In the present study, we aimed to understand the landscape of gut microbial diversity and function across geographic populations of mainland *A. cerana* with metagenome sequencing. We also examined the effects of host genetics and diet variation on the honeybee gut symbionts. In addition, we explored the adaptive mechanisms of the microbes in response to selective pressures.

Materials and methods

Sample collection

A total of 99 worker bees of *A. cerana* were obtained from inside the hives at 15 sites in 13 provinces of China (Hainan, Yunnan, Taiwan, Fujian, Jiangxi, Hunan, Tibet, Sichuan, Shaanxi, Gansu, Qinghai, Hebei, and Jilin), between April 2017 and January 2019. For each population, ≥5 gut samples were sequenced from at least two hives to represent the diversity of each population (**Supplementary Table 1**). We collected nurse bees based on their morphology (Seeley, 1982). The nurse bees are generally characterized by relatively lightened color and apparently intact hairs and wings. Bees that were newly emerged (with shiny hair and slow-moving capability) or aged (with visible wing wear and hair loss) were excluded from sampling. Our sampling covered the main natural distribution range of *A. cerana* in China, from 19.2°-43.5°N, 95.7°-128.7°E, representing drastically different altitudes (12-3,325 m, **Supplementary Table 1**). The guts (including the midgut and hindgut) were dissected from the abdomen and stored in 100% ethanol or directly frozen at −80°C. To preserve live gut bacteria for strain isolation, a subset of guts was suspended in 100 μl of 25% glycerol (v/v, dissolved in PBS buffer), homogenized, and then frozen at −80°C.

Isolation, cultivation, and identification of gut microbe strains

The gut homogenates were plated on different cultivation media, respectively, for various honeybee gut bacteria following Engel et al. (2013), including heart infusion agar (HIA) with 5%

(v/v) de-fibrinated sheep blood, Columbia agar with 5% (v/v) de-fibrinated sheep blood, De Man, Rogosa and Sharpe (MRS) agar, and trypticase–phytone–yeast (TPY) agar supplemented with 1% mupirocin. The plates were incubated at 35°C in a 5% CO_2 or anaerobic atmosphere.

When bacterial colonies became visible on the plates, they were identified by sequences of their 16S rRNA gene. The isolates were picked and dissolved with H_2O, then boiled at 100°C for 1 min, which was used directly as a DNA template in PCR. PCR amplicons were generated using the universal 16S primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) with 25 cycles of amplification (94°C for 30 s, 60°C for 40 s, and 72°C for 60 s) after an initial incubation for 1 min at 95°C. Amplicons were sequenced using Sanger sequencing and identified using blastn against annotated sequences in GenBank.

DNA extraction for genome and metagenome sequencing

The gut DNA was extracted following Kwong et al. (2017b). Briefly, the crushed gut was suspended in a capped tube with 728 μl of CTAB buffer, 20 μl of proteinase K, 500 μl of 0.1-mm Zirconia beads (BioSpec), 2 μl of 2-Mercaptoethanol, and 2 μl of RNase A cocktail. The mixtures were bead-beaten for 2 min for 3 times. After digested overnight at 50°C, the mixtures were added with 750 μl of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and centrifuged to obtain the aqueous layer. After being precipitated at −20°C, spun at 4°C, and washed with −20°C ethanol, the DNA pellets were dried at 50°C, and then re-suspended in 50 μl of nuclease-free H_2O. Final DNA samples were stored at −20°C.

Genomic DNA of honeybee gut bacterial isolates was also extracted using the phenol-chloroform protocol. The bacterial cells were re-suspended in 500 μl of lysis buffer [50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM EDTA, nuclease-free H_2O, 2% SDS, proteinase K (20 mg/ml)], then added with 500 μl of CTAB extraction buffer [50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 1% PVP 40000, nuclease-free H_2O; pre-heated at 56°C]. The mixtures were incubated for 30 min at 65°C before the addition of 500 μl of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0). Then, the mixture was centrifuged at 14,000 *g* at room temperature (RT) for 5 min. The aqueous layer was transferred to a new tube, added with 5 μl of RNase (100 mg/ml), incubated at RT for 20 min, and added with 600 μl of chloroform: isoamyl alcohol (24:1). After spinning at 14,000 *g* at RT for 5 min, the aqueous layer was transferred to a new tube and added with 5 μl of ammonium acetate (final concentration 0.75 M), 1 μl glycogen solution (20 mg/ml), and 1 ml of cold 100% ethanol. DNA was precipitated at −20°C for 30 min. Precipitations were spun at 14,000 *g* at 4°C for 15 min, and the supernatant was decanted. DNA pellets were washed with 80 and 70% ethanol pre-cooled at −20°C, respectively, and spun for an additional 10 min at 4°C. The supernatant was discarded and the DNA pellet was air dried. The pellet was re-suspended in 50 μl nuclease-free H_2O and kept at 4°C overnight before being stored at −20°C.

Genome and metagenome sequencing

A total of 99 honeybee gut samples were used for metagenome sequencing (**Supplementary Table 1**). In total, 83 representative core bacterial strains obtained from *A. cerana* were selected and sequenced to construct a reference genome library for phylotype, SDP, and single nucleotide variation (SNV) analyses (**Supplementary Table 2**). DNA samples were paired-end sequenced at the Beijing Genomics Institute Shenzhen Branch (BGI-Shenzhen) using the BGISEQ-500 platform (200–400 bp insert size; 100 bp read length; paired-ended [PE]) and at Novogen company using the Illumina Hiseq X Ten platform (350 bp insert size; 150 bp read length; PE). One *Gilliamella* strain (B3022) was sequenced on the PacBio RS II platform at NextOmics company.

Bacterial genome assembly and annotation

Low-quality reads from the Illumina Hiseq X Ten platform were filtered out using fastp (Chen et al., 2018) (version 0.13.1, -q 20 -u 10) before subsequent analyses. For isolated bacterial strains, clean data were assembled using SOAPdenovo (Luo et al., 2012) (version 2.04, -K 51 -m 91 –R for PE 150 reads; -K 31 -m 63 –R for PE 100 reads), SOAPdenovo-Trans (Xie et al., 2014) (version 1.02, -K 81 -d 5 -t 1 -e 5 for PE 150 reads; -K 61 -d 5 -t 1 -e 5 for PE 100 reads), and SPAdes (Bankevich et al., 2012) (version 3.13.0, -k 33,55,77,85) based on contigs assembled by SOAPdenovo (only for PE 150 reads) or SOAPdenovo-Trans. The assembly with the longest N50 was retained for each strain as the draft genome. Then clean reads were mapped to the assembled scaftigs using minimap 2-2.9 (Li, 2018) and the bam files were generated by samtools (Li H. et al., 2009) (version 1.8). Genome assemblies were processed by BamDeal[1] (version 0.19) to calculate and visualize the sequencing coverage and GC content of the assembled scaftigs. Scaftigs with aberrant depths and GC contents were then removed from the draft genome. Next, the remaining scaftigs were filtered taxonomically. Scaftigs assigned to eukaryote by Kraken2 (Wood et al., 2019) using the standard reference database were removed, and the ones aligned to a wrong phylum by blastn (megablast with $e < 0.001$) were further removed. The remaining genome assemblies were

1 https://github.com/BGI-shenzhen/BamDeal

used as bacterial genome references. The *Giliamella* strain (B3022) sequenced on the PacBio RS II platform was assembled using a hierarchical genome assembly method (HGAP2.3.0) (Chin et al., 2013).

The protein coding regions of bacterial genomes were predicted using Prokka version 1.13 (Seemann, 2014). The KEGG orthologous groups (KOs) annotation was carried out using KofamKOALA (Aramaki et al., 2020) based on profile HMM and adaptive score threshold with default parameters. Programs KEGG Pathway and Brite Hierarchy were used to screen the annotation results. Finally, dbCAN2 version 2.0.11 (Zhang et al., 2018) was applied to annotate CAZymes and CAZyme gene clusters (CGCs) using embedded tools HMMER, DIAMOND, and Hotpep with default parameters.

Genetic variation of *A. cerana* hosts

Metagenomes were filtered by fastp (-q 20 -u 10) (Chen et al., 2018). Clean reads were then mapped to the *A. cerana* reference genome (ACSNU-2.0, GCF_001442555.1) (Park et al., 2015) using the BWA-MEM algorithm (v 0.7.17-r1188) (Li and Durbin, 2010), with default settings and an additional "-M" parameter to reach compatibility with Picard. Read duplicates were marked using Picard MarkDuplicates 2.18.9[2]. GATK HaplotypeCaller in the GVCF mode (McKenna et al., 2010) (v4.0.4) was used to call variants for each sample. All of the per-sample GVCFs were joined using GenotypeGVCFs. Then, the final variant file retained SNPs that met all of the following criteria: (1) average depth $>5\times$ and $<40\times$; (2) quality score (QUAL) $>$ 20; (3) average genotype quality (GQ) $>$ 20; (4) minor allele frequency (MAF) $>$ 0.05; (5) proportion of missing genotypes $<$ 50%; and (6) bi-allelic SNP sites.

The identity by state (IBS) distance matrices were performed and constructed with the filtered SNPs using functions "snpgdsIBS" in the R package SNPRelate (Zheng et al., 2012). A neighbor-joining tree was reconstructed based on the IBS distance matrix using the function "nj" in the R package Ape (Paradis et al., 2004). Node support values were obtained after 1,000 bootstrap replicates.

Reference-based metagenome composition analyses

Shotgun reads generated from the whole honeybee gut were first mapped against the *A. cerana* genome (ACSNU-2.0, GCF_001442555.1) using BWA aln (version 0.7.16a-r1181, -n 1) (Li and Durbin, 2010) to identify host reads, which were subsequently excluded. For taxonomic assignments of bacterial sequences, we used Kraken2 (Wood et al., 2019) and Bracken version 2.0 (Lu et al., 2017) to profile bacterial phylotype composition and used MIDAS (Nayfach et al., 2016) to profile strain composition for metagenomic samples. The reference database contained 390 bacterial genomes, including 307 published genomes and 83 newly-sequenced *A. cerana*-derived strains from this study (**Supplementary Table 2**). The majority of the reference strains belonged to six core phylotypes (*Gilliamella, Snodgrassella, Bifidobacterium, Lactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Apibacter*) of honeybee gut bacteria. The analyses of public gut metagenome data of *A. cerana* from Japan (Ellegaard et al., 2020) and *A. mellifera* (Ellegaard and Engel, 2019; Ellegaard et al., 2020) followed the same pipeline.

Identification and profiling of sequence-discrete population

We defined SDPs for each core gut bacterium (*Gilliamella*, *Snodgrassella*, *Bifidobacterium*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Apibacter*) using a 95% gANI threshold (Richter and Rosselló-Móra, 2009). Pairwise average nucleotide identities were calculated using the pyani Python3 module[3]. To generate the whole-genome tree for each core bacterium, we used Roary version 3.12.0 (Page et al., 2015) with the parameter -blastp 75 to obtain core single-copy genes shared among all strains. The alignments of nucleotide sequences were concatenated, from which a maximum-likelihood tree was inferred using FastTree version 2.1.10 (Price et al., 2010) with a generalized time-reversible (GTR) model and then visualized using iTOL (Letunic and Bork, 2019).

We used the 'run_midas.py species' script of MIDAS (Nayfach et al., 2016) with default parameters to estimate SDP relative abundances in each sample. The script 'merge_midas.py species' with the option '–sample_depth 10.0' was used to merge SDP abundance files across samples. The SDPs with a relative abundance of less than 1% were filtered out.

Detection of single nucleotide variation and copy number variations across populations

CheckM version 1.0.86 (Parks et al., 2015) was used to estimate the completeness and contamination of genomes. The genome with the highest completeness and lowest contamination was selected as the reference sequence for each SDP. The metagenomic reads were mapped against reference genomes and the SNVs were quantified along the entire genome using MIDAS (Nayfach et al., 2016) and the

2 http://broadinstitute.github.io/picard/

3 https://github.com/widdowquinn/pyani

script 'run_midas.py snps' with default parameters. For each SDP, the script 'merge_midas.py snps' pooled data across multiple samples with options '–snp_type bi –site_depth 5 –site_prev 0.05 –sample_depth 5.0 –fract_cov 0.4 –allele_freq 0.01' to obtain the minor allele (second most common) frequency file. Thus, bi-allelic SNVs prevalent in more than 5% of profiled samples were predicted and rare SNVs with abnormally high read depth were excluded. The matrix files of SNVs remaining polymorphic were obtained after filtering steps.

We used the 'run_midas.py genes' script in MIDAS (Nayfach et al., 2016) to map metagenomic reads to pangenomes of each SDP and quantified gene copy numbers with default parameters. Then, we merged results from pangenome profiling across samples with the option '–sample_depth 5.0' from the 'merge_midas.py genes' module. The gene coverage was normalized by the coverage of a set of 15 universal marker genes to obtain the estimated copy number for genes of each SDP. The coverage of each KO term was obtained by summing up all genes annotated as the same KO for each SDP. *p*-values were calculated using the Kruskal–Wallis one-way analysis across populations with the 'compare_means' function in the R package 'ggpubr.' KO copy number variation and SNV of each SDP were detected as highly variable when an adjusted $p < 0.05$.

De novo assembly of metagenomes

The metagenome was also *de novo* assembled using MEGAHIT (Li et al., 2016) (version 1.1.2, -m 0.6 –k-list 31,51,71 –no-mercy) for each gut sample. Assemblies longer than 500 bp were blasted against the NCBI nr database using DIAMOND (Buchfink et al., 2015) (version 0.9.22.123, blastx -f 102 -k 1 -e 1e-3) and were assigned to fungi, bacteria, archaea, virus, or plants (Viridiplantae). Only assemblies assigned as bacteria were retained for further analyses.

A customized bacterial genome database was constructed to enable taxonomic assignments for the bacterial assemblies. The database included all bacterial genomes available on NCBI[4] up to Jan 2019 (167,172 genomes), 83 genome assemblies of newly sequenced *A. cerana* gut bacteria (**Supplementary Table 2**), and 14 *Apibacter* genomes from *A. cerana* (Zhang et al., 2022). Taxonomical assignments were conducted using blastn, and an e-value of 1e-5 was observed. The assemblies were assigned to the genus of the best hit, while those without any hits were defined as unassigned bacteria.

For each metagenome sample, all clean reads were mapped against bacterial assemblies using SOAPaligner (Li R. et al., 2009) (version 2.21, -M 4 -l 30 -r 1 -v 6 -m 200). The results were summarized using the soap.coverage script (version 2.7.7[5]). Only assemblies with ≥90% coverage were considered true bacteria. Shannon index and Bray–Curtis dissimilarity were calculated using the vegan R package (Philip, 2003). The analyses of public gut metagenome data of *A. cerana* from Japan (Ellegaard et al., 2020) and *A. mellifera* (Ellegaard and Engel, 2019) followed the same pipeline.

Gene prediction and functional annotation for metagenomes

Gene prediction was conducted using MetaGeneMark (Zhu et al., 2010) (GeneMark.hmm version 3.38) with the *de novo* metagenome assemblies, and those longer than 100 bp were clustered using CD-HIT (Li and Godzik, 2006) (version 4.7, -c 0.95 -G 0 -g 1 -aS 0.9 -M 0) to obtain a non-redundant gene catalog for *A. cerana* metagenomes. For each individual metagenome sample, clean data were aligned onto the non-redundant gene catalog using SOAPaligner (Li R. et al., 2009) (version 2.21, -M 4 -l 30 -r 1 -v 6 -m 200). The gene abundance was calculated using soap.coverage script (version 2.7.9, see Text Footnote 5). For each sample, only assemblies of ≥90% coverage were retained for further annotation. The analyses of public gut metagenome data of *A. cerana* from Japan (Ellegaard et al., 2020) and *A. mellifera* (Ellegaard and Engel, 2019) followed the same pipeline.

Functional annotation of the gene catalog was performed by GhostKOALA (Kanehisa et al., 2016) using the genus_prokaryotes KEGG GENES database and KofamKOALA (Aramaki et al., 2020) with an e-value threshold of 0.001. Genes were first assigned with KO ID predicted by KofamKOALA, and the remaining unassigned genes were then annotated using GhostKOALA. KOs were mapped onto KEGG pathways using the KEGG Mapper online[6].

The abundances of KOs and pathways were calculated as the sum of the abundances of all genes annotated to them using custom scripts. Population dissimilarities (Bray–Curtis distance) of KO function among the 15 bee populations were tested by the ANOSIM test included in the vegan package (Philip, 2003) with 999 permutations. Linear discriminant analysis (LDA) was performed using LEfSe (Segata et al., 2011) with default parameters to identify KO biomarkers in different populations. Function enrichment of featured KOs was estimated by one-sided Fisher's Exact Test using the stats R package at both module and pathway levels.

For each featured KO, the abundances for all bacterial species encoding the KO-related genes were listed for all of the 99 samples. In each population, the median abundance was used as the abundance of bacterial species encoding the respective KO. Then, the contributions by different

4 https://ftp.ncbi.nlm.nih.gov/genomes/refseq/

5 https://github.com/aquaskyline/SOAPcoverage

6 https://www.kegg.jp/kegg/tool/map_pathway2.html

bacterial species to the corresponding KO were estimated. If the KO term was identified in > 50% of individual bee guts of the same population, the KO was considered to be present in the population. To compare the gene numbers among different populations, we standardized metagenome data by randomly extracting 400 Mb bacterium-derived data from each gut sample, which were mapped to the gene assemblies. The assemblies were retained only if the coverage was ≥90%.

The glycoside hydrolase (GH) and polysaccharide lyase (PL) genes were functionally assigned to the dbCAN2 database (Zhang et al., 2018). In each population, the median abundance was used as the abundance of bacterial species encoding respective GH/PL gene clusters. Then, the contributions by different bacterial species to the corresponding GH/PL gene clusters were estimated.

Diet profiling of gut and honey metagenomes

A customized chloroplast genome database was first constructed for flowering plants (4,161 from NCBI and 271 newly sequenced ones generated by our group) for KrakenUniq version 0.5.5 (Breitwieser et al., 2018). For gut metagenome data, we filtered out reads mapped to the *A. cerana* genome or to the *de novo* bacterial assemblies and used the remaining reads for pollen diet profiling based on chloroplast DNA found in the gut annotated at the family level. The remaining reads were first aligned to the customized chloroplast genomes with KrakenUniq (Breitwieser et al., 2018) with default parameters. Those mapped reads were aligned to nt database with blastn with an e-value setting as 1e-5, and the best alignment was retained. Then, the reads from the alignments with similarity >95% and query coverage >90% to reference sequences from plants were kept and used to estimate the pollen abundance at the family level. The families with a relative abundance of less than 1% were filtered out.

The geographical variation in pollen composition was also conducted with the assembled metagenome data from honey samples collected from five representative regions of this study (SC_AB, SC_GB, SX_QL, QH_GD, JL_DH) (Liu et al., 2021). The assemblies with similarity >95% and query coverage >90% to reference plant sequences were retained. Clean reads were then aligned to these assemblies using Minimap2 (Li, 2018), and the mapped reads were used to estimate the pollen abundance at the family level with SamBamba (Tarasov et al., 2015). The families with a relative abundance of less than 1% were filtered out.

The gut bacterial phylotype and KO composition from *de novo* assembly and annotation were used in the correlation analysis with pollen composition at the family level.

Heritability of bacterial diversity

The rank-based inverse normal transformation of the relative abundance with the reference-based method was used in the heritability analysis. The heritability was defined as the Percentage of Variance Explained (PVE) and estimated with Genome-wide Efficient Mixed Model Association (GEMMA, v0.94) (Zhou and Stephens, 2012). To control the effects of individual relatedness, population structure, and diet variation, we regressed the transformed gut bacteria abundance with the first three PCs from the PCA of the host genotypic data and the pollen Shannon index from the gut. Then, PVE estimation was performed with the residuals using GEMMA (with relatedness matrices and the HE regression algorithm). A phylotype or SDP was considered heritable if the PVE measurements did not show overlaps with zero.

The association between host genetic variation and bacterial diversity

The rank-based inverse normal transformation of the relative abundance of core gut bacteria was used in the Genome-Wide Association Studies (GWAS) analysis. We used the Linear Mixed Model in rMVP v1.0.0 (Yin et al., 2021). In the GWAS analysis, the kinship between individuals, the first three PCs in host PCA, and the diet (Shannon index of pollen family composition) were used for correction. We used the 'EMMA' method to analyze variance components in rMVP. The statistical significance level was set to $p < 5 \times 10^{-8}$ for the GWAS association.

The effects of diet on the abundance of *Gilliamella* and *Lactobacillus* Firm-5

A *Gilliamella* strain (B2889, belonging to the SDP Gillia_Acer_2) was cultivated with HIA, and a *Lactobacillus* Firm-5 strain (B4010) was cultivated with MRS with 0.02 g/ml D-fructose (aladdin F108331) and 0.001 g/ml L-cysteine (aladdin C108238). The microbiota-free *A. cerana* workers were obtained following Zhang et al. (2022). Pupae in the late stage were removed from brood frames and incubated in sterile plastic bins at 35°C. Both bacterial strains of OD_{600} = 1 were mixed at equal volumes and then mixed with 50% (v/v) sterilized sucrose syrup, which was fed to newly emerged microbiota-free honeybees. After 3 days, cellobiose (Shanghai Yuanye Bio-Technology Co., Ltd. S11030, final concentration 5 mg/ml) and solutions with different proportions of pectin (Sigma, P9135) and cellulose (Megazyme, P-CMC4M) (1:10, 10:1, final mixed concentration 5.5 mg/ml) mixed with sterilized 50% sucrose syrup were fed to honeybees, respectively. Honeybees fed with only 50% sucrose syrup were used as control. After feeding for

4 days, DNA was extracted from bee guts and used for the qPCR assay.

qPCR assay

We conducted real-time qPCR experiments to determine bacterial loads for both *Gilliamella* and *Lactobacillus* Firm-5 after the feeding experiments. 16S-F-Gillia (5′-TGAGTGCTTGCACTTGATGACG-3′) and 16S-R-Gilla (5′-ATATGGGTTCATCAAATGGCGCA-3′) primers were used for *Gilliamella* 16S rRNA gene amplification. 16S-F-Firm5 (5′-GCAACCTGCCCTWTAGCTTG-3′) and 16S-R-Firm5 (5′-GCCCATCCTKTAGTGACAGC-3′) primers (Kešnerová et al., 2017) were used for *Lactobacillus* Firm-5 16S rRNA gene amplification. Actin-AC-F (5′-ATGCCAACACTGTCCTTTCT-3′) and Actin-AC-R (5′-GACCCACCAATCCATACGGA-3′) primers were used to amplify the *actin* gene of the host *A. cerana* (Park et al., 2020), which was used to normalize the bacterial amplicons (Kešnerová et al., 2017). The 16S target sequences were cloned into vector pEASY-T1 (Transgen), and the Actin target sequence was cloned into pCE2 TA/Blunt-Zero Vector (Vazyme), then confirmed by Sanger sequencing. The copy number of the plasmid was calculated, serially diluted, and used as the standard. qPCR was performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme) and QuantStudio 1 (Thermo Fisher) in a standard 96-well block (20-μl reactions; incubation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 20 s). The data were analyzed using the QuantStudio Design & Analysis Software v1.5.1 (Thermo Fisher) and Excel (Microsoft). *p*-values were calculated using the Mann–Whitney test.

Results

Bacterial composition significantly varied across Asian honeybee populations at multiple levels

A total of 99 nurse bees from 15 geographic populations covering 13 provinces across China were analyzed (**Figure 1A** and **Supplementary Table 1**). SNPs derived from honeybee reads were used to construct a neighbor-joining tree (**Figure 1B**), which confirmed the geographic origin of the sampled populations. This result was consistent with the reported genetic structure and geographic distribution of *A. cerana* populations (Ji et al., 2020), thereby excluding the possibility of colony translocation.

Bacterial reads were then *de novo* assembled and aligned against the GenBank nr database to recover the phylotype composition for individual nurse bees. In congruence with previous studies (Kwong et al., 2017b; Ellegaard et al., 2020), the core gut microbiota in *A. cerana* included six groups of bacteria, i.e., *Gilliamella* and *Snodgrassella* from Proteobacteria, *Bifidobacterium* from Actinobacteria, *Lactobacillus* Firm-4 and Firm-5 from Firmicutes, and *Apibacter* from Bacteroidetes (**Figure 1C**). This result was further confirmed by the reference-based method (**Supplementary Figures 1**, **2**), which employed a customized database containing 307 public and 83 newly sequenced bee gut bacterial genomes (**Supplementary Table 2**). However, apparent variations in phylotype composition were observed among individual bees (**Figure 1C**), and the composition of core microbes appeared to be less stable than in *A. mellifera* (Regan et al., 2018; Ellegaard and Engel, 2019; Ge et al., 2021).

Both Shannon index (**Figure 1D**, Kruskal–Wallis, $P = 0.0022$) and phylotype diversity (ANOSIM, $r = 0.29$, $P = 0.001$) showed noticeable differences across populations of *A. cerana*. Nine of the 15 investigated populations could be defined by featured bacteria in the LEfSe analysis (Segata et al., 2011), which showed significantly higher relative abundances in the focal population than all remaining populations (**Figure 1E**).

The distinct gut variation across host populations was also reflected at finer taxonomic scales. Among all six core phylotypes in *A. cerana*, *Gilliamella* contained the most diverse host-specific sequence-discrete populations (SDPs) (**Figures 1F–H** and **Supplementary Figures 3–6**), which were defined as strains sharing a genome-wide average nucleotide identity (gANI) >95% within each phylotype. Our results revealed varied presence and abundance in SDPs of core phylotypes among gut samples (**Figures 1I–K** and **Supplementary Figure 7**), whereas *Gilliamella* showed significant SDP differences among geographical populations (**Supplementary Figure 8**, ANOSIM $r = 0.14$, $p = 0.001$). We also identified genome sites showing single nucleotide variation (SNV) for major SDPs in each sample, to detect gut variations at the strain level (**Supplementary Figure 9**). The results demonstrated significant variations in SNV composition across populations (**Supplementary Figure 10**). Thus, the gut bacterial composition of Asian honeybees varied significantly across geography at phylotype, SDP, and strain levels.

Progressive changes in the honeybee microbial community were related to diet shift

Gut compositions showed extensive overlaps among populations, forming continuous groups in PCoA analyses (**Figure 2A**), indicating progressive changes in microbial community structure among endemic honeybee populations. Interestingly, a continuous variable contributing to the separation along the first principal coordinate axis (PCoA) reflected antagonistic dynamics in abundances of *Gilliamella*

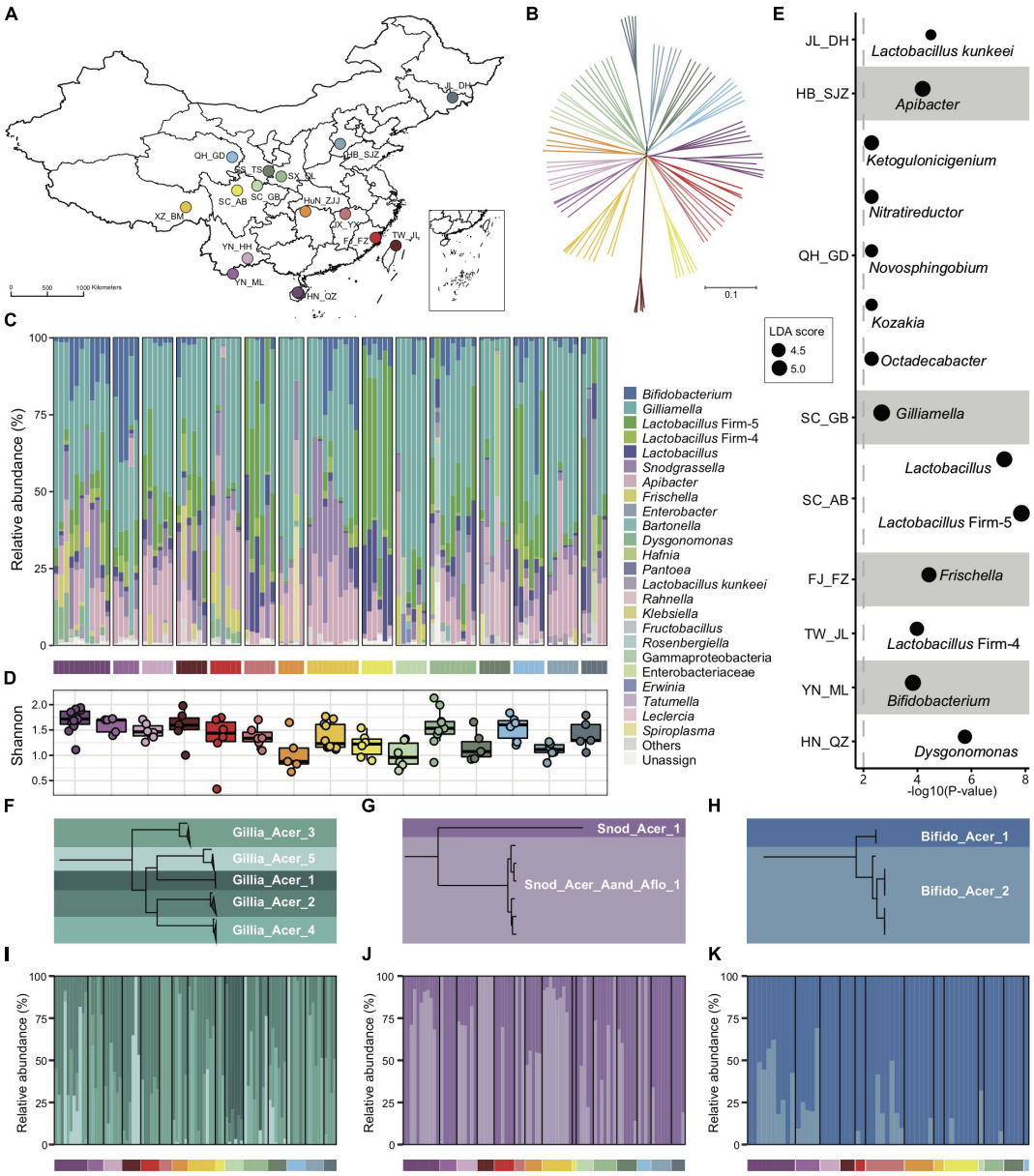

FIGURE 1
Bacterial composition of gut microbiota in geographic populations of *Apis cerana*. **(A)** Sampling sites of 15 *A. cerana* geographic populations. **(B)** Neighbor-joining tree reflecting the honeybee population structure, based on genome-wide SNPs. Bacterial relative abundance **(C)** and Shannon index **(D)** are based on gut metagenomes of different populations. Phylotypes with at least 5% abundance in any sample or 0.5% abundance in more than 6 samples were shown, otherwise included in "Others." *Lactobacillus*: *Lactobacillus* that is not assigned to any known groups. **(E)** Featured gut microbe phylotypes in each geographic population were revealed by LEfSe analyses. The size of the bubbles represents LDA score. Phylogenetic relationships of SDPs within *Gilliamella* **(F)**, *Snodgrassella* **(G),** and *Bifidobacterium* **(H)**. Maximal-likelihood phylograms, reconstructed using core genes present in all strains of the corresponding phylotype. The SDP compositions of *Gilliamella* **(I)**, *Snodgrassella* **(J)**, and *Bifidobacterium* **(K)** in gut samples, with those of abundances <1% excluded. Horizontal bars under panels **(C,I–K)** indicate population origins of the guts, with colors corresponding to those in **(A,B)**.

and *Lactobacillus* Firm-5 (Figure 2B). Among all six core phylotypes, the relative abundance of *Gilliamella* (Spearman's *rho* = −0.85, $p = 2.14e\text{-}28$) and *Lactobacillus* Firm-5 (Spearman's *rho* = 0.79, $p = 4.47e\text{-}22$) showed the most significant correlation with the PCoA1 value.

In the 99 samples, the populations from XZ_BM, SC_AB, HN_QZ, JL_DH, TW_JL, and QH_GD represent each of the peripheral six subspecies, and the others are from the central ancestral population (Ji et al., 2020). We first tested whether the gut compositions showed differences at the subspecies level. We

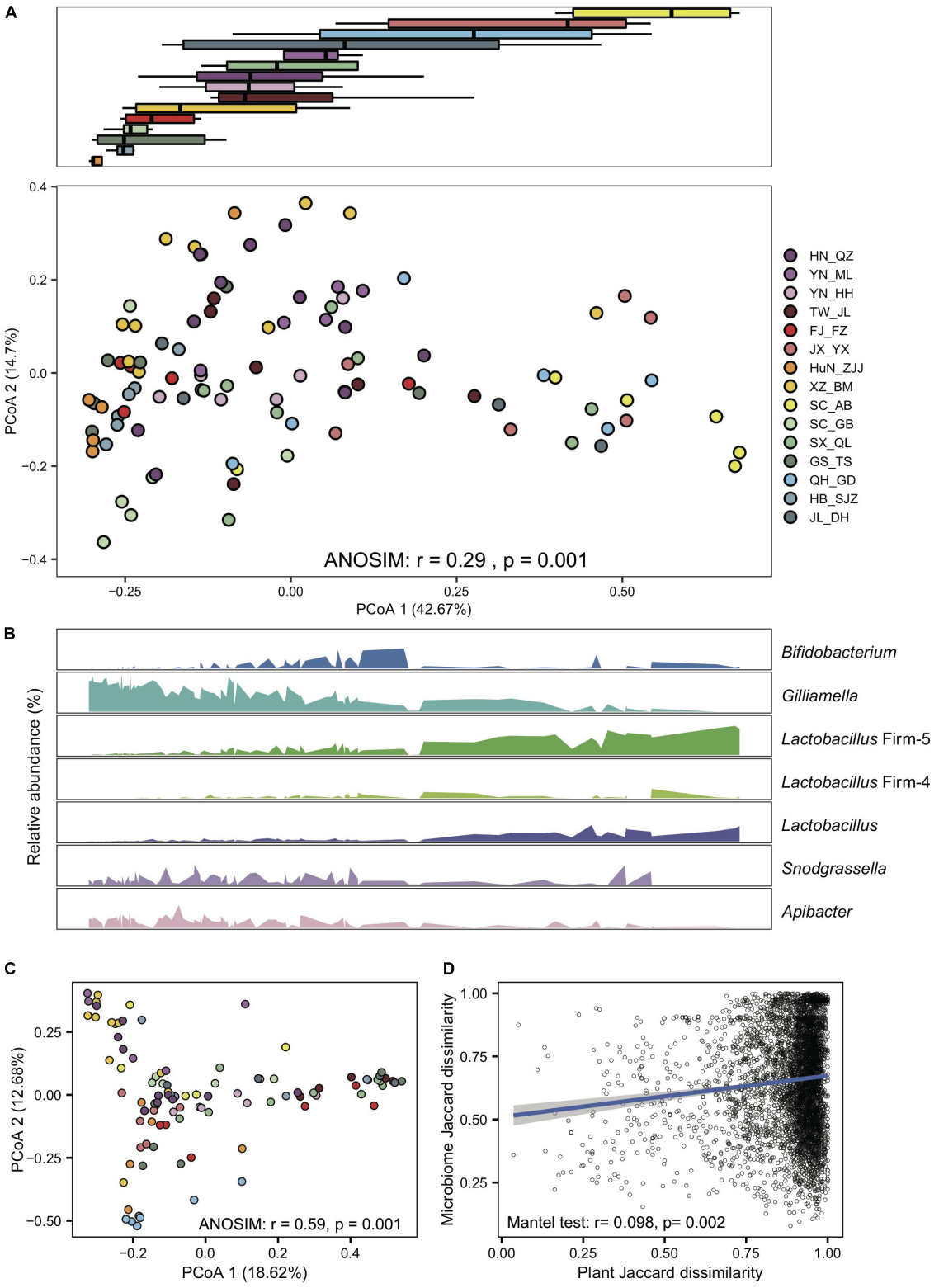

FIGURE 2
Gilliamella and *Lactobacillus* Firm-5 showed antagonistic trends in compositional turnover of honeybee gut microbiomes. **(A)** Overall variation of the gut microbial community at the phylotype level, revealed by Bray–Curtis dissimilarity PCoA (bottom panel). Boxplots (top panel) indicate the distribution of each population along the first principal coordinate axis (PCoA1). Boxplot center values represent the median and error bars represent the SD. Colors correspond to the population origin of the gut samples. **(B)** Relative abundances of core bacterial phylotypes along PCoA1. **(C)** The pollen composition at the family level varied in gut metagenomes from populations of *A. cerana*. **(D)** The Jaccard distances of the gut bacterial phylotype and the pollen composition at the family level were significantly correlated.

compared the Bray–Curtis distance between each of the central populations and between central and peripheral populations. The gut variations among subspecies were significantly more prominent than those within subspecies at the phylotype level (Wilcoxon test, p = 1.4e-7) but not at the SDP level (Wilcoxon test, p = 0.87). The results indicated that the gut microbiome changed along host differentiation, which might be related to the host genetic differentiation and diet variation.

Next, we estimated the heritability of the relative abundance of core bacteria at both phylotype and SDP levels. The heritability was overall low. Among the core phylotypes, *Gilliamella* abundance showed the highest heritability (**Supplementary Figure 11**), while that of *Snodgrassella* was not obvious. The abundances of about one-third SDPs were not heritable. The GWAS analysis did not detect any apparent site variation that had determined bacterial composition, as no genomic region of *A. cerana* was found significantly associated with the bacterial abundance (with a threshold as $p < 2e\text{-}8$) at either the phylotype or SDP level. These results indicated that gut microbial diversity at the population level was not likely driven by single-site nucleotide variations. The complex genetic heterogeneity and limited sample size might also mask the effect of host genetics.

To examine the effect of diet on the gut microbiome, we first extracted pollen reads from the metagenome data and identified flower composition for each gut sample (details in Section "Materials and methods"). Honeybee populations from different regions showed significant variation in pollen diet at the family level (ANOSIM, r = 0.59, P = 0.001, **Figure 2C** and **Supplementary Table 3**). Such a diet shift was further confirmed by pollen variation in honey samples extracted from five of the representative populations (SC_AB, SC_GB, SX_QL, QH_GD, and JL_DH), where pollen composition again showed significant differences at the family level (ANOSIM, r = 0.35, p = 0.007, **Supplementary Figure 12** and **Supplementary Table 4**). Most importantly, the Jaccard distances of the gut bacterial phylotype and the pollen composition were significantly correlated (mantel test, r = 0.098, p = 0.002, **Figure 2D**). Among the core phylotypes, the abundances of *Gilliamella* showed a significant correlation with the Shannon index of pollen composition in the gut (Spearman's $rho = -0.23$, p = 0.020). Therefore, the pollen diet showed a correlation with the composition of the honeybee gut microbiome.

KEGG orthology function was correlated with diets and characterized by carbohydrate metabolism and transport

To understand whether gut microbes in *A. cerana* showed idiosyncratic regional traits on the function level, we *de novo* assembled the metagenomes and annotated genes for each of the 99 gut samples. As with bacterial compositions, the number of gene clusters per gut varied significantly among populations (Kruskal–Wallis test, p = 6.2e-4) (**Figure 3A**). The gene cluster number in different individuals was significantly correlated with the Shannon index of gut bacteria (Pearson's r = 0.64, p = 8.28e-13), suggesting that bacterial diversity is the basis for gene varieties among individual bees. We also quantified the rate of novel gene accumulation for each population. The results demonstrated distinct differences in the genetic diversity among populations (**Figure 3B**).

We assigned predicted gene clusters from all metagenome data to the KEGG database to reveal the diversity of functions among populations. A total of 1,965 functional orthologs (KOs) shared among all populations were enriched in genetic information processing and signaling and cellular processes (**Supplementary Figure 13**). The KO category compositions (**Figure 3C**) also showed extensive overlap and were distinctively differentiated among populations (ANOSIM, r = 0.33, p = 0.001, **Supplementary Figure 14**). The LEfSe analyses showed that 11 of the 15 geographic populations had noticeably enriched KO categories (**Supplementary Figure 15**), which showed significantly higher relative abundances in the focal population than all remaining populations.

We also tested whether the KO compositions showed a difference at the subspecies level. The gut bacterial KO composition among subspecies was significantly more prominent than those within subspecies (Wilcoxon test, p = 1.6e-4). Furthermore, the Jaccard distances of the gut bacterial KO composition and pollen diversity at the family level showed a significant correlation (mantel test, r = 0.12, p = 0.001, **Figure 3D**). The results indicated that not only bacterial composition but also their functions changed along host differentiation and were associated with diets.

The top population-enriched KOs ($p < 1e\text{-}5$) mainly included functions in metabolism and membrane transport (**Supplementary Figure 15**). At the KO term level, we identified 83 KO terms showing inter-population differences (**Supplementary Table 5**), in which they were significantly more abundant in only one of the geographic populations. Interestingly, 37 of 83 of the enriched KO terms were transporter pathway genes (all belonging to ko02000) (**Figure 4A**), whereas the pathway was also enriched in some local populations (e.g., SC_AB and YN_ML, **Figure 4B**). Most featured transporters were related to carbohydrates (**Figure 4C**) and six of the enriched KO terms belonged to the glycoside hydrolase (GH) family (**Supplementary Table 5**), in concert with the fact that polysaccharides are one of the major nutritional components derived from pollen. Therefore, the population-enriched gut microbe KOs were mainly associated with carbohydrate metabolism and transport and were significantly correlated to pollen composition in a given local environment.

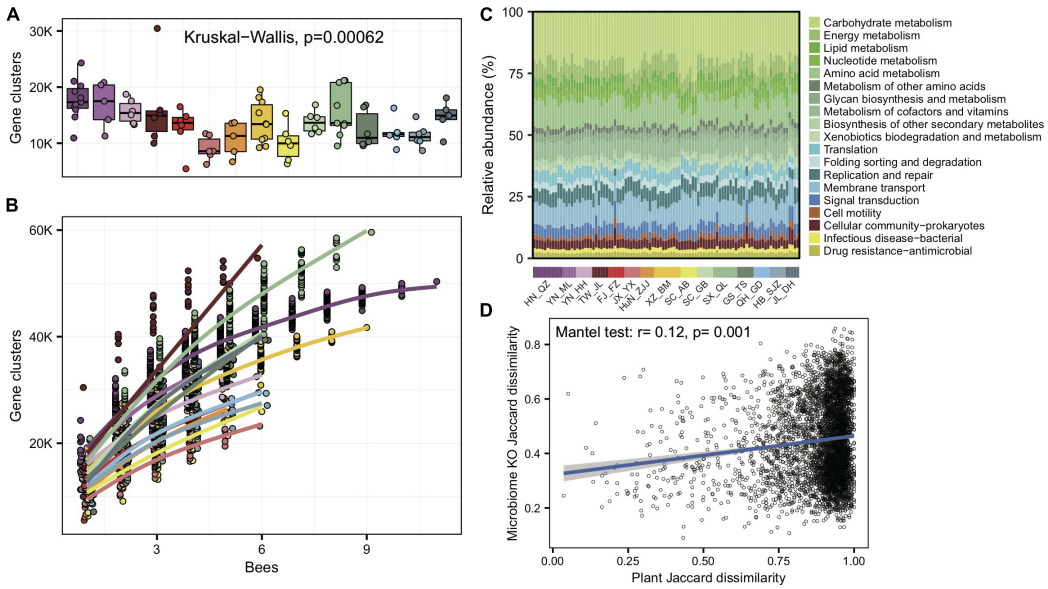

FIGURE 3
Significant variations in gene cluster and functional annotation among populations. **(A)** Gene cluster numbers per gut sample, based on 400 Mb bacteria-mapped reads. **(B)** Accumulation curves for gene clusters of each population of *A. cerana*, based on 400 Mb bacteria-mapped reads. **(C)** Relative abundance of KEGG annotations in each gut sample, based on all bacteria-mapped reads in metagenomes. **(D)** The Jaccard distances of the gut bacterial KO composition and the pollen composition at the family level were significantly correlated.

Phosphotransferase system, ATP binding cassette transporters, and glycoside hydrolases contributed by *Gilliamella*, *Lactobacillus* Firm-5 and *Bifidobacterium* were hotspot genes involved in local adaptation

In congruence with the finding that carbohydrate metabolism and transport play important roles in adapting to local diets, key genes of such pathways, such as phosphotransferase system (PTS) transporters and ATP binding cassette (ABC), were often characterized in distinct honeybee populations. For instance, a total of 17 PTS and 16 ABC transporters were identified from the 37 enriched transporter pathway genes (**Supplementary Table 5**). All featured PTS genes were only found in the SC_AB population, while the featured ABC transporters were present in several populations (XZ_BM, SC_AB, and YN_ML). PTS serves as one of the major mechanisms in carbohydrate uptake, particularly for hexoses and disaccharides. In SC_AB, the 17 featured PTS genes included some that are specific for ascorbate, beta-glucoside, cellobiose, fructoselysine/glucoselysine, galactitol, mannose, and sucrose (**Supplementary Table 5**). The mapping of relevant gene clusters against the bacterial nr database suggested that these featured PTS genes were mainly contributed by *Gilliamella* and *Lactobacillus* Firm-5 (**Supplementary Table 6**). The dominant role of these two bacteria in coding PTS genes was further confirmed by analyses of 81 individually sequenced and annotated genomes, where *Gilliamella* and *Lactobacillus* Firm-5 were the major phylotypes encoding PTS genes (**Supplementary Table 7**). At the SDP level, *Lactobacillus* Firm-5 had a higher copy number of PTS transporters for cellobiose, fructoselysine/glucoselysine, and galactitol than *Gilliamella* (**Figure 5A**). Many of these PTS transporters were found in the featured genes in the SC_AB population, which was dominated by *Lactobacillus*. Thus, the enrichment of featured PTS genes could at least be partially explained by the elevated abundance of the contributing bacteria in local populations (**Figure 1E**).

The featured ABC transporters included transporters for amino acids, iron, and carbohydrates (**Supplementary Table 5**). Besides *Gilliamella* and *Lactobacillus* Firm-5, *Bifidobacterium* also contributed unique ABC transporters (**Supplementary Table 6**). For example, the *Bifidobacterium*-unique transporters for raffinose/stachyose/melibiose (msmE, msmF, and msmG) (genome annotation results in **Supplementary Table 7**) were featured in the YN_ML population, in which *Bifidobacterium* was also the featured phylotype (**Figure 1E**). The elevated *Bifidobacterium* and its unique ABC transporters characterized in YN_ML might be attributed to the presence of raffinose and stachyose in specific pollen or nectar, which are toxic to the honeybees (Barker, 1977).

At a finer taxonomic scale, 14 of the 17 featured PTS genes had significant population-distinct SNV sites coded by SDP from *Lactobacillus* Firm-5, and 9 of the 16 ABC transporters

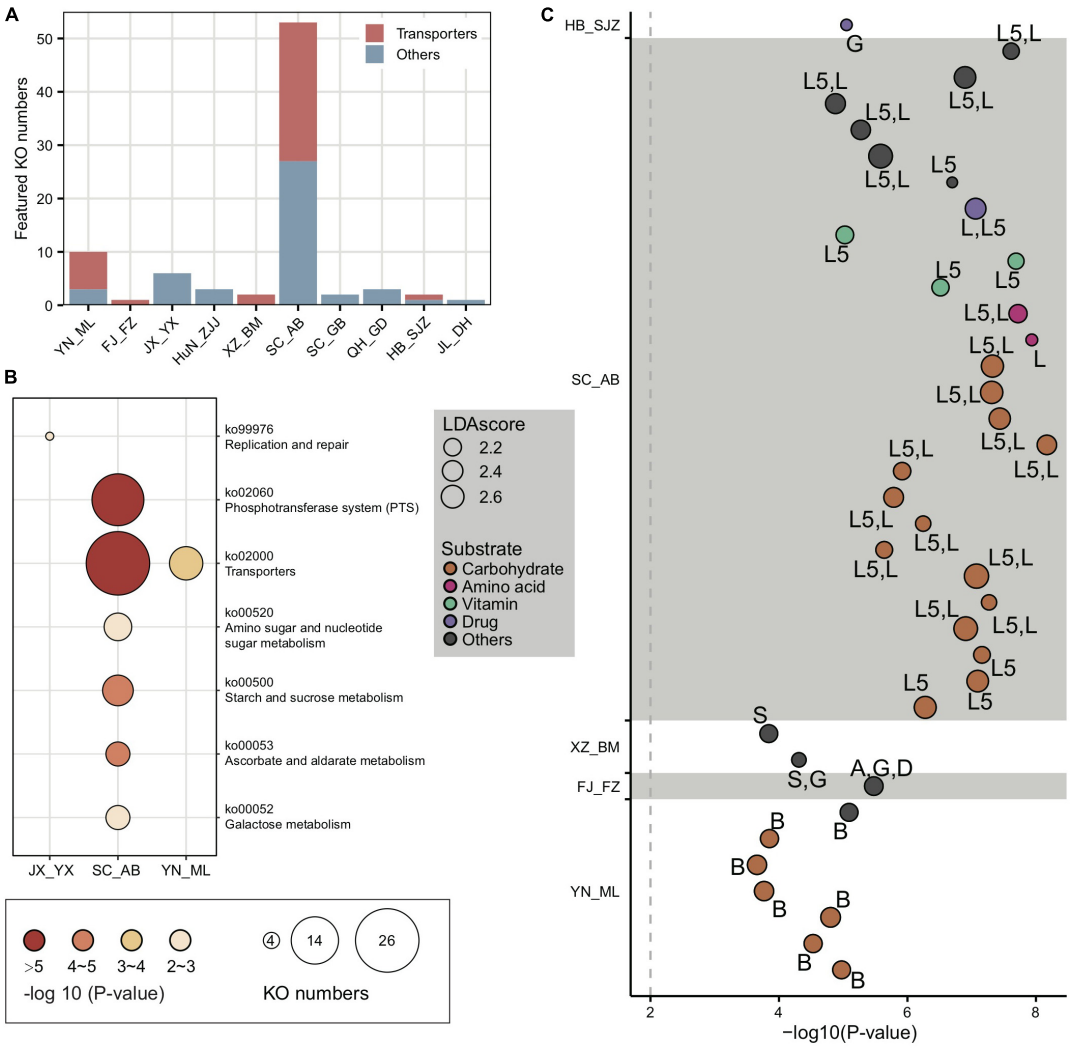

FIGURE 4
Locally featured KOs were enriched in carbohydrate transporters. **(A)** Featured KOs in geographic populations were enriched in transporters. **(B)** Featured KEGG pathways in gut microbiota from *A. cerana* populations. The size of the bubbles represents KO numbers. **(C)** Transporters in featured KOs were mainly specialized for carbohydrates. The size of the bubbles represents the LDA score. The codes marked next to each bubble indicate the main contributing bacteria species, where only those with >10% contribution were listed: A, *Apibacter*; B, *Bifidobacterium*; D, *Dysgonomonas*; G, *Gilliamella*; L, *Lactobacillus* that is not assigned to any known groups; L5, *Lactobacillus* Firm-5; S, *Snodgrassella*.

harbored significant population-distinct SNV sites coded by SDPs from *Lactobacillus* Firm-5 and *Apibacter* (**Supplementary Table 8**). One featured gene *ulaC* (ascorbate PTS system EIIA or EIIAB component, K02821), coded by SDP from *Lactobacillus* Firm-5 showed significant population-distinct copy number variations (CNVs) (**Supplementary Table 9**). Thus, the variations in functional genes seemed to have been caused by changes in the featured bacterial composition at both phylotype and strain levels.

Besides PTS and ABC genes, six GH genes were featured in *A. cerana* populations (from GH1, GH3, GH29, GH36, GH43, and GH78 family) and were mainly contributed by *Gilliamella*, *Lactobacillus*, and *Bifidobacterium* (**Figure 5B** and **Supplementary Table 10**). To construct the profile for major gene families involved in glycoside breakdown, we used dbCAN2 (Zhang et al., 2018) to annotate all GH and polysaccharide lyase (PL) genes. We discovered that the GH/PL profiles varied across populations (**Supplementary Figure 16**). Additionally, the non-core bacterium also encoded novel 1265 GH genes. For instance, *Dysgonomonas* contributed unique GH gene families in *A. cerana*, including GH57, GH92, GH133, and GH144 (**Supplementary Table 10**). This non-core bacterium was featured in the HN_QZ population (**Figure 1E**), likely due to its contribution to unique GH gene sets.

Some of the six featured GH genes were positioned together with featured PTS or ABC transporters on the genome.

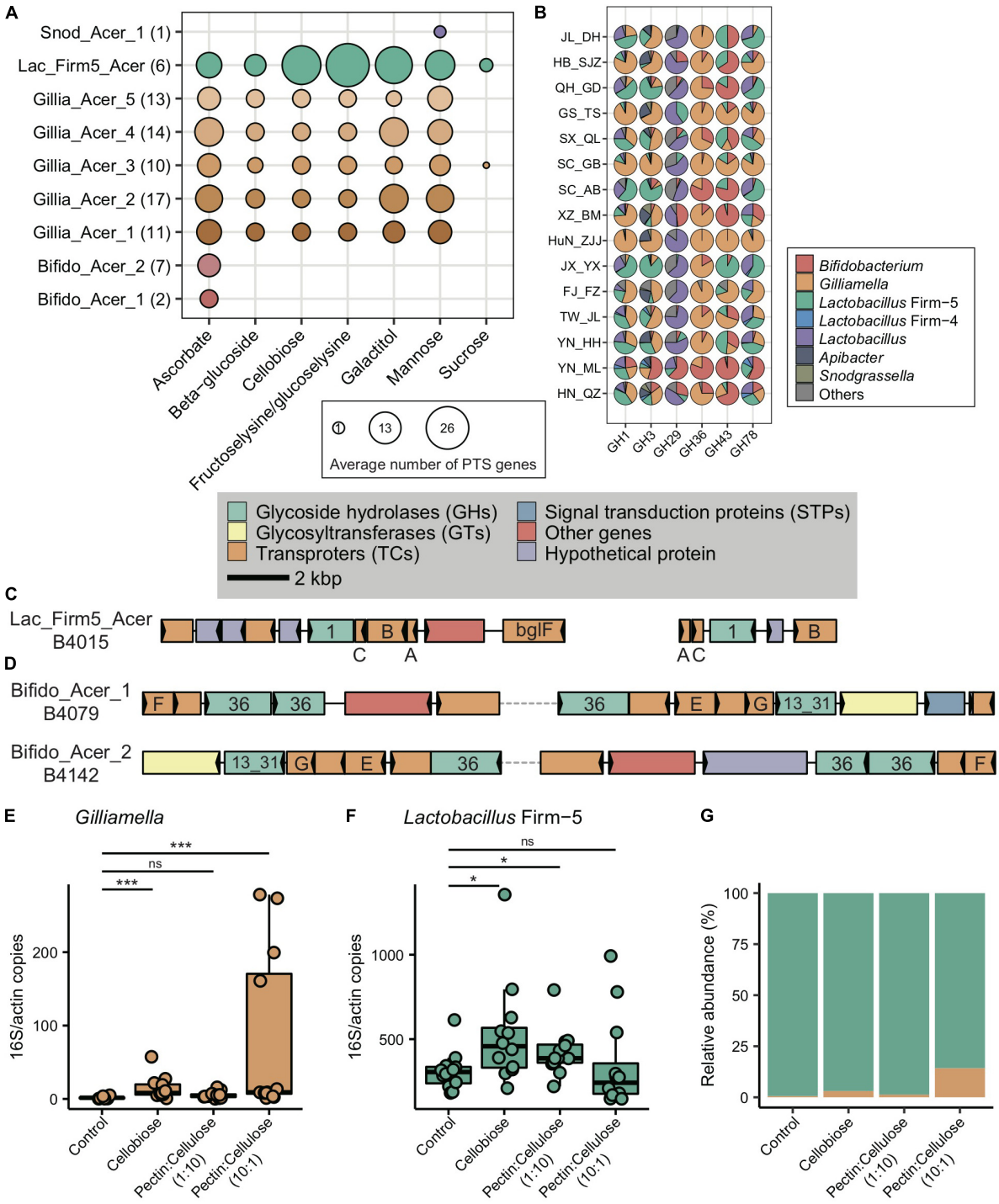

FIGURE 5
Main bacterial phylotypes coding for PTS and GHs. **(A)** Gene copy numbers in population-featured PTS pathways identified in all SDPs. Numbers in parentheses represent SDP strain numbers. Genes were annotated from the genomes of newly isolated microbial strains from *A. cerana* guts. **(B)** Featured GHs were coded by different bacterial phylotypes from metagenome of 15 geographic populations of *A. cerana*. **(C)** PTS transporters (*celA*/*celB*/*celC*/*bglF*), 6-phospho-beta-glucosidase (*bglA*) from the GH1 family were often found located together in genomes, which were represented here by *Lactobacillus* Firm-5 SDP. **(D)** ABC transporters (*msmE*/*msmF*/*msmG*), alpha-galactosidase from the GH36 family, and alpha-glucosidase from the GH13_31 family were often found located together in genomes, which were represented here by two *Bifidobacterium* SDPs. The change in the absolute abundance of *Gilliamella* **(E)**, *Lactobacillus* Firm-5 **(F)**, and the percentage of *Gilliamella* and *Lactobacillus* Firm-5 **(G)** after feeding cellobiose and mixtures of pectin and cellulose with different concentrations. PTS, phosphotransferase system; GH, glycoside hydrolase. ns, not significantly different, $*p < 0.05$, $***p < 0.001$.

Together, these genes formed CAZyme gene clusters (CGCs), performing sequential functions in polysaccharide degradation and transportation. For example, in *Lactobacillus* Firm-5, the featured 6-phospho-beta-glucosidase (*bglA*) from the GH1 family, PTS system genes for beta-glucoside, and cellobiose were usually clustered and formed CGCs (**Figure 5C**), and all these genes were enriched in the SC_AB population. In *Bifidobacterium*, the raffinose/stachyose/melibiose transport system msmEFG and alpha-galactosidase from the GH36 family involved in raffinose/melibiose degradation were usually located together (**Figure 5D**). These genes were all featured in the YN_ML population, which had *Bifidobacterium* as the featured phylotype.

The feeding experiment verified the contribution of pollen polysaccharide composition to the trade-off of *Gilliamella* and *Lactobacillus* Firm-5

Our investigation of *A. cerana* guts from its natural range revealed antagonistic abundance between the two core-bacteria *Gilliamella* and *Lactobacillus* Firm-5 across geographic populations (**Figure 2B**). As both lineage and function diversities of honeybee gut bacteria were correlated to pollen diets (**Figures 2D**, **3D**), we speculate that characteristic traits in local food resources may have led to bacterial community shifts observed at the grand scale. To test this hypothesis, we conducted feeding experiments to verify whether functional adaptations observed in metagenomes can lead to adaptive advantages in bacterial competition.

As the main structural components of the pollen wall, pectin and cellulose were chosen as representative nutritional contents to examine the impacts of food on the abundance variation between *Gilliamella* and *Lactobacillus* Firm-5 in co-feeding experiments. In the main gut microbe phylotypes in the honeybee, only *Gilliamella* are able to degrade the polygalacturonic acid (PGA), the backbone of pectin (Engel et al., 2012). On the other hand, cellobiose (the key metabolite of cellulose) related PTS genes (**Supplementary Table 5**) and the metabolic pathway (ko00500, starch, and sucrose metabolism) were highly enriched in the SC_AB population, as revealed by the metagenome data. The newly assembled *Lactobacillus* Firm-5 genome also showed elevated copy numbers in cellobiose PTS (**Figure 5A**). As such, we anticipated that local food with a higher proportion of pectin would increase the fitness of *Gilliamella*, and food with a higher proportion of cellulose would favor *Lactobacillus* Firm-5 in the community.

We fed *A. cerana* workers that were colonized with an equal abundance of *Gilliamella* and *Lactobacillus* Firm-5, with cellobiose, pectin, and cellulose mixture with different concentrations (1:10 and 10:1, respectively) and examined corresponding changes in bacterial composition after 4 days. Interestingly, the absolute abundance of *Lactobacillus* Firm-5 was always higher than *Gilliamella* in the control group, which was only fed with sucrose (**Figures 5E–G**), indicating a predominant role of *Lactobacillus* over *Gilliamella* in the given condition. The absence of pollen in food, and the absence of sucrose PTS genes in the strain we used (belonging to Gillia_Acer_2 SDP, **Figure 5A**) might explain the low abundance of *Gilliamella* in the control group. The absence of *Snodgrassella* might also affect the colonization of *Gilliamella* (Martinson et al., 2012; Kwong et al., 2014).

After feeding cellobiose, the absolute abundance of both *Gilliamella* and *Lactobacillus* Firm-5 significantly increased relative to the control group (**Figures 5E,F**), which was in accordance with the presence of cellobiose PTS genes in both phylotypes (**Figure 5A**). As expected, *Gilliamella* and *Lactobacillus* Firm-5 showed different responses to the mixed food with varied concentrations of pectin and cellulose. The absolute abundance of *Gilliamella* did not show significant variation after feeding food of pectin:cellulose (1:10), but the abundance of *Lactobacillus* Firm-5 significantly increased (**Figures 5E,F**). On the other hand, the absolute abundance of *Gilliamella* showed a significant increase after feeding food of pectin:cellulose (10:1), but the abundance of *Lactobacillus* Firm-5 did not vary significantly (**Figures 5E,F**). The varied proportion of pectin and cellulose impacted the antagonistic of *Gilliamella* and *Lactobacillus* Firm-5. These results suggested that diet, pollen polysaccharide, in particular, is an important factor in shaping gut bacterial composition and functions in *A. cerana*.

Discussion

The progressive change of gut microbiome in Asian honeybee populations

In this study, we carried out comprehensive investigations on the gut microbiomes of the widespread Asian honeybee *A. cerana* at the population level. While many studies have contributed to our knowledge of the honeybee gut microbiota, little is understood about how this essential symbiont system evolves with the host. In agreement with previous studies on both *A. mellifera* (Rothman et al., 2018; Ellegaard and Engel, 2019) and *A. cerana* (Ge et al., 2021), our results revealed variations in gut microbes among *A. cerana* individuals, even among those from the same hive. The intra-colony variation might be related to differed social interactions among honeybee individuals (Powell et al., 2014) or varied exposure to the stored pollens and other hive materials (Anderson et al., 2022) in the honeybee.

Our studied system involved 15 geographic populations of *A. cerana*, and we were particularly interested in understanding

gut variations among the seven genetically distinctive populations, which we showed in our recent study (Ji et al., 2020) that had diverged genetically and morphologically at a subspecies level. These subspecies have been confined to drastically different habitats (e.g., mountain valleys of >3,000 m, tropical islands, temperate plains, hills, etc.). In contrast to the abrupt distinction between *A. mellifera* and *A. cerana*, the gut microbiome of honeybee populations showed progressive change within *A. cerana* (**Supplementary Figure 17**). The bacterial compositions across populations showed significant variations at phylotype, SDP, strain, and gene content levels, albeit with extensive overlaps. The strain composition of *Gilliamella* and *Snodgrassella* was largely similar among populations of western honeybees from four different states in the United States (Bobay et al., 2020). The gut microbiota community from 18 different human populations across geography also showed extensive overlap (Smits et al., 2017), implying a common trend in gut microbiome evolution for hosts exhibiting a continuous and wide-range distribution.

In the western honeybee, host genetics influenced the gut microbe composition, where *Bifidobacterium* abundance was associated with the genotype of the host glutamate receptor (Wu et al., 2021). Different from Wu et al. (2021), the complex background heterogeneity combined with a limited sample size might have masked apparent host genetic influence on gut bacteria at the local scale in our study, which may explain the weak signal reported in our GWAS analysis. However, our findings genuinely reflect the host genetic background and associated microbiota, which could not have been discovered without a broad scale sampling. With an in-depth understanding of the molecular mechanisms underlying honeybee host-gut bacteria interactions in the future, we expect that a more focused genomic screening on these target genes would reveal their specific contributions in widely distributed native bee populations.

Gut microbiome evolution under local diet shift in Asian honeybee

The honeybees consume relatively simple but consistent food, i.e., pollen and honey, and pollen is especially important to the gut microbes. Controlled experiments on the diet with or without pollen influenced the total and specific gut bacteria abundance (Kešnerová et al., 2020; Ricigliano and Anderson, 2020). Pollen diet also facilitates the co-existence of closely related *Lactobacillus* species by using different pollen-derived carbohydrate substrates (Brochet et al., 2021). Although controlled experiments conducted on *A. mellifera* have built the foundation on diet influence on honeybee gut microbiota, we knew little about how natural diets influenced honeybee gut microbiota in their native range.

Floral shifts are a common theme during range expansion and habitat adaptation of the honeybees (Ji et al., 2020). The change of locally flowering plants inevitably alters nutrients for honeybees and the associated gut microbiome, because nutritional components vary in both pollen and nectar across plant species. Pollen walls are enriched in polysaccharides in the forms of cellulose, hemicelluloses, and pectin, which serve as major food resources for the gut bacteria (Engel et al., 2012; Zheng et al., 2019). Previous studies have shown the contents of cellulose and hemicellulose varied in pollen of different species (McLellan, 1977) and in bee pollen collected from different regions (Herbert and Shimanuki, 1978). Similarly, sugar composition in nectar varied among flowers (Chalcoff et al., 2006). Particularly, nectar may contain low doses of sugars that are toxic to the honeybee, such as raffinose and mannose (Barker, 1977). Thus, both the general floral configuration and specific flower traits could serve as determining factors for the formation of a local honeybee gut profile.

Our recent work on the evolution of mainland *A. cerana* revealed that the changing floras led to a convergent adaptation of the honeybee (Ji et al., 2020). Here, we showed that both microbial composition and function of the honeybee gut microbiota exhibited progressive change throughout the studied natural range. The variation could be partially explained by the pollen diet, which is closely related to changing flora in the habitat. Such an intra-species transition in the gut microbiome reflects the evolutionary consequence of collective adaptation of both the honeybee and its symbionts.

Besides amino acids, lipids, and vitamins, pollen is a source of diverse carbohydrate sources. Carbohydrate metabolism is the second most abundant functional class of bacterial transcripts (Lee et al., 2015). Different honeybee gut bacteria species showed varied GH transcripts (Lee et al., 2015) and activities (Ricigliano et al., 2017). The PTS and ABC transporters, genes involved in the transportation of multiple types of polysaccharides, were also associated with different gut bacteria species (Lee et al., 2015). Accordingly, the PTS and ABC transporters were primarily encoded by *Gilliamella* and *Lactobacillus* Firm-5, representing the most enriched transporters among all bacterial genes featured in local populations of *A. cerana*. Our feeding and inoculation assays further showed that pollen polysaccharides determined the abundance of the two core bacteria, *Gilliamella* and *Lactobacillus* Firm-5. The role of core bacteria in local adaptations was reinforced by evidence showing their dominant contributions to genes related to pollen and nectar digestions.

Unexpectedly, non-core bacteria sometimes became abundant in local honeybee populations. For instance, *Dysgonomonas* was typically low in abundance among *A. cerana* individuals, as reported in both *Apis nigrocincta* (Lombogia et al., 2020) and *A. mellifera* (Erban et al., 2017). But this bacterium contributed a series of unique GH genes in FJ_FZ and HN_QZ populations, thereby becoming abundant

and common in the corresponding gut microbiome. This observation suggested that local food resources might trigger bacterial species turnover when non-core bacteria became more suited to new diets, which again highlights the significance of diet on the gut profile.

Population heterogeneity needs to be considered for the evolution and adaptation of honeybee microbiomes

A recent study suggested that both lineage and function diversities of the gut microbes were significantly lower in *A. cerana* when compared with *A. mellifera* (Ellegaard et al., 2020). However, this conclusion was drawn based on two *A. mellifera* colonies from Switzerland, two colonies of both *A. mellifera* and *A. cerana* from two sites in Japan, it is difficult to anticipate whether such a distinct pattern could be generalized when population gradients of both honeybee species are taken into consideration. Although the present study was not designed for comprehensive analyses of inter-species comparisons, our results provided insights into how intra-species variations in gut microbiota might affect interpretations of differences between honeybee species.

Although the per-bee gene diversity was generally lower in *A. cerana* microbiota than *A. mellifera*, individual bees of different *A. cerana* populations showed variation (**Supplementary Figure 18A**). In addition, the divergence of the accumulated gene diversity between the two species was much less significant than previously suggested. The Japanese populations representing *A. cerana* in the earlier study (Ellegaard et al., 2020) were one of the least variable populations among all *A. cerana* populations investigated in this study (**Supplementary Figure 18B**). Given the large variations observed among *A. cerana* populations, it is unknown whether a similar difference might also be common within *A. mellifera* and how that might influence the distinctions between these two widely distributed honeybee species. Additionally, other confounding factors should also be taken into consideration to gain a comprehensive understanding of honeybee gut microbiomes. In particular, the evolutionary pathways and phylogenetic relationships of focal populations, the specifics in honeybee management (such as colony merging and artificial diet additions), and other human interventions, may all have significant impacts on the honeybee gut profile. As the gut symbiont profile is a signature of the natural adaptation of the host to specific habitats, it would seem that comparisons between microbiomes of intra- and inter-host honeybee species should always be placed in a context of specific environments.

Since worker age (Hroncova et al., 2015) and seasonality (Almeida et al., 2022) showed effects on honeybee gut microbiome, these factors need to be considered in the data interpretation. Additionally, the limited sampling for each local population might also under-estimate the gut microbe diversity and bring bias into intra-colony variation (Rothman et al., 2018). However, season control and simultaneous age marking are admittedly difficult for sampling honeybees from a wide geographic range. In our study, we chose a practical method to specifically sample nurse bees based on their morphology. Admittedly, such criteria are not as accurate as individual marking and errors are possible. Despite the potential influence of seasonality, populations sampled in the same month did not show any elevated affinity to those sampled from different months (**Figure 2** and **Supplementary Figure 14**). Nevertheless, with honeybee age and season controlled, collecting enough individual bees at the intra- and inter- colony levels could improve future research initiatives on investigations of native honeybee colonies. Notably, season control is to collect bees with the same circadian activity and probably not the same month for different populations located across temperate and tropical regions.

Our study took a first step toward understanding the relative contribution of diet and host genetics on the gut microbiota of widely ranged honeybee populations. Our results detected localized gut features at both species and functional levels throughout the distribution range. However, the gut microbiome showed unexpected extensive overlap across the investigated ranges, which covered temperate to tropical regions. These results suggested that progressive change is the foundation of gut microbiome evolution in the Asian honeybee and specialized bacterial traits help to adapt to local diets. In this regard, regional floral diversity could serve as a key to maintaining characteristic repertoires of honeybee gut microbes, which is tremendously important for honeybee health as a whole. Therefore, a sustaining plant community containing diverse endemic flower species should be considered a key part of a honeybee conservation plan. On the other hand, the fitness of gut microbiomes of the honeybee populations may play an unforeseen role in the survival of colonies, during honeybee introduction, hybridization, and especially translocation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA705951.

Author contributions

XiZ designed the study. XiZ, SL, and XeZ organized and coordinated the study. QS coordinated sample collection, bacterial isolation, and genome annotation, reference-based metagenome mapping, and SDP analysis. MT conducted

de novo assembly of bacteria and metagenome and metagenomics analysis. SL conducted genetic variation analysis of *A. cerana*, heritability, and GWAS analysis. JH conducted diet profiling. QS and JT conducted feeding experiments and qPCR assay. XiZ, QN, and XL organized sampling. SL, XiZ, and XgZ wrote the first draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.934459/full#supplementary-material

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Aspergillus-bees: A dynamic symbiotic association

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Besides representing one of the most relevant threats of fungal origin to human and animal health, the genus *Aspergillus* includes opportunistic pathogens which may infect bees (Hymenoptera, Apoidea) in all developmental stages. At least 30 different species of *Aspergillus* have been isolated from managed and wild bees. Some efficient behavioral responses (e.g., diseased brood removal) exerted by bees negatively affect the chance to diagnose the pathology, and may contribute to the underestimation of aspergillosis importance in beekeeping. On the other hand, bee immune responses may be affected by biotic and abiotic stresses and suffer from the loose co-evolutionary relationships with *Aspergillus* pathogenic strains. However, if not pathogenic, these hive mycobiota components can prove to be beneficial to bees, by affecting the interaction with other pathogens and parasites and by detoxifying xenobiotics. The pathogenic aptitude of *Aspergillus* spp. likely derives from the combined action of toxins and hydrolytic enzymes, whose effects on bees have been largely overlooked until recently. Variation in the production of these virulence factors has been observed among strains, even belonging to the same species. Toxigenic and non-toxigenic strains/species may co-exist in a homeostatic equilibrium which is susceptible to be perturbed by several external factors, leading to mutualistic/antagonistic switch in the relationships between *Aspergillus* and bees.

KEYWORDS

Aspergillaceae, saprophytic fungi, fungal entomopathogens, pollinators mycobiota, mycotoxins, bee immunity

Introduction

Pollination by insects is one of the most important mechanisms involved in maintenance and promotion of biodiversity on Earth and has a direct effect on agricultural activities, contributing to about one third of the global crop production (Klein et al., 2007). Wild bee species, central to the agro-ecosystem service of pollination (Garibaldi et al., 2013), have been declining in many parts of the world (Goulson et al., 2015), attracting the attention of the public opinion, which stimulated government policies aimed at protecting these species (Laursen, 2015).

Due to the great variety of the visited floral species, honey bees (*Apis mellifera* L.) are at the center of several pollination networks and represent the most widespread pollinating species. Although honey bee world population has increased in recent decades, along with beekeeping activities (Geldmann and González-Varo, 2018), a high proportion of colony losses has been reported at a local scale by several monitoring programs (Kulhanek et al.,

2017). Besides climatic and anthropogenic factors, colony losses have been related to the incidence of biotic adversities caused by parasites and pathogens, including protozoans, viruses, bacteria and fungi (Genersch et al., 2010; Schwarz et al., 2015; Flores et al., 2021; Ribani et al., 2021; Lannutti et al., 2022; Schäfer et al., 2022). Among the latter pathogens, the most relevant are represented by microsporidia (*Nosema* spp.; Grupe and Quandt, 2020) and *Ascosphaera apis* (Eurotiomycetes, Ascosphaeraceae), the causal agent of chalkbrood (Aronstein and Murray, 2010). Interactions between bees and other fungi, particularly species in the genus *Aspergillus* (Eurotiomycetes, Aspergillaceae), are less definite, and range from mutualistic to parasitic.

Aspergillus spp. are ubiquitous in terrestrial habitats due to their ability to disperse globally with air currents and to grow on many different substrates. These fungi are commonly isolated from soil, particularly from plant litter. Indeed, many species of *Aspergillus* can abundantly grow as saprophytes on decaying vegetation and are adapted for the degradation of complex plant polymers (Bennett, 2010). Thus, the association of several *Aspergillus* species with bees and bee products, particularly with pollen, is not surprising. Pollen represents an entry for fungal pathogens in the hive. Indeed, spores of *Aspergillus* spp. may contaminate pollen on plants (Sainger et al., 1978; Gilliam et al., 1989); once collected, stored and consumed by bees, these spores reach the gut, which is the primary site of infection for bee pathogens (Foley et al., 2014). According to the prevalent point of view, several *Aspergillus* spp. are considered as opportunistic pathogens which may infect bees in all developmental stages (Foley et al., 2012). Intriguingly, infection with *Aspergillus* species can provoke symptoms very similar to the colony collapse disorder firstly described in 2006, with no or very few adult bees remaining in the hive (Burnside, 1930; Hamzelou, 2007; Leska et al., 2021). Nevertheless, *Aspergillus flavus*, the causal agent of stonebrood, is considered of minor importance and is poorly studied in the framework of the honey bee pathosphere (Foley et al., 2014; Schwarz et al., 2015).

On the other hand, *Aspergillus* spp. are considered one of the most relevant threats of fungal origin to human and animal health (Seyedmousavi et al., 2015). Indeed, some *Aspergillus* species are mycotoxigenic and represent a sanitary risk related to contamination in the feed and food production chains (Ráduly et al., 2019). Moreover, they are zoonotic pathogens that can cause aspergillosis in humans, with symptoms ranging from allergic reactions to true infections of the respiratory system, primarily in immune-compromised patients or those already suffering from other lung diseases (Dagenais and Keller, 2009). Considering the known effects on human health of species such as *A. fumigatus* and *A. flavus* (de Graaf et al., 2008), an accurate knowledge of the association between honey bees and *Aspergillus* spp. is also relevant for the safety of beekeepers.

The present work is aimed at reviewing the currently available literature concerning the interactions between *Aspergillus* species and both wild and managed bees, focusing on pathogenic and mutualistic interactions.

Species of *Aspergillus* reported in association with bees and bee products

The genus *Aspergillus* consists of six subgenera and 18 sections, which accommodate over 250 species (Gams et al., 1986; Tsang et al., 2018). Microscopic examination of conidial structures and macroscopic characteristics of the colony (texture, growth rate, degree of sporulation, conidial and mycelial colors) can be used for species differentiation. However, DNA sequencing and phylogenetic analysis of *calmodulin* and *β-tubulin* loci have become the gold standard for accurate identification at the species level (Tsang et al., 2018; Houbraken et al., 2020). Recent studies reported that subtle phenotypic variation between cryptic *Aspergillus* spp. can have strong implications with their pathogenicity toward bees, highlighting the importance of an accurate identification of the isolates (Foley et al., 2014).

Based on data available in literature and GenBank, so far at least 30 different species of *Aspergillus* have been isolated from bees (Table 1), mostly belonging to the sections *Flavi, Fumigati* and *Nigri*. In particular, honey bees resulted associated with 25 identified *Aspergillus* species, while wild bees resulted associated with 14 *Aspergillus* spp. However, one should consider that many studies cited in Table 1 were conducted before the more recent description of new species, and before the spread of DNA sequencing and other accurate identification methods, such as those based on specific antibodies (Schubert et al., 2018). Thus, species such as *A. nomius*, which was described in 1987 and is phenotypically similar to *A. flavus* (Kurtzman et al., 1987), are probably underrepresented. In the cited studies, most of which were conducted in North and South America, the sources of isolation were highly diverse, with a prevalence of mummified and diseased brood, dead and living adults (Table 1). Notably, in the cited studies external sterilization of the samples has been rarely carried out, making it impossible to establish if the isolated fungi were developing internally, or were just contaminating the integument.

Other arthropods associated with bees are considered as vehicles of *Aspergillus* species. *Aspergillus niger* and *A. flavus* have been found on the surface of females of *Varroa destructor* (Parasitiformes, Varroidae), indicating that this parasitic mite can be a vector for their spread in hives (Benoit et al., 2004). Whether or not *V. destructor* itself can be damaged by these fungi requires further assessments, considering that reproduction of another parasitic mite, *Imparipes apicola* (Acariformes, Scutacaridae), has been reported to be inhibited by *A. flavus* (Cross and Bohart, 1992). Another potential vector of *A. flavus* is the wax moth *Galleria mellonella* (Lepidoptera, Pyralidae), which is a common beekeeping pest. Indeed, a polyethylene-degrading strain of *A. flavus* has been recently isolated from the gut of *G. mellonella*, revealing a certain degree of plasticity of this fungal species in terms of adaptive capacity to different pH conditions, from the acid gut of bees to the extremely alkaline gut of Lepidoptera (Zhang et al., 2020). Moreover, *A. flavus* and *A. fumigatus* have been isolated from dead adults and living larvae of *Vespula* spp.,

TABLE 1 Occurrence of *Aspergillus* species reported as bee associates.

Aspergillus species	Bee species	Source	Location	Reference
A. alliaceus	*Nomia melanderi*	Brood cells	Michigan, United States	Burnside (1930)
A. amstelodami (= *A. montevidensis*?)	*Apis mellifera*	Gut of adult workers	Arizona, United States	Gilliam and Prest (1972)
A. aureoterreus	*N, melanderi*	Brood	Northwestern United States	Batra et al. (1973)
	A. mellifera	Hive	Michigan, United States	Burnside (1930)
	Nomia triangulifera	Brood	Northwestern United States	Batra et al. (1973)
A. caelatus	Stingless bee	Unknown	Malaysia	GenBank: MW040902
A. calyptratus	*A. mellifera*	Dead adults	Michigan, United States	Burnside (1930)
A. candidus	*A. mellifera*	Dead adults	Michigan, United States	Burnside (1930)
	A. mellifera	Adult gut	Poland	Kaznowski et al. (2005)
A. clavatus	*A. mellifera*	Dead adults	Michigan, United States	Burnside (1930)
A. flavus	*Augochlora pura*	Dead adults	Michigan, United States	Burnside (1930)
	A. mellifera	Diseased brood, comb	Michigan, United States	Burnside (1930)
	A. mellifera	Mummified larvae	Northwestern United States	Batra et al. (1973)
	N. melanderi	Diseased prepupae	Northwestern United States	Batra et al. (1973)
	Anthophora pacifica	Diseased brood	Northwestern United States	Batra et al. (1973)
	Anthophora occidentalis	Diseased brood	Northwestern United States	Batra et al. (1973)
	N. triangulifera	Diseased brood	Northwestern United States	Batra et al. (1973)
	Lasioglossum zeiphyrum	Diseased brood	Northwestern United States	Batra et al. (1973)
	Megachile rotundata	Diseased adults; crop; excreta	Northwestern United States	Batra et al. (1973)
	A. mellifera	Gut of adult workers	Arizona, United States	Gilliam et al. (1974)
	Apis florea	Mummified brood	Iran	Alizadeh and Mossadegh (1994)
	A. mellifera	Mummified brood; healthy larvae and adults	Egypt	Shoreit and Bagy (1995)
	A. mellifera	Gut of adult workers	Slovakia	Kačániová et al. (2012)
	A. mellifera	Homogenized larvae; adult gut	England	Foley et al. (2014)
	A. mellifera adansonii	Adult gut and integument	Nigeria	Ayo Fasasi (2018)
	A. mellifera	Adult hemolymph	Italy	DellaGreca et al. (2019)
	A. mellifera	Homogenized larvae and adults	Turkey	Bayrakal et al. (2020)
*A. fresenii**	*N. melanderi*	Diseased larvae and prepupae	Nortwestern United States	Batra et al. (1973)
	A. mellifera	Hive	Michigan, United States	Burnside (1930)
A. fumigatus	*N. melanderi*	Diseased prepupae	Northwestern United States	Batra et al. (1973)
	A. mellifera	Diseased adults and brood	Michigan, United States	Burnside (1930)
	M. rotundata	Excreta of chalkbrood-infected larvae	Alberta, Canada	Inglis et al. (1993)
	A. florea	Mummified brood	Iran	Alizadeh and Mossadegh (1994)
	A. mellifera	Mummified brood; healty larvae and adults	Egypt	Shoreit and Bagy (1995)
	A. mellifera	Homogenized larvae; adult gut; hive airborne	England	Evison et al. (2013), Foley et al. (2014)
	A. mellifera adansonii	Adult gut and integument	Nigeria	Ayo Fasasi (2018)
	A. mellifera	Homogenized larvae and adults	Turkey	Bayrakal et al. (2020)
A. glaucus	*A. mellifera*	Mummified adults and larvae	Michigan, United States	Burnside (1930)
	M. rotundata	Larval cadavers	Saskatchewan, Canada	Goerzen (1991)
A. nidulans	*A. mellifera*	Diseased adult and brood	Michigan, United States	Burnside (1930)

(Continued)

TABLE 1 Continued

Aspergillus species	Bee species	Source	Location	Reference
A. niger	*A. mellifera*	Mummified adults and larvae	Michigan, United States	Burnside (1930)
	A. mellifera	Queen larva; Gut of adult workers	Arizona, United States	Gilliam and Prest (1972), Gilliam et al. (1974)
	Anthophora abrupta	Brood	Maryland, United States	Norden and Scarbrough (1982)
	M. rotundata	Living adults; larval cadavers; spoiled cell; larval excreta	Saskatchewan, Canada	Goerzen (1991)
	A. florea	Mummified brood	Iran	Alizadeh and Mossadegh (1994)
	A. mellifera	Mummified brood; healty larvae and adults	Egypt	Shoreit and Bagy (1995)
	Melipona subnitida	Dead adults	Brazil	Morais et al. (2013)
	A. mellifera	Homogenized larvae; adult gut	England	Foley et al. (2014)
	A. mellifera adansonii	Integument; gut	Nigeria	Ayo Fasasi (2018)
	A. mellifera	Gut	Italy	Callegari et al. (2021)
	A. florea	Gut	Saudi Arabia	Callegari et al. (2021)
	A. mellifera jemenitica	Gut	Saudi Arabia	Callegari et al. (2021)
A. nomius	*A. mellifera*	Adult gut	England	Foley et al. (2014)
	Bombus transversalis	Floral visiting adults (abdomen)	Amazonas, Brazil	Massi et al. (2015)
	Centris denudans	Floral visiting adults (abdomen)	Amazonas, Brazil	Massi et al. (2015)
	Centris ferruginea	Floral visiting adults (abdomen)	Amazonas, Brazil	Massi et al. (2015)
	Epicharis flava	Floral visiting adults (abdomen)	Amazonas, Brazil	Massi et al. (2015)
	Xylocopa frontalis	Floral visiting adults (abdomen)	Amazonas, Brazil	Massi et al. (2015)
A. ochraceopetaliformis	Bees	Unknown	Egypt	GenBank: MN966663
A. ochraceus	*A. mellifera*	Diseased adults and brood	Michigan, United States	Burnside (1930)
	A. mellifera	Mummified brood; healty larvae and adults	Egypt	Shoreit and Bagy (1995)
	A. mellifera	Hive airborne	England	Foley et al. (2014)
	A. mellifera	Adult midgut	Maryland, United States	GenBank: MT472089
A. oryzae	*A. mellifera*	Mummified brood	Egypt	Shoreit and Bagy (1995)
	A. mellifera	Homogenized larvae; hive airborne	England	Foley et al. (2014)
A. parasiticus	*N. melanderi*	Diseased pupae and prepupae	Northwestern United States	Batra et al. (1973)
	A. mellifera adansonii	Adult gut and integument	Nigeria	Ayo Fasasi (2018)
A. phoenicis	*A. mellifera*	Adult gut	England	Foley et al. (2014)
A. proliferans	*A. mellifera*	Larva	South Africa	GenBank: MK451496
A. rugulosus	*A. mellifera*	Larval faeces	Arizona, United States	Gilliam and Prest (1987)
A. sclerotiorum	*A. mellifera*	Adult gut	England	Foley et al. (2014)
Aspergillus sp.	*A. mellifera*	Adult gut	Poland	Kaznowski et al. (2005)
	Bombus griseocollis	Adults (abdomen)	Ontario, Canada	Macfarlane (1976)
	M. subnitida	Dead adults	Brazil	Morais et al. (2013)
	Nomia oxybeloides	Cell wall and faeces	India	Batra (1966)
	Osmia cornifrons	Adults (abdomen)	New York, United States	Hedtke et al. (2015)
	Osmia lignaria	Whole foragers	California, United States	Cohen et al. (2020)
	A. mellifera	Homogenized foragers	China	Ye et al. (2021)
	A. mellifera	Faeces	Australia	GenBank: MK402099
A. subversicolor	Bee	Unknown	South Korea	GenBank: MZ687463
A. sydowii	*A. mellifera*	Dead adults; combs	Michigan, United States	Burnside (1930)
	A. mellifera	Gut of adult workers	Arizona, United States	Gilliam et al. (1974)
	M. rotundata	Larval excreta	Alberta, Canada	Inglis et al. (1993)
A. tamarii	*N. melanderi*	Faeces; all stages	Northwestern United States	Batra and Bohart (1969)

(Continued)

TABLE 1 Continued

Aspergillus species	Bee species	Source	Location	Reference
A. terreus	*A. mellifera*	Gut	Arizona, United States	Gilliam et al. (1974)
	M. rotundata	Larval excreta	Alberta, Canada	Inglis et al. (1993)
	M. subnitida	Dead adults	Brazil	Morais et al. (2013)
	A. mellifera	Gut	Italy	Callegari et al. (2021)
	A. cerana indica	Unknown	India	GenBank: KY800395
A. tubingensis	*A. mellifera*	Adult gut	England	Foley et al. (2014)
	A. mellifera	Chalkbrood mummies	China	Cheng et al. (2022)
A. unguis	*A. florea*	Gut	Saudi Arabia	Callegari et al. (2021)
A. ustus	*N. melanderi*	Cell content	Northwestern United States	Stephen (1959)
A. versicolor	*A. mellifera*	Bees; hive	Michigan, United States	Burnside (1930)
	M. rotundata	Pre-defecation larvae	Alberta, Canada	Inglis et al. (1993)
	A. mellifera	Homogenized larvae	England	Foley et al. (2014)

*This species originally reported as *A. sulphureus*.

which are well known predators of bees (Glare et al., 1996; Quinn et al., 2018; Loope et al., 2019), suggesting a likely mechanism of fungal spores transmission from bees to wasps.

The close association of *Aspergillus* spp. with honey bees is confirmed in the analysis of bee provisions, such as pollen (Gilliam et al., 1989). Concerning species, *A. flavus, A. fumigatus, A. niger, A. terreus* and *A. versicolor* were isolated from corn pollen, *A. niger* again from almond pollen (Gilliam et al., 1989), while *A. flavus, A. luchuensis* (= *A. niger*), *A. nidulans, A. sulphureus* (= *A. fresenii*) and *A. versicolor* were isolated in pollen collected from three herbaceous annual plants (Sainger et al., 1978). By manipulating and storing pollen inside the hive, bees alter its mycoflora composition, which is likely the result of microbial inoculations by bees and chemical changes resulting from additions of honey sac contents and secretions of glands, as well as microbial fermentation, which allow some fungal species, but not others, to survive (Gilliam et al., 1989). As a whole, *Aspergillus* spp. have a higher incidence in analysis concerning bee bread when compared to corbicular pollen in both *A. mellifera* and *A. cerana* (Table 2), which may indicate that contamination of bee bread is internal to the hive rather than deriving from the pollen sources (Gilliam et al., 1989; Disayathanoowat et al., 2020). When commercialized, pollen may still contain *A. flavus* spores as reported by several studies (González et al., 2005; Bucio Villalobos et al., 2010; Deveza et al., 2015), highlighting the potential risk for human health in bee pollen consumption due to the high contamination level by these moulds and their mycotoxins. As a matter of fact, it has been demonstrated that bee pollen is a substrate stimulating production of ochratoxin A by *A. ochraceus* (Medina et al., 2004); this mycotoxin is highly cytotoxic and is reported for insecticidal effects (Boguś et al., 2021). A few studies showed contamination of honey. In Northern Italy, *A. flavus* and *A. japonicus* have been identified in a shotgun sequencing of DNA contained in honey (Bovo et al., 2020). In Turkey, *A. flavus* and *A. fumigatus* were, respectively, found in 4.4 and 6.4% of the honey samples examined (Dümen et al., 2013). The species *A. flavus, A. niger, A. fumigatus, A. candidus, A. terreus, A. versicolor, A. ochraceus* were recovered in an investigation carried out on 50 honey samples in Slovakia (Kačániová et al., 2009), while the first two were found in honey samples analysed in Brazil (Pires et al., 2015). In the latter country, *A. flavus* was also reported to occur in honey of the stingless bee *Melipona scutellaris* (Gois et al., 2010).

Pathogenic interactions

Pathogenic interactions in honey bees

In 1896 a new disease was described in Texas which was called 'pickled brood' or 'white fungus', caused by an approximately described *Aspergillus pollini*, which was found to occur on both larvae and adults of *A. mellifera* (Burnside, 1930). In Europe, the incidence of *A. flavus* as the causal agent of stonebrood was already known at the beginning of the 20th century (Burnside, 1930). Recent findings indicate that different species of *Aspergillus* can be pathogenic to bees (Foley et al., 2014). A comprehensive study carried out in England showed that, out of 10 species recovered in apiaries (Table 1), three species (*A. flavus*, *A nomius* and *A. phoenicis*) resulted pathogenic to honey bee larvae; as tested for pathogenicity towards adult bees, following diet administration, *A. flavus* proved to be pathogenic at all the tested doses, while *A. niger* and *A. fumigatus* were not infectious (Foley et al., 2014).

Aspergillus spp. are known to cause stonebrood, turning honey bee broods into hard mummies. As discussed above, one of the main sources of *Aspergillus* in the hive is probably pollen, as nectar is not thought to significantly harbor fungal conidia (González et al., 2005). Conidia present in the air may also colonize hive substrates saprophytically and be transmitted via physical contact or food sharing by adult bees (Foley et al., 2014). In these ways conidia may in turn be fed to larvae in the cells, where infection through the alimentary tract occurs. Spores germinate in the gut

TABLE 2 *Aspergillus* species reported from pollen collected by honey bees.

Aspergillus species	Location	Reference
A. amstelodami (= *A. montevidensis*?)	Arizona, United States	Gilliam et al. (1989)
A. carbonarius	Argentina	Patiño et al. (2005)
	Brazil	Deveza et al. (2015)
	Spain	Patiño et al. (2005)
A. flavus	Arizona, United States	Gilliam et al. (1989)
	Egypt	Shoreit and Bagy (1995)
	Argentina	Patiño et al. (2005)
	Slovakia	Kačániová et al. (2009)
	Mexico	Bucio Villalobos et al. (2010)
	Brazil	Deveza et al. (2015)
	Taiwan	Hsu et al. (2021)
A. fumigatus	Egypt	Shoreit and Bagy (1995)
	Spain	Patiño et al. (2005)
	Slovakia	Kačániová et al. (2009)
	Brazil	Deveza et al. (2015)
A. japonicus	Egypt	Shoreit and Bagy (1995)
A. niger	Arizona, United States	Gilliam et al. (1989)
	Egypt	Shoreit and Bagy (1995)
	Argentina	Patiño et al. (2005)
	Slovakia	Kačániová et al. (2009)
	Brazil	Deveza et al. (2015)
	Spain	Patiño et al. (2005)
	Georgia	Janashia et al. (2018)
	France	GenBank: KY886458
A. ochraceus	Argentina	Patiño et al. (2005)
	Slovakia	Kačániová et al. (2009)
	Brazil	Deveza et al. (2015)
	Spain	Patiño et al. (2005)
A. oryzae	Brazil	Deveza et al. (2015)
A. parasiticus	Argentina	Patiño et al. (2005)
	Spain	Patiño et al. (2005)
A. terreus	Slovakia	Kačániová et al. (2009)
	Brazil	Deveza et al. (2015)
A. tubingensis	Argentina	Patiño et al. (2005)
	Spain	Patiño et al. (2005)
A. ustus	Arizona, United States	Gilliam et al. (1989)
A. versicolor	Slovakia	Kačániová et al. (2009)
	Brazil	Deveza et al. (2015)
Aspergillus sp.	Slovakia	Kačániová et al. (2009)
	Mexico	Bucio Villalobos et al. (2010)
	Thailand	Sinpoo et al. (2017)
	Germany	Friedle et al. (2021)

leading to an invasive mycosis and host death (Burnside, 1930). Although this is likely the primary entry point, other infection routes may occur. Indeed, external infection of larvae by cuticle penetration is possible but rare (Burnside, 1930). The ectoparasitic mite *V. destructor* may also potentially act as vector of *Aspergillus* spp., facilitating the infection through the opening of feeding wounds on the bee integument (Benoit et al., 2004). When infection occurs through wounds on the cuticle rather than through ingestion, physical barriers are by-passed and usually death is more rapid (Burnside, 1930; Leger et al., 2000). Symptoms of aspergillosis in the brood were accurately described in one of the first published studies about fungal diseases of bees (Burnside, 1930). After few hours from infection, the larvae show increased firmness and dryness. Then, a collar of hyphae emerges from the sutures around the head, and a white mycelium covers the integument. Before mummy formation, a colored sporulation usually occurs, starting from the posterior part of the abdominal tergites (Burnside, 1930).

Aspergillus spp. can also infect adult bees, although in this case germination of spores within the alimentary tract is the only way of infection to be considered. Indeed, the experimental application of spores and germinated conidia on the body surface of adult bees did not lead to mycosis (Burnside, 1930). The first sign of infection by *Aspergillus* in adults is restlessness, followed by weakness and paralysis. In artificially infected colonies, bees start crawling and try to leave the hive by flying, usually dying at a considerable distance (Burnside, 1930). This can be interpreted as a self-isolation behavior aimed at limiting disease transmission within the colony (Stockmaier et al., 2021).

Aspergillosis is not limited to *Apis mellifera*, but can also affect other *Apis* species. Indeed, *Aspergillus* spp. were frequently found in association with drone broods of *Apis florea* and may represent a potential threat to this bee. Out of 600 mummies sectioned and examined microscopically, 252 (42%) were infected with *A. flavus*, 138 (23%) with *A. niger*, 72 (12%) with *A. fumigatus* and 102 (17%) with either two or three of these species (Alizadeh and Mossadegh, 1994).

Pathogenic interactions in wild bees

Aspergillus spp. infections may impact wild bees too. However, only a handful of mycological studies focusing on wild bees have been carried out so far. In their seminal work Batra et al. (1973) investigated fungal occurrence in the alkali bee (*Nomia melanderi*). *Aspergillus flavus, A. tamarii,* and *A. sulphureus* were isolated from all samples in all sampling periods; the first two species, in that order, were also the most abundant species in 16 of the 20 sites investigated, and they caused the heaviest damage to bees (Batra et al., 1973). *Aspergillus alliaceus* was another species occurring at some extent. *Aspergillus flavus, A. tamarii, A. aureoterreus* attacked larvae and prepupae and killed 15.68% of alkali bee population, with *A. flavus* being the most common. However, the presence of *Aspergillus* spp. in alkali bee nest cells does not necessarily result in an invasion of the larvae. Healthy prepupae are frequently found to be completely surrounded by mycelium growing from the faecal material (Batra et al., 1973). Interestingly, when living prepupae and pre-defecating mature larvae of alkali bees were plated with *A. flavus* for 24 h, spore germination was inhibited over a 1.5–3 cm zone surrounding each larva (Batra et al., 1973). The interesting practice of sealing cells containing infected broods with compact soil has also been

reported in this species, emphasizing the protective role of cleaning practices in bee colonies (Batra and Bohart, 1969).

Also Megachilidae seem to be affected by *Aspergillus*, as attested by the occurrence of *A. glaucus* and *A. niger* in larval cadavers of *Megachile rotundata* (Goerzen, 1991). Moreover, a recent work reported the occurrence of *Aspergillus* sp. in adult workers of *Osmia lignaria* (Cohen et al., 2020; Table 1).

Mycotoxins and other virulence factors

Besides causing a direct damage and depleting important nutrients, fungal entomopathogens may be lethal to insects also by producing toxic secondary metabolites. In *Aspergillus*, the production of these compounds is largely influenced by the substrate and growth conditions (Vega and Kaya, 2012; Salvatore et al., 2018; Frisvad et al., 2019). Several *Aspergillus* metabolites revealed antiinsectan effects, resulting in competitive biocenotic interactions. Uka and colleagues (2020) delineated different groups of secondary metabolites produced by *A. flavus* with a known antiinsectan effect: polyketides (aflatoxins, aflavarins), polyketide-non ribosomal peptides (leporins), indole-diterpenoids (aflatrem) and other metabolites (kojic acid) (Uka et al., 2020). The effects of these metabolites on bees, which have been largely overlooked, may include growth retardation, reduced pupal and adult size, lower fecundity, loss of fertility, mortality, repellency, and genetic changes, as observed in other insects (Wicklow et al., 1994).

Burnside (1930) reported that a toxin contained in the ether extract from liquid cultures of a strain of *A. flavus* could kill adult bees. Later on, the toxicity of aflatoxin B_1 was evaluated in assays on adult worker bees (Hilldrup and Llewellyn, 1979), revealing a high tolerance towards this compound due to P450-mediated metabolic detoxification (Niu et al., 2011; Johnson et al., 2012). Aflatoxins are major secondary metabolites produced by *Aspergillus* species in the section *Flavi* which are particularly considered for their occurrence as mycotoxins in food products (Cary and Ehrlich, 2006). A wide variation has been observed in the production of aflatoxins and other secondary metabolites in *A. flavus*; at least in part, this plasticity could be influenced by the horizontal transfer of gene clusters encoding biosynthesis of secondary metabolites, a phenomenon which is likely to generally occur in *Aspergillus* species (Pires et al., 2015, Uka et al., 2020).

Aflavarins and aflatrem have been reported for antifeedant and growth reducing effects, respectively, on diverse insect species (TePaske et al., 1992). Leporins form iron complexes which revealed antifeedant and antiinsectan effects on fall armyworms (*Spodoptera frugiperda*), corn earworms (*Helicoverpa zea*) and the Freeman sap beetle (*Carpophilus freemani*) (Cary et al., 2015). Moreover, some *Aspergillus* spp. produce ochratoxins, at varying extents depending on species, strains and growth substrates (Medina et al., 2004), which may exert antifeedant and lethal effect on insects (Paterson et al., 1990). Another secondary metabolite with a putative role as virulence factor is kojic acid, the dominant product in cultures of strains of *A. flavus* (DellaGreca et al., 2019), which may display a regulatory impact on the immune system of honey bees, by interfering with the melanization response, as described in other insects (Shelby and Popham, 2006).

Furthermore, *Aspergillus* species secrete hydrolytic enzymes, which have a role in pathogenesis and digestion of the host tissues. In particular, pectinases and proteinases are important virulence factors involved in plant and insect host colonization, respectively (Leger et al., 2000). The capacity to infect organisms belonging to different kingdoms is widespread among fungi and is influenced by such diverse repertoire of virulence factors. Although *A. flavus* is considered as a saprophytic species, it has been suggested that it can routinely infect both plants and animals with insects acting as vectors (Leger et al., 2000). By infecting pollinator insects, *Aspergillus* spp. can create a very large inoculum to infect flowers and colonize seeds (Klich and Chmielewski, 1985; Leger et al., 2000). However, this hypothesis of a dispersal mechanism exploiting interkingdom host jumps deserves to be examined more in depth.

In general, the role of virulence factors in pathogenesis of *Aspergillus* spp. has yet to be examined, but it is possible that larval mortalities are in part due to toxicity rather than fungal invasion (Foley et al., 2012). Indeed, the stronger virulence displayed by *A. flavus* in honey bees (Foley et al., 2014) matches well with the greater toxicity towards mosquitos of *A. flavus* toxins, as compared to *A. niger* and *A. parasiticus* (Maurya et al., 2011). Such highly toxic and abundant toxins of *A. flavus*, and the fact that *Aspergillus* spp. are opportunistic pathogens with a loose coevolutionary history with bees, may explain the lack of genotypic variation in differently resistant honey bee populations, which has been pointed out in recent studies (Evison et al., 2013, 2016).

Bee defenses

Opportunistic pathogens, such as *Aspergillus* spp., reveal their pathogenicity only under particular circumstances, especially when host defenses are suppressed or by-passed. Honey bee defenses include physical barriers, immune responses and behavioral responses. Physical barriers against the external invaders are represented by the integument, which covers the bee body, and peritrophic matrix, which protects the midgut (Boucias and Pendland, 2012). Fungal pathogens can adhere to these barriers and penetrate using a mix of physical pressure and lytic enzymes. If these barriers are crossed, honey bees can rely on very efficient cellular and humoral responses (Morfin et al., 2021). Humoral responses include blood clotting and melanization, which are activated, by a proteolytic cascade, when a non-self object is recognized. Fungal cells invading the hemocoel are usually encapsulated and killed by immune cells (hemocytes). Fat body cells synthesize potent antimicrobial peptides which are secreted in the hemolymph, where they act synergistically to kill the remaining microorganisms (Hoffmann, 1995; Ilyasov et al., 2012). This efficient and apparently simple innate immune system is finely regulated through a series of control mechanisms, based on molecular cross-talks and pathways activations (Evans et al., 2006).

However, the main stressors affecting bees, such as the decline in abundance and variety of flowers, the chronic exposure to

agrochemicals and the viruses vectored by parasitic mites, negatively impact the immune response (Goulson et al., 2015; Nazzi and Pennacchio, 2018). Deformed wing virus (DWV) is an endemic pathogen which occurs asymptomatically in nearly all hives and can generate an escalating immunosuppression in the infected bees (Brutscher et al., 2015). Considering that immunosuppressed hosts can be more susceptible to pathogens, it should be interesting to study the effect of the interaction between DWV and *Aspergillus* spp. In one of the few studies reporting viral and fungal pathogens co-occurrence in bees, DWV resulted associated with *Aspergillus* in western yellowjacket wasps (*Vespula pensylvanica*) exposed to honey bees infested by *V. destructor* (Loope et al., 2019), suggesting that immune suppression is beneficial to the opportunistic pathogen, as observed in other co-infection studies. Indeed, in mixed infections on ants *Aspergillus* outcompeted *Metarhizium anisopliae*, which is a virulent entomopathogen able to suppress the host's immune defenses (Hughes and Boomsma, 2004). When defenses are negated, the opportunistic pathogen can supersede the specialized pathogen through a rapid exploitation of host tissues (Boomsma et al., 2014). *Aspergillus*-virus interactions deserve further studies, considering that some honey bee viruses have been recently detected in *A. tubingensis* mycelia and spores, and can be transmitted both horizontally and vertically (Cheng et al., 2022).

Any stress factor competing for metabolic resources may negatively affect immune response and turn an opportunistic pathogen into a deadly invader. The reduction in the availability and diversity of nutritional resources (pollen and nectar) affects immunocompetence (Alaux et al., 2010) and increases susceptibility to *A. flavus*, *A. phoenicis* and *A. fumigatus* (Foley et al., 2012). Honey bee larvae were more susceptible to *A. fumigatus*, when royal jelly is reduced by 20%, highlighting the importance of this component of the larval diet, which contains fundamental nutrients and antimicrobial peptides (Foley et al., 2012; Bílikova et al., 2015).

In response to the selective pressure exerted by the pathogens which affect the hive, honey bees evolved individual and social defenses based on collective actions or on altruistic behaviors performed by infected individuals (Cremer et al., 2007). Bees detect cues of fungal pathogens, avoid direct contact with contaminated individuals, clean the body surface of nestmates by allogrooming, sanitize the nest with antimicrobials and remove dead individuals, reducing the probability of epizootic spread (Cremer et al., 2018). However, behavioral responses, such as the removal of stonebrood infected individuals and the self-isolation of infected bees, which leave the hive by crawling or flying, negatively affect the chance to diagnose the pathology, and may contribute to the underestimation of stonebrood importance (Burnside, 1930; Jensen et al., 2013). Indeed, it is frequently reported that stonebrood has a lower prevalence in the field when compared to chalkbrood, although virulence of *A. flavus* is higher than *A. apis*, with respect to speed of kill and sporulation (Vojvodic et al., 2011; Evison and Jensen, 2018). On the other hand, other *Aspergillus* species, such as *A. tubingensis*, can be considered cryptic pathogens characterized by a low growth rate and can be isolated from chalkbrood mummies (Cheng et al., 2022).

The role of bee-associated microbiota

Besides intrinsic defense abilities, a relevant role in contrasting fungal infections is reported to derive from symbiotic interactions with other microorganisms (Daisley et al., 2020; Khan et al., 2020). A role in this respect has been inferred for lactic acid bacteria (Janashia et al., 2018; Iorizzo et al., 2021), and for miscellaneous bacteria isolated from honey bee gut (Vojvodic et al., 2011; Borges et al., 2021). In particular, *Apilactobacillus kunkeei*, *Sphingomonas paucimobilis* and *Pseudomonas aeruginosa* showed inhibitory activity against *A. niger,* while cell-free supernatant extracted from culturing strains of *Staphylococcus aureus* and *A. kunkeei* produced inhibitory halo zones around colonies of *A. flavus* (Shehabeldine et al., 2021).

Bee broods supplemented with the acetobacterium *Bombella apis* (formerly known as *Parasaccharibacter apium*) were significantly less infected by *A. flavus* (Miller et al., 2021). Additionally, the presence of this symbiont, known to be associated in the gut and the hypopharyngeal glands, reduced sporulation of *A. flavus* in the few bees that were infected (Miller et al., 2021). Analysis of biosynthetic gene clusters across *B. apis* strains provided indications for their capacity to synthesize antifungal compounds, including a type 1 polyketide, a terpene and an aryl-polyene. The secreted metabolites were effective in suppressing fungal growth, supporting the hypothesis that they mediate fungal inhibition (Miller et al., 2021).

Several methods are under consideration in view of improving the capacity by honey bees to contrast these noxious biotic agents, including the administration of probiotics based on microbial consortia (Borges et al., 2021), or single strains of bacteria and fungi, such as *Aureobasidium melanogenum* (Hsu et al., 2021). Although beneficial fungi may be transient passengers and less important than bacteria as gut symbionts (Decker et al., 2022), they can inhibit growth of other species (Gilliam et al., 1988) and mediate detoxification (Bush et al., 2018), thus enhancing a general honey bee resistance towards pathogens (Yoder et al., 2017).

Inhibitory effects of bee products

Behaviors that increase sanitation of the nest (Wilson-Rich et al., 2009) include the use of propolis as an antimicrobial against hive pathogens (Bastos et al., 2008). Propolis is a mixture of resinous substances collected from various plants, partially digested by β-glycosidase enzyme of their saliva and added to bee wax to form the final product (Silva et al., 2012). Analysis of propolis of the Australian stingless bee *Tetragonula carbonaria* showed the presence of myrtucommulone and other identified and unidentified alkylated phloroglucinols known for their antibacterial properties (Massaro et al., 2015; Nicoletti et al., 2018). The presence of propolis in all hives acts as a chemical barrier against the establishment of harmful fungi, resulting in the downregulation of immune gene expression, which emphasizes the role of this bee product in disease resistance (Simone et al., 2009).

Propolis and its ethanolic extract (EPE) have been found to inhibit *in vitro* growth and mycotoxin production in *A. flavus*

(Ghaly et al., 1998), *A. fumigatus* (Kačániová et al., 2012), *A. parasiticus* (Hashem et al., 2012), and *A. sulphureus* (Pepeljnjak et al., 1982). A more recent study showed that EPE is also able to decrease the expression of genes involved in the aflatoxin biosynthetic pathway (Hosseini et al., 2020). Notably, propolis methanolic extract was shown to promote detoxification of aflatoxin B_1, as mediated by cytochrome P450 (Niu et al., 2011). Furthermore, dimethylsulfoxide extract of propolis inhibited *A. fumigatus in vitro* (Netíková et al., 2013). In other studies, several organic extracts of propolis proved to be ineffective or have limited efficacy against *A. fumigatus, A. flavus* and *A. niger* (Garedew et al., 2004a; Agüero et al., 2010, 2011, 2014; Kačániová et al., 2012; Falcão et al., 2014). Some extent of inhibition against the same species was also observed to be induced by bee pollen (and beeswax) ethanolic extracts (Kačániová et al., 2012), and by honey against *A. fumigatus, A. flavus, A. parasiticus* and *A. niger* (Wellford et al., 1978; Radwan et al., 1984; Efem and Iwara, 1992; Boukraâ et al., 2008; Tenore et al., 2012; Fahim et al., 2014; Samad et al., 2016). The latter species also proved to be sensitive to honey produced by stingless bees of the genus *Trigona* (Garedew et al., 2004b).

Due to the evident importance of propolis usage, it has been theorized that honey bees may have developed a dependence on the medicinal properties of plant secondary metabolites. Self-medication in honeybees based on the properties of propolis, honey, etc. is still largely unexplored. However, some studies have suggested that honey bee colony declines may depend on the decreased availability of some forage plants with essential medical properties (Tihelka, 2018).

Environmental fungi, commensals or mutualists

Despite the association with stonebrood, some studies have shown that *Aspergillus* spp., particularly *A. fumigatus, A. flavus* and *A. niger*, equally occur in both diseased and non-diseased colonies (Shoreit and Bagy, 1995). This is not surprising if we consider that the same species includes toxigenic and non-toxigenic strains (Ehrlich, 2014).

Aspergillus species are generally considered to be environmentally adaptable, occasionally interacting with their bee hosts. Indeed, they are stress-resistant saprophytes which enter the hive as conidia (resting spores), basically waiting for conditions that favor germination and spoilage of stored pollen (Friedle et al., 2021), which also involves other microbial partners of bee bread (Gilliam et al., 1989; Goerzen, 1991). *Aspergillus* occurrence in honey bee gut may just be the result of pollen ingestion, although further studies are needed to assess if these species can stably colonize bee gut or are just a transient passengers. As matter of fact, spores of *Aspergillus* spp. can germinate at low pH and high temperatures (above 30°C; Araujo and Rodrigues, 2004), which are typical features of honey bee gut/bee bread and hive, respectively.

However, considering that fungal biomass in bee bread increases with storage time (Gilliam et al., 1989; Friedle et al., 2021), we may hypothesize a nutritional benefit resulting in a better fitness for *Aspergillus* species. In this context, *Aspergillus* spp. obtain food without damaging or benefiting bees, which is indicative of a mere association as commensals.

Although a direct evidence of mutualistic symbiosis is lacking, several hints suggest that *Aspergillus* spp. can be beneficial to bees in multiple ways. In fact, they may play a role in competition with pathogenic and/or mycotoxigenic *Aspergillus* species/strains (Bhandari et al., 2020), or produce inhibitory effects towards bee pathogens and parasites (Vojvodic et al., 2011). Moreover, they might enhance honey bee resistance to xenobiotics through detoxification (Berenbaum and Johnson, 2015), or transform and stabilize pollen and bee bread through the production of enzymes, vitamins, antibacterial substances, organic acids and lipids (Kieliszek et al., 2018).

Considering that aflatoxin occurrence in corn and other crops can be deleterious to humans and animals consuming their products, the spread of atoxigenic strains of *A. flavus* has been considered as a possible means to reduce product contamination based on competition with the wild strains. In this context, a field study carried out in Texas showed that using atoxigenic strains of *A. flavus* to replace toxigenic ones has no detrimental effect on the abundance of honey bees and other Apidae belonging to the genera *Ceratina, Diadasia, Melissodes* and *Svastra* (Bhandari et al., 2020). In the field, *A. flavus* is an assemblage of aflatoxigenic and non-aflatoxigenic strains, which lack the ability to produce G-aflatoxins due to a gap in the gene cluster that includes a required cytochrome P450-encoding gene (*cypA*; Ehrlich, 2014). Such equilibrium can be altered by extrinsic factors, such as climate change and fungicide exposure, and intrinsic factors, like genetic recombination derived from sexual reproduction between strains (Ehrlich, 2014). Clearly, these factors have an impact on any biocontrol strategy based on the release of atoxigenic strains (Ehrlich, 2014). Another key issue in such strategies is the absence of a simple method to discriminate between aflatoxigenic and non-aflatoxigenic *Aspergillus* strains (European Food Safety Authority et al., 2022), highlighting the importance of developing sensitive, fast and affordable molecular tools.

The presence of *Aspergillus* spp. in the gut of honey bees seems to be positively correlated with their health status concerning key diseases, such as chalkbrood and American foulbrood, suggesting that lower levels of these fungi may represent a condition of dysbiosis (Gilliam et al., 1988; Ye et al., 2021). Besides representing effective competitors of the chalkbrood fungus *A. apis* (Gilliam et al., 1988; Vojvodic et al., 2011), some *Aspergillus* spp., *A. fumigatus* in particular, can produce antibiotics such as fumagillin, which is used as an effective product against microsporidian pathogens (Bailey, 1953; Guruceaga et al., 2019; Steenwyk et al., 2020; Glavinic et al., 2021). The mutualistic hypothesis is also supported by studies on other insects. As an example, *A. flavus* is helpful to the navel orangeworm (*Amyelois transitella*; Lepidoptera, Pyralidae) in the detoxification of

xenobiotics (phytochemicals) added to the artificial diet of larvae reared in the laboratory (Bush et al., 2018).

Considering that *Aspergillus* spp. are mycotoxin producers, toxin extraction, identification and investigation on non-targeted organisms should be performed before their use in biological control. Besides the direct use of fumagillin implying possible effectiveness of natural spread of *A. fumigatus*, so far few studies have directly explored *Aspergillus* spp. as biopesticides against beekeeping pests. *Aspergillus niger* and *A. flavus* have been evaluated as potential biocontrol agents of the small hive beetle (*Aethina tumida*; Coleoptera, Nitidulidae) with limited evidence of efficacy (Sammataro and Yoder, 2011). In the wax moth *G. mellonella*, *A. fumigatus* causes immunosuppression through the production of fumagillin and gliotoxin, which play a critical role in enhancing virulence (Reeves et al., 2004; Fallon et al., 2011).

Fermentation by microorganisms converts stored pollen into bee bread that is fed to honeybee larvae. Although the role of non-aflatoxigenic *Aspergillus* spp. in preserving or enhancing the nutritional value of bee provisions has been poorly investigated, the spread of fungicide use has been suggested as one of the detrimental factors leading to honey bee colony collapse (Yoder et al., 2017). In this context, fungicides negatively affect *Aspergillus* abundance, reducing their beneficial effects. Indeed, bee bread collected from colonies showing chalkbrood symptoms was found to be contaminated by fungicides and contained a reduced number of beneficial fungi, including *Aspergillus* spp. (Yoder et al., 2013). By reducing the abundance of these fungi, fungicides can holistically reduce honey bee immunocompetence and expose the colony to pathogens and parasites.

Conclusion

Despite the rapid accumulation of documented occurrences of *Aspergillus* spp. in association with wild and domesticated bees in the last decades, the symbiotic relationship between the fungi and pollinators is not clearly defined. The occasional spread of stonebrood counteracts recognized antagonistic properties against some key hive pathogens, which support the conclusion that these mycobiome components are constantly associated to bees at all developmental stages, in a homeostatic equilibrium which is susceptible to be perturbed by several external factors (Figure 1). Current literature supports a dynamic range of symbiotic relationships between *Aspergillus* and bees, from mutualism to

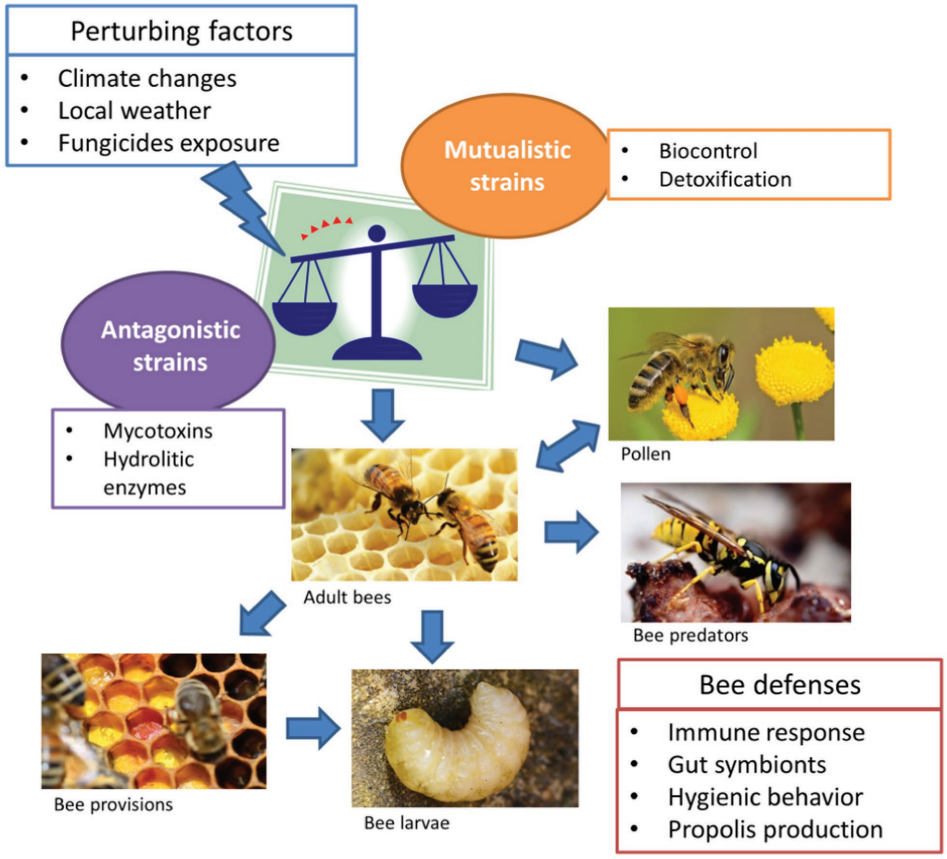

FIGURE 1
Aspergillus spp. infection routes in bees. *Aspergillus* is a species assemblage, including pathogenic strains and atoxigenic strains, which can be perturbed by many extrinsic factors. Such disequilibrium may result in the spread of large inoculums of antagonistic strains, leading to contamination (blue arrows) of pollen, bees and associated species.

antagonism. Antagonistic interactions are basically related to pathogenicity of particular species/strains which are able to produce secondary metabolites acting as virulence factors. Although well documented, stonebrood seems to be underestimated by beekeepers because bees perform hygienic and altruistic behaviors which negatively affect the chance to diagnose the pathology. On the other hand, commensalistic and mutualistic hypotheses have received very little attention. We highlighted diverse beneficial effects of *Aspergillus* presence in the hive: competition with pathogens and parasites, detoxification, stabilization of pollen and bee bread. Regarding the dynamism of such interactions, much of the uncertainty depends on the heterogeneous assemblage of species associated with bees. The recent progresses in techniques for taxonomic identification of fungi have shown that actually this assortment is wider than previously inferred. In fact, common species such as *A. flavus* and *A. niger* have been reconsidered to represent species aggregates including a variable number of taxa, which could perform different ecological roles. Even within a single species, the existence of a variation in the ability to synthesize mycotoxins and other virulence factors might imply different functional relationships with bees, both at individual and colony level.

Author contributions

RN: conceptualization. AB: data curation. AB and RN: writing—original draft preparation and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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The effects of urban land use gradients on wild bee microbiomes

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Bees and their microbes interact in complex networks in which bees form symbiotic relationships with their bacteria and fungi. Microbial composition and abundance affect bee health through nutrition, immunity, and fitness. In ever-expanding urban landscapes, land use development changes bee habitats and floral resource availability, thus altering the sources of microbes that wild bees need to establish their microbiome. Here, we implement metabarcoding of the bacterial 16S and fungal ITS regions to characterize the diversity and composition of the microbiome in 58 small carpenter bees, *Ceratina calcarata,* across urban land use gradients (study area 6,425km^2). By categorizing land use development, green space, precipitation, and temperature variables as indicators of habitat across the city, we found that land use variables can predict microbial diversity. Microbial composition was also found to vary across urban land use gradients, with certain microbes such as *Acinetobacter* and *Apilactobacillus* overrepresented in less urban locations and *Penicillium* more abundant in developed areas. Environmental features may also lead to differences in microbe interactions, as co-occurrences between bacteria and fungi varied across percent land use development, exemplified by the correlation between *Methylobacterium* and *Sphingomonas* being more prevalent in areas of higher urban development. Surrounding landscapes change the microbial landscape in wild bees and alter the relationships they have with their microbiome. As such, urban centres should consider the impact of growing cities on their pollinators' health and protect wild bees from the effects of anthropogenic activities.

KEYWORDS

bacteria, fungi, urbanization, microbial diversity, land use, Apidae, Ceratina

Introduction

Research on bee microbiomes uncovered their vital role in many aspects of bee health, including improving immunity (Engel et al., 2012; Mockler et al., 2018; Rubanov et al., 2019), nutrient utilization (Engel et al., 2012; Raymann and Moran, 2018), and reducing metalloid toxicity (Rothman et al., 2019). For example, the presence of non-pathogenic microbial symbionts in honey bees upregulates antimicrobial peptides in the bee and prepares the immune response against pathogenic microbes (Kwong et al., 2017a). The

importance of microbes can also be implicated more broadly, with the sterilization of larval mason bee microbiomes negatively impacting bee fitness through declining growth rates, biomass, and survivorship (Dharampal et al., 2019). The gut microbiome has even been associated with different behavioural tasks in honey bees and memory retention in bumble bees (Jones et al., 2018; Li et al., 2021). With the common consensus that microbes engage in beneficial interactions with their bee hosts, research continues to examine the factors that influence the establishment and maintenance of the microbiome.

The core microbiome is described as the microbes that are consistently found within many individuals of a species (Turnbaugh and Gordon, 2009; Danko et al., 2021). Social and solitary bees acquire their microbiome in different ways, with honey and bumble bee microbiome composition directly influenced by social interactions with their colony members (Martinson et al., 2012; Tarpy et al., 2015; Kwong and Moran, 2016; Elijah Powell et al., 2018; Su et al., 2021), whereas less social wild bees inherit their microbiome from their surrounding environment and from their diet (McFrederick and Rehan, 2016; Dew et al., 2020; Voulgari-Kokota et al., 2020; Figueroa et al., 2021; Kapheim et al., 2021). Much work has been done on social bees such as *Apis* and *Bombus* to characterize and examine the health effects of an altered core microbiome (Martinson et al., 2011; Raymann and Moran, 2018; Rothman et al., 2019; Su et al., 2021). For solitary wild bees, this research is in its infancy; however, it is known that wild bees do not always maintain the same consistent core microbiome seen in social bees (McFrederick et al., 2012, 2014; Kwong and Moran, 2016; McFrederick and Rehan, 2016; Graystock et al., 2017). An example on how solitary wild bees can have differing core microbiomes can be found in *C. calcarata*, a wild bee displaying different core microbiomes from other bee species and across different regions (Graystock et al., 2017; Dew et al., 2020; Nguyen and Rehan, 2022; Shell and Rehan, 2022).

A range of variables, including developmental stage (McFrederick et al., 2014; Kapheim et al., 2021; Nguyen and Rehan, 2022), sociality of host species (Mohr and Tebbe, 2006; McFrederick et al., 2014; Graystock et al., 2017), climate (McFrederick and Rehan, 2019), geographical location (Almeida et al., 2022), and landscape features (Cohen et al., 2020), have been examined to determine their effects on the microbiome. Examining microbiomes across host developmental stages has allowed for closer examinations of the establishment of microbes, showing that diet is the main source of bacteria and fungi for developing solitary bees (Dew et al., 2020; Pozo et al., 2020; Kapheim et al., 2021; Nguyen and Rehan, 2022). Sociality can influence the bee microbiome by impacting how solitary and social bees interact with food resources, the environment, and other bees to transmit different microbes (Mohr and Tebbe, 2006). Across various bee species, environmental factors such as climate (McFrederick and Rehan, 2019), agriculture (Motta et al., 2018; Muñoz-Colmenero et al., 2020), natural habitat, floral resources, and wild bee diversity in the landscape can all shape microbe composition (Cohen et al., 2020; Shell and Rehan, 2022). In previous studies of *Osmia lignaria,* the presence of some microbes, such as *Apilactobacillus* sp., was associated with increased green spaces and an increased relative rarified ASV abundance in bees from less developed landscapes as opposed to urban and highly developed sites (Cohen et al., 2020). Thus, microbial members within the microbiome are subject to many different factors that can change their abundance, composition, and diversity.

The impact of a changing environment on bees and their microbiome needs to be studied as urbanization and anthropogenic activities continue to alter bee habitats in growing cities (Ritchie and Roser, 2018; Wilson and Jamieson, 2019; Ayers and Rehan, 2021; Prendergast et al., 2022). With more than half of the world currently living in urban areas and projections predicting this number to increase to two thirds of the global population living in a city centre by 2050 (Ritchie and Roser, 2018), the growth of large, developed areas is undeniable. Decreased availability of green space and the urban heat island effect tends to result in increased temperatures and reduced precipitation in these areas (Rinner and Ussain, 2011; Steensen et al., 2022). Urbanization affects the availability of green space, abundance and richness of floral resources, microclimate, and habitat quality for bees, changing the landscape features that can shape bee microbiomes (Goulson et al., 2015; Buchholz et al., 2020; Cohen et al., 2020, 2022; Ayers and Rehan, 2021; Danko et al., 2021). Floral abundances and garden sizes have a direct, positive effect on parasite and pathogen richness that is harmful to bumble bees, attributable to increased transmission from more resource provisioning (Cohen et al., 2022). Wild bumble bees have also been shown to harbour pesticides in both agricultural and urban landscapes (Botías et al., 2017), potentially jeopardizing microbial composition (Kakumanu et al., 2016; Rothman et al., 2020). Characterizing the microbiome of urban bees and how its composition and diversity varies across different landscapes offers an essential step towards understanding contributing factors to changes in bee health.

This study examines the small carpenter bee *C. calcarata* Robertson (Hymenoptera: Apidae). These subsocial bees nest within pithy stems, laying eggs on mass provisions that will provide brood the total nutrition required until they are fully grown (Michener, 2007; Rehan and Richards, 2010). Numerous studies have characterized diversity and composition of the microbiome and pollen provisions in *C. calcarata* (McFrederick and Rehan, 2016; Graystock et al., 2017; Dew et al., 2020; Nguyen and Rehan, 2022). In adult bees this core microbiome consists of 13 bacterial phylotypes, including *Lactobacillus, Acinetobacter, Methylobacterium, Pseudomonas,* and *Gilliamella* (Graystock et al., 2017; Shell and Rehan, 2022), several of which are common in other bee microbiomes as well (Vásquez et al., 2012; Voulgari-Kokota et al., 2019; Kapheim et al., 2021). The *C. calcarata* fungal microbiome includes members such as *Alternaria, Ascosphaera* and *Penicillium* (Nguyen and Rehan, 2022). However, despite various characterizations of this small carpenter bee bacterial and fungal microbiome, closer investigations into the specific factors

driving differences in microbial composition and diversity, as well as the functional role of different microbial taxa on maintaining bee health, are fundamental.

The aim of this study is to determine whether the microbiome of adult *C. calcarata* differs across an urbanization gradient including local environmental features: percent land use development, percent green space, temperature, and precipitation. Using 16S and ITS metabarcoding, we examined the respective bacterial and fungal composition and diversity within 58 female small carpenter bees collected across a densely urban landscape, with different levels of urbanization. Here, we hypothesize that bees living under different environmental conditions across an urban land use gradient will result in varying microbial composition. We predict lower microbial diversity and the underrepresentation of beneficial microbes in more urban and developed areas with less available green space, increased temperatures, and reduced precipitation. This research aims to understand the differences in the microbiomes of wild bees living under different levels of urbanization.

Materials and methods

In June–July 2019 and 2020, 58 female individuals of *C. calcarata* Robertson (Hymenoptera: Apidae) were collected across 29 sites within Toronto, Canada (43.6532° N, 79.3832° W) (Figure 1). Between one and three bees were selected from each site and sites were chosen to cover a widespread area across the city. *Ceratina calcarata* is a native small carpenter bee commonly found in urban and rural contexts across eastern North America, including within the city of Toronto (Packer et al., 2007; City of Toronto, 2016; Shell and Rehan, 2016; Dew et al., 2020; Kelemen and Rehan, 2021). Nests established in the pithy stems of sumac, *Rhus typhina,* were opened with the lone adult female being removed from each collected nest, flash frozen in liquid nitrogen, and stored at-80°C until DNA extractions.

The collection map was created using the Sentinel-2 land use/land cover timeseries from 2017 to 2021 by Impact Observatory, Microsoft and Esri at a resolution of 10 m (Karra et al., 2021; Figure 1). Collection sites were characterized into five different levels of urban intensity using measurements of the developmental percent, percent green space, mean annual temperature, and annual precipitation (Supplementary Table S1). These categories were assigned by evenly dividing the range of values for each environmental variable into five categories ranging from 1 (very low) to 5 (very high). Landscape features of developmental percent and percent green space were calculated at each collection site using the Ontario Land Cover Compilation (OLCC) v.2.0 in ArcGIS as a percentage of landscape cover within a 500 m radius from the collection point (Land Information Ontario, 2019). Climate data, including mean annual temperature and annual precipitation, were calculated using the same process with WorldClim v.2.0 data at a resolution of 30 s (Fick and Hijmans, 2017). These features provide an overall characterization of urban land use gradients in the study region and were divided into the five categorical levels for later analyses (Supplementary Table S1).

DNA extractions were performed using the Omega-Biotek E.Z.N.A. Soil DNA kit, following the manufacturer's protocol for 100–250 mg samples, with some modifications as described in Nguyen and Rehan (2022). This included the addition of 100 µg of 1xPBS, 30 µl of proteinase K, 5 µl of RNAse and manual crushing of the bees using a sterile pestle. DNA concentrations were checked using a QuBit HS DNA assay (Invitrogen) prior to submission to the Génome Québec Centre D'Expertise et de Services (Montreal, Canada), who conducted library preparation and sequencing. Illumina MiSeq amplicon sequencing with 300 bp paired-end reads was conducted using the 16S rRNA region for bacteria with the V5-V6 fragment (forward primer 799F-mod3 CMGGATTAGATACCCKGG and reverse primer modified 1115R AGGGTTGCGCTCGTTG) as in McFrederick and Rehan (2019) and the ITS region for fungi with the ITS1 fragment (forward primer ITS1F CTTGGTCATTTAGAGGAAGTAA and reverse primer ITS2 GCTGCGTTCTTCATCGATGC).

Qiime2 was then used to process reads for microbiome analysis (Bolyen et al., 2019). Demultiplexed sequences underwent sequence quality control using the DADA2 pipeline, which filters phiX reads, chimeric sequences, and joins paired ends (Callahan et al., 2016). Sequences were omitted when quality scores dropped below 30 and read lengths fell below 283 bases for forward reads and 260 bases for reverse reads. Qiime2 was also used to generate feature tables, representative sequences, and taxonomy tables (Price et al., 2010; McDonald et al., 2012; Katoh and Standley, 2013; Weiss et al., 2017; Bolyen et al., 2019). ASVs were tested against the SILVA 128 99% OTUs full length sequences classifier for 16S bacterial sequences and the UNITE 99% OTUs classifier for ITS sequences using the q2-feature-classifier and classify-sklearn pipeline (Pedregosa et al., 2011; Yilmaz et al., 2014; Bokulich et al., 2018; Abarenkov et al., 2021). The SILVA database with 99% sequence identity was used for its refinement and removal of duplicate sequences (Glöckner et al., 2017). Taxonomic classifications were then cross referenced against the NCBI nt database using BLAST, where classifications from the NCBI database were used to clarify and prioritized when there were any discrepancies within the two classifiers (Johnson et al., 2008, 2021).

Resultant amplicon sequence variants (ASVs) read counts and taxonomic classification tables for each ASV were imported into R (version 3.6.1) for further statistical analysis (R Core Team, 2019). ASVs of the genera *Wolbachia* and *Sodalis* were removed as they are common intracellular endosymbionts present due to mite contamination (Graystock et al., 2017). While one blank did not contain any reads, ASVs identified in the other two of blanks were reagent or human-sourced contaminants and either absent in all samples or had low read counts of less than 50 reads. Using the "phyloseq" package, reads from three blanks were proportionally removed (McMurdie and Holmes, 2013).

Alpha and beta diversity analyses, measured using the Shannon diversity index and Bray–Curtis dissimilarity respectively, were conducted using the "phyloseq" package

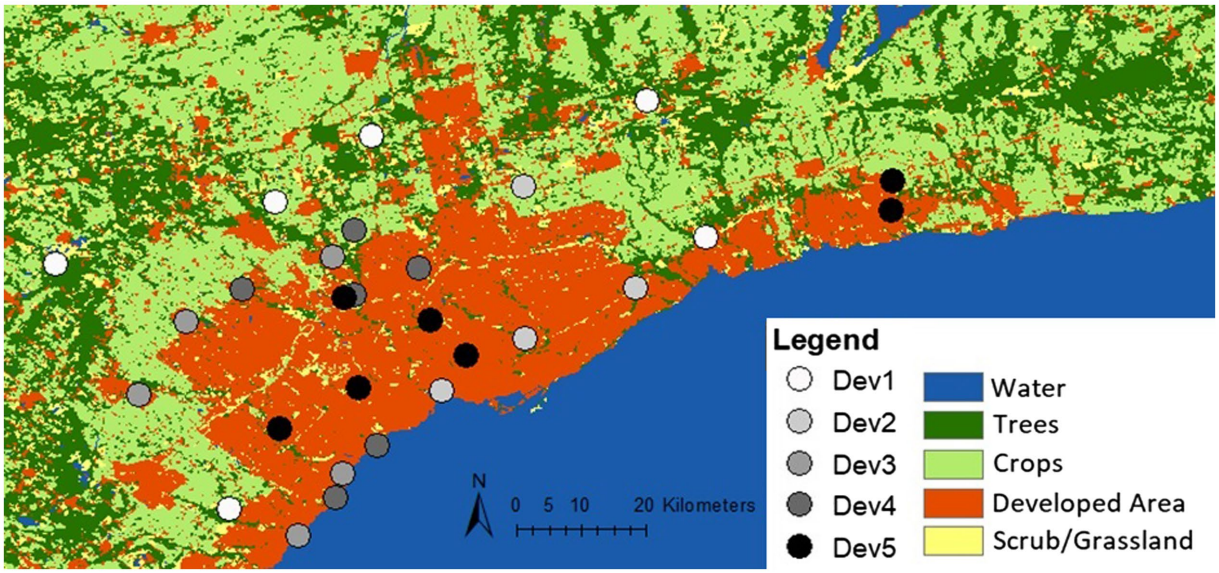

FIGURE 1
Development distribution and collection locations in and around Toronto, Canada using the Sentinel-2, 10m land use time series from 2017 to 2021 by ESRI. Circles represent 29 collection sites. The trees class includes trees and flooded vegetation. Crops include human planted grass and crops below tree height. Scrub/grassland consists of bare ground and rangeland, or open areas with homogenous grasses. Developed areas are built areas with human made structures, roads, and impervious surfaces.

(McMurdie and Holmes, 2013). The adonis function was used to conduct permutational multivariate analyses (PERMANOVA) that test whether microbial composition varies significantly in the different levels of urbanization (Oksanen et al., 2020). Assumptions required for the PERMANOVA test were validated using the betadisper function and significant results followed up with Tukey's HSD test (Oksanen et al., 2020).

Bipartite networks were created using the "bipartite" package in R, examining associations between the top 18 bacterial and fungal taxa and land use development gradients (Dormann et al., 2008, 2009; Dormann, 2011). Statistics were calculated at the species level, examining the degree of connectance, effective number of interacting partners, Shannon diversity of interactions, and closeness centrality in a weighted network across five categories of land use development (Dormann et al., 2008). Redundancy analyses (RDA) were conducted using the rda function from the "vegan" package (Oksanen et al., 2020). Using the decostand function in "vegan," the Hellinger transformation was applied to taxa abundances and the environmental variables were standardized prior to RDA analyses (Legendre and Gallagher, 2001). An ANOVA like permutation test was performed with the anova.cca function to determine the significance of which environmental features could model microbe abundance.

Similarity percentage (SIMPER) values were calculated within the PAST (version 4.07) program to identify taxa predominantly leading to differences in diversity (Hammer et al., 2001). Furthermore, correlation analyses using CoNet and SparCC were conducted to find co-occurring bacterial and fungal taxa amongst all the bees. CoNet was performed using the package "CoNetinR" and edge scores calculated with Spearman, Bray, Pearson, and Kullback–Leibler (Faust and Raes, 2016). The package "SpiecEasi" was used to conduct SparCC analyses with 100 bootstrap replicates (Friedman and Alm, 2012).

Results

Metabarcoding of the 58 adult *C. calcarata* resulted in an average of 31,394 reads, ranging from 19,860 to 43,593 paired-end reads per sample. The average quality of these reads was 34.5. A total of 192 bacterial and 367 fungal amplicon sequence variants (ASVs) with a mean sequence length of 317 bp were found and compared across 58 bee samples.

Diversity

Microbial community composition did not reveal differences across urban land use gradients through alpha diversity or due to sample collection date over the range of 2 years (Supplementary Figure S1). Sample collection year did not associate with any differences in the alpha diversity, beta diversity, or relative abundance of bacterial and fungal taxa. Shannon diversity index comparisons across each environmental variable revealed no overall significant differences in microbial alpha diversity among the five categorical levels of developmental percent, green space percent, temperature, or precipitation (Supplementary Figure S1).

Bray-Curtis dissimilarities revealed bacterial and fungal differences in beta diversity across three environmental variables including land use development, precipitation and temperature (Figure 2; Supplementary Figure S2). Land use development percent was associated with both bacterial and fungal beta diversity (PERMANOVA; bacteria, $R^2 = 0.10$, $df = 4$, $p = 0.017$; fungi, $R^2 = 0.10$, $df = 4$, $p = 0.023$; Figure 2). Figure 2A indicates that individuals from moderate to very high levels of development were similar in microbial composition and dissimilar to individuals from sites with very low to low levels of development. Samples from sites with very high development had more bacterial genera richness than sites with low development (Supplementary Table S2), corroborating that development positively associates with bacterial richness. However, green space percent was not a significant factor in determining differences in Bray-Curtis diversity (PERMANOVA; bacteria, $R^2 = 0.07$, $df = 4$, $p = 0.18$; fungi, $R^2 = 0.09$, $df = 4$, $p = 0.056$; Supplementary Figures S2A,B).

Temperature explained variations in fungal beta diversity (PERMANOVA; fungi, $R^2 = 0.09$, $df = 4$, $p = 0.021$; Supplementary Figure S2D), while bacterial beta diversity did not pass the test for homogeneity of multivariate dispersions with temperature (betadisper; bacteria, $F = 3.13$, $df = 4$, $p = 0.018$; fungi, $F = 0.33$, df = 4, $p = 0.855$; Supplementary Figure S2C). As for precipitation gradients, fungal beta diversity differences were detected, while bacterial beta diversity differences were insignificant (PERMANOVA; bacteria, $R^2 = 0.08$, $df = 4$, $p = 0.181$; fungi, $R^2 = 0.12$, $df = 4$, $p = 0.002$; Supplementary Figures S2E,F). Clear clustering was less evident for the fungal PCoA, suggesting increased dissimilarity between individuals (Figure 2B). One group of fungal samples that were clustered, indicating similar beta diversity, tended to have moderate to high annual temperatures, and low to moderate annual precipitation (Supplementary Figures S2D,F). This was also consistent comparing the average number of genera across the environmental variables, which saw that the low to moderate development, high temperature, and low precipitation categories had the highest fungal genera richness (Supplementary Table S3). However, the individuals grouped closely on the PCoA were spread across different levels of land use development and green space, suggesting the environmental variables are not always correlated with each other. As temperature, green space, and precipitation were not significant environmental variables for bacteria, this comparative analyses between the environmental variables could not be performed for the bacterial PCoA.

An RDA was conducted and analyzed using an ANOVA with 999 permutations on all four environmental variables to determine which variables were associated with bacterial and fungal taxa (Figure 3). Development was significant (ANOVA; bacteria, $F = 1.86$, $df = 1$, $p = 0.032$; Figure 3A) in associations between urbanization level and bacterial taxa. Green space was significantly associated with both bacterial and fungal taxa (ANOVA; bacteria, $F = 1.86$, $df = 1$, $p = 0.037$; fungi, $F = 2.45$, df = 1, $p = 0.002$; Figures 3A,B). Precipitation was also key in the RDA analyses for fungal taxa (ANOVA; fungi, $F = 1.86$, $df = 1$, $p = 0.012$; Figure 3B) with variation in precipitation explaining variation in fungal taxa. In addition to the RDA with all environmental variables, forward selection modelling was performed to select the driving environment variables that could predict diversity. Bacterial taxa revealed a significant model (ANOVA; bacteria, $F = 1.80$, $df = 1$, $p = 0.016$, adjusted R-squared = 0.017) associated with development (ANOVA; bacteria, $F = 1.86$, $df = 1$, $p = 0.036$) and temperature (ANOVA; bacteria, $F = 1.74$, $df = 1$, $p = 0.046$). The fungal model resulted in a different significant model (ANOVA; $F = 1.97$, $df = 1$, $p = 0.007$, adjusted R-squared value of 0.17) involving only temperature (ANOVA, fungi, $F = 1.97$, $df = 1$, $p = 0.005$) predicting fungal taxa.

Taxonomy

Across the 58 samples, the bacterial genera *Acinetobacter, Apilactobacillus*, *Nocardia,* and *Saccharibacter* had the greatest summed relative abundances amongst all the bacterial genera (Supplementary Table S2). Particularly notable, *Apilactobacillus* had a relative abundance of over 50% of the total reads in 25 samples (Figure 4A). A low amount of bacterial diversity is noticed amongst the adults, as 26 samples contained reads from only one genus and 12 samples had two bacterial genera (Figure 4A; Supplementary Table S2). Overall, there was an average of 3.6 bacterial genera associated with each bee. In terms of fungi, *Alternaria, Ascosphaera*, and *Penicillium* had the greatest summed relative abundances and were common genera in the bee microbiome (Supplementary Table S3). Fungal genera richness was higher than bacterial, with an average of 6.5 genera per individual (Figure 4B; Supplementary Table S3).

Similarity percentage (SIMPER) analyses corroborated bacterial and fungal relative abundances were driven by environmental features (Supplementary Table S4). Some bacteria and fungi are typically overrepresented at either high levels of development or green space, suggesting patterns along an urbanization gradient (Supplementary Table S4). For example, *Acinetobacter* and *Saccharibacter* had high abundances in very low levels of development (Supplementary Table S4). On the contrary, *Lactobacillus* bacteria were found mostly in areas with moderate to high levels of development (Supplementary Table S4). *Apilactobacillus* was simultaneously overrepresented in areas with the highest amount of green space, least amount of development, and high levels of development (Supplementary Table S4). In terms of fungi, *Ascosphaera* was similarly abundant at both ends of the spectrum at low, high, and very high levels of development (Supplementary Table S4). *Taphrina* fungi were overrepresented in areas with very low levels of development, whereas the opposite was true for *Zygosaccharomyces* being overrepresented in areas of high development (Supplementary Table S4). Differences in taxa abundances were also apparent across varying precipitation and temperature gradients, with *Alternaria* being underrepresented with increased levels of precipitation and *Apilactobacillus* most

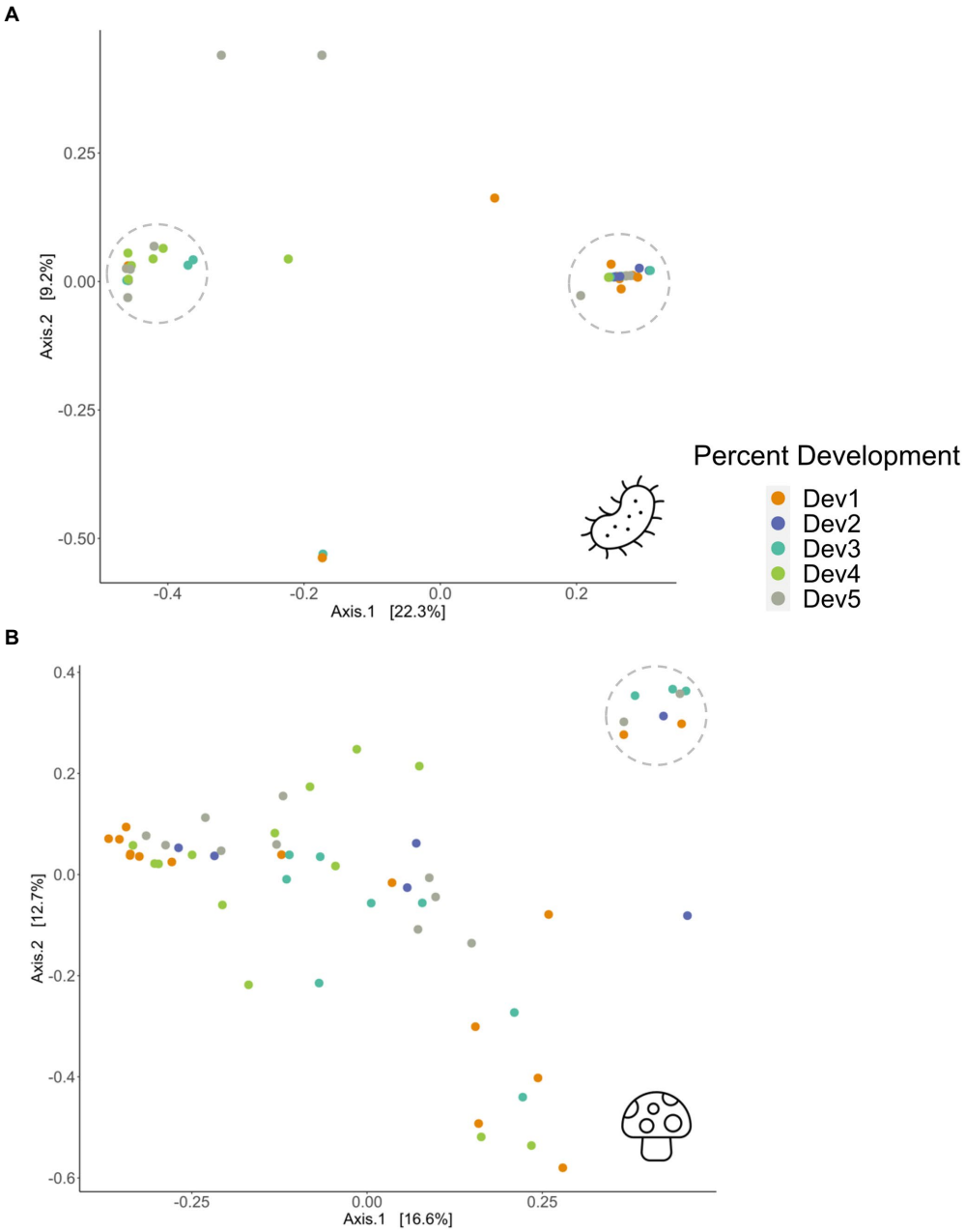

FIGURE 2
PCoA plots of Bray–Curtis dissimilarity matrices showing **(A)** bacterial (p= 0.017) and **(B)** fungal (p =0.023) beta diversity in 58 adult *Ceratina calcarata* across five levels of percent development, ranging from Dev 1 (very low development) through Dev 5 (very high development). For exact development percentages, see Supplementary Table S1. Dotted circles represent clusters of individuals with similar beta diversity.

abundant in environments with low annual temperature (Supplementary Table S4). In examining the clustered individuals on the fungal PCoA from sites with low precipitation and high temperatures (Supplementary Figures S2D,F), *Alternaria* was present in all these samples, with other common fungi including *Mortierella* and *Ascosphaera*. Thus, urbanization markedly characterizes disparate overrepresentations in bacteria and fungi.

To examine the uniqueness and connectedness of the microbiome across an urbanization gradient, bipartite networks were used to examine associations between different levels of development and microbial genera (Table 1; Supplementary Table S5; Supplementary Figure S3). The bacterial network resulted in overall lower levels of connectance (bacteria, 0.54; fungi, 0.82), links per species (bacteria, 2.13; fungi, 3.26), and Shannon diversity (bacteria, 1.56; fungi, 2.53) when compared to the fungal network. Bees from sites with the greatest percent development were found to have a higher degree of bacterial connectance, Shannon diversity of

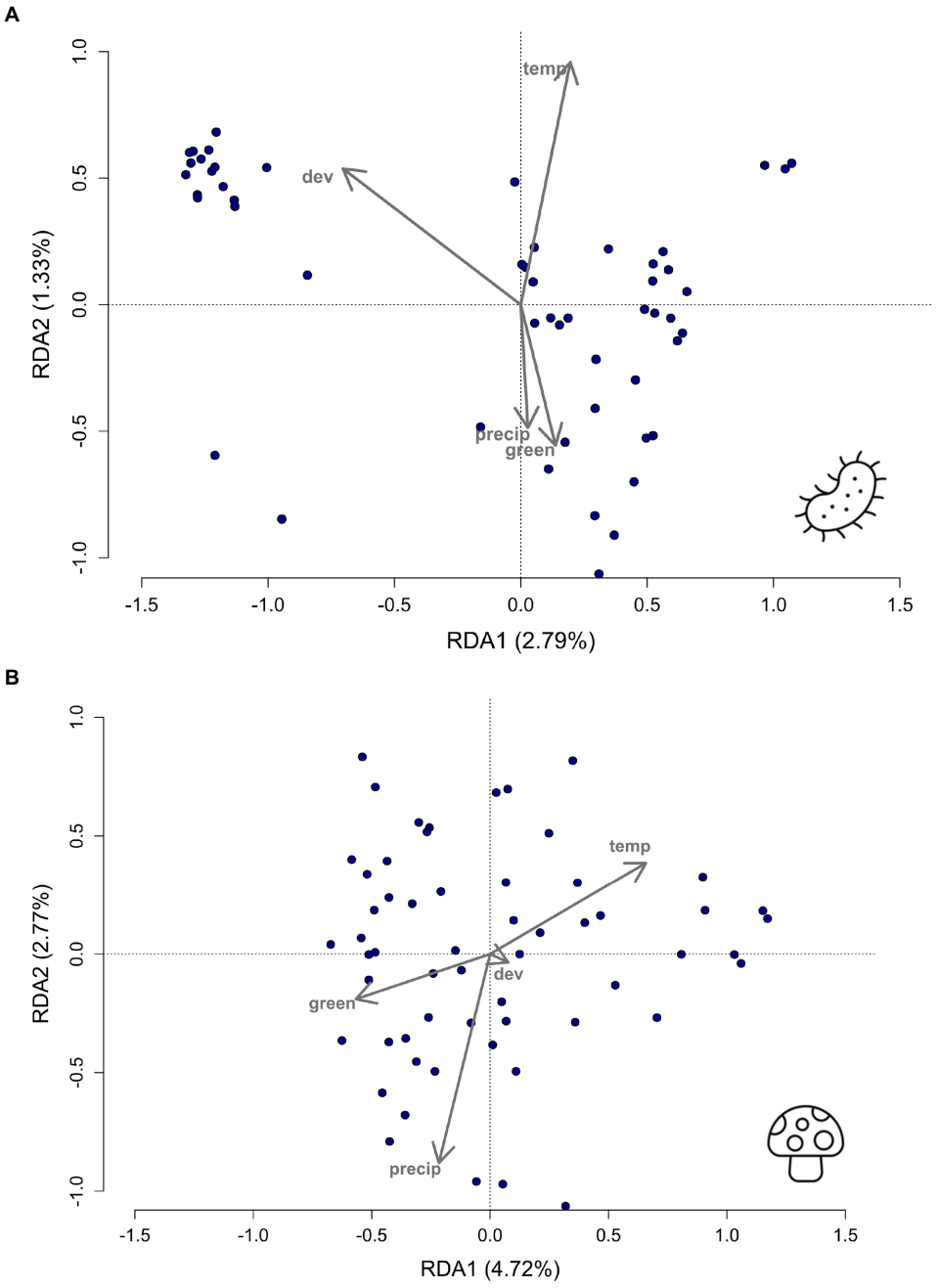

FIGURE 3
Redundancy analyses (RDA) plot showing whether **(A)** bacterial (p =0.030) and **(B)** fungal (p =0.001) taxa are associated with the environmental variables of dev=development, green=green space, temp=temperature, and precip=precipitation. Bacterial taxa are influenced by the development (p= 0.032) and green space (p =0. 037) variables. Fungal taxa are driven by green space (p= 0.002) and precipitation (p =0.012).

interactions, and effective partners, while also inversely showing low weighted closeness, when compared to areas with the least percent development (Table 1). Sites with low to moderate and very high percent development showed more abundant but less specialized relationships between bacteria and bees. This pattern held for fungi at a moderate level of land use development (Table 1). Thus, fungal networks maintained more consistency across the urban land use gradients. Networks revealed certain microbes like *Apilactobacillus, Alternaria, Ascosphaera*, and *Penicillium* had high degrees of connectance across all five development levels, whereas others had low degrees of connectance and were associated with an urbanization level. For example, *Clostridium* and *Saccharibacillus* were only found in very high levels of development and *Enterobacter* and *Samsoniella* were associated with very low levels of development.

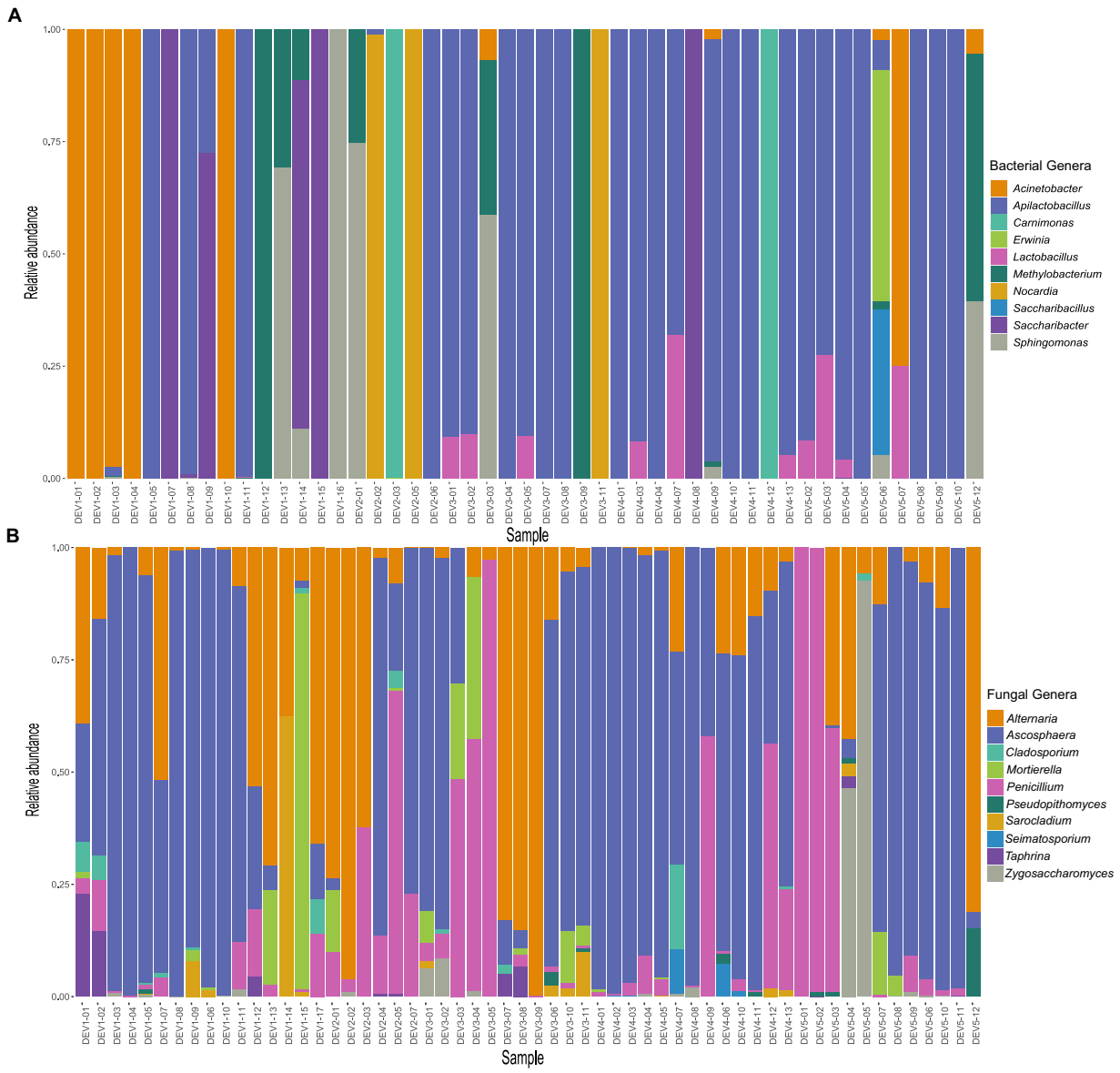

FIGURE 4
Top 10 **(A)** bacterial and **(B)** fungal genera found in 58 *Ceratina calcarata* from different levels of development across Toronto. The five categories range from Dev 1 (very low development) through Dev 5 (very high development).

Bacterial and fungal co-occurrences

CoNet and SparCC analyses were used to determine if any bacterial and fungal associations were found among the top 10 bacterial and fungal taxa across all 58 samples (Table 2; Supplementary Tables S6, S7). Using all individuals, CoNet revealed 17 associations (Supplementary Table S6), while SparCC presented 28 total co-occurrences of positive and negative correlations (Supplementary Table S7). Interestingly, a positive association between bacteria-bacteria was found to be significant across both statistical analyses in *Sphingomonas* and *Saccharibacillus* (CoNet, correlation = 0.31, $p = 0.019$; SparCC, correlation = 0.53, $p < 0.01$; Supplementary Tables S6, S7). Additionally, separate analyses for each land use development level revealed patterns in co-occurrences. Through CoNet analyses, *Metholybacterium* was only found not correlated to *Sphingomonas* in the very low and moderate development levels, while *Acinetobacter* and *Sphingomonas* were associated only in the moderate to high development levels (Table 2). Associations between fungi-bacteria and fungi-fungi were absent in densely urban areas and only seen in sites with the lowest land use development, with the rest of the associations only existing between bacteria (Table 2).

TABLE 1 Summary of bipartite network level statistics comparing the association between the top 18 bacterial and fungal taxa across five levels of development.

	Network level statistics	Dev 1	Dev 2	Dev 3	Dev 4	Dev 5
Bacteria	Degree	8	6	10	10	15
	Effective partners	1.45	2.15	2.11	1.06	2.33
	Shannon diversity	0.34	0.77	0.75	0.06	0.85
	Weighted closeness	0.55	0.03	0.06	0.51	0.12
Fungi	Degree	14	14	17	14	16
	Effective partners	1.97	3.43	4.50	2.07	3.80
	Shannon diversity	0.68	1.23	1.50	0.73	1.33
	Weighted closeness	0.35	0.08	0.19	0.34	0.28

The five categories range from Dev 1 (very low development) through Dev 5 (very high development).

Discussion

This study examines the bacterial and fungal microbiome in 58 *C. calcarata* individuals across an urban land use gradient. Percent development, percent green space, annual temperature, and annual precipitation were examined to determine how environmental factors may drive differences in microbial diversity and composition. While alpha diversity did not differ across the city, beta diversity and redundancy analysis modelling could be predicted by percent development and temperature. Taxonomic comparisons also revealed some bacterial and fungal taxa were more commonly found in either very low or highly developed areas of the city, indicating differences in the microbiome between urban land use gradients. Different levels of land use development also result in varying degrees of connectance in networks and different co-occurrences between microbes.

Microbial diversity

Shannon's diversity indices, a measure of alpha diversity, did not vary with environmental variables of development, green space, precipitation, or temperature and yielded low values of bacterial diversity, where more than half of the samples only contained one or two genera (Supplementary Figure S1). This aligns with a previous study of *C. calcarata* in Toronto, which found little change and overall low microbial alpha diversity as bees matured from brood to adults (Nguyen and Rehan, 2022). Similarly, a study comparing the stingless bee *Tetragonula carbonaria* microbiome between two different sites also showed alpha diversity remaining consistent, despite climatic and floral resource differences (Hall et al., 2021). Although studies with stingless bees have revealed the presence of environmental bacteria in the microbiome (Kwong et al., 2017b; Cerqueira et al., 2021), social bees often have a core microbiota and low diversity (Kwong and Moran, 2016). However, this is not representative of solitary wild bees, such as in a study with *Osmia lignaria* across different environmental contexts, which found that environmental factors drove differences in relative ASV abundances and alpha diversity (Cohen et al., 2020). Yet, as many factors affect microbiome alpha diversity it remains difficult to segregate how factors are affecting overall diversity in isolation and how a combination of environmental or situational variables co-occurring can affect alpha diversity.

Beta diversity, represented by Bray–Curtis dissimilarity matrices (Figure 2), was able to capture more of the microbial differences driven by urban land use gradients. Percent development was the most significant factor, with development associating with both bacterial and fungal Bray-Curtis dissimilarities, high development showing increased bacterial genera richness, and with development able to predict bacterial diversity through the redundancy modelling analyses (Figures 2, 3; Supplementary Table S2). Annual temperature was another considerable variable, yielding a significant ability to model differences in both bacterial and fungal microbiomes and associate with fungal beta diversity (Supplementary Figure S2F). Collectively, the interplay between these environmental characteristics may be dynamically changing microbial diversity, as features such as high temperature and low precipitation can act together to foster higher fungal genera richness and clustered Bray-Curtis dissimilarities, as seen in the circled individuals on the PCoA plots (Supplementary Table S3; Supplementary Figures S2D,F). However, these samples did not cluster with either land use development or green space, suggesting that the correlation between environmental variables is unclear (Figure 2; Supplementary Figure S2). Environmental features were expected to be a factor determining differences in beta diversity, as was initially seen in a study comparing two colonies of stingless bees at different locations (Hall et al., 2021), and in *O. lignaria* when dissimilarity matrices could be predicted by percent natural cover, number of trees and shrubs, bee species richness, and bare soil (Cohen et al., 2020). Similarly, McFrederick and Rehan (2019) found different species richness of fungi and bacteria when comparing subtropical, temperate and grassland zones across Australia, suggesting that climate shapes the *C. australensis* microbiome. Thus, environmental characteristics describing both land use and climate affect the microbial diversity of individual *C. calcarata* microbiomes.

TABLE 2 CoNet correlations between bacteria-bacteria, fungi-fungi, and fungi-bacteria within the top 10 bacterial and fungal genera found in 58 *Ceratina calcarata* across five levels of land use development.

Development level	Correlated taxa	Correlation	*p*-value
DEV-1 ($n = 16$)	*Alternaria – Penicillium*	0.81	0.013
	Sarocladium – Saccharibacter	0.75	0.015
DEV-2 ($n = 6$)	*Carnimonas – Erwinia*	1.00	<0.01
	Methylobacterium – Sphingomonas	1.00	<0.01
DEV-3 ($n = 11$)	*Acinetobacter – Sphingomonas*	1.00	<0.01
DEV-4 ($n = 13$)	*Acinetobacter – Methylobacterium*	1.00	<0.01
	Acinetobacter – Sphingomonas	1.00	<0.01
	Methylobacterium – Sphingomonas	1.00	<0.01
DEV-5 ($n = 12$)	*Acinetobacter – Saccharibacillus*	1.00	<0.01
	Acinetobacter – Erwinia	1.00	<0.01
	Erwinia – Saccharibacillus	1.00	<0.01
	Methylobacterium – Sphingomonas	0.98	<0.01

The five categories range from Dev 1 (very low development) through Dev 5 (very high development). Sample sizes at each land use development level are provided in brackets. A full representation of all correlations is available in the supplement (Supplementary Table S6).

Microbial composition

Apilactobacillus, *Alternaria*, *Penicillium* and *Ascosphaera* were the most prevalent and abundant bacterial and fungal genera found across the city (Supplementary Tables S2, S3). *Apilactobacillus* are common beneficial bee symbionts (Tlais et al., 2022) and were established as part of the core microbiome in *C. calcarata* in New Hampshire, a more rural landscape (McFrederick and Rehan, 2016; Graystock et al., 2017). In urban cities such as Toronto, *Apilactobacillus* was previously largely absent in adult *C. calcarata* (Nguyen and Rehan, 2022) and was found to be underrepresented at sites with moderate levels of land use development, overrepresented in sites with the most green space, and overrepresented at sites with lower annual temperatures in this study (Supplementary Table S4). Thus, urban bees reveal a different microbiome from those in rural contexts and of particular concern is the varying abundance of *Apilactobacillus*.

The fungal genus *Ascosphaera* contains both pathogenic and apathogenic fungi (Klinger et al., 2013), and the species *A. major* was common in *C. calcarata* (Supplementary Table S3). This species has caused chalkbrood-like diseases in *Megachile centuncularis* and *Apis mellifera*, but can also live relatively harmlessly as a facultative parasite within bee nests on pollen provisions and larval feces, including other wild bees such as *Osmia bicornis* (Holm and Skou, 1972; Bissett, 1988; Wynns et al., 2013). Therefore, the abundance of *Ascosphaera* may indicate a commensalism between *C. calcarata* and these bee specialist fungi. Future studies are needed to determine the fitness effects of *Ascosphaera* on this species.

Overrepresentations of certain bacterial and fungal taxa at sites of varying land use development may indicate such factors are affecting microbial composition. Areas with a low percentage of development were found to have a greater abundance of *Acinetobacter, Ascosphaera, Saccharibacter,* and *Taphrina* (Supplementary Table S4). *Acinetobacter* is a flower-associated species of bacteria also commonly associated with yeasts in nectar which can induce germination and pollen bursting that then benefits pollinators by way of improved nutrition from nectar (Christensen et al., 2021; Rering et al., 2021). Another flower-associated bacteria, *Saccharibacter*, is closely related to the bacteria *Bombella apis* which is known to protect developing honey bees from fungal pathogens and contains genetic loci involved with nutrition, microbial and host interactions, and immunity (Smith and Newton, 2020). Thus, the overrepresentation of beneficial microbes in areas with low land use development is promising for these pollinators. On the contrary, the two fungal genera found in high abundance in more rural areas, *Ascosphaera* and *Taphrina,* are facultative bee and plant pathogens, respectively, (Cissé et al., 2013; Wynns et al., 2013). However, these genera were also previously seen in immature *C. calcarata* and it is unclear if they pose any threat to this species (Nguyen and Rehan, 2022). *Ascosphaera* was also overrepresented at high and very high development levels (Supplementary Table S4; Figure 4B), suggesting this fungi may not be limited to rural areas.

The overrepresented genera present in sites with a high percentage of development, such as *Lactobacillus*, *Penicillium,* and *Zygosaccharomyces* (Supplementary Table S4), were not microbes that are known to be harmful to bee health. *Lactobacillus* spp., such as *L. crispatus* and *L. intestinalis*, have been seen in *A. mellifera*, *Bombus terrestris*, and *O. bicornis* (Mohr and Tebbe, 2006), and many studies have uncovered the important and beneficial relationship between *Lactobacillus* and bees (Rothman et al., 2019; Li et al., 2021). *Penicillium* molds are commonly found in *Melipona scutellaris* (Barbosa et al., 2018) and *A. mellifera* bee bread and is of importance as it produces enzymes involved in lipid, protein, and carbohydrate metabolism that can even protect bees against fungicides (Gilliam et al., 1989; Yoder et al., 2013). *Zygosaccharomyces* sp. are fungi that have been shown to provide steroid precursors crucial for the pupation of the stingless bee

Scaptotrigona depilis (Paludo et al., 2018). Hence, while differing from areas of low land use development, bacteria and fungi found in urban bees may be supported by their own beneficial properties to their bee hosts. Regardless of urbanization, different overrepresentations of bacteria and fungi may serve varying, but equally beneficial, purposes. Areas with low development seem to harbour plant associated microbes that may be associated with natural plant availability, whereas high development sites contain microbes more associated with bee development and digestion.

Co-occurrences between microbes have been studied in bees and pollen to examine how microbial members interact and establish the microbiome (Russell et al., 2011; Graystock et al., 2017; Manirajan et al., 2018; Dew et al., 2020). This study found a strong positive relationship between *Sphingomonas* and *Saccharibacillus* when examining adults (Supplementary Tables S6, S7). While *Saccharibacillus* has been found in commercial bee pollen from Europe, little is known about its interactions and presence in bee microbiomes (Andrade et al., 2019). Bacteria of the genus *Sphingomonas* have been shown to be negatively correlated with *Fusarium* species that cause Fusarium Head Blight in maize crops (Cobo-Díaz et al., 2019). In *C. calcarata*, *Sphingomonas* co-occurred positively with the fungal genera *Pantoea* (Nguyen and Rehan, 2022), a genus prevalent in *C. australensis* (Shell and Rehan, 2022), *A. mellifera* (Wright et al., 2001), and stingless bees (Leonhardt and Kaltenpoth, 2014). Additionally, *Sphingomonas* is a dominant bacteria found in the nests of stingless bees *Frieseomelitta varia*, *Melipona quadrifasciata*, and *Tetragonisca angustula* (de Sousa, 2021) and is also found in *A. mellifera* (Anjum et al., 2018; Muñoz-Colmenero et al., 2020) and *O. bicornis* microbiomes (Mohr and Tebbe, 2006), thus this bacteria co-occurs naturally with wild bees. In regards to urban and agricultural bees, this bacteria may be particularly beneficial as it contains enzymes that degrade organochlorides in insecticides (Russell et al., 2011). Therefore, commonly occurring bacteria, such as *Sphingomonas*, may be playing an underappreciated role in the wild bee microbiome.

Microbe correlations can be examined considering land use development to determine if environmental factors may be affecting the stability of these associations. *Methylobacterium* was correlated with *Sphingomonas* in all but the low and moderate percent development sites (Table 2). *Methylobacterium* have shown beneficial relationships with plants and bacteria, sometimes even relying on growth factors produced by other microbes (Iguchi et al., 2015; Manirajan et al., 2018). The number of correlations present also increased with percent developed area, suggesting that urbanized areas may be associated with more positive co-occurrences between bacteria. This has been seen in urban soils, where environmental features altered microbial networks (Wang et al., 2018). Therefore, landscape features may be changing the way bacteria and fungi are supported, which can in turn affect the presence and abundance of these and other microbes. Further examination into the functional role of specific microbes as well as how they exist in symbioses is needed to explain how these networks are maintained.

Effects of a changing environment

Changes in microbial diversity and composition are of potential concern because bee-microbe symbioses play a key role maintaining bee health (Engel et al., 2012; Mockler et al., 2018; Dharampal et al., 2019, 2020; Rothman et al., 2019). While this study described several abundant bacterial and fungal genera dominating the microbiome, this is a large contrast to previous studies of *C. calcarata* that revealed 13 core bacterial phylotypes in bees and much more diversity (Graystock et al., 2017). The reason for bacteria showing decreased diversity compared to fungi, as seen in lower Shannon diversity measures, lower degrees of network connectance, and fewer effective partners (Table 2), remains unclear. This low bacterial diversity in adults was also found in a previous study of *C. calcarata* in Toronto, particularly when compared to developing brood, and suggests a persistent and concerning decrease in microbial diversity in urban landscapes (Nguyen and Rehan, 2022). Ongoing long-term and additional studies are needed to examine whether the few bacterial genera currently making up the microbiome are excluding other bacteria and/or whether bees in cities have generally less diverse microbiomes in this and other wild bee species.

As the urban land use gradients such as percent development and temperature reveal their effects on the bee microbiome, rapid urbanization becomes increasingly alarming. Urbanization and anthropogenic activities are a worsening problem driving declines in bee populations, altering bee community compositions, and negatively affecting certain bee species (Ritchie and Roser, 2018; Wilson and Jamieson, 2019; Ayers and Rehan, 2021; Prendergast et al., 2022). In addition, the presence of pesticides in urban areas may be driving declines in bee health and in bee microbiome structure, composition, and diversity (Kakumanu et al., 2016; Botías et al., 2017; Hotchkiss et al., 2022). Future work comparing a wider range of rural and agricultural landscapes across multiple regions will help determine how bee microbiomes change with land use. Additional studies examining pesticides present in bee habitats in urban and rural areas and how these accumulate in bees and their pollen provisions will also be important (Pisa et al., 2014; Kakumanu et al., 2016; Botías et al., 2017; Hotchkiss et al., 2022). As research continues to untangle the variables that work together to establish and maintain the wild bee microbiome, the ever-changing landscape in cities adds new considerations for possible environmental stressors.

In conclusion, this study examines the bacterial and fungal composition and diversity in adult *C. calcarata* across an urban land use gradient, revealing differences explained by percent land use development, green space, precipitation, and temperature. Individuals from low to moderate development levels tended to share similar bacterial composition within one cluster, while those from moderate to high development levels grouped separately. In examining fungal taxa, individuals showed greater

dissimilarity in beta diversity, with climatic variables as possible drivers. The interplay of environmental factors across urbanization gradients act on microbial community composition, with overlapping characteristics such as annual temperature and annual precipitation coinciding with fungal beta diversity. Microbial composition in rural areas were dominated by genera such as *Acinetobacter* and *Apilactobacillus*, beneficial microbes that support bee health and may affect bee survival. Specific taxa varied across different levels of urbanization, which may be explained by co-occurrences between bacteria and fungi that varied amongst different land use development, and suggests that microbial relationships are dependent on changes in environment. These complex networks reveal that urban areas may exhibit a stronger degree of connectance in bacteria, while lower levels of urbanization foster greater connectance within the fungal microbiome. Overall, increased urbanization has led to a significant impact on microbial composition and diversity. As cities continue to expand and urbanization rises globally, it is increasingly important to understand how landscapes affect bee health through their microbiome.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/ BioProject: PRJNA805022 BioSamples: SAMN25891990-SAMN25892379.

Author contributions

PN: sample preparation, DNA extraction, bioinformatics, and analyzed and visualized data. SR: conceived study, supervised, and provided funding. PN and SR: wrote and approved final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.992660/full#supplementary-material

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Stably transmitted defined microbial community in honeybees preserves *Hafnia alvei* inhibition by regulating the immune system

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The gut microbiota of honeybees is highly diverse at the strain level and essential to the proper function and development of the host. Interactions between the host and its gut microbiota, such as specific microbes regulating the innate immune system, protect the host against pathogen infections. However, little is known about the capacity of these strains deposited in one colony to inhibit pathogens. In this study, we assembled a defined microbial community based on phylogeny analysis, the 'Core-20' community, consisting of 20 strains isolated from the honeybee intestine. The Core-20 community could trigger the upregulation of immune gene expressions and reduce *Hafnia alvei* prevalence, indicating immune priming underlies the microbial protective effect. Functions related to carbohydrate utilization and the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS systems) are represented in genomic analysis of the defined community, which might be involved in manipulating immune responses. Additionally, we found that the defined Core-20 community is able to colonize the honeybee gut stably through passages. In conclusion, our findings highlight that the synthetic gut microbiota could offer protection by regulating the host immune system, suggesting that the strain collection can yield insights into host-microbiota interactions and provide solutions to protect honeybees from pathogen infections.

KEYWORDS

***Apis mellifera*, colonization resistance, *Hafnia alvei*, immune system, gut microbiota**

Introduction

The host intestinal tract is a complex ecosystem offering niches for beneficial symbionts that aid in food digestion and disease resistance (Engel and Moran, 2013b; Pereira and Berry, 2017). Imbalanced gut microbiota driven by the antibiotic treatment could lead to metabolism changes, potentially pathogenic bacteria blooming, epithelial barrier

disruption, and increased susceptibility to infections (Buffie et al., 2015; Raymann et al., 2017; Fünfhaus et al., 2018; Lang et al., 2022). Therefore, the gut microbiota can preclude infections of enteric pathogens, which is one of the most widespread benefits to its host (Spees et al., 2013; Kim et al., 2017). Considering the complexity of interactions between the microbiota and the host, the underlying basis of this protection, or 'colonization resistance', is still insufficiently understood.

Honeybees (*Apis mellifera*) harbor about five core host-specific bacterial genera, which probably have co-evolved with social bees for over 80 million years (Koch et al., 2013; Kwong and Moran, 2016). They include *Snodgrassella*, *Gilliamella*, *Bifidobacterium*, *Bombilactobacillus* Firm-4, and *Lactobacillus* Firm-5 (Martinson et al., 2011; Kwong and Moran, 2016). Additionally, the genus *Apilactobacillus*, *Frischella*, *Commensalibacter, Bartonella*, and *Bombella* are less prevalent, which occupy particular niches and engage in host health maintenance (Engel et al., 2016; Liu et al., 2022). With relatively simple gut microbiota, honeybees present opportunities to investigate gut community dynamics and host–microbe interaction as an experimental system (Zheng et al., 2018). Recent research has demonstrated the honeybee gut microbiome contributes to metabolism, development, and protection against pathogens (Engel et al., 2016; Raymann and Moran, 2018). Some species belonging to *Bombilactobacillus* Firm-4, *Lactobacillus* Firm-5, and *Bifidobacterium* can inhibit the growth of other microorganisms *in vitro* (Forsgren et al., 2010; Vásquez et al., 2012; Butler et al., 2013; Killer et al., 2014). Members of bee gut microbiota, such as *Snodgrassella alvi* and *Gilliamella apis*, could lower gut lumen pH and oxygen levels (Zheng et al., 2017), compete for nutrients (Martinson et al., 2012; Wu et al., 2021), and antagonize with type VI secretion system (Steele et al., 2017) to inhibit pathogen virulence and growth.

The colonization resistance conferred by the gut microbiota through stimulating the host's innate immune system was supported by increasing evidence (Lawley and Walker, 2013). The innate immune system of honeybees comprises the Toll and Imd pathways (Lourenço et al., 2013, 2018; Danihlík et al., 2015), which primarily regulate the production of antimicrobial peptides (AMPs), such as abaecin, apidaecin, defensin, and hymenoptaecin, during pathogen infection (Evans et al., 2006; Guo et al., 2021). When honeybees were colonized with conventional gut microbiota or mono-colonized with strains from *S. alvi*, the immune system of honeybees was stimulated to inhibit potential pathogens such as *Serratia marcescens* (Horak et al., 2020). However, substantial strain-level diversity was found within the bee gut microbiota, where individual strains harbor unique genes and distinct functional capabilities (Ellegaard et al., 2019; Brochet et al., 2021; Lang et al., 2022). In addition to understanding individual strains involved in interactions determining colonization resistance, how bacterial combinations by multiple strains from different species control colonization resistance still need to be investigated.

Hafnia alvei, a specific pathogen in bees, could cause septicemia with a mortality rate of over 90% by injection and inflammation of the intestinal tract by oral (Møller, 1954; Erban et al., 2017; Grabowski and Klein, 2017). Leveraging previous work, *Lactobacillus apis* W8171 could inhibit *H. alvei* infection and prevent severe mucosal architecture damage in the honeybee rectum (Lang et al., 2022). In this study, we established a consortium based on phylogeny analysis, the 'Core-20' community, consisting of 20 strains isolated from the honeybee intestine that provide colonization resistance against *H. alvei*. Interestingly, the higher complex and biodiversity community displays advantages in promoting the expression of regulators and AMPs of the immune system. The comparative genomic analysis revealed that the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS system) could potentially be involved in manipulating immune responses. In addition, we transmitted the Core-20 community for four passages and found that the Core-20 could colonize steadily. Thus, the Core-20 community serves as a stable and functional microbiota that can be used for detailed investigation of host-microbe and microbe-microbe interactions in honeybees.

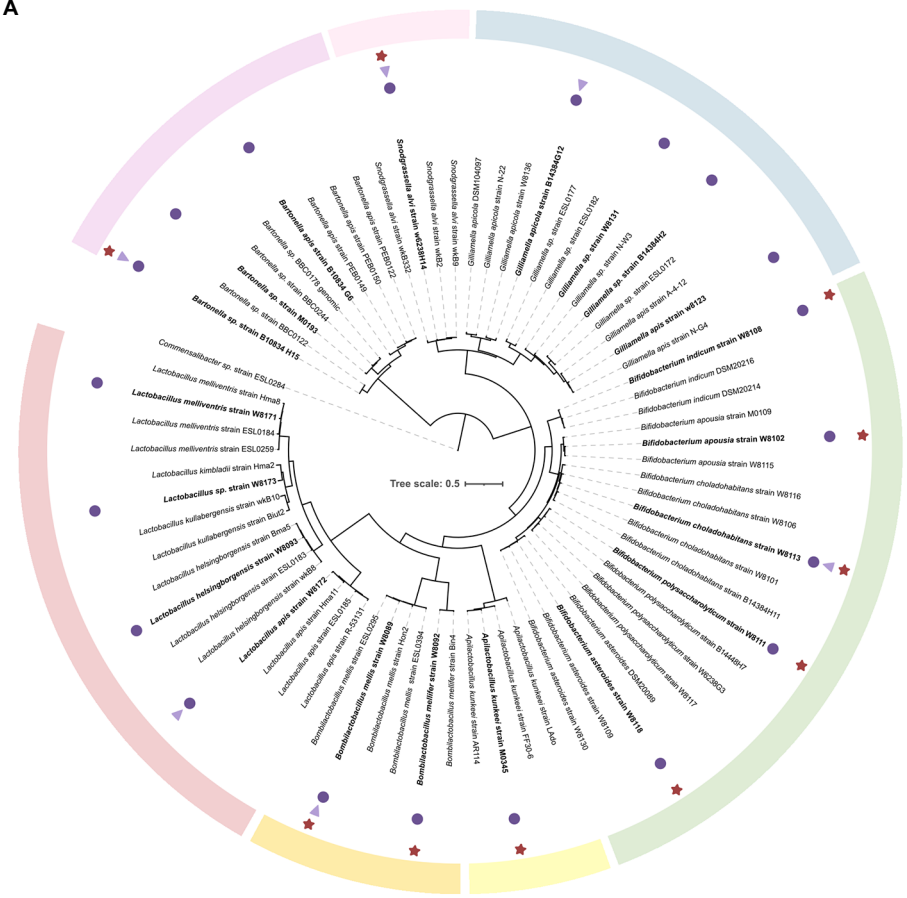
Materials and methods

Characterization of stains in the Core-20 community designed by the phylogeny of honeybee gut microbiota

To establish defined minimal microbiota that recapitulates healthy honeybee gut microbiota stably and functionally, the integral intestine homogenization of conventional honeybee was cultured on a rich, non-selective culture medium. About 110 strains were mono-cloned and identified by whole genome sequencing (WGS), representing conventional bacterial strains. The quality-controlled reads were assembled with the SOAPdenovo2 genome assembler. The completeness and contamination of genomes were assessed by CheckM (>96% completeness, <0.6% contamination). Phylogenetic analysis by WGS shows that strains assorted into different clusters according to gANI identities referred to as species-level (Su et al., 2021; Wu et al., 2021). Six strains representing the six most prevalent and abundant genera of honeybee gut microbiota are selected for a bacterial consortium named "Core-6," and 20 strains at the species-level form "Core-20" bacterial community (Figure 1).

Within the *Proteobacteria* phylum, four members of the Core-20 community were assigned to the genus *Gilliamella*, one strain to *Snodgrassella*, and three strains to *Bartonella*. Two abundant species clusters in the *Firmicutes* phylum are *Bombilactobacillus* Firm-4 and *Lactobacillus* Firm-5, including two strains and four strains, respectively. Additionally, *Apilactobacillus kunkeei*, which proved its ability to protect honeybees from pathogens, was added as an essential functional part (Daisley et al., 2020a,b).

A



B

Genus	Species	Strains	Core-20	Core-6	Mono-colonization
Gilliamella	*apicola*	B14448G12	●	▲	
	sp.	W8131	●		
	sp.	B14384H2	●		
	apis	W8123	●		
Snodgrassella	*alvi*	W6238H14	●	▲	★
Bifidobacterium	*indicum*	W8108	●		★
	apousia	W8102	●		★
	choladohabitans	W8113	●	▲	★
	polysaccharolyticum	W8111	●		★
	asteroides	W8118	●		★
Bartonella	***choladocola***	B10834H15	●	▲	★
	apihabitans	M0193	●		
	apis	B10834G6	●		
Bombilactobacillus	*mellis*	W8089	●	▲	★
	mellifer	W8092	●		★
Apilactobacillus	*kunkeei*	M0345	●		★
Lactobacillus	*apis*	W8172	●	▲	
	helsingborgensis	W8093	●		
	melliventris	W8171	●		
	sp.	W8173	●		

FIGURE 1
Composition of honeybee gut microbiota and strains of the Core-20 and Core-6 community. **(A)** Maximum-likelihood tree inferred by GTDB-tk based on the amino acid sequences of bacterial marker genes. **(B)** Detailed information on strain classification and grouping. The Core-6 community consists of six strains representing the six most prevalent and abundant genera of honeybee gut microbiota, and the Core-20 is composed of 20 strains at the species level. Rounds mark the strains of the Core-20, triangles mark the members of the Core-6 and stars mark strains used in the mono-colonization experiments. Color bars indicate the classification of honeybee gut microbiota.

Bacterial strains were isolated from the guts of *A. mellifera* and stored at −80°C with 25% (v/v) glycerol PBS solution. The glycerol stocks were plated on heart infusion agar supplemented with 5% (vol/vol) defibrinated sheep's blood (Solarbio, Beijing, China), MRS agar (Solarbio, Beijing, China) or TPY agar (Solarbio, Beijing, China) incubated at 35°C in 5% CO2 for 2–3 days. The culture conditions of strains used in this study were described by Wu et al. (2021). Confirmed by PCR with universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGACTTAACCCCAATCGC-3′), individual strains were mixed with 25% glycerol PBS solution. The defined bacterial communities were generated by mixing equal volumes of bacterial suspensions with adjusted $OD_{600} = 1$.

Honeybee collection, containment, and experiment

Microbiota-free (MF) bees were obtained as described by Zheng et al. (Zheng et al., 2018). All bees were kept in an incubator (35°C, RH 50%). For the *H. alvei* challenging experiment, newly emerged MF bees (Day 1) were divided into several groups, with 25 MF bees in one cup cage. For each colonization group, bees lived on the 1 ml bacterial suspensions mixed with 1 ml sucrose solution (50%, w/v) and 0.5 g sterilized pollen for 24 h. For the MF group, 1 ml of 1 × PBS was mixed with 1 ml of sucrose solution (50%, w/v) and 0.5 g sterilized pollen. After 24 h inoculation, all groups were fed regular diets, sucrose solution (50%, w/v), and sterilized pollen. To precisely control the infection amount of *H. alvei* cells, bees from the colonization and MF groups were all orally inoculated with *H. alvei* SMH01 individually on Day 7 (Lang et al., 2022). After five-day regular diets, the load of *H. alvei* was determined by qPCR.

For inoculation and sampling in passaging line, newly emerged MF bees (Day 1) were randomly assigned to three cups and living on the 1 ml the Core-20 bacterial suspension mixed with 1 ml sucrose solution (50%, w/v) and 0.5 g sterilized pollen for 24 h, with 25 MF bees in one cup cage. Five days after the final oral inoculation, the whole gut of each bee was sampled, immediately placed into a sterile 1.5 ml tube individually, and ground with sterile 25% glycerol PBS solution. Three guts from each cup were pulled together to prepare inoculation for the following passage, and the other guts were stored at −80°C for DNA extraction and sequencing.

16S rRNA gene amplicon sequencing and processing

DNA was extracted from gut homogenates using the CTAB method (Powell et al., 2014; Zheng et al., 2018). Targeted amplicons of the V3-4 region of the 16S rRNA gene were generated with primers 341F and 806R (Caporaso et al., 2011). Sequencing libraries were generated with NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, United States). They were sequenced at Novogene Bioinformatics Technology Co. Ltd., Beijing, China, on the Illumina NovaSeq6000 platform (2 × 250 bp). Bioinformatic analysis was implemented using Mothur (version 1.40.5; Schloss et al., 2009; Kozich et al., 2013; Schloss, 2020). After primer trimming and quality control, sequences were split into groups corresponding to their taxonomy at the level of species and then assigned to operational taxonomic units (OTUs) at a 1% dissimilarity level based on the reference database consisting of aligned 16S rRNA sequences of our 20 strains (Supplementary Figure S1; Xue et al., 2019). Relative abundances were then calculated based on the read numbers. Principal coordinates analysis (PCoA) and alpha diversity indices were visualized in R (version 3.6.1). Raw sequence reads have been deposited at the NCBI SRA database under the BioProject accession number PRJNA891025.

Quantitative PCR of bacterial 16S rRNA genes and immune-related genes

DNA was extracted from gut homogenates using the CTAB method (Powell et al., 2014; Zheng et al., 2018). DNA concentration was determined with the Qubit 4 Fluorometer (Thermo Fischer Scientific; Waltham, MA, United States). *H. alvei* loads and immune-related gene expressions were determined by qPCR using the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China). Primer sets specific to *H. alvei* and immune-related genes are listed in Supplementary Table S1 (Horak et al., 2020; Lang et al., 2022). The primers of spaetzle 4 (*Spz4*; XM_028668966.1) were designed based on the nucleotide sequence available in GenBank: forward 5′-CAACGAATTCAGGGACGAGG-3′, reverse 5′-AGTAGTGCCGGGGAAATTCA-3′. All qPCRs were performed in 96-well microplates on a QuantStudio 1 real-time PCR system (Thermo Fischer Scientific). Melting curves were generated after each run (95°C for 15 s, 60°C for 20 s, and increments of 0.3°C until reaching 95°C for 15 s). Each reaction was performed in triplicates on the same plate. The data was analyzed using the QuantStudio Design and Analysis Software. After calculating gene copies, normalization was performed to reduce the effect of gut size variation and extraction efficiency using the host's actin gene (Kešnerová et al., 2020).

Functional genomics analysis

Input files were assembled and annotated genomes of the Core-20 (Su et al., 2021; Wu et al., 2021). *H. alvei* reference protein sequence (GCF_011617105.1) was downloaded from NCBI and annotated by KAAS[1] (Moriya et al., 2007). Artificial metagenomes were created by merging the contigs of each genome into a

1 https://www.genome.jp/kegg/kaas/

multi-fasta file (Brugiroux et al., 2016). KEGG mapping was performed using the online version[2] (Kanehisa et al., 2022). The comparison and analysis of orthologous clusters among genomes were performed at[3] (Xu et al., 2019).

Statistical analysis

Statistical analysis was performed using one-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (honestly significant difference) using package "multcomp" in R (version 3.6.1). *p*-value of less than 0.05 (two-tailed) was considered statistically significant ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Results

Resistance of the Core-20 community against honeybee opportunistic pathogen *Hafnia alvei*

To evaluate the potential of the defined communities to protect against *H. alvei* infection, we first colonized MF honeybees with the Core-20, the Core-6, and strains from the genus *Snodgrassella*, *Bartonella*, *Bombilactobacillus* Firm-4, *Apilactobacillus* and *Bifidobacterium* (Figure 1). At Day 7, all honeybee were orally infected with *H. alvei* individually (10^6 CFU per bee; Figure 2A). Successful microbiota colonization was confirmed by 16S rRNA gene V3-V4 amplicon sequencing at Day 12. Compositional analysis showed that observed species in the Core-20, compared to the Core-6, were increased, and the relative abundance of each taxonomy group differs (Figure 2B). Strains W8131, B14384H2 and W8123 from the genus *Gilliamella* and strains W8093, W8171, and W8173 from the genus *Lactobacillus* Firm-5, which are specific to the Core-20, exhibit substantial improvement in species abundances, showing their fitness in honeybee gut environment and ability to coexist with the complex bacterial community.

After 5 days of infection, *H. alvei* loads were measured by qPCR. Among mono-colonized bees, only *B. choladocola* B10834H15 and *B. choladohabitans* W8113 significantly inhibited the growth of *H. alvei in vivo* compared with the MF group at Day 12 (Figure 2C). According to our previous research, *H. alvei* loads in the bees with *L. apis* W8172, and *Gilliamella apicola* W8136 (the same species as *G. apicola* B14384G12) were also significantly lower than MF bees (Lang et al., 2022). Interestingly, bees treated with the Core-6, including all these strains demonstrating the ability to inhibit pathogens, did not show a significant reduction of *H. alvei*, while the Core-20 reduced the *H. alvei* loads by 78 times. Taken together, the presence of particular species did inhibit *H. alvei*. Still, this microbiota-induced prevention of pathogen infection possibly changes with the gut microbiota composition, suggesting a complex dynamic balance between microbe-host and microbe-microbe interaction.

Immune expression response induced by the defined community

Intestinal homeostasis maintenance depends on dynamic interactions between gut bacteria and the host's innate immune systems (Yoo et al., 2020). Commensal gut microbiota could prevent pathogen colonization and infection by enhancing the mucosal barrier and promoting innate immune responses. The gut microbial symbionts of the honeybee can induce antimicrobial immune responses in the host, like AMPs (Kwong et al., 2017). We assessed the relative expression of genes from Toll and Imd pathways by qPCR 24 h following inoculation with the Core-20 and Core-6. The Toll and Imd pathways include the receptors (spz4, toll; pgrp-lc), the regulators (cactus; dredd), and the transcription factors (dorsal; relish), respectively. On Day 2, bees colonized with the Core-20 significantly upregulated *pgrp-lc*, *dredd,* and *relish* from the Imd pathway as well as *toll* and *cactus-2* from the Toll pathway relative to MF bees (Figure 3A). Furthermore, we focused on the expression of genes encoding AMPs, and remarkably, we discovered that bees with the Core-6 exhibited a notable reduction of AMPs *abaecin*, *apidaecin*, *hymenoptaecin* and *defensin-1* (Figure 3B), which might indicate an immunosuppressive ability of the Core-6. To find out whether the Core-20 could consistently upregulate host-producing AMPs in response to *H. alvei* infection, the expression of AMPs genes was measured again on Day 7, right before *H. alvei* inoculation. Interestingly, bees with the Core-20 showed a significant increase of AMPs *abaecin*, *apidaecin*, *hymenoptaecin,* and *defensin-1* (Figure 3C), indicating abilities of the Core-20 to stimulate innate immune response preventing the colonization by pathogenic *H. alvei*.

Our findings showed the Core-6 community exhibited diminished production of antimicrobial peptides, while the Core-20 community upregulated the host immune system, including regulators in innate immune pathways and AMPs expression. *Apidaecin*, the most susceptible AMP against *H. alvei in vitro* (Lang et al., 2022), expressed much higher in the Core-20 condition. Overall, our findings suggested that a primary mechanism by which Core-20 provides colonization resistance is that it can trigger host immune responses.

Potential to regulate host immune system through phosphoenolpyruvate-dependent sugar phosphotransferase system

Protection against *H. alvei* by the Core-20 community supports immune regulation as a factor in pathogen defense. To gain insights

2 https://www.genome.jp/kegg/mapper/

3 http://orthovenn2.bioinfotoolkits.net

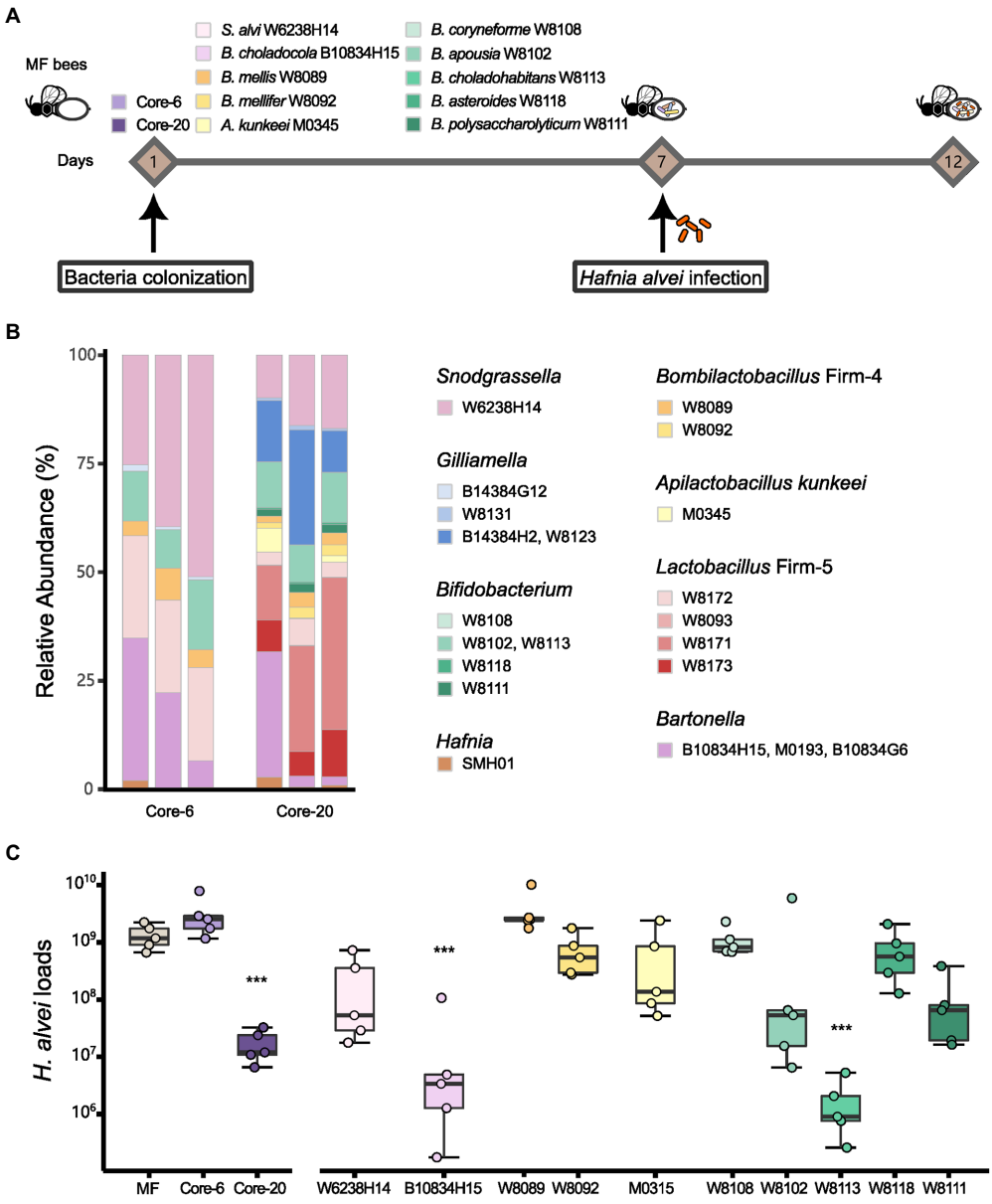


FIGURE 2
Core-20 leads to protection against oral *H. alvei* infection. **(A)** Experimental design for honeybees colonized with specific microbes challenging with *H. alvei*. **(B)** Relative abundance of the Core-6 and Core-20 community on Day 12. 16S rRNA V3-V4 amplicons were sequenced and analyzed, showing successful microbiota colonization and composition differences between the Core-6 and the Core-20. **(C)** Absolute abundance of *H. alvei* in different treatment groups 5days post-infection. Single strains, such as *B. choladocola* B10834H15 and *B. choladohabitans* W8113, significantly inhibited the growth of *H. alvei*. The Core-20 community, which is much more complex than the Core-6, significantly reduced the *H. alvei* loads.

into the potential functional capabilities of the Core-20 to activate immunologic responses, we sequenced and annotated the individual genomes of the 20 strains and mapped the predicted protein sequences against the KEGG database. Artificial metagenomes of the Core-6 and Core-20 were generated by merging the contigs of individual strains. The presence and completeness of KEGG modules were determined for individual genomes of 20 strains, the Core-6 and Core-20 (Figure 4). After hierarchical clustering, we observed different functional groups depending on their phylogenetic relatedness incidentally. The majority of strains share highly conserved modules, including phosphate acetyltransferase-acetate kinase pathway (M00579), PRPP biosynthesis (M00005), F-type ATPase (M00157), various carbohydrate metabolism pathways and multiple amino-acid and nucleoside biosynthesis pathways. Additionally, modules more prominent in *Gilliamella* strains comprised pyridoxal-p biosynthesis (M00916) and carbohydrate degradation modules, such as ascorbate, D-glucuronate, and D-galacturonate (M00550, M00061, M00631). We also found

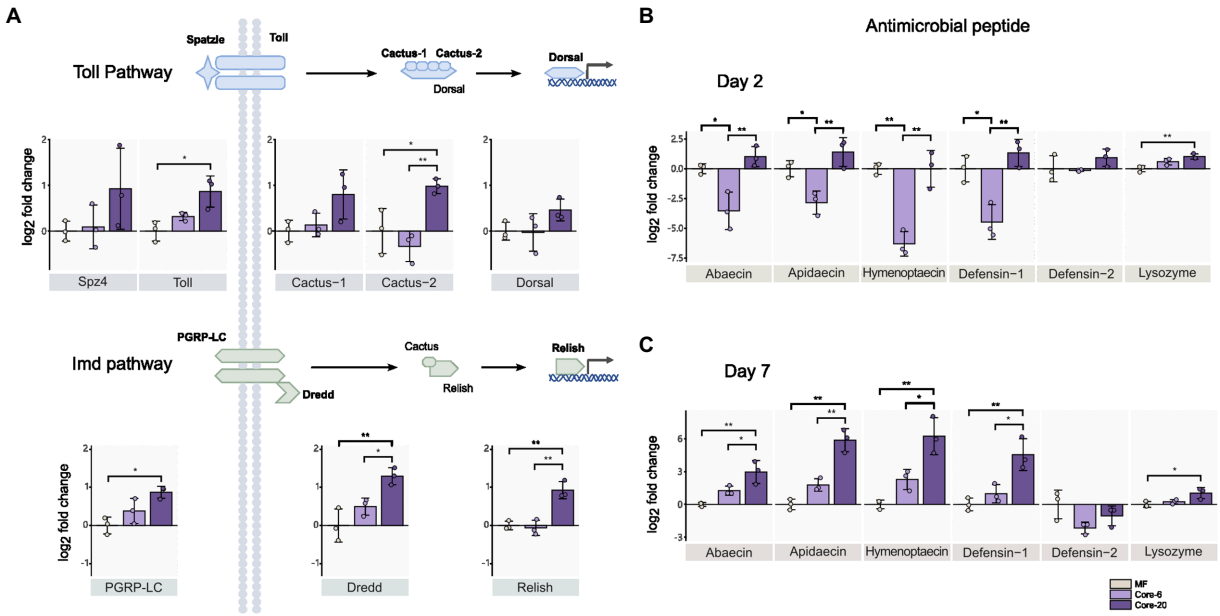


FIGURE 3
Core-20 triggers host immune gene expression in Imd and Toll pathways **(A,B)** at 24 h post-colonization and **(C)** 7 days post-colonization. The Core-20 displayed a significant promotion in regulators of the Toll and Imd pathways on Day 2 and potential ongoing upregulation in AMPs expression on Day 7. Besides, the Core-6 significantly reduced the expression of AMPs on Day 2. All results indicated that the gut microbiota could stimulate the host's innate immune system. Gene expression was normalized relative to the housekeeping gene *actin*. **p* <0.05; ***p* <0.01 (Tukey honest method).

nucleotide sugar biosynthesis (M00554), galactose degradation (M00632), and beta-oxidation (M00086) modules enriched in *Bifidobacterium* strains. In total, the comparison of the Core-6 and Core-20 shows functional similarity. However, there were still several modules enriched in the Core-20, including cobalamin anaerobic biosynthesis (M00924), beta-oxidation (M00087), propanoyl-CoA metabolism (M00741), d-galactonate degradation(M00552), pectin degradation (M00081), and hydroxyproline degradation (M00948). Additionally, we also estimated the complement of KEGG modules for the genome of *H. alvei*, and we found highly overlapping functions with the Core-20 community, indicating its fitness and potential virulence. At the same time, several pathways were found enriched in *H. alvei*, such as glycogen biosynthesis (M00854), undecaprenylphosphate alpha−L−Ara4N biosynthesis (M00761), fumarate reductase (M00150), cysteine biosynthesis (M00338), menaquinone biosynthesis (M00116), ubiquinone biosynthesis (M00117), and multiple pathways of biotin biosynthesis. Overall, we speculated that receptors or products from carbohydrate, fatty acid, and amino acid metabolism could probably display a key role in regulating the immune system.

Next, we investigated the genes specific to the Core-20 but not present in the Core-6, potentially associated with the capacity to trigger the immune system. The comparative analysis found that 3,206 genes unique to the Core-20 were enriched in 1,231 Gene Ontology clusters (Figure 5A). Notably, the enriched GO among all identified clusters was the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS, GO:0009401), a complex enzyme system functioning in the detection, transport, and phosphorylation of various sugar substrates (Kotrba et al., 2001; Gabor et al., 2011). The PTS is comprised of two general cytoplasmic components, enzyme I (EI) and histidine phosphoryl carrier protein (HPr), and membrane-bound sugar-specific multidomain enzymes II (EII). Each EII complex consists of one or two hydrophobic integral membrane domains (domains C and D) and two hydrophilic domains (domains A and B; Figure 5B). Mannose/fructose/sorbose family PTS system was observed, and four genes, including EIIAB, EIIB, EIIC, and EIID, were shared in four strains from the genus *Lactobacillus* Firm-5 (Figure 5C). Interestingly, W8173, W8093, and W8171, three stains specific to the Core-20, harbored their unique clusters of EIIA, EIIB, EIIC, and EIID (Figure 5D). Taken into account that both IIC and IID components of the mannose phosphotransferase system are involved in recognition of antimicrobial peptides (Kjos et al., 2010; Zhou et al., 2016), our results indicated that membrane-bound EII of phosphotransferase system could probably trigger an immune response, causing protection in the Core-20 bees.

Stability transmission of Core-20 community during successive passaging *in vivo*

Due to the potential of the Core-20 to inhibit pathogens and shape the host immune system, we wonder whether the Core-20

FIGURE 4
The presence and completeness of KEGG modules analysis of individual strains, the Core-6, and the Core-20 community. A hierarchical clustering heat map of KEGG module distribution in the draft genomes and artificial metagenomes. The comparison of the Core-6 and Core-20 shows functional similarity. We also found highly overlapping functions between *H. alvei* and the Core-20 community, indicating its fitness and potential virulence. The color code indicates the presence and completeness of each KEGG module, expressed as a value between module complete (dark blue) and module absent (white).

community can stably colonize the honeybee gut over several passages. Microbiota-free bees were inoculated with the frozen mixtures of the Core-20 colony and sampled the whole gut on Day 7. The gut microbiota was mixed and passed on throughout four passages: passage 1 (P1), P2, P3, and P4. At the end of each passage, bacterial communities were sequenced by amplicon

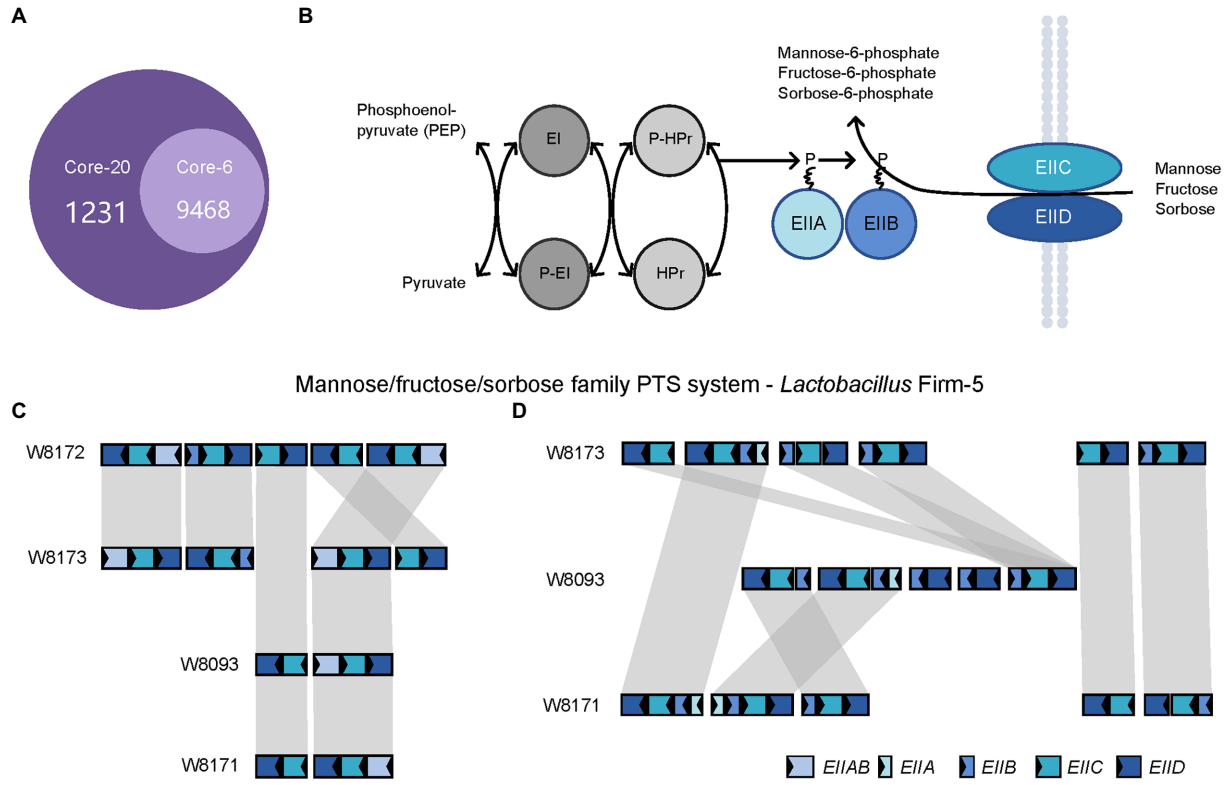


FIGURE 5
The PTS system enriched in the Core-20 community might trigger the immune response offering protection. **(A)** The Venn diagram was generated using OrthoVenn2. Results showed the number of shared orthologous clusters of protein-coding genes between artificial metagenomes. **(B)** Diagrammatic representation of the bacterial phosphotransferase signal transduction pathway (Mannose/fructose/sorbose family PTS system as an example). General phosphoryl and sugar transport reaction catalyzed by the PTS. Sugars are transported and concomitantly phosphorylated by the PTS. **(C,D)** In the genus *Lactobacillus* Firm-5, gene loci of mannose/fructose/sorbose family PTS system **(C)**, shared in 4 strains or **(D)**, unique to strains in the Core-20. Homologous genes are connected by gray bars. We found that W8173, W8093, and W8171, three stains specific to the Core-20, harbored their unique clusters of EIIA, EIIB, EIIC, and EIID, indicating that membrane-bound EII of phosphotransferase system could probably trigger an immune response.

sequencing of the variable regions 3 and 4 of the 16S rRNA gene (Figures 6A,B). All strains except *G.* sp. W8131 were detected individually in bee gut samples among passages, indicating W8131 either is below the detection limit or does not colonize. The relative abundance of *Bifidobacterium*, *Snodgrassella*, and *Apilactobacillus* increased during passaging. Notably, *G. apicola* W14384G12 and *L. melliventris* W8171 were dominant within their genus, respectively. The relative abundance of *Bartonella* was maintained at a relatively stable level during transmission, suggesting the restriction and regulation of honeybee hosts to gut microbiota. We also found an overall decrease in alpha diversity over time across the four passages (Figure 6C, Tukey honest method, $p < 0.05$ for P1-P4, P2-P4) and a significant difference between P1 and the other passages in beta-diversity measured by Bray–Curtis dissimilarity (Figure 6D, PERMANOVA, $p = 0.013$ for P1-P2, $p = 0.004$ for P1-P3, $p = 0.001$ for P1-P4). Our findings indicated that strains of the Core-20 community display stable coexistence after slight fluctuations in species abundances and biomass during P1. In summary, these data suggest that the Core-20 community maintains stability despite fluctuations over the course of passage.

Discussion

While early culture-based studies demonstrated that honeybee gut symbionts could be cultured *in vitro*, induce host immune response, and confer protection against pathogens after inoculation, little is known about the capacity of these isolates deposited in one colony. In this study, we assembled a defined microbial consortium of honeybees (the Core-20 community) based on the phylogeny analysis, which strongly inhibits *H. alvei*. Following exposure, *H. alvei* can grow to high loads (10^9 CFU per gut), produce inflammatory reactions, and potentially result in host mortality. We focused on the expansion of *H. alvei* infection, which is primarily influenced by the gut microbiota, and carried out comprehensive investigations on the mechanism of colonization resistance by the gut microbiota. The Core-20 community could trigger upregulation of AMPs and precise *H. alvei* prevalence, indicating immune priming underlies part of the defined community protective effect. Functions related to carbohydrate utilization and the PTS system were represented in genomic analysis of the Core-20 community, which might play a role in immune

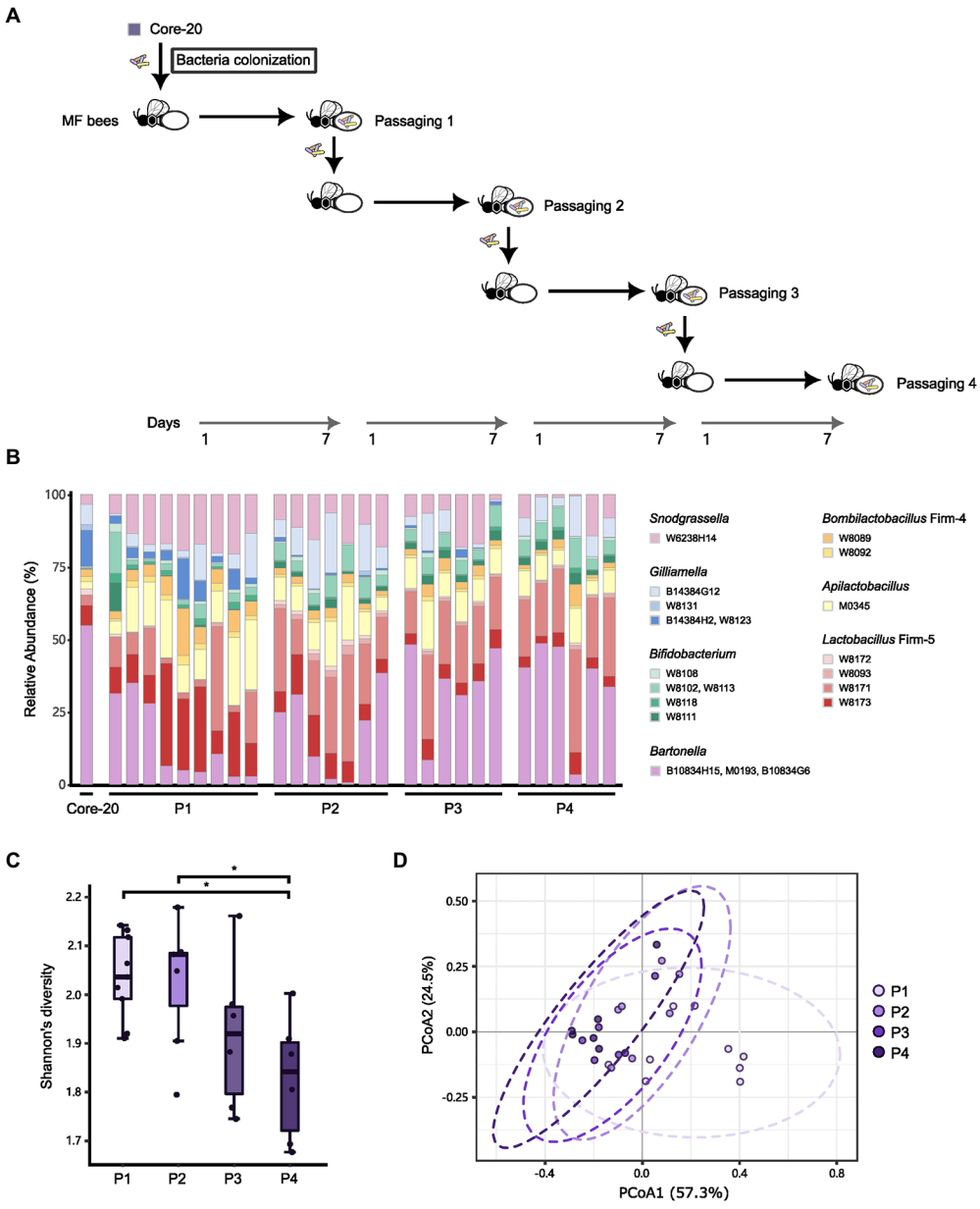


FIGURE 6
The Core-20 community stably colonized honeybees for four passages. **(A)** Experimental design for passaging transmission. **(B)** Relative abundance of strains in the Core-20 during four passages. All strains except *G.* sp. W8131 were detected individually in bee gut samples among passages. **(C)** Box plots of Shannon's alpha diversity index at each passage. Results showed a slight decrease in alpha diversity. **(D)** Principal coordinates analysis (PCoA) plot of Bray–Curtis dissimilarity among samples. We found that the last three passages showed community similarity except for P1.

system stimulation. Additionally, we found that the Core-20 community is able to colonize the honeybee gut over four passages stably. Our findings highlight a defined microbial community could offer protection *via* host–microbe interaction (for example, regulating the host immune system), suggesting that the Core-20 community could be used for gut microbiota research in honeybees.

A major function of the stable gut microbiota is to provide colonization resistance, preventing pathogens from colonizing and causing long-term infection and even mortality. Ghimire et al. identified *Clostridioides difficile*-inhibiting strains through single strain versus pathogen coculture assays *in vitro*. However, when they came to investigate how changes in the combinatorial assembly of bacteria might affect the inhibition capacity, their results demonstrated that new phenotypes masking the individual strain phenotype could emerge depending on the composition of the mix. For instance, bacterial consortia, where all the strains individually showed inhibition, display the enhancement of *C. difficile* growth (Ghimire et al., 2020). Moreover, germ-free mice colonized with

members of the altered Schaedler flora (ASF), a bacterial consortium consisting of eight mouse-derived strains, provided insufficient colonization resistance to *Salmonella enterica* serovar Typhimuriumthe. However, enforced with three facultative anaerobes in Oligo-MM^{12} mice prevent infection completely (Brugiroux et al., 2016). Here, *B. choladocola* B10834H15 from *Bartonella* and *B. choladohabitans* W8113 from *Bifidobacterium* significantly inhibited the growth of *H. alvei*. In previous studies, *Bifidobacterium* of honeybees could produce antimicrobial substances *in vitro* to inhibit other microorganisms, contributing to the resistance of pathogenic bacteria for the host (Forsgren et al., 2010; Vásquez et al., 2012; Butler et al., 2013). In addition, *Bombella apis* has been evidenced to benefit the larval development of honey bees and protect larvae against fungal pathogens (Liu et al., 2022). Notably, the Core-6 community could increase the growth of *H. alvei*. In contrast, single strains and the Core-20 effectively inhibited *H. alvei* (Figure 2C), demonstrating that a defined bacterial community could offer the inhibition capacity as individual strains. The microbe-microbe interaction needs to be concerned with designing defined pathogen-inhibiting bacterial mixtures *in vivo*.

The mechanisms that regulate the ability of the microbiota to restrain pathogen growth are complex, including induction of host immune responses, localization to intestinal niches, and competitive metabolic interactions (Kamada et al., 2013). AMPs can maintain gut microbiota homeostasis by selectively inhibiting foreign bacteria and keeping native symbionts from over-proliferating (Kwong et al., 2017). The synthesis and secretion of AMPs is a highly regulated process, mainly controlled by the Toll and Imd pathways (Lourenço et al., 2013, 2018; Danihlík et al., 2015). Specific gut symbionts, such as *S. alvi*, *A. kunkeei*, *Frischella perrara*, and *L. apis*, have been confirmed to induce honeybee innate immune response. They upregulate the Toll and Imd pathway, leading to AMPs expression (Emery et al., 2017; Daisley et al., 2020b; Lang et al., 2022). Considering that the Core-6 consisted of microbes that were able to induce the immune response, the whole gut microbiota balance composition could be more important for regulating the immune system. The Core-20, a high-species-diversity colony, had more significant upregulation of the immune regulatory genes and AMPs genes encoding abaecin, apidaecin, hymenoptaecin, and defensin-1 (Figure 3), suggesting the ability of the Core-20 community in stimulating host innate immune system through their regulators and effectors.

Biofilm and the outer membrane protein, such as the S-layer protein unique to *L. apis* W8172, could be potential drivers of the host immune response. We used KEGG modules to character gene sets linked to specific metabolic capacities and OrthoVeen2 to compare and annotate orthologous gene clusters among multiple genomes (Figures 4, 5). Results showed that the PTS system was significantly enriched in the Core-20 community. The PTS system is a highly conserved phosphotransfer cascade whose components modulate many cellular functions in response to carbohydrate availability (Houot et al., 2010). Previous studies have elucidated the importance of bacterial PTS system for honeybees, including detoxifying specific nectar components (Engel and Moran, 2013a), nutrient metabolic transformations (Lee et al., 2015), and adaptation to the diet and gut environment of the honeybee. PTS system of *Enterococcus faecalis* could increase proinflammatory cytokine secretion by colon tissue and macrophages to enhance colonization in mice (Fan et al., 2019). Besides, the PTS system of *Vibrio cholerae* display control of carbohydrate transport and activation of biofilm formation on abiotic surfaces (Houot et al., 2010). Additionally, EIIC and EIID from the mannose/fructose/sorbose family PTS system, the membrane-banding proteins, is responsible for specific targeting by antimicrobial peptides, indicating their potential to regulate the immune system (Diep et al., 2007; Kjos et al., 2010; Zhou et al., 2016).

According to Rolf Freter's nutrient niche theory, a pathogen can only invade if it is able to use a specific limiting nutrient more efficiently than the rest of the community, which means colonization resistance against pathogens is affected by efficient restriction of all available nutrient niches by a complex microbial community (Freter et al., 1983). Invasion theory Figures out that biotic selection could be the critical determinant (Dillon et al., 2005; van Elsas et al., 2012; Mallon et al., 2015; Ketola et al., 2017). Higher diversity communities can competitively exclude an invader by reducing the availability of ecological niches and efficiently utilizing resources (Hromada et al., 2021). Thus, the protective effect is probably provided through antagonism between microbes (Chiu et al., 2017; Ubeda et al., 2017). In the case of an animal pathogen, three facultative anaerobes potentially prevent infection in Oligo-MM^{12} mice by filling up the niche space that is preferred by *S.* Tm (Brugiroux et al., 2016). Previous studies showed that *H. alvei* reduced nitrates and fermented l-arabinose, glycerol, maltose, d-mannitol, d-mannose, l-rhamnose, trehalose, and d-xylose (Møller, 1954; Janda et al., 2005; Tian and Moran, 2016; Erban et al., 2017). Genomic analysis reveals that *H. alvei* harbors various carbohydrate degradation modules and has similar functions as the Core-20 (Figure 4), suggesting its ability to grow in the honeybee gut and compete for multiple carbohydrates. *Gilliamella*, a primary polysaccharide degrader in the honeybee gut, utilizes mannose, arabinose, xylose, or rhamnose (monosaccharides that can cause toxicity in bees; Zheng et al., 2016, 2017, 2019). Functions for carbohydrate use and PTS systems are represented in genomic analysis of the Core-20 community, which may also promote colonization resistance by competition for limited nutrients that *H. alvei* presumably depends on. Our findings implied that protection by the *Gilliamella* and the Core-20 bees occurs *via* occupation of niche space (for example, consumption of carbohydrates) that can no longer be exploited by *H. alvei*. Loss of microbial diversity might create ecological niches that pathogens can use, underlying why bees colonized with low-complexity gut microbiota, such as the Core-6, are more susceptible to *H. alvei* infection. Whereas, because the Core-20 had 14 strains more than the Core-6, it is conceivable that the Core-20 community could

actually fill up the niche space that is preferred by *H. alvei* and thereby prevent infection.

The honey bee gut microbiota is dominated by limited numbers of bacterial phylotypes, commonly with species from the *Gilliamella*, *Snodgrassella*, *Lactobacillus* Firm-5, *Bombilactobacillus* Firm-4, *Bifidobacterium*, and *Bartonella* genera. Gut microbial communities influence host health in many ways, including food digestion, defense against pathogens, and modulation of behavior, development, and immunity (Engel and Moran, 2013a,b). Therefore, dysbiosis (microbial imbalance) may impact honeybee health and susceptibility to disease. Honeybees treated with tetracycline severely altered both the size and composition of the gut microbiome, decreasing the survival rate of bees and increasing susceptibility to opportunistic pathogens (Raymann et al., 2017; Lang et al., 2022). Here, the Core-20 consisted of typical isolates representing species in honeybee gut microbiota, which demonstrated transmission stability and functional redundancy during passages. Potentially, consequences of dysbiosis, such as nutritional impacts or heightened susceptibility to toxins, could be reduced through the development of alternative treatment methods, for example, adding the Core-20 to the bee hive.

In conclusion, we have assembled a minimal community of 20 bacterial strains that provided colonization resistance against *H. alvei*, elucidating the underlying molecular and functional mechanisms. The native gut symbionts are essential in the resistance to pathogen invasion. Such strain collections can yield insights into host-microbiota interactions, hoping to offer solutions to protect honeybees from pathogen infection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JW and HZ designed the research. JW, HL, and WZ collected the samples. JW, HL, and WZ performed the experiments and analyzed the data with contributions from YZ, LZ, HL, and YL. JW and HZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1074153/full#supplementary-material

SUPPLEMENTARY TABLE S1
qPCR primer sequences for gene expression and bacterial load.

SUPPLEMENTARY FIGURE S1
Differences of the V3–V4 region of 16S rRNA genes of strains in the relevant genus. Our results indicated the feasibility of using 16S rRNA genes V3–4 region for species classification.

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Experimental inheritance of antibiotic acquired dysbiosis affects host phenotypes across generations

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Microbiomes can enhance the health, fitness and even evolutionary potential of their hosts. Many organisms propagate favorable microbiomes fully or partially *via* vertical transmission. In the long term, such co-propagation can lead to the evolution of specialized microbiomes and functional interdependencies with the host. However, microbiomes are vulnerable to environmental stressors, particularly anthropogenic disturbance such as antibiotics, resulting in dysbiosis. In cases where microbiome transmission occurs, a disrupted microbiome may then become a contagious pathology causing harm to the host across generations. We tested this hypothesis using the specialized socially transmitted gut microbiome of honey bees as a model system. By experimentally passaging tetracycline-treated microbiomes across worker 'generations' we found that an environmentally acquired dysbiotic phenotype is heritable. As expected, the antibiotic treatment disrupted the microbiome, eliminating several common and functionally important taxa and strains. When transmitted, the dysbiotic microbiome harmed the host in subsequent generations. Particularly, naïve bees receiving antibiotic-altered microbiomes died at higher rates when challenged with further antibiotic stress. Bees with inherited dysbiotic microbiomes showed alterations in gene expression linked to metabolism and immunity, among other pathways, suggesting effects on host physiology. These results indicate that there is a possibility that sublethal exposure to chemical stressors, such as antibiotics, may cause long-lasting changes to functional host-microbiome relationships, possibly weakening the host's progeny in the face of future ecological challenges. Future studies under natural conditions would be important to examine the extent to which negative microbiome-mediated phenotypes could indeed be heritable and what role this may play in the ongoing loss of biodiversity.

KEYWORDS

microbiome, antibiotics, honey bees, experiments, dysbiosis, transgenerational effects

Introduction

The Anthropocene provides many novel selection pressures on organisms, such as climate change and the application of agrochemicals and antibiotics (Sánchez-Bayo and Tennekes, 2017; Cavicchioli et al., 2019). Organisms respond in various ways to these pressures, ranging from the evolution of resistance to extinction. When animals are exposed to nutritional disturbance (e.g., by chemicals), in addition to potential direct effects on the organism itself, their gut microbiome may be affected. Dwelling at the interface between host epithelia and the external environment, microbial symbionts (microbiomes) can affect host health by influencing traits such as nutrition, immunity and behavior (Round and Mazmanian, 2009; Flint et al., 2012; Tremaroli and Bäckhed, 2012). Microbial communities can change rapidly in composition or in gene-expression patterns when responding to ecological forces. Therefore, a microbiome can extend host evolutionary potential and may facilitate rapid host acclimation to environmental change (Alberdi et al., 2016; Henry et al., 2021). Specific gut microbial communities can provide hosts with novel functions, such as mediating insecticide resistance (Kikuchi et al., 2012; Wang et al., 2020) or promoting tolerance to thermal stress (Zare et al., 2018; Zhang et al., 2019; Raza et al., 2020). Such microbial rescue effects have the potential to stabilize host dynamics and may explain population persistence in changing environments (Mueller et al., 2020). Due to the wide range of functional benefits they provide, microbiomes are often tightly curated by the host, for example by management and vertical transmission between generations (Foster et al., 2017; Rosenberg and Zilber-Rosenberg, 2021). In general, transmission of microbiomes across generations will transmit the community and its associated functions – which may be positive or negative for the host depending on the conditions.

Indeed, a microbiome is not always beneficial for the host. Some organisms even completely lack it (Hammer et al., 2019) and the functional benefit provided by a microbiome may also be dependent on environmental conditions. For example experiments in mice show that adapted microbiomes efficiently harvest energy from food but causing obesity in recipient individuals when being transferred (Turnbaugh et al., 2006). While such efficiency may be beneficial under food restriction, it could lead to health problems in times of plenty. Importantly, evolved cooperation between hosts and symbionts can result in wide reciprocal functional inter-dependencies. In such cases, disturbances to the microbiome can compromise host health and development by, e.g., loss of important microbiome-mediated functions, or microbial production of harmful substances as a response to environmental change (Littman and Pamer, 2011; Soen, 2014). As a result, vertical transfer of such sub-optimal microbiomes could compromise host health transgenerationally. Hypothetically, in extreme cases, a host population that is unable to escape a mal-adapted microbiome may face extinction.

Dysbiotic (defined by a loss of beneficial microbes, expansion of pathobionts or loss of diversity of the healthy, homeostatic gut condition (Petersen and Round, 2014)) parental microbiomes can affect the microbiome composition and phenotypes of offspring across systems. For example, female mice inoculated with antibiotic-disturbed microbiomes will transfer this dysbiosis to the offspring causing enhanced colitis (Schulfer et al., 2018). In fish, chemical exposure causes dysbiosis which persists in F1 offspring with correlating intestinal problems (Chen et al., 2018) and even result in alterations in the F2 intestinal epigenome, transcriptome and morphology (Guzman, 2021). Diet induced microbiome changes modulate transgenerational cancer risk in mice (Poutahidis et al., 2015). In addition, another interesting study in flies showed antibiotic-mediated depletion of a commensal bacterial genus can cause non-Mendelian, transgenerational inheritance of a stress-induced phenotype (Fridmann-Sirkis et al., 2014).

By their design, antibiotics pose particular threats to microbiomes. Antibiotic pollution is omnipresent in ecosystems due to heavy usage in medicine and agriculture (Kraemer et al., 2019) and they are known to decrease microbial diversity, to compromise host-microbiome interactions, to weaken immune system homeostasis (Modi et al., 2014) and impair colonization resistance (Bäumler and Sperandio, 2016). Still so far, the focus in most studies on stress factor effects on microbiomes usually lays on immediate effects during an individual's life (Francino, 2016), and in such cases direct effects of stressors on the host cannot clearly be disentangled from indirect effects *via* a disturbed gut microbiome.

Here, we set out to examine whether the deleterious effects of a disrupted microbiome can persist transgenerationally, using honey bees as a tractable model system. Honey bee microbiomes are socially transmitted between worker 'generations', whereby newly eclosed workers acquire microbiomes from their colony-mates and the direct hive environment. While this is a different vertical transmission approach from the classical parent-to-offspring one, it was successfully leading to strong co-evolution between corbiculate bees and their microbiomes (Koch et al., 2013; Kwong et al., 2017). The adult honey bee microbiome consists of ~8 bacterial phylotypes that are involved in key biological functions such as nutrition, digestion, and immunity (Engel et al., 2016; Emery et al., 2017; Kešnerová et al., 2017; Raymann and Moran, 2018). Because young adults emerge from pupation without a microbiome, they can reliably be inoculated with a microbiome of choice in the lab (Powell et al., 2014; Zheng et al., 2018; Kowallik and Mikheyev, 2021). Thus, it is possible to serially transfer microbiomes across worker 'generations' to study how microbial changes in response to environmental stressors affect host phenotypes and health. In addition, honey bees are important pollinators and are exposed to diverse chemicals in the agricultural landscape as well as by beekeepers. It could be shown that antibiotics have strong effects on the honey bee microbiome (Powell et al., 2021; Tian et al., 2012; Moullan et al., 2015; Li et al., 2017; Raymann et al., 2017; Baffoni et al., 2021; Jia et al., 2022) and that such dysbiosis can even be experimentally transferred between workers (Jia et al., 2022).

In our study we used controlled lab experiments passaging microbiomes affected by antibiotics from one worker cohort to the next and examined mediated effects on host physiology by exposing naïve bees receiving these microbiomes to high levels of antibiotic stress. This design allowed us to isolate changes in the microbiome from host responses and from environmental changes. We found that the microbiome was disturbed after antibiotic exposure leading to compositional and functional changes. These were both transmitted to subsequent host generations, leading to some changes in host gene expression and to high mortality under stress.

Materials and methods

To test how honey bee microbiomes respond under antibiotic pressure and how this affects host phenotypes across generations, we conducted experiments in which microbiomes were transferred over two host cycles (worker "generations") under sub-lethal chemical administration. In the third cycle, to examine whether past chemical exposure affects host survival, we applied lethal levels of the chemicals to which prior "generations" had been exposed. We quantified changes in both host gene expression and microbial composition using RNA-seq and 16S amplicon sequencing, respectively.

Experimental setup

The first experiment (Figure 1) was conducted in February/March 2019 at Australian National University in Canberra, Australia. See also the Supplementary information for more methodological details. The same, chemically untreated *Apis mellifera ligustica* colony was used throughout the whole experiment to avoid host genetic background changes. We started with a cohort of microbiome depleted individuals of the same age in each cycle. Late-stage pupae (dark eyes but lacking movement) were carefully removed from brood frames and allowed to develop under sterile conditions in the lab. Workers eclosing within 24 h were randomly distributed into six cages (three independent cages per treatment with ~25 bees/cage) and provided with filter-sterilized 0.5 M sucrose solution (Supplementary Figure S1). When all bees were distributed, the sucrose feeders were replaced with sterile sucrose or antibiotic-infused sucrose. We used a tetracycline hydrochloride concentration previously published in a honey bee microbiome study (450 µg tetracycline / mL sucrose (Raymann et al., 2017)). Concurrently, 10 nurse bees from the same hive were surface sterilized, and their dissected hindguts were macerated in 1:1 PBS/sucrose solution, mixed with gamma-irradiated bee bread (previously collected from colonies from the same apiary and then sterilized with 35kGY) and equally distributed across all six cages. On the following day, the remaining food was discarded and the microbiome feeding method was repeated for a second time for 24 h using again 10 nurse bee guts. On both days, small amounts of the microbiome pools were kept for later determination of the start microbiome. After the inoculation period the bees received sterile pollen and sucrose with or without antibiotics. Daily, the tetracycline solution was freshly prepared, dead bees were removed and fresh sucrose and sterile bee bread were offered *ad libitum*. Bees were maintained under these conditions for 6 days in cycle one and 10 days in cycle two, differences due to the need to have enough pupae of the same age and hive background ready for the next cycle. However, the aim was to provide enough time that the microbiome can be fully established. We previously experienced that when newly emerged bees receive a microbiome pool for 48 h, they show the full adult bee microbiome in composition and abundance after 7 days (Kowallik and Mikheyev, 2021). It is also known that under natural conditions, adult bees get colonized within the first 2 days after emergence which is followed by rapid establishment within 4 to 6 days post-eclosion (Powell et al., 2014). We therefore gave a minimum of 6 days to allow inoculation, internal growth and establishment of the microbiome. For microbiome transfer in cycle two the newly emerged bees received the microbiome from the previous cycle to mimic

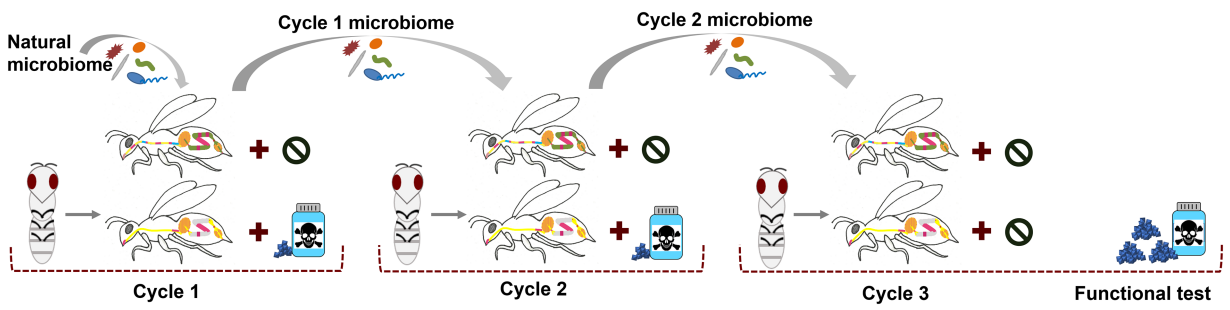

FIGURE 1
Design of the main experiment. Pupae emerge in the lab and are first inoculated for 48h with a natural microbiome from hive siblings. Three cages per treatment were used. Throughout cycle 1 and 2, bees are continuously fed with sterile pollen and sucrose containing tetracycline or not (control). These exposed and control microbiome communities get passed to the following cycle of lab-emerged bees (cage to cage transfer). In cycle 3, bees that received control or pre-exposed microbiomes are kept naïve toward the chemical until they are administered high doses of tetracycline at the end.

generation-spanning microbiome transmission. For this, three bees in each cage were sacrificed, surface sterilized, and their dissected hindguts were mixed with sterile pollen and administered to one bee cage of the next cycle for 48 h (cage to cage transfer provided three independent cage replicates per treatment). We always kept small amounts of these transfer pools for later sequencing. All other surviving bees in each cage at the end of cycle 1 and 2, as well as small amounts of the gut transfer pools were snap-frozen in liquid nitrogen and stored in a −80° C freezer until further processing. In the beginning of the third cycle, control and exposed microbiomes from the previous cycle were transferred again to newly emerged bees as stated above. However, in cycle three, all cages received sterile food without toxins for 6 days. On day six, three individuals per cage were collected and snap frozen to examine the established microbiome community ("cycle 3 before stress") at this time point. Subsequently, all cages were then challenged with a high dose of the stressor (20 mg tetracycline per mL sucrose), a concentration identified to cause 50% mortality in 24 h (LD50) during a pilot study (see Supplementary Methods). Due to the high mortality in the "exposed microbiome" cages, we counted survival after 20 h, with the surviving bees ("cycle 3 after stress") being snap-frozen and stored at −80° C until further extractions.

We calculated the survival proportion for each day of the experiment before high stress application and plotted the mean of the three cages for both treatments for each cycle with standard deviations. To compare the control and tetracycline treatment we performed two-sided Fisher's exact tests on alive/dead count data of the three cages for each day. For statistical analysis of the final survival data after high stress application, we used a Bayesian logistic regression approach to examine effects of past chemical exposure on survival in the face of lethal stress levels. To account for between-cage heterogeneity within treatments, we first estimated mortality levels for each cage regardless of treatment (survival ~ cage) using the *brms* package (Bürkner, 2017). We chose standard minimally informative priors and verified adequate model performance using diagnostic plots and statistics provided by the package. We then tested the hypothesis that cage mortality coefficients were the same in control vs. experimental treatment, using the *brms* hypothesis function, which computes the posterior distribution of the difference between Bayes factor levels in the contrast. This approach parallels planned linear contrasts in regression analysis. In addition, we conducted a non-parametric analysis using two-sided Fisher's exact tests on alive/dead count data (altogether 53 control-gut and 47 tetracycline-gut individuals).

Mechanisms underlying phenotypic effects of tetracycline-exposed microbiome transfer

To exclude leftover tetracycline or derived by-products inside the transferred guts as proximal drivers of stress-induced mortality we ran an additional control experiment. In March 2021 in Okinawa Japan, we started the experiment as described before by grafting pupae. Experimental procedures were generally identical to the previous experiment. After sterile emergence, bees were distributed equally to eight cages with ~28 bees each. Microbiome transfer from nurse bees of the same hive was done as before. Four cages received tetracycline and the other sterile food only. After 6 days, the volume of four macerated guts (one more to account for any loss in the filter) per cage was filtered using a 0.2 um syringe filter to remove microbial cells. After surface-sterilizing and dissecting 20 nurse bees from the same colony, we pooled the hindguts to receive a healthy microbiome pool as base for the next cycle's bees. This pool was equally split into eight parts, and each got mixed with the filtered gut solution of one cage from cycle 1 (Figure 2). For the next cycle, this resulted in four cages of microbiome + filtered control (supernatant of cycle 1 bee guts receiving sterile food) and four cages of microbiome + filtered tetracycline-exposed (supernatant of cycle 1 tetracycline-exposed guts) solution. All bees received sterile food for 6 days and high tetracycline dose on day six. After 15 h, mortality was recorded. The same statistical approach as described above was used by applying Bayesian logistic regression and Fisher's exact tests (N = 4 cages; altogether 60 control-filter-gut and 50 tetracycline-filter-gut individuals).

Extractions and sequencing

For extractions of bees from the first experiment we used the Qiagen AllPrep PowerFecal DNA/RNA Kit on abdomens of frozen bees. Every bee was first rinsed with ethanol and three subsequent rinsing steps in sterile water to clean the surfaces and then the whole abdomen or the microbiome transfers were processed following the recommended settings of the protocols, including bead beating using the Geno/Grinder®. DNA was eluted in 30 µl TE buffer. For 16S sequencing we examined the microbial community composition of 75 samples. These were one sample of start microbiome composed of the nurse microbiome pool (day 1 and day 2 pooled together), six nurse bees from the same hive as natural controls, two ZymoResearch Mock DNA controls, 12 microbiome transfer pools (one for each cage being composed of three pooled guts) for the cycle to cycle microbiome transfers in the beginning of cycle 2 and 3 (=24 pools together). In addition, we sequenced 54 individual bee abdomen from four different time points during the experiment (end of cycle 1 (9 control, 3 tetracycline), end of cycle 2 (9 control, 9 tetracycline), cycle 3 before high tetracycline application (9 control, 9 tetracycline) and after (8 control, 1 tetracycline)). We aimed to sequence three individuals per cage and time point, however, as the number of sampled individuals relied on the numbers of bees surviving, minus the ones used for gut transfer and sometimes a dissection may have gone wrong or a bee escaped, we ended up with fewer numbers of sequenced samples in some cases.

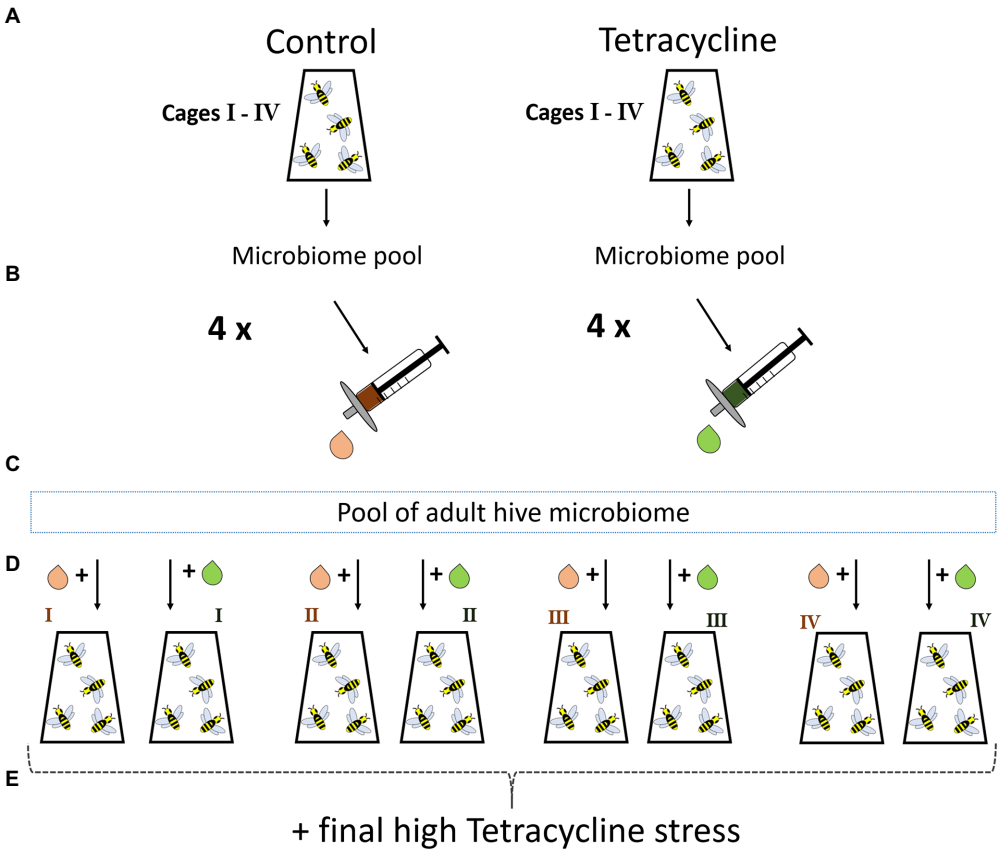

FIGURE 2
Control experiment with filtered gut solution. Emerged bees with transferred natural nurse microbiome are raised in four cages with control or tetracycline diet for 6 days **(A)**. On day six a bee gut pool for each cage is prepared as done in the first experiment and filtered to exclude microbes but to keep all potential tetracycline and derivates potentially present in the guts **(B)**. A microbiome pool of hive sibling guts is generated to allow a healthy background microbiome for newly emerged bees for the next cycle **(C)**. This microbiome pool is equally split and each part gets mixed with the filtrate of one control or tetracycline cage **(D)**. The bees receive sterile food for 6 days and are exposed to high tetracycline stress in the end and mortality is recorded **(E)**.

DNA of samples was submitted to DNA Sequencing Section at the Ramaciotti Centre for Genomics in Sydney Australia. Library preparation was performed based on Illumina protocol with 25-µl reactions. Illumina barcoded primers (Klindworth et al., 2013) were used to create a single amplicon of approximately 460 bp encompassing the V3-V4 region of bacterial 16S rRNA. Samples were pooled to equimolar concentration and sequenced on Illumina MiSeq v3 2 × 300 bp platform. Reads were demultiplexed on the basis of barcode sequences, allowing for one mismatch.

16S amplicon sequence analysis

Demultiplexed reads were processed using QIIME2 version 2019.1 (Bolyen et al., 2019), denoising of the fastq files was performed using the denoise-paired command from the DADA2 software package (Callahan et al., 2016), wrapped in QIIME2, including removal of chimeras using the "consensus" method. Decreased quality scores (below 20) of the sequences at the beginning to remove primers and end were truncated (trim-left-*f* = 17, trim-left-r = 21, trunc-len-*f* = 275, trunc-len-r 225). This resulted in a remaining overlap of ~40 bases in merged sequences. The result is an amplicon sequence variant (ASV) table, a higher-resolution analog of the traditional OTU table. For taxonomic assignment, the QIIME2 q2-feature-classifier plugin (Bokulich et al., 2018) and the Naïve Bayes classifier (Wang et al., 2007), which we trained with our primers previously, were used on the SILVA release 132 (Quast et al., 2013; Yilmaz et al., 2014).

All following graphical and statistical comparisons were performed in R using the phyloseq package (McMurdie and Holmes, 2013). In short, we first removed all non-bacterial sequences, mitochondrial and chloroplast sequences, and ASVs not present in any sample (likely artifacts) from the datasets using the *subset_taxa* and *prune_taxa* functions. We plotted rarefaction curves of all samples using the ranacapa function *ggrare* (Kandlikar et al., 2018) on the minimum sample depths (12,351 reads). Alpha diversity of the rarefied samples was explored by plotting Observed species numbers and Shannon's diversity index. Pairwise, two-sided Wilcoxon rank sum tests were used to test for

significant alpha diversity differences between treatments in each cycle. As rarefying sample counts is not recommended, unless necessary, (McMurdie and Holmes, 2014) we converted data to proportions for normalization purposes. On these proportions, non-metric Multidimensional Scaling (NMDS) was performed on Bray-Curtis distances for ordination plots.

To test for variation within groups, we used the *betadisper* function in the Vegan package, version 2.5–5 in R on the Bray-Curtis distance matrix on proportion data to calculate distances to group centroids per treatment for each cycle. Subsequently, output was plotted as ordination for visualization and *permutest* was run for each cycle to check for homogeneous distribution of samples across the two treatments. Multifactor permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis distances with 999 permutations using the ADONIS function were performed to test for effects of experimental factors on the gut community. As we sequenced single bees as well as microbiome transfers after each cycle, we first tested whether there is a difference according to method for each treatment. We also tested for cage effects in the data set in each cycle and treatment. In addition, we compared each treatment against the respective controls for all 3 cycles. Finally, we tested whether the microbiomes of each treatment changed across cycles. For taxonomic visualization we plotted the relative abundances of all genera accounting for at least 1% of the abundance across treatments and cycles. We then extracted the seven dominant taxa from the rarefied sample set and plotted their individual, total abundances across cycles with subsequent two-sided Wilcoxon rank sum tests between treatments and the respective controls. To further investigate response variation in species as well as ASV level (also see Supplemental results for more details), we pooled all cycles after checking that no cycle-specific differences could be observed and extracted the abundant species for each core genus (>1,000 reads) and plotted their abundances across the two treatments. We used online megablast against the full NCBI Nucleotide collection database on abundant ASVs (>1,000 reads) for each genus for better taxonomic resolution (sequences and alignment output in supplements). Similarly, we also plotted the total abundances of ASVs across the two treatments.

RNA-sequencing and analysis

To understand the molecular basis of physiological effects that the microbiome's antibiotic treatment history has on hosts, we conducted RNA-sequencing of six honey bees in cycle 3 before high stress application. We sequenced one individual per cage (three per treatment), comparing bees with tetracycline-stressed and control microbiomes.

For RNA library preparation, the QIAseq® Stranded mRNA Select Kit was used following the standard protocol. Sequencing was done on a Nextseq 2000 with V2 75 cycles (75-bp Single Read). Reads were quantified using *kallisto* (Bray et al., 2016) with the honey bee transcriptome (version Amel_HAv3.1) as a reference, using default parameters. The R package DESeq2 was used to normalize and determine which genes were differentially expressed among control and treatment samples, setting the control group as reference to be compared against. Genes were considered differentially expressed at an FDR adjusted *value of p* <0.05. To visualize the differences in expression profile between the samples, the *plotPCA* function in DESeq2 was used to generate principal component analyses. MA plots visualizing base-2 log fold-change (LFC) (y-axis) versus normalized mean expression (x-axis) in the tetracycline treatment against the control were plotted using the *ggmaplot* function on previously shrinked effect sizes using the *lfcShrink* function for better visualization and ranking of genes. To study the amount by which each of the significantly different determined genes deviates in a specific sample from the gene's average across all samples we created a heatmap using the *pheatmap* function on regularized logarithm *rlog()* transformed data. Gene ontology (GO) enrichment analysis of the significantly differentially expressed genes were carried out using GOstats, GSEABase and Category R packages (Falcon and Gentleman, 2007). Biological processes associated with these GO terms were summarized and visualized using REVIGO (Supek et al., 2011).[1] The semantic similarity was measured using the Resnik's measure (SimRel) (Resnik, 1999) and the threshold used was C = 0.7 (medium). The results were then used to produce a scatter plot using the ggplot2 package in R.

Results

Microbiomes affect bee immunity and survival under high toxin stress

Bee guts were transferred three times to new hosts after exposure to sub-lethal doses of tetracycline. Bee survival during the 3 cycles showed higher mortality under tetracycline in all cycles in comparison to respective control (Supplementary Figure S2). At the end of these transfers, in cycle 3, naïve recipient bees were given lethal doses of the tetracycline. Survival was compared between bees receiving chemical-exposed microbiomes and those receiving unexposed control microbiomes. The microbiomes with previous tetracycline exposure significantly decreased the survival of the host bees (Bayes Factor (BF) comparing survival in control vs. dysbiotic treatments 95% CI -26.84 – −4.38) (−34% survival, $p < 0.001$, *Fisher's exact test* on alive/dead count data) (Figure 3).

We further experimentally investigated if the microbiome itself or rather tetracycline residues inside the transferred guts affected the bee survival. We found no support for the latter hypothesis, as the filtered gut solutions did not decrease survival under high stress (BF 95% CI -4.14 – 3.88) (*Fisher exact test*, $p = 0.64$).

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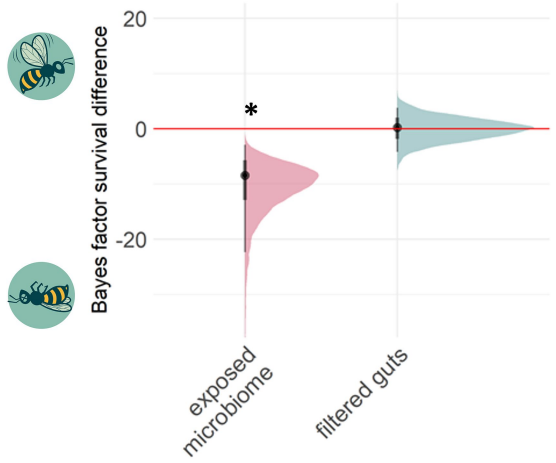

FIGURE 3
Past chemical exposure of a microbiome can affect future host survival. The Bayes factor difference between treatment and control groups measures whether survival in treatments was higher (positive axis) or lower (negative) under a high dose of tetracycline relative to the respective control group. 95% posterior distribution confidence intervals lying outside zero are highlighted by asterisks. Transferring tetracycline pre-exposed guts (N=3 cages; altogether 53 control-gut and 47 tetracycline-gut individuals) negatively affected bee health under high stress, while transferring filtered gut solution together with a healthy adult microbiome (N=4 cages; altogether 60 control-filter-gut and 50 tetracycline-filter-gut individuals) did not affect the survival.

Tetracycline affects the bacterial community composition

Challenging bees with tetracycline over two cycles ("worker generations"), affected microbial community composition. We examined the gut microbial community composition of 54 individual bees from four different time points during the experiment as well as six hive nurse bees, the start microbiome, 12 microbiome transfer samples and two mock DNA controls. The V3-V4 region of the bacterial 16SrRNA gene was amplified and sequenced on the Illumina MiSeq platform, generating an average of 30,462 reads per sample (range, 14,253 to 65,293). The total number of ASVs was reduced from 1717 to 460 after filtering out mitochondria, chloroplasts, artifacts and reads not assigning to the kingdom Bacteria. The two mock community control DNA samples (ZymoResearch cat D6306) sequenced in this study showed no qualitative differences compared to expected theoretical proportions provided by the mock community manufacturer (Supplementary Figure S3). ASVs matching non-mock taxa belonged to honey bee core symbionts but accounted for only 0.23% of the abundance, representing neglectable cross-contamination during library preparation or sequencing. Rarefaction plots on the minimum sample count (Supplementary Figure S4) show quickly reaching converged lines in all samples, indicating sufficient depth. We observed no significant differences between whole bee and microbiome transfer samples for control as well as tetracycline treatments (PERMANOVA; control: $p = 0.52$, $R^2 = 0.03$, $F = 0.75$; tetracycline: $p = 0.55$, $R^2 = 0.03$, $F = 0.72$). Based on these results we continued analyzing the transfer and bee samples together.

Microbial alpha diversity was much lower in the tetracycline treated individuals at all time points, as measured with the Shannon index (Figure 4) and numbers of observed species (Supplementary Figure S5). This effect could be seen using Non-metric Multidimensional Scaling (NMDS) with tetracycline-treated samples being distinct from control samples (Figure 4). PERMANOVA on Bray-Curtis distances identified tetracycline-stressed microbiomes as being significantly different from controls (cycle 1: $p = 0.003$, $F = 31.2$, $R^2 = 0.71$; cycle 2: $p < 0.001$, $F = 62.5$, $R^2 = 0.74$; cycle 3; $p < 0.001$, $F = 41$, $R2 = 0.72$). Treatments did show significant effects on groups dispersion in cycle 1 (permutest; $p < 0.001$, $F = 11.4$), and 3 ($p = 0.01$, $F = 8.5$) but not in cycle 2 ($p = 0.64$, $F = 0.19$) (Supplementary Figure S6) indicating that high dispersion may affect the PERMANOVA statistical output.

At the end of the first cycle, several bacterial core genera disappeared from guts of antibiotic-fed bees, namely *Frischella, Bartonella, Snodgrassella* and *Commensalibacter* (Figure 5). The abundances of almost all core symbionts were significantly affected by tetracycline (Supplementary Figure S9 and Supplementary Table S1 for stats). On a finer scale, we observed in several bacterial species some ASVs being susceptible to antibiotic treatment and getting eliminated, while others were unaffected or even increased in relative abundance (Supplementary Figure S10).

Tetracycline affected microbial communities affect host gene expression

We sequenced mRNA of one honey bee per cage (three per treatment and control respectively) in cycle three before high stress application, with an average of 99.5 million (min 4.8 million, max 567 million) raw reads. While most of these reads mapped to bees, the pathogen Nosema could be detected as a higher percentage of the control reads (0.35, 0.95, 0.11 percent aligned) in comparison to the tetracycline treated bees (0, 0, 0.04 percent aligned) in the taxonomy analysis of NCBI on the submitted raw reads. The pseudoalignment rates of the samples were 64 ± 5.1% (s.d.).

Differential gene expression analysis showed that receiving the antibiotic-disturbed microbiomes affects host gene expression. Altogether 30 genes were significantly differently expressed ($p > 0.05$) after FDR adjustment for multiple comparisons (Figure 6). Surprisingly, only three genes were down-regulated and are mainly involved in lipid metabolism such as *phospholipase A2-like* (LOC724436) and *fatty acyl-CoA reductase 1* (LOC724560). Some of the up-regulated genes have likely functions in immunity such as *apidermin 1* (GeneID_551367) or *lysozyme-like* (LOC113218576), transport activities, e.g., NPC

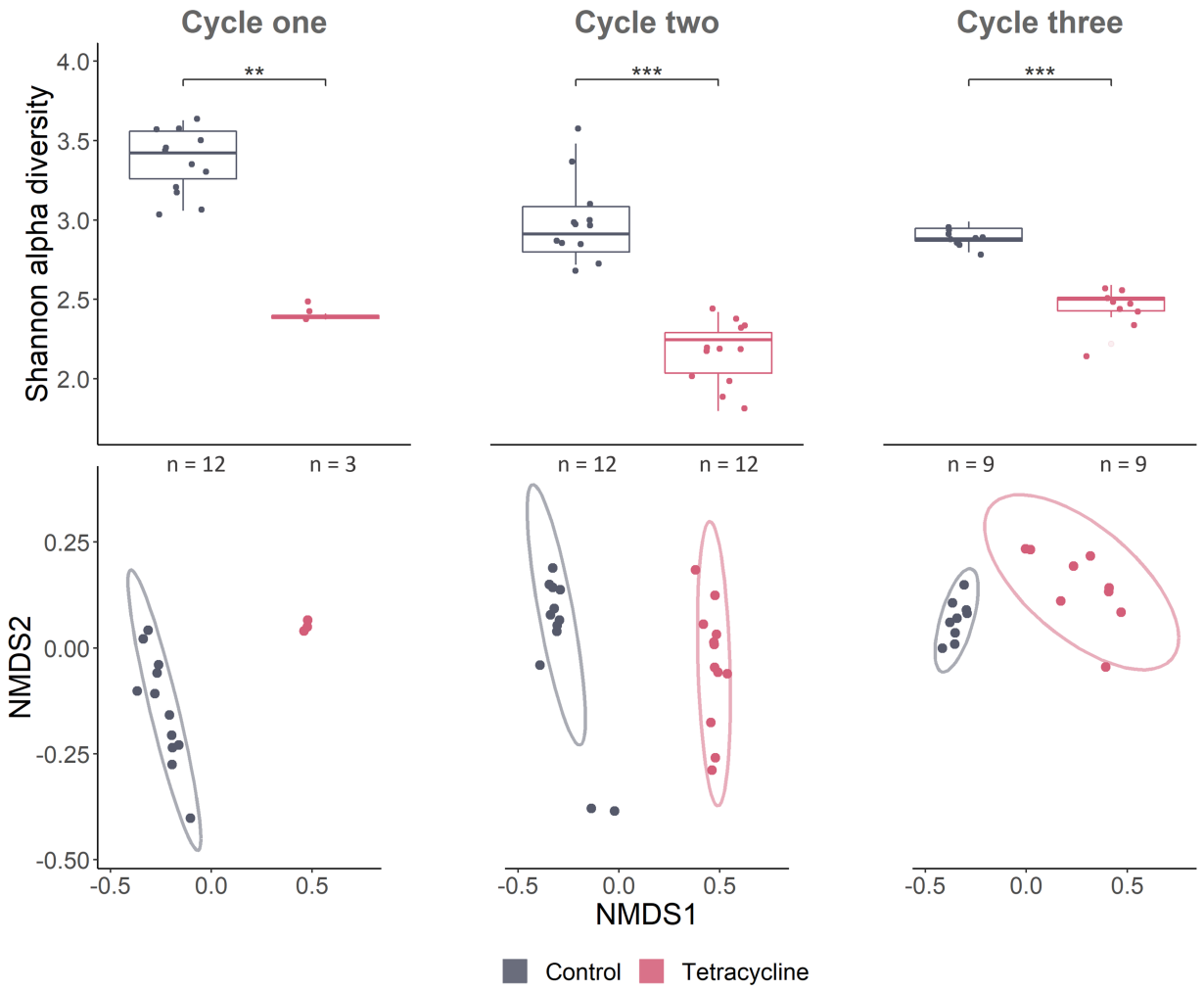

FIGURE 4
Gut microbial community composition responds to tetracycline treatment. Alpha- and beta-diversity as well as taxonomy show tetracycline leading to a strong dysbiosis, decreasing several taxa. Alpha diversity Shannon index accounts for abundance and evenness of ASV in samples. Pairwise Wilcoxon rank sum tests were used for statistical comparisons between treatments and controls (***p 0.001; **p 0.01) (cycle 1: W=36, p=0.004; cycle 2: W=144, p<0.001; cycle 3 before stress: W=81, p<0.001). NMDS on Bray–Curtis dissimilarity which considers presence/absence as well as abundances of ASVs, represents compositional differences between samples (beta diversity). Stress of NMDS was 0.069. Ellipses represent 95% confidence intervals around treatment centroids.

intracellular cholesterol transporter 2 (LOC724386) and metabolism such as *lipase member H-A* (LOC727193) or *chitooligosaccharidolytic beta-N-acetylglucosaminidase* (LOC725178) (Figure 6 and Supplementary Figure S11). As we sequenced only three individuals per treatment it is important to be cautious about generalizations. However, while the tetracycline-pre-treated-gut bees showed more within-group variation in their expression profiles comparison to the control (Supplementary Figure S12), the significantly different genes showed relatively similar expression patterns within the two groups although both coming from three individual cage communities (Figure 6). See additional information such as the lists of up- and down regulated genes with information on gene description, GO term and beebase IDs in the Github folder.

Discussion

Considering the worldwide increase in variety and abundance of anthropogenic stresses together with the loss of biodiversity (Barnosky et al., 2011, 2012), there is urgent need to understand all potentially contributing effects. This includes consideration of interactions between organisms. How microbiomes affect host's responses to such selection remains underexplored (Cavicchioli et al., 2019). Associated microbial symbionts and their functional relationships with their hosts are sensitive to disturbance. Given that microbiomes are vertically inherited, wholly or in part, in many organisms, any changes in composition and associated second-order effects on organismal health may be propagated across generations.

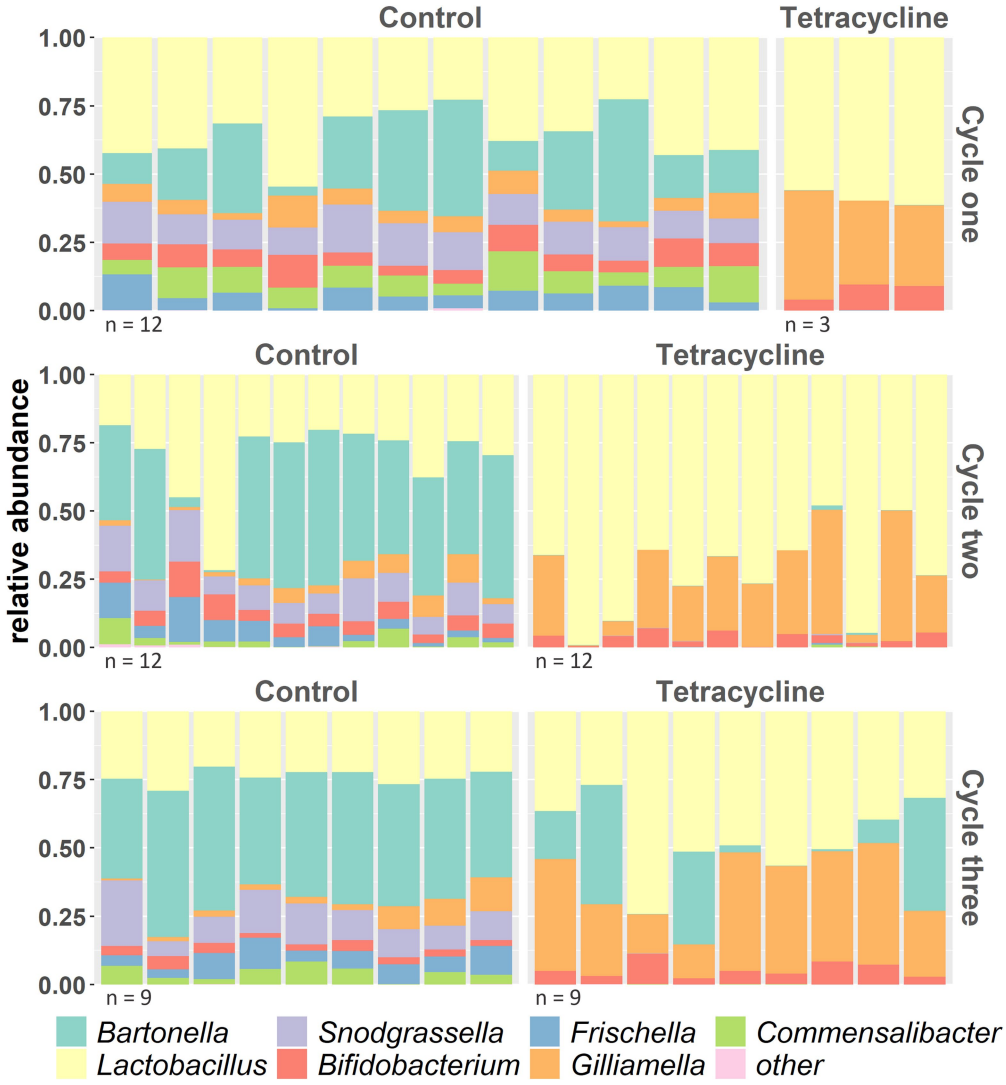

FIGURE 5
Taxonomy of bacterial genera across the 3 cycles (cycle three before high stress application) with at least 1% relative abundance across samples (everything else is combined to "others") shows several taxa disappearing under tetracycline.

Here, we used controlled lab experiment to show that deleterious effects of antibiotics on the microbiome can be passed across generations and affect host health decoupled from any direct toxicity of the antibiotic.

Antibiotics reduce microbiome diversity on genus- species- and strain level

Consistent with the direct action of antibiotics on bacteria, we observed substantial changes in the honey bee gut community after tetracycline exposure. While in previous studies, antibiotics were shown to affect the honey bee microbiome (Powell et al., 2021; Tian et al., 2012; Moullan et al., 2015; Li et al., 2017; Raymann et al., 2017; Baffoni et al., 2021; Jia et al., 2022), it rarely led to the total collapse of bacterial species as we observed in our design. At the end of the first cycle, four bacterial genera disappeared from guts of antibiotic-fed bees (Figure 5 and Supplementary Figure S9). In general, it may be difficult to compare different studies as they differ in methodology. Also, honey bees used in the studies may differ genetically, in their surrounding environment and likely in their colony's chemical exposure histories which can affect the microbial strain composition (Tian et al., 2012; Ellegaard and Engel, 2019; Wu et al., 2021). Low antibiotic intake (10 ug/mL) after emergence did not show to affect the later establishment of the microbiome in honey bees (Jia et al., 2022). However, we used previously published higher concentrations (Raymann et al., 2017) which were administered immediately after emergence, which could affect the uncolonized gut environment and the overall response of a microbiome. Cox et al. introduced early life as "critical developmental window" when antibiotics have greatest impact on

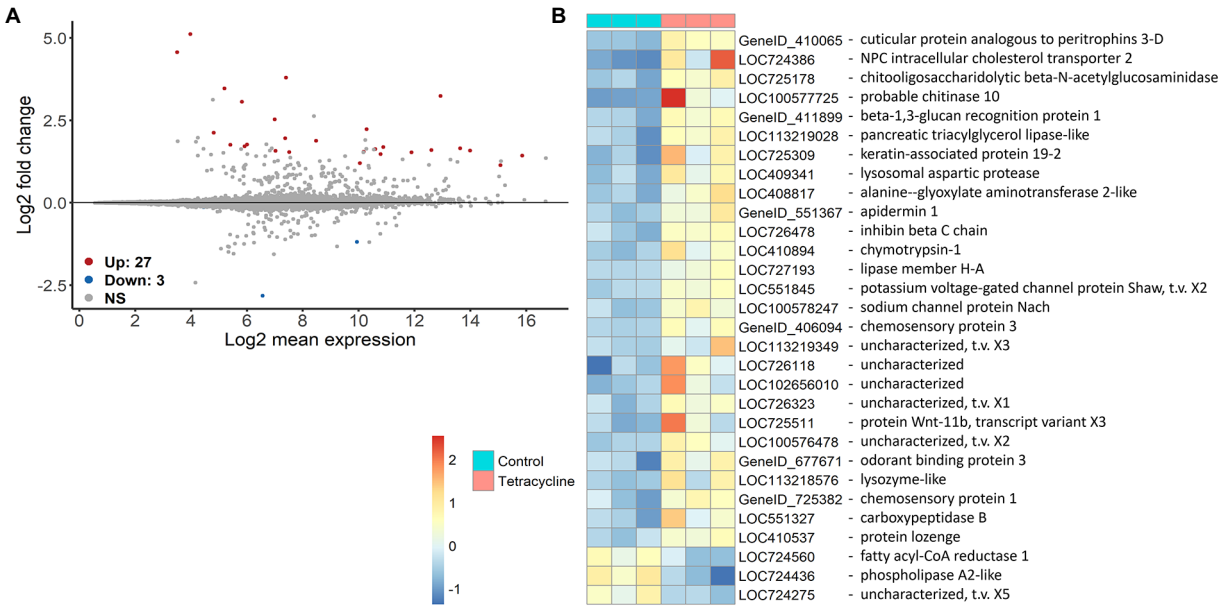

FIGURE 6
Differential gene expression of genes in naïve bees that received tetracycline-exposed in comparison to individuals that received control microbiomes. MA plots show the differential expression of the tetracycline-gut against the control-gut treatment (n=3 (one bee per cage for both treatments respectively)) **(A)**. The x axis shows the average expression over the mean of normalized counts, and the y axis shows the gene-wise dispersion estimate's shrunken log2 fold change. Red and blue points indicate significant up- or downregulation (FDR≤0.05 determined by DESeq2) of individual genes. Heatmap on *rlog()* transformed data shows the expression difference of each significantly different gene in a specific sample from the gene's average across all samples. In addition the gene descriptions are shown **(B)**.

the gut microbiome of mice, leading to lasting metabolic consequences (Cox et al., 2014). Such time-dependent response difference has also been demonstrated in honey bees (Motta and Moran, 2020), which eclose largely without microbes and then acquire them from the surrounding environment and nestmates (Powell et al., 2014). After the high tetracycline exposure in cycle three, the microbiome composition did not change (Supplementary Figure S7). For the tetracycline-treated microbial communities this could be explained by the fact that we selected for antibiotic resistant strains in the previous cycles, however we also did not observe changes in the controls. While this seems surprising considering the extreme effects of lower antibiotic dosages in the cycles before, this may be caused by the fact that we firstly applied tetracycline to fully colonized adults in cycle three and secondly that we sampled 20 h after antibiotic exposure and DNA sequencing will also capture dead material. Other studies also detected a more prominent effect of antibiotics on the honey bee gut community several days after treatment was stopped which may be a result of a delayed effect of the antibiotic (Raymann et al., 2017). In addition, while 16S sequencing has limitations when it comes to fine-scale taxonomic identification (Ellegaard and Engel, 2016), we found extensive response variation at the generic, species, and ASV levels (Supplementary Figure S10). This is consistent with other studies that found effects of antibiotics (Raymann et al., 2018) and other pesticides (Cuesta-Maté et al., 2021) vary across bee gut bacterial species and strains. These data together with the increase in resistance genes in antibiotic exposed bee microbiomes (Tian et al., 2012; Sun et al., 2022) indicate adaptation to chemical selection factors.

Negative effects of antibiotic-disturbed microbiomes can be transferred to following generations

In general, perturbations of a healthy gut environment can affect gene expression, protein activity, and the overall metabolism of a host associated gut microbiota (Franzosa et al., 2015). Antibiotic exposure causes dysbiosis, with effects on host health (Francino, 2016; Neuman et al., 2018), the resistome (genes involved in resistance responses), and gut bacterial diversity (Li et al., 2019; Xu et al., 2020). We found that short-term dysbiosis could be transferred to subsequent worker bee generations which is in line with previous experiments in honey bees and flies (Ourry et al., 2020; Jia et al., 2022). In our experiment, tetracycline disrupted the normally stable bee gut community, which did not recover over subsequent generations even after antibiotic administration was ceased. In cycle three only *Bartonella* could recover in some samples, while the other antibiotic-affected genera appeared permanently eliminated from the community (Figure 5). This transmitted dysbiosis was likely the reason of the higher mortality under subsequent tetracycline stress at the end of the experiment in naïve bees that inherited the disturbed microbiome (Figure 3).

Feeding macerated honey bee guts to other bees is an established method of microbial transfer in laboratory studies (Powell et al., 2014; Zheng et al., 2018; Kowallik and Mikheyev, 2021). However, since bees do not defecate in captivity, toxins such as tetracycline could conceivably accumulate in the hindgut. It is therefore imaginable that small amounts of left tetracycline or derivates may negatively affect the health of the following worker bee generation. We excluded this possibility by an additional experiment transferring tetracycline-exposed guts filtered to remove bacteria and seeing no effects on mortality (Figures 2, 3). This supports the interpretation that the detrimental effect was indeed caused by the disturbed microbial community. In general, we cannot exclude that we also transmitted non-bacterial pathogens during microbiome transfer in our design which may affect host health. For the fungal pathogen Nosema a potential correlation has been reported between infection load and gut microbiome structure (Rubanov et al., 2019). However, we do not see a higher Nosema load in the antibiotic treated bees in our RNA data but rather the opposite. In addition, none of the significant genes in our design are common pathogen-response genes. The humoral immunity in honeys bees involves synthesis of antimicrobial peptides (AMPs) from which *abaecin*, *apidaecin*, defensin and *hymenoptaecin* usually respond to bacterial, viral and fungal infection (Evans et al., 2006; Chaimanee et al., 2012; Flenniken and Andino, 2013; Doublet et al., 2017).

Gut bacteria function as a protective barrier, enhancing nutritional provisioning and affecting the host immune system across animal systems (Hooper et al., 2012; Tremaroli and Bäckhed, 2012; Kamada et al., 2013) including honey bees (Kešnerová et al., 2017; Raymann and Moran, 2018). Administration of antibiotics has been shown to reduce gene expression of antimicrobial peptides in bees (Li et al., 2017; Motta et al., 2022). We observed a significant up-regulation of genes having functions in immunity, biotic responses, carbohydrate metabolism and transport for all kind of molecules (e.g., metal ion, sodium ion, sterol transport) in bees receiving dysbiotic microbiomes (see Figure 6 and Supplementary Figure S11). Only three genes showed to be down-regulated which were mainly involved in lipid metabolism. As our cycle three bees did not consume tetracycline themselves, we can conclude that the differential gene expression was most likely caused by the microbial community changes. Changes in community structure such as those observed in our study can alter the provided microbiome function such as provision of nutrients or removal of toxic metabolites across systems (Willing et al., 2011). In general, interactions between symbionts can be as important as the individual species in gut microbiomes, therefore the effects of a disturbed microbiome go far beyond the loss of functions attributable to single taxa (Gould et al., 2018). In our design, a disturbed cross-talk between host and microbiome could have affected host gene expression as the host may have had to compensate for missing functions. However, as we sequenced only one bee per cage and the expression of bees receiving tetracycline pre-exposed microbiomes shows higher within treatment variation than the control (Supplementary Figure S12) we should be cautious with generalizations.

The honey bee as model system

Previous work characterized the honey bee microbiome and developed methods such as artificial microbiome transmission (Engel et al., 2013; Powell et al., 2014; Kwong and Moran, 2015). We built on this foundation using honey bees as a model to study stress-induced, microbiome-mediated effects on subsequent generations. In our experiments we performed a purely vertical microbiome transfer between individuals, a rate at the extreme end of a continuum of strategies. While in most systems microbes are acquired both vertically and horizontally, high rates of vertical transfer are typical in honey bees (Engel and Moran, 2013). We did not provide the opportunity to recruit different strains through the environment or social contact inside the hive which could have led, for instance, to some recovery from the dysbiotic state induced by tetracycline or could have led to colonization of opportunistic pathogens. Although a previous study did not find that honey bees with antibiotic-induced dysbiosis recovered their microbiomes to a healthy state when being put back to the hive environment and that they also suffered from higher mortality in this natural environment compared to the control (Raymann et al., 2017). Beside chemically induced changes to the microbiota, even communities in our control treatment were also gradually changing in the lab. For instance, we observed an increase of *Bartonella* abundance in all treatments in comparison to hive nurse siblings and the starting microbiome pool (Supplementary Figure S8). These changes likely reflect lab adaptations and emphasize the need to run proper lab controls in microbiome experiments (Arora et al., 2020), but also a need to run more natural experiments in the future. Additionally, the high tetracycline dosage over two worker generations may not reflect natural conditions, though mimicking nature was not our intent.

Controlled laboratory experiments such as microbiome transplants, provide the most convincing insights into functional host-microbiome relationships (Greyson-Gaito et al., 2020). They are invaluable because they can simplify the complexity and disentangle factors to achieve fundamental understanding which is still lacking in the field. However, these experiments trade control for natural complex conditions, which is important for drawing ecological and evolutionary conclusions (Carrier and Reitzel, 2017).

In addition to being a tractable model for microbiome research, honey bees are important pollinators in natural and agricultural ecosystems (Hung et al., 2018). They are exposed to diverse agricultural chemicals including those applied to plants making up their diet but also the ones used by beekeepers to prevent infection or suppress parasites (Ortiz-Alvarado et al., 2020). Antibiotics have been experimentally demonstrated to disturb the core microbial bee microbiome, lowering diversity on species and strain level and leading to negative health effects (Raymann et al., 2017, 2018; Powell et al.,

2021; Jia et al., 2022). Facilitated by social transmission between workers, changes in the microbiome could theoretically quickly go to fixation in a population. Indeed, antibiotic resistance genes have accumulated in bacterial symbionts in managed honey bee colonies, demonstrating long-term impacts with unknown consequences (Tian et al., 2012; Ludvigsen et al., 2017; Daisley et al., 2020; Piva et al., 2020). Considering the social-vertical transfer of the microbiome between worker generations in honey bee colonies with the fact that chemicals including antibiotics accumulate and persist in the hive environment over longer periods (Martel et al., 2006), the damage on the bee microbiome could theoretically go beyond one individual's health affecting a whole population. In mice, diet-induced progressive loss of taxonomic diversity is cumulative over generations and indicate that taxa driven to low abundance are inefficiently transferred to the next generation, and are at increased risk of becoming extinct within an isolated population making this change eventually irreversible (Sonnenburg et al., 2016). This suggests that multigenerational environmental exposure could indeed cause a stable transgenerational alteration of organism physiology *via* the microbiome.

Conclusion

Co-evolved microbiomes can offer a range of benefits to their hosts and vice versa. However, under disturbance this picture may change, and the dependent partner could suffer negative consequences. While it is often difficult to disentangle cause and consequences of chemical-induced microbiome disruption on host health, we provide evidence that a disturbed microbiome and its mediated effects on host phenotypes can get transmitted across generations in a lab environment. This "dark side" of a specialized, vertically transferred microbiome could, likewise as negative mutations, theoretically go into fixation affecting the health of a whole population if no refreshing is possible. This is particularly true if the whole population is affected by chemical stress, for example in an agricultural context. For instance, agrichemical degradation of microbiomes may be a plausible, silent factor underlying global insect declines. Future studies would be important to examine the extent to which negative microbiome-mediated phenotypes are really heritable in the field. Examining whether such heritable dysbiosis has the potential to threaten host populations or which potential rescue mechanisms may play a role to prevent such scenario under natural conditions would be relevant to further understand organism health and conservation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA863631. All data files and codes used for processing and analysis can be found following this link: https://github.com/kowallik/inheritance_microbiome_disturbance. RNA and 16S raw reads are available under NCBI Bioproject PRJNA863631.

Author contributions

VK and AM wrote the manuscript. AM processed raw RNA sequences. VK designed research, conducted experiments, processed 16S sequences, and analyzed 16S, RNA and survival data. AD extracted samples and prepared RNA libraries. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1030771/full#supplementary-material

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Changes in gut microbiota and metabolism associated with phenotypic plasticity in the honey bee *Apis mellifera*

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Honey bees exhibit an elaborate social structure based in part on an age-related division of labor. Young workers perform tasks inside the hive, while older workers forage outside the hive, tasks associated with distinct diets and metabolism. Critical to colony fitness, the work force can respond rapidly to changes in the environment or colony demography and assume emergency tasks, resulting in young foragers or old nurses. We hypothesized that both task and age affect the gut microbiota consistent with changes to host diet and physiology. We performed two experiments inducing precocious foragers and reverted nurses, then quantified tissue-specific gut microbiota and host metabolic state associated with nutrition, immunity and oxidative stress. In the precocious forager experiment, both age and ontogeny explained differences in midgut and ileum microbiota, but host gene expression was best explained by an interaction of these factors. Precocious foragers were nutritionally deficient, and incurred higher levels of oxidative damage relative to age-matched nurses. In the oldest workers, reverted nurses, the oxidative damage associated with age and past foraging was compensated by high Vitellogenin expression, which exceeded that of young nurses. Host-microbial interactions were evident throughout the dataset, highlighted by an age-based increase of *Gilliamella* abundance and diversity concurrent with increased carbonyl accumulation and CuZnSOD expression. The results in general contribute to an understanding of ecological succession of the worker gut microbiota, defining the species-level transition from nurse to forager.

KEYWORDS

phenotypic plasticity, gut microbiota, precocious foragers, immunity, oxidative stress, vitellogenin, age-polyethism

Introduction

The ecological success of eusocial insects is attributed to an organized and efficient division of labor (Oster and Wilson, 1978). Social insects solve complex problems with individual behaviors, resulting in emergent group properties (Hölldobler and Wilson, 2008). The numbers of workers performing a particular task is optimized by feedback loops to efficiently collect, process, and distribute resources among colony members (Fewell, 2003). During normal ontogeny, individual workers transition among various tasks during their lifetime, and exhibit a broad range of phenotypic plasticity. More simply, colony demography is socially regulated (Huang and Robinson, 1996), allowing a proximate internal response to unpredictable external environments. Various worker tasks involve different physiological and behavioral demands, producing strong selection on social phenotypes. Social insects are well suited to the study of sociality and phenotypic plasticity because they represent a complex adaptive system or "superorganism" from which the functional parts can be manipulated and measured (Hölldobler and Wilson, 2008).

Honey bees are highly social insects that live in complex societies consisting of one reproductive queen and thousands of facultatively sterile workers. While the queen spends a preponderance of her life laying eggs, workers build and maintain all aspects of the hive. Under normal conditions, worker bees display age polyethism, performing tasks within the hive for the first 2–3 weeks of adult life before transitioning to outside tasks (Seeley, 1982). Specifically, young adults function as "nurse bees" that feed growing larvae, then act as food processors in middle age. Near 20 days of age, middle-age bees transition into foragers that then procure nectar (carbohydrates), pollen (protein and lipids), propolis (antimicrobial plant resins), and water (Seeley, 1982) from the pollination environment. Despite this well-established pattern, adult workers can decouple age from behavioral task in response to social cues from other workers and temporal effects in the pollination environment (Huang and Robinson, 1992; Johnson, 2010). Thus, the ontogeny of an adult bee is extremely plastic and nursing/foraging behaviors can be accelerated, slowed, or reversed (Robinson, 1992).

Phenotypic plasticity in honey bees workers is directly associated with the availability of nutrition and storage proteins, vitellogenin in particular (Amdam et al., 2003). Vitellogenin (Vg) is a phospholipoglyco-protein evolved to serve many functions; as an antimicrobial, antioxidant, and to produce brood food in the nurse worker head (hypopharyngeal) glands (Seehuus et al., 2007; Amdam, 2011). Associated with changes in the gut microbiota, foragers switch to a diet of simple sugars to support the metabolic demands associated with foraging (Anderson et al., 2018). This labor transition is associated with reduced lipid stores (Toth and Robinson, 2005), reduced Vg titers (Fluri et al., 1982), decreased nutritional status (Ament et al., 2008), differential gene expression (Byhrø et al., 2019), and protein oxidation; a direct measure of biological aging (Fedorova et al., 2014). Many differences contribute to foraging success; a decrease in body mass and a proportional increase in flight capacity (Vance et al., 2009). However, orientation to the pollination environment is the riskiest time of an adult bee's life. A recent study documented that 40% of bees die during the pre-foraging stage of life, a time where bees perform exploratory and learning orientation flights (Prado et al., 2020). Bees that survive this training face a constant increase in extrinsic mortality risk per unit time that increases to 100% after 18 days of foraging activity (Dukas, 2008), yet only ~20% of foragers will live past 10 days of foraging (Visscher and Dukas, 1997). Therefore, the age a worker initiates foraging has a strong impact on an individual's lifespan and colony fitness.

Foraging also has direct consequences for intrinsic senescence, including increased sensitivity to physiological stressors (Remolina et al., 2007) and a decrease in innate immune defenses (Amdam et al., 2004, 2005; Schmid et al., 2008; Lourenço et al., 2019). Foragers also show an increased susceptibility to oxidative stress (Seehuus et al., 2006), including oxidative damage to the brain (Rueppell et al., 2007), trophocytes, and fat cells (Hsieh and Hsu, 2011). The accumulation of oxidative damage from reactive oxygen species (ROS) is proposed as the main cause of aging (Harman, 1956). Thus, a precocious transition to foraging is predicted to result in premature aging. Flight and the associated ROS accumulation from muscle usage and attrition may surpass the capacity for antioxidant enzymes to remove them. Indeed, the honey bee's innate antioxidant enzymes: e.g., various superoxide dismutases, catalase, and glutathione S-transferase, reach their greatest expression in older workers (Corona et al., 2005). While the physiology of behavioral plasticity and aging has been explored in honey bees, the role of the gut microbiome in this process is poorly known (Vonaesch et al., 2018). A compendium of results characterizing the transition to foraging found that the worker hindgut microbiota is depleted of core hindgut *Lactobacillus* firm4, firm5, and *Bifidobacterium asteroides,* and can be enriched for Acetobacteraceae Alpha 2.1 and *Bartonella apis* (Anderson et al., 2018), but results were inconclusive for core ileum species perhaps reflecting a lack of tissue-specific sequencing.

The honey bee gut microbiota is remarkably consistent and dominated by five omnipresent, highly co-evolved phylotypes representing >95% of bacterial cells; *Lactobacillus*, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and *Bifidobacterium* (Martinson et al., 2012; Sabree et al., 2012; Kwong and Moran, 2016). Recent work has revealed a strong association of the microbiome with worker physiology including the expression of insulin-like peptides and vitellogenin (Engel et al., 2016; Maes et al., 2016; Kešnerová et al., 2017; Raymann et al., 2017; Ricigliano et al., 2017; Zheng et al., 2017; Powell et al., 2021). Although the microbiota of nurses and foragers is taxonomically similar (Corby-Harris et al., 2014), composition differs by behavioral task and may impact host physiology and health (Anderson et al., 2018; Jones et al., 2018).

Here we investigate changes in the gut microbiota and host gene expression associated with typical and atypical ontogeny. Individual worker behavior and physiology can be manipulated *via* the perturbation of social structure (Huang and Robinson,

1996). We manipulated colony social structure to test two hypotheses: (1) Premature foraging comes with a physiological costs reflected in the gut microbiota, and (2) a return to nursing behavior in old age restores youthful physiology and associated microbiome characteristics. To test if gut microbiota differences are associated with ontogeny (atypical vs. typical) or age, we (1) generated "single-cohort colonies" (SCC) comprised of bees that were all the same age (Robinson et al., 1989) and (2) generated observation hives where we induced foragers to return to nursing behaviors. These perturbations of colony demography induce portions of the population to assume behavioral tasks independent of age. For the first experiment, we assessed differences in nurses and precocious foragers (PF) midgut and ileum gut microbiota of the same age, monitoring fat body gene expression related to immunity and oxidative stress. Likewise, we assessed protein oxidation in the fat body resulting from precocious foraging. In the second experiment, we assessed the hindgut (ileum and rectum) microbiota and fat body gene expression of reverted nurses relative to normal worker ontogeny. PF midgut and ileum microbiotas were explained by both age and ontogeny, but gene expression of the fat body tissue was best explained by an interaction of ontogeny and age. Precocious foragers lacked key ileum species *Gilliamella*, and accrued more oxidative damage relative to age-matched nurses and foragers that experienced normal ontogeny. The hindgut microbiotas of reverted nurses were remarkably stable, but relative to young nurses, vitellogenin expression was significantly elevated, and carbonyl accumulation increased by an order of magnitude.

Materials and methods

Colony manipulations and sampling

To investigate changes in host physiology and gut microbial composition associated with the range of worker phenotypic plasticity, we designed and implemented two experiments in June 2019 at the USDA-ARS Carl Hayden Bee Research Center in Tucson Arizona (Figure 1A). The first experiment utilized a "single-cohort colony" (SCC) design, which was previously shown to uncouple behavioral task from chronological age (Robinson et al., 1989). We induced a subset of the population to become precocious (young) foragers (PFs). In the second experiment we removed the young (nurse) bee population, forcing older foragers to revert back to nursing behaviors.

Experiment 1 (Precocious foragers PF): Closed brood frames were sourced from 30 honey bee (*Apis mellifera, linguistica*) hives. Frames were incubated overnight (30°C, 75% relative humidity, and 24 h dark cycle) and newly emerged adult workers were collected and combined into a mixed cohort to implement into experiment. To construct the two SCCs, newly emerged workers (4,500 and 3,500) were added to a small hive box, each containing a naturally mated queen, one frame with pollen and honey, and one frame with eggs and open brood (Figure 1B). Bees assigned to each SCC were <24 h old and differentiated into separate behavioral tasks, i.e., nurses and atypically, PFs. Additionally, a marked cohort (MC) with 4,100 newly emerged workers was constructed to serve as a control for sampling normal ontogeny throughout both experiments (Figure 1B). Newly emerged workers were marked with paint on their thorax and transferred into a healthy double-deep colony free from visible signs of disease. This allowed us to sample natural age nurses (7D, 8D, and 13D) and 27 day (27D) old natural foragers, complementing both experiments. On day 6 of the experiment, SCC PFs we observed with corbicular pollen loads were marked with paint on their thorax.

At the peak of foraging activity in the summer, a workers lifespan is ~30 days (Fluri et al., 1982). Over the course of the experiment, we sampled marked foragers and nurses from the SCCs at 7, 13, and 19-days old. By design, SCC nurses and foragers performed the same behavioral tasks for the duration of the experiment. Nurses were identified by observing the brood nest and sampling bees that spent 3 s with their head in a cell containing brood. Part way through the experiment, we replaced the brood frame in the SCC's to ensure no newly emerged workers replaced the current nurses. For the MC sampling, we sampled age-right nurses at 7, 8, and 13-days old and age-right foragers at 27-days based on well-established honey bee worker ontogeny (Seeley, 1982). Thus all samples could be categorized as typical (age-right) or atypical (PFs) ontogeny (Figure 1F). Sampled bees were collected with sterile soft forceps, snap frozen with dry ice, and stored in −80°C for processing.

Experiment 2 (Reversion REV): Three putatively healthy double-deep colonies were used to induce foragers to revert to nurse behaviors (Figure 1C). A mobile shed was retrofitted with 6 stalls, 3 north facing and 3 south facing to accommodate the colonies. Each stall was provided a single entrance by drilling a hole through the sheds wall. A 0.5 m flexible plastic tube was inserted through the hole and attached to the colony's bottom board. Colonies were installed in the north facing stalls and given 1 week to acclimate to the new location. The night prior to the beginning of the experiment, each colony was moved to south facing stalls (Figure 1D). The vacant north facing stalls were replaced with three-frame observation colonies containing the queen from the source colony, one frame of food (stored honey and pollen), one frame of uncapped brood (to ensure no emergence would occur), and an empty frame to provide room for the queen to lay. A one-way entrance reducer was installed on the south facing source colony. The next morning, foragers leaving the source colonies returned to the observation colonies at the northern entrance.

We sampled groups of initial foragers (IF), defined by having corbicular pollen loads on their legs that returned to the north facing observation hives. The observation colonies composed of a queen and foragers were given 24 h to adjust to the new colony demography and redistribute into nurse and foragers. Next, individuals observed engaged in nursing behavior (head in cell with larvae >3 s) were painted on the thorax. After 1 week,

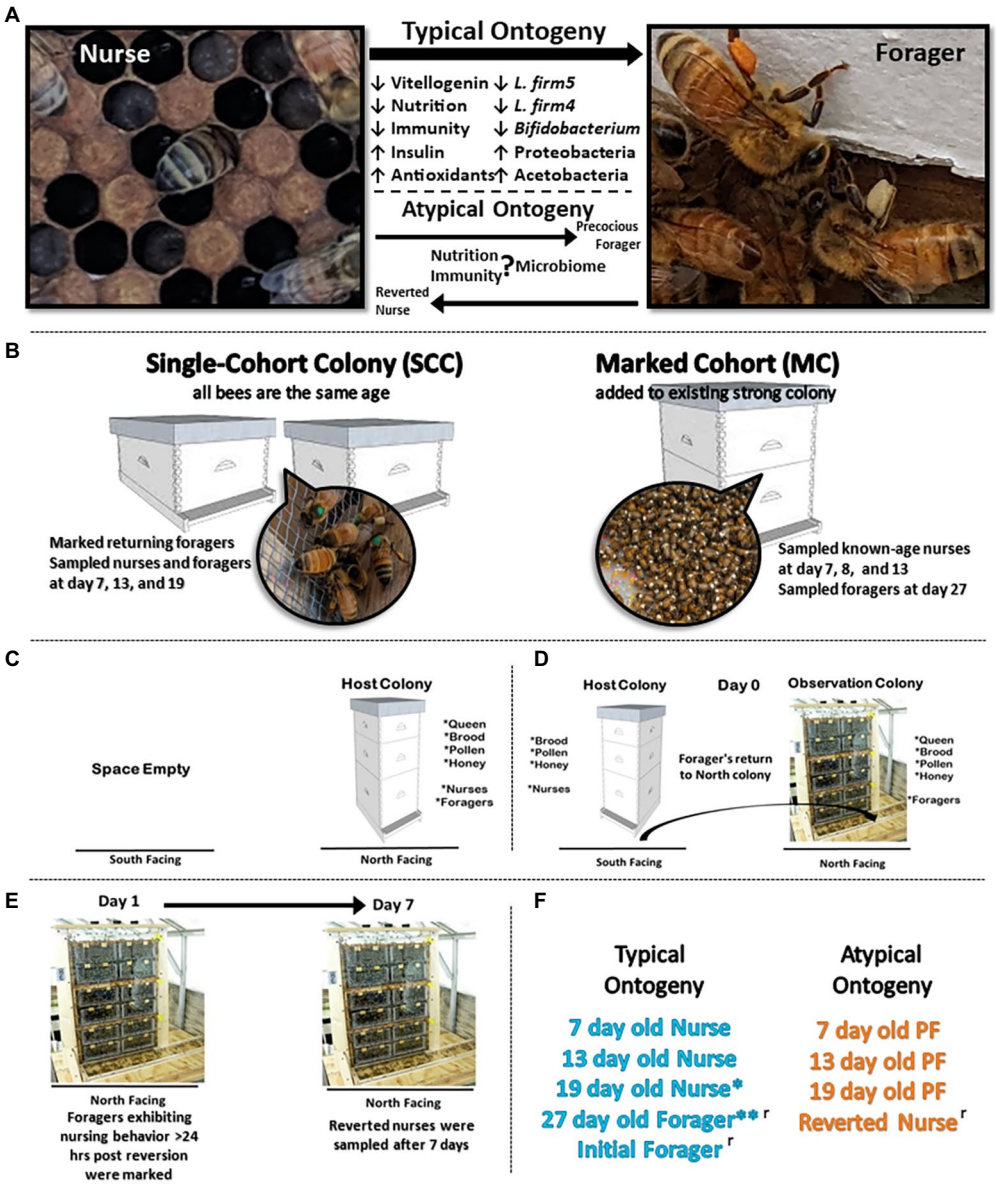

FIGURE 1
Experimental design. **(A)** During typical ontogeny workers transition from in-hive tasks like nursing to riskier outside the hive tasks like foraging as they age. We designed two experiments to explore this relationship of ontogeny, phenotypic plasticity, physiology, and the gut microbiota. **(B)** Experimental design for precocious forager experiment: to decouple age and behavioral task, we created two single-cohort colonies (SCC) with 4,500 and 3,500 bees. In a SCC, all bees are the same age but as an emergent property of sociality, some will differentiate by task into precocious foragers. We also established a marked cohort to sample normal age bees following classic ontogeny patterns. **(C–E)** Experimental design for reverted nurse experiment: a host colony was switched to an observation colony so that returning foragers returned to a colony with no nurses. By the same social mechanism, a proportion of old foragers will revert to nursing behaviors. **(F)** Typical (age-right and blue color) ontogeny occurs under normal colony demography. Atypical (orange color) designates a decoupling of age and behavior in response to colony needs. *19day old nurses can be considered as overage nurses, especially in a busy pollination season. These data present 19 are typical considering a long transition in-hive nursing before transitioning to foraging. **27day old foragers were used in both experiments. r designates categories used in reversion experiment.

previously marked individuals that were still observed engaged in nursing behaviors were sampled as reverted nurses (RN; Figure 1E).

Worker dissections occurred under sterile conditions. The sting was discarded and the fore and hindguts were removed from the abdomen. Gut tissues were dissected in 95% ethanol to wash and separate the midgut, ileum, and rectum before being added to a bead-beating tubes with 0.2 g of 0.1-mm silica beads and 600 µl of 1X TE buffer. Experiment 1 focused on the midgut and ileum tissues. Experiment 2 focused on the ileum and rectum. Both experiments utilized the abdominal fat body and attached dorsal sclerites as a single unit for gene expression

and protein oxidation (carbonyl) assay to quantify biological aging.

DNA/RNA extractions

In preparation for DNA/RNA extractions, samples were bead-beaten for 2 min at 30-s intervals and centrifuged to recover the supernatant. Gut tissue DNA was extracted with Thermo Scientific GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Fat body supernatant was split into two aliquots of 300 µl, one used for RNA extraction [Thermo Scientific™ GeneJET Genomic RNA Purification Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States)] and the other reserved for carbonyl assay. The extracted fat body RNA was converted into cDNA with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). DNA fractions for each sample were used for 16S rDNA amplicon sequencing and bacterial quantification *via* real-time quantitative-PCR (qPCR). RNA fractions were used to create cDNA and examine gene expression *via* qPCR.

We quantified total bacterial abundance for gut tissues with a qPCR assay of bacterial 16S and fungal 18S rRNA gene copies (Liu et al., 2012a,b). The bacterial 16S gene template was amplified using forward primer 27F (5′-AGAGTTTGATCCCTCAG-3′) and reverse primer 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′). The fungal 18S gene template was amplified using forward primer PanFungal_18S_F (5′-GGRAAACTCACCAGGTCCAG-3′) and reverse primer PanFungal_18S_R (5′-GSWCTATCCCCA KCACGA-3′). Quantitative PCRs for 16S rRNA genes were carried out in triplicate on a BioRad CFX96 thermocycler in 12 µl reactions containing 5 µl of New England Biolabs – Luna® Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, Massachusetts, United States), 0.5 µl forward primer, 0.5 µl reverse primer, 4 µl of H_2O and 2 µl of DNA template. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. The qPCR results were expressed as the total number of 16S and 18S rRNA gene copies per DNA extraction (100 µl volume elution).

To provide absolute quantification of 16S and 18S rRNA copy number and ensure inter-run comparability, in-run standard curves and no-template controls were included on each run. Invitrogen's pCR™2.1 TOPO™ (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used to produce plasmid vectors, which were then transformed into DH5α™ cells (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Successfully transformed colonies were selected and grown overnight in broth. Plasmid DNA was purified using the Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The purified plasmid cells were measured using an Implen nanophotometer P300, and the known mass of plasmid plus PCR insert was used to calculate 16S plasmid-standard copies per µl. A 10-fold serial dilution of the plasmid standards was included on each plate, and these data were pooled across all plates to calculate a single standard curve used to interpolate all sample Cq values. To determine the total number of 16S rRNA-gene copies present in each sample extraction, Cq values were adjusted for elution volume and any subsequent dilution(s).

16S rRNA gene amplicon sequencing and analysis

A 466-bp fragment in the V3–V4 region of the 16S rRNA gene was amplified using PCR primers (forward primer, 341F 5′-CCTACGGGNGGCWGCAG-3′; reverse primer, 805R 5′-GACT ACHVGGGTATCTAATCC-3′). DNA library preparation was performed following Illumina MiSeq DNA library preparation protocol. Sequencing was performed at the University of Arizona Genetics Core (UAGC) on a MiSeq following the manufacturer's guidelines. The sequence data for this study have been deposited in GenBank, Sequence Read Archive no. as (PF) PRJNA801240 and (Reversion) PRJNA885470.

The 16S rRNA gene sequences were processed by gut tissue using MOTHUR v.1.44.3 (Schloss et al., 2009). Briefly, forward and reverse reads were joined using the make.contigs command. After the reads were joined, the first and last five nucleotides were removed using the SED command in UNIX. Sequences were screened to remove ambiguous bases, using the screen.seqs command. Unique sequences were generated using the unique.seqs command. A count file containing group information was generated using the count.seqs command. Sequences were aligned to BEExact (Daisley and Reid, 2021) database using the align.seqs command. Sequences were filtered to remove overhands at both ends and gaps using filter.seqs. The unique.seqs command was ran again to remove new redundancies from filtering. A precluster step using pre.cluster was performed. Chimeras were removed using chimera.uchime command (Edgar et al., 2011). Sequences were classified with the BEExact database using classify.seqs command. Sequences that were not bacterial origin were removed using the remove.seqs command. All unique sequences with one or two members (single/doubletons) were removed using the AWK command in UNIX. A distance matrix was constructed for the aligned sequences using the dist.seqs command. Sequences were classified at the unique level with the BEExact database. Uniques were merged at the species-level with the merge.otus command. Samples with <5,000 reads were excluded from downstream analyses. ASVs at the species-level that were left unclassified by BEExact but matched unambiguously at 100% identity to genus were assigned as "*genus* unclassified."

Gene expression

The fat body is a main metabolic tissue of the honey bee and is functionally analogous to vertebrate liver or adipose tissue (Liu

et al., 2009). Comparisons of fat body gene expression can relay information on immunocompetence and overall health. A list of genes used in both experiments can be found in Supplementary Table S1. Quantitative PCR reactions for immune gene expression were performed in triplicate as follows: initial denaturation at 95°C for 5 min; 40 cycles with denaturation at 95°C for 15 s; and a primer-pair-specific annealing and extension temperature for 30 s. To confirm the absence of contaminating genomic DNA and primer dimers in the qPCR assay, we monitored amplification and melting curves. Relative gene expression was determined based on standardized Ct values (Δ Ct; Livak and Schmittgen, 2001) using the mean of two reference genes: *β-actin* and *RPS18*. *β-actin* and *RPS18* are constitutively expressed in different honey bee tissues and has been previously established as an effective control for calibrating less constitutive gene expression in adult workers (Evans et al., 2013; Jeon et al., 2020).

Carbonyl assay

To measure protein damaged by oxidative stress, we quantified the accumulation of protein carbonyl groups *via* another well-validated assay (Reznick and Packer, 1994). To determine carbonyl content of fat body homogenates, we used Protein Carbonyl Content Assay Kit (MAK094; Sigma-Aldrich, Burlington, Massachusetts, United States). Briefly, samples were treated with a 10 mg/ml streptozocin solution and incubated for 15 min to precipitate nucleic acids. Keeping the supernatant, 2,4-dinitrophenylhydrazones (DNPH) was added to samples to form stable dinitrophnyl hydrozone adducts. Derivatized proteins were precipitated with trichloroacetic acid and were followed by three successive ice-cold acetone washes. Samples were resuspended in 100 µl of 6 M guanidine (pH 2.3). The total protein concentration of each sample was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States; Smith et al., 1985). Protein oxidation was expressed as nanomoles of carbonyl groups per mg of protein.

Statistical analysis

We evaluated both relative and absolute abundance to emphasize different properties of the microbiome data (Anderson et al., 2018). ASVs were normalized by 16S rRNA gene copy number *via* ribosomal RNA operons (*rrn*) database (Stoddard et al., 2015) and total bacterial 16S rRNA gene copies from qPCR prior to analysis. In this case, qPCR-normalized abundance is extrapolated from relative abundance of amplicons, so remains compositional, so may be best referred to as normalized abundance. To allow the use of parametric multivariate analyses (Pearson, 1897), we converted the qPCR-normalized bacterial abundances to ratios among all ASVs (Gloor and Reid, 2016) using the software CoDaPack's centered log ratio (CLR) transformation (Comas and Thió-Henestrosa, 2011). After conversion, nearly all bacterial species followed normal distributions. Thus, a MANOVA on CLR transformed data considers changes of ASV abundance relative to the entire community, and the Wilcoxon results analyze absolute abundance of OTUs without reference to other community members (Anderson et al., 2018).

For the PF experiment a two-way MANOVA was performed on the CLR-adjusted abundances and Log10 normalized gene expression which allows for comparisons between dependent (ASVs or genes) and independent (age, ontogeny, and age*ontogeny) variables. For REV experiment we evaluated relative microbiota structure using a one-way MANOVA. We applied Pillai's Trace test statistic; robust to violations of multivariate normality and homogeneity of covariance, followed by a False Discovery Rate (FDR) to account for multiple comparisons. We also performed principle component analysis (PCA) on the matrix of CLR scores for each gut tissue, to visualize the relationship of bacterial community composition with behavioral task and age-associated succession. To determine differences in absolute abundance of the microbial communities, we used Wilcoxon rank sum tests corrected for multiple comparison with FDR. Absolute abundance was used to determine correlations between bacteria using Spearman's ρ, corrected by FDR for multiple comparisons.

We evaluated bacterial and fungal copy numbers by ontogeny (atypical versus typical) using one-way ANOVA with Tukey's HSD post-hoc. Gene expression was log10 transformed to normalize variation and analyzed by sample type using one-way ANOVA with Tukey's HSD post-hoc. PCAs of normalized gene expression was used to plot the relationship of immunity and oxidative stress genes for each experiment. For the REV experiment, a canonical correlation analysis was performed on log10 transformed gene expression. We compared carbonyl content by sample type using one-way ANOVA with Tukey's HSD post-hoc. To validate the marked cohort (MC) as a control for typical ontogeny, we compared SCC age-right nurse microbiota and gene expression with the MC using Wilcoxon 2-sample *t*-test with FDR corrections (Supplementary Table S2). Multivariate analyses were conducted on ASVs with gene expression and carbonyl contents using Spearman's ρ to find significant correlations after correcting for multiple comparisons with an FDR. All analyses were conducted in JMP_v14.3.0 (JMP_1989–2007) and/or SAS_v9.4 (Institute, SAS, 2015). We considered values of $p < 0.05$ statistically significant.

Results

16S rRNA gene sequencing

Next-generation sequencing returned 23.9 million quality trimmed reads (455 bp assembled) across 469 libraries. Libraries used in downstream analyses were sampled to exhaustion

according to goods coverage (>0.99%). The worker midguts (PF experiment) represented 5.0 million reads averaging 36.6 K per library. The worker ileums (used in both experiments) represented 13.2 million read averaging 55.9 K per library. The worker rectums (REV experiment) represented 5.6 million reads averaging 58.8 K per library. To examine the effect of community size in the midgut, the top 13 ASVs and a sum of remaining ASVs were used for downstream analysis. It should be noted that ASV 1–13 accounted for 84.5% of all sequences, the 14th group consisted of "SumOther" (Σ ASVs 14–740) accounted for the remaining 15.5%. In the ileum and rectum, the top 15 ASVs and a sum of remaining ASVs were also calculated for downstream analysis. In the ileum, ASV 1–15 accounted for 94.7% of sequences, while the 16th group of "SumOther" (Σ ASVs 16–214) accounted for the remaining 5.3%. ASV 1–15 in the rectum accounted for 88.9% of sequences, and the 16th group of "SumOther" (Σ ASVs 16–123) accounted for the remaining 11.1%.

Based on classification with BEExact, the PF midguts (Figure 2A; Supplementary Figure S1) the *Lactobacillus* Firm5 species cluster separated into 4 species: *L. apis*, *L. kimbladii*, *L. melliventris*, and *L. helsingborgensis*. Also common in the midgut, *Gilliamella* and *Apilactobacillus* separated into two species, and *G. apicola*, and *Gilliamella* sp., *A. kunkeei*, and *A. apinorum, respectively. Bifidobacterium asteroides*, *Bombilactobacillus mellis* (*L.* firm4), *Snodgrassella alvi*, *Frischella perrara*, and *Fructobacillus fructosus* were each represented by single species, however *F. fructosus* is the only species traditionally not considered core to the microbiome (Martinson et al., 2012; Sabree et al., 2012; Kwong and Moran, 2016). PF and REV ileum microbiotas (Figure 2A; Supplementary Figures S1, S2) were clustered together which resolved the same 4 *Lactobacillus* species and four distinct groups of *Gilliamella*: *G. apicola*, *G. apis*, *Gilliamella* sp., and *Gilliamella* unclassified. *Bifidobacterium* and *Snodgrassella* clustered into 2 species each: *B. asteroides*, *Bifidobacterium indicum*, *S. alvi*, and *S.* unclassified respectfully. The remaining species were represented by a single species each: *B. mellis*, *F. perrara*, and *A. kunkeei*. REV rectums (Supplementary Figure S3) were represented by the same four *Gilliamella* and *Lactobacillus* in the ileum with the exception of a 5th *Lactobacillus* that was included as *Lactobacillus* unclassified. *Bombilactobacillus* was represented by both traditionally known Firm4 species: *B. mellis* and *B. mellifer*. *Bifidobacterium asteroides*, *S. alvi*, *F. perrara*, and *Bartonella apis* were each represented by a single species.

For the PF experiment, the MC and SCC age-right nurses gut microbiota were compared and did not differ statistically at 7 and 13 days of age in the midgut and ileum (Supplementary Table S2). Additionally, a comparison between both SCC1 and SCC2 colonies and ASVs found some statistically significant differences in the midgut, but none for the ileum (Supplementary Table S3). Specifically, PFs from SCC1 had higher absolute abundances of *A. kunkeei*, *F. perrara*, *F. fructosus*, and *A. apinorum* at 7 days old and *F. fructosus*, *A. apinorum*, and "SumOther" at 13 days old. At 19 days, nurses in SCC1 had less *A. kunkeei*, *F. fructosus*, and *A. apinorum* than SCC2.

Microbiota and gene associations with age and ontogeny

The two-way MANOVA performed on PF experiment midguts and ileums revealed significant variation due to age and ontogeny, but not as an interaction factor (Supplementary Table S4). In the midgut, the MANOVA revealed significance for six of the bacterial species analyzed. The independent variables (IV) of age (F value 3.23, $\Pr > F = 0.0001$) and ontogeny (F value 2.57, $\Pr > F = 0.0047$) were significant for six and five species, respectively. Specifically, *Gilliamella* sp. was abundant with age only and highest in 27D age-right foragers and 19-day nurses (Supplementary Figure S6) which also had the largest proportions and the largest microbiotas based on 16S rRNA gene copies (Supplementary Figure S4A). The fungal loads were more similar between sample types and tissues but bifurcated by age and task in the midguts; the oldest nurses and 27D foragers had the greatest fungal loads (Supplementary Figure S4B). *Apilactobacillus kunkeei* was explained by ontogeny and in greater abundances than atypical PF. *Fructobacillus fructosus* was strongly correlated with *A. kunkeei* (Supplementary Table S5: Spearman's ρ, $r_s = 0.81$, $p = 0.0002$), but was explained by both age and ontogeny; seemingly increasing with age and in greater proportions during typical ontogeny. *Bifidobacterium asteroides*, *B. mellis*, and *L. melliventris* were highly correlated across all samples (Supplementary Table S5: Spearman's ρ, $r_s < 0.77$, $p = 0.0002$), but had higher relative abundance in PFs versus age-matched nurses as well as increasing with age. PCA on CLR adjusted midgut microbiota groups well by ontogeny, with 33% of the variation captured by the first component and 13.4% by the second component (Figure 2B). Grouping is also consistent on microbial based age-association (Supplementary Figure S5). Patterns predicted by MANOVA (Supplementary Table S4) are represented in the midgut PCA (Figure 2B) with *Apilactobacillus* and *F. fructosus* being more associated with typical ontogeny and the highly correlated species, *Lactobacillus*, *Bifidobacterium*, and *Bombilactobacillus* were more associated with the guts of atypical ontogeny bees.

A visual inspection of the ileum's relative abundances shows remarkable stability across age and ontogeny compared to the midgut (Figure 2A). The size of the ileum microbiota was mostly consistent across treatment groups (Supplementary Figure S4C). Nevertheless, the two-way MANOVA revealed significant variation by age (F value 2.48, $\Pr > F = 0.0001$) and ontogeny (F value 4.47, $\Pr > F = 0.0001$), but not as an interaction term (Supplementary Figure S4). There were 12 bacteria that differed significantly by relative abundance in the overall model, seven best explained by age and 10 by ontogeny. *Frischella perrara* was significant in the model, but failed to meet significant thresholds after FDR correction. The bacteria *B. mellis*, *G. apis*, *Bifidobacterium indicum*, *B. asteroides*, *L. melliventris*, and *L. helsingborgensis* were explained by both factors in the model. *Bombilactobacillus mellis*, *B. asteroides,* and the two *Lactobacillus* were also very strongly correlated across all samples

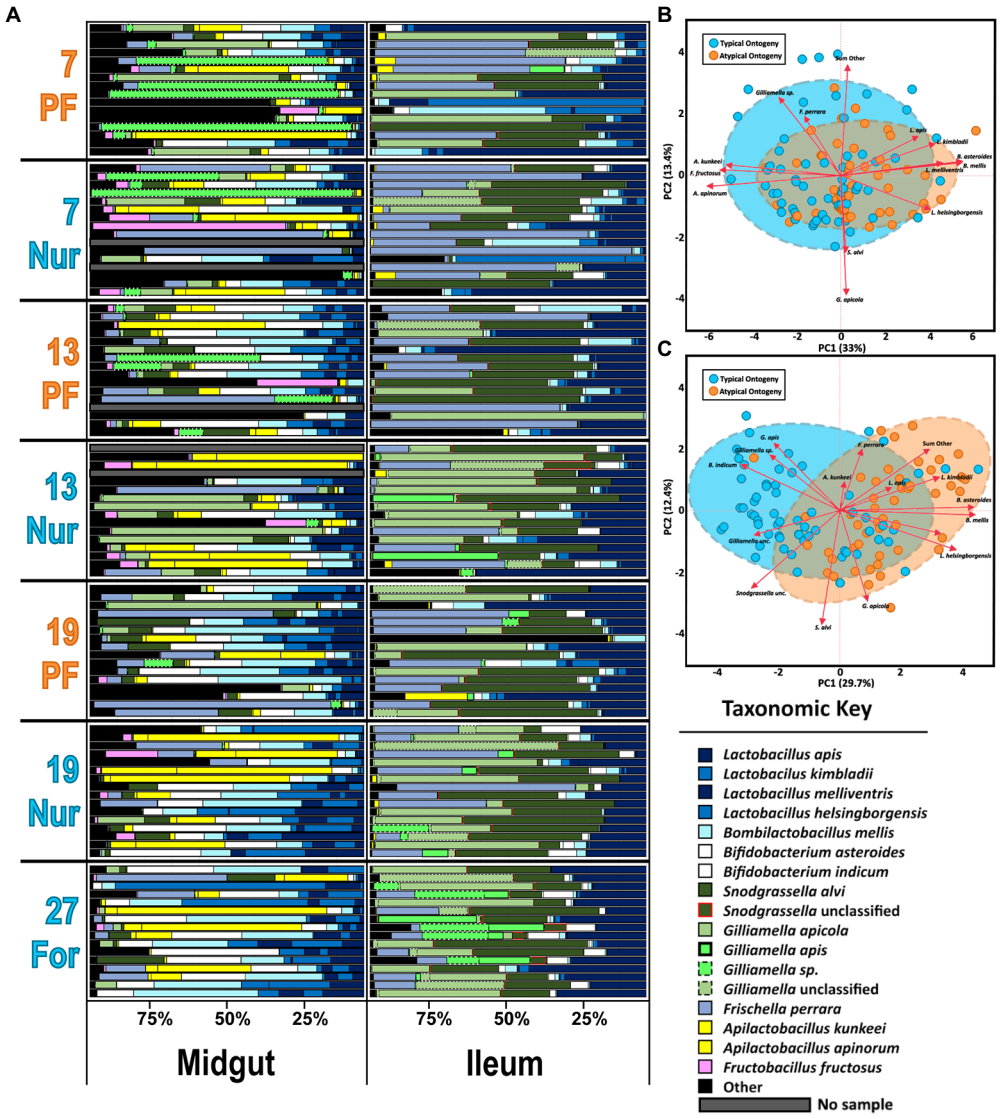

FIGURE 2
(A) The honey bee microbiota of the midgut and ileum by age and task. Color-coded bars represent relative abundance corrected by species-specific 16S rRNA gene copy number. Black represents diversity abundance, midgut: ASV's 14–740 and ileum: ASV's 16–214. **(B,C)** Principal component analysis of the midgut and Ileum, respectively, based on the most abundant ASVs. Clustered groups of points contain similar groupings of taxa with similar microbiota ratio abundances. Longer ASV vectors result from greater variation in CLR-adjusted scores. Density ellipses cover 90% of plots for each group.

(Supplementary Table S5: Spearman's ρ, $r_s > 0.60$, $p = 0.0004$). These bacteria were negatively correlated with *G. apis* (Supplementary Table S5: Spearman's ρ, $r_s < -0.19$, $p < 0.048$) and *B. indicum* (Supplementary Table S5: Spearman's ρ, $r_s < -0.44$, $p < 0.0004$). *Gilliamella* sp. was explained by age in the MANOVA model and was most abundant in the 19 and 27-day-old bees (Supplementary Figure S7). Following patterns of typical or atypical ontogeny, an unclassified *Gilliamella*, an unclassified *Snodgrassella*, *L. kimbladii*, and the group of "SumOther" differed significantly. A Wilcoxon test of absolute abundance reveals the unclassified *Gilliamella* had greater abundance under conditions of typical ontogeny, while *L. kimbladii* and "SumOther" were greatest in atypical bees (Supplementary Figure S7). In contrast to the midgut, 16S/18S rRNA gene copy number in the ileum was relatively stable regardless of age or ontogeny (Supplementary Figures S4C,D). A PCA on CLR adjusted ileum microbiota shows more separation than the midgut for ontogeny, with 29.7% and 12.4% explained for the first and second component, respectively, (Figure 2C). Age-associated groupings are also consistent with the MANOVA where 27D bees break out from the other age groupings, especially in respect to *G. apis*, *Gilliamella* sp., and *B. indicum* (Supplementary Figure S5).

Correlations among the major gut species also reflects typical and atypical ontogeny. In the midgut, the abundance of *S. alvi* and

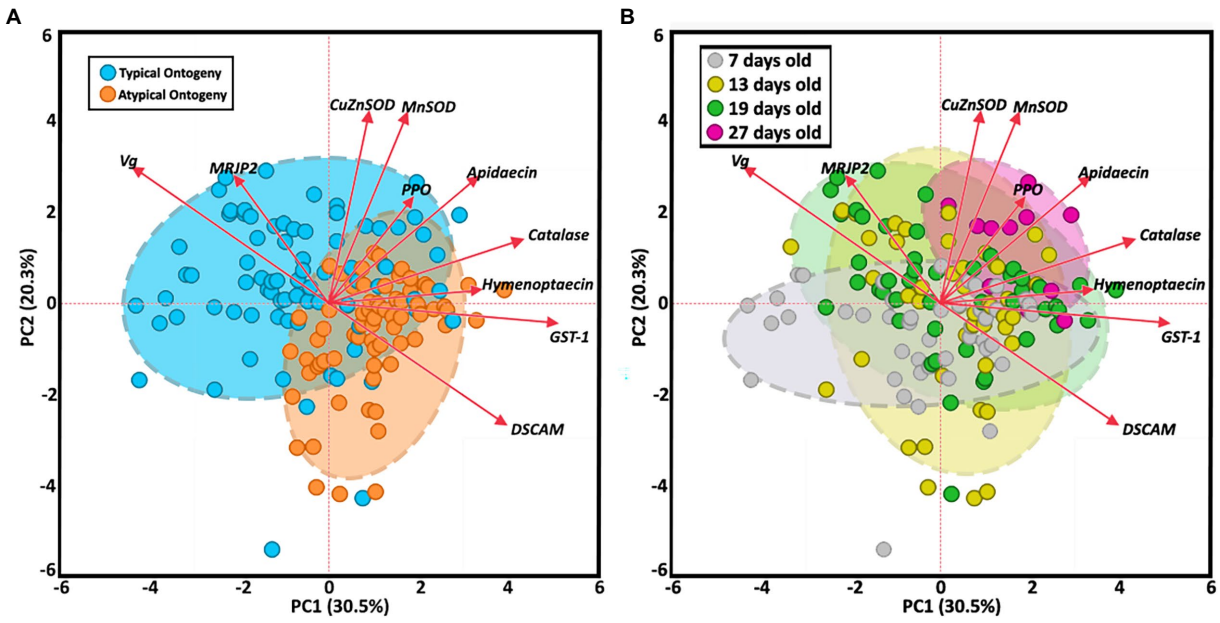

FIGURE 3
Principal component analysis of fat body gene expression by both ontogeny **(A)** and age **(B)**. Clustered groups of points contain similar gene expression. Longer vectors result from greater variation in gene expression. Density ellipses cover 90% of plots for each group.

G. apicola was positively correlated in workers with natural ontogeny (Supplementary Table S5), but showed no relationship in atypical PFs. *Gilliamella* sp. in PFs was also positively correlated with *F. fructosus* and *A. apinorum*, but in typical ontogeny these correlations were shared as well as strong positive correlations with *A. kunkeei*, *B. asteroides*, *L. apis*, *L. kimbladii*, and *S. alvi*. *Gilliamella apicola* and *Gilliamella* sp. absolute abundance did not correlate together significantly. In the ileum there were strong negative correlations between *G. apicola* and *G. apis* in all samples and groups (atypical vs. typical; Supplementary Table S6). An association with *S. alvi* and the unclassified *Gilliamella* group was found in typical ontogeny but missing in atypical PFs.

The two-way MANOVA revealed a significant interaction effect of age and ontogeny for fat body gene expression (Supplementary Table S7). However, there were greater effects seen for individual gene differences by age or ontogeny. Two PCAs illustrate the relationship of immune gene expression with ontogeny and/or age (Figure 3). The first principal component (30.5%) maximizes the explained variance of immune gene expression, with gene vectors along the horizontal explaining greater variance associated with ontogeny (Figure 3A). In contrast, variation in gene expression distilled by the second component (20.3%) is more closely associated with age (Figure 3B). Overall, gene expression differed significantly by ontogeny or age or a combination of both factors (Figure 4). *Vitellogenin* and *MRJP2* are highly expressed in all age nurses and downregulated in age-matched and age-right foragers (Figures 4A,B). *Catalase*, *hymenoptaecin*, *GST-1*, and *DSCAM* had increased expression in atypical PFs relative to age-matched nurses following typical ontogeny (Figures 4C–F). Hymenoptaecin was significant by MANOVA for an interaction factor between ontogeny and age (Supplementary Figure S7), while *GST-1* and *DSCAM* were not. However, *GST-1* and *DSCAM* could be explained by either ontogeny or age independently and expression patterns show gene expression being controlled by both factors (Figures 4D–F). *CuZnSOD* and apidaecin followed age-associated gene expression patterns with age-right 27D foragers having the highest transcript levels (Figures 4G,H). *MnSOD* was also explained by an interaction factor in the MANOVA full model and *PPO* was the same across groups regardless of ontogeny or age (Supplementary Table S7).

We looked at Spearman's ρ correlations between the midgut and ileum bacterial absolute abundances with fat body gene expression to investigate potential relationships between these tissues and overall immune health (Supplementary Tables S7–S10). In the midgut of atypical PFs, there was a negative correlation of *MRJP2* with *F. fructosus*, *A. kunkeei*, *Apilactobacillus apinorum*, and *Gilliamella* sp. For nurses (age 7, 13, and 19), there were significant correlations between *CuZnSOD* and *Gilliamella* sp., *A. kunkeei*, and *A. apinorum* and when considering typical ontogeny including age-right 27D foragers there were many additional positive relationships with *CuZnSOD*; *Gilliamella* sp., *A. kunkeei*, *A. apinorum*, *L. apis*, *L. melliventris*, *F. fructosus*, *S. alvi*, *B. mellis*, *B. asteroides*, and the collective sum of remaining bacteria "SumOther". *Gilliamella* sp. was also negatively correlated with *MRJP2* and *Vg*, while positively correlated with *GST-1*, *apidaecin*, and *MnSOD*. *Hymenoptaecin* was also strongly correlated with *F. fructosus* and *A. kunkeei*.

In the ileum *Gilliamella* sp., *G. apis*, and the unclassified *Gilliamella* present significant correlations among bacteria and fat

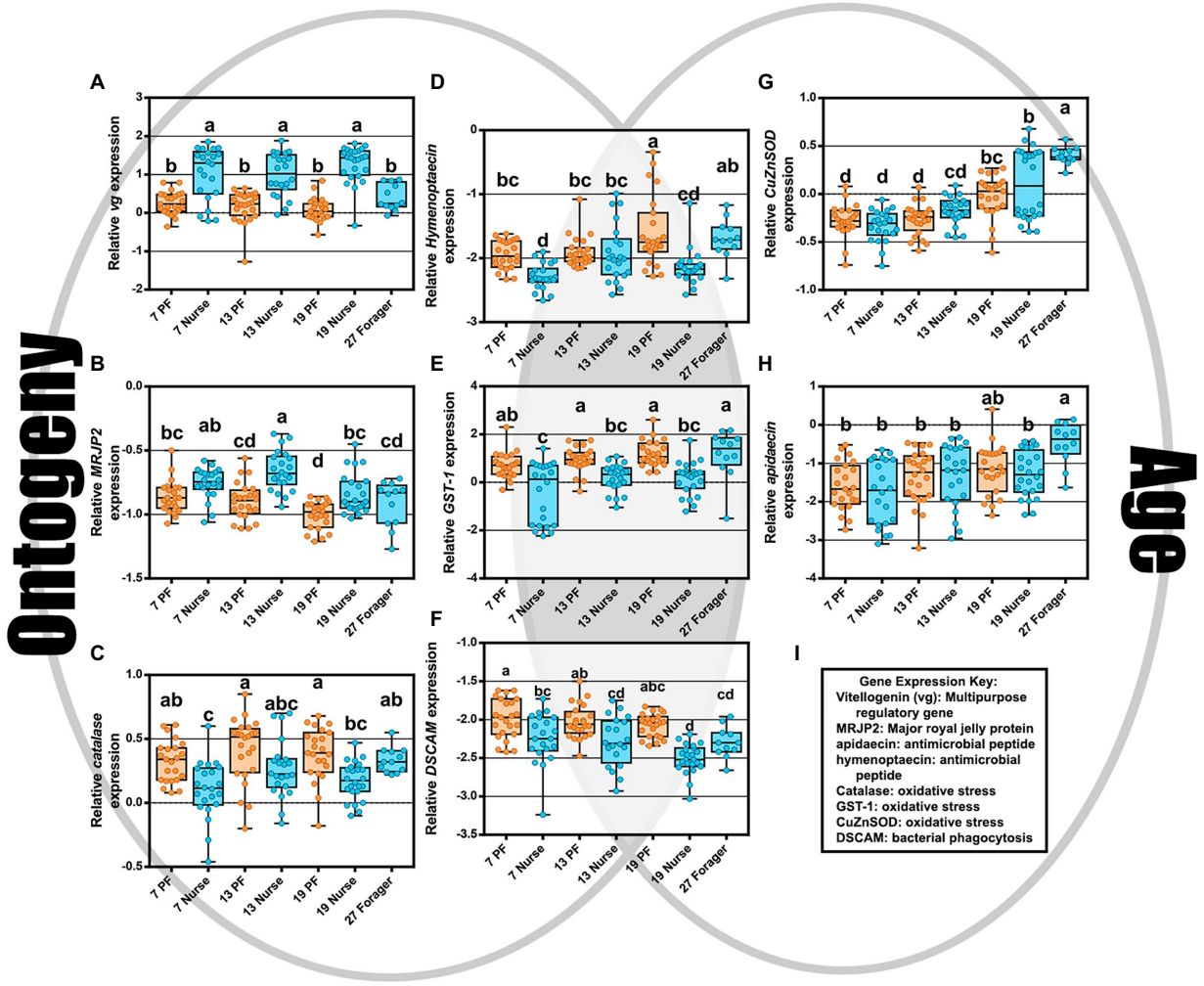

FIGURE 4
As depicted by the Venn diagram (gray background), gene expression reflects ontogeny, age, or an interaction of the two. **(A–C)** Vitellogenin, MRJP2, and catalase expression were associated with ontogeny. **(D–F)** hymenoptaecin, GST-1, and DSCAM expression were explained by either an interaction factor or combination of both ontogeny and age. **(G,H)** CuZnSOD and apidaecin expression increased with age regardless of ontogeny. **(I)** Key explaining functional roles of gene expression. Different letters indicate significant differences ($p<0.05$).

body gene expression (Supplementary Tables S9, S10). *Gilliamella* sp. was positively correlated with *CuZnSOD* in atypical PFs while *MnSOD* was positively correlated with *G. apis*. Again when considering typical ontogeny bees (including 27D) there were many more significant correlations. *CuZnSOD* was strongly correlated with *G. apis* and *Gilliamella* sp. *MnSOD* expression was strongly correlated with *G. apis*, *B. indicum*, and negatively correlated with *L. helsingborgensis*. Other significant negative correlations were *MRJP2* with *Gilliamella* sp. and *GST-1* with *L. helsingborgensis*. When considering all samples together there were more significant correlations, such as *Gilliamella* sp. being negatively correlated with *DSCAM* as well as the unclassified *Gilliamella*. The antimicrobial peptide *apidaecin* was also positively correlated with *Gilliamella* sp. and *B. indicum*. Interestingly, *B. asteroides* and *B. indicum* had a negative and positive correlation with *Vg* respectfully.

Microbiota and gene associations with reversion

A one-way MANOVA comparing the ileums and rectums of 8D and 27D old bees with the reversion experiment's initial foragers (IF) and reverted nurses (REV) had some significant differences (Supplementary Figure S4). The overall MANOVA model in the ileum indicates significant differences by phenotype (F value 2.01, $Pr > F = 0.0004$) and for dependent variables: *S. alvi*, *F. perrara*, *L. kimbladii*, *L. melliventris*, and an unclassified *Snodgrassella*. Despite these significant values, the Wilcoxon results were not significant for any bacteria. The MANOVA model for the rectum was also significant (F value 2.1, $Pr > F = 0.0002$) with significant dependent variables *L. melliventris*, *L. apis*, *Bartonella apis*, *B. mellifer*, and an unclassified *Lactobacillus* group. The Wilcoxon found significant differences in the rectum when comparing all phenotypes together,

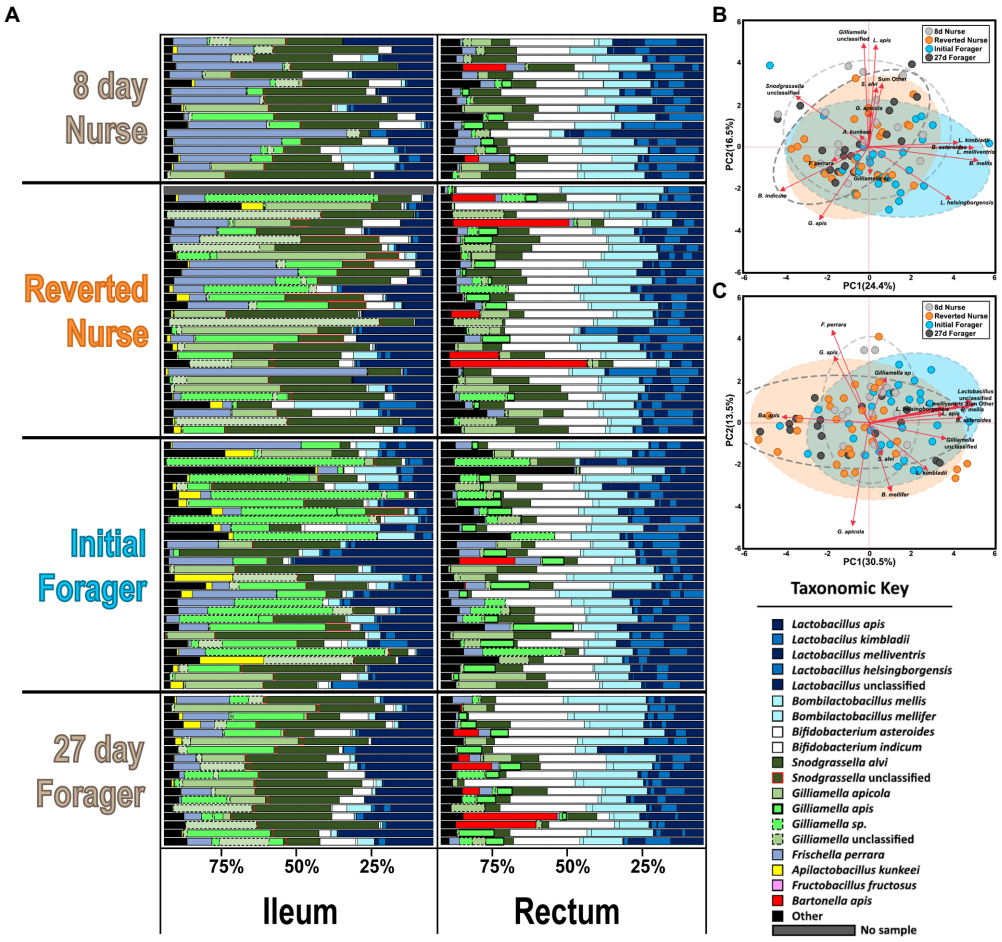

FIGURE 5
(A) Honey bee microbiota of the ileum and rectum. Results for the reversion experiment compare 8-day-old control nurses, 27-day-old control foragers, initial foragers (pre-reversion), and reverted nurses (initial foragers after 1week of nursing). Color-coded bars represent relative abundance corrected by species-specific 16S rRNA gene copy number. Black represents diversity abundance (ileum: ASV's 16–214, rectum: ASV's 16–123). **(B,C)** Principal component analysis of the ileum and rectum, respectively, based on the most abundant ASVs. Clustered groups of points contain similar groupings of taxa with similar microbiota ratio abundances. Longer ASV vectors result from greater variation in CLR-adjusted scores. Density ellipses cover 90% of plots for each group.

but not when comparing reverted nurses to 8D nurses. Wilcoxon comparing REV with IF found a significant difference in the absolute abundance of *Ba. apis*, with REV having larger proportions. PCA on CLR adjusted ileum and rectum microbiota did not show as much separation in phenotypes as PF PCAs (Figures 5B,C). Groups shared significant overlap, but some differences seen in the rectum are explained solely by the presence of *Ba. apis* in several samples (Figure 5C). Bacterial load evaluated by total 16S copy number did not vary in the ileum or rectum (Supplementary Figure S4). Fungal load was also stable across phenotypes in the ileum, but were statistically significant in the rectum with 8D nurses and reverted nurses having the highest loads compared to 27D foragers and IF.

An LDA of fat body gene expression shows overlap of both forager phenotypes (27D and initial) but separation between 8D and REV (Figure 6A). Reverted nurse gene expression matched 8D nurses for increased *vitellogenin* expression and decreased for antimicrobial peptides *hymenoptaecin* and *defensin1* (Figures 6B–D). Other gene expression, including immune pathways or pathway–adjacent genes were either statistically lower relative to 8D, IF, and/or 27D bees or met a pattern of general depression (Figures 6E–L). Correlations between bacteria and gene expression in reverted nurses (Supplementary Tables S11, S12) had several positive interactions in the ileum. *Toll* expression positively correlated with *B. mellis*, *B. asteroides*, and *L. melliventris*, while *abaecin* expression was associated with *L. helsingborgensis* and *L. kimbladii* abundances. *Lactobacillus melliventris* was negatively correlated with *abaecin* in the rectum of REV, but generally across all phenotypes there were less correlations in the rectum.

Carbonyl contents of abdomen

Oxidative damage in the abdomen was measured by carbonyl assay of the fat body and attached abdominal sclerites. PFs

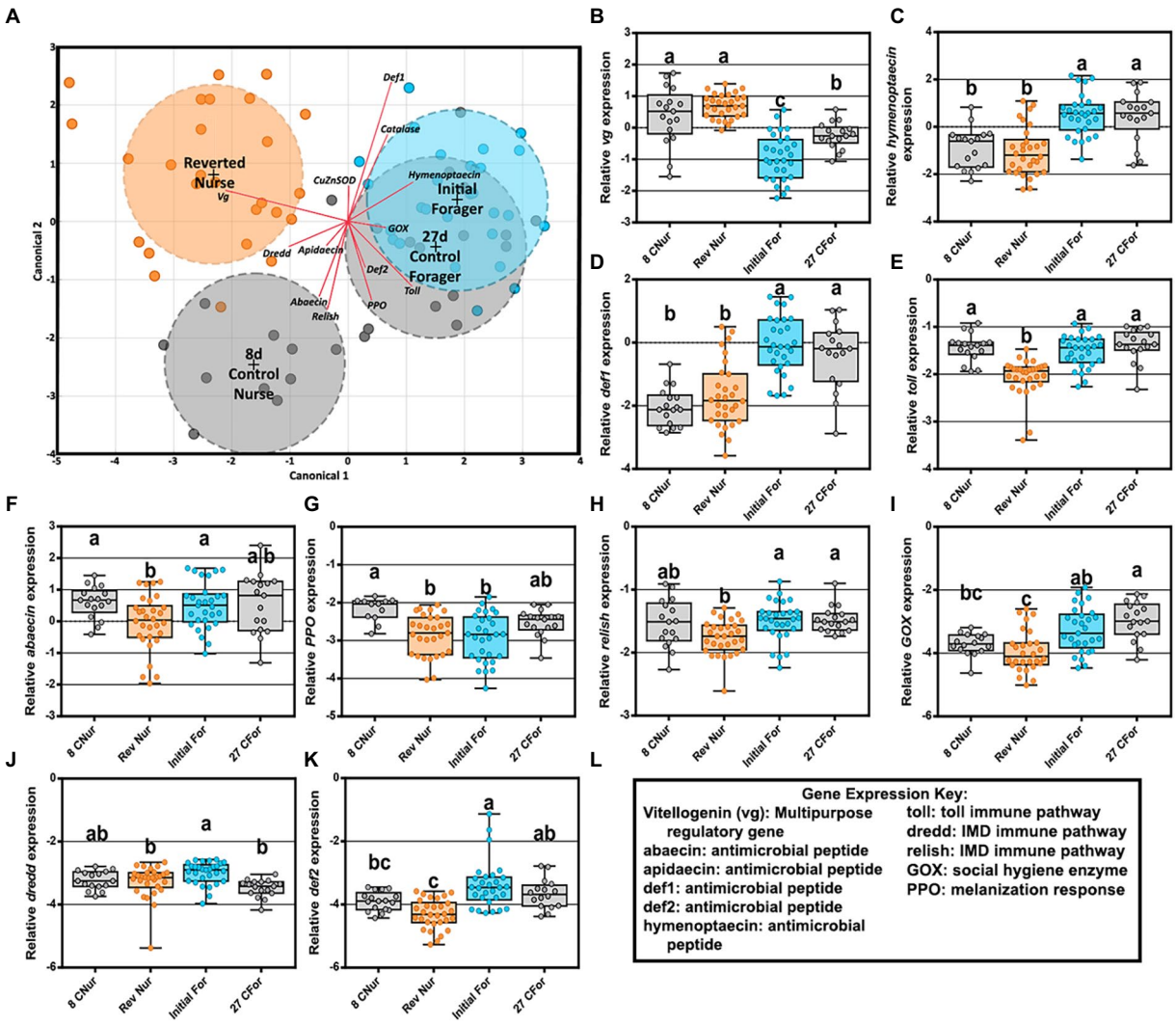

FIGURE 6
Reversion experiment gene expression comparing 8-day-old control nurses, 27-day-old control foragers, initial foragers (pre-reversion), and reverted nurses (initial foragers after 1week of nursing) **(A)** Canonical correlation analysis of fat body gene expression by age and behavioral phenotype. Ellipses demarcate 95% confidence and phenotypes are displayed at the group's centroid. **(B–D)** Fat body gene expression of reverted nurses was similar to that of 8-day-old control nurses. **(E–K)** Reverted nurse gene expression was reduced compared 8-day-old control nurses or foragers. **(L)** Key explaining functional roles of gene expression. Different lower case letters indicate significant differences ($p<0.05$).

accumulated more oxidized proteins than age-matched nurses, a finding that became statistically meaningful after 19-days but started to trend in this direction at 13 days old (Figure 7). 19D PF had accrued significantly more carbonylation than 27D foragers following the typical ontogeny. Although we did not control for age in the REV study, IF and REV were biologically the oldest bees sampled. IF were presumed to follow typical ontogeny and 19D PF (known foragers since at least 7D) had reached similar levels of oxidative damage. As predicted Carbonyl contents decreased with increased Vg expression ($r_s = -0.30$ $p < 0.0005$; Supplementary Table S12). To consider whether fat body gene expression or tissue bacterial absolute abundance had a relationship with carbonyl content accumulation we ran Spearman ρ correlation analyses (Supplementary Tables S7–S10). In the midgut, carbonyl contents were positively correlated with *S. alvi* in atypical PFs but also *Gilliamella* sp., *L. apis*, *F. fructosus*, *F. perrara*, *B. mellis*, and the "SumOther" across all samples. In the ileum, atypical PFs had carbonyl positively correlated with *Gilliamella* sp. and *L. apis*. The strong correlation with *Gilliamella* sp. also carried over to correlations across all samples, but interestingly there were also many correlations with carbonyl with typical ontogeny. Carbonyl had strong positive correlations with *Gilliamella* sp., *L. apis*, *G. apis*, and "SumOther" and a strong negative correlation with *L. helsingborgensis*.

Discussion

Division of labor and phenotypic plasticity are the hallmarks of social insect success (Hölldobler and Wilson, 1990; Fewell, 2003). In the experiments detailed here, we manipulated colony-level task

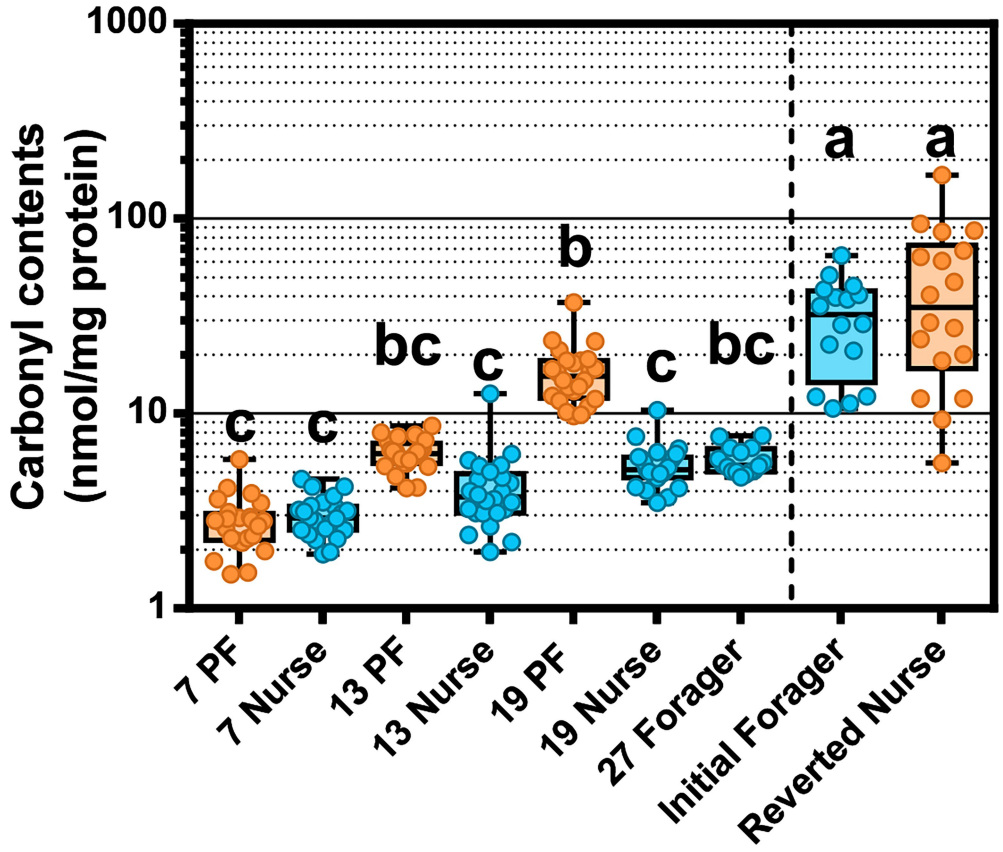

FIGURE 7
Oxidative stress measured as protein carbonylation in the worker fat body. Different letters indicate significant differences ($p<0.05$). The line separates PF and REV experiments. REV initial foragers and reverted nurses have unknown ages, but were presumed to be the oldest bees across both experiments.

allocation to investigate effects on tissue-specific microbiome succession and associated physiology (Figure 1). During typical adult ontogeny, worker honey bees undergo an age-based division of labor, transitioning from in-hive tasks to riskier foraging behavior as they age. The natural acquisition, and succession, of the gut microbiome has been described throughout this process, a data set biased toward young nurse-aged bees (Sabree et al., 2012; Corby-Harris et al., 2014; Kwong and Moran, 2016; Anderson et al., 2018; Copeland et al., 2022). Our results provide a new perspective on the aging microbiota, detailing tissue-specific results from a large sample of workers aged >19 days. In the reversion (REV) experiment, we induced a social reversion of foragers back to a nursing behavioral phenotype, requiring a second round of pollen consumption by older aging workers. The precocious forager (PF) experiment induced workers to forage much earlier than normal, highlighting a common colony-level phenotype associated with various forms of colony dwindling (Perry et al., 2015).

Early nutritional state is associated with typical adult ontogeny in many social insects including honey bees (Amdam et al., 2004; Anderson et al., 2008). In early adult workers, the somewhat constitutive expression of vitellogenin (Vg) following pollen consumption is associated with insulin-like peptide signaling and the presence of the hindgut microbiome (Zheng et al., 2017). In turn, the Vg rich environment found in the hive provides social immune function and extended resistance to oxidative stress in the hemolymph (Amdam et al., 2003; Seehuus et al., 2006; Corona et al., 2007). A worker that does not engage in foraging can live for many months to nearly a year (Sakagami and Fukuda, 1968). This extended period within the hive also allows partner choice among core gut bacterial species of *Gilliamella* for *Snodgrassella* providing favorable microbiome function (Kwong et al., 2017; Zheng et al., 2017). Unlike the other core microbiota members, *Gilliamella* establishment is strongly influenced by diet and social interaction, perhaps even social immunity (Powell et al., 2014; Anderson et al., 2016, 2022). The factors that contribute to increased *Gilliamella* dominance in the aging ileum require more investigation. Past work had assumed complete hindgut assembly by 7–9 days of age, a time point defined by a high frequency of nurse duties, but our results here (Figure 2) and in the literature (Moran et al., 2012; Horton et al., 2015) indicate that *Gilliamella* establishment in the ileum can require >13 days.

Our experimental design was successful in quantifying the typical physiology and microbiota of adult worker development capturing the natural transition from nurse to forager. Associated with the control of complex social behaviors in honey bees, Vg is

tightly linked to division of labor, patterns of oxidative stress and immune gene expression, and succession of the gut microbiota. As revealed by the carbonyl assay, the accumulation of oxidative stress was a function of Vg expression. As the primary exemplar, mated queens can withstand extreme oxidative stress without loss of function. Mated queen phenotypes are constantly fed royal jelly, and thus high Vg titers circulate constantly throughout their hemolymph providing protection from oxidative damage (Amdam et al., 2003; Seehuus et al., 2006; Corona et al., 2007). Here we found a similar function for Vg in reverted nurses, wherein investment in oxidative stress and immunity was significantly depleted in the high Vg environment (Figure 6B).

A number of significant relationships between microbial species abundance and fatbody gene expression support our post-hoc hypothesis; that oxidative stress or immune gene expression predicts microbial taxonomy and abundance. Beyond relationships demonstrated previously (Zheng et al., 2016, 2017; Emery et al., 2017; Kwong et al., 2017), we note a relationship of *Gilliamella apis* abundance in the ileum with the expression of CuZnSOD, and carbonyl accumulation in precocious foragers (Table 1). Giving context to this relationship, *Gilliamella apis* is also associated with old age, becoming the dominant *Gilliamella* in the ileums of workers aged 27 days and older (Figure 5). In contrast, early ileum colonization was dominated by *G. apicola*. While *G. apicola* maintains its numbers with age, both *G. apis* and an unclassified *Gilliamella* species begin to reproduce in older workers beginning at 13 and 19 days, respectively (Figure 2). Competition between *G. apicola* and *G. apis* is supported by significant negative correlations within individual ileums, but in general, all four *Gilliamella* species either remain stable or increase in absolute abundance with age. We suggest that larger and more diverse *Gilliamella* populations are supported by changes in host physiology and/or diet that accompany aging.

Reversion experiment

Given the established relationship between vitellogenin expression and the hindgut microbiome (Zheng et al., 2017), we predicted that old foragers reverting to a nursing phenotype would harbor a healthy nurse-like hindgut microbiome. During this transition, we found that the ileum and rectum (hindgut) microbiotas remained remarkably stable (Figure 5) suggesting that the established biofilm had attained a climax community resistant to host physiological changes. Given their high nutritional state, the reverted nurses may not require the expression of oxidative stress genes or immune genes at levels matching a young nurse. Foragers subsist primarily on simple sugars; nectar and honey. For a forager to revert to a nurse, a second round of pollen consumption is required to meet the physiological demands of nursing. Gene expression profiles were distinct between reverted nurse, young nurse and older forager phenotypes (Figure 6A) reflecting differences in the expression of antimicrobial peptides (AMPs), oxidative stress, and innate immunity genes. Both forager groups shared significant gene expression by task at 27 days. Although reverted nurses were the oldest bees in the study, they had the greatest Vg expression (Figure 6B), even higher than 8 day old nurses. This study and others show that foragers typically have increased expression of AMPs and oxidative stress genes relative to nurses (Vannette et al., 2015; Cervoni et al., 2017). In contrast, with the exception of Vg, gene expression from reverted nurses was mostly depleted or depressed in relation to both younger nurses and foragers (Figures 6C–K).

The expression differences between young and old nurses are best explained with reference to vitellogenin expression and carbonyl content. The carbonylation in worker fat body reflects the oxidation of Vg as compared to other storage proteins, such that a direct relationship is evident in our results (Figure 7). Reverted nurses showed the greatest Vg expression and the greatest ratio of oxidized carbon atoms relative to general protein. Similar to mated queen phenotypes, Vg provides major long-term oxidative stress management for the insect (Fedorova et al., 2014; Salmela et al., 2016).

Precocious foragers experiment

At the other phenotypic extreme, precocious foragers (PFs) experience a rapid depletion of Vg and were less likely to possess *Gilliamella* spp. partnered with *Snodgrassella* spp. in the ileum contributing to gut dysbiosis. Based on results obtained with the genes selected for this analysis, host physiology was best explained by an interaction of age and ontogeny, with the greatest variation explained by ontogeny (Supplementary Table S7). This result is likely a reflection of differential nutrition and Vg titers associated with both age and task performance (Figure 4). We found that Vg was already differentially expressed by day 7 for same-age nurses and PFs (Figure 4A). Thus, Vg expression and task specialization influenced the trajectory of microbiome succession and fat body gene expression in typical versus atypical ontogeny paths. PFs with low Vg expression and poorly developed ileum microbiomes incur oxidative damage *via* the accumulation of carbonyl contents in the hemolymph at a significantly greater rate than both age-matched nurses and age-right (27D) foragers (Figure 7). Young bees transition to foraging faster when there is limited social contact with older bees (Huang and Robinson, 1996; Pankiw, 2004). This can occur in response to various biotic (predators, pathogens) or abiotic pressures including pesticides and anthropogenic factors. The nutrient deficient physiology of PFs may be poorly suited for tasks outside the hive (Vance et al., 2009) and evidence suggest that PF individual risk of death increases relative to older foragers (Prado et al., 2020). Precocious foraging is also less productive (Chang et al., 2015), factors that conspire to accelerate colony loss (Perry et al., 2015).

Antimicrobial peptides (AMPs) are expressed as part of the innate immune system of the honey bee (Alberoni et al., 2016). Explained primarily by ontogeny, we found high levels of hymenoptaecin in all ages of foragers and strong upregulation of apidaecin in 27D foragers. This pattern is supported by studies

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TABLE 1 Summary of Spearman rank correlations of ileum OTU absolute abundance, carbonyl, and fat body gene expression that were determined significant after FDR correction ($p<0.05$) for various phenotypes.

Phenotypes included in spearman ρ correlations	Gene expression or carbonyl	Bacterial species	Spearman ρ	Prob > $\lvert\rho\rvert$	Plot
All samples 7PF, 7Nu, 13PF,	Carbonyl	*Lactobacillus apis*	0.4457	0.0008	++++
13Nu, 19PF, 19Nu, 27For	GST-1	*Lactobacillus apis*	0.4295	0.0008	++++
	CuZnSOD	*Gilliamella* sp.	0.4105	0.0008	++++
	CuZnSOD	*Gilliamella apis*	0.3913	0.0008	++++
	Vitellogenin	*Lactobacillus apis*	−0.3497	0.0015	−−−
	Carbonyl	SumOther	0.3335	0.0021	+++
	Carbonyl	*Gilliamella* sp.	0.3122	0.0048	+++
	MNSOD	*Gilliamella apis*	0.3167	0.0048	+++
	Apidaecin	*Lactobacillus apis*	0.3093	0.0062	+++
	MRJP2	*Bifidobacterium asteroides*	−0.3057	0.0069	−−−
	MRJP2	*Lactobacillus apis*	−0.3036	0.0073	−−−
Atypical ontogeny 7PF, 13PF,	Carbonyl	*Gilliamella* sp.	0.6307	0.0035	++++++
19PF	CuZnSOD	*Gilliamella* sp.	0.4437	0.0281	++++
	Carbonyl	*Lactobacillus apis*	0.4347	0.0305	++++
	MRJP2	*Apilactobacillus kunkeei*	−0.4525	0.0222	−−−−−
Typical ontogeny 7Nu, 13Nu,	Vitellogenin	*Lactobacillus apis*	−0.5714	0.0023	−−−−−−
19Nu	GST-1	*Lactobacillus apis*	0.4911	0.0067	+++++
	MnSOD	*Gilliamella apis*	0.4628	0.0113	+++++
	Carbonyl	*Lactobacillus helsingborgensis*	−0.4532	0.014	−−−−
Typical ontogeny 7Nu, 13Nu,	Vitellogenin	*Lactobacillus apis*	−0.5595	0.0013	−−−−−−
19Nu, 27For	MnSOD	*Gilliamella apis*	0.4779	0.0013	+++++
	CuZnSOD	*Gilliamella apis*	0.4562	0.0025	+++++
	GST-1	*Lactobacillus apis*	0.4398	0.0041	++++
	Carbonyl	*Lactobacillus helsingborgensis*	−0.4218	0.005	−−−−
	Carbonyl	*Gilliamella* sp.	0.4097	0.0074	++++
	Carbonyl	*Lactobacillus apis*	0.4034	0.0088	++++
	MnSOD	*Bifidobacterium indicum*	0.3796	0.0193	++++
	CuZnSOD	*Gilliamella* sp.	0.3628	0.0281	++++
	Carbonyl	SumOther	0.3449	0.031	+++
	MRJP2	*Gilliamella sp.*	−0.3519	0.0328	−−−−
	GST-1	*Lactobacillus helsingborgensis*	−0.3439	0.0378	−−−
	CuZnSOD	*Snodgrassella* unclassified	0.3342	0.0444	+++
	Carbonyl	*Gilliamella apis*	0.3211	0.046	+++

that show foragers express genes encoding AMPs in greater abundance than nurses (Vannette et al., 2015). Similar to our findings, honey bees inoculated with gut microbiota or mono-colonized with *S. alvi*, upregulated apidaecin and hymenoptaecin constitutively in the fat body (Kwong et al., 2017). The core microbiota tends to have increased tolerance for host AMPs compared to non-native microorganisms (Kwong et al., 2017), therefore it's advantageous to constitutively express AMPs as a prophylactic measure given that foragers are exposed to more pathogen pressure outside the hive. Younger foragers also expressed DSCAM higher than age-matched nurses, with an age-associated decline by 19D (Figure 4F). In *Drosophila*, DSCAM is a highly diverse Ig-superfamily receptor that may affect phagocytic uptake of bacteria by host hemocytes (Watson et al., 2005). The number of honey bee hemocytes decrease in relation to age and behavioral task (Amdam et al., 2004, 2005; Schmid et al., 2008), thus, foragers have decreased hemocyte counts in the hemolymph and a higher number of pycnotic cells than nurse bees. The honey bee DSCAM gene has the potential to generate as many as 12,000 splice variants which may allow them to target specific microorganisms (Graveley et al., 2004). The expression of DSCAM shows a strong negative association with Vg expression (Supplementary Table S13), suggesting it is not governed by nutritional state. Taken together, higher DSCAM expression in nutrient depleted foragers may serve to increase efficiency of the decreased number of hemocytes.

We also considered the effects of oxidative stress in relation to aging and ontogeny. Oxidative stress, produced by intensive foraging flights, is likely mitigated by host enzyme expression within the limits of host physiology. We found that CuZnSOD

increased with age, and catalase and GST-1 were highest in precocious foragers. CuZnSOD detoxifies the free radical superoxide (O_2·-) into the less reactive hydrogen peroxide (H_2O_2), which is then processed by catalase into water and oxygen (Lei et al., 2016). Mitochondrial activity during aerobic respiration (flight) is the main cause of ROS generation, which tends to generate more H_2O_2. Fat body respiration is greater in nurses due to the continuous metabolic function needed to sustain brood rearing. Foragers experience ROS production in the flight muscles of the thorax that would circulate throughout the hemolymph. A previous study found that abdominal H_2O_2 levels were elevated in forager flight muscles as a likely result of increased mitochondria density (Cervoni et al., 2017). Foragers also have decreased abdominal lipid stores (Toth and Robinson, 2005), less developed fat body (Ament et al., 2008; Wilson-Rich et al., 2008), and a decrease in Vg expression compared to nurses (Seehuus et al., 2006). While young bees have generally more resistance to oxidative stress, foragers incur a gradual accumulation of tissue damage reflecting age-associated declines in the efficiency and degradation of ROS (Williams et al., 2008). We showed that 19D PF that had been foraging since at least 6-days old, had a higher level of fat body protein carbonylation relative to age-matched nurses and 27D foragers (Figure 7). The age-right 27-day foragers had the highest oxidative stress gene expression, which could explain their low levels of protein carbonylation. These were the bees that remained nurses the longest and most recently transitioned to foraging (see Vg expression relative to 7–13–19-day foragers Figure 4A). The 19D PFs antioxidant capacity may have reached its physiological limit. Paradoxically, ROS can have positive effects such as acting in redox signaling pathways (Lei et al., 2016) or modulating the microbiota (Engel and Moran, 2013b). Our results suggest that the physiological cost of early foraging is extreme, and highlight the progression of colony dwindling, a common but poorly understood process.

The midgut microbiota varied in composition based on age and ontogeny (Figure 2; Supplementary Table S4). In agreement with past results (Anderson and Maes, 2022) we report the aging midgut as a potential niche for microbial invasion (Figures 3, 4). It has been suggested that the midgut is inhospitable to microbial colonization due to the continual shedding of the peritrophic membrane, however, recently it was shown that the peritrophic membrane is absent or greatly reduced in foragers (Harwood and Amdam, 2021), which may leave the tissue vulnerable to microbial opportunism. We saw a massive increase in midgut microbiome size in 27D foragers, an order of magnitude larger than younger bees including significantly more (non-core) bacterial diversity (Supplementary Figure S4); a trend observed as early as 7D (Supplementary Figure S4). *Gilliamella apicola* and *Gilliamella* sp. absolute abundance in the midgut was greatest in age-matched nurses vs. foragers and comprised nearly half of bacterial cells in 27D foragers. Likewise, CuZnSOD expression levels were associated with both bacterial cell abundance and *Gilliamella* spp. in the midgut (Figure 4; Supplementary Tables S7, S8). *Gilliamella* often dominates the midgut (Ludvigsen et al., 2015), but can be lacking in the ileums of young nurse workers, becoming better established at middle age (Anderson and Ricigliano, 2017). Strains of *Gilliamella* have varying capabilities to degrade pollen cell wall components (Engel and Moran, 2013a), metabolize toxic monosaccharides (Zheng et al., 2016), and encode partner compatibility genes such as type VI secretion systems (Steele et al., 2017). *Gilliamella apis* and the unclassified *Gilliamella* abundances in the ileum showed the strongest relationship with ontogeny, establishing more efficiently in nurses than precocious foragers. As hypothesized previously (Anderson and Maes, 2022) performing tasks within the hive improves the chance of compatible *Gilliamella* establishment, or fortifies its establishment *via* other mechanisms.

Midgut bacterial growth of 27 day old foragers was characterized by increased diversity abundance coupled with blooms of *A. kunkeei* and *F. fructosus* and an unclassified species of *Gilliamella*. Coculture assays demonstrate that *A. kunkeei* and *F. fructosus* support the growth of other honey bee symbionts considered "core hindgut bacteria" (Rokop et al., 2015). Together, this suggests that some so-called transient microbes likely have co-evolved functional roles within the honey bee gut as more ubiquitous microbial members. In the midgut, samples often had a dynamic mix of *Lactobacillus* comprised of at least 3–4 strains in varying proportions. However, the ileum was largely dominated by *Lactobacillus apis* which should be considered the *de facto* ileal strain. This is supported by previous research indicating there are a variety of host adapted strains of *Lactobacillus* Firm5 in the system, some more proximately available to colonize niches during age-based succession (Anderson et al., 2016). Additionally, *L. apis* is a pioneer species able to populate the honey bee ileum and maintain dominance even as its relative abundance gives way to other core members. *Lactobacillus apis* was also associated with carbonyl accumulation and negatively associated with Vg expression suggesting a direct relationship with the aging ileum and overall host senescence.

The ileal *Gilliamella/Snodgrassella* relationship is one primary metric of a healthy gut microbiome (Zheng et al., 2017). For the adult worker, *S. alvi* is considered a keystone species in the ileum/pylorus, interfacing with host epithelium and creating a protective biofilm with *G. apicola* and *Lactobacillus apis* (Martinson et al., 2012). *Snodgrassella alvi* protects the host from opportunism (Maes et al., 2016), while consuming oxygen and producing nutrients to support other gut bacteria (Kwong and Moran, 2016). Both *G. apicola* and *S. alvi* increase with age and stabilize in ratio abundance in the midgut and ileum, succession apparently accelerated and reinforced by an extended nursing role in early life (Supplementary Tables S6, S7). The succession of gut bacteria in honey bee workers is typically considered a climax community at 7–9 days of adult life. However, we found that variation of the three major ileum bacteria was minimized, and evenness of the entire microbiome maximized at 27 days of age, suggesting the attainment of a climax community that

provides the greatest protection for new foragers. The different successional patterns documented between midgut and ileum might suggest an important function of the gut microbiome in mitigating gut opportunism and dysbiosis. Following the decreased production of midgut peritrophic membrane, bacteria populate the midgut, a process that may rely on early bacterial succession and strong establishment of core ileum species, as seen with normal ontogeny. We speculate that the co-evolved character of ileum bacteria present at this transition may contribute to longevity or accelerate opportunistic diseases like *Nosema*. Natural or premature immunosenescence provides context for how immunity shapes and is shaped by the host microbiota. Host regulation/dysregulation of the microbiome resulting in the reduction of *Lactobacillus* spp. and expansion of *Gilliamella* spp. with age requires more investigation.

Conclusion

Here we found that the social structure of honeybee colonies affects composition of the gut microbiota and associated metabolism. Honey bees are challenged continuously by environmental and agricultural factors that alter colony demography. Occurring with *Nosema* disease, pollen dearth, pesticide exposure and viral disease, precocious foraging is a widespread colony-level deficiency. Worker aging in honey bees can be defined as the ratio between vitellogenin levels and oxidative stress. High vitellogenin titers in reverted nurse phenotypes compensated for decreased gene expression associated with immunity and oxidative stress. Premature foragers quickly accrue oxidative damage as a result of intense foraging activity and low Vg levels. The physiological demands of foraging are best met by older workers that have transitioned to foraging following extended development within the hive. The ratio abundance of keystone ileum species *S. alvi* and *G. apicola* is refined with an extended nursing period within the hive, setting the stage for long-lived foragers. Our study highlights the importance of tissue-specific microbiome sampling, revealing niche specialization of bacterial species. In agreement with previous hypotheses (Anderson and Ricigliano, 2017), our results indicate that the aging midgut becomes a niche for rapid microbial colonization, with potential consequences for both individual and colony survival.

Data availability statement

The data presented in the study are deposited in GenBank, Sequence Read Archive, accession numbers PRJNA801240 and PRJNA885470.

Author contributions

DC, PM, BM, and KA contributed to experimental design and commented on the manuscript. DC and PM performed laboratory work with the input of KA and BM. DC and KA analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1059001/full#supplementary-material

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Diversity and antibacterial potential of the Actinobacteria associated with *Apis mellifera ligustica*

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Insect-associated Actinobacteria are a potentially rich source of novel natural products with antibacterial activity. Here, the community composition of Actinobacteria associated with *Apis mellifera ligustica* was investigated by integrated culture-dependent and independent methods. A total of 61 strains of *Streptomyces* genera were isolated from the honeycomb, larva, and different anatomical parts of the honeybee's body using the culture-dependent method. Amplicon sequencing analyses revealed that the actinobacterial communities were dominated by the family of Bifidobacteriaceae and Microbacteriaceae in the honeybee gut, and Nocardiaceae and Pseudonocardiaceae in the honeycomb, whereas only *Streptomyces* genera were isolated by the culture-dependent method. Culture-independent analyses showed more diverse actinobacterial communities than those of culture-dependent methods. The antibacterial bioassay showed that most crude extracts of representative isolates exhibited antibacterial activities. Among them, the crude extract of *Streptomyces* sp. FCF01 showed the best antibacterial activities against *Staphylococcus aureus*, *Micrococcus tetragenus*, and *Pseudomonas syringae* pv. *actinidiae* (Psa) with the disc diameter of inhibition zone diameter (IZD) of 23.00, 15.00, and 13.33mm, respectively. Chemical analysis of *Streptomyces* sp. FCF01 led to the isolation of three secondary metabolites, including mayamycin (**1**), mayamycin B (**2**), and N-(2-Hydroxyphenyl) acetamide (**3**). Among them, compound **1** displayed strong antibacterial activity against *S. aureus*, *M. tetragenus*, and Psa with minimum inhibitory concentrations (MIC) values of 6.25, 12.5, and 6.25μg/ml, respectively. In addition, two novel derivative compounds **1a** and **1b** were synthesized by acetylation of compound **1**. Both compounds **1a** and **1b** displayed similar antibacterial activities with those of metabolite **1**. These results indicated that *Streptomyces* species associated with honeybees had great potential in finding antibiotics.

KEYWORDS

honeybee, Actinobacteria, antibacterial activity, secondary metabolites, community composition

Introduction

Bee-associated microorganisms play an important role in nutritional function, pathogen protection, host behavior regulation (Menezes et al., 2015; Paludo et al., 2018, 2019; Zheng et al., 2018; Paxton, 2020; Zhang et al., 2022). These microorganisms are not only sourced from the gut of bees, but also from other anatomical parts of bees, food sources (pollen, beebread, and honey), and honeycombs (Khan et al., 2020). Bacteria are common bee-associated microorganisms and have also been the focus of attention (Zheng et al., 2018). In contrast to Gram-negative bacteria, Gram-positive Actinobacteria associated with bees are less well studied (Promnuan et al., 2021). Bee-associated Actinobacteria have been isolated from diverse bee species, including honeybees (*Apis mellifera*, *A. cerana*, *A. florae*, *A. dorsata*, and *A. andreniformis*), stingless bees, and wasps and other key components of bees (including larvae, adults, brood cells, hive, pollen, beebread, honey, and honeycomb; Promnuan et al., 2009, 2020, 2021; Poulsen et al., 2011; Anjum et al., 2018; Cambronero-Heinrichs et al., 2019; Grubbs et al., 2021). Isolated Actinobacteria have mainly belonged to the genera *Streptomyces*, and some other rare genera, such as *Micromonospora*, *Nonomuraea*, *Nocardiopsis*, *Actinomadura*, and *Saccharopolyspora*. Furthermore, some bee-associated Actinobacteria have good antimicrobial potential against the pathogen of *Paenibacillus*, human pathogens, and plant-pathogenic bacteria (Cambronero-Heinrichs et al., 2019; Rodríguez-Hernández et al., 2019; Promnuan et al., 2021). Previous studies have found that bee-associated Actinobacteria produced antibiotics to inhibit pathogens of bees (Engl et al., 2018; Rodríguez-Hernández et al., 2019; Menegatti et al., 2020; Grubbs et al., 2021). Thus, bee-associated Actinobacteria harbor the biosynthetic potential to produce antimicrobial compounds. Although antibiotics have been found in some bee-associated Actinobacteria, they are still a huge and underexplored repository to search for novel antibiotics or natural products.

Honeybee (*A. mellifera ligustica*) is a kind of eusocial insect, which is widely distributed in primary beekeeping areas of China (Xiao et al., 2021). To the best of our knowledge, fewer studies have focused on Actinobacteria associated with *A. mellifera ligustica* compared with other bee species. In this study, we investigated the diversity of Actinobacteria from honeybee (*A. mellifera ligustica*) by using culture-dependent and independent approaches, and assessed the antibacterial activity of culturable Actinobacteria. Additionally, we described the isolation, structural elucidation, and derivatization of secondary metabolites produced by one *Streptomyces* strain with antibacterial activity.

Materials and methods

Sample collection

Honeybee samples (including 35 larvae, 49 adults, and honeycomb) were collected from the Institute of Apicultural Research, Anhui Agricultural University, Hefei, China (GPS: 31°53′ N, 117°20′ E) between November 2021 and April 2022. The honeybee larvae and adults starved for 24 h. Some honeybee samples were stored at −20°C for isolation and −80°C for DNA extraction, respectively.

Actinobacteria isolation

Initially, seven adult honeybees were separately placed into 10 ml of sterile water in an autoclaved tube to obtain Actinobacteria from external isolation. Then, seven individuals of honeybee larvae and adults were separately placed in an autoclaved 50 ml tube with 10 ml 75% ethanol for 2 min (Xu et al., 2020), followed by rinsing in 10 ml of sterile water three times (30 s each). For the honeycomb, one gram sample was also processed using the same method. After external sterilization, sterile forceps were used to divide samples of the adult honeybee to get the head, gut, and abdomen. According to the earlier report (Chevrette et al., 2019), each body part of the adult honeybee, larvae, and honeycomb was fully homogenized separately in 10 ml of sterile water. Finally, the homogenates were diluted in a 10-fold series (i.e., 10^{-1}, 10^{-2}, 10^{-3}), and an aliquot of 100 µl suspension was spread to six different Actinobacteria-selective media types (Supplementary Table S1), including cellulose-casamino acid (CC), starch casein agar (SCA), Reasoner's 2A agar (R2A), Gause's No. 1 (GS), modified HV (M-HV), and Actinobacteria isolation agar (AIA). All isolation media were amended with nystatin (50 mg/L), nalidixic acid (25 mg/L), cycloheximide (25 mg/L), and potassium dichromate (25 mg/L) to suppress the growth of Gram-negative bacteria and fungi (Li et al., 2021). The cultures were incubated at 28°C for 1–4 weeks. The actinobacterial colonies obtained after incubation were transferred onto Gause's No.1 agar and then preserved on slants at 4°C or as glycerol suspensions (25%, v/v) at −80°C until use.

Molecular identification and phylogenetic analysis of isolates

Isolates were cultivated on Gause's No.1 medium at 28°C, and then preliminarily identified according to their distinct morphological characteristics. DNA extraction of each isolate was performed as described by Jiang et al. (2018). The specific primer pair 27F (5′-TCCTCCGCTTATTGATATGC-3′)/1492R (5′-GGTTACCTTGTTACG ACTT-3′) were used to amplify 16S rRNA based on the actinobacterial genomic DNA, and all PCR reactions were conducted according to the previous method (Long et al., 2022). Then, each successful product was sent to Tsingke Biotechnology Co., Ltd. (Beijing, China) for sequencing. All achieved sequences were compared with those of closely related reference strains and obtained the top hits (described species) with type material sequences using the EzTaxon-e server (Kim et al., 2012; https://www.ezbiocloud.net/). Neighbor-joining

phylogenetic tree was constructed using the MEGA software version 5.0, and bootstrap replication (1,000 replications) was used to assess the topology of the phylogenetic tree (Felsenstein, 1985). The obtained gene sequences were deposited in the GenBank database under accession numbers OP491886-OP491954.

Culture-independent community analysis

The external sterilization of seven adult honeybees and one gram honeycomb were the same as those mentioned above method to obtain the honeybee gut and honeycomb. The total community DNA of the honeybee samples was performed using the Fast DNA Extraction Kit referring to the manufacturer's instructions. Then, the yield and purity of DNA were detected with electrophoresis on a 2% agarose gel. Each sample was repeated three times. The hypervariable regions V4 of the 16S rRNA gene were targeted for amplification by PCR with primers 515F and 806R. The PCR reaction was carried out with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 μM of forward/reverse primers, and about 10 ng of template DNA. The reaction conditions of PCR were performed as described method (Cui et al., 2022). Mixture PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The PCR products were pooled in an equimolar ratio and purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The sequencing library was generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States) according to the manufacturer's instructions, and index codes were added. The library quality was evaluated on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2,100 system. Finally, the library was sequenced on an Illumina NovaSeq platform using 250 bp paired-end reads.

Raw data obtained from sequencing were merged using FLASH (V1.2.7; Magoč and Steven, 2011). Then, quality filtering on the raw tags was performed to obtain high-quality clean tags according to the QIIME (V1.9.1; Caporaso et al., 2010). Subsequently, the clean tags were compared with the Silva database using UCHIME Algorithm to detect and remove chimera sequences (Edgar et al., 2011; Haas et al., 2011). The sequences with ≥97% pairwise identity were assigned to the same operational taxonomic units (OTUs) by Uparse software (Uparse v7.0.1001; Edgar, 2013). For each OTU, the Silva Database was used based on the Mothur algorithm to annotate taxonomic information (Quast et al., 2013). Raw data were available from the NCBI Short Read Archive under accession numbers PRJNA883759 and PRJNA882994.

Extracts preparation and antibacterial assay

Based on morphological characteristics and molecular identification, 49 isolates were selected for small-scale fermentation to screen isolated actinobacterial strains with antibacterial activity. Strains were cultivated in a 250 ml Erlenmeyer flask containing 150 ml of Gause's No.1 liquid medium and incubated at 28°C under 180 rpm for 7 days. The culture was passed through four layers of cheesecloth to get the supernatant. Then, the supernatant was extracted three times by using a separatory funnel with ethyl acetate (EtOAc, 1:1, v/v). The upper organic layer was condensed by a vacuum to obtain the crude extract for further experimental use.

The antibacterial activity of crude extracts of isolated strains was determined by using the filter paper disc method (Xu et al., 2020). Specifically, crude extracts were dissolved separately in acetone to get a concentration of 10 mg/ml. 5 μl of the tested crude extract was dripped on a sterile paper disk (diameter, 6 mm), then the paper disk was placed on the Luria broth (LB) agar plates containing the tested strains. Four bacterial strains including *Staphylococcus aureus* (ATCC6538), *Micrococcus tetragenus* (ATCC35098), *Escherichia coli* (ATCC8739), and *Pseudomonas syringae* pv. *actinidiae* (Psa) were used as indicator pathogens, three of which (*E. coli*, *M. tetragenus*, and *S. aureus*) were cultivated at 37°C for 24–36 h. Psa was cultivated at 28°C for 24–36 h. In addition, 5 μl of pure acetone alone and gentamicin sulfate with a concentration of 10 mg/ml served as the blank control and positive control, respectively. The plates were prepared in triplicate. Lastly, the diameters of inhibition zone diameter (IZD, in mm) were measured for evaluating antibacterial activity.

Isolation and characterization of secondary metabolite

One strain FCF01 with the best antibacterial activity was selected for the purification and identification of compounds in this study. The strain FCF01 was inoculated into a 250 ml Erlenmeyer flask containing 150 ml of Gause's No. 1 liquid medium and incubated at 28°C under 180 rpm for 3 days. Then, aliquots (15 ml) of the seed culture were transferred into 1,000 ml Erlenmeyer flasks filled with 400 ml of the same medium and cultured at 28°C for 7 days with shaking at 180 rpm. The fermentation broth (16 L) was filtered, and the supernatant was extracted with EtOAc (3 × 16 L). The EtOAc phase was concentrated by a rotary evaporator under reduced pressure to obtain 2.5 g of crude extract.

The crude extract was divided into six fractions using column fractionation packed with silica gel (200–300 mesh) eluting with dichloromethane (CH_2Cl_2)/methanol (MeOH; 100,0, 100,1, 100,2, 100:4, 100:8, and 100:16, v/v; fractions 1–6). Fraction 6 (CH_2Cl_2/MeOH, 100:16, v/v) was further fractionated on a silica gel column, eluting with CH_2Cl_2/MeOH (100,16, v/v) to yield compound **1** (310 mg) and subfraction (R1). The subfraction R1 was loaded onto a Sephadex LH-20 column (MeOH) to give compound **2** (1.6 mg). Fraction 3 (CH_2Cl_2/MeOH, 100:2, v/v) was loaded onto a Sephadex LH-20 column (MeOH) to give compound **3** (2 mg).

The structure of the secondary metabolites was determined by using spectroscopic analysis. NMR spectra were measured with Agilent 600 MHz DD2 spectrometers (Agilent, United States). HR-ESI-MS data were obtained by using a TripeTOF 4,600 mass analyzer (Bruker, United States).

Acetylation of compound 1

According to the previous method with some modifications (Park et al., 2019), a solution of 0.075 mmol of compound **1** was added in 2.0 ml of dimethylformamide (DMF) and 21 µl of Acetic anhydride (Ac_2O). After stirring the mixture for 4 h at 25°C, distilled water was added and the mixture was extracted with EtOAc (3 × 15 ml). The resulting mixture was concentrated *in vacuo* and purified by analytical HPLC (XBridge C18 column, 250 × 10 mm i.d., 5 µm, 1.0 ml/min, 0.0–30.0 min, and CH_3OH: H_2O = 90:10) to obtain compounds **1a** (t_R = 15.8 min, 6.0 mg) and **1b** (t_R = 18.2 min, 2.0 mg).

Antibacterial activities of compounds

The antibacterial activity of the compounds was determined by the methods of minimum inhibitory concentrations (MICs; Li et al., 2014) and filter paper disc method (Xu et al., 2020). Four bacteria including *S. aureus*, *M. tetragenus*, *E. coli*, and Psa were used to assess the antibacterial activity. MICs of compounds were measured in disposable 96-well microtiter dishes. Specifically, a stock solution of each tested compound (200 µg/ml) was further 2-fold diluted in LB liquid medium and added separately into individual wells (100 µl/well) with a series of concentrations ranging from 100 to 3.13 µg/ml. Then, a 100 µl standard amount of the tested bacteria (1.0×10^6 CFU/ml) were added per well. The 96-well plates were incubated at 37°C for 12–14 h. The control wells contained the same amount of culture broth and bacteria without the compound. The lowest concentration of compounds that inhibit bacterial growth was defined as MIC, as shown by no turbidity. Gentamicin sulfate was used as the positive control. Each test was performed three times. The diameters of IZD (in mm) of the compounds were determined by using the filter paper disc method as previously described for the antibacterial activity of crude extracts of isolated strains.

Results

Isolation and identification of Actinobacteria

In this study, a total of 61 isolates were obtained from the honeycomb, larvae, and different parts of the adult honeybee on six different media. Among them, 15 isolates were isolated from the honeycomb, 12 from larvae, 12 from the honeybee gut, 11 from the honeybee head, six from the honeybee cuticle, and five from the honeybee abdomen (Figure 1A). The majority of isolates were recovered from CC (17 isolates, 27.9%) and SCA medium (17 isolates, 27.9%), followed by R2A (11 isolates, 18.0%), GS (six isolates, 9.8%), AIA (five isolates, 8.2%), and M-HV (five isolates, 8.2%; Figure 1B). Thus, the CC and SCA media favored the isolation of *Streptomycetes*.

All isolates were identified using 16S rRNA sequencing and analyzed by BLAST. The results showed that all isolates had high similarity to members of the genus *Streptomyces* belonging to the family Streptomycetaceae (Supplementary Table S2; Supplementary Figure S1). Especially, EzTaxon analysis of the 16S rRNA gene sequences revealed that some isolates showed relatively low similarities to the type strains of the corresponding genera. For example, two isolates (BTF01 and BTF07) showed only 98.67% similarity to *S. cavourensis* NBRC 13026^T, which indicated a potential new species. One isolate BTF12 also showed similarity to *S. cavourensis* NBRC 13026^T with a low identity of 98.74%. Moreover, some similar actinobacterial strains were isolated from different parts of the honeybee, larva, and honeycomb. For instance, BTF27, BFF03, YCF15, and BCF05, which were isolated from the head, abdomen, larva, and gut, respectively, showed 99.86% similarity to *S. cavourensis* NBRC 13026^T.

Culture-independent community

The bacterial communities in the honeybee gut and honeycomb were analyzed by sequencing the V4 region of the bacterial 16S rDNA gene. Amplicon sequencing yielded a total of 430,065 high-quality bacterial clean reads distributed across 1918 OTUs. According to taxonomic classifications of OTUs, a total of 29 known phyla were identified in the samples of honeybee gut, wherein the Proteobacteria (59.53%) was the most abundant phylum, followed by the phylum Firmicutes (34.95%) and Actinobacteria (4.05%; Figure 2A). Proteobacteria (60.54%) was also the dominant phylum in the honeycomb. However, Actinobacteria was the fifth most prevalent phylum in the honeycomb with a relative abundance of 2.08% (Figure 2B). The actinobacterial communities were further analyzed at the family level, in which 15 families were identified from the honeybee gut, and 23 families from the honeycomb (Supplementary Table S3, S4). Among them, Pseudonocardiaceae (20.38%), Nocardiaceae (12.68%), Nocardioidaceae (12.02%), Micrococcaceae (11.19%), and Intrasporangiaceae (10.72%) had higher abundance in the honeycomb (Figure 2D). However, the relative abundance of the family Bifidobacteriaceae in honeybee gut was very high (97.24%), followed by the family Microbacteriaceae (0.77%), Mycobacteriaceae (0.35%), and Micrococcaceae (0.35%; Figure 2C; Supplementary Table S3). In addition, the family Streptomycetaceae showed lower relative abundance in both the honeybee gut (0.10%) and honeycomb (0.26%).

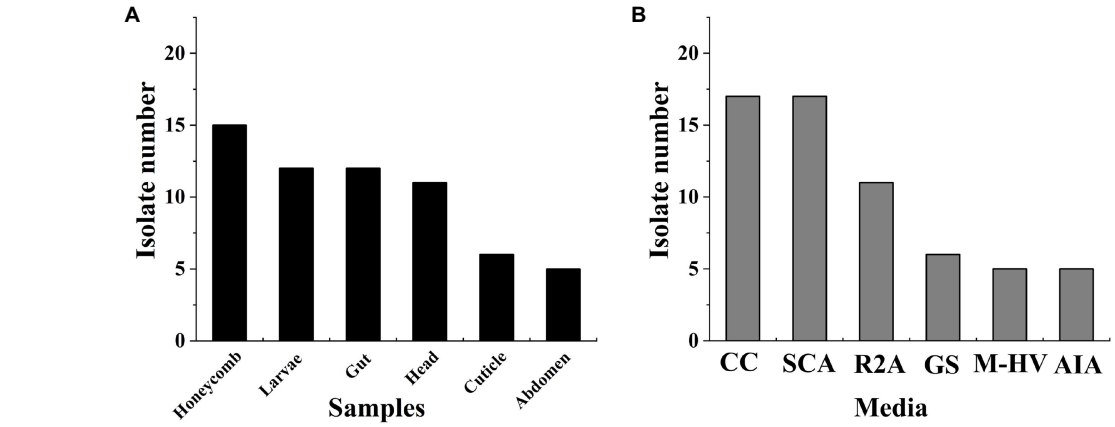

FIGURE 1
Statistics of Actinobacteria isolated from honeybee samples. **(A)** Different isolation parts of samples; **(B)** Different isolation media.

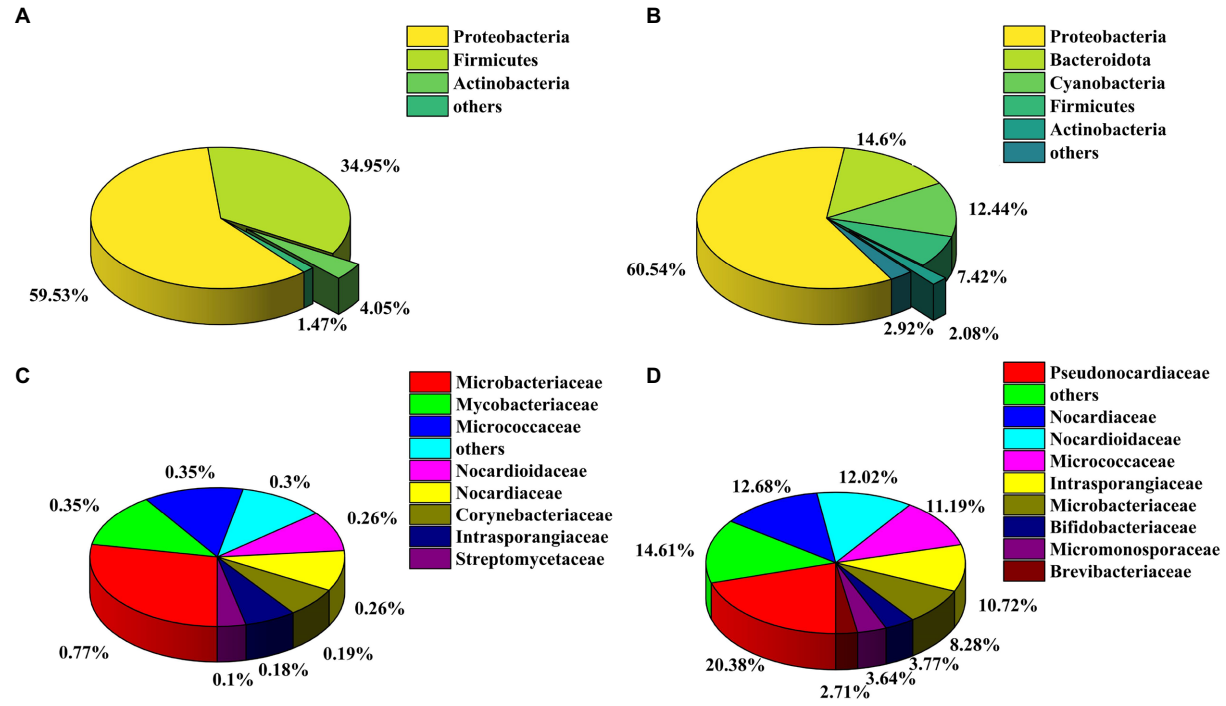

FIGURE 2
Analysis of culture-independent bacterial communities. Relative abundance of OTUs at the phylum level of honeybee gut **(A)** and honeycomb **(B)**; Relative abundance of OTUs at the family level from the phylum Actinobacteria of honeybee gut **(C)** and honeycomb **(D)**.

Screening for antibacterial activities

The antibacterial activities of crude extracts were performed by the filter paper disc method. The results showed that 38 of the 49 isolates (77.6%) exhibited antibacterial activities against at least one of the tested bacterial strains (Supplementary Table S5). Especially, three isolates (BTF05, YCF09, and BCF02) exhibited antibacterial activities against both Gram-positive and Gram-negative bacteria. FCF01 and BFF04 showed moderate to excellent antibacterial activities against *S. aureus* with an IZD of more than 12.00 mm, which was slightly weaker than the positive gentamicin sulfate with an IZD of 21.67 mm. BTF05, BTF15, and BCF02 exhibited remarkable inhibitory activities against *M. tetragenus*

with an IZD of more than 25.00 mm, which was slightly weaker than the positive gentamicin sulfate with an IZD of 37.67 mm. Furthermore, the strain FCF01 exhibited moderate antibacterial activity against *M. tetragenus* with an IZD of 15.00 mm. In addition, eight and 20 isolates exhibited antibacterial activities against *E. coli* and Psa, respectively.

Identification of secondary metabolites and derivative compounds

Three compounds were purified from Gause's No. 1 liquid fermentation product of *Streptomyces* sp. FCF01 and their structures were determined to be mayamycin (**1**; Bo et al., 2018), mayamycin B (**2**; Bo et al., 2018), and N-(2-Hydroxyphenyl) acetamide (**3**; Shang et al., 2012; Figure 3A) by spectroscopic data analyses and comparison of their data in the literature. The synthesis pathways of derivative compounds based on compound **1** are shown in Figure 3B. The structures of derivatives (**1a** and **1b**) were identified based on the 2D-NMR spectroscopic analysis and (HR)-ESI-MS data.

Mayamycin (**1**): brown solid; HR-ESI-MS: *m/z*: 464.1677 $[M+H]^+$, calculated for $C_{26}H_{25}NO_7$ 463.1631; ^{1}H NMR (600 MHz, CD_3OD) δ: 8.00 (1H, s, 4-H), 7.75 (1H, td 7.62, 10-H), 7.58 (1H, s, 11-H), 7.29 (1H, s, 9-H), 6.74 (1H, s, 2-H), 5.73(1H, d 11.04, 1′-H), 3.61 (1H, m, 5′-H), 3.52 (1H, td 9.48, 4′-H), 3.43 (1H, m, 3′-H), 2.75 (3H, s, 3′-N-CH_3), 2.48 (3H, s, 3-CH_3), 2.35 (2H, d 12.36, 2′-H), 1.47 (3H, d 5.94, 5′-CH_3); ^{13}C NMR (150 MHz, CD_3OD): 194.2 (C7), 188.0 (C12), 162.9 (C8), 156.6 (C1), 143.5 (C3), 139.9 (C4a), 138.9 (C6a), 137.8 (C11a), 124.8 (C9), 120.3 (C11), 119.4 (C12a), 117.8 (C12b), 117.4 (C4), 116.4 (C7a), 114.7 (C2), 79.2 (C5′), 74.2 (C4′), 72.8 (C1′), 62.9 (C3′), 32.5 (C2′), 31.1 (3′-N-CH_3), 22.64 (3-CH_3), and 18.6 (C5′-CH_3).

Mayamycin B (**2**): brown solid; HR-ESI-MS: *m/z*: 450.1550 $[M+H]^+$, calculated for $C_{25}H_{23}NO_7$ 449.1475; ^{1}H NMR (600 MHz, CD_3OD) δ: 7.99 (1H, s, 4-H), 7.75 (1H, td 7.92, 10-H), 7.58 (1H, d 7.38, 11-H), 7.28 (1H, d 8.46, 9-H), 6.74 (1H, s, 2-H), 5.71(1H, d 11.7, 1′-H), 3.58 (1H, m, 5′-H), 3.43 (1H, m, 4′-H), 3.43 (1H, m, 3′-H), 2.52 (1H, m, 2′-H), 2.45 (3H, s, 3-CH_3), 2.20 (1H, d 13.08, 2′-H), and 1.43 (3H, d 6.12, 5′-CH_3).

N-(2-Hydroxyphenyl) acetamide (**3**): white powder; HR-ESI-MS: *m/z*: 152.0708 $[M+H]^+$, calculated for $C_8H_9NO_2$ 151.0633; ^{1}H NMR (600 MHz, acetone-d_6) δ: 9.38 (1H, s, 1-NH), 9.26 (1H, s, 2-OH), 7.37 (1H, d 7.92, 6-H), 7.02 (1H, td 7.98, 4-H), 6.89 (1H, d 7.86, 3-H), 6.79 (1H, td 7.86, 5-H), 2.20 (3H, s, NHAc); ^{13}C NMR (150 MHz, acetone-d_6): 171.2 (C7), 149.5 (C2), 127.8 (C1), 126.7 (C4), 122.9 (C6), 120.5 (C5), 119.1 (C3), and 23.5 (NHAc, CH_3).

Compound **1a** was obtained as red powder, and its molecular formula $C_{32}H_{31}NO_{10}$ was deduced from HR-ESI-MS data (*m/z*: 590.2018 $[M+H]^+$ and 612.1837 $[M+Na]^+$, calculated for $C_{32}H_{32}NO_{10}$ 590.2027 and $C_{32}H_{31}NO_{10}Na$ 612.1846, respectively). The structure of compound **1a** was established through comparison with compound **1** and the detailed NMR data analysis of 2D-NMR (Supplementary Figures S2–S8). The ^{1}H NMR spectrum of **1a** exhibited the presence of three acetyl groups at δ_H 2.18 (s, 3H), 2.30 (s, 3H), and 2.47 (s, 3H), respectively. ^{13}C NMR (Table 1) and DEPT spectrum displayed 32 carbon resonances that were grouped into 16 aromatic carbons, 2 carbonyl carbons signal (δ_C 184.8, 188.5), 3 methyl groups carbons signal (δ_C 18.7, 22.8, and 31.3), 3 acetyl groups carbons signal (δ_C 21.2, 21.3, 22.6, 168.5, 169.5, 173.9), and 1 glycosyl carbon signal (δ_C 33.7, 57.1, 72.6, 73.8, 79.2). The HMBC correlations from 3′-NCOCH_3 (δ_H 2.18) to 3′-NCOCH_3 (δ_C 173.9, 22.6), from 1-OCOCH_3 (δ_H 2.30) to 1-OCOCH_3 (δ_C 168.5, 21.2) and C-1 (δ_C 147.5), and from 8-OCOCH_3 (δ_H 2.47) to 8-OCOCH_3 (δ_C 169.5, 21.3) and C-8 (δ_C 150.3) indicated the location of the three acetyl groups.

Compound **1b** was obtained as yellow powder, and its molecular formula $C_{34}H_{33}NO_{11}$ was deduced from HR-ESI-MS data (*m/z*: 632.2133 $[M+H]^+$ and 654.1946 $[M+Na]^+$, calculated for $C_{34}H_{34}NO_{11}$, 632.2121, and $C_{34}H_{33}NO_{11}Na$ 654.1952, respectively). The structure of compound **1b** was established through comparison with compound **1** and the detailed NMR data analysis of 2D-NMR (Supplementary Figures S9–S15). The ^{1}H NMR (Table 1) spectrum of **1b** presented four acetyl groups signal δ_H 2.18 (s, 3H), 2.33 (s, 3H), 2.44 (s, 3H), and 2.50 (s, 3H). The ^{13}C NMR and DEPT spectrum exhibited 34 carbon resonances including 16 aromatic carbons, 2 carbonyl carbon signal (δ_C 180.6, 185.3), 3 methyl groups carbons signal (δ_C 18.6, 22.6, 31.2), 4 acetyl groups carbons signal (δ_C 21.2, 21.2, 21.5, 22.6, 168.3, 169.3, 169.7, 174.0), and 1 glycosyl carbon signal (δ_C 34.0, 56.8, 73.7, 73.7, 79.3). The HMBC correlations from 3′-NCOCH_3 (δ_H 2.18) to 3′-NCOCH_3 (δ_C 174.0, 22.6), from 1-OCOCH_3 (δ_H 2.33) to 1-OCOCH_3 (δ_C 168.3, 21.2) and C-1 (δ_C 147.6), from 8-OCOCH_3 (δ_H 2.44) to 8-OCOCH_3 (δ_C 169.3, 21.2) and C-8 (δ_C 149.7), and from 6-OCOCH_3 (δ_H 2.50) to 6-OCOCH_3 (δ_C 169.7,21.5) and C-6 (δ_C 149.7) indicated the location of the four acetyl groups.

Antibacterial activities of compounds

The MIC values and IZD of four compounds (**1**, **1a**, **1b**, and **3**) against different bacteria are presented in Table 2. The results showed that compound **1** exhibited strong antibacterial activities against *S. aureus*, *M. tetragenus*, and Psa in the MIC tests with the MIC values of 6.25, 12.50, and 6.25 μg/ml, which were comparable to those of positive gentamycin sulfate with the MIC values of 6.25, 12.50, and 3.13 μg/ml, respectively. In the filter paper disc tests, compound **1** also presented strong antibacterial activities against *S. aureus*, *M. tetragenus*, and Psa with the IZD of 16.33, 30.00, and 15.00 mm, which were slightly weaker than those of positive control with the IZD of 18.00, 36.33, and 19.67 mm, respectively. Compound **1a** displayed potent inhibitory activities against *S. aureus*, *M. tetragenus*, and Psa with MIC values of 12.50, 12.50, and 6.25 μg/ml, and the IZD of 15.00, 27.67, and 10.00 mm, respectively. Similarly, compound **1b** also showed potent inhibitory activities against *S. aureus*, *M. tetragenus*, and Psa with MIC values of 25, 12.50, and 12.50 μg/ml and the IZD of 14.67,

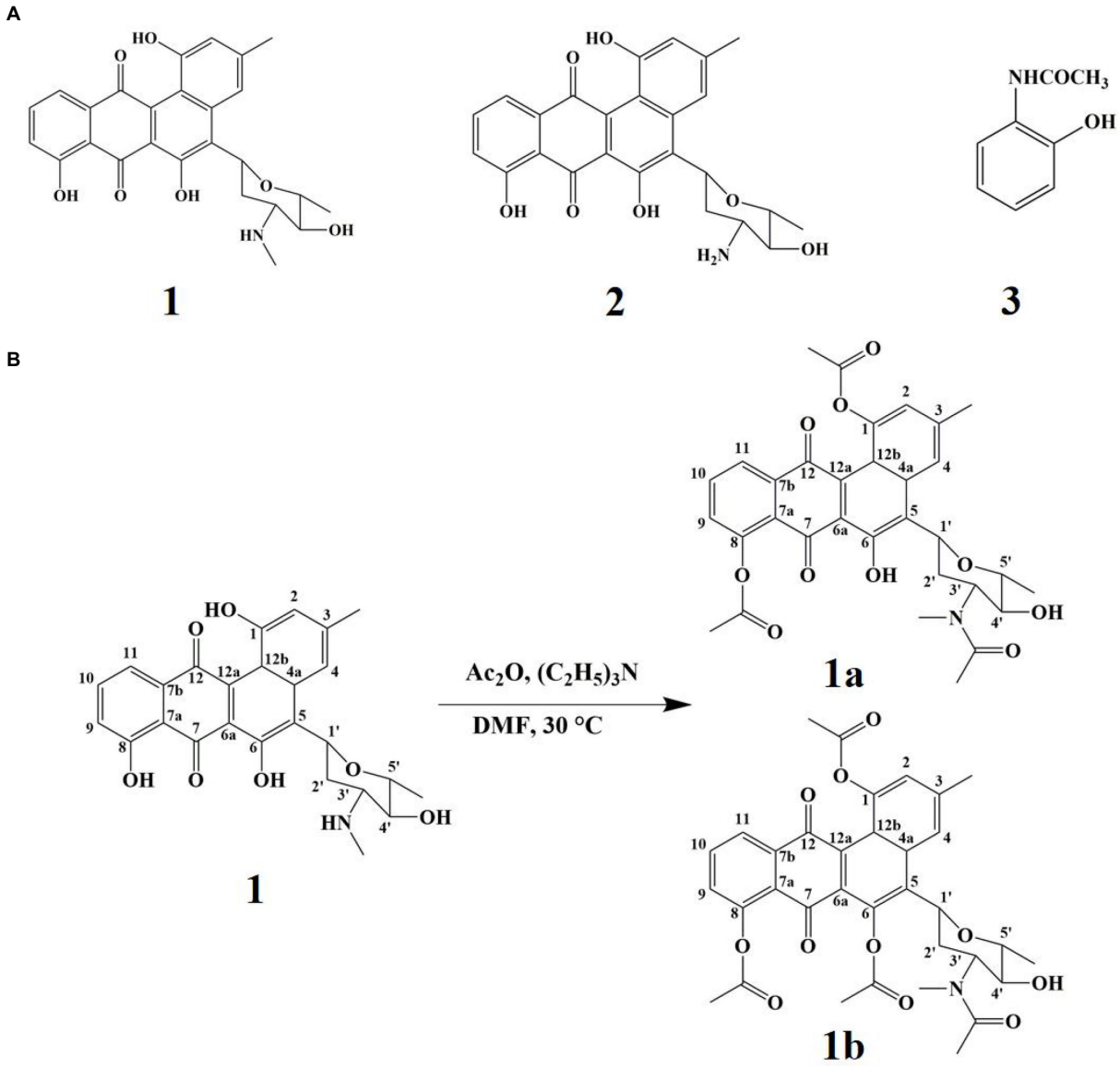

FIGURE 3
The secondary metabolites and derivative compounds of strain FCF01. **(A)** The structure of compounds 1–3; **(B)** Derivatization pathways of compound 1.

22.00, and 9.00 mm, respectively. Compound **3** displayed moderate antibacterial activities against *S. aureus*, *M. tetragenus*, and Psa with MIC values of 25, 25, and 12.5 μg/ml and the IZD of 8.33, 12.00, and 10.00 mm, respectively. However, the remaining *E. coli* was not susceptible to all compounds.

Discussion

Actinobacteria, especially of the genus *Streptomyces*, has been one of the most essential sources for the discovery of antibiotics (Genilloud, 2017). Due to the continuing development of antibiotic resistance and the discovery of new antibiotics decreases, researchers were starting to search for *Streptomycetes* in other habitats rather than soil, such as insects, and plants (Jose et al., 2021). Compared to soil and plant-associated Actinobacteria, insect-associated Actinobacteria showed significant antimicrobial activity (Chevrette et al., 2019). Furthermore, insect-associated Actinobacteria have been a significant source of new microbial resources and novel natural products (Promnuan et al., 2011; Beemelmanns et al., 2017; Wang et al., 2020; Zhang et al., 2020). Here, 61 Actinobacteria, including two potential new species, were isolated and identified by culture-dependent and molecular biological methods. A 16S rRNA gene sequence similarity of 98.7% was considered a threshold value for species delimitation (Chun et al., 2018). The strains BTF01 and

TABLE 1 ^{1}H NMR and ^{13}C NMR data of compounds 1a and 1b in $CDCl_3$.

Position	1a		1b	
	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	147.5		147.6	
1-$OCOCH_3$	168.5, 21.2	2.30	168.3, 21.2	2.33
2	123.0	7.14	125.1	7.35
3	140.7		140.8	
3-CH_3	22.8	2.54	22.6	2.59
4	122.3	8.41	125.3	8.26
4a	138.4		136.8	
5	126.9		126.4	
6	154.1		149.7	
6-OH		12.61		
6-$OCOCH_3$			169.7, 21.5	2.50
6a	119.1		120.5	
7	188.5		180.6	
7a	124.1		125.3	
8	150.3		149.7	
8-$OCOCH_3$	169.5, 21.3	2.47	169.3, 21.2	2.44
9	129.4	7.40 (d, J = 7.92)	129.3	7.38 (d, J = 7.98)
10	136.2	7.81 (td, J = 7.68)	134.9	7.75 (td, J = 7.80)
11	124.4	7.95 (d, J = 7.44)	123.8	7.91 (d, J = 7.62)
11a	137.6		136.1	
12	184.8		185.3	
12a	134.8		134.3	
12b	118.0		120.5	
1′	72.6	5.71 (d, J = 10.2)	73.7	5.41
2′	33.7	1.90	34.0	1.25 (td, J = 7.32)
3′	57.1	4.91	56.8	4.86
3′-N-CH_3	31.3	2.98	31.2	2.97
3′-$NCOCH_3$	173.9, 22.6	2.18	174.0, 22.6	2.18
4′	73.8	3.46	73.7	3.42 (td, J = 9.48)
5′	79.2	3.66	79.3	3.60
5′-CH_3	18.7	1.47 (d, J = 5.40)	18.6	1.44 (d, J = 5.70)

BTF07 showed less than 98.7% similarity to the closest species and thus were considered as potential new species. Meanwhile, the community composition of the honeybee gut and honeycomb was further analyzed by the culture-independent method. Moreover, three compounds and two novel derivative compounds, which had good antibacterial activities, were purified and characterized from *Streptomyces* sp. FCF01. Therefore, *Streptomyces* species associated with honeybees have great potential in finding new antibiotics.

To obtain extensive Actinobacteria from honeybee samples, we used six different types of isolation media, which have been found effective in the isolation of Actinobacteria. Among them, the CC and SCA media were the most effective as regards the number of obtained isolates. Both media have been also used to isolate rare Actinobacteria from caves and soils (Fang et al., 2017; Li et al., 2021). Chitin agar (CA) and ISP2 media were also widely used for the isolation of insect-associated Actinobacteria (Rodríguez-Hernández et al., 2019; Menegatti et al., 2020; Grubbs et al., 2021; Ortega et al., 2021). Therefore, these media can be further considered for the isolation of honeybee-associated Actinobacteria.

Actinobacteria isolated from the honeycomb, larvae, and different parts of adult honeybees (gut, head, cuticle, and abdomen) were investigated in this study. The result showed that *Streptomyces* was the predominant genus, which was consistent with other reports (Cambronero-Heinrichs et al., 2019; Grubbs et al., 2021). *Streptomyces* associated with bees might be the strains collected by many bees through pollen (Kim et al., 2019). Previous studies have focused on the isolation of Actinobacteria from the honeybee gut, honeycomb, pupae, pollen, and honey (Promnuan et al., 2009; Khan et al., 2017; Grubbs et al., 2021). However, the isolation of Actinobacteria from different parts of *A. mellifera* was neglected, such as the head and abdomen. Moreover, Poulsen et al. found the potential role of *Streptomyces* isolated from different parts of the wasp as antibiotic-producing symbionts (Poulsen et al., 2011). *A. mellifera* has been emerging as a potential source of novel species of Actinobacteria (Promnuan et al., 2011). In this study, two potentially new species were isolated from honeybee head. Considering the limitations of isolation methods, the culture-independent method was used to evaluate the actinobacterial community composition of insects in recent years (Wang et al., 2020).

Actinobacterial community structure was analyzed in both honeybee gut and honeycomb using the culture-independent method in this study. The phylum Actinobacteria was detected in the honeybee gut and honeycomb at 4.05 and 2.08% relative abundance respectively, which was a similarity to the results of the previous study (Liu et al., 2021). Fifteen and twenty-three actinobacterial families were detected by culture-independent method from the honeybee gut and honeycomb, respectively. However, only the family Streptomycetaceae was isolated, and some rare actinobacterial families, for instance, Nocardiaceae, Nocardioidaceae, Micrococcaceae, etc., were not detected by the culture-dependent method. A greater diversity of actinobacterial communities was detected using the culture-independent method compared to those of the culture-dependent method. This result provided the impetus to continue developing cultivation methods and strategies to culture rare Actinobacteria in future studies. For example, the treatment of samples and organism-media pairings could increase the recovery of rare Actinobacteria (Subramani and Aalbersberg, 2013; Oberhardt et al., 2015). Combined methods encompassing culture-dependent and independent techniques to retrieve broader actinobacterial communities have been used for different sources

TABLE 2 Minimum inhibitory concentration (MIC) values (μg/mL) and inhibition zone diameter (IZD, mm) of compounds against four tested bacteria.

Compounds	*S. aureus*		*M. tetragenus*		*E. coli*		Psa	
	MIC	IZD[a]	MIC	IZD[a]	MIC	IZD[a]	MIC	IZD[a]
1	6.25	16.33 ± 0.47	12.5	30.00 ± 0.00	>100	NI	6.25	15.00 ± 0.00
1a	12.5	15.00 ± 0.00	12.5	27.67 ± 0.47	>100	NI	6.25	10.00 ± 0.00
1b	25	14.67 ± 0.47	12.5	22.00 ± 0.00	>100	NI	12.5	9.00 ± 0.00
3	25	8.33 ± 0.47	25	12.00 ± 0.00	>100	NI	12.5	10.00 ± 0.00
PC[b]	6.25	18.00 ± 0.00	12.5	36.33 ± 0.47	12.5	18.33 ± 0.47	3.13	19.67 ± 0.47

[a]Results are presented as the mean ± standard; "NI" means not inhibited; the concentration for the test is 30 μg/filter paper.
[b]Gentamycin sulfate as the positive control.

of the samples, such as dung beetle, desert sandy soils, and soybean (Liu et al., 2019; Kim et al., 2021; Li et al., 2021). Therefore, it was critical to use a combination of culture-dependent and independent methods to accurately assess the composition of the actinobacterial communities.

To validate that honeybee-associated Actinobacteria have antibacterial activity against pathogenic bacteria, 49 isolates were conducted antibacterial assay using three different human food-borne bacteria and one plant pathogenic bacterium. The results revealed that a high proportion of strains (77.6%) had antibacterial activities. There was also evidence that honeybee-associated Actinobacteria had potent antimicrobial activity against pathogens, including human food-borne bacteria (*S. aureus*), insect pathogen (*Beauveria bassiana*, *P. larvae*), plant pathogenic bacteria (*Ralstonia solanacearum*, *Xanthomonas campestris* pv. *Campestris*), and plant fungal pathogen (*Fusarium oxysporum*; *Botrytis cinerea*; Promnuan et al., 2020; Grubbs et al., 2021; Santos-Beneit et al., 2022). Actinobacteria associated with other insects had also been reported to have good antibacterial activities, such as termites, ants, and beetle (Scott et al., 2008; Wang et al., 2020; Long et al., 2022). Furthermore, some insect-associated Actinobacteria could produce substances with antibacterial activity (Zhang et al., 2020; Zhou et al., 2021).

Many antimicrobials with unique structures were identified from honeybee-associated Actinobacteria (Rodríguez-Hernández et al., 2019; Grubbs et al., 2021; Santos-Beneit et al., 2022). We investigated the secondary metabolites from one *Streptomyces* strain FCF01 with good antibacterial activity, which resulted in the isolation of mayamycin (**1**), mayamycin B (**2**), and N-(2-Hydroxyphenyl) acetamide (**3**). Among them, antibacterial compounds **1** and **2** have been reported to be produced by *Streptomyces* species and showed activity against *S. aureus* with the same MIC value of 64 μM (Bo et al., 2018; Alam et al., 2022). Furthermore, two novel derivatives (**1a** and **1b**) were further identified by acetylation of compound **1**. However, their antibacterial activities were slightly weaker than those of compound **1**, which indicated that the hydroxyl group of metabolite **1** might play a vital role in antibacterial activity. A similar study has shown the replacement of the phenolic hydroxyl group by aldehyde groups of the 15-copaenol resulted in weaker antibacterial activity (Espinoza et al., 2019).

Conclusion

Here, the actinobacterial diversity of the honeybee samples was analyzed using both culture-dependent and independent methods. The results demonstrated the honeybee-derived sample harbored an excellent source of culturable actinobacterial strains. Antibacterial activity assays showed that most of these honeybee-associated Actinobacteria exhibited antibacterial activities. In addition, three known metabolites were purified from *Streptomyces* sp. FCF01 and two novel derivatives were identified by acetylation of compound **1**. Both compound **1** and its novel derivatives displayed potent antibacterial activity. These results suggest that honeybee-associated Actinobacteria represent a promising and underexplored resource for exploring antibiotics.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, OP491886-OP491954 https://www.ncbi.nlm.nih.gov/, PRJNA883759 https://www.ncbi.nlm.nih.gov/, PRJNA882994.

Author contributions

PC, HW, TJ, JT, ZZ, PL, and LY performed the experiments and analyzed the data. PC wrote the manuscript. YZ designed the experiments and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Solitary bee larvae modify bacterial diversity of pollen provisions in the stem-nesting bee, *Osmia cornifrons* (Megachilidae)

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Microbes, including diverse bacteria and fungi, play an important role in the health of both solitary and social bees. Among solitary bee species, in which larvae remain in a closed brood cell throughout development, experiments that modified or eliminated the brood cell microbiome through sterilization indicated that microbes contribute substantially to larval nutrition and are in some cases essential for larval development. To better understand how feeding larvae impact the microbial community of their pollen/nectar provisions, we examine the temporal shift in the bacterial community in the presence and absence of actively feeding larvae of the solitary, stem-nesting bee, *Osmia cornifrons* (Megachilidae). Our results indicate that the *O. cornifrons* brood cell bacterial community is initially diverse. However, larval solitary bees modify the microbial community of their pollen/nectar provisions over time by suppressing or eliminating rare taxa while favoring bacterial endosymbionts of insects and diverse plant pathogens, perhaps through improved conditions or competitive release. We suspect that the proliferation of opportunistic plant pathogens may improve nutrient availability of developing larvae through degradation of pollen. Thus, the health and development of solitary bees may be interconnected with pollen bacterial diversity and perhaps with the propagation of plant pathogens.

KEYWORDS

bees, brood cell, Megachilidae, development, pollen, microbiome, larvae–development, plant pathogen

Introduction

Both solitary and social bees have been shown to host diverse communities of microbial taxa both in their guts, as well as in their pollen/nectar provisions (Gilliam et al., 1989; Mattila et al., 2012; Anderson et al., 2014; Kwong and Moran, 2016; Dharampal et al., 2020). While the adult gut microbiome may play an important role in adult fitness (Koch and

Schmid-Hempel, 2011; Kwong and Moran, 2015; Raymann et al., 2017; Rutkowski et al., 2021), it is the bacterial community of the pollen/nectar provisions that plays a key role in larval growth and development (Dharampal et al., 2020). Mounting evidence across diverse bee species suggests that the pollen/nectar provisions in both solitary and social bees host diverse bacterial and fungal taxa (Gilliam, 1979a,b, 1997; Gilliam et al., 1990; Rosa et al., 2003; Pimentel et al., 2005; Vannette et al., 2013; McFrederick et al., 2014) and that these microbes are vital to larval development (Vannette et al., 2013; Steffan et al., 2019; Dharampal et al., 2020), immune function (Mattila et al., 2012; Kaltenpoth and Engl, 2014; McFrederick et al., 2014), resistance to disease (Raymann and Moran, 2018), and overall fitness (Steffan et al., 2017; Dharampal et al., 2019, 2020; Voulgari-Kokota et al., 2019a,b; Cohen et al., 2020; Rothman et al., 2020).

The bacterial diversity from brood cell provisions of species of Megachilidae are particularly well-studied because megachilids construct above-ground, stem- and cavity-nests that can be easily sampled. For example, Mohr and Tebbe (2006) documented the bacterial community in pollen provisions of *Osmia bicornis* and found several bacterial genera including *Sphingomonas*, *Ralstonia*, *Burkholderia*, and *Acinetobacter*. Studies on the nest chambers of *Osmia bicornis* found bacterial families Burkholderiales, Clostridiaceae, Enterobacteriaceae, and Acetobacteraceae, and bacterial genera *Bacillus* and *Paenibacillus* (Keller et al., 2013; Voulgari-Kokota et al., 2019c), whereas, a study of cultured bacteria from nest contents of *Osmia cornuta*, revealed seven prevalent bacterial genera: *Bacillus*, *Lactobacillus*, *Paenibacillus*, *Clostridium*, *Serratia*, *Pantoea*, and *Curtobacterium* (Lozo et al., 2015). Additionally, a comparative study of larvae and pollen provisions from three genera of Megachilidae found a monophyletic *Lactobacillus* clade shared by this group (McFrederick et al., 2017). Thus, there is both overlap and variable bacterial diversity across the pollen provisions of related megachilid species. These bacterial taxa are largely considered to be of environmental origin, obtained primarily through foraging for pollen and nectar on host-plant flowers (Vannette, 2020; Keller et al., 2021). Furthermore, through comparisons of field collected samples, several studies have documented changes in the bacterial community in both the pollen provisions and larvae (Mohr and Tebbe, 2006; Keller et al., 2013; Voulgari-Kokota et al., 2019b), but these studies lacked a standardized experimental design and a direct comparison of pollen provisions with and without larvae. Therefore, the influence of developing bee larvae on the microbial environment of pollen provisions has yet to be fully assessed.

Bees have been shown to depend on microbial symbionts that colonize their gut and on microbes fermenting and metabolizing pollen provisions. Notably, past studies using trophic biomarkers have shown that microbes may be direct prey for bees making bees omnivorous (feeding on both plant and microbial-derived food) rather than strictly herbivorous (Steffan et al., 2019). This appears to be true for solitary bees where microbes have been shown to be an important source of larval nutrition and the microbial activity in pollen provisions helps unlock nutrients, trapped within the rigid, indigestible exine of the pollen (Steffan et al., 2019). Experiments in which microbes are eliminated from the pollen provisions through sterilization indicate that the presence of the naturally occurring microbial community is essential for larval growth and development (Dharampal et al., 2019, 2020).

Given the importance of the brood cell microbiome to larval growth and development, one might expect solitary bee adults and larvae to influence the richness and composition of the brood cell microbiome. However, while adult derived inputs, such as glandular secretions, have been shown to produce select antimicrobial properties in pollen microbiome (Cane, 1983), no previous study has directly examined the impact of larval feeding on the microbial community of pollen provisions. In this study, we set out to explore how solitary bee larvae impact the brood cell microbiome in a common, easily manipulated, solitary, stem-nesting bee, *Osmia cornifrons* (Megachilidae). We conducted an experiment to determine (1) the temporal shift of larval and pollen bacterial communities through larval development and (2) whether actively feeding larvae modify the brood cell bacterial community through feeding.

Materials and methods

Preparation for bee experimentation

Long term nesting aggregations of *Osmia cornifrons* in the vicinity of Ithaca, NY were used as a source of brood cells for our experiments. In Spring 2018, local populations of adult *O. cornifrons* established nests in wooden nesting shelters housing collections of 70–140 empty cardboard tubes with paper nest inserts purchased from Crown Bees (Woodinville, WA, United States). In June 2018, completed nests were overwintered at ambient conditions. In February 2019, nests were examined *via* x-ray imaging, and nests with parasites or high levels of mortality were excluded from the experiment (Pitts-Singer, 2004). Parasite-free nests were established in the field in March 2019 alongside unused nesting tubes. Following emergence of adult males and females, nesting shelters were surveyed daily for nest completion. Once unused nesting tubes were closed by a female, we brought these tubes into the laboratory for our experiments. Closed nests were collected between May 6 and May 27th from two localities.

Bee sampling and processing

We opened recently completed nests of *Osmia cornifrons* containing freshly provisioned pollen and recently laid eggs. Each nest was carefully opened by slicing the paper nest inserts horizontally on each side with a sterile scalpel and removing the top portion to reveal the nest contents. Damaged eggs or larvae were excluded from the experiment. Pollen provisions (approximately 10 per tube; see Figure 1A), were extracted with sterile forceps. Tools were flame sterilized between each sample

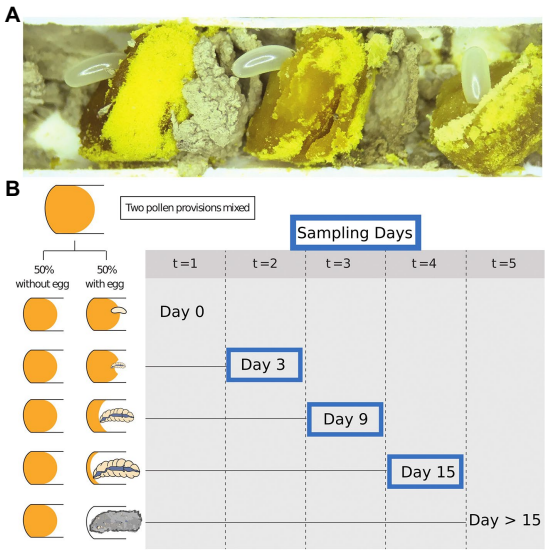

FIGURE 1
Samples and study design. **(A)** The exposed cells of *Osmia cornifrons*. **(B)** Experimental overview shows sampling time during our experiment: points; (t=0=day 0; t=1=day 3; t=2=day 6; t=3=day 9; t=4=day 12; and t=5=day 15). Only Day 3, 9, and 15 were selected for bacterial diversity analysis.

and working areas were cleaned before and after dissections with 10% bleach.

Undamaged pollen provisions were collected in pairs. Eggs were gently removed and then the two pollen provisions from neighboring cells in the nest were homogenized together using a sterile micro-spatula (Kimura spatula; World Precision Instruments). Pollen balls were combined from brood cells of the same sex, which was determined visually by the mass of the pollen/nectar provision, the size of the brood cell, and the position within the nest. Pollen provisions destined for female offspring are much larger and placed farther from the nest entrance than those destined for males (Figure 1A). The homogenized pollen ball was split in two equal parts and placed into 48-well tissue culture plates (purchased from Falcon). The pollen was gently compacted into the bottom of the well to minimize desiccation and empty wells were used to separate pairs of samples. Subsequently, a male or female egg was returned to one of the pair (pollen from the same sex as the egg), and the other was left without the egg (later described as "pollen with larvae" and "pollen without larvae," respectively). Plates were stored in an incubator (Percival 500,365) at 27°C. We tracked larval development daily. Pollen and larvae were sampled from the tissue culture plates at 3-day intervals, and we analyze samples for bacterial diversity on day 3, 9, and 15. Most larvae completed development and began spinning cocoons on day 15, so the last sample was taken at the beginning of the spinning larval stage. Samples of pollen and larvae were stored in sterile 2.0 ml screw-cap vials, immediately frozen in liquid nitrogen, and then stored at −80°C for later analysis and amplicon sequencing.

From the pairs of pollen samples described above, we selected representative pairs (no contamination, no parasites, or death) for downstream processing. Sample sizes per group are as follows: day 3, pollen with larvae and pollen without larvae ($N = 24$, 12 pairs) and larvae only ($N = 12$); day 9, pollen with larvae and pollen without larvae ($N = 23$, 11 pairs) and larvae ($N = 12$); day 15, pollen with larvae and pollen without larvae ($N = 7$, and $N = 11$, respectively) and larvae only ($N = 11$; See Figure 1B for experimental design). Total sample numbers by type: pollen with larvae $N = 30$, pollen without larvae $N = 35$, and larvae only $N = 35$. Sample numbers were slightly reduced at the final observation day, due to the number of individuals that had completely consumed pollen provisions on, or before, sample collection day 15.

DNA extraction

We extracted DNA from each sample (approximately 100 mg) using Qiagen PowerPlant kits, following manufacturers protocol, including the recommended 10 min of bead beating with the lysis buffer (Galimberti et al., 2014). We homogenized the sample using the Bead Ruptor Elite, set at 1.15 m/s, for 10 min. We found this setting was sufficient for complete mixing and mechanical disruption of the pollen sample and the larvae. An extraction control was added during each extraction event, approximately once every 48 extractions (once per 50 reaction kit), and these extraction controls were included in library preparation and sequencing. Our final elution volume was 60 µl, chosen to increase DNA concentration and improve quantification and downstream sequencing.

Sequence processing

To assess the microbiome of samples collected throughout our experiment, 100 pollen and bee larval samples were submitted for 16S amplicon sequencing. Library preparation and sequencing was performed at the UC Davis Department of Medical Microbiology laboratory using the following protocol. Primers 799F (CMGGATTAGATACCCKGG) and 1193R (AGGGTTGCGCTCGTTG) were used to amplify the V5–V7 domain of the 16S rRNA using a two-step PCR procedure. This region was chosen to minimize amplification of plant chloroplasts (Beckers et al., 2016; Thijs et al., 2017). A detailed description of our two step PCR procedure is provided in the Supplementary materials. The final product was quantified on a Qubit instrument using the Qubit High Sensitivity dsDNA kit (Invitrogen), and individual amplicons were pooled in equal concentrations. The pooled library was cleaned utilizing Ampure XP beads (Beckman Coulter) and then checked for quality and proper amplicon size on an Agilent 2100 Bioanalyzer (Agilent Technologies). The library was quantified *via* qPCR followed by 300-bp paired-end sequencing using an Illumina

MiSeq instrument (Illumina) in the Genome Center DNA Technologies Core, University of California at Davis, CA, United States.

The amplicon sequence data was exported as Fastq files and were demultiplexed with dbcAmplicons from https://github.com/msettles/dbcAmplicons using miniconda. Then, the input files barcode sheet, primer sheet, and sample metadata were validated. The resulting amplicon sequence data was imported into QIIME2 (v2021.4, Bolyen et al., 2019). We truncated forward reads at 260 bp and reverse reads at 160 bp, based on the length of our fragment and visual inspection of the error profiles and quality scores. We used DADA2 to join reads, de-noise, and dereplicate sequences, including the removal of chimeric sequences, quality filtering, and joining of paired ends (Callahan et al., 2016). Taxonomy was assigned using the vsearch referencing SILVA version silva138 with 99% identity (Bokulich et al., 2018). We extracted reference ASV with "classify-sklearn" and aligned sequences with MAFFT (align-to-tree-mafft-fasttree) generating a rooted phylogenetic tree (Bolyen et al., 2019). Following taxonomy assignments all sequences matching to chloroplast, mitochondria, and any sequences left as unspecified were removed. Bee larval samples experienced moderate host amplification and these sequences were identified in the 16S dataset and filtered from those samples. DNA extraction control samples returned little to no amplification, did not exhibit evidence of contamination and were subsequently excluded from downstream analyses. We filtered out ASVs with <10 sequences per sample.

Richness, evenness, and composition analyses

Alpha and beta diversity metrics were calculated using QIIME2 (2021.4) and computed using the diversity plugin in QIIME2. To assess differences in alpha diversity and evenness we report and visualize "observed features," the total number of unique ASVs calculated by sample type. We utilize this metric to capture shifts in richness that consider nearly all microbes found in pollen, including changes in the presence and absence of comparatively rare microbes between and across sample types. The significance of differences for all alpha diversity and evenness metrics were calculated using Kruskal-Wallis tests, followed by pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg FDR (BH) corrections, when significant differences were observed (Kruskal and Wallis, 1952; Benjamini and Hochberg, 1995).

To analyze compositional differential abundance between groups, we investigated the bacterial community structure, using weighted and unweighted UniFrac metrics (Lozupone and Knight, 2005; Lozupone et al., 2007). Significant differences for beta diversity metrics were calculated using a permutational multivariate ANOVA (PERMANOVA) followed by pairwise PERMANOVAs with BH correction when significant differences were observed over more than two factors. To visualize differences in beta diversity metrics, we used principal coordinate analysis (PCoA).

Proportional abundance tables of pollen without larvae, pollen with larva, and larvae

To visualize and compare the overall taxonomic structure of the bacterial communities in our experiment, we plotted the relative abundance of ASVs matching to the top 10 most abundant bacterial genera by sample type (pollen without larvae, pollen with larvae, and larvae only). We consider the top 10 most abundant bacterial genera by sample type to be the dominant bacterial taxa. To compare proportional abundance by sample type and by experimental time point, taxonomic tables were grouped in QIIME2. While we summarize the data tables at the level of genera, several taxa were not identified to the level of genus and remain described at the order and family level. The dominant bacterial taxa were generally shared across sample types. To visualize the bacterial composition of pollen and larvae across time, we plotted the relative abundance of the combined 12 most dominant bacterial taxa in R.

Differential abundance testing

To analyze differential abundance of bacterial taxa between pollen samples with and without larvae, we first compare samples from day 9 and day 15. These sampling days independently reveal significant differences in bacterial diversity between sample types (pollen with and without larvae) and combined they offer a more robust analysis of differential features between the two sample types.

We analyze differential features using a compositionally aware method Songbird QIIME2 plugin (Morton et al., 2019). This approach works from our unrarefied dataset and includes all 41 relevant samples: pollen with larva ($N = 18$) and pollen without larvae ($N = 23$). First, the sample data were split into a test set and a training set. Songbird trains a null model and a multinomial model on the training data for each set of metadata explored then predicts and tests this against the test dataset. We quantify the model's performance, compare the models, and visualize the model's ability to differentiate between the pollen groups in question. Microbes that significantly contribute to differences between pollen with and without larvae were extracted. Next, we use DESeq2 package in Phyloseq available in R (R Core Team, 2021) following Kapheim et al. (2021). This approach uses a rarified data table and calculates the differential abundance of taxa specified at desired taxonomic levels and significance thresholds. Here, we calculate the maximum log fold change for differential taxa and visualize the data (R Core Team, 2021).

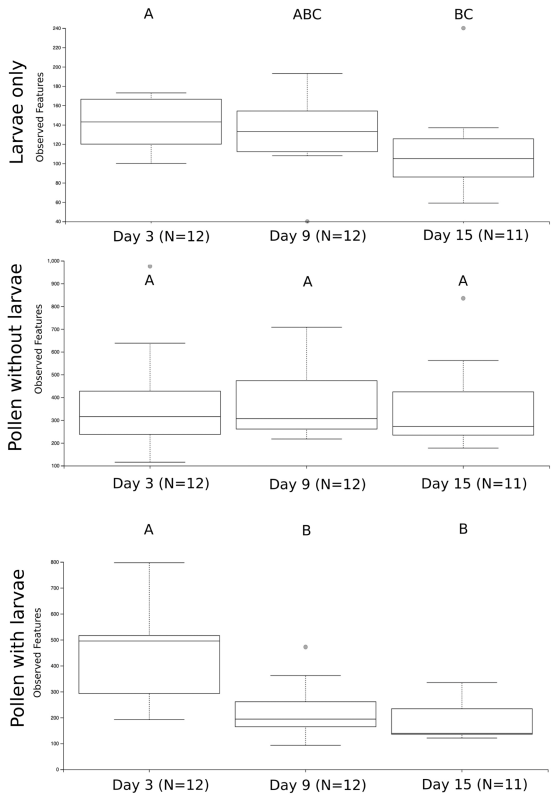

FIGURE 2
The number of unique bacterial ASVs summarized by the three treatment groups and sampling time points. Significance is denoted by **(A–C)**. Significant differences in bacterial richness across time points were found using a Kruskal Wallis test for larvae only (H=4.69, p =0.096) and pollen with larvae (H=11.53, p =0.003), but not for pollen without larva (H=0.358, p =0.836).

Results

Research questions and objectives overview

The goal of our experiment was to document the natural progression of microbial community growth over time in bee brood cells for: pollen with larvae, pollen without larvae, and for larvae only (Figure 1). Our analyses of pollen with larvae, pollen without larva, and larvae only fell into five primary sets of comparisons. To determine the temporal change in bacterial diversity in the absence of larvae we compared (1) pollen only through the experiment. To determine the temporal change in bacterial diversity in the presence of larvae we compared (2) pollen with larvae through the experiment. To determine temporal change in bacterial diversity in larvae we compared (3) larvae only through the experiment. To assess whether actively feeding larvae modify the brood cell bacterial community through time, we compared (4) alpha and beta diversity metrics of bacterial diversity of pollen, both with and without larvae, through the experiment. Finally, we (5) identified bacterial taxa with differential abundance between samples of pollen with and without larvae.

We find that the presence of a bee larva exerts a selective force on the bacterial diversity of pollen throughout bee development (Figures 2–4). After initial filtering, our dataset comprised 99 samples and the resulting 16S ASV table held 4,751,528 sequences, with a median frequency of 38,221 sequences per sample and 13,518 unique bacterial features. After reviewing alpha rarefaction plots, we were able to capture most of the bacterial diversity using a rarefaction depth of 4,690 sequences per sample which only resulted in one sample (pollen without larvae on day 3) dropping below that threshold (Supplementary Figure 1). This rarefaction depth maximizes our exploration of community composition while excluding only a minimal number of samples from our experiment.

Richness and compositional assessment of brood cell bacterial diversity

We first compare the richness of pollen with larvae, pollen without larvae and larvae only by combining all samples and all time points in our experiment (Supplementary Figure 2A). We calculate the richness of observed ASVs and evaluated them with Kruskal-Wallis and then pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg corrections. At this level of comparison, we see significant differences in the richness of all three sample types (Kruskal Wallis; H = 53.45, p = 2.44) and all pairwise comparisons are significant: pollen with larvae compared with pollen without larvae (H = 3.01, p = 8.26 e−12), pollen with larvae compared with larvae only (H = 29.97, p = 4.38 e-8), and lastly pollen without larvae compared with larvae only (H = 45.51, p = 1.52 e−11). We calculate the richness of sample types across three timepoints (day 3, day 9, and day 15) and find that the richness of pollen with larvae and larvae only change significantly throughout larval development, but pollen without larvae is unchanged throughout our experiment (Figure 2). Pollen with larvae was significantly different between day 3 and day 9 (H = 8.02, p = 0.0046), and day 3 and day 15 (H = 7.78, p = 0.005), but not significantly different between day 9 and day 15 (H = 0.593, p = 0.441). The overall difference of pollen with larvae across the three time points was significant (H = 11.53, p = 0.003). Pollen without larvae was not significantly different across any of the three time points, and the overall difference across groups was not significant (H = 0.358, p = 0.836). Larvae only were significantly differentiated between day 3 and day 15 (H = 4.908, p = 0.027), however, overall differences between all three-time points were not significant (H = 4.69, p = 0.096).

To compare and visualize the bacterial composition of the bees in our study, we plot the proportional abundance of the 12 most abundant bacterial taxa (referred to as "dominant" bacteria) and grouped all additional taxonomic groups into the category "other" (Figure 3). We found that most dominant taxa identified remain relatively equivalent and consistent across sample types and through time. However, the proportional abundance of *Ralstonia* increases considerably over time in pollen with larvae. Conversely, the

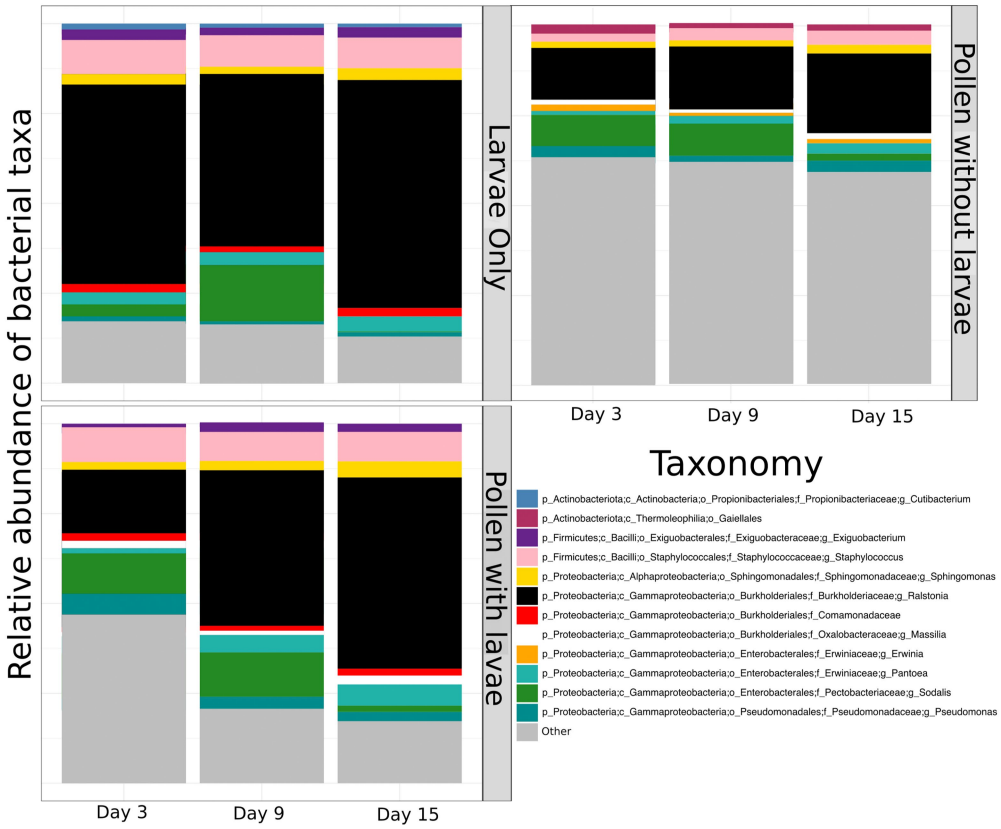

FIGURE 3
The proportional abundance of dominant bacterial ASVs of pollen and *Osmia cornifrons* summarized across sampling days. Proportional abundances are displayed for three treatment groups, larvae only, pollen with larvae, and pollen without larvae.

proportional abundance of *Sodalis* decreases with time in all groups. One bacterial taxon, identified only to the level of Comamonadacae was considered dominant only in samples with larvae, and another bacterial taxon, *Erwinia*, was considered dominant only in samples without larvae. Most importantly, the proportional abundance of all "other" taxa decreases in time for all groups and this is particularly evident for pollen with larvae (Figure 3). Additionally, we confirm that *Ralstonia*, a dominant member of our pollen microbial community, is a valid member, and we verified that larval feces were not a significant contributor to bacterial shifts that occur in the pollen microbiome during larval development. For brevity, results regarding the diversity of *Ralstonia* and feces in pollen samples are available in the Supplementary results.

Composition assessment of pollen with and without larvae, and larvae only

To assess the bacterial composition of our sample types and differences in beta diversity between and among groups, we analyzed weighted UniFrac distance matrices and visualized the beta diversity using PCoA. We use weighted UniFrac because it is robust against modest changes in the abundance of individual bacterial taxa. When we compare overall differences in our sample types (pollen with larvae and without larvae, and larvae only), we observed significant differences in sample type (Supplementary Figure 2B; $F = 10.93$, $p = 0.001$). Next, we compared our sample types across three sampling events in our experiment (day 3, day 9, and day 15). We find that pollen with and without larvae, and larvae only, all change across sampling days (Supplementary Figure 3). However, pollen with larvae changed the most ($F = 5.560$, $p = 0.001$) and larvae only changed the least ($F = 2.42$, $p = 0.012$).

Pollen with and without larvae at each sampling day

To better assess the direct effect of larval development on pollen masses with and without larval development, we paired these samples at each time point (Figure 4; see methods for homogenized pollen masses). First, we compare the bacterial richness (observed features) for these paired samples at each of the three time points (day 3, day 9, and day 15). We find no significant difference at day 3 ($H = 0.592$), but significant differences between pollen with and without larvae on day 9 ($H = 6.06$, $p = 0.0138$) and day 15 ($H = 4.93$, $p = 0.0264$). Second, we compared Pielou evenness between paired samples.

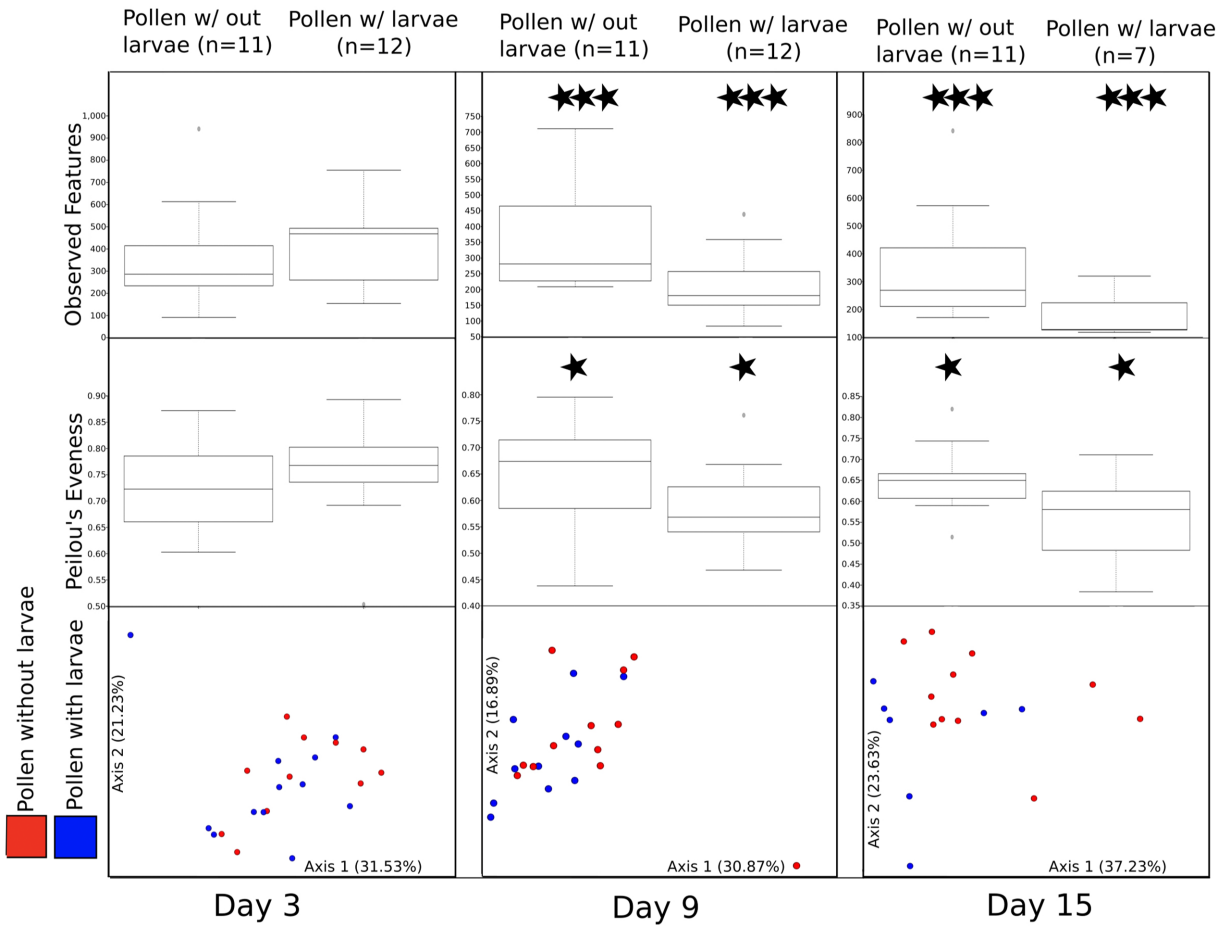

FIGURE 4
A comparison of bacterial diversity between pairs of pollen masses that were combined and then separated returning one larva to each pair. All figures (observed features, Peilou's evenness, and beta diversity) are summarized by three experimental time points (day 3, day 9, and day 15). Moderately significant is denoted by one star (*) and significant differences are denoted by three stars (***).

We detect no significant difference between the bacterial evenness on day 3 ($H = 0.592$, $p = 0.442$), and only a moderately significant reduction in evenness of pollen with, compared to pollen without larvae, for time point day 9 and day 15 ($H = 2.97$ $p = 0.085$: and $H = 2.81$, $p = 0.094$), respectively. Third, we compared the bacterial composition (beta diversity) using weighted UniFrac, and we detect no significant difference between pairs of samples using PERMANOVA at day 3, or at day 9 ($F = 0.86$, $p = 0.5$ and $F = 1.40$, $p = 0.1$, respectively). However, marginally significant differences were detected between sample pairs at day 15 ($F = 2.03$, $p = 0.06$). A similar pattern is observed for unweighted UniFrac, which considers all taxa equally, regardless of their abundance. Again, we detected no significant difference between pairs of samples using PERMANOVA at day 3 ($F = 0.79$, $p = 0.82$). However, significant differences were detected between pairs of samples at day 9 and day 15 ($F = 1.52$, $p = 0.011$ and $F = 1.70$, $p = 0.012$, respectively). Differences between pollen without larvae and pollen with larvae are even more evident when samples collected on days 9 and 15 are combined (Supplementary Figure 4).

Taxonomic differences between pollen with and without larvae

We employ two approaches to explore taxa that significantly and non-significantly differentiate samples of pollen with and without larvae. Because differences between these groups are detected at day 9 and consistent on day 15, we combine these days to increase our ability to detect bacterial taxa with differential abundance between these two-sample types. A detailed justification is provided in the Supplementary materials. In our first approach, we extracted the taxa from the numerator of the balance table generated using Qurro plots (Quantitative Rank/Ratio Observations) that integrate the Songbird model differentials that predicted samples of pollen with and without larvae. The predictive model explains 22 percent more variation than the null model. The differential table produced with Qurro highlights 27 taxa that contribute to the predictive power of the multinomial model. The bacterial taxa identified are summarized in Supplementary Figure 5. Using a second approach, DESeq2 in phyloseq, we again identified the taxa that differed between

samples of pollen with and without larvae. This approach assigns a direction to the features differential abundance, and we present this data with two figures: (Supplementary Figure 6A) shows taxa that are significantly different ($p = 0.05$) between the two groups and (Supplementary Figure 6B) shows the general pattern ($p = 0.5$) observed across diverse bacterial families. While there is significant overlap in the taxa that contribute most to differences between the groups identified using Qurro and DESeq2 (e.g., genera *Gaiella*, *Massilia*, *Pseudomonas*, and *Bradyrhizobium*, as well as unidentified; 17–14, and MND1), each approach also reveals additional bacterial taxa that separately contribute to differences in the sample types. Importantly, the results of DESeq2 suggest *Ralstonia* had only a modest increase in abundance, and the results of Qurro confirm that *Ralstonia* contributes little to differences between the sample types. Thus, *Ralstonia* is more of a constant than it appears and observed differences in its proportional abundance are, in part, the result of decreases in other taxa.

Discussion

General overview

Solitary bees, and their associated mutualistic and beneficial microbes, support plant diversity and the health of diverse ecosystems (Frison et al., 2011; Vanbergen and Initiative, 2013). Understanding how microbes interact with developing bee larvae, and vice versa, is essential for understanding how microbes impact bee health (Engel et al., 2016). General principles regarding interactions between pollen and larval microbes through bee development are limited yet needed to better understand the biology of solitary bees. To the best of our knowledge, no previous studies have evaluated the bacterial diversity of pollen provisions through time while controlling for the presence of larvae. Furthermore, no previous study has described the bacterial diversity of pollen provisions or the larval microbiome of *Osmia cornifrons*, an agriculturally important pollinator.

Our study utilized a controlled experimental design to assess the impact of larval development on pollen microbes and that of pollen microbes on larval gut microbes. By characterizing the bacterial diversity of pollen provisions with and without larvae, as well as the bacteria within larvae, sampling repeatedly throughout larval bee development, we obtained important insights into the microbial ecology within the closed "mini ecosystem" of the bee brood cell (Biani et al., 2009). Our study, like all other published work on *Osmia* microiomes, did not amplify fungal and micro eukaryotic members of the brood cell. However, only a limited scope of published work, pertaining to a specialized social stingless bee, found fungi to be a critical component to larval nutrition (Menezes et al., 2015). Rather we contend, apart from several fungal pathogens, bacterial diversity of the pollen provision is most relevant to *Osmia* developmental biology. Here, we found abundant bacterial diversity in pollen provisions of *Osmia cornifrons*, higher than what has previously been reported for bees in the family Megachilidae, and we found that the bacterial diversity in larvae is a reduced subset of what is available in the pollen provision. Additionally, we found evidence suggesting that developing larvae exert a selective pressure on the pollen microbiome through time—larval feeding appears to diminish the rare bacterial taxa in the pollen community. Furthermore, we discovered many bacterial taxa in the pollen provisions of *O. cornifrons* that correspond to known plant pathogens, suggesting that the bee brood cell provisions may serve as a repository for plant pathogens (Rothman et al., 2019; Kapheim et al., 2021).

A comparison of the bacterial diversity across *Osmia* species

The bacterial diversity within pollen provisions of *Osmia corniforns* partially matches what is known about the bacterial diversity of other *Osmia* species, as well as bees within the family Megachilidae more broadly. For example, we find higher bacterial richness in the provisions of *O. corniforns* compared with several other *Osmia* species (Keller et al., 2013; McFrederick et al., 2014; Lozo et al., 2015; Voulgari-Kokota et al., 2019a,b), yet, similar to richness found in *O. lignaria and O. ribifloris* (Rothman et al., 2020). Like our central finding, that larvae reduce bacterial diversity of pollen provisions, decreasing bacterial diversity in the pollen provisions of *O. caerulescens* through larval development was reported (Voulgari-Kokota et al., 2019a) Additionally, bacterial structure across sample types was paralleled in our results, such that some proportionally abundant bacteria were present in pollen (e.g., *Erwinia*), but not in larvae (Voulgari-Kokota et al., 2019b). Indeed, bacterial taxonomy of *O. corniforns* pollen provisions was generally congruent with the aforementioned studies of Osmia species. Specifically, bacterial phylum Actinobacteria, Firmicutes, and Proteobacteria were considered dominant in *O. cornifrons* and abundant in other *Osmia*, and at higher resolution, bacterial orders Burkholderiales, Enterobacteriales, Clostridiales, and Pseudomonadales, as well as bacterial genera *Pantoea*, *Sodalis*, and *Massilia* were also shared (Keller et al., 2013; Lozo et al., 2015; Rothman et al., 2019, 2020; Voulgari-Kokota et al., 2019b; Cohen et al., 2020). Further, *Paenibacillus*, a bacterial pathogen of honey bees (Ebeling et al., 2016) and a potential pathogen of *O. bicornis* (Keller et al., 2013, 2021) was also consistently found in low abundance across samples of *O. cornifrons* pollen and larvae.

Bacterial taxa and diversity patterns of *Osmia* not found in *Osmia cornifrons*

Despite general similarities in bacterial diversity patterns and bacterial taxa found across species of *Osmia*, there exist notable differences in the results from this study compared to previous,

primarily field-based, studies. For example, one study reported increasing bacterial diversity in the larvae of *O. caerulescens* and the pollen and larvae of *O. bicornis through time* (Voulgari-Kokota et al., 2019b). This is dissimilar to our experimental results, which showed decreasing bacterial diversity through time, and thus inconsistent with our view of a closed mini-ecosystem, and our findings that microbial diversity is lost in the presence of a larva.

In our study, more bacterial orders contribute to the total bacterial diversity of pollen provisions, compared with other studies. Nevertheless, several bacterial groups were underrepresented compared with other studies of *Osmia* (Keller et al., 2013; Lozo et al., 2015; McFrederick et al., 2017; Rothman et al., 2019; Vuong and McFrederick, 2019). Specifically, *Acinetobacter* (a common flower bacteria), reported in *Osmia* (Keller et al., 2013; Cohen et al., 2020), was generally absent in our experimental samples. Such differences could arise from biological differences of species of bees and their microbes as well as in their pollen provisions. Similarly, *Lactobacillus* a common microbial member in bee nest environments and adult bees was minimal in *O. cornifrons* when compared with microbial studies of other *Osmia* and other Megachilidae (McFrederick et al., 2017; Vuong and McFrederick, 2019; Voulgari-Kokota et al., 2019c). The lack of a dominant *Lactobacillus* and the more specifically *Apilactobacillus* is curious, and perhaps calls into question the generality of this bee-microbe association across diverse groups of solitary bees. Likewise, bacterial genera *Bartonella* and *Bacillus* which include both symbionts and generalist pathogens (Bulla et al., 1975; Gilliam, 1997; Segers et al., 2017), found frequently in both social and solitary bees (Engel et al., 2012; Keller et al., 2013; Lozo et al., 2015), were absent in *O. cornifrons*.

Conversely, many bacterial taxa found in our experimental analysis of *O. cornifrons* are absent from published studies of *Osmia* or related Megachilidae. However, there is limited utility in reporting all bacterial taxa not identified elsewhere, and rather, we emphasize that the diversity of *O. cornifrons* is robust, and potential plant pathogens make up a substantial proportion of the bacterial composition in pollen provisions. This is particularly evident in the presence of a feeding larva, a result not highlighted elsewhere. While we have yet to determine the consequences of these microbes on larval development or the transmission of these taxa back to plants in the spring, we provide a synthesis of the relevant taxa below.

Bacterial diversity assessment of *Osmia cornifrons* brood cells

We compared both the richness and the composition of the bacterial community for pollen with larvae, pollen without larvae and of larvae only (Supplementary Figures 2A,B). We found significant differences in the richness and composition across these groups of samples and all pairwise comparisons. These results suggest that larvae feeding on pollen significantly reduce the richness of bacteria in the pollen provisions. The nearly 2-fold reduction in bacterial richness between pollen with and without larvae, compared to the richness found in the larval samples themselves, suggests that larvae are not taking up and maintaining pollen bacteria in their gut indiscriminately. Rather, only a portion of the total bacterial diversity is detected in their gut. While it is possible that *O. cornifrons* gains little from the microbial environment of the brood cell, existing evidence from a congeneric species suggests that most bacterial taxa are consumed and microbe-derived amino acids and lipids are detectible at high levels in the musculature and fat body of adult bees (Dharampal et al., 2020). Due to the way larvae feed, consuming nearly all pollen in the stored pollen provisions, it is unlikely that they are selectively feeding on certain microbial taxa. Rather, we hypothesize that (1) rare taxa are lost during metabolism, (2) that their gut is selective against nearly all microbes and only the dominant bacterial taxa are recovered, and/or, (3) the chemistry of the larval gut selects against certain bacterial taxa, favoring others. When we compared the species richness of pollen with and without larvae, and larvae only through our experiment we also see evidence that larvae exert a selective force on the pollen microbiome. This may occur due to selection against microbes caused by larvae, perhaps through secretions, or because some bacterial taxa are able to replicate in this mini-ecosystem and other are not. Reduced pollen mass through feeding may also haphazardly remove rare microbes, but this is likely insufficient to fully explain the decrease in pollen bacterial diversity, as sufficient pollen material was recovered at all time points, and we presume the distribution of bacterial diversity in the pollen mass is generally homogeneous. Taken together, we found that the bacterial richness of larvae only and pollen with larvae are significantly reduced through time. This was not the case with pollen provisions incubated without larvae, which remained unchanged over time.

Pollen with and without larvae at each time point

To better understand the influence of developing larvae on the microbiome of pollen provisions, we utilized direct comparisons of pollen with and without larvae through time. We found a significant reduction in the bacterial richness of pollen with larvae, compared to pollen without larvae by day 9 and, while still declining through day 15, the difference between day 9 and 15 was non-significant. This suggests that the larval effect on the bacterial diversity of pollen is minimal at first but is substantial by day 9. It also suggests that the reduction in pollen bacterial diversity, driven by larval development is bounded, and that at least 1/3rd of the bacterial diversity is resilient and can withstand the impact of larval feeding. Similarly, we found a moderately significant reduction in the evenness of the bacterial community of pollen with feeding larvae, compared to pollen without larvae by day 9. A decline in evenness implies that some bacterial taxa are becoming increasingly common, and we observe that the diverse

rare taxa are declining or disappearing; a result supported by our comparisons of bacterial richness in pollen provisions with and without larvae. We find that bacterial composition (weighted UniFrac) shows only a marginally significant difference between pollen with and without larvae by day 15. Taken together the bacterial composition of pollen with larvae appears to be increasingly differentiating from that of pollen without larvae over time, but the largest shifts occur around the mid-point of larval development.

Taxonomic differences between pollen with and without larvae

We combined samples of pollen with and without larvae by day 9 and 15 to increase our ability to detect differences in the bacterial community composition in the presence and absence of feeding larvae. Utilizing this more robust approach, we detected 16 taxa that are significantly differentiated (Supplementary Figure 6A), and only three taxa that were enhanced. Thus, the presence of larvae does not enhance very many microbes, in fact only two taxa strongly benefit from larval presence. One of these, the genus *Cutibacterium* (known anaerobic bacteria), likely colonizes the larvae itself, and the other, the genus *Massilia* (an aerobic bacteria previously found to be plant associated Ofek et al., 2012), may increase as the result of reduced competition within the pollen microbiome. The differential abundance of microbial species reflects the central finding of our experiment—larvae are selecting against diverse groups of bacteria, many of which are rare to begin with. This would explain why individual taxa are not significantly differentiated, but the sum of these reduced taxa is significantly driving the bacterial diversity between these two sample types. To further illustrate this effect, we plotted the families of bacteria that are nonsignificantly ($p = 0.5$) differentiated between pollen with and without larvae (Supplementary Figure 5). Indeed, most bacterial families are reduced in the presence of a larva, while comparatively few are increasing.

Composition assessment of pollen with and without larvae, and larvae only

We examined the taxonomic composition of bacteria in pollen provisions with and without larvae and larvae only to compare the dominant 12 bacterial taxa found across all sample types. Here, we observed similar taxonomic structure of pollen with larvae and larvae only, and we did not observe several dominant taxa shared by these two groups in pollen without larvae. Specifically, we do not find bacteria in the genera *Exiguobacterium* and *Comamonadaceae* to be dominant in pollen alone. These results suggest that the influence of larval feeding is observable both inside and outside the larva. In larvae only, we observe little to no change in the taxonomic composition of bacteria across sampling days, except for a modest reduction in the proportional abundance in the genus *Sodalis*, which is a common insect endosymbiont (see below; Dale et al., 2001; Chari et al., 2015). Additionally, bacteria in the genus *Cutibacterium* are only found in the larvae, and the isolated nature of *Cutibacterium* suggests that this bacterium is utilizing the host itself. Conversely, bacteria in the genus *Massilia* are only found in the pollen and do not appear to persist in the larvae.

In pollen without larvae, we again saw a relatively modest difference in composition across sampling days and a slight reduction in the proportional abundance of *Sodalis*. This reduction in the proportional abundance of *Sodalis*, as well as its presence in all sample types, suggests that it was specifically introduced by the adult mother at the time of pollen provisioning. We detected 20 unique sequences of *Sodalis*. When we blast the most abundant sequences, we detected sequences closely related to several endosymbionts of insects including an endosymbiont of a chestnut weevil (*Curculio sikkimensis*; Higaki, 2005), an endosymbiont of a parasitic wasp (*Spalangia cameroni*; Betelman et al., 2017), an endosymbiont of a neotropical mealybug (*Puto barberi*; Szklarzewicz et al., 2018), and endosymbionts of stinkbugs (*Nezara antennata* and *Piezodorus hybneri*; Hosokawa et al., 2015, 2016). The specificity of *Sodalis* in solitary bees is currently unknown, and future work may uncover strains of *Sodalis* to be bee specific or even bee species specific. Furthermore, the near complete absence of *Wolbachia*, a common insect endosymbiont, may suggest potential within-host competition occurs between the two genera.

One notable difference in pollen without larvae is the constant presence of *Erwinia* (a common plant associate and pathogen). *Erwinia* is a genus within the bacterial family Enterobacteriaceae and is generally the sole genus within the family found to be commonly associated with Megachilidae (Voulgari-Kokota et al., 2019b). This group of bacteria may be suppressed by the presence of the larvae, which may aid in preserving the pollen provision. Alternatively, or perhaps in concert with the timing of developing larvae, *Erwinia* may be degrading pollen, thus providing additional or accelerated nutritional value to pollen, a process has been documented for several pollen and flower associated bacteria (Christensen et al., 2021).

By contrast, in pollen with larvae, we see a dramatic increase in the proportional abundance of bacteria in the genera *Ralstonia* and *Pantoea*, and a decrease in *Sodalis*. Most other dominant taxa in pollen with larvae remain relatively stable. The proportional increase in *Ralstonia*, and, to a lesser degree *Pantoea*, appears to result from the significant decrease in non-dominant bacterial taxa, especially in the presence of larvae.

Plant pathogens in the pollen microbiome

Our analysis of the pollen microbiome of *Osmia cornifrons* revealed the presence of bacterial sequences that match closely to diverse plant pathogens. These presumed pathogens make up a

substantial portion of the total bacterial sequences in pollen provisions, and are much higher in their proportional abundance when compared with related bee species (Keller et al., 2013; McFrederick et al., 2014; Lozo et al., 2015; Voulgari-Kokota et al., 2019a,b; Dharampal et al., 2020). In our study, we identify 17 unique sequences matching to bacteria in the genus *Erwinia*, 443 matching to the genus *Pantoea*, 68 matching to the genus *Ralstonia*, and 240 matching to the genera *Pseudomonas*. To improve our understanding of pollen-associated bacterial sequences that match plant pathogenic bacterial genera, we blasted the dominant representative sequences from *Erwinia*, *Pantoea*, *Pseudomonas*, and *Ralstonia*. In doing so, we uncovered sequence matches to previously studied isolates of plant pathogens. We provide a summary for the top five most abundant sequences of each genus known to contain plant pathogens in Supplementary Table 1.

Several of the potential pathogens identified in our study have been reported in other studies of *Osmia* and related bees. Most notably, *Pantoea agglomerans* (a pathogen of pome fruit including apples, pears, nashi, and quince), and a causal agent in fire blight was a dominant species in *O. cornuta* pollen provisions (Lozo et al., 2015). Additionally, *Pantoea* more generally was also detected in previous studies of *O. bicornis*, *O. lignaria*, *O. ribifloris*, and *Megachile rotundata* (Keller et al., 2013; Rothman et al., 2019, 2020). *Erwinia*, was also found separately in association with *Osmia lignaria* (Cohen et al., 2020), as well as a small carpenter bee, *Ceratina calcarata* (McFrederick and Rehan, 2016; Dew et al., 2020). *Ralstonia* was present in *O. lignaria*, *O. ribifloris* (Rothman et al., 2020), and *Osmia bicornis* (Mohr and Tebbe, 2006) as well as *Megachile rotundata* (Rothman et al., 2019). *Pseudomonas* was present in *Osmia bicornis* (Keller et al., 2013) and in *Megachile* and *Osmia* (McFrederick et al., 2017). Since *Osmia cornifrons* is native to Japan, novel pathogenic bacterial associations are likely to exist, and may have been co-introduced into the new range of *Osmia cornifrons* (Hedtke et al., 2015). Indeed, *O. cornifrons* has already been implicated in the introduction of *Ascosphaera naganensis* a fungal pathogen of bees that may contribute to declines in related native species (Hedtke et al., 2015; LeCroy et al., 2020).

The discovery of diverse putative plant pathogens accumulating in pollen provisions is intriguing, as the ability of solitary bees to transmit pathogens of plants in orchards and in natural environments represents a substantial knowledge gap. Additionally, how plant pathogens may function in both detrimental or beneficial ways for bees within pollen provisions, and how bees may act in both detrimental and beneficial ways for pathogen transmission dynamics in the spring, are understudied areas. While we currently lack data to test these interactions, we offer several hypotheses. First, plant pathogens may help facilitate pollen degradation that could improve nutritional quality of pollen for feeding larvae. Indeed, experimental evidence of bacteria acting like an external rumen, pre-digesting and enhancing the nutritional quality of pollen for *O. ribifloris* was laid out in Steffan et al. (2019) and Dharampal et al. (2019, 2020), and bacterial induced germination of pollen was further detailed by Christensen et al. (2021). It is, therefore, reasonable to assume that a similar mechanism may be at work in *O. cornifrons*, and probable that these plant pathogens could possess enzymes or metabolites that may help liberate nutrients from pollen grains. Second, acquisition of potential plant pathogens by *O. cornifrons* may simultaneously serve as a sink and/or a source for plant pathogen transmission. By collecting plant pathogens and storing them within brood cells, *O. cornifrons* may alter transmission dynamics among plants—a hypothesis also explored in *Megachile rotundata* (Rothman et al., 2019). If these microbes are sequestered, consumed, or do not survive or replicate, pathogen burden of plants may be reduced. If however, *O. cornifrons* emerges from their brood cells and carries spores or live cells (perhaps by climbing through infected brood cells) in contact with plants, they may be in part responsible for re-establishing transmission dynamics in the spring. It is conceivable that both processes may be taking place and should be considered when assessing the microbial diversity of pollen provisions in solitary bees. A discussion of horizontal transmission of microbes that occur at flowers and subsequent microbial filtering that may influence *Osmia* pollen bacterial diversity can be found in the Supplementary materials.

Conclusion

Solitary bees are important pollinators of agricultural crops and diverse flowering plants in natural landscapes. Unlike many floral visitors (flies, moths, and beetles), solitary bees collect and store pollen and nectar as food for their developing offspring. Our experimental design sought to uncover the direct effects of larval feeding on the pollen bacterial community of one solitary bee species, *Osmia cornifrons* that may be representative of bee species in the larger bee family Megachilidae. We find that the bacterial community of developing larvae are relatively stable over the course of larval development, and that the larval microbiome consists of a subset of the dominant bacterial taxa found initially in the pollen provisions. Our results confirm that contact with developing larvae results in a dramatic decrease in bacterial richness of the pollen provision, a decrease in microbial evenness of the pollen provision, and shift in the bacterial composition. These changes in bacterial diversity through larval development are likely the result of selection against the rare bacteria in the system. Indeed, there appear to be very few microbes that benefit from close association with a developing larva and many bacterial taxa are lost along the way. Thus, it does not appear that larval *Osmia cornifrons* utilize specific bacteria internally to support their development, however larvae may benefit from bacteria as a

source of nutrition, either directly or indirectly through degradation of pollen. Lastly, bee-microbial interactions likely confer substantial implications for plant pathogen propagation, and still unknown are the feedback mechanisms and reciprocal consequences of plant pathogen propagation for bee health, development and ultimately bee conservation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject: PRJNA893014.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1057626/full#supplementary-material

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Environment or genetic isolation? An atypical intestinal microbiota in the Maltese honey bee *Apis mellifera* spp. *ruttneri*

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Introduction: *Apis mellifera* evolved mainly in African, Asian, and European continents over thousands of years, leading to the selection of a considerable number of honey bees subspecies that have adapted to various environments such as hot semi-desert zones and cold temperate zones. With the evolution of honey bee subspecies, it is possible that environmental conditions, food sources, and microbial communities typical of the colonized areas have shaped the honey bee gut microbiota.

Methods: In this study the microbiota of two distinct lineages (mitochondrial haplotypes) of bees *Apis mellifera ruttneri* (lineage A) and *Apis mellifera ligustica* and *carnica* (both lineage C) were compared. Honey bee guts were collected in a dry period in the respective breeding areas (the island of Malta and the regions of Emilia-Romagna and South Tyrol in Italy). Microbial DNA from the honey bee gut was extracted and amplified for the V3-V4 regions of the 16S rRNA gene for bacteria and for ITS2 for fungi.

Results: The analyses carried out show that the Maltese lineage A honey bees have a distinctive microbiota when compared to Italian lineage C honey bees, with the most abundant genera being Bartonellaceae and Lactobacillaceae, respectively. Lactobacillaceae in Maltese Lineage A honey bees consist mainly of *Apilactobacillus* instead of *Lactobacillus* and *Bombilactobacillus* in the lineage C. Lineage A honey bee gut microbiota also harbors higher proportions of *Arsenophonus*, *Bombella*, *Commensalibacter*, and *Pseudomonas* when compared to lineage C.

Discussion: The environment seems to be the main driver in the acquisition of these marked differences in the gut microbiota. However, the influence of other factors such as host genetics, seasonality or geography may still play a significant role in the microbiome shaping, in synergy with the environmental aspects.

KEYWORDS

honey bees, microbiome, *Bartonella*, *Lactobacillus*, environment, *Apis mellifera* spp. *ruttneri*, *Bombella apis*, mitochondrial haplotype

Introduction

A new subspecies of honey bees, *Apis mellifera* subsp. *ruttneri*, was identified 25 years ago by Sheppard et al. (1997) in the Maltese Islands. It belongs to the African bee subgroup and is classified close to *Apis mellifera* subsp *intermissa, Apis mellifera* subsp *siciliana* and is distantly related to the European subspecies, as revealed by the morphometric analysis and the mitochondrial haplotype of the tRNAleu-Cox2 region (Zammit-Mangion et al., 2017). The Maltese honey bee shows peculiar characteristics of adaptation to drought as well as very hot and windy weather. It is slightly smaller in size, dark in color with no apparent yellow bands, highly active and resistant to varroosis (Sheppard et al., 1997). These characteristics have developed after thousands of years of isolation on the Maltese Islands.

Honey bees have been classified into five main lineages discriminated according to the mitochondrial haplotype used to characterize evolutionary diversity between and within populations: (a) lineage A (Africa) to which *A. mellifera ruttneri* belongs; (b) lineage Y (Yemen and Ethiopia); (c) lineage O (Oriental, from Turkey to Kazakhstan); (d) lineage C (Carnica, from Central/South Europe) to which *A. mellifera ligustica* and *carnica* belong, and (e) lineage M (Mellifera, from West/North Europe) which comprises over 28 different subspecies, with many others expected to be discovered (Miguel et al., 2007). Lineages have also been divided into subcategories and *A. mellifera ruttneri*, at present, belongs to the mitochondrial sub-haplotypes A4, A8, and A9 (Zammit-Mangion et al., 2017).

Described honey bee subspecies have shown behavioral and morphological adaptations to their native environments, allowing them to better exploit available food resources. Considering how crucial the gut microbiota is for food exploitation in bee nutrition, it is hypothesized that environment, behavior and food quality shapes the microbial community composition at honey bee subspecies level. In fact, recent studies demonstrated how seasonality, landscape (environment and nutrient availability) and host genetic background can impact the microbial profile of different caste of honey bees (Mattila et al., 2012; Kešnerová et al., 2020; Wang et al., 2020).

The main available studies report that the honey bee gut harbors a simple microbial community (Martinson et al., 2011) composed of a limited number of core bacterial species (Sabree et al., 2012), which include both Gram negative and Gram positive groups (Moran, 2015). These bacteria are specific to the bee gut and can be directly transmitted among individuals through social interactions (Zheng et al., 2018). The honey bee gut microbial community is relatively stable over time and space, unless honey bees are subjected to anthropogenic pressures such as the use of antibiotics (Raymann et al., 2017; Baffoni et al., 2021) and/or pesticide treatments in agricultural practices, including glyphosate (Motta et al., 2018) and neonicotinoids (Alberoni et al., 2021a). These studies have generally only addressed the domesticated *A. mellifera* and as such, a description of gut microbial profiles looking at honey bee subspecies have never been convincingly reported. Some studies have regarded the characterization of cultivable lactic acid bacteria and bifidobacteria in different *A. mellifera* subspecies, e.g., *scutellata, mellifera*, and *monticola* (Olofsson et al., 2011), revealing that all share the same Lactobacillaceae and *Bifidobacterium* phylotypes. Sharifpour et al. (2016) isolated and characterized lactic acid bacteria and bifidobacteria from the gut of *A. mellifera* subspecies of West Azerbaijan showing that there is low sequence divergence in comparison with other lactic acid bacteria.

Given the huge interest in honey bee gut microbiota and the relevant papers published on the European *A. mellifera*, this study investigates the gut microbiota of *A. mellifera ruttneri* (lineage A), looking at its core composition and abundance. High throughput sequencing gave an overview of the overall abundance of bacteria and yeast communities; moreover, investigation of the lactobacilli population was also performed with culture-dependent techniques and PCR-DGGE. Data based on the 16S rRNA gene sequencing were used for comparative analysis with data obtained from *A. mellifera* subsp. *ligustica* and *carnica* (lineage C) and for metagenome functional prediction.

To the best of our knowledge, this is the first deep analysis of the Maltese honey bee gut microbiota. The study investigates whether there are distinctive differences in the gut microbiota of the honey bees prevalent in Italy (*A. mellifera ligustica* and *carnica*) and *A. mellifera ruttneri*, since these subspecies have been sampled from niches with different climate conditions and possibility of exchange of genetic resources, in addition to their different mitochondrial haplotypes (C and A) and consequent different phylogenesis. To date, the Maltese honey bee is considered an endangered subspecies due to the importation of different honey bees from the European continent, thus representing a threat to the one hundred pure beehives still present on the Maltese Islands (Jansen, 2018). The investigation was carried out in Malta in three different apiaries with different beekeeping management practices. In one of the test apiaries, the Maltese honey bee is still being reared in terracotta hives called "Migbha," dating back to Punic times (**Supplementary Figures 1A, B**), a unique case in Europe.

Materials and methods

Sampling location and samples collection

Guts from *Apis mellifera ruttneri* were sampled from three different apiaries located in Malta during April 2016. Sampled honey bees, picked off the brood surface, were between 15–20 days old. The apiary in Għargħur (GH) had been established for more than 80 years as it belongs to a beekeeping family who still rear some of their colonies in terracotta hives, a practice unique to the Maltese Islands and other southern European countries (**Supplementary Figure 1**). This apiary is located in an urban location (35° 92′22.58″ N, 14° 45′39.58″ E) overlooking a small valley system. The apiary Campus Msida (CM) is located on the University of Malta grounds (35° 90′40.36″ N, 14° 48′33.56″ E) in Wied Għollieqa (Valley) and represents a recently established apiary with around 20 colonies of bees. The environment surrounding CM is best described as abundant agricultural land now dominated by carob trees (*Ceratonia siliqua*) and prickly pear (*Opuntia ficus-indica*). The apiary in Żejtun (ZT) is located at the outskirts of the village (35° 85′98.35″ N, 14° 53′74.71″ E), in an agricultural dwelling where occasional use of pesticides is practised. The main crops cultivated in the area include potatoes, tomatoes and courgettes. For bacteria isolation, a pool of 20 honey bee guts per sampling

location were smashed and mixed. Following this, 0.5 mg of each pool was mixed with 4.5 ml of sterilized glycerol broth (meat extract 2.7 g/L, peptone 4.5 g/L, glycerol 100 ml/L) and 1:10 serial dilutions were carried out. For metagenomic analysis, 20 individual guts (both midgut and hindgut) were sampled from each apiary. All samples were immediately shipped on dry ice to the University of Bologna, Italy.

For comparative analysis, data obtained from *Apis mellifera* lineage C were used, samples of both subspecies *ligustica* and *carnica*. The *ligustica* data referred to samples collected in the Emilia-Romagna region (Italy) at Valsamoggia (Bologna, 44°29′45.3″N 11°06′10.4″E) and San Lazzaro di Savena (Bologna, 44°27′28.2″N 11°23′45.8″E) (Alberoni et al., 2021a,b; Baffoni et al., 2021), whereas the *carnica* data referred to samples previously collected in the South Tyrol region, Bolzano (46°22′47.7″N 11°14′14.6″E) (Baffoni et al., 2021). The full list of samples deriving from these studies can be found in **Supplementary Table 1**.

DNA extraction, 16S rRNA gene, and ITS library preparation

Genomic DNA from honey bee gut samples was extracted from 20 single honey bee guts per site with the Quick-DNA™ Insect Microbe Miniprep Kit-Zymo Research (ZYMO, Irvine, CA, USA), according to the manufacturer's instructions. DNA concentration and purity were analyzed with Tecan Infinite 200 PRO reader (Tecan Group Ltd., Mannedorf, Switzerland). DNA was then stored at −20°C. The microbial gut community was determined using tag-encoded 16S rRNA gene MiSeq-based (Illumina, San Diego, CA, USA) high throughput sequencing for bacteria and the variable internal transcribed spacer (ITS)-2 rDNA region for yeast and fungi. The bacterial (V3-V4) and eukaryotic (ITS2) sequencing libraries were prepared according to Takahashi et al. (2014) and Haastrup et al. (2018), respectively. The amplified fragments with adapters and tags were purified and normalized using custom-made beads, pooled and subjected to 250 bp pair-ended MiSeq sequencing. Of the 60 Maltese honey bee guts individually extracted, 30 samples (10 samples from each Maltese testing apiary) were run on a Next Generation Sequencing (NGS) Illumina MiSeq platform for bacterial (V3-V4) sequencing, while the remaining 30 samples were processed for eukaryotic (ITS2) sequencing. The raw dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using Trimmomatic v 0.39 with the following settings, -fastq_minovlen 100, -fastq_maxee 2.0, -fastq_truncal 4, and -fastq_minlen of 160 bp. De-replicating, purging from chimeric reads, and constructing *de novo* zero-radius Operational Taxonomic Units (zASV) were conducted using the UNOISE pipeline Edgar (2018) and taxonomically assigned with –sintex Edgar (2016) coupled to the EZtaxon (Kim et al., 2012) for 16S rRNA gene and UNITE (Kõljalg et al., 2013) for ITS2 as references. A total of 1,25 million reads were obtained for both 16S rRNA genes sequencing. Following assembling and quality filtering (low quality reads, chimeric sequences and unaligned sequences), with an average of 42 thousand sequences per sample. One sample, GH7, failed the sequencing and was therefore removed. The ASVs assigned were 5,513.

Lactobacilli isolation and identification

For lactic acid bacteria enumeration, serial dilutions were prepared and plated on man rogosa sharpe (MRS) agar (VWR, Milano, Italy) containing 0.01% l-Cysteine-HCl (Merck, Darmstadt, Germany), 0.1% fructose (Sigma-Aldrich, Milano, Italy) and 0.1% cycloheximide (Sigma-Aldrich, Milano, Italy). Analyses were performed in triplicate. Plates were incubated anaerobically at 35°C for 72–120 h, the number of colony forming units (CFU) were recorded and counts were made. Around 100 isolated colonies were re-streaked and purified. For long term storage, purified isolates were stored at −80°C with their respective liquid medium containing 20% glycerol. DNA extraction from pure cultures was performed with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Fingerprinting was then obtained using BOX-PCR, as in Gaggìa et al. (2015). Cluster analysis and grouping BOX profiles was carried out with Bionumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) using Dice's Coefficient of similarity and the un-weighted pair group method arithmetic averages clustering algorithm (UPGMA). Based on the genotypic grouping, representative isolates were selected, the 16S rRNA gene amplified with primers 8-fw and 1520-rev and sequenced according to Gaggìa et al. (2015). Sequences were then deposited to GenBank®[1] with the following accession number: MT381710-MT381736 and MG649988-MG650060. The obtained 16S rRNA gene sequences were used to generate a phylogenetic tree together with sequences of *A. kunkeei* retrieved from the National Center for Biotechnology Information (NCBI) Genomes RefSeq database (**Supplementary Table 2**) especially from Germany, Sweden (Tamarit et al., 2015), and Switzerland (Crovadore et al., 2021). The phylogenetic tree was generated with MEGA11 (Tamura et al., 2021) inferred by using the Maximum Likelihood method (K2 + G substitution model) with rate variation among sites. *Lactobacillus melliventris* MT53, *Lactobacillus apis* MT61, and *Gilliamella apicola* MT1 and MT6 were used as outgroups.

PCR-DGGE analysis of lactobacilli population

PCR-DGGE analyses were performed to investigate lactobacilli populations; for each sampling location, 17 (out of 20) DNA extracted from individual guts were processed. The PCR and subsequent denaturing gradient gel electrophoresis (DGGE) analysis, using the Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA), were performed as described by Alberoni et al. (2018). Denaturing gradient was established at 35–65%. Fingerprinting analyses were carried out using the Bionumerics v 7.1 (Applied Maths, St. Martens-Latem, Belgium) and the UPGMA algorithm based on the Pearson correlation coefficient with an optimization of 1% was applied. Microbial diversity was analyzed with the following parameters: Shannon–Wiener index (H), Simpson index (S), and band evenness (EH), calculated according to Hill et al. (2003). Moreover, principal

1 www.ncbi.nlm.nih.gov/genbank/

components analysis (PCA) was carried out by using Bionumerics. Relevant bands were excised from the gels and processed to achieve purified amplicons to be sequenced (Gaggìa et al., 2015). Sequencing was carried out by Eurofins Genomics (Ebersberg, Germany) and obtained sequences were assigned to bacterial species using megablast algorithm.[2]

Statistical analysis

Bioinformatic analysis was performed using R open-source statistical software v 4.2.1 (R Core Team, 2022) with phyloseq (McMurdie and Holmes, 2013), metagenomeSeq (metagenomeSeq: Statistical analysis for sparse high-throughput sequencing, Paulson, 2014), vegan (Dixon, 2003), ggpubr v 0.4.0 (Kassambara and Kassambara, 2020), and ggplot2 v 3.5.5 (Wickham, 2011) packages. Raw reads were filtered and low-abundance ASVs (below 0.5%) were removed across all samples. The sequencing depth was, on average, 40,103 reads per sample for 16S amplicons and 92,456 for ITS amplicons before filtering. After filtering, 36,109 and 84,396 sequencing were, respectively obtained. For diversity analysis, all samples were rarefied to mean-read depth and cumulative sum scaling (CSS) normalization was used for beta diversity analysis. PICRUSt 2.0 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, Douglas et al., 2020) was used to predict functional abundances based on 16S amplicon sequences. Comparisons of alpha diversity was performed using analysis of variance (ANOVA) with Tukey Honest Significant Differences (Tukey HSD) multiple testing correction. Permutational ANOVA (PERMANOVA) was used to evaluate group comparisons of bacterial community composition, using the Bonferroni–Holm method for multiple testing correction. Statistical significance was determined at $p < 0.05$. LEfSe analysis on microbiome data was performed comparing the sampling sites using Galaxy (Blankenberg et al., 2011).

Climate data elaboration

The monthly climatic data for precipitations (cumulative millimeters of rainfall), average minimum and maximum temperatures, and absolute lower temperatures were retrieved from local repositories. Data from Malta were obtained from the local international Airport,[3] approximately midpoint of all samplings carried out in the apiaries of Għargħur–GH, Wied Għollieqa–GH, and Campus Msida–CM. The climatic data of the Sud Tirol province (Apiary of Bozen–BZ) were retrieved from the "Südtirol Open Data Alto Adige,"[4] whereas the climatic data of the Emilia-Romagna region (Municipalities of Valsamoggia–VS) and (San Lazzaro di Savena–SLS) were retrieved from Agenzia regionale per la prevenzione e l'ambiente dell'Emilia Romagna (ARPAE) Emilia-Romagna Environmental Agency database (Dext3r Platform),[5] using as midpoint of the locality of Zola Predosa (approximately equidistant from the two sampling points), as no data were available for VS and SLS. Retrieved data were used to generate Walter and Lieth climate diagrams (improved Bagnouls and Gaussen climate diagram) of the three main sampling areas. Moreover, to better understand the climatic trend, data from the year prior to sampling were also analyzed. Walter and Lieth climate diagrams were generated with the R statistic package "climatol" (Guijarro and Guijarro, 2019).

Results

Results on 16S rRNA gene sequencing on *Apis mellifera ruttneri* gut bacterial communities

Overall, at phylum level, the most representative members were α-proteobacteria (41.70%), γ-proteobacteria (26.70%), Firmicutes (15.60%), β-proteobacteria (7.50%), and Actinobacteria (5.70%) (**Supplementary Figure 2**), these accounted for 97.30% of the total reads. **Supplementary Figures 3, 4** also report the relative abundances at Order and Class level. Among α-proteobacteria, the most representative family was Bartonellaceae accounting for 32.50%, followed by Acetobacteraceae (8.10%). Within γ-proteobacteria, Orbaceae (13.60%), and Morganellaceae (8.80%) were the most abundant families. Finally, Firmicutes, β-proteobacteria, and Actinobacteria, mostly corresponded to the Lactobacillaceae, Neisseriaceae, and the Bifidobacteriaceae families, respectively (**Supplementary Figure 5**).

At genus level (**Figure 1** and **Supplementary Figure 6**), 32.50% of the assigned reads could be ascribed to *Bartonella*, 9.90% to *Arsenophonus*, followed by 9.30% to *Lactobacillus*, 7.40% to *Snodgrassella*, 5.90% to *Commensalibacter*, and 5.50% to *Bifidobacterium*. Less abundant genera were *Apilactobacillus*, *Bombella*, *Bombilactobacillus*, *Pseudomonas*, *Spiroplasma,* and *Acinetobacter* (1–3.00%). In addition, within the Orbaceae family, 8.80% was assigned to *Gilliamella* and 3.70% to *Frischella*. ASVs species assignment among Lactobacillaceae (**Figure 2**) allowed the detection of the following genera and species: *Lactobacillus apis* 19.55%, *Lactobacillus kimbladii* 13.79%, *Lactobacillus helsingborgensis* 4.46%, and *Lactobacillus melliventris* 3.74%. 15.55% of *Lactobacillus* remained unassigned. The *Apilactobacillus* genus showed up as only two species: *Apilactobacillus apinorum* 3.25% and *Apilactobacillus kunkeei* 23.00%. Within the *Bombilactobacillus* genus, the species *Bombilactobacillus mellis* 8.54% and *Bombilactobacillus mellifer* 8.10% were identified. Interestingly, within *Bartonella*, only 3.80% of ASVs was taxonomically identified as *Bartonella apis*, while the majority of them (96.20%) remained unassigned at species level. Comparing the three Maltese sampling sites (CM, ZT, and GH), the core microbial composition of sampled honey bees did not show appreciable variation in composition.

Results on ITS gene sequencing on *Apis mellifera ruttneri* gut yeasts community

Results for the fungal gut community of the Maltese honey bee revealed the phylum Ascomycota to be, by far, the most abundant,

2 http://www.ncbi.nlm.nih.gov/BLAST/

3 https://weatherspark.com/y/148306/Average-Weather-at-Malta-International-Airport-Malta-Year-Round

4 https://data.civis.bz.it/it/dataset/misure-meteo-e-idrografiche

5 https://simc.arpae.it/dext3r/

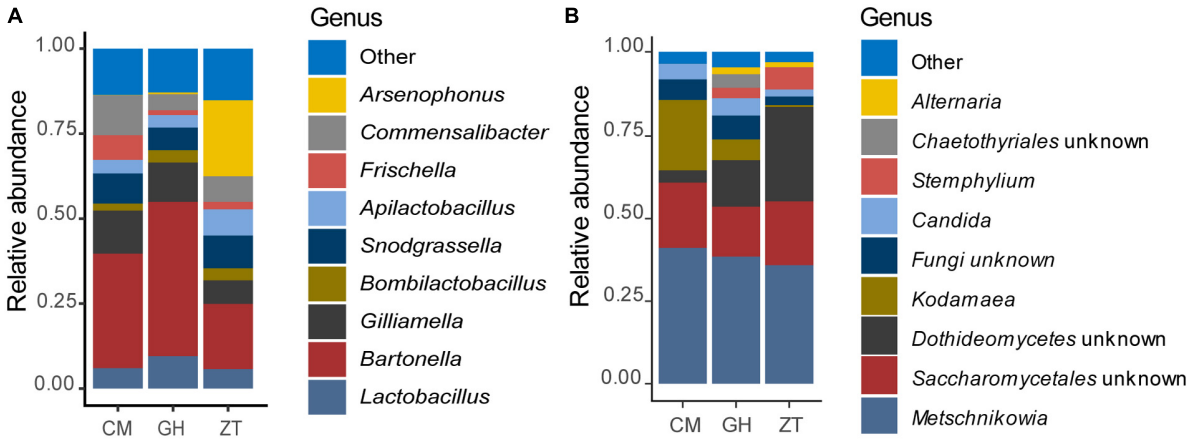

FIGURE 1
Relative abundance of the gut bacterial **(A)** and fungal **(B)** populations determined by NGS. Bar charts are reporting the major microbial genera cumulated by sampling site in Malta: CM, University of Malta–Campus Msida; GH, Għargħur; ZT, Żejtun.

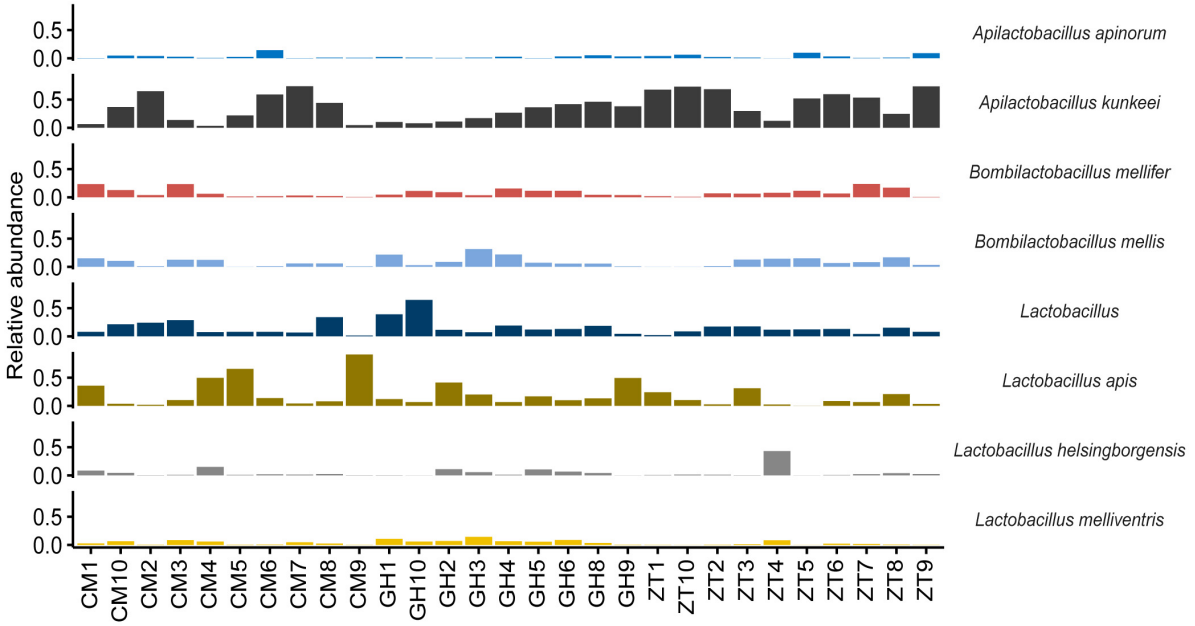

FIGURE 2
Bar chart showing the relative abundance of the major species belonging to the family Lactobacillaceae in every sampled honey bee gut in Malta determined by NGS.

making up 87.26% of the total reads. Basidiomycota counted only 1.53% of the total reads and about 11.20% of the reads remained unassigned at phylum level. The most abundant orders in the Ascomycota phylum were Saccharomycetales and Pleosporales, respectively 65.60 and 4.09%. Saccharomycetales comprised the family Metschnikowiaceae (45.74%–**Supplementary Figure 7**), followed by unclassified Saccharomycetales family (17.54%). Pleosporales' most representative family was Pleosporaceae (3.23%). Metschnikowiaceae, at genus level, was represented by *Kodamaea* (8.10%) (with only a specie identified, *Kodamaea ohmeri*) and *Metschnikowia* (34.57%), comprising mostly unidentified species together with *Metschnikowia cibodasensis* (2.19%) and *Metschnikowia chrysoperlae* (0.13%). Pleosporaceae was accounted by *Stemphylium* and *Alternaria* at 2.83 and 0.93%, respectively. Members of the *Candida* genus (assigned to *Saccharomycetales incertae sedis*) accounted for up to 3.33% of the relative abundance, although the relevance of this genus was low amongst samples. The detected species were *C. versatilis* (1.82%), *C. primensis* (0.70%), and *C. kofuensis* (0.52%). Relative abundances, at genus level, are shown in **Figure 1B** and **Supplementary Figure 8**. No significative differences were detected in within-sample eukaryotic microbial diversity for neither Shannon and Observed ASVs α-diversity indexes (**Figure 3A**), whereas between-group comparisons of community composition using Bray–Curtis dissimilarity index (**Figure 3B**) and Sorensen–Dice indexes for β-diversity showed significant

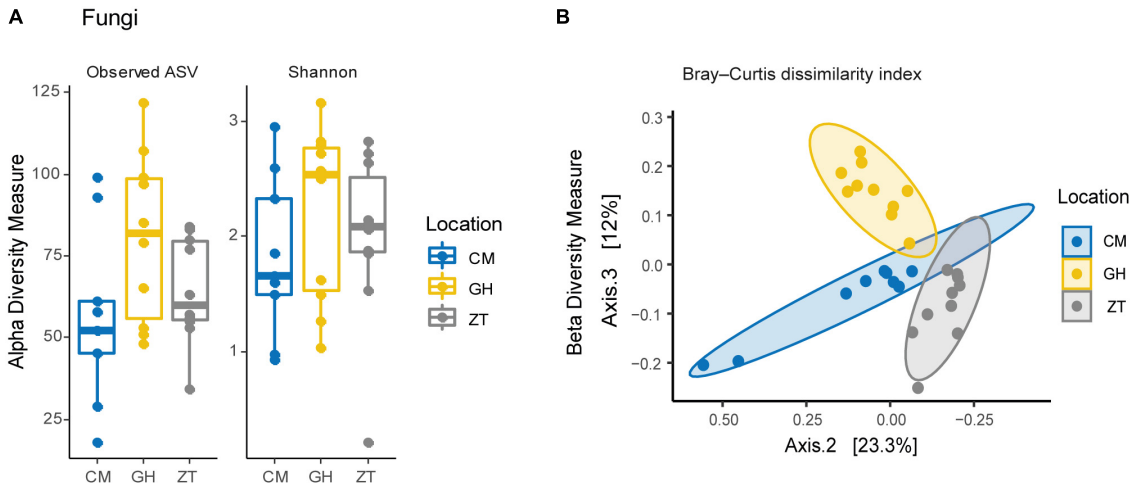

FIGURE 3
(A) Fungal α-diversity within the three sampling sites in Malta: CM, University of Malta–Campus Msida; GH, Għargħur; ZT, Żejtun. **(B)** β-diversity Bray–Curtis dissimilarity index per sampling site on the yeasts microbial community in Malta.

differences between all three Maltese localities ($p \leq 0.001$ for all comparisons).

Comparison of the bacterial communities of honey bees sampled in Malta (lineage A) and Italian honey bees (lineage C)

The gut microbiota composition of honey bees in Malta (lineage A) showed major differences when compared to the Italian honey bees (lineage C) with significant differences detected in microbial diversity within locations at genus level Figures 4A–K. Honey bees collected in Malta showed significant increases in microbial groups such as *Bartonella* (31.26% in lineage A *vs.* 4.82% in lineage C), *Bombella* (2.280% in A *vs.* 0.005% in C) and *Commensalibacter* (5.59% in A *vs.* 0.73% in C) (Figures 4B, E, F, $p < 0.01$). Notably, *Bartonella* was found to be the most highly represented genus in almost all sampled Maltese honey bees' guts, with the sole exception of ZT2 and ZT10 which were dominated by *Arsenophonus* (89.34 and 98.77% in ZT2 and ZT10, respectively Figure 4A) and CM9, GH10, and ZT5 which were dominated by *Snodgrassella* (from 27.10 to 63.76%, Figure 4J). On the contrary, major core microbial groups *Bombilactobacillus* and *Lactobacillus* for Lactobacillaceae (Lactobacillaceae: 14.86% in lineage A *vs* 61.50% in lineage C, Figures 4D, I and Supplementary Figure 9), *Frischella* (3.60% in A *vs.* 5.92% in C, Figure 4G), and *Gilliamella* (10.17% in A *vs.* 14.12% in C, Figure 4H) were found at a significantly lower proportion in honey bees collected from Malta ($p < 0.05$). Other core microbial groups like *Bifidobacterium* (Figure 4C) and *Snodgrassella* (Figure 4J) did not significantly vary among honey bees sampled in Malta and in Italy. Figures 5A, B report the bar charts and the differentially abundant genera, comparing the composition of the Malta and Italy sampling sites. Comparison of the major microbial genera per sampling site (BZ, CM, GH, VS, SLS, ZT) are reported in Supplementary Figures 10A–K, among samples in Supplementary Figure 11 and raw data per for the major microbial taxa per sample are reported in Supplementary Table 3. Bacterial within-sample diversity of the Maltese sampling sites (lineage A, localities CM, GH, and ZT) or the Italian ones (lineage C, localities BZ, SLS, and VS) did not significantly differ for neither observed ASVs nor Shannon α-diversity indexes (CM *vs.* GH *vs.* ZT and BZ *vs.* SLS *vs.* VS). However, when the sampling sites of Italy and Malta (BZ, SLS, and VS *vs.* CM, GH, and ZT) were compared, observed ASVs and Shannon indexes resulted in significant differences ($p < 0.01$, Figures 5C, D). Additionally, the bacterial community compositions were significantly different when comparing honey bees sampled in Italy to those sampled in Malta, as evidenced by the Bray–Curtis dissimilarity index (Figures 5E–G) and the Unweighted Unifrac β-diversity metrics (Supplementary Figure 12). LEfSe analysis (Figure 6) confirmed the significant fold change of some ASVs between Malta and Italy: *Bombilactobacillus* and *Lactobacillus* are more abundant in honeybees sampled in Italy (Lineage C) whereas *Commensalibacter*, *Acinetobacter,* and *Arsenophonus* resulted with an increased abundance in Maltese honey bees (Lineage A).

Comparison of the predicted metabolic pathways of the honey bee microbiomes in Malta and Italy showed a clear separation between the two mitochondrial haplotypes (lineage A and C) (Supplementary Figure 13). In more details, Italian bees showed increased predicted abundance of genes involved in terpene biosynthesis, formaldehyde oxidation as well as lactose and galactose degradation. Maltese bees had increased predicted abundance of genes involved in tryptophan metabolism and B12 vitamin production (Supplementary Figure 14).

Lactobacillaceae counts, grouping, and identification

Lactobacillaceae from the three sampling locations in Malta were detected in high numbers and plate count enumeration

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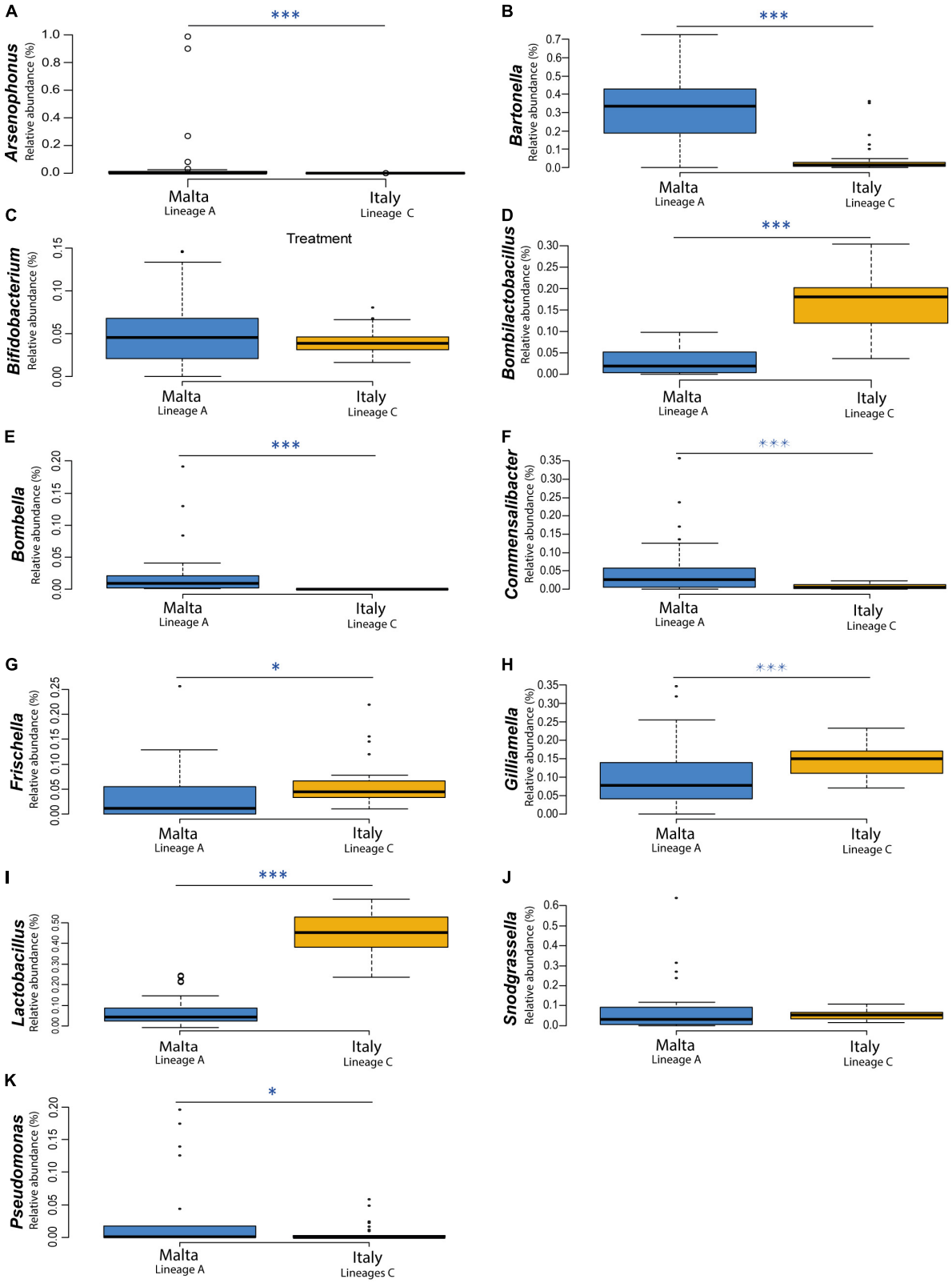

FIGURE 4
The boxplot chart shows the relative abundance of the gut bacterial populations determined by NGS of the 11 major microbial taxa populating the sampled honey bee guts: **(A)** *Acinetobacter,* **(B)** *Arsenophonus,* **(C)** *Bartonella,* **(D)** *Bifidobacterium,* **(E)** *Bombella*, **(F)** *Commensalibacter*, **(G)** *Frischella*, **(H)** *Gilliamella*, **(I)** *Lactobacillus*, **(J)** *Snodgrassella,* and **(K)** *Pseudomonas* compared for mitochondrial haplotypes. Sampled honey bees mitochondrial haplotype were "A" for the Maltese honey bees and "C" for the Italian honey bees. The box plots compares the average relative abundance values at genus level of 30 sampled honey bees in Malta (Campus Msida, Gharghur, Żeitun) with 30 sampled honey bees in Italy (Bozen, San Lazzaro di Savena, Valsamoggia). Asterisks indicate statistically significant differences (*$p < 0.05$; ***$p < 0.01$).

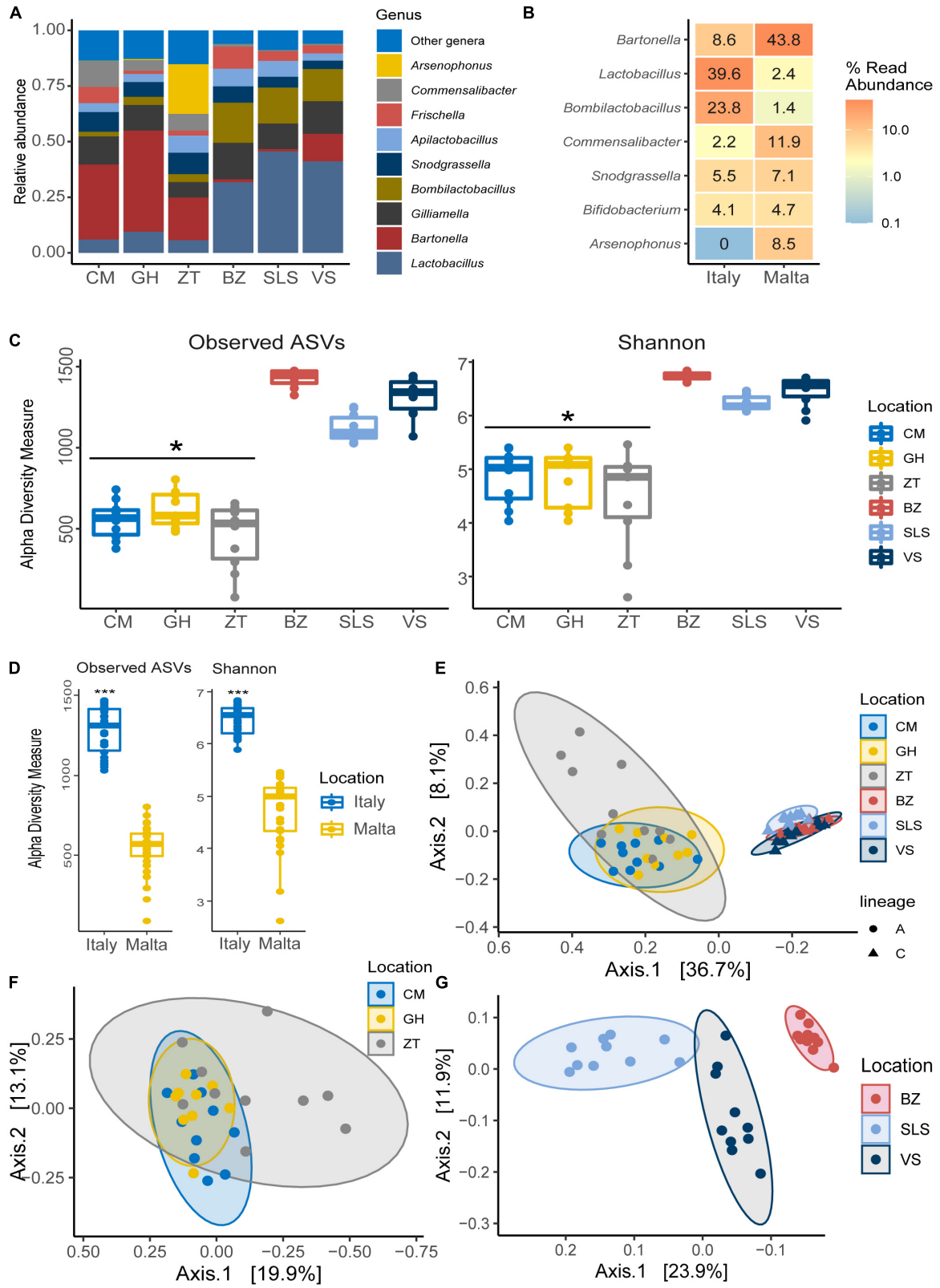

FIGURE 5
(A) Bar chart showing the relative abundance of the major microbial genera in both Malta and Italy determined by NGS. **(B)** Differential abundance heatmap highlighting significantly differentially abundant ($p < 0.05$) microbial genera between honey bees sampled in Italy and in Malta, showing the relative abundance of the genus. **(C)** Boxplot of α-diversity indexes for Observed ASVs and Shannon indexes per sampling site in Italy and Malta. **(D)** Boxplot of α-diversity indexes for Observed ASVs and Shannon indexes per nation (Italy and Malta). **(E)** β-diversity Bray–Curtis dissimilarity index per mitochondrial haplotype (lineage) and sampling site. **(F)** β-Diversity Bray–Curtis dissimilarity index per sampling site in Malta. **(G)** β-diversity Bray–Curtis dissimilarity index per sampling site in Italy. *$p < 0.05$; ***$p < 0.01$.

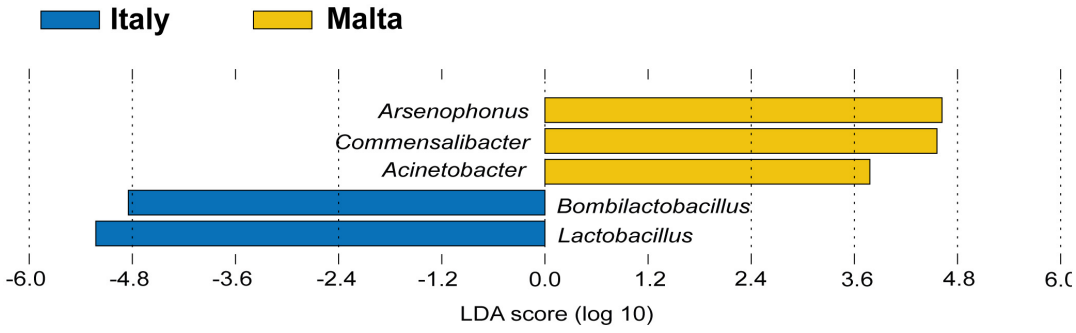

FIGURE 6
LEfSe analysis highlighting possible microbial biomarkers of the Maltese (lineage A) and Italian (lineage C) honeybees.

showed the following: 8.67 ± 0.03 Log cfu/g (GH), 6.67 ± 0.03 Log cfu/g (ZT) and 7.28 ± 0.02 Log cfu/g (CM) of gut content. The cluster analysis of random amplification of polymorphic DNA (RAPD) profiles of 184 isolated colonies showed a large heterogeneity, although most of the isolates belonging to the same sampling site, to some extent, clustered together. In some cases, the cluster similarity was over 90% (**Supplementary Figure 15**); overall, 36 lactobacilli belonging to the corresponding different clusters were processed for sequencing and the taxonomic identification is shown in **Supplementary Table 4**. Based on the percentage identity of the 16S rRNA gene of the isolates with the sequences in the NCBI database, the majority of Lactobacillaceae strains isolated from the modified MRS agar showed the greatest similarity to *A. kunkeei* (the nucleotide identity was over 99%). The obtained phylogenetic tree (**Supplementary Figure 16**) showed three main clusters of *A. kunkeei*. However, the *A. kunkeei* isolates from Malta and other countries (especially Sweden and Germany) did not group into specific clusters but mixed into the three distinct clusters.

PCR-DGGE results

The DGGE profiles obtained from each sample had several major PCR bands and a characteristic pattern of bands was detected in each locality. The cluster analysis (**Supplementary Figure 17**) highlighted three major clusters (cut off at 56%). GH samples clustered together, and the similarity was over 85% for most profiles. The biggest cluster, divided in different sub-clusters, comprises all the profiles from ZT and half from CM (similarity was less than 80%). Similarity above 90% was associated with only a few profiles belonging to the same sampling site. Finally, the third cluster was related to nine profiles from CM with six of them having a very similar visual profile. The Shannon–Wiener diversity index and the Simpson index did not differ among samples and the evenness was significantly lower in GH samples when compared to CM and Z. DNA sequences of 45 bands corresponded to different Lactobacillaceae (**Supplementary Figure 15**).

Results of the climate analysis

The results of the climatic analysis are shown in **Figure 7**. Climate data show that the island of Malta is affected by severe and long-lasting periods of drought, quantifiable to 6 months in 2015 and 9 months in 2016 (year of sampling of the Maltese honey bees). The drought period was shorter in the Emilia Romagna region of Italy, with 2 months of drought in 2015, 1 month in 2016, and 3 months in 2017 (2016 and 2017 are the years of sampling). Finally, in the Italian province of South Tyrol, no drought was detected in either 2016 or 2017.

Discussion

In honey bees, the core gut bacterial microbiota is relatively stable, comprising five to eight bacterial taxa specialized in terms of metabolic capabilities (Maes et al., 2016; Motta et al., 2020). Variations within core bacterial taxa proportion are usually driven by environmental or rearing conditions such as seasonality (Kešnerová et al., 2020; Castelli et al., 2022), diet and feed additives (Maes et al., 2016; Alberoni et al., 2021b), xenobiotics (Motta et al., 2020) or pathogens (Alberoni et al., 2022; Jabal-Uriel et al., 2022). The proportions of the core microbial genera, or their presence/absence, directly influence the functionality of the gut microbiome, affecting honey bees' behavior through impairment of the gut-brain axis (Zhang et al., 2022a,b) and efficiency in nutrient digestion (Alberoni et al., 2022). In addition, Powell et al. (2016) showed how lineages of gut bacteria often include many closely related strains, not distinguishable at species level but highly specialized and restricted to a single host species or subspecies. Recently, Su et al. (2022) studied the impact of both host genetics and diet on the gut microbial populations of different *Apis ceranae* subspecies. The results showed extensive overlapping of the gut microbial strains among different subspecies and suggested an effect of the floral diet in maintaining specialized bacterial traits.

The relationship between microbial population and the environment is therefore a new frontier in the understanding of the honey bees' microbiome's structure and functionality. In this study we tried to contribute to this understanding by focusing on a unique Mediterranean habitat, the Maltese Islands, characterized by (i) a semi-desert climate with intense drought periods, (ii) the presence of Mediterranean plants producing nectars with high amount of essential oils (*e.g., Thymus*), (iii) the proximity to the sea of the entire territory with the impact of salinity and high humidity, and (iv) the isolation of the honey bee ecosystem characterized by an African lineage population resistant to *Varroa destructor*.

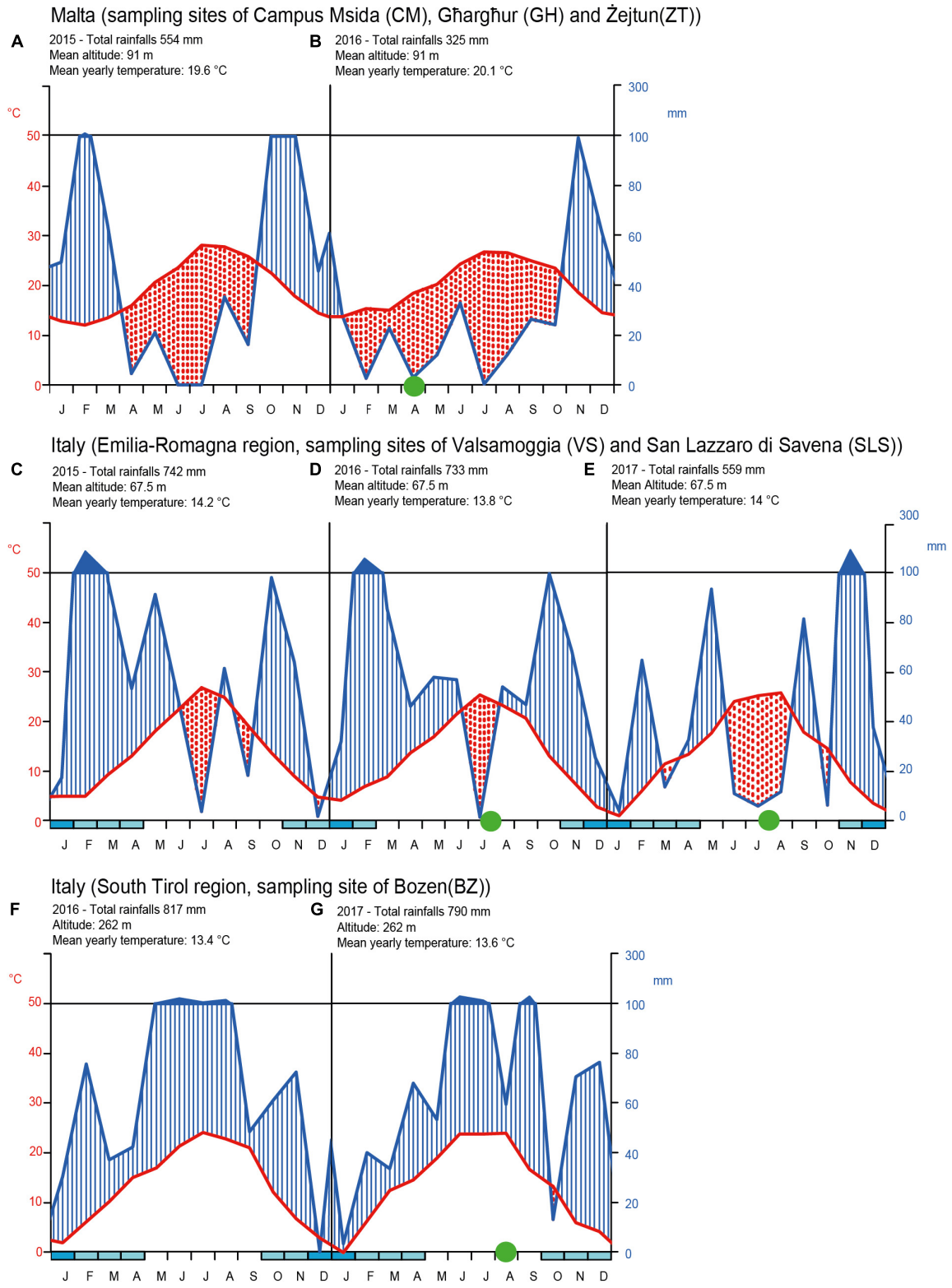

FIGURE 7
Walter and Lieth climate diagrams for drought periods in Malta (sampling sites CM, GH, ZT) in the years 2015 **(A)** and 2016 **(B)**. In Italy for the Emilia-Romagna region sampling zones of VS and SLS in the years 2015 **(C)**, 2016 **(D)**, and 2017 **(E)**. In Italy for the South Tirol region sampling zone of BZ the years 2016 **(F)** and 2017 **(G)**. The diagrams shows on the x-axis the months of the year and on the ordinate the rainfall amount (on the right) and temperatures (on the left). The temperature values are shown on a scale double that of precipitation (1°C = 2 mm). When the precipitation curve (blue line) drops below that of the temperature (red line) the period concerned is considered as drought. Finally, if monthly rainfall values exceed 100 mm, the rainy period is represented ten times smaller than that previously adopted scale for rainfall lower than 100 mm. Blue marks in the x-axis represents period of intense frost. Green circles represent the honey bee sampling period for this work and for retrieved data from Alberoni et al. (2021a,b) and Baffoni et al. (2021).

These contexts are probably the reasons why this study has identified marked differences in the core gut microbial community of Maltese honey bees (lineage A) when compared to the Italian honey bees (lineage C). Even though all the eight core microbial taxa were present in both the Maltese and Italian honey bees, the proportions were different. The Maltese honey bees showed an inverse proportion of Lactobacillaceae and Bartonellaceae when compared to Italian samples. In European honey bees (both C and M lineages), *Lactobacillus* and *Bombilactobacillus* altogether are much more represented, whereas *Bartonella*, although still considered a core member, is only present as a minor group. In the Maltese honey bees (lineage A), these proportions are inverted to such an extent that *Lactobacillus* and *Bombilactobacillus* are highlighted as biomarkers of lineage C honeybees in the LEfSe analysis. The same concept can be applied to *Bartonella* and Acetobacteraceae (*Commensalibacter* and *Acinetobacter*) for the Maltese honey bees. However, to consider some taxa as biomarkers, a further validation with additional analyses is envisaged. Recent publication focusing on the gut microbiota of another lineage A honey bee, *Apis mellifera scutellata* (Kenya), did not highlight a similarly predominant population of *Bartonella* (Tola et al., 2020). As such, we suggest that the preponderance of *Bartonella* in *Apis mellifera ruttneri* is related to the Maltese environmental conditions rather than the lineage itself, even if additional factors such as host genetics, seasonality, or geography, in synergy with each other or interacting with environmental factors, may still be plausible. Regarding the environmental conditions that may play a major role in the microbiome acquisition, the influence of nectar and pollen composition and climatic conditions are hypothesized as driving factors in the shaping of the core microbiota. It is known that environmental conditions characterized by high solar irradiance, high temperature and humidity can strongly increase the polyphenolic content of plant tissues (Spayd et al., 2002) and, consequently, also the polyphenolic content in honey (Tenore et al., 2012). *Bartonella apis* was found to harbor genes for the degradation of secondary plant metabolites, such as 4-hydroxybenzoate and quinate (Segers et al., 2017), but also hydrocarbons in crude oil (Bacosa et al., 2015) and organophosphorus insecticides like fenitrothion (Tago et al., 2006). It can therefore be postulated that *Bartonella* can degrade a large array of aromatic compounds and terpenes, leading to a positive selection in the Maltese honey bees as adaptation to nectars with higher content of phenolic compounds (Mannina et al., 2015). *Bartonella*, therefore, provides crucial functions for its host and might be considered a typical trait of the Maltese honeybees. Further studies are envisaged to isolate and characterize *Bartonella* strains from this source. Another factor that might have led to an increased abundance of *Bartonella* is the scarcity of available nectar. During the sampling season, the Maltese Island was in a condition of severe drought with scarcity of nectar. Kešnerová et al. (2020) highlighted that *Bartonella* population increases in wintering bees in Switzerland, that is during a period of absence of nectar. Although a detailed metabolic analysis of the single detected taxa has not been performed in this work, a separation of the predicted metabolic functionality of the Italian and Maltese honeybee gut bacteria has been observed and appears to be related to the unique Maltese habitat.

While *Bartonella*, *Bombella* and *Commensalibacter* in Maltese honey bees were observed with high abundance, *Bombilactobacillus*, *Frischella*, *Gilliamella,* and *Lactobacillus* were low in abundance. Our results report not only a low abundance of total Lactobacillaceae, but also a significant change within the Lactobacillaceae genera. *Bombilactobacillus* population in Maltese honey bees was very low when compared to the Italian honey bees. Previous works have correlated this reduction to antibiotic treatments or xenobiotic stressors (Raymann et al., 2017; Motta et al., 2018, 2020; Alberoni et al., 2021a,b; Baffoni et al., 2021). Also, *Lactobacillus* abundance was significantly lower in Maltese honey bees in comparison to the Italian honey bees analyzed, whereas *Apilactobacillus*, whose members are typical colonizers of the honey bee's honey stomach (not analyzed in this work), was found abundant in the Maltese honey bee midgut and rectum. To the best of our knowledge, the high abundance of *Apilactobacillus* is atypical in any analyzed western honey bees. NGS results were also confirmed by plate isolation in MRS medium of Lactobacillaceae, where most isolated strains belonged to *A. kunkeei*. Moreover, DGGE analysis showed a noteworthy strain variability within *A. kunkeei* despite the low abundance in the gut microbiome. Strain variability within the same microbial taxon in samples of different geographical locations was also highlighted by Moran et al. (2012), Anderson et al. (2013), and Engel et al. (2014).

Commensalibacter, *Bombella* and *Pseudomonas* were found in higher abundance in the Maltese honey bees when compared to the Italian honey bees. *Bombella* and *Pseudomonas* are usually occasional colonizers of the honey bee gut in European honey bees. *Commensalibacter* is a controversial non-obligatory core member of the honey bee microbiota or even classified as core hive microorganisms rather than core gut microorganism of adult bees (Corby-Harris et al., 2014). The definition of core microbiome considers different variables such as frequency and abundance (Ainsworth et al., 2015; Risely, 2020). In the case of honey bees, *Bifidobacterium,* the prevalent genus within Actinobacteria in *A. mellifera ligustica* gut using a culture-independent analysis (Cui et al., 2022a), is classified as a core microbial taxon despite its low relative abundance (usually around 2% reaching 5% in some cases) because of its prevalence. Therefore, the separation between core and non-core taxa remains challenging in insects. Our results suggest that *Pseudomonas* still shows a low prevalence within the gut microbiome of the Maltese honey bee and cannot be considered as a core taxon even if its relative abundance in some samples is high. On the contrary, *Bombella* and *Commensalibacter* showed a relative abundance similar to *Bifidobacterium* in most of the samples, therefore they might be considered as core members of the Maltese honey bee. These results also find a confirmation in *Apis mellifera scutellata* in which *Bombella* and *Commensalibacter* are also described as core microbiome taxa (Tola et al., 2020). Higher occurrence of *A. kunkeei* and *Bombella* has been correlated with diet change (presence, absence, or degraded pollen) and stress (Anderson and Ricigliano, 2017) and recently it has been shown to be negatively correlated with yeasts abundance in the honey bee ileum and rectum (Anderson et al., 2022). This highlights the possible influence the Maltese climate and environment has on the local honey bees' gut microbial population. *Bifidobacterium* did not significantly vary among the different honey bees subspecies and its relative abundance was in overall agreement with Cui et al. (2022a).

Arsenophonus is a horizontally transmitted symbiont in honey bees (Drew et al., 2021) that, in this study, was detected only in four Maltese honey bee samples although with relevant abundance.

Arsenophonus can be an insect reproduction manipulating parasite (Elston et al., 2022) that can potentially colonize off-target microbial niches; therefore, it should be intended as a non-core gut bacterial community member. Little is known about this genus, however, recently, a novel species, *Arsenophonus apicola*, was isolated and characterized in honey bees (Nadal-Jimenez et al., 2022). Its abundance is linked with seasonality, increasing in honey bees during winter while almost disappearing in the spring (Drew et al., 2021). *Arsenophonus* also correlates with areas of anthropogenic pressure and intensive agriculture (Gorrochategui-Ortega et al., 2022), which are reflective of the Maltese Islands. In many insects, *Arsenophonus* is a harmful intracellular parasite, for instance negatively influencing reproduction in *Nasonia* wasp (Darby et al., 2010). There is little evidence supporting the pathogenicity of *Arsenophonus* in honey bees, although analyses of the gut microbiome of honey bees with colony collapse disorder symptomatology showed an increase of this taxon (Cornman et al., 2012). Also, Budge et al. (2016) associated *Arsenophonus* with poor honey bee health due to high viral load, however, this does not prove its pathogenicity. Yet its presence was found in *V. destructor*, a possible vector of infection for honey bees (Hubert et al., 2015).

The Walter and Lieth climatic analysis confirmed a persistent and very dry climatic conditions on the Maltese island, which, also based on historical data, has determined the selection of a spontaneous Mediterranean flora. Although the Emilia-Romagna region undergoes periods of drought, these are shorter and consequently, the spontaneous flora differs in the two areas (Galuzzo et al., 2021). In the Emilia-Romagna region, the spontaneous vegetation is continental (large latifolia plants) and in the two sampling areas, not of the Mediterranean type. Crops and fruit trees are also very different in the two areas. The Emilia-Romagna region spontaneous flora resembles more the alpine vegetation rather than the Mediterranean one and this may explain the results on the bacterial community analysis of honeybees sampled in Italy, which all cluster close, highlighting a well-defined and stable core microbiota despite differences in climatic and environmental conditions of the two sampling areas (Emilia-Romagna and the South Tyrol regions). Therefore, sampling sites that are hundreds of kilometers in distance and with different prevalent honey bees subspecies (South Tyrol = *A. mellifera carnica*; Emilia-Romagna = *A. mellifera ligustica*), show remarkable stability of the core microbial groups between sites and over time. On the other hand, the intestinal microbial communities of the Maltese bees (lineage A) showed a dispersed spatial distribution. The microbiota seemed less consistent in the abundance of core microbial taxa although differences among sites were not significant and it harbored a relevant number of low-abundant microbial genera (below 1%), similar to honey bees treated with antibiotics (Baffoni et al., 2021) and suffering gut dysbiosis.

Finally, the yeast community found in the Maltese honey bees showed an important presence of Metschnikowiaceae members, mainly represented by the genera *Metschnikowia* and *Kodamaea*. Little is known about the effect of yeasts on honey bee health, but recent studies have shown that yeasts, when supplied as additives to the honey bee diet, may have an immunomodulatory function controlling the transcription of immune-related genes and they can also alter the bacterial composition of the gut with unpredictable effects (Tauber et al., 2019). Although studies in the literature are not conclusive on this point, it has been highlighted that yeasts are likely associated with both negative and positive aspects of every stage of the honey bee's life that needs to be further explored (Ptaszyńska et al., 2016; Tauber et al., 2019). Anderson et al., 2022 suggested that fungi or fungal associated factors contribute to core-hindgut microbiota assembly especially in the ileum, however, the abundance and prevalence of *Bombella* and *A. kunkeei* found in this work suggest a sparse yeast population at the sampling time of Maltese honey bees. The antagonisms of yeasts and Lactobacillace is already well known in nectar (Álvarez-Pérez et al., 2019) and may also occur in the gut microbiome. *Metschnikowia* genus is reported as a nectar-specialist yeast that, living in the flower nectar, plays an important role in honey bees' attraction and thus in flower and crop pollination (Good et al., 2014; Colda et al., 2021). When consumed by pollinators, the nectar microorganisms, in particular yeasts, may serve as an additional source of nutrition (*e.g.,* vitamins and steroids), that may have positive effects on the flower visiting insects (Martin et al., 2022), although this mechanism has been poorly studied. *Metschnikowia* species, although different from those identified in this work, have been isolated from the honey bee gut (Good et al., 2014). However, no isolation of the species detected in our work has been documented so far. A recent work by Cui et al. (2022b) explored the phylogenetic diversity and community composition of *A. mellifera ligustica* associated fungi in honey bees and the colony environment, including the gut and bee-derived products using a combination of culture-dependent and culture-independent approaches. The relative abundances of ASVs showed data similar to ours at the phylum level, with a highest abundance of Ascomycota followed by a lower proportion of Basidiomycota. Data at genus level (Cui et al., 2022b) showed a relative abundance of *Kodamaea* higher than 80%, different from our results that recorded this genus at 8%, on the other hand, *Metschnikowia* was not detected at all (threshold 0.1%). The *Metschnikowia* genus was, on the contrary, detected at 18% of relative abundance in honeycomb in the same study. Our study has considered a different bee subspecies and it is difficult to extrapolate conclusions considering the small amount of data present in the literature on the yeast gut population. Our study highlights the need to further explore the impact of yeasts in honey bee physiology and gut microbial population.

In conclusion, the Maltese honey bee was found to host a peculiar core microbiome, where *Apilactobacillus*, *Bartonella*, *Commensalibacter,* and *Bombella* were among the major taxa at the expense of *Frischella*, *Gilliamella,* and *Lactobacillus*. With currently available data on gut microbes in Maltese honey bees, obtained over a single sampling time point, it cannot be clearly assumed that the peculiar gut microbial composition of the Maltese honey bee is ascribed to the different evolutionary phylogenesis of this subspecies. Multiple samplings along the season are needed to separate the contributions of honey bee genetics and environmental influence. The environment seems the major driving factor shaping the local flora, food availability and therefore the honeybee microbial population although other co-occurring factors cannot be excluded. In particular, the combination of environment and genetic evolution already shown in plants (Oyserman et al., 2021) is the most likely also in honeybees, although further studies are necessary to understand this combined effect. This work opens to

future research that focuses on the ability of different honey bee subspecies to select and co-evolve with specific microbial taxa and strains, adapting to the local environment. This work also evidences the importance of research on honey bees' microbiome adaptation to climate conditions (especially drought), in a world facing strong climate changes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

DA, FG, and DM collected honey bees samples. DA performed DNA extraction. DA, FG, and LB prepared NGS sequencing on bacteria. RJ and FG prepared NGS sequencing on yeasts. FG carried out DGGE analysis and the isolation of microbial strains. LB and RJ performed bioinformatics analysis. RJ, DA, and LB were involved in statistics and figures plotting. FG, DA, RJ, SC, and DD were involved in the manuscript writing. DM, DD, and DN were involved in the research funding. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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Gut microbiota is a potential factor in shaping phenotypic variation in larvae and adults of female bumble bees

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Host symbionts are often considered an essential part of the host phenotype, influencing host growth and development. Bumble bee is an ideal model for investigating the relationship between microbiota and phenotypes. Variations in life history across bumble bees may influence the community composition of gut microbiota, which in turn influences phenotypes. In this study, we explored gut microbiota from four development stages (early-instar larvae, 1st instar; mid-instar larvae, 5th instar; late-instar larvae, 9th instar; and adults) of workers and queens in the bumble bee *Bombus terrestris* using the full-length 16S rRNA sequencing technology. The results showed that morphological indices (weight and head capsule) were significantly different between workers and queens from 5th instar larvae (p <0.01). The alpha and beta diversities of gut microbiota were similar between workers and queens in two groups: early instar and mid instar larvae. However, the alpha diversity was significantly different in late instar larvae or adults. The relative abundance of three main phyla of bacteria (Cyanobacteria, Proteobacteria, and Firmicutes) and two genera (*Snodgrassella* and *Lactobacillus*) were significantly different (p <0.01) between workers and queens in late instar larvae or adults. Also, we found that age significantly affected the microbial alpha diversity as the Shannon and ASVs indices differed significantly among the four development stages. Our study suggests that the 5th instar larval stage can be used to judge the morphology of workers or queens in bumble bees. The key microbes differing in phenotypes may be involved in regulating phenotypic variations.

KEYWORDS

***Bombus terrestris*, worker, queen, development, sociality**

Introduction

Microbial symbionts are often considered an important part of the host phenotype, participating in host health maintenance, nutrition uptake, energy release, and regulation of host physiology (Tremaroli and Bäckhed, 2012; Archie and Tung, 2015). Similarly, gut bacterial communities reflect changes in host phenotype and are influenced by the host's diet and physiology (Chandler et al., 2011; Koch and Schmid-Hempel, 2011). Whether phenotypic diversity among individuals results from host–microbe interactions deserves further exploration.

Compared with the gut microbiota of many other animals, the social bumble bees and honey bees harbor a relatively simple yet specialized gut microbiota, including *Snodgrassella alvi*, *Lactobacillus,* and *Gilliamella apicola* (Neveling et al., 2012; Kwong and Moran, 2013; Zheng et al., 2016). It is reported that these microbes have many beneficial interactions with bumble bees and honey bees, including increasing metabolic function and protection from invading pathogens (Kwong et al., 2017).

The eusocial Hymenoptera provides an excellent opportunity to explore the relationship between microbiota and host phenotypic variation. In many social species of ants, bees, and wasps, individuals in the same colony show differences in the division of labor, accompanied by changes in nutritional status and physiology (Wilson, 1971). For example, in bumble bees, queens lay fertilized eggs (one set of chromosomes from the drone, one from the queen) that mature into workers and new queens; Alaux et al., 2007). Morphology, physiology, behavior, longevity, and other life-history traits significantly differ between queens and workers, although they are derived from the same genome (Weiner and Toth, 2012). Compared to workers, the queen is larger with more developed reproductive organs and a longer life span (Bloch and Hefetz, 1999). Also, since they share a nested environment and transfer food using trophallaxis during development, the larvae of workers and queens have a similar diet (Pereboom et al., 2003).

Shared social communities and environments generally contribute to common gut microbiota (Degnan et al., 2012; Zhang and Zheng, 2022). In support of this view, the gut microbiota sampled from social species of bees, such as honey bees and bumble bees, tend to be host-specific (Koch et al., 2013). Similar gut microbiota has also remarkably been observed for workers from different colonies of honey bees (Martinson et al., 2011). The physical interactions in social species create a potential for colony-wide transmission of gut microbiota, suggesting little microbiome variation among individual members in a colony, such as between soldiers and workers in termites (Otani et al., 2019).

However, some gut microbiotas may be involved in phenotypic variation by improving digestion and enhancing metabolism (Engel and Moran, 2013). These gut microbiota characteristics in turn correspond to their roles in the division of labor (Engel et al., 2012). Specifically, workers harbor more complex gut communities than queens, presumably more suited to process food than queens. In *Apis mellifera*, queen microbiomes may enhance the metabolic conversion of energy from food to egg production (Aupinel et al., 2005). Within the worker caste, young (nurse) honey bees that perform tasks inside the hive, such as brood care, have more diverse gut microbial communities than foragers (Jones et al., 2018). Furthermore, age has an influence on gut microbial communities during the development of bees (Hroncova et al., 2019). For example, the genera *Bartonella* and *Enterobacter* are mainly founded in the first instar larvae, while *Acinetobacter* and *Rhodococcus* are mainly founded in the fifth instar larvae of the bumble bee (*Andrena camellia*; Kou et al., 2022).

Bombus terrestris, one of the eusocial Hymenoptera species, is an ideal model to investigate the relationship between microbiota and phenotypes (Koubová et al., 2019). Although the diet of queens and workers is similar, the gut microbial composition may differ. In this study, we first measured morphological changes between workers and queens during larval development. Then, we explored changes in gut microbiota in larvae and adults of bumble bee workers and queens. We hypothesized that (i) the richness of some key microbiotas is different between workers and queens, (ii) the diversity of microbiota changes with the development of bumble bees. The findings of this study will improve our understanding of possible relationships between gut microbial communities and the phenotypic variation of bumble bees.

Materials and methods

Sample collection

Bombus terrestris colonies were reared under standard laboratory conditions (27 ±2°C, 60 ±5% relative humidity; Gurel and Karsli, 2013). Fresh pollen and sugar solutions (1:1, w/v) were provided *ad libitum* as a diet (Zhang et al., 2021).

To collect the worker and queen larvae accurately, we monitored the whole development period of the larvae. Monitoring began when the colonies had about 100 workers, which is close to the time for new queens to develop. At this stage, we recorded the location and time of each batch of eggs laid by queens daily, then took one larva on the 1st, 5th, and 9th, whereas the remaining were left to develop into adults. We determined whether the collected larvae were queens or workers based on the adult bees that emerged from the remaining larvae. Moreover, to explore the development of the queens and workers throughout the larval span, we took the larvae of each instar of workers and queens to determine the morphological index. The larval body weight was measured using a digital electronic scale (accurate to within 0.1 mg) (BSA124S, Sartorios, Gottingen, Germany), and the head capsule was observed using a microscope (SZ2ILST, Olympus Corporation, Tokyo, Japan; Cnaani et al., 1997).

Gut microbiota was sampled from four stages, three of which were larval stages and one was the adult bumble bee stage (Figure 1). These samples consisted of: (a) early-instar queen-destined larvae (EQ, 1 instar larvae, 80 larvae from six colonies, representing four biological replicates) and early-instar worker-destined larvae (EW, 1 instar larvae, 80 larvae from six colonies, representing four biological replicates); (b) mid-instar queen-destined larvae (MQ, 5 instar larvae, 32 larvae from six colonies, representing eight biological replicates) and mid-instar worker-destined larvae (MW, 5 instar larvae, 20 larvae from six colonies, representing 5 biological replicates); (c) late-instar queen-destined larvae (LQ, 9 instar larvae, 8 larvae from 6 colonies, representing 8 biological replicates), and late-instar worker-destined larvae (LW, 9 instar larvae, 8 larvae from 6 colonies, representing 8 biological replicates); (d) adult queens (AQ, 7 instar adults, 8 individuals from 6 colonies, representing 8 biological replicates), and adult workers (AW, 7 instar adults, 8 individuals from 6 colonies, representing 8 biological replicates). Harvested samples were kept at −80°C until the total 16S rRNA was extracted.

DNA extraction and sequencing of 16S rRNA

Following the manufacturer's instructions, DNA from fecal contents was extracted by a QIAamp DNA Stool Mini Kit from Qiagen

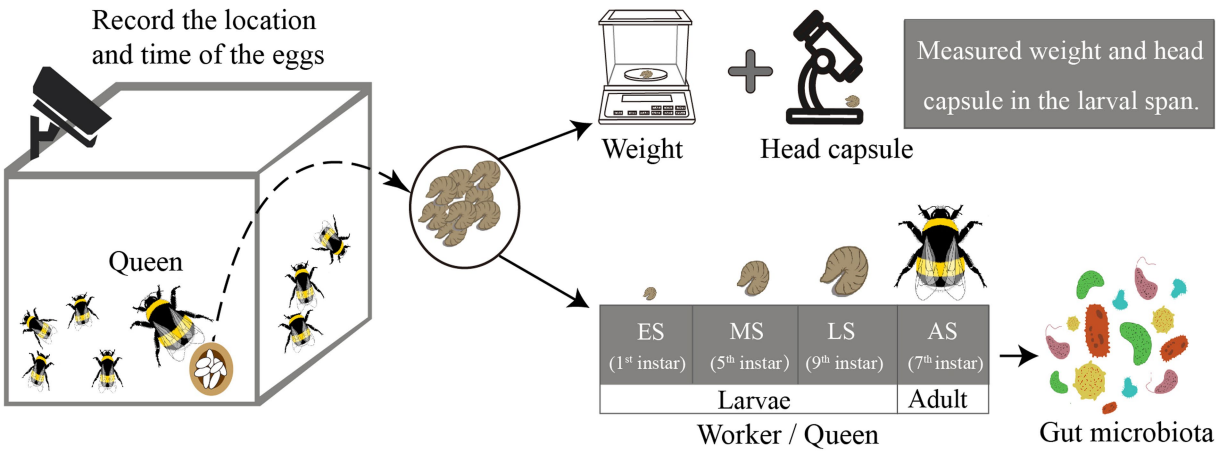

FIGURE 1
Schematic overview of this study. We monitored the development of larval in *Bombus terrestris*. Some larvae were used to measure morphological indicators while others were used to determine microbiota. The gut microbiota was sampled at four stages (ES represents early larvae stage, MS represents mid larvae stage, LS represents late larvae stage, and AS represents adult stage).

(Germany). The primer sequences of the full-length 16S rRNA were as follows: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT3'; He et al., 2020). The PCR reaction conditions were 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with 25 cycles in a reaction volume of 10 μl. PCR products were purified by adding Genome DNA Clean magnetic beads of equal volume. DNA concentration was measured using Qubit® DNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, United States). The samples with DNA concentrations greater than 20 ng/μL were used for constructing the DNA library (Pacific Biosciences SMRT bellTM Template Prep Kit 1.0), sequencing by PacBio SMRT RS II instrument P6 - C4 reagent computer, and the movie times were 240 min by CCS mode. Demultiplexed CCSs were generated using the RS_ReadsOfInsert.2.1 protocol in SMRT Analysis software version 2.3.0 with the following settings: Minimum Full Passes = 2, Minimum Predicted Accuracy = 90, and Minimum Barcode Score = 22 in Symmetric Barcode Mode (Callahan et al., 2019). All subsequent library building and sequencing work were conducted at Berry Genomics Biotech Co., Ltd. (China).

Data processing of gut samples

The data from different samples were identified according to the barcode sequence and converted into fastq datasets. We used the DADA2 pipeline within the QIIME2 (version 2021.8) package[1] to filter low-quality and chimera errors and generate unique sequence variants. Because the "operational taxonomic units (OTUs)" resulting from DADA2 are created through the grouping of unique sequences, these sequences are the equivalent of 100% OTUs, and are generally referred to as amplicon sequence variants (ASVs; Straub et al., 2020). The obtained ASVs were taxonomically annotated in the Greengene reference database (Smith et al., 2020).

1 https://qiime2.org

Statistical analysis

The alpha diversity measures such as the observed ASVs (i.e., the total number of ASVs detected per sample) and Shannon index (i.e., the number of taxa and evenness of their distribution, more influenced by the richness and rare species) were compared (Delbeke et al., 2022). The Kruskal–Wallis test was used to compare the differences among groups as some of the variables were not normally distributed. The effects of age and phenotype in the 2-by 2-factor design on alpha diversity were analyzed by linear mixed model in R (implemented in R package limerTest; Bates and Pinheiro, 1998).

Bray–Curtis dissimilarity was used to visualize beta diversity to examine the difference in microbial composition among the sampled groups. The principal component analysis (PCA) was visualized in R (implemented in R package vegan) (Segata et al., 2011). We used pairwise permutation multivariate analysis of variance (PERMANOVA) with 999 random permutations to test the significance of the differences among groups (Anderson, 2001).

Also, we used PICRUSt2 to predict the functional capacity of the gut microbial community. The ASVs table was supplied to PICRUSt2, and then predicted functional genes were categorized into MetaCyc pathways (Low et al., 2021).

Results

Fifth-instar is the key point for morphological difference between worker and queen bumble bees

The whole development period of worker-destined larvae was different from queen-destined larvae. Queen larvae took more time (11 days) than worker larvae (9 days) for development. In addition, significant body weight differences were found between queen and worker bees at 5th - 9th instar larvae (Figure 2A; *t*-test, $p < 0.01$). Similarly, the head capsules were significantly larger in queens than in workers from 5th - 9th instar larvae (Figure 2B; *t*-test, $p < 0.01$), while

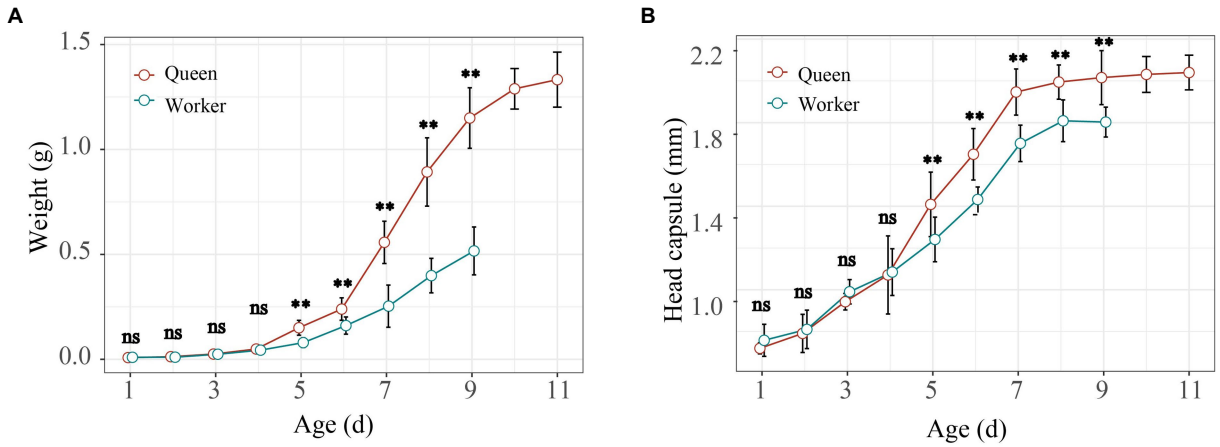

FIGURE 2
Larval growth and development curve of larvae in *Bombus terrestris*. The weight **(A)** and head capsule **(B)** of worker and queen larvae during their entire development (*t*-test; ns represents $p > 0.05$, * represents $p < 0.05$, ** represents $p < 0.01$).

there were no differences from the 1st – 4th instar larvae (Figure 2B; *t*-test, $p > 0.05$). Thus, our results showed that the 5th instar larval stage was the key period at which the weight and head capsule began to show significant variation between workers and queens of bumble bees.

Variation of microbial compositions between worker and queen bumble bees

To identify whether gut microbiota exhibited variations in response to workers and queens, we analyzed 16S rRNA sequences from gut samples. After quality control, a total of 267,307 high-quality sequences were retained for all samples and an average of 5,043 sequences were obtained per sample.

Overall, there was no significant difference in microbial diversity between workers and queens. We found a total of 205 shared ASVs, and 310 and 270 ASVs that were specific to worker and queen samples, respectively (Figure 3A). In addition, the linear mixed-effects model analysis indicated that phenotype could not significantly affect microbial alpha diversity within the bumble bees (Table 1; $F_{ASVs} = 0.79$, $p = 0.3783$; $F_{Shannon} = 1.99$, $p = 0.1646$). Based on PERMANOVA of the Bray-Curtis distance matrix, we revealed that microbial communities were not significantly different between workers and queens ($p > 0.05$). The principal-coordinate analysis (PCoA) graphs clearly illustrated that worker and queen samples were clustered together (Figure 3B).

However, the ASVs index was significantly different between workers and queens, indicating significant differences in the microbial richness at the late stage (Figure 3C; Wilcoxon test, $p < 0.05$). The Shannon index was higher in workers than in queens, revealing a significant difference in the abundance of bacteria at the adult stage (Figure 3C; Wilcoxon test, $p < 0.05$). Moreover, we found that the relative abundance of three main phyla (Cyanobacteria, Proteobacteria, and Firmicutes) significantly differed between queens and workers. The relative abundance of Cyanobacteria was significantly higher in queens compared to workers of the late larvae group (Figure 3D; Wilcoxon test, $p < 0.01$) while the relative abundance of Proteobacteria was significantly high in queens than in workers of the adult group (Figure 3D; Wilcoxon test, $p < 0.05$). Firmicutes were significantly less abundant in queens than in workers of both late larvae and adult groups (Figure 3D; Wilcoxon test, $p < 0.05$).

Moreover, the taxonomic composition and distribution of microflora at the genus level revealed that the relative abundance of *Snodgrassella* was significantly higher in queens than in workers of the adult group (Figure 3D; Wilcoxon test, $p < 0.05$). In contrast, the relative abundance of *Lactobacillus* genus was significantly lower in queens than in workers of both late larvae and adult groups (Figure 3D; Wilcoxon test, $p < 0.05$).

Microbial diversity varied as the age increased in worker and queen bumble bees

Linear mixed-effects model analysis indicated that age was an important factor for changes in microbial diversity within bumble bees (Table 1; $F_{ASVs} = 2.63$, $p = 0.06$; $F_{Shannon} = 4.54$, $p = 0.0067$). In worker bumble bees, ASVs and Shannon indices were not significantly different (Figure 4A; Kruskal–Wallis test, $p = 0.22$, $p = 0.072$), but the values were higher in the three late groups (MW, LW, and AW) than in the EW group. Additionally, the PERMANOVA analysis showed that microbial communities (beta-diversity) were significantly different between groups (Figure 3B; $p < 0.05$). The PCoA plot showed that larval samples (EW, MW, and LW) were clustered together, different from adult bumble bee samples (AW; Figure 4B). Furthermore, the relative abundance of the top five phyla (Proteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, and Actinobacteriota) differed in the four groups (Figure 4C). The relative abundance of Firmicutes was significantly higher in both LW and AW than in EW and MW groups, whereas the relative abundance of Cyanobacteria was significantly lower in LW and AW groups (Figure 4D; Wilcoxon test, $p < 0.05$). The relative abundance of Bacteroidetes was significantly high in the AW than in other groups (Figure 4D; Wilcoxon test, $p < 0.05$). Thus, the diversity of microbiota was affected by the age of workers.

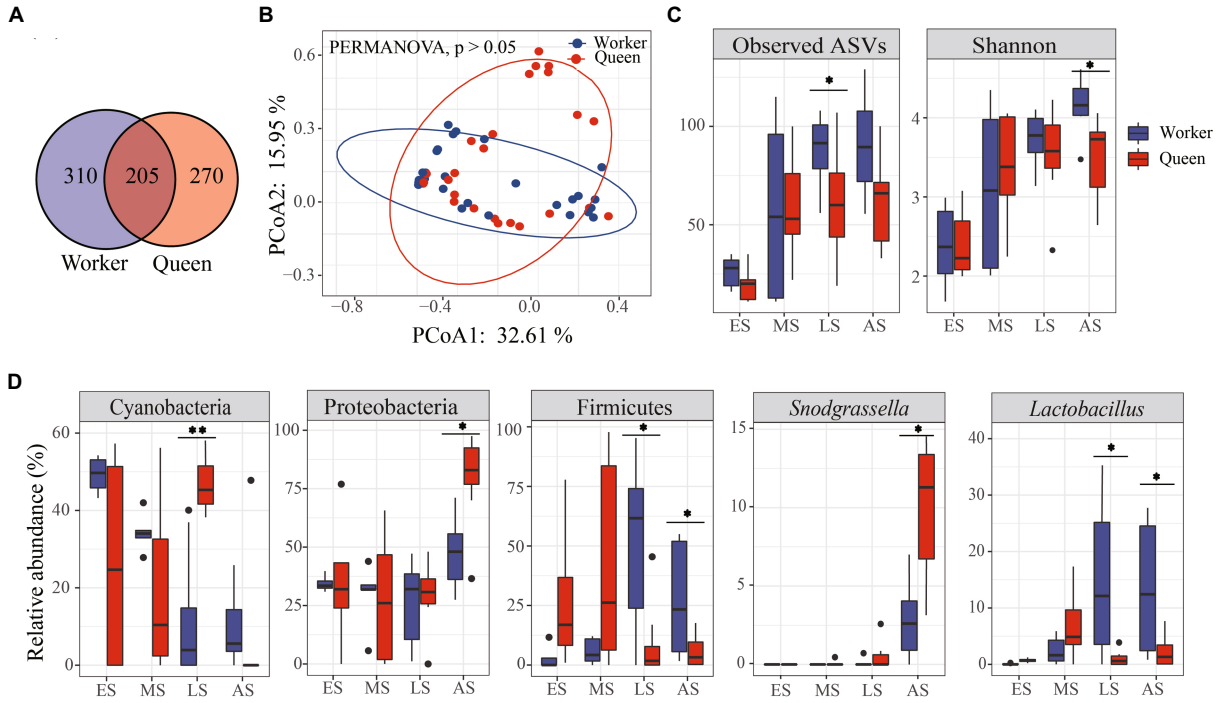

FIGURE 3
The composition and diversity of microbiota in *Bombus terrestris*. **(A)** Venn diagram of common amplicon sequence variants (ASVs). Circle A represents worker samples; circle B represents queen samples. **(B)** PCoA plot based on Bray-Curtis distance metrics depicting the differences in microbial community structure between worker and queen bumble bees. **(C)** Alpha diversity (ASVs and Shannon indices) of microbiota between workers and queens (Wilcoxon test, * represents $p < 0.05$). **(D)** The relative abundance of phyla and genera significantly differed between workers and queens (Wilcoxon test, * represents $p < 0.05$).

TABLE 1 Linear mixed-effects model by restricted maximum likelihood (REML) for alpha diversity of ASV and Shannon indices in *Bombus terrestris*.

	d.f.	*F*	Value of *p*
Main effects – ASV			
AIC = 558.685			
Age	50	2.63	0.06
Phenotype	50	0.79	0.3783
Main effects – Shannon			
AIC = 134.9644			
Age	50	4.54	0.0067
Phenotype	50	1.99	0.1646

d.f., degrees of freedom; AIC, Akaike information criterion.

Likewise, in queen bumble bees, we found both ASVs and Shannon indices significantly differed between the four groups (Figure 5A; Kruskal–Wallis test, $p = 0.024$, $p = 0.023$). Both ASVs and Shannon indices were significantly higher in MQ, LQ, and AQ than EQ group (Figure 5A; Wilcoxon test, $p < 0.05$). The PERMANOVA analysis revealed that microbial communities (beta-diversity) were significantly different between the four groups ($p < 0.05$). The PCoA plot showed that larval samples (EQ, MQ, and LQ) were clustered together, which were also different from adult bumble bee samples (AQ; Figure 5B). In addition, the relative abundance of the top five phyla (Proteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, and Actinobacteriota) differed in the four groups (Figure 5C). Proteobacteria were significantly more abundant in AQ than in other groups (Figure 5D; Wilcoxon test, $p < 0.05$), while Cyanobacteria were significantly less abundant in AQ than in MQ and LQ groups (Figure 5D; Wilcoxon test, $p < 0.05$). Therefore, our results showed that the richness and abundance of microbiota were also influenced by age in queens.

Furthermore, bacterial functions were predicted and 'mapped' in the MetaCyc database. A total of 220 metabolic pathways encoded were predicted, 67 and 26 of which were significantly different in queens and workers, respectively (Supplementary Table S1; Kruskal–Wallis test, $p < 0.01$). The worker bacteria-encoded functions that were active throughout the life cycle mainly included amino acid metabolisms and biosynthesis (arginine ornithine and proline interconversion, L-histidine degradation I and L-lysine biosynthesis III ; Figure 4E; Kruskal–Wallis test, $p < 0.01$). Also, the queen bacteria-encoded functions also mainly included amino acid biosynthesis (arginine ornithine and proline interconversion, L-methionine biosynthesis I and L-lysine biosynthesis III) and generation of precursor metabolites and energy (hexitol fermentation to lactate, pyruvate fermentation to acetate and lactate II and aerobic respiration I; Figure 5E; Kruskal–Wallis test, $p < 0.01$). Overall, our results revealed that more pathways were enriched at the adult stage than at the larval stages of both worker and queen bumble bees.

Discussion

The gut microbiota of eusocial Hymenoptera is highly conserved and represented by a few phylotypes (Cornet et al., 2022). Its

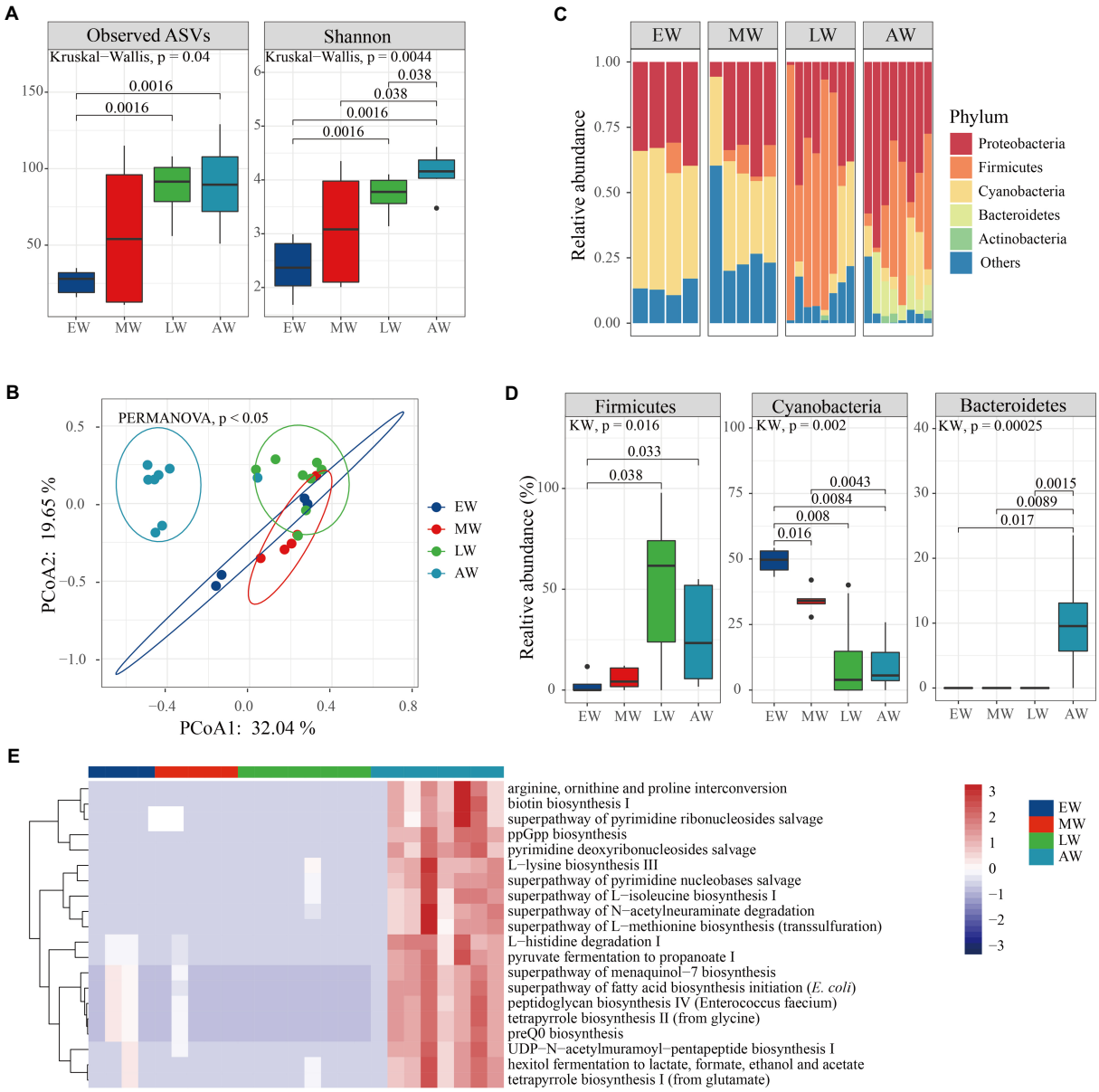

FIGURE 4
The variation of microbial diversity between the four stages of workers in *Bombus terrestris*. **(A)** Alpha diversity (ASVs and Shannon indices) of microbiota in worker bumble bees using Kruskal–Wallis test. **(B)** PCoA plot based on Bray–Curtis distance metrics depicting the differences in microbial community structure (ES represents early larvae stage, MS represents mid larvae stage, LS represents late larvae stage. AS represents adult stage). **(C)** Phylum-level microbial composition of bumble bees. **(D)** The relative abundance of phyla significantly differed between the four groups (Kruskal–Wallis test, p <0.05). **(E)** The relative abundance of the top 20 metabolic pathways using PICRUSt-predicted Metacyc orthologs.

composition tends to be dominated by phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes in *Amdrena camellia* (Kou et al., 2022). Likewise, Proteobacteria and Firmicutes are the most abundant taxa in Pheidole rugaticeps Emery (Hymenoptera: Formicidae) (Ashigar and Ab Majid, 2021). The study found that Proteobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, and Actinobacteria were the main phyla in queen and worker bumble bees. The alpha and beta diversity of these microbial communities was not significantly different between workers and queens (Table 1; Figure 3C). One potential explanation is that *B. terrestris* shares nectar *via* honeypots inside the colony, thus the diversity of microbiota shows similarity (McFrederick et al., 2013).

Gut bacteria increase weight gain in young adult bees, affect the expression of genes governing insulin and vitellogenin levels, and increase sucrose sensitivity (Zheng et al., 2017). Additionally, gut bacteria produce short-chain fatty acids, with acetate and propionate as the major metabolites, as in the guts of humans and other animals (Lee et al., 2015). In our study, significant body weight differences were observed between queen and worker bees at 5th – 9th instar larvae (Figure 2A). The 5th and 9th instar larvae stages showed differences in alpha diversity between worker and queen bees. Also, these instar larvae stages exhibited differences in the relative abundance of bacterial groups, such as Cyanobacteria, Proteobacteria, and Firmicutes (Figures 3C,D), which play an important role in the digestion and

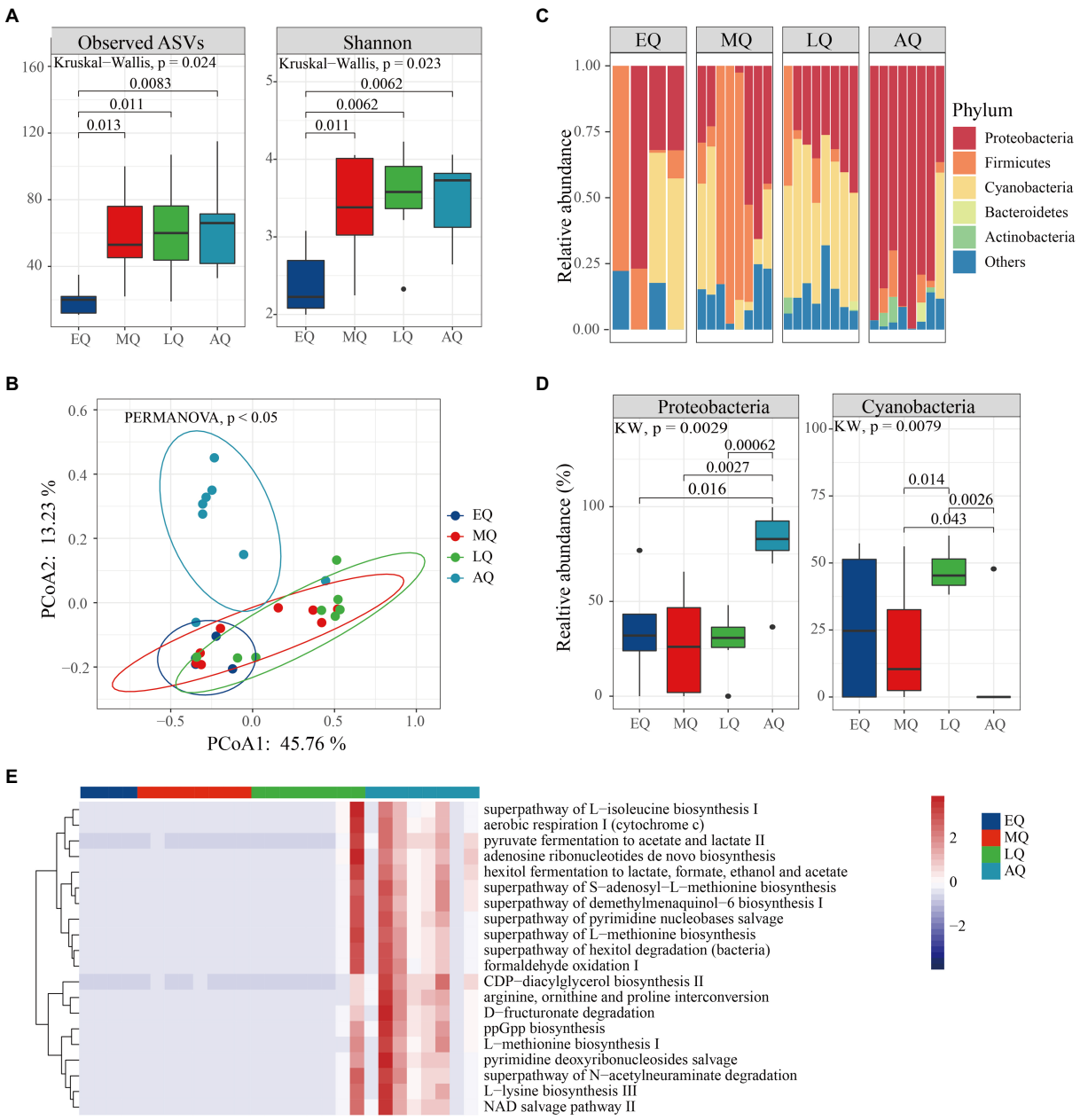

FIGURE 5
The variation of microbial diversity between four stages of queens in *Bombus terrestris*. **(A)** Alpha diversity (ASVs and Shannon indices) of microbiota in queen bumble bees using Kruskal–Wallis test. **(B)** PCoA plot based on Bray-Curtis distance metrics depicting the differences in microbial community structure (ES represents early larvae stage, MS represents mid larvae stage, LS represents late larvae stage. AS represents adult stage). **(C)** Phylum-level microbial composition of bumble bees. **(D)** The relative abundance of phyla was significantly different among the four groups (Kruskal–Wallis test, p <0.05). **(E)** The relative abundance of the top 20 metabolic pathways using PICRUSt-predicted Metacyc orthologs.

absorption of food. Cyanobacteria are photosynthetic prokaryotes that use light energy to split water and transfer electrons to produce ATP in insects (Kang et al., 2021). Proteobacteria can digest secondary metabolites (such as terpenes, alkaloids, glycosides, and phenolic compounds) of insect hosts and help to maintain the growth and development of insects; its absence leads to slower development in insects (Shah et al., 2021). Studies show that Firmicutes play a role in energy absorption in insects (Zhang et al., 2022).

Moreover, we found *Snodgrassella* and *Lactobacillus* significantly differed in worker and queen bumble bees (Figure 3D). *Snodgrassella* is the core genus in honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.) (Cornet et al., 2022). Functional analyses revealed the importance of small proteins, defense mechanisms, amino acid transport, and metabolism in *Snodgrassella* genus (Hammer et al., 2021). Honey bees also associate with *Lactobacillus* from flowers and may therefore obtain these bacteria from flowers (McFrederick et al., 2017). A recent study shows that *Lactobacillus plantarum* influences mate preferences in *Drosophila melanogaster*, possibly through alterations of cuticular hydrocarbon sex pheromones affecting the phenotype (Sharon et al., 2010). Thus, our results elucidate the important role of bacteria in bumble bee developments.

Microbial composition is significantly affected by age during bee development (Tarpy et al., 2015). Linear mixed-effects model analysis indicated that age was the major factor shaping microbial community alpha diversity within bumble bees (Table 1). This is consistent with the findings on *Apis mellifera* that there is an increase in the number of isolated bacterial colonies and diversity as the larvae age (Evans and Armstrong, 2006). One possible explanation is that the absence of defecation during the larval stage contributes to increasing diversity and abundance with successive larval instar. In addition, our results showed that the alpha diversity was lower in early larvae stage but was higher in other stages of bumble bees. Bacterial diversity may also have increased due to the increase in pollen in the larval food (Voulgari-Kokota et al., 2020). When glandular secretions of bees are mixed with increasing amounts of sugary crop contents, the larval food becomes more susceptible to microbial inoculation from pollen grains (Vojvodic et al., 2013).

Finally, microbial communities in the larval gut can differ from those in adults of Hymenoptera (Li et al., 2021). For example, Gammaproteobacteria, Acetobacteraceae, Firmicutes, and *Bacillus* spp. differ between larvae and adult honey bees (Vojvodic et al., 2013). Our results showed that the microbial diversity of larval samples exhibited much higher similarity, different from adult bumble bees (Figures 4B, 5B). The bacteria-encoded functions that were active throughout the life cycle included mainly biosynthesis and generation of precursor metabolites and energy in adults than in larval bumble bees (Figures 4E, 5E). Bacteria play a role in metabolism processing during the early and fragile stages of bumble bees.

Conclusion

Fertilized eggs produced by the bumble bee queen develop into two phenotypes, the new queen (large size) and the worker (small size). This study explore the relationship between microbiota and phenotypic variation in bumble bees. The results show that alpha and beta diversity of gut microbiota is similar in workers and queens. However, the relative abundance of three main phyla of bacteria (Cyanobacteria, Proteobacteria, and Firmicutes) and two genera (*Snodgrassella* and *Lactobacillus*) are significantly different. Furthermore, results show microbiota diversity is significantly affected by age in bumble bees. The findings of this study give insights for further studies on the relationships between gut microbiota and phenotypic variation in female bumble bees.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JA conceived the research project. BG collected the samples, analyzed the data, and sequenced the samples. BG, JT, GD, SM, JH, and JA wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1117077/full#supplementary-material

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Bee breweries: The unusually fermentative, lactobacilli-dominated brood cell microbiomes of cellophane bees

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Pathogens and parasites of solitary bees have been studied for decades, but the microbiome as a whole is poorly understood for most taxa. Comparative analyses of microbiome features such as composition, abundance, and specificity, can shed light on bee ecology and the evolution of host–microbe interactions. Here we study microbiomes of ground-nesting cellophane bees (Colletidae: Diphaglossinae). From a microbial point of view, the diphaglossine genus *Ptiloglossa* is particularly remarkable: their larval provisions are liquid and smell consistently of fermentation. We sampled larval provisions and various life stages from wild nests of *Ptiloglossa arizonensis* and two species of closely related genera: *Caupolicana yarrowi* and *Crawfordapis luctuosa*. We also sampled nectar collected by *P. arizonensis*. Using 16S rRNA gene sequencing, we find that larval provisions of all three bee species are near-monocultures of lactobacilli. Nectar communities are more diverse, suggesting ecological filtering. Shotgun metagenomic and phylogenetic data indicate that *Ptiloglossa* culture multiple species and strains of *Apilactobacillus*, which circulate among bees and flowers. Larval lactobacilli disappear before pupation, and hence are likely not vertically transmitted, but rather reacquired from flowers as adults. Thus, brood cell microbiomes are qualitatively similar between diphaglossine bees and other solitary bees: lactobacilli-dominated, environmentally acquired, and non-species-specific. However, shotgun metagenomes provide evidence of a shift in bacterial abundance. As compared with several other bee species, *Ptiloglossa* have much higher ratios of bacterial to plant biomass in larval provisions, matching the unusually fermentative smell of their brood cells. Overall, *Ptiloglossa* illustrate a path by which hosts can evolve quantitatively novel symbioses: not by acquiring or domesticating novel symbionts, but by altering the microenvironment to favor growth of already widespread and generalist microbes.

KEYWORDS

bacteria, symbiosis, microbiota, *Apilactobacillus*, Colletidae, Diphaglossinae, *Ptiloglossa*

Introduction

Bees are a diverse group of key pollinators (Michener, 2007; Danforth et al., 2019). As many species are declining (Ollerton et al., 2014; Goulson et al., 2015; LeBuhn and Vargas Luna, 2021), there is an urgent need to understand ecological factors that influence bee health. One such factor is the microbiome, the assemblage of beneficial, neutral, and harmful microbes associated with a host. Though molecular studies of bee microbiomes began over 15 years ago (Jeyaprakash et al., 2003; Mohr and Tebbe, 2006), and culture-based studies much earlier (Batra et al., 1973; Gilliam, 1979), our understanding of bee microbiomes is far from comprehensive. Only a small fraction of the ~20,000 described bee species (Michener, 2007) have been microbially characterized to date. The majority of studies are on the social corbiculate bees and a handful of solitary bee groups (Voulgari-Kokota et al., 2019). Moreover, as bees are holometabolous and nest-dwelling insects, multiple life stages and microhabitats need to be characterized for a complete picture of bee-microbe interactions. The brood cell, which contains pollen provisions and larvae, is much harder to sample than adults, but has a central role in bee biology. In social corbiculate bees, larvae and adults have very different microbial associations (Kwong and Moran, 2016). In solitary bees—which make up the majority of bee diversity (Danforth et al., 2019) — brood cell microbiome composition and function are poorly understood.

16S rRNA profiling-based studies have provided an initial picture of microbiome composition in solitary bee brood cells. Lactobacilli tend to be dominant members of the bacterial community, across distantly related bee species (McFrederick et al., 2012, 2017; Kapheim et al., 2021). However, for large swaths of bee diversity—including entire families—brood cell microbiomes remain uncharacterized. Moreover, potential variation in the absolute abundance and activity of microbes cannot be inferred from 16S rRNA profiles (Hammer et al., 2019). Here, the decades of research on bee nesting biology provide some clues. In many bee taxa, larval provisions are semi-solid or dough-like, and sometimes quite dry (Danforth et al., 2019; Cane and Love, 2021). Microbial growth in these substrates could be limited by low water potential (Bartlett and Roberts, 2000), as well as other factors. However, in the family Colletidae, provisions are liquid or semi-liquid (Rozen, 1984; Michener, 2007; Almeida, 2008; Sarzetti et al., 2013). The brood cell of colletid bees is lined by an impermeable "cellophane" coating, produced by the Dufour's gland, that both protects the larva during development and prevents the liquid provisions from leaking into the surrounding substrate (Rozen, 1984; Almeida, 2008; Danforth et al., 2019). Unusually for solitary bees, the colletid subfamily Diphaglossinae also have open brood cells, which have been suggested to facilitate gas exchange (Roberts, 1971). Brood cell venting could be particularly important when there are large numbers of metabolically active microbes alongside the developing bee larva.

Within Diphaglossinae, the genus *Ptiloglossa* has provisions that are particularly watery, and have an obvious odor and taste characteristic of fermentation. This trait was first observed by D. H. Janzen in Veracruz, Mexico in the early 1960s (pers. comm.) and reported by Roberts for a Costa Rican species, *P. costaricana*, in 1971 (Roberts, 1971). Vigorous microbial growth in healthy brood cells appears to be characteristic for this genus, and not a case of sporadic microbial spoilage (which is not uncommon among bees; Batra et al., 1973). Strong fermentation odors have also been noted in *Ptiloglossa* brood cells in Brazil and Arizona, United States (Rozen, 1984; de Araujo et al., 2020). *Ptiloglossa*, and perhaps other colletids (Michener, 1960), may have similarities with insects such as *Drosophila*, for which microbes make up an important part of the larval diet (Markow and O'Grady, 2008). But colletid bee brood cells have not been studied using molecular methods, limiting our ability to infer the ecological function and evolutionary history of this symbiosis. There are many open questions. Which microbes grow in the larval provisions? Are they unique species domesticated by the bee host? How do they vary between closely related bee hosts and between habitats? Are brood cell microbes vertically transmitted, or acquired from the environment? How do they vary over host development? And what traits might bees be using to "brew" fermenting larval provisions?

Here we explore these questions using microbiome sequencing of field-collected brood cells of three diphaglossine bee species: *Ptiloglossa arizonensis* and *Caupolicana yarrowi* in Arizona, United States, and *Crawfordapis luctuosa* in western Panama. We used 16S rRNA gene sequencing to characterize the composition of bacteria in a range of microhabitats in which they may contribute to bee biology, and among which they may be transmitted: larval provisions, larvae of different developmental stages, pupae, adult guts, and floral nectar. For a subset of *P. arizonensis* larval provisions, we also sequenced shotgun metagenomes. These data were used to measure the relative abundance of nonbacterial microbes such as fungi, infer ratios of bacterial to plant biomass, and assess strain-level diversity. We also constructed a phylogeny, using assembled 16S rRNA gene sequences, to evaluate host- and habitat-specialization of the dominant lactobacilli.

Materials and methods

Collections

We collected a small number of bee and nectar samples from southeastern Arizona, United States from August 25–26, 2018. For convenience we refer to this as the "*Caupolicana* dataset" although two bee individuals of other species were also included. We caught three adult *Caupolicana yarrowi* foraging from *Solanum elaeagnifolium*, near Portal, AZ. An adult *Protoxaea gloriosa* (Andrenidae) and *Ptiloglossa arizonensis* were also collected in the same area, both from *S. elaeagnifolium*. We dissected and stored the entire gut, from crop to hindgut. From each of three flowers from the same *Agave palmeri* individual, collected near Portal, AZ, we sampled nectar using sterile swabs. We sampled larval provisions from two *Caupolicana yarrowi* brood cells (from two separate nests at the same site) near Paradise, AZ. More information on this site is given in Rozen et al. (2019). For one sample, 50 µl of liquid was pipetted from the brood cell. For the other brood cell, a sterile swab was used to collect the larval provisions since they were more viscous. The latter brood cell contained a larva of *Triepeolus grandis*, a brood parasitic bee described in Rozen et al. (2019). All samples were transported to the laboratory in a dry-shipping liquid nitrogen dewar, where they were frozen at −20°C.

On August 28 2019, we collected *Ptiloglossa arizonensis* brood cell samples, all from a single aggregation of nests at ~5,200' elevation, near Portal, AZ. Brood cells were carefully excavated from the soil matrix, and liquid provisions were pipetted into sterile tubes. As reported previously (Rozen, 1984), provisions (especially in early stages) are stratified into a more nectar-rich and liquid top layer, and

a more pollen-dense bottom layer. In two brood cells we collected the upper and bottom layers separately for analysis (PA.LP.2 [upper] and PA.LP.3 [lower]; PA.LP.4 [upper] and PA.LP.5 [lower]). In the others, the entire volume of the provisions was collected and mixed. Brood cells varied in how recently they had been provisioned by the adult female. Some (e.g., PA.LP.7) had no egg or larva and were presumably still in the process of being provisioned. Other brood cells contained an egg (e.g., PA.LP.4, PA.LP.5) or developing larvae (e.g., PA.LP.6), which were also collected. Larvae were sampled at different developmental stages, with fresh weights ranging from 9.8–99.2 mg. Mature larvae were sampled from capped brood cells; these larvae had finished consuming the pollen/nectar provisions. One of these larvae was later (during homogenization in the laboratory) observed to lack any visible trace of pollen, and had therefore likely completed defecation. We refer to this as a prepupa (following: Michener, 2007; Danforth et al., 2019). Other mature larvae still had pollen in their gut. Both developing and mature larvae were rinsed twice in 70% ethanol before storage, in order to remove soil or provisions on the surface. All provisions and larvae were collected in sterile tubes in a dry-shipping liquid nitrogen dewar for transport to the laboratory.

On August 29, 2019, near dawn, we collected seven adult *P. arizonensis* as they exited nests of the same aggregation. Entire guts were dissected; all bees had empty crops, likely because they were just beginning to forage. From August 28–30, 2019, we also obtained *Agave palmeri* nectar and *Solanum eleagnifolium* flowers, as potential microbial inputs into the brood cell. Flowering stalks from three *Agave* plants in the vicinity of the *Ptiloglossa* nest aggregation were harvested and brought to the field station. From each plant, 500 µl nectar samples were collected from replicate flowers (six in total). Whole *S. elaeagnifolium* flowers (four from each of two plants) were collected in Portal. Nectar and flower samples were also stored in the dewar for transport to the laboratory for long-term storage at −80°C, along with the bee samples.

We collected samples of *Crawfordapis luctuosa* from high-elevation premontane rainforest in Western Panama. A single population, in the vicinity of Mount Totumas near Los Pozos, was sampled in both 2019 (adults) and 2020 (brood cells). Adult bees were caught with an insect net while entering and exiting nests and preserved directly in 100% ethanol. To sample larval provisions and immature stages, we carefully excavated brood cells and collected their contents. Provisions were pipetted into sterile tubes. We used sterilized tweezers to remove developing larvae, mature larvae, pupae, and pharate adults (i.e., those which have completed metamorphosis but not emerged from the cocoon). Adult and brood cell samples were collected and stored at −18-20°C. Adult bees were collected into 50 ml tubes with 15 ml of 100% EtOH (to euthanize immediately), whereas brood cell samples were directly frozen; both freezing and ethanol have been shown to be suitable for insect microbiome characterization, and do not introduce significant bias (Hammer et al., 2015).

Sample prep and sequencing

For the *Caupolicana* and other bee samples collected in 2018, DNA was extracted using the Qiagen PowerSoil DNA isolation kit. The manufacturer's instructions were followed, with one modification: samples were heated with Solution C1 for 10 min at 65°C in a dry heat block prior to bead beating. We conducted PCRs in duplicate using GoTaq Colorless Master Mix (Promega) and the 16S rRNA gene primers 515F and 806R (Supplementary methods, Table S1) with Illumina sequencing adapters and unique 12-bp barcodes. Amplification was verified by gel electrophoresis. Amplicons were cleaned and normalized with the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific) following the manufacturer's instructions, and then pooled. Libraries were sequenced on an Illumina MiSeq using a v2 300 cycle kit (paired-end reads, 2 × 150) at the University of Colorado BioFrontiers Institute Next-Gen Sequencing Core Facility. Negative controls were included during both DNA extraction and PCR amplification.

We also used the Qiagen PowerSoil kit to extract DNA from the *Ptiloglossa* and associated plant samples. For liquid samples (provisions, *Agave* nectar), 100 µl was loaded into bead tubes. *Solanum* flowers were added directly. Larvae and adult guts were weighed, homogenized with a pestle in 100 µl molecular-grade water, and up to 100 µl of homogenate was added to bead tubes. Extractions followed the manufacturer's protocol, including blanks as negative controls. We then prepared amplicon libraries using 16S rRNA gene primers 799F and 1115R that amplify the V5-V6 region (Supplementary methods, Table S1). We have previously used these primers for paired-end sequencing with inline barcodes (Figueroa et al., 2021), as they minimize amplification of plant chloroplasts and mitochondria (Hanshew et al., 2013; Kembel et al., 2014). PCR conditions are detailed in the Supplementary methods. To normalize the amount of DNA in each library, we used SequalPrep normalization plates (Invitrogen) following the manufacturer's protocol. We combined 5 µl of each normalized library to create a library pool. To remove primer-dimers and excess master mix components, we cleaned the library pool with AMPure XP beads (Beckman Coulter). We checked the quality and concentration of the pooled libraries using a Bioanalyzer (Agilent). Libraries were sequenced on an Illumina MiSeq using the V3 2 × 300 reagent kit at the Genomics Core of UC Riverside.

For gut microbiome characterization of *Crawfordapis* adults, we used whole abdomens, which contain most of the gut—from the crop to the hindgut. DNA for all samples was extracted using the Qiagen PowerSoil DNA isolation kit, following the manufacturer's instructions, with one modification: adult abdomens were homogenized for two additional minutes using the beads and lysis solution included in the Qiagen PowerSoil kit. Barcoded 16S rRNA primers (V4-V5 region; 515F/926R) were used for PCR amplification (Supplementary methods, Table S1). Amplicons were cleaned using MoBio UltraClean PCR Clean-Up Kit, following the manufacturer's instructions. Cleaned, normalized and pooled amplicons were submitted for 2 × 250 bp sequencing on an Illumina HiSeq 2500 at the University of New Hampshire.

To characterize bacterial diversity at finer phylogenetic scales, and to characterize non-bacterial organisms, we conducted shotgun metagenomic sequencing for two *Ptiloglossa* larval provisions samples (PA.LP.1 and PA.LP.9). From the genomic DNA used for amplicon sequencing, we constructed metagenomic libraries using the QIAseq FX DNA Library kit (Qiagen). Library prep methods are described in the Supplementary methods. We also included a commercially available microbial community DNA standard (ZymoBIOMICS D6305) as a positive control, and extraction blanks as negative controls. We checked the quality and concentration of the pooled libraries using a 2100 Bioanalyzer (Agilent). Libraries were sequenced

on an Illumina NovaSeq at the UCSF Genomics Core using the S4 2 by 150 bp reagent kit.

Amplicon data analysis

Raw amplicon data are available from NCBI BioProject PRJNA925568. Demultiplexed sequence libraries from all three datasets were processed separately but using the same methodology. For quality control, chimera removal, and read denoising and binning into amplicon sequence variants (ASVs), we used DADA2 with default parameters (Callahan et al., 2016) except for the number of bases trimmed and truncated (see Supplementary methods). To assign taxonomy to ASVs, we trained the QIIME2 sklearn classifier to the primer set used for each bee species in reference to the SILVA 138 SSURef NR99 full-length sequences and taxonomy databases (Quast et al., 2013; Bokulich et al., 2018): 799–1,115 region for *Ptiloglossa*, 515–806 region for *Caupolicana*, and 515–926 region for *Crawfordapis*.

As different regions of the 16S rRNA gene were targeted in the three datasets, they were analyzed separately (but following the same general approach). In each, ASVs with <100 total sequences across all samples were removed, following (Hammer et al., 2020). ASVs classified as chloroplast, mitochondria, or unidentified Eukaryote were removed. Some bee samples had very high proportions of eukaryotic sequences, potentially indicative of relatively low bacterial biomass (Hammer et al., 2017). *Solanum* flower samples were 98.4–99.9% eukaryotic. The *Ptiloglossa* prepupa sequence library was 97.8% eukaryotic, comprising mostly bee sequences. As these samples were left with a low number of bacterial sequences (below the rarefaction cutoff) they were excluded from further analysis. In the *Crawfordapis* dataset, all of the pupal ($N = 3$) and pharate adult ($N = 4$) samples, four mature larvae, and one adult had low bacterial sequence counts and were subsequently excluded. Contaminants were identified with the tool decontam, applying the prevalence-based method (Davis et al., 2018). In the *Ptiloglossa* dataset, six replicate blanks yielded sequences. Decontam identified six contaminant ASVs, which belong to typical reagent- or human-associated taxa (Salter et al., 2014; Eisenhofer et al., 2019): *Ralstonia, Dietzia*, *Staphylococcus*, *Cutibacterium*, *Micrococcus*, and *Streptococcus*. These were removed from the dataset. In the *Caupolicana* dataset, decontam was not able to identify any contaminants because only one blank yielded any sequences. In the *Crawfordapis* dataset, three blanks yielded sequences. Seven ASVs were identified as contaminants and removed: *Escherichia*, *Streptococcus*, *Klebsiella*, *Cutibacterium*, *Lactococcus*, *Corynebacterium*, and *Staphylococcus*. After filtering and contaminant removal, sequence libraries were rarefied (randomly subsampled) to different depths in each dataset: 34957 reads (*Caupolicana*), 1728 reads (*Ptiloglossa*), and 1809 reads (*Crawfordapis*). Finally, we also evaluated taxonomic classifications (using blastn searches) for the most abundant ASVs classified as *Lactobacillus*. This genus has recently undergone a major revision (Zheng et al., 2020), and changes are not yet fully implemented in the SILVA reference database we used.

To visualize microbial taxonomic composition, we summarized each sample's read counts at the genus level. Only dominant genera—those with >2% mean relative abundance across samples within the dataset—are labeled and colored in the stacked bar plot; all other genera are shown in white. To analyze patterns of beta diversity, we square-root transformed the ASV data table before calculating Bray-Curtis dissimilarities using the vegan package (Oksanen et al., 2022). We visualized these patterns with non-metric multidimensional scaling (NMDS) plots. The effect of sample type on community composition was first tested with a global permutational multivariate analysis of variance (PERMANOVA). Then, pairwise differences were tested using the pairwiseAdonis package, which applies a correction for multiple comparisons. We used the betadisper function in the vegan package to test for potential variation among sample types in within-group dispersion (i.e., heterogeneity). Differences in alpha diversity (Shannon diversity) among sample types were tested with a one-way ANOVA. After a significant global test we then used Tukey *post hoc* tests for pairwise differences.

Metagenomic and phylogenetic data analysis

Raw shotgun metagenomic data are available from NCBI BioProject PRJNA925568. We removed adapters and quality-filtered metagenomic reads using cutadapt (Martin, 2011), with a minimum read length of 50, and a minimum phred score of 20. Single-sample assemblies were performed using megahit (Li et al., 2015), with a minimum contig length of 1 kb. The assembly from sample PA.LP.1 contained 1880 contigs with an N50 of 3,541 bp. The assembly from sample PA.LP.9 contained 4,410 contigs with an N50 of 1,405 bp. In preparation for binning, we mapped each sample's reads to its assembly using bowtie2 (Langmead and Salzberg, 2012, 2) with the – very-sensitive-local setting. Overall alignment rates were low (33.3 and 47.2%), possibly because of a large number of plant sequences that were not assembled (see phyloFlash results below). Assemblies were then binned using MetaBAT 2 (Kang et al., 2019). We used checkM (Parks et al., 2015) to evaluate completeness, contamination, and strain heterogeneity of the bins, and GTDB-tk (Chaumeil et al., 2019) to classify them (Table 1). FastANI (Jain et al., 2018), implemented within GTDB-tk, was used to calculate ANI. Two low-quality bins with ≤20% completeness were discarded (quality defined following: The Genome Standards Consortium et al., 2017).

We also used phyloFlash to identify and classify SSU rRNA sequences from the quality-filtered, paired-end reads (Gruber-Vodicka et al., 2020). phyloFlash was run with the SILVA SSU Ref NR99 reference database for classification (Quast et al., 2013) and other default settings. Taxonomic composition of the metagenomes was then measured using the mapping-based phylotypes identified by phyloFlash. To compare the ratio of bacterial to plant sequences between *Ptiloglossa* and other bees, we included data from 84 bee larval provision metagenomes, sequenced as described above. These data represent eight species of Apidae and one species of Andrenidae, with 5–17 replicate samples each: *Amegilla dawsoni*, *Andrena asteris*, *Anthophora bomboides*, *Centris caesalpiniae*, *Centris cockerelli*, *Centris pallida*, *Diadasia australis*, *Melissodes druriella*, and *Xylocopa micheneri* (unpublished data from MA-G, QM, SB, and BD).

To reconstruct a phylogeny of the dominant lactobacilli, we used the full-length sequences assembled by SPAdes (Bankevich et al., 2012) within phyloFlash. Each sample had a single 16S rRNA sequence with high sequence identity to various *Apilactobacillus* species. We collected sequences of the close matches and outgroup taxa from NCBI Genbank. Sequences were aligned using MUSCLE (Edgar, 2004) and manually trimmed in Jalview (Waterhouse et al., 2009). A

TABLE 1 Metagenome-assembled genomes (MAGs) from two *Ptiloglossa* brood cell samples.

Sample	% Completeness	% Contamination	% Strain heterogeneity	Reference classification	ANI to reference
PA.LP.1	92.62	2.97	90.91	*Apilactobacillus micheneri*	98.54%
PA.LP.9	76.45	0.31	100.00	*Apilactobacillus timberlakei*	99.08%

Completeness and contamination are metrics calculated by checkM that use lineage-specific, single-copy marker genes to evaluate genome quality. Strain heterogeneity measures the contribution of intraspecific diversity (versus heterospecific diversity) to the reported contamination. MAGs were classified using GTDB-tk.

maximum-likelihood phylogeny was inferred with IQ-TREE[1] using the Auto substitution model finder (Kalyaanamoorthy et al., 2017) (which determined TVMe+I as the best-fit model) and standard nonparametric bootstraps (Felsenstein, 1985). The tree was visualized using iTol[2] and rooted on *Holzapfelia floricola* (Zheng et al., 2020).

Natural history

Here, we summarize new and published natural history observations relevant to interactions between diphaglossine bees and microbes. At our field site in southeastern Arizona, we observed *Ptiloglossa arizonensis* and *Caupolicana yarrowi* foraging for pollen from *Solanum elaeagnifolium* and *S. rostratum* (Figure 1), matching earlier observations (Linsley, 1962; Rozen et al., 2019). *Solanum* is a commonly reported pollen source across the range of *Ptiloglossa*, though non-Solanaceous plants are also used (Janzen, 1968; Roberts, 1971; Sarzetti et al., 2013). We also have evidence that *P. arizonensis* collects nectar from *Agave palmeri* flowers. First, bees returning from foraging are often dusted with pollen that is morphologically identical to *Agave* pollen. This pollen is concentrated on the dorsum of the mesosoma, a location that matches the position of exserted *Agave* anthers. Second, *Agave palmeri* nectar has a powerful and unique melon (*Cucumis melo*)-like odor. We detected the same odor from *P. arizonensis* brood cells. *Caupolicana yarrowi* were observed collecting nectar from creosote (*Larrea tridentata*). Earlier studies from the southwestern US report that *P. arizonensis* visit *Larrea* as well (Hurd and Linsley, 1975). We do not have information about food plants of the specific *Crawfordapis luctuosa* population we studied, but another population in Western Panama was reported to collect pollen from dozens of flowering plant species, mainly belonging to Solanaceae, Melastomataceae, and Begoniaceae (Roubik and Michener, 1984), all of which (except Begoniaceae species) have poricidal anthers and require buzz pollination (Buchmann, 1983).

The timing and duration of foraging influence the availability and quality of floral resources and the risk of parasitism (Wcislo and Tierney, 2009). These factors could alter the types of microbes to which bees are exposed, and could select for different microbially mediated nutritional or defensive strategies. *Ptiloglossa* are dim-light foraging (crepuscular) bees, with activity concentrated from before, to shortly after dawn (Linsley, 1962; Janzen, 1968; Roberts, 1971; de Araujo et al., 2020). This is the case at our field site in Arizona, where we observed *P. arizonensis* foraging beginning roughly an hour before dawn and ending just after dawn. *Caupolicana* has a longer foraging window. In Arizona, we and others (Rozen et al., 2019) have observed *Ca. yarrowi* foraging later in the morning than *P. arizonensis*, and as late as mid-afternoon; another *Caupolicana* species has been collected near mid-day (Michener, 1966). Our *Crawfordapis luctuosa* study population forages throughout the day, as reported earlier (Roubik and Michener, 1984).

We observed that *P. arizonensis* larval provisions—particularly those in older cells with large larvae—have a sour taste and a strong odor characteristic of microbial fermentation. According to earlier reports from the neotropics, the odor of fermentation is "unmistakable" (Costa Rica; Roberts, 1971), a "strong sour smell" (Brazil; de Araujo et al., 2020) similar to "fermenting beer or mead" (Venezuela; D. H. Janzen, pers. comm.). *Caupolicana yarrowi* brood cells did not have a noticeable fermentation odor, in agreement with published descriptions (Rozen, 1984; Rozen et al., 2019). We observed fermentation odors during collection of *Crawfordapis luctuosa,* although they were not observed in an earlier study (Roubik and Michener, 1984), perhaps due to seasonal or fine-scale temporal differences in fermentation within brood cells. Although these odors are likely to be a useful proxy for overall microbial activity, there are caveats. It is difficult to infer which microbes are abundant, as very different microbes can have overlapping volatile profiles (e.g., *Saccharomyces* yeast and *Lactobacillus* bacteria; Hansen and Hansen, 1994), and as different volatiles have different salience to the human nose. Also, unusual odors in bee brood cells are not necessarily a product of microbial metabolism. They can be derived from plant volatiles or, as in the case of the cheesy-smelling brood cells of *Anthophora*, from glandular substances produced by the bee (Norden et al., 1980).

Results

Microbiomes of diphaglossine bee larval provisions are dominated by lactobacilli (mean relative abundance +/− SEM: *Ptiloglossa*, 94.3 +/− 3.61%, $N = 16$; *Caupolicana*, 99.2 +/− 0.645%, $N = 2$; *Crawfordapis*, 97.2 +/− 0.627%, $N = 10$; Figure 2). *Lactobacillus* is dominant even in brood cells inferred to have been recently provisioned (i.e., no egg or larva). For two *Ptiloglossa* brood cells in which we separately sampled the top (more nectar-rich) and bottom (more pollen-rich) stratified layers of the larval provisions, we did not observe a difference in microbial composition (Figure 2). In *Ptiloglossa* and *Caupolicana*, the dominant ASVs have 100% sequence identity to various strains of *Apilactobacillus*, particularly *A. micheneri* and *A. timberlakei*. In contrast, *Crawfordapis* larval provisions are dominated by an ASV that may represent a new bacterial species, with <97% identity to isolates belonging to *Lactobacillus*, *Pediococcus*, *Nicolia*, and related genera. For consistency, we use the default classification provided by the SILVA database (*Lactobacillus*) in the text and plots describing 16S

1 http://iqtree.cibiv.univie.ac.at/

2 https://doi.org/10.1093/nar/gkab301

FIGURE 1
An overview of the ecology of *Ptiloglossa arizonensis* in southeastern Arizona, showing major nectar (*Agave*) and pollen (*Solanum*) sources used to provision the larvae.

rRNA amplicon data. Other bacteria, namely *Saccharibacter*, *Fructobacillus*, and *Acinetobacter*, are only sporadically present, but can be abundant in individual brood cells (Figure 2).

Whole-body microbiomes of developing *Ptiloglossa* larvae are also dominated by *Lactobacillus*, similarly to their diet (compare larvae to provisions samples with matching letters [a, b, d, e] in Figure 2). Secondary bacterial taxa (most notably, *Saccharibacter*) that appear in larval provisions (sample PA.LP.6) also appear at a similar relative abundance in the larva from the same brood cell (sample PA.DL.4; Figure 2), suggesting dietary acquisition. (Larvae of most bees, including diphaglossines, only defecate after they finish feeding and immediately before entering the last larval instar, or prepupal stage (Danforth et al., 2019). These larvae cannot contaminate the provisions through defecation). Microbiomes of *Ptiloglossa* larval provisions and developing larvae are not significantly different in Shannon diversity (*post hoc* test, adjusted $p = 0.98$; Figure 3) or in composition (pairwise PERMANOVA, adjusted $p > 0.05$; Figure 4). In contrast, developing *Crawfordapis* larvae have high relative abundances of *Wolbachia* in addition to *Lactobacillus* (Figure 2), leading to a clear division between larval provisions and developing larval microbiome composition (pairwise PERMANOVA, $R^2 = 0.55$, adjusted $p = 0.003$; Figure 4) and Shannon diversity (posthoc test, adjusted $p < 0.01$; Figure 3), though not within-group heterogeneity (betadisper, $F = 0.067$, $p = 0.79$).

In *Ptiloglossa*, microbiomes shift, diverging from the diet, as larvae approach the prepupal stage. Mature larvae—those which have

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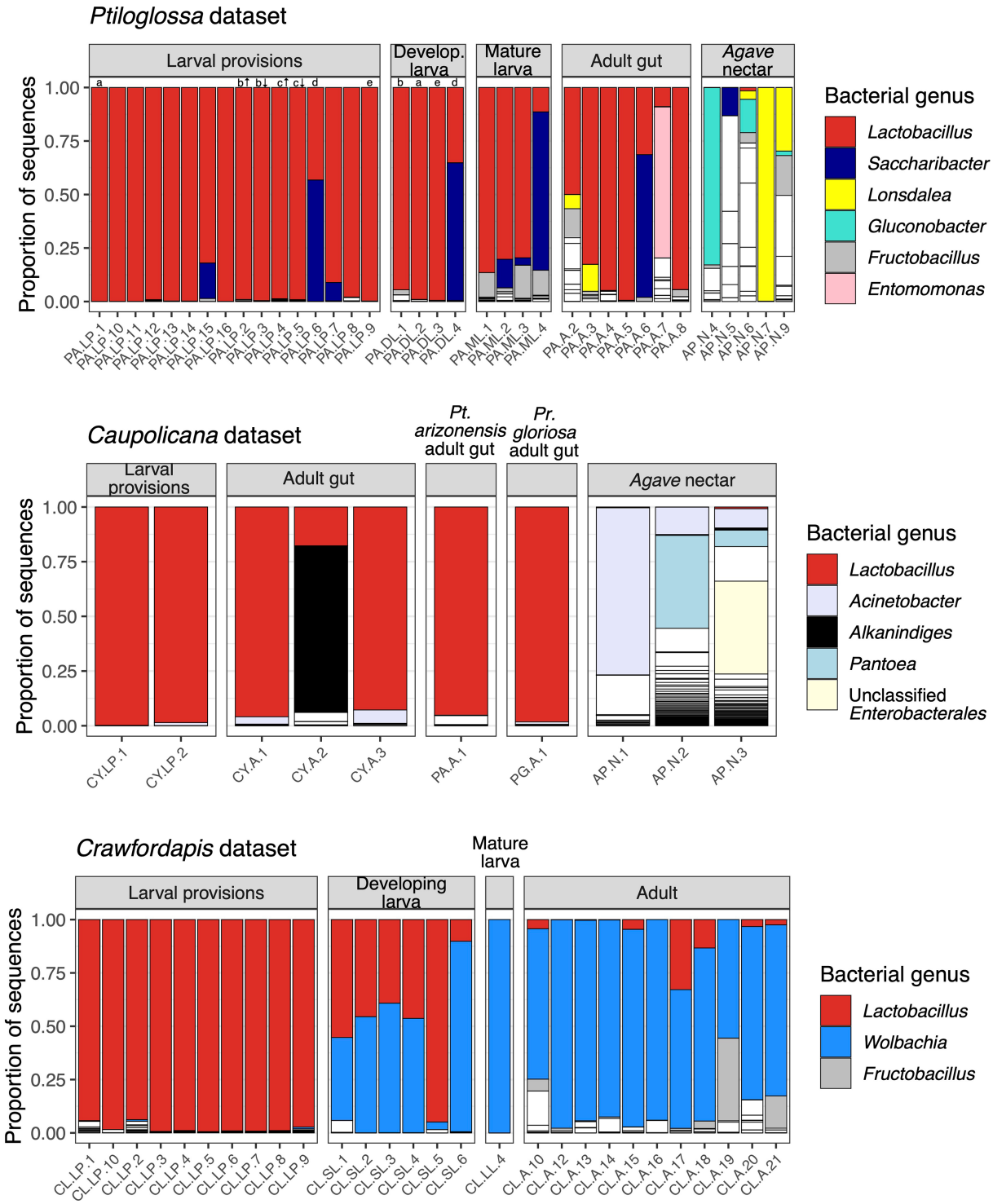

FIGURE 2
Bacterial composition of bees and nectar. Each column along the x axis is a different sample. Each bar, outlined in black, represents the relative abundance of different bacterial genera within samples. For clarity, only genera with ≥2% mean relative abundance across samples are colored; all others are white. In the *Ptiloglossa* dataset, letters above bars indicate samples that came from the same brood cell (a-e). For two brood cells (b and c), the top (indicated by ↑) and bottom (indicated by ↓) layers were sampled separately. Developing larvae are ordered left-to-right by increasing fresh weight, a proxy for age. In the *Caupolicana* dataset, all samples are of *Caupolicana yarrowi* unless otherwise noted.

consumed all of the provisions, but have not yet defecated—have about twice as high alpha diversity as developing larvae (Figure 3), although this difference is not statistically significant (adjusted $p > 0.05$). (Note that sample sizes are low: N = 4 per sample type.) Mature and developing larvae also appear to have somewhat distinct microbiome composition (Figures 2, 4), though again this difference is not statistically significant (adjusted $p > 0.05$). For *Crawfordapis*, only one out of six mature larvae had enough 16S rRNA reads for

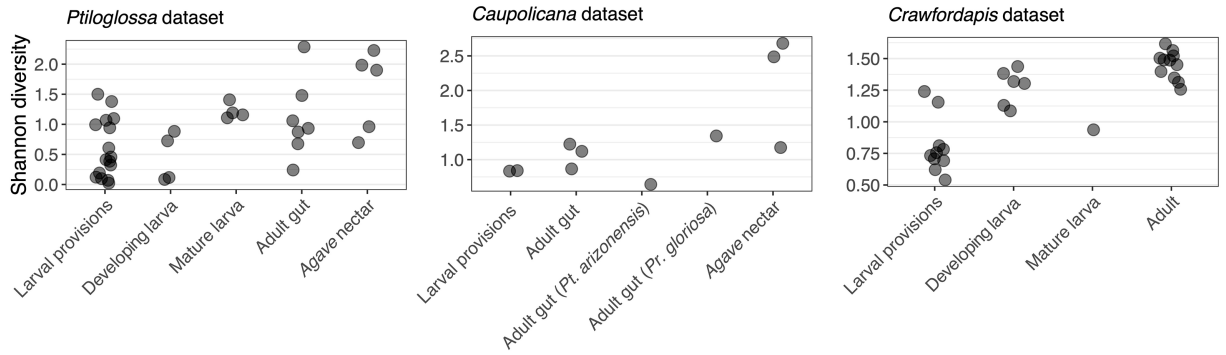

FIGURE 3
Shannon diversity, a measure of alpha diversity, of bee and nectar-associated bacterial communities. Each point represents a unique sample. In the *Caupolicana* dataset, all samples are of *Caupolicana yarrowi* unless otherwise noted.

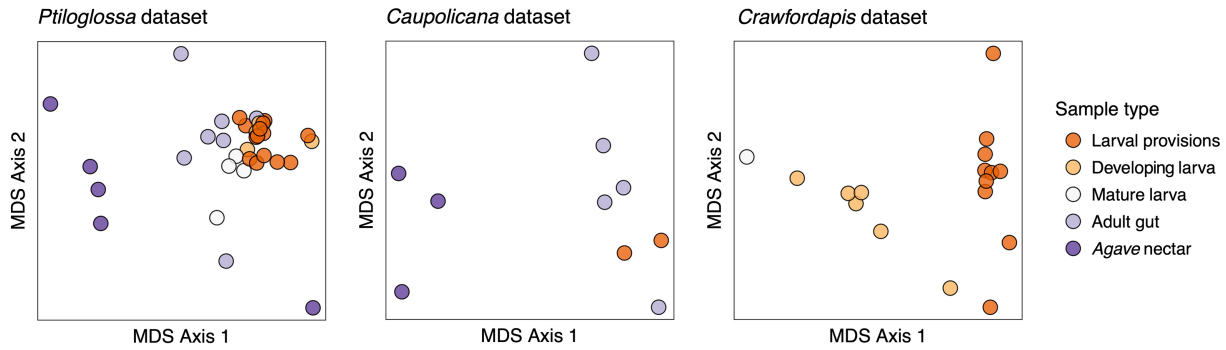

FIGURE 4
Ordinations (non-metric multidimensional scaling) representing differences in community composition (Bray-Curtis dissimilarities) among samples. Note that in the *Caupolicana* dataset, three bee species are represented within the 'Adult gut' group (see Figure 2).

analysis. *Wolbachia* is the only bacterium detectable in this larva (Figure 2).

Lactobacillus, and potentially all bacteria, are cleared from the gut before larvae enter diapause as a prepupa. We sequenced one *Ptiloglossa* prepupa in which pollen was visibly absent from its gut, confirming that it had already defecated. This sample is nearly devoid of bacteria. 97.8% of the 16S rRNA reads are eukaryotic; based on blastn searches, these are likely to originate from the bee as opposed to plant or other eukaryotic DNA. The remaining bacterial reads do not include any *Lactobacillus*. Rather, these ASVs are all very rare (<100 total reads) or absent in the other *Ptiloglossa* and nectar samples, and hence may be transient or spurious. In *Crawfordapis*, eukaryotic reads are not abundant (<10%) in pupae and pharate (pre-eclosion) adult samples—possibly because of a lower identity to bee rRNA with this primer pair. However, sequencing depth was very low (2–198 reads, mean 47), suggesting low amounts of bacterial DNA in these life stages. *Lactobacillus* is also absent.

Adult *Ptiloglossa arizonensis* collect *Agave* nectar, regurgitating it from the crop into the larval provisions and likely consuming it themselves (see Natural History). Bacterial communities in *Agave* nectar, *Ptiloglossa* adult guts, and *Ptiloglossa* larval provisions are distinct. Shannon diversity is much higher in *Agave* nectar than in larval provisions (adjusted $p = 0.011$; Figure 3). Microbiome composition also differs between each of these habitats (pairwise PERMANOVAs, $R^2 = 0.17–0.45$, adjusted $p = 0.01–0.02$; Figure 4). As evident in the ordination (Figure 4), within-group microbiome heterogeneity varies among sample types (betadisper, $F = 2.78$, $p = 0.043$), with higher heterogeneity for adult guts and *Agave* nectar. *Lactobacillus* is present in adult guts as well as *Agave* nectar, but at much lower and more variable relative abundances than in larval provisions (Figure 2). In addition to *Lactobacillus*, adult gut microbiomes contain a variety of bacterial genera common among bees and other pollinating insects, such as *Entomomonas* and *Fructobacillus* (McFrederick et al., 2017; Hammer et al., 2020; Wang et al., 2020; Handy et al., 2023). In a smaller set of samples, collected in 2018 from the same site in Arizona, adult guts of *Ptiloglossa*, *Caupolicana*, and *Protoxaea gloriosa* (Andrenidae) were all dominated by *Lactobacillus* (Figure 2). *Alkanindiges*, a gammaproteobacterium detected in other Hymenoptera (Suenami et al., 2019; Koto et al., 2020), was also abundant in one *Caupolicana* adult. *Lactobacillus* and *Fructobacillus* are again dominant bacterial genera in *Crawfordapis* adult microbiomes (whole abdomen samples), alongside *Wolbachia* (Figure 2).

There are multiple *Lactobacillus* amplicon sequence variants (ASVs) in our three datasets, representing distinct species or strains. We analyzed the distribution of these ASVs to further investigate transmission and potential host-symbiont specificity. In the *Ptiloglossa* dataset, *Lactobacillus* populations across provisions, larvae, and adult

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guts predominantly belong to a single ASV (Figure 5). However, many of the samples contain one or more additional *Lactobacillus* ASVs. General patterns are similar in the *Caupolicana* and *Crawfordapis* datasets, with 1–2 dominant *Lactobacillus* ASVs (alongside a few rarer ASVs) in provisions, larvae, and adult guts (Figure 5). Within the 2018 *Caupolicana*-focused dataset, we also see evidence of *Lactobacillus* ASV sharing among co-occurring bee species. The dominant ASV in *Ptiloglossa* is present in *Caupolicana*, as well as *Protoxaea gloriosa* (Figure 5), a distantly related bee in the subfamily Oxaeinae, which also produces liquid provisions (Sarzetti et al., 2014).

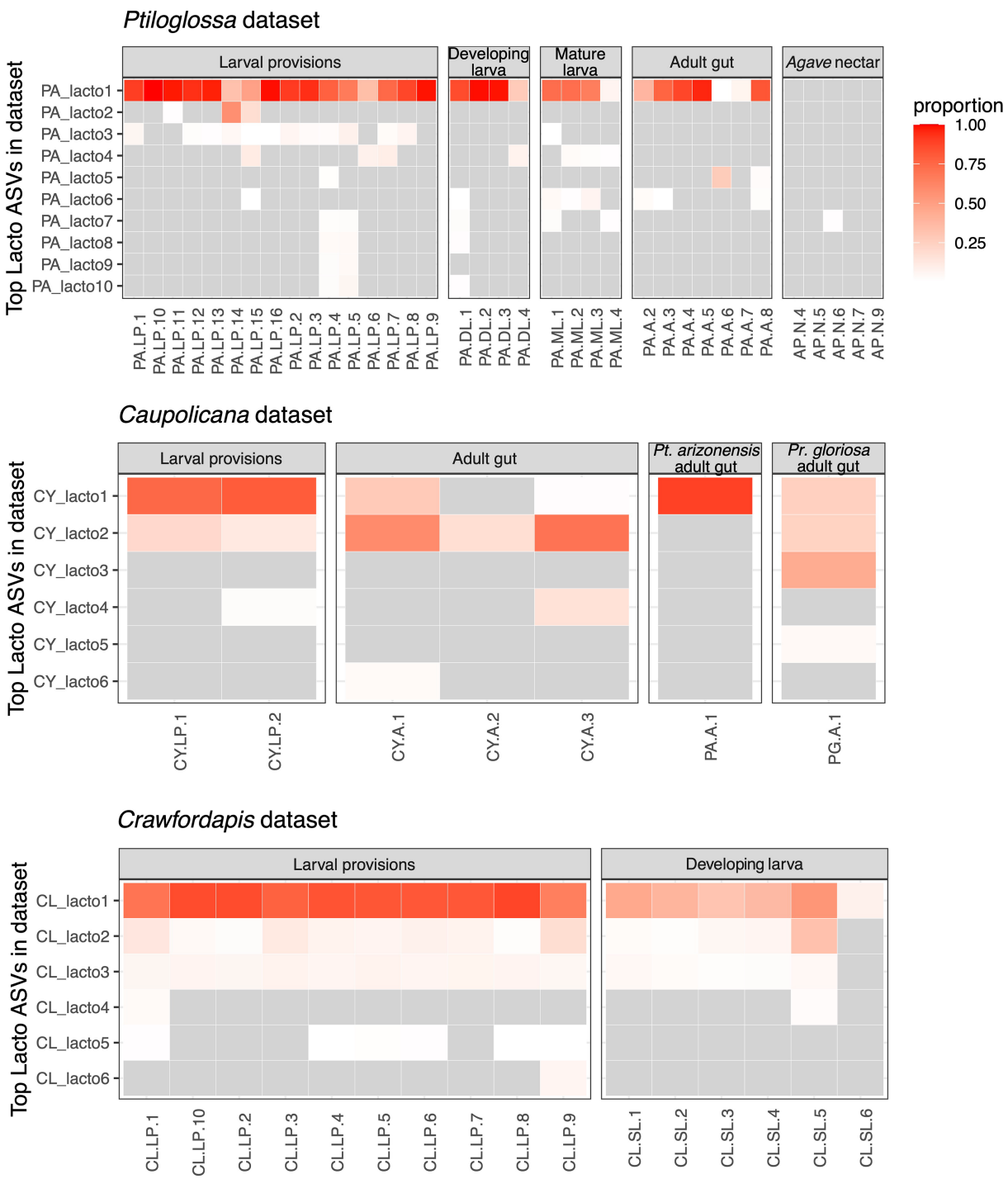

FIGURE 5
Heatmaps of the dominant ASVs classified as *Lactobacillus* for the three datasets. Columns represent individual bee or nectar samples. Rows represent up to 10 of the top ASVs (ranked by mean proportion across samples). Grey cells represent relative abundance values of <1%. Other cells are colored according to their relative abundance (proportion of sequences). *Agave* nectar is not shown in the *Caupolicana* dataset because no *Lactobacillus* ASVs were detected at ≥1% relative abundance. In the *Caupolicana* dataset, all samples are of *Caupolicana yarrowi* unless otherwise noted.

Shotgun metagenomic data from larval provisions of two *Ptiloglossa* brood cells corroborate the 16S rRNA gene amplicon data. Based on taxonomic classification of all SSU rRNA genes identified in the metagenomes, microbial community structure is dominated by *Lactobacillus* (Figure 6). Bacterial sequences not classified as *Lactobacillus* mostly (78.7%) belong to the order Lactobacillales; these either belong to related genera or lacked a genus-level classification. We also used these data to quantify the relative abundances of non-bacterial microbes such as fungi. The latter are rare (Figure 6). Fungi occur at 0.502 and 0.120% in the two larval provisions samples. 87.7% of the unidentified eukaryotic sequences—those not classified as host (Metazoa), plant, or fungi—lacked any classification below the Domain level and may be artifactual. *Acanthamoeba* and *Plasmodium* were present, but at very low levels ($\leq$ 32 sequences total). As a control, a mock community (Zymo) was also sequenced and processed alongside the larval provisions. The proportion of *Lactobacillus* in this sample is fairly accurate (13.1% versus expected 12%) and fungi are somewhat overrepresented (13.5% versus expected 4%).

As amplicon sequencing and metagenomics are compositional methods, they do not directly quantify the absolute abundance (biomass) of microbes in a sample. However, variation in the ratio of bacterial to plant SSU rRNA sequences may be used as a proxy for variation in bacterial biomass (relative to plant biomass). We find that the bacteria:plant ratio in *Ptiloglossa* larval provisions is substantially higher than nine non-colletid bee species assayed with the same methodology (Figure 6). This suggests there is likely more bacterial biomass per brood cell in *Ptiloglossa* as compared with many other bee species, in line with inferences of microbial activity based on the presence or absence of fermentation odors. Note it is possible that *Ptiloglossa* females provision brood cells with less pollen than other bees, which would also increase the bacteria:plant ratio.

We further used the shotgun metagenomes to explore sub-ASV-level diversity of lactobacilli in the two *Ptiloglossa* brood cell samples. Single-sample assemblies resulted in one medium-quality and one low-quality metagenome-assembled genome (MAG) per sample (see The Genome Standards Consortium et al., 2017, for quality definitions; Table 1). The medium-quality MAGs are classified as *Apilactobacillus*, matching results from blastn searches of dominant ASVs in the 16S rRNA amplicon dataset. One sample contains an *A. micheneri* MAG, while the other contains an *A. timberlakei* MAG, with high average nucleotide identity (98.5–99%) to previously sequenced genomes (Table 1). As measured using single-copy core genes, bins have high levels of strain heterogeneity (Table 1). This metric evaluates the degree to which reported contamination comes from conspecific strains, versus heterospecifics (Parks et al., 2015). High values suggest that multiple closely related strains were co-assembled into the same MAG. Thus there is likely intrageneric *Apilactobacillus* diversity within each of the two brood cells, including sample PA.LP.9, which contains only a single ASV classified as *Lactobacillus* in the 16S rRNA amplicon data (Figure 5).

To investigate possible host-specificity between *Ptiloglossa* and *Apilactobacillus*, we constructed a phylogeny using full-length 16S rRNA gene sequences assembled from the metagenomes. Only a single *Apilactobacillus* sequence was reconstructed from each of the two brood cells using phyloFlash. This does not necessarily conflict with the evidence of within-sample *Apilactobacillus* diversity (Figure 5; Table 1), as the assembler tends to collapse strain-level variability into a single, approximately species-level, consensus sequence (Gruber-Vodicka et al., 2020). Closely matching sequences and outgroups were collected from GenBank. The phylogeny (Figure 7) generally agrees with prior phylogenomic analysis of *Apilactobacillus*, with two sister clades corresponding to *A. micheneri* and *A. timberlakei* (Vuong and McFrederick, 2019). Matching the assembly data (Table 1), one *Ptiloglossa* brood cell (PA.LP.1) has a consensus 16S sequence belonging to *A. micheneri*, while the other (PA.LP.9) has a consensus sequence belonging to *A. timberlakei*. Thus, *Ptiloglossa* exhibit some degree of species-level flexibility in their symbiosis with *Apilactobacillus* (note that these two species are very closely related; Vuong and McFrederick, 2019; Wittouck et al., 2019). Conversely, *Apilactobacillus* are not specialized to particular bee hosts.

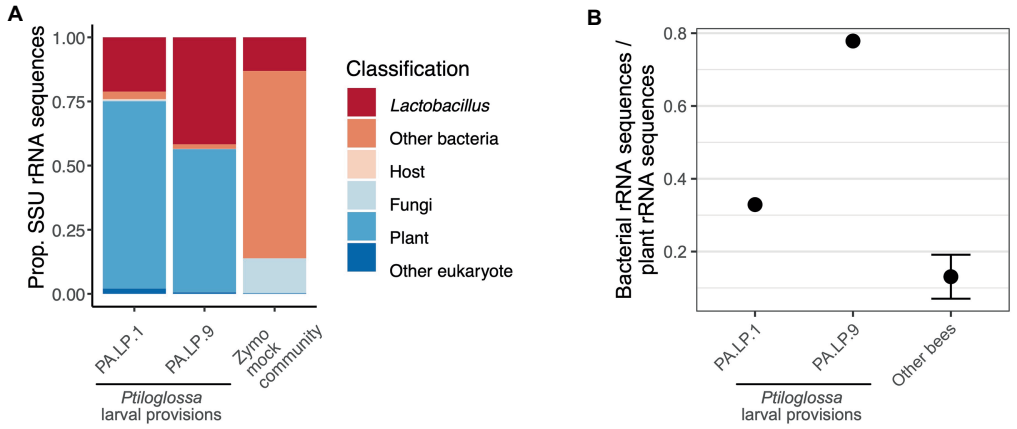

FIGURE 6
(A) Taxonomic composition of small subunit rRNA sequences in shotgun metagenomes of two *Ptiloglossa* larval provisions, and a mock community (Zymo). Sequences classified as "Other eukaryote" lacked any classification below the Domain level and may be artifactual. Note that among *Ptiloglossa* provisions sequences labeled "Other bacteria," 79% are Lactobacillales (without a lower taxonomic classification). **(B)** The ratio of bacterial:plant rRNA sequences for metagenomes of two *Ptiloglossa* larval provisions, compared with metagenomes of larval provisions of apid and andrenid bees (9 bee species, 84 samples; unpub. data). For the latter, the mean +/− SEM is shown.

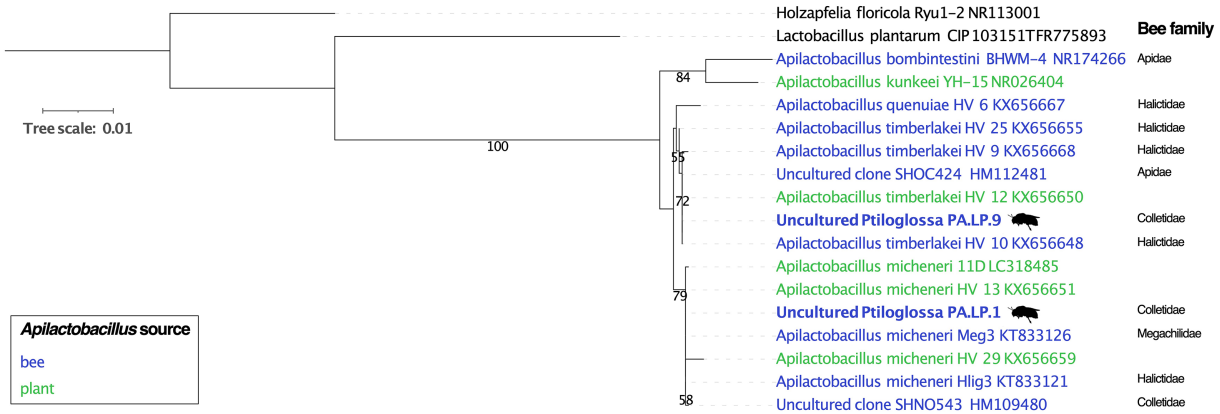

FIGURE 7
Phylogeny of *Apilactobacillus* and outgroups, including reconstructed 16S rRNA gene sequences from *Ptiloglossa* larval provisions (tip labels in bold, with bee silhouette), and close matches from Genbank. Only >50% bootstrap values are shown. NCBI accession numbers are given in the tip labels. The source (bee versus plant material) from which *Apilactobacillus* were isolated or sequenced is shown. For bee-derived *Apilactobacillus*, the family classification of the source bee is shown.

The *Ptiloglossa*-associated *A. micheneri* has 99.9–100% sequence identity to *A. micheneri* strains isolated from non-colletid bees and from nectar (McFrederick et al., 2018), and to a clone from the gut of a *Caupolicana yarrowi* adult collected in 2006 from southeastern Arizona (Martinson et al., 2011; Figure 7). Likewise, the *Ptiloglossa*-associated *A. timberlakei* has 99.9–100% sequence identity to additional isolates from non-colletid bees and nectar, and to a clone from the gut of a *Diadasia opuntiae* (Apidae) adult bee collected in 2012 from Sonora, Mexico (Martinson et al., 2011; Figure 7). *Diadasia* and *Ptiloglossa* co-occur in Arizona but use temporally non-overlapping floral resources. *Diadasia opuntiae* forage nearly exclusively on cactus (*Opuntia* and *Carnegiea*) flowers for nectar and pollen (Ordway, 1984). Therefore, sharing of lactobacilli between these bees is likely mediated by other flower visitors.

Discussion

Across all three diphaglossine bee species, in both the Sonoran desert (Arizona, United Sates) and premontane tropical rainforest (Western Panama), microbial communities of larval provisions are near-monocultures of lactobacilli. Larval provisions of *Ptiloglossa* and *Caupolicana* specifically harbor *Apilactobacillus* while those of *Crawfordapis* harbor unique and potentially novel *Lactobacillus*-related taxa. Thus, diphaglossine bees fit an emerging pattern of symbiotic interactions between bees and lactobacilli, spanning solitary species, social species, the adult stage, and brood cells (Kwong and Moran, 2016; McFrederick et al., 2017; Voulgari-Kokota et al., 2019; Tang et al., 2021; Handy et al., 2023).

Consistency of the diphaglossine bee brood cell microbiome is unlikely to come from vertical transmission. Lactobacilli disappear from larvae before metamorphosis—a common phenomenon in gut symbionts of holometabolous insects (Hammer and Moran, 2019). Furthermore, phylogenetic evidence from *Ptiloglossa* brood cells indicates that *Apilactobacillus* are not host-specific; host specificity (or host restriction) is a common feature of vertically transmitted symbionts (Moran et al., 2019). *Apilactobacillus* appear to be frequently exchanged with other bees via flowers, as is the case in other solitary bee-lactobacilli associations (McFrederick et al., 2014, 2017). Brood cell microbiome consistency is likely mediated instead by horizontal transmission coupled with strong ecological filtering (or partner choice; Sachs et al., 2004). *Agave palmeri* nectar (visited by *Ptiloglossa*) does contain detectable levels of lactobacilli, but amid a diverse and heterogeneous background of bacteria. Similarly, a culture-based study in southern Arizona found that *Apilactobacillus* is present in *Agave palmeri* nectar, but at a low abundance relative to other bacteria and yeasts (von Arx et al., 2019). The traits that allow *Apilactobacillus* to colonize brood cell microbiomes are not known, but may include tolerance of low pH, osmotic stress, and oxidative stress, and the ability to metabolize nectar carbohydrates (particularly fructose) and attach to host tissues (Vuong and McFrederick, 2019). Where and when filtering takes place are also unknown, but given that *Apilactobacillus* dominates even freshly provisioned brood cells, one possibility is that it begins in the female bee's crop. Microbes have been observed at higher densities in the bee crop as compared with nectar (Batra et al., 1973), and the crop of *Xylocopa sonorina* bees is strongly enriched in *Apilactobacillus* (Handy et al., 2023). We cannot directly address this hypothesis because our adult bee samples include the midgut and hindgut in addition to the crop, and these regions may have highly distinct microbiomes (Kwong and Moran, 2016).

Although brood cell microbiomes are homogenous at the level of bacterial genera—*Apilactobacillus* in *Ptiloglossa* and *Caupolicana*, unclassified *Lactobacillus* in *Crawfordapis*—there is diversity at finer phylogenetic scales. Most individual brood cells of all three bee species harbor lactobacilli comprising multiple amplicon sequence variants (ASVs). In *Ptiloglossa*, there is evidence of further diversity not detectable at the ASV level. Across brood cells, *Ptiloglossa* cultures at least two closely related species of *Apilactobacillus—micheneri* and *timberlakei*. Within a brood cell, there is also strain-level diversity (Table 1). Thus, ecological filtering in *Ptiloglossa* brood cells is somewhat porous, permissive of multiple species and strains of *Apilactobacillus*. Whether sub-generic diversity of *Apilactobacillus* has

functional consequences for bee development is not known. *A. micheneri* and *A. timberlakei* are sister species, and gene sets involved in carbohydrate metabolism generally overlap between their genomes (Vuong and McFrederick, 2019). But there is also evidence for divergence, particularly in genes mediating tolerance of environmental stressors (Vuong and McFrederick, 2019). Partnering with multiple symbiont species and strains can be advantageous to hosts (Batstone et al., 2018). For example, the ability to culture multiple microbial partners with distinct niches could buffer the brood cell symbiosis against environmental variation and against a heterogeneous distribution of *Apilactobacillus* species in flowers. At the same time, bees may not be able to discriminate among closely related lactobacilli. For example, legumes are often unable to selectively exclude certain strains of rhizobia from colonizing root nodules, even those that are ineffective mutualists (Hahn and Studer, 1986; Simms and Taylor, 2002).

Microbiomes of developing *Ptiloglossa* larvae are statistically indistinguishable from those of their diet. Further, microbial variation among provisions is reflected in the corresponding larvae. A similar pattern occurs in caterpillars, where ingested bacterial communities pass transiently through the gut (Whitaker et al., 2016; Hammer et al., 2017). This result suggests that: (i) there are no substantial populations of unique symbionts inhabiting the gut of *Ptiloglossa* larvae; (ii) there is no additional ecological filtering of the provisions once ingested. Larvae of several other solitary bees, as well as honey bee larvae, appear to be similar in these respects (Martinson et al., 2012; McFrederick et al., 2014, 2017; Kapheim et al., 2021). A strategy of suppressing bacterial colonization makes sense in light of the fact that most developing solitary bee larvae do not defecate until after they have completed feeding (Danforth et al., 2019); it may be too risky to allow microbial proliferation without a way of expelling excess cells. In contrast, we do observe a strong difference between provisions and whole-body larval microbiomes in *Crawfordapis*, mediated by the presence of *Wolbachia* in larvae. *Wolbachia* is also abundant in adult *Crawfordapis*. *Wolbachia* is fairly common in temperate bees (Gerth et al., 2015), and in at least some tropical bees such as *Megalopta centralis* (McFrederick et al., 2014).

Although dominated by lactobacilli, diphaglossine bee brood cells do harbor a variety of rarer bacteria. *Saccharibacter* is sporadically abundant in *Ptiloglossa* brood cells and adults, and in *Agave* nectar. *Saccharibacter* and other aerobic, acid-tolerant, and osmophilic Acetobacteraceae are commonly found in insects with sugar-rich diets (Crotti et al., 2010), on pollen (Jojima et al., 2004), and in the crop (Handy et al., 2023) and pollen provisions of bees (McFrederick et al., 2012). Fungal sequences are also present, but at very low relative abundances, in shotgun metagenomes from *Ptiloglossa* larval provisions. The lack of a substantial fungal community contrasts with earlier reports of yeasts in neotropical *Ptiloglossa* brood cells (Roberts, 1971; Batra et al., 1973). One possible explanation is that the presence of yeasts differs between *Ptiloglossa* in temperate deserts (studied here) versus tropical forests. Another explanation, which cannot be fully excluded, is a technical bias against fungi. However, our mock community metagenome harbors more yeast sequences than expected, and the DNA extraction protocol we used is similar to that used for fungal sequencing in sourdough starters, soil and other habitats (Rousk et al., 2010; Landis et al., 2021).

Although similar to related diphaglossines and to other solitary bee groups in terms of composition and host specificity (Figure 2; McFrederick et al., 2013, 2017; Kapheim et al., 2021), the abundance and activity of *Ptiloglossa* brood cell microbiomes appear to be unusual. First, larval provisions of *Ptiloglossa* have a much higher ratio of bacterial to plant DNA as compared with several other ground-nesting solitary bee species. Second, they are consistently observed to exhibit strong fermentation odors, which are only rarely reported from healthy brood cells of solitary bees. These two lines of evidence suggest that there has been a quantitative, but not qualitative, microbiome shift within diphaglossine bees. As more abundant beneficial microbes will generally confer stronger benefits to hosts (up to a point; Hammer et al., 2019), this shift may constitute a functionally novel form of symbiosis in *Ptiloglossa*.

Novel host-microbe symbioses often evolve through a process analogous to domestication, with capture and vertical transmission of host-restricted symbionts (Moran et al., 2019; Ganesan et al., 2022). Humans also domesticate microbes, propagating starter cultures used for fermentation of certain foods and beverages (Gibbons and Rinker, 2015; Steensels et al., 2019). In contrast, *Ptiloglossa* appear to culture undomesticated bacteria in their larval provisions. If confirmed, this result would demonstrate that quantitative microbiome shifts can evolve without changes in host specialization. A caveat is that symbiont domestication can occur rapidly (Stallforth et al., 2013; Bodinaku et al., 2019), without concomitant changes in 16S rRNA gene sequences. Another caveat is that our results are limited to *Ptiloglossa arizonensis*. The common ancestor of *Ptiloglossa* was most likely a tropical species, given that most extant diversity is in the tropics and subtropics (Michener, 2007), and the sister genus *Crawfordapis* (following, Velez-Ruiz, 2015) is also tropical. Hence it is not yet clear whether the undomesticated nature of *P. arizonensis* brood cell symbionts is ancestral or derived (e.g., related to adaptation to desert environments).

Our results suggest that the unusually fermentative brood cell microbiomes of *Ptiloglossa* evolved not through domestication, but simply by modification of the culturing environment. This path to symbiotic novelty has parallels with animals such as *Riptortus* bugs, which use modified gut structures to enrich non-host-specialized *Burkholderia* from the diet (Kikuchi et al., 2007; Ohbayashi et al., 2015). Some other animals behaviorally modify their environment to promote the growth of undomesticated "crops" (Zhu et al., 2016; Selden and Putz, 2022). Analogously, spontaneous (or natural) fermentation of certain foods and beverages relies on wild, undomesticated microbes. Lactobacilli often participate in this process; for example, *Apilactobacillus micheneri* (strain 11D in Figure 7) is a dominant member of the bacterial community in kôso, a fermented vegetable drink (Chiou et al., 2018). What traits enable *Ptiloglossa* to culture *Apilactobacillus* at high densities in their brood cells? Facilitated by waterproof brood cell linings, colletid bees in general, and *Ptiloglossa* in particular, tend to have more liquid larval provisions than other solitary bees (Roberts, 1971; Rozen, 1984; Almeida, 2008; Cane and Love, 2021). We hypothesize that this relatively high water content may facilitate microbial growth. How exactly highly liquid provisions are achieved is not known, but one possibility is that *Ptiloglossa* collect a large volume of nectar relative to pollen. *Ptiloglossa* often forage from flowers (like *Agave*) that produce large quantities of nectar (see Natural History). Another possibility is that by foraging in the early morning, *Ptiloglossa* collect particularly dilute nectar. Nectar is generally more dilute when first produced (Cane and Love, 2021).

As hypothesized by Roberts (1971), high bacterial densities in larval food likely benefit *Ptiloglossa* development. The elevated ratio of bacterial to plant DNA suggests that bacterial biomass is a major nutrient source for larvae, alongside pollen and nectar. Although almost all bee species are herbivorous (Michener, 2007), *Ptiloglossa* may have commonalities with insects that derive nutrition from microbes, such as *Drosophila* and dung beetles (Markow and O'Grady, 2008; Holter, 2016). Pollen and nectar contain all of the nutrients required for bee development (Roulston and Cane, 2000), but a "brewing" strategy could be economical for *Ptiloglossa*, a bee that almost exclusively forages from just before, to just after dawn. If bacteria upgrade the nutritional quality of the provisions, *Ptiloglossa* females may be able to rear more offspring despite a highly constrained foraging window. Brood cell bacteria could also play a role in defense. Ground-nesting, immature Hymenoptera (and their food) are vulnerable to attack by soil-borne microbes. Hence, both endogenous and bacterially based defenses are common (Kaltenpoth et al., 2005; Fernández-Marín et al., 2009; Strohm et al., 2019). For *Ptiloglossa*, the organic acids (and potentially ethanol) resulting from *Apilactobacillus* fermentation may suppress microbial invaders (McFrederick et al., 2018; Vuong and McFrederick, 2019) analogously to the anti-spoilage properties of lacto-fermented food. Indeed, despite the common presence of fungi in nectar and soil, fungal growth in *Ptiloglossa* brood cells appears to be kept to a minimum. Enrichment for lactobacilli occurs even before eggs are laid, potentially acting as a prophylactic antimicrobial defense for offspring, as occurs in some animals (Flórez et al., 2017; Kerwin et al., 2019).

To understand whether the diphaglossine bee brood cell symbiosis constitutes a mutualism, effects on bacterial fitness also need to be determined. Many insects have mechanisms to transmit symbionts vertically (Buchner, 1965), which more closely align fitness interests between hosts and symbionts (Ewald, 1987; Sachs et al., 2004). Many food fermentation practices also involve the reuse of starters or culture vessels, allowing domestication to occur. But, while the diphaglossine bee brood cell is clearly a highly favorable environment for local and short-term growth of lactobacilli, the bacteria may ultimately be digested by the larva or otherwise fail to escape alive. Indeed, lactobacilli consumed by larvae do not persist internally through the prepupal and pupal stages; we hypothesize that adults acquire them anew each generation from flowers. Other mechanisms by which bees could propagate lactobacilli need to be tested but seem unlikely. In theory, adults emerging from brood cells could acquire lactobacilli externally. But first, lactobacilli would need to survive for potentially several months *ex vivo*, as *Ptiloglossa arizonensis*, like many bees, overwinter as post-defecating prepupae (Rozen, 1984; Michener, 2007). Opportunities for emerging adults to contact residual lactobacilli in the brood cell are also limited. Postdefecating larvae of most diphaglossines pierce the cell lining such that feces drains into the soil; once the cocoon is spun, there is little direct exposure to remaining fecal material or the cell lining (Rozen, 1984).

If the dead-end hypothesis is correct, it implies a strong contrast with horizontally transmitted mutualisms in which symbionts benefit from their associations with hosts. For example, in the legume-rhizobia and bobtail squid-*Vibrio* mutualisms, hosts release the symbionts they culture back into the environment in large numbers (Lee and Ruby, 1994; Simms and Taylor, 2002). Indeed, adult diphaglossine bees harbor lactobacilli in their gut, and are likely important for their dispersal and persistence in the bee-flower niche. But the brood cell association may be more exploitative, with little to no long-term benefit to the lactobacilli.

In sum, the nesting biology of these bees appears to create favorable conditions for spontaneous fermentation or "brewing" of generalist lactobacilli: underground, temperature-stable brood cells; maintenance of high water content in provisions; suitable sugars (especially fructose); protection from contamination by the cellophane-like cell lining; open cells, possibly to allow venting; and, potentially, pre-enrichment of lactobacilli in the adult crop. Given the consistency of brood cell fermentation, particularly in *Ptiloglossa*, the bees likely benefit from culturing lactobacilli. On the other hand, lactobacilli may not benefit from being cultured, as they seem unable to escape the brood cell. While many details remain speculative, our findings provide an initial picture of the microbiology and ecology of a remarkable feat of fermentation.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA925568.

Author contributions

TH, JK, QM, SB, and BD designed the study. JK, QM, WW, and BD helped acquire grant funding. TH, JK, SB, and BD conducted fieldwork. TH, JK, MA-G, and LG conducted labwork. TH and MA-G performed bioinformatic and statistical analyses. TH created the figures and drafted the manuscript, which was then read and edited by all authors. All authors contributed to discussion and interpretation of results.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1114849/full#supplementary-material

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Xylocopa caerulea and *Xylocopa auripennis* harbor a homologous gut microbiome related to that of eusocial bees

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Background: Eusocial bees, such as bumblebees and honey bees, harbor host-specific gut microbiota through their social behaviors. Conversely, the gut microbiota of solitary bees is erratic owing to their lack of eusocial activities. Carpenter bees (genus *Xylocopa*) are long-lived bees that do not exhibit advanced eusociality like honey bees. However, they often compete for nests to reproduce. *Xylocopa caerulea* and *Xylocopa* auripennis are important pollinators of wild plants on Hainan Island. Whether they have host-specific bacteria in their guts similar to eusocial bees remains unknown.

Methods: We targeted the bacterial 16S rRNA V3-V4 region to investigate the diversity of bacterial symbionts in the fore-midgut and hindgut of two carpenter bees, *X. caerulea* and *X. auripennis*.

Results: A maximum of 4,429 unique amplicon sequence variants (ASVs) were detected from all samples, belonging to 10 different phyla. *X. caerulea* and *X. auripennis* shared similar bacterial community profiles, with Lactobacillaceae, Bifidobacteriaceae, and Orbaceae being dominant in their entire guts. *X. caerulea* and *X. auripennis* harbor a highly conserved core set of bacteria, including the genera *Candidatus Schmidhempelia* and *Bombiscardovia*. These two bacterial taxa from carpenter bees are closely related to those isolated from bumblebees. The LEfSe analysis showed that Lactobacillaceae, Bifidobacteriaceae, and the genus *Bombilactobacillus* were significantly enriched in the hindguts of both carpenter bees. Functional prediction suggested that the most enriched pathways were involved in carbohydrate and lipid metabolism.

Conclusions: Our results revealed the structure of the gut microbiota in two carpenter bees and confirmed the presence of some core bacterial taxa that were previously only found in the guts of social bees.

KEYWORDS

carpenter bees, gut microbiota, bacteria symbiosis, *Candidatus Schmidhempelia*, *Bombiscardovia*, eusocial bees, *Bombilactobacillus*

Introduction

Bees are well-known for their abundant biodiversity, with more than 17,500 identified species (Danforth et al., 2013). Among them, honey bees, bumblebees, and stingless bees are domesticated by humans to provide essential pollination services for crops and fruit trees (Brittain et al., 2013). These bees exhibit social behaviors and have a complete social structure, whereas most other bee species are solitary. Solitary bees

have broad geographic distribution, varying body sizes, and diverse foraging preferences, making them irreplaceable pollinators in the ecological environment with flowering plants, even in places where domestic honey bee populations exist (Hedtke et al., 2013). However, the ecosystem roles of solitary bees have often been overlooked by researchers. Most studies have focused on eusocial bees, such as the honey bee *Apis mellifera*, while the study of solitary bees has been scarcely investigated.

The microbiota represents a vital indicator of the fitness and health of numerous insect species. Moreover, microorganisms play a critical role in many interactions between insect hosts and their habitats. For example, in the camellia weevil *Curculio chinensis*, the microbiota was responsible for tea saponin degradation in the insect's feeding (Zhang et al., 2020). Even though symbionts can be beneficial for their host, they can also bring negative effects to some insects. Among arthropods, *Wolbachia* spp. has been identified as a bacteria symbiont that distorts the reproductive cells, thereby enhancing its maternal transmission into subsequent progenies. Consequently, it has been deemed a novel pest biocontrol bacterium (Ali et al., 2018). The hispid leaf beetle *Octodonta nipae* is naturally infected with *Wolbachia*, which has been identified as an obligate endosymbiont present in all life stages, body parts, and tissues that were tested (Ali et al., 2019). Similar to other insects, the bacteria symbiont of honey bees plays a critical role in their survival. Massive losses of honey bee colonies and the decline of many bumblebee species have elicited global concern in recent decades (Lee et al., 2015; Hammer et al., 2021). A large body of evidence suggests that gut microbiota is critical in maintaining bee health (Engel et al., 2016; Jones et al., 2017; Zheng et al., 2018). Previous studies have shown that honey bees and bumblebees harbor distinctive core gut bacterial communities that are transmitted through social behaviors such as oral trophallaxis and fecal–oral pathway (Khan et al., 2020; Hammer et al., 2021). The abundance and species of core bacteria have been found to be remarkably stable, showing little effect across various habitats (Anderson et al., 2015; Kwong and Moran, 2016; Hammer et al., 2021). Host-specific bacteria have diverse functions in the digestion and absorption of nutrients, as well as in defending against pathogen colonization and reinforcing the host's immune system (Zheng et al., 2017; Kešnerová et al., 2019; Ribière et al., 2019). However, most studies have focused on interactions between gut bacteria and social bees, such as *A. mellifera* and *Bombus terrestris*, and the study of solitary bees has been scarcely investigated.

Carpenter bees belong to one taxon of wild bees (genus: *Xylocopa*) and are known for their large body size. They play a crucial role in crop pollination due to their greater pollination efficiency compared to honey bees in some cultivated large-flower plants such as passion fruit *Passiflora edulis* (Junqueira and Augusto, 2016; Alberoni et al., 2019; de Farias-Silva and Freitas, 2021). The *Xylocopa* genus comprises about 470 species worldwide (Michener, 2000) and 40 species mainly described in tropical and subtropical China (Wu, 2000). The social behavior of the genus *Xylocopa* is not well-understood. Although incipient social behaviors have been observed in some species in the wild, knowledge is lacking for most species (Handy et al., 2023). Female *Xylocopa* excavates their nests in dry plant tissues, such as trees, dead trunks, and bamboo canes (Junqueira et al., 2012), and lay eggs in cells with pollen and nectar (Keasar et al., 2007). Unlike social bees, nests of *Xylocopa* are often reused for several years (Yamamoto et al., 2014). Newly emerged female *Xylocopa* leave their old nest and find other abandoned nests to reproduce during the nesting seasons. Nest reusing is more common in an environment of limited nesting materials. In addition to the aforementioned characteristics, fighting between female *Xylocopa* for nests can also result in the reuse of old nests (He et al., 2013). Nest reusing is generally a common behavior in the genus *Xylocopa*.

At present, the composition of microbiota in *Xylocopa* species is rarely studied, with most of the microbiota species of these bees remaining unclear. However, a few available studies suggest that carpenter bees have consistent relationships with specific bacterial taxa. *Xylocopa micans*, *Xylocopa mexicanorum*, and *Xylocopa tabaniformis parkinsoniae*, which are carpenter bees from central Texas, were found to share similar gut bacterial communities, including Bifidobacteriaceae, Orbaceae, Lactobacillaceae, Pseudomonadaceae, and Enterobacteriaceae. Meanwhile, *Xylocopa virginica* had a distinct microbiota dominated by the genus *Bombilactobacillus*, a group of bacteria abundant in the guts of eusocial bees (Holley et al., 2022). In *Xylocopa tenuiscapa*, the diversity of bacteria in the foregut and hindgut were found to be different, and certain species, such as *Gilliamella*, *Lactobacillus*, and *Bifidobacterium,* were found to be related to those found in honey bees (Subta et al., 2020).

In the present study, we investigated the gut bacteria of two *Xylocopa* species (*X. caerulea* and *X. auripennis*) from different ecological environments. *X. caerulea* is found in Jianfengling National Forest Park of Hainan (~838 m above sea level), which is a tropical forest area rich in natural resources and one of the best protected in the region. On the other hand, *X. auripennis* inhabits mountainous villages with ample farmland (~650 m above see level). Symbionts are spatially organized within specific gut regions (Zheng et al., 2017, 2018). Here, we used 16S rDNA sequencing to investigate the gut bacterial communities in different parts of the intestinal tract of two *Xylocopa* species. Our results showed that these two carpenter bee species have consistent gut communities and specific gut symbionts that are commonly found in bumblebees. This finding provides novel insights into the symbiotic gut communities of solitary bees.

Materials and methods

Sample collection

Pollinating carpenter bee samples (*X. caerulea* and *X. auripennis*) were used for gut microbial composition analysis. Six *X. caerulea* were collected from the Jianfengling National Forest Park in Hainan (18°44′28″N, 108°51′39″E) and six *X. auripennis* were collected by sweep nets in April 2022 in Hainan Province from Zhahan village of Hongmao town, Qiongzhong Li, and Miao Autonomous County (19°4′58″N, 109°38′59″E; Figure 1). The collected carpenter bees were transported to the laboratory in a bubble chamber with ice packs.

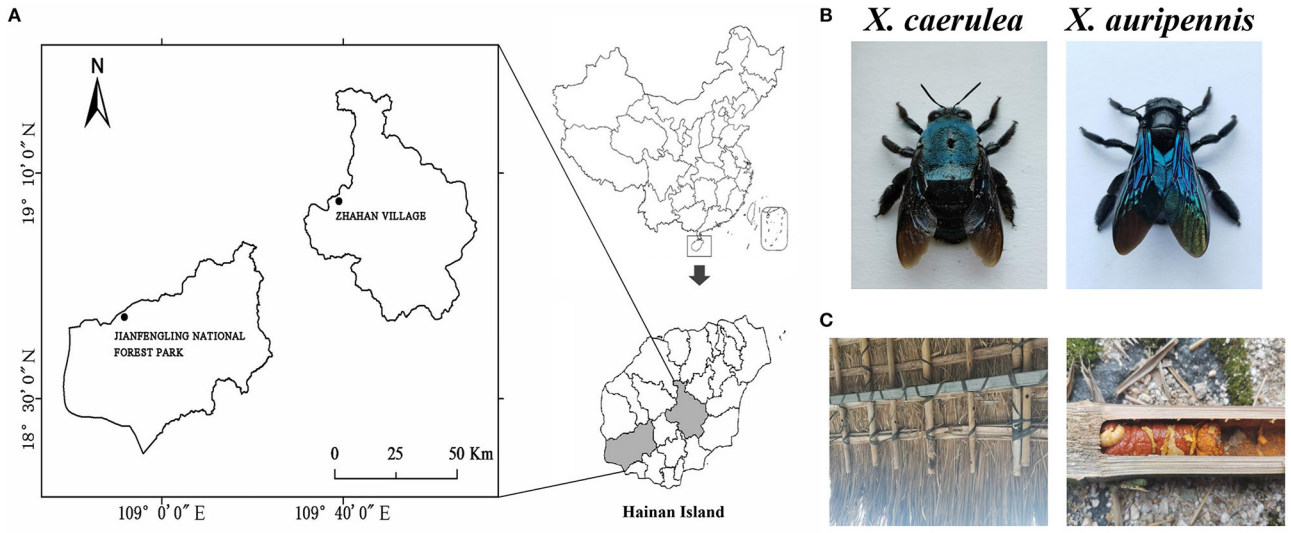

FIGURE 1
Collecting locations of carpenter bees. **(A)** Jianfengling National Forest Park in Hainan (18°44′28″N, 108°51′39″E) and Zhahan village of Hongmao town in Qiongzhong Li and Miao Autonomous County (19°4′58″N, 109°38′59″E), **(B)** *Xylocopa caerulea* and *Xylocopa auripennis*, and **(C)** nest and larvae of *X. auripennis*.

Insect dissection and DNA extraction

To extract bacterial DNA, *X. caerulea* and *X. auripennis* were dissected immediately upon arrival at the laboratory. After freezing the live carpenter bees in a −20°C refrigerator, each bee was washed three times with 75% alcohol and then several times with sterile water. The whole gut of each carpenter bee was carefully removed using sterile tweezers and scissors. Then the gut was divided into two parts: hindgut and fore-midgut (including crop), which were immediately placed in a 1.5 ml centrifuge tube, respectively, frozen in liquid nitrogen, and stored at −80°C refrigerator until further analysis. Six replicates of the dissected intestinal tract of the carpenter bee (one individual/sample) were processed for DNA extraction.

DNA extraction was carried out using the E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer's instructions. Nuclear-free water was used as a blank. The total DNA was eluted in 50 μl of elution buffer (Tris–hydrochloride buffer, pH 8.0, containing 1.0 mM EDTA) and stored at −80°C until usage for the PCR.

16S rRNA amplification

For each gut DNA sample, PCR was conducted for the V3–V4 region of bacterial 16S rRNA gene using the primer set 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). The 5′ ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. PCR amplification was performed in a total volume of 25 μl of reaction mixture containing 25 ng of template DNA, 12.5 μl of PCR Premix, 2.5 μl of each primer, and PCR-grade water to adjust the volume. The PCR conditions are divided into two steps: initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, and then the final extension at 72°C for 7 min. In the second step, there was an initial denaturation at 98°C for 30 s, followed by seven cycles of denaturation at 98°C for 10 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s, then the final extension at 72°C for 5 min.

After amplification, the PCR products were confirmed with imaging of 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water was used instead of a sample DNA to exclude false-positive PCR results as a negative control.

Library preparation and sequencing

The PCR products underwent purification using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantification using Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed using an Agilent 2100 Bioanalyzer (Agilent, USA) and with the library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on the NovaSeq PE250 platform. Sequencing and bioinformatics analyses were performed by a commercial company (Biotree, Shanghai, China).

Sequence analysis

Paired-end reads were assigned to samples based on their unique barcode and primer sequence. The paired-end reads were merged using FLASH software. Quality filtering was performed on the raw reads under specific filtering conditions to obtain high-quality clean tags according to the fqtrim (v0.94). Chimeric sequences were filtered using V search software

(v2.3.4). After dereplication using DADA2, we obtained a feature table and associated sequences. After that, amplicon sequence variants (ASVs) were clustered and annotated at a 97% similarity threshold. Sequences with ambiguous, chloroplast, or mitochondrion assignments were removed. Alpha diversity and beta diversity were calculated by normalizing them to the same sequences randomly. According to the SILVA (release 132) classifier, feature abundance was normalized using the relative abundance of each sample. Alpha diversity was applied in analyzing the complexity of species diversity for a sample through five indices, including Chao1, Observed species, Goods coverage, Shannon, and Simpson.

Bacterial phylogenetic reconstruction

Amplicon sequence variants were subjected to the BLAST approach against the NCBI nucleotide collection database for phylogenetic construction. The phylogenetic tree was built based on the sequence alignment using the neighbor-joining (NJ) algorithm in the software of Mega X program (Kumar et al., 2018). The reliability of the branching was tested by bootstrap resampling (1,000 pseudo-replicates).

Putative functional profiling

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2; https://github.com/picrust/picrust2) was adopted for the functional prediction of gut microbiota (Douglas et al., 2020). Functional community profiling was predicted based on the bacterial 16S rDNA gene ASVs associated with different parts of the intestinal tract. Sequenced prokaryotic genomes of 16S rDNA gene sequences were linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog for functional annotation.

Statistical analysis

In this study, QIIME2 was used to compare sample complexity and diversity. The basic analysis of ASVs was performed, including the generation of a Venn diagram of ASVs distribution and ASV cluster analysis. Principal coordinate analysis (PCoA) was performed on unweighted UniFrac distance matrices to study the similarities of differences in sample community composition. The differences in the community structure of the gut microbiota at five levels (ranging from phylum to genus) between two species and hindgut and fore-midgut were analyzed using non-parametric factorial Mann–Whitney *U*-test ($P < 0.05$) and estimated LDA score using linear discriminant analysis effect size (LEfSe), with an LDA threshold of ≥ 3. PCoA analysis was calculated and visualized using R statistical software (Lockstone, 2011). The STAMP software (version 2.1.3) was employed to identify the significant differences in the relative abundance of predicted gene proportion between the fore-midguts and hindguts of two carpenter bee species (Welch's *t*-test, $P < 0.05$).

Results

Bacterial diversity estimation

A total of 1,695,348 valid sequences of the16S rDNA gene were acquired from six *X. caerulea* and six *X. auripennis* samples after stringent quality checking (Supplementary Table S1). A maximum of 4,429 unique amplicon sequence variants (ASVs) were clustered based on a 97% similarity cutoff. Among these ASVs, there were 1,946 ASVs unique to *X. caerulea* and 1,569 ASVs unique to *X. auripennis*. The shared ASVs (914) accounted for 20.64% of total ASVs. These ASVs were presented in a Venn diagram (Figure 2A) and classified into 42 phyla, 126 classes, 258 orders, 411 families, 821 genera, and ~1,088 species. The rarefaction curves of bacterial diversity estimators (observed OTUs and Shannon) for all samples reached a plateau phase, indicating that most microbial species had been captured in all samples (Figures 2B, C). Meanwhile, Good's coverage was used to check the completeness of sequencing. The results showed that the coverage of each sample was above 99.99%, indicating that most species in the sample were identified. The alpha diversity indices were estimated to uncover the bacterial diversity (Simpson and Shannon), species richness (Chao1 and observed OTUs), and bacterial coverage (Good's coverage) (Table 1). Based on these indices, no significant differences were detected between *X. caerulea* and *X. auripennis* comparisons. Similarly, there was no significant difference between the hindgut and fore-midgut of *X. caerulea* and *X. auripennis*. The beta diversity estimates were calculated by computing unweighted UniFrac and visualized by principal coordinates analysis (PCoA). The results indicated that the gut bacterial communities of *X. caerulea* were not significantly different from those of *X. auripennis* (Figure 2D). Moreover, the bacterial communities in the hindgut were not significantly different from the fore-midgut in *X. caerulea* or *X. auripennis* (Figures 2E, F).

Relative abundance of gut bacterial communities

The two carpenter bee species *X. caerulea* and *X. auripennis* shared similar bacterial community profiles. At the phylum level (Figure 3A), Firmicutes was the dominant phylum, with a relative abundance of 67.26% in *X. caerulea* and 79.88% in *X. auripennis*. Actinobacteria, Proteobacteria, and Bacteroidota were also dominant in both species, with relative abundances of 17.94 and 9.29%, 12.32 and 8.41%, 0.99 and 1.37%, respectively.

At the family level (Figure 3B), *X. caerulea* and *X. auripennis* also shared similar bacterial community profiles. Lactobacillaceae was the most abundant family (44.17 and 64.12%), followed by Lactobacillales unclassified (20.80 and 13.18%), Bifidobacteriaceae (10.08 and 7.35%), Coriobacteriaceae (5.30 and 0.76%), Orbaceae (3.19 and 2.50%), and Acetobacteraceae (0.65 and 4.64%) in *X. caerulea* and *X. auripennis*, respectively.

At the genus level, the top 12 genera in relative abundance (>1%) were *Leuconostoc*, *Lactobacillales unclassified*, *Apilactobacillus*, *Bombilactobacillus*, *Bifidobacteriaceae unclassified*, *Lactobacillus*, *Coriobacteriaceae unclassified*, *Commensalibacter*,

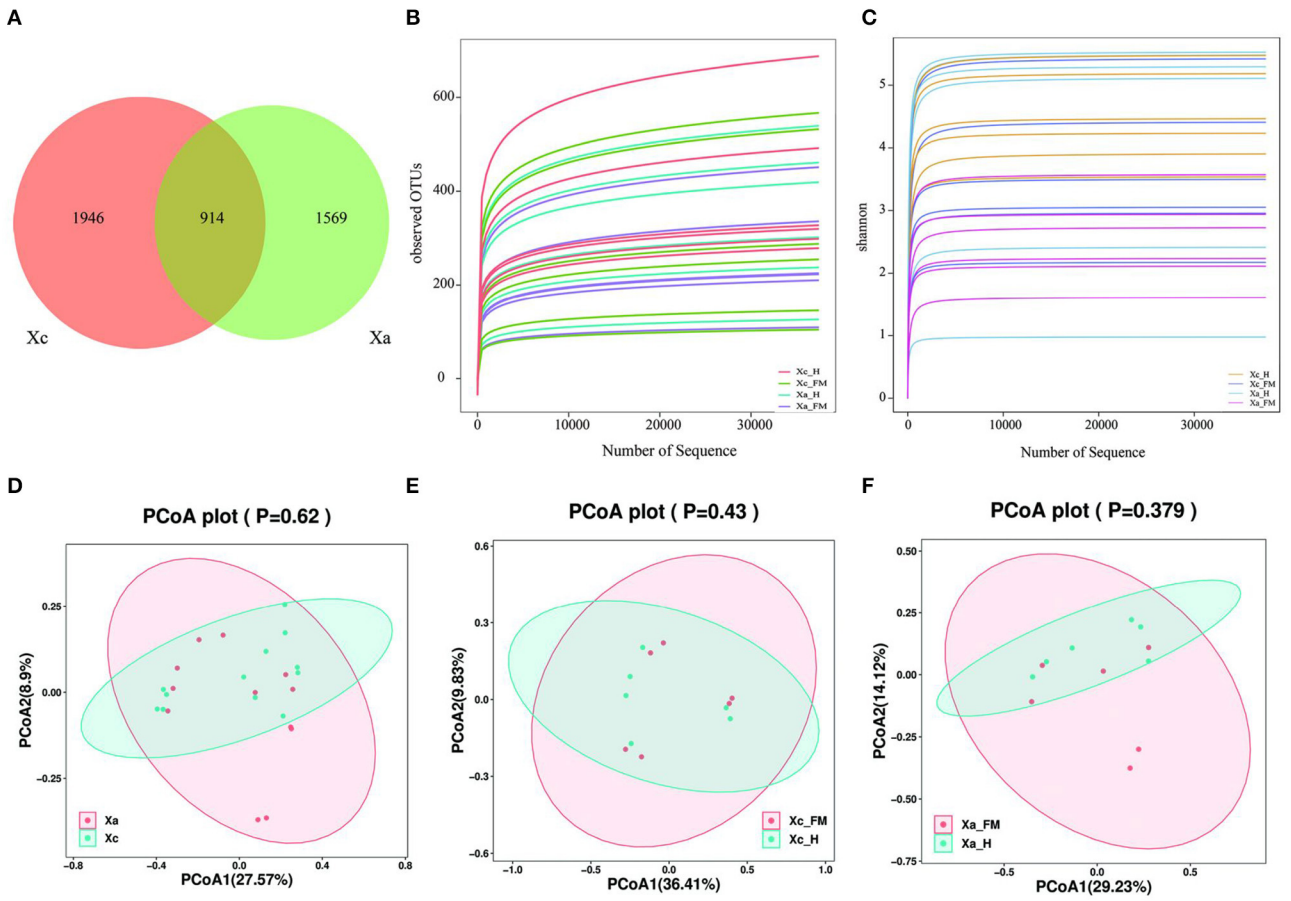

FIGURE 2
Gut bacterial diversity of carpenter bees from different locations in Hainan Island of China. **(A)** Venn diagram of the distribution of ASVs in the two carpenter bees. **(B)** Rank-abundance curves. **(C)** Shannon index rarefaction curves. **(D–F)** Unweighted UniFrac principal component analysis (PCoA) estimates for the gut bacteria of **(D)** *X. caerulea* and *X. auripennis* **(E)** fore-midgut and hindgut of *X. caerulea* **(F)** fore-midgut and hindgut of *X. auripennis*. Xa, *X. auripennis*; Xc, *X. caerulra*. Xc_FMG, the fore-midgut of *X. caerulra*; Xc_HG, the hindgut of *X. caerulra*; Xa_FMG, the fore-midgut of *X. auripennis*; Xa_HG, the hindgut of *X. auripennis*. The same is as follows.

TABLE 1 Alpha diversity indices of the gut microbiota of carpenter bees *X. caerulea* and *X. auripennis*.

Group	Diversity indices (mean ± standard deviation)				
	Observed OTUs	Shannon	Simpson	Chao1	Goods coverage (%)
Xc	370.50 ± 190.51	4.02 ± 1.04	0.80 ± 0.11	381.70 ± 195.73	>99.99
Xa	317.00 ± 146.88	3.33 ± 1.62	0.64 ± 0.27	327.00 ± 153.74	>99.99
Xc_FMG	324.50 ± 184.86	3.57 ± 1.05	0.76 ± 0.12	331.87 ± 189.65	>99.99
Xc_HG	413.67 ± 164.78	4.46 ± 0.68	0.84 ± 0.08	426.38 ± 176.69	>99.99
Xa_FMG	285.17 ± 124.34	2.53 ± 0.63	0.56 ± 0.17	286.35 ± 124.68	>99.99
Xa_HG	364.00 ± 152.14	4.13 ± 1.78	0.73 ± 0.30	364.25 ± 152.18	>99.99

Xa, *X. auripennis*; Xc, *X. caerulea*. Xa and Xc stand for the sequence data of whole guts in *X. caerulea* and *X. auripennis*, respectively. Xc_FMG, the fore-midgut of *X. caerulea*; Xc_HG, the hindgut of *X. caerulea*; Xa_FMG, the fore-midgut of *X. auripennis*; Xa_HG, the hindgut of *X. auripennis*. The same is as follows. There were no significant differences detected between Xa and Xc, Xc_FMG and Xc_HG, Xa_FMG, and Xa_HG. Data (mean ±SD) were analyzed by Mann–Whitney U-test.

Candidatus Schmidhempelia, *Comamonadaceae unclassified*, *Bifidobacterium*, and *Bombiscardovia*. A clustering analysis of species abundance similarity among the top 12 genera was performed and presented in a heat map (Figure 4). In the whole guts, *Leuconostoc* was 2.21-fold more abundant in *X. auripennis* (42.87%) compared to *X. caerulea* (19.40%), and *Apilactobacillus* was 0.35-fold abundant in *X. auripennis* (5.18%) compared to *X. caerulea* (14.92%). *Candidatus Schmidhempelia* and *Bombiscardovia* were first identified in carpenter bees and their relative abundances in *X. caerulea* and *X. auripennis* were similar. The proportion of *Candidatus Schmidhempelia* and *Bombiscardovia* was

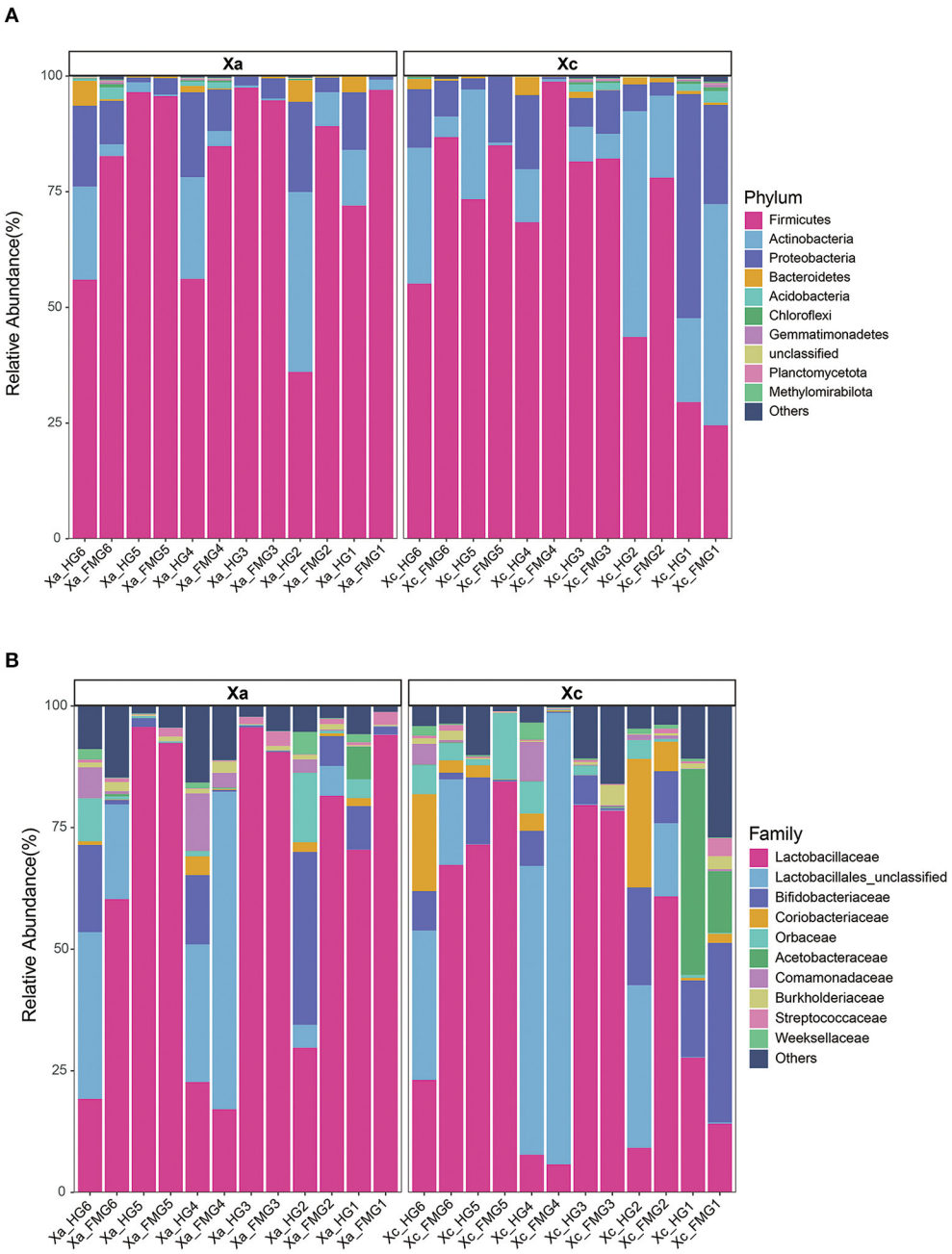

FIGURE 3
The gut bacterial composition of the carpenter bees on **(A)** phylum and **(B)** family. The top 10 taxa in abundance were shown in the bar charts. Each color represents a species, and the height of the color block indicates the proportion of the species in relative abundance. Other species are incorporated as "Others" shown in the diagram.

1.66% and 1.37% in *X. caerulea* and 1.47% and 1.33% in *X. auripennis*, respectively.

Comparison of the bacterial community between fore-midgut and hindgut

The relative abundance difference of bacterial sequences at the genus level between the fore-midgut and hindgut of two carpenter bees was analyzed (Figure 5). The results showed that the abundance of *Bombilactobacillus*, *Lactobacillus*, *Candidatus Schmidhempelia*, *Bifidobacterium*, *Bombiscardovia*, *Gilliamella*, *Apibacter*, *Atopobium*, and *Bacilli unclassified* in the hindgut was significantly higher than that detected in the fore-midgut.

Linear discriminant analysis effect size analysis confirmed abundance differences of specific taxa between the hindgut and fore-midgut. In the *X. caerulea*, LEfSe analysis revealed that Bacteroidota (phylum), Bacteroidia (class), Flavobacteriales and Bifidobacteriales (order), Weeksellaceae,

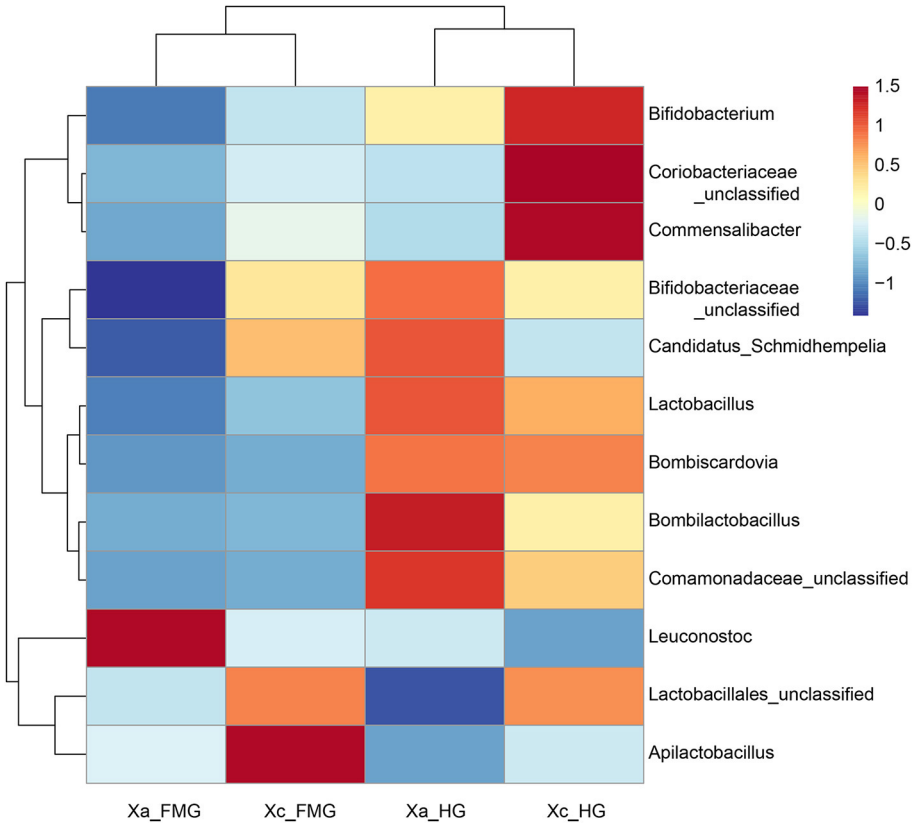

FIGURE 4
Heatmap of the 12 most abundant genera in the bacterial community at the genus level. Dendrograms of hierarchical cluster analysis grouping genera and samples are shown on the left and at the top, respectively.

Dysgonomonadaceae, Bifidobacteriaceae, and Bacilli unclassified (family), *Bombilactobacillus*, *Lactobacillus*, *Bombiscardovia*, *Bifidobacterium*, *Apibacter*, *Bacilli unclassified*, and *Dysgonomonas* (genera) were predominant in the hindgut, while *Cyanobacteriales* (order), *Paenibacillus*, *Paracoccus*, and *Methylibium* (genera) were predominant in the fore-midgut (Figures 6A, B). In the *X. auripennis*, LEfSe analysis identified that Enterobacterales and Bifidobacteriales (order), Orbaceae and Bifidobacteriaceae (family), *Candidatus Schmidhempelia*, *Bifidobacterium*, *Bombilactobacillus*, *Lactobacillus*, *Apibacter*, and *Atopobium* (genera) were rich in the hindgut, while *Brochothrix*, *Lentilactobacillus*, *Stenotrophomonas*, *Actinomyces*, and *Secundilactobacillus* (genera) were rich in the fore-midgut (Figures 6C, D). Notably, we found that most bacteria enriched in the hindgut of carpenter bees were host-specific bacteria previously known only to bumblebees and honey bees.

Phylogenetic analyses

In Figure 7, the phylogenetic tree of three non-core and four core gut bacteria within carpenter bees-associated ASVs is denoted by an asterisk. *Apilactobacillus* from *Xylocopa* clustered closely with *Apilactobacillus micheneri* and *Apilactobacillus quenuie*, which were isolated from various bees including *Augochlorella* sp, *Dialictus* sp, *Halictus* sp, and *Megachile* sp. Two *Lactobacillus* from *Xylocopa* clustered closely with *Cephalotes*. *Candidatus Schmidhempelia* from *Xylocopa* clustered closely with five uncultured gamma proteobacterium isolated from *Bombus* sp, which were identified in the previous study and renamed *Candidatus Schmidhempelia* (Martinson et al., 2014). *Leuconostoc* from *Xylocopa* clustered with *Leuconostoc mesenteroides*, commonly found in vegetables and fermented food such as potatoes and taros. *Bombilactobacillus* from *Xylocopa* first clustered closely with *Bombilactobacillus bombi* isolated from *Xylocopa violacea*, then with *Bombilactobacillus bombi* isolated from *B. terrestris*. *Bombiscardovia* from *Xylocopa* clustered with *Bombiscardovia coagulans* isolated from *Bombus* sp. *Bifidobacteriaceae unclassified* from *Xylocopa* clustered with *Bifidobacterium aemilianum* and *Bifidobacterium coryneforme* from *X. violacea* and *Osmia bicornis*.

Functional prediction of the gut microbiota

To better understand the important role of the gut microbiota of carpenter bees, we used PICRUSt2 software to predict the compositions of functional genes in samples based on the 16S rDNA sequencing data. The predicted functions were closely related to genetic information processing, cellular processes,

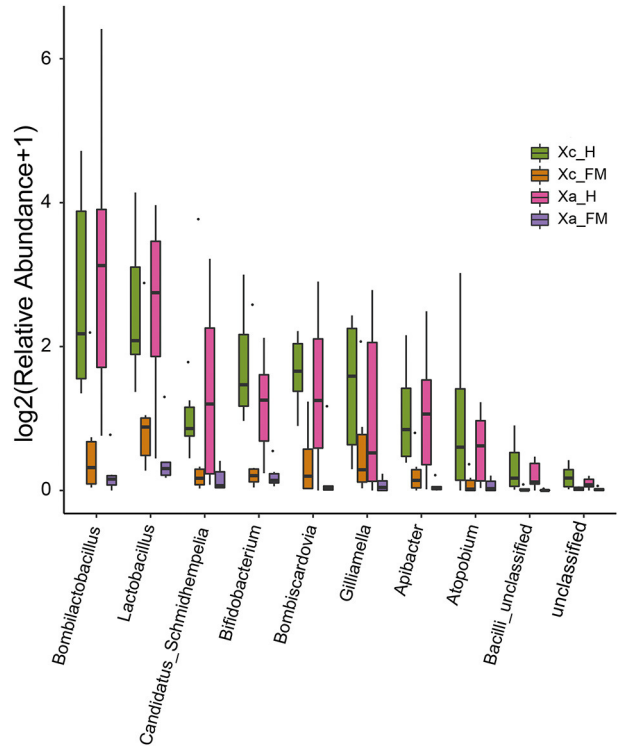

FIGURE 5
Comparison of the relative abundance of bacterial sequences at the genus level between fore-midguts and hindguts of two carpenter bees. The data were analyzed by the Kruskal–Wallis test ($P < 0.05$), and all the species with $P < 0.05$ are shown in the figure.

organismal systems, environmental information processing, and human diseases and metabolism.

The functional profile between the fore-midgut and hindgut of *X. caerulea* exhibited significant differences. In the hindgut tract, functions related to the bacterial secretion system, base excision repair, fructose and mannose metabolism, and caprolactam degradation were significantly higher than those in the fore-midgut. In contrast, ubiquinone and other terpenoid–quinone biosynthesis and methane metabolism were significantly lower than those in the fore-midgut (Figure 8A). In the hindgut tract of *X. auripennis*, only lipid metabolism was significantly lower than those in the fore-midgut (Figure 8B).

Discussion

Carpenter bees are a type of plant pollinator that is covered in thick fur clumps and has a larger body size than honey bees, allowing them to carry more pollen on their bodies. They play a critical role in pollinating fruits with large flowers in tropical regions. It was previously reported that large solitary bees of the genus *Xylocopa* are the main pollinators for yellow passion fruit *P. edulis* (Barrera et al., 2020), and the supply of nests of *Xylocopa frontalis* in crop areas was shown to be effective for the boost of the production and quality of fruits in southeastern Brazil (Toledo et al., 2017). Beyond their commercial value in crop farming, *Xylocopa* bees are also recognized for their ecological importance in tropical rainforest and mangrove forests. In particular, in Malaysia's mangrove forests, *Xylocopa varipuncta* has been identified as a critical pollinator alongside bats and birds and plays a crucial role in carrying pollen for these ecosystems (Hodgkison et al., 2003). Generally, carpenter bees have great potential to increase fruit production and maintain the stability of the ecological system.

In the long-term evolution process, microorganisms harbor in the gut of insects with a mutually interdependent symbiotic relationship. Insects, including Apidae, rely on a mutualistic gut microbial community to digest food, detoxify harmful molecules, provide essential nutrients, protect them from pathogen and parasite invasions, and modulate development and immunity (Engel and Moran, 2013; Douglas, 2015). Eusocial bees, including honey bees (*Apis*) and bumblebees (*Bombus*), harbor host-specific and beneficial microbiota, which play multiple roles in biological activities (Kwong and Moran, 2016; Hammer et al., 2021). However, bee species vary in microbiota composition, including the presence of specialized taxa and the relative bacteria from the environment (McFrederick et al., 2017). The factors that predict this variation in microbiota composition between bee species, as well as the microbial functions that they perform, are poorly understood. However, sociality has been considered a critical driver of gut microbiota evolution in bees (Moran et al., 2019). Here we studied the gut bacterial diversity and community composition of two carpenter bees collected from a tropical rain forest, providing a more comprehensive understanding of the structure of the bacteria community in carpenter bees *Xylocopa*. The results reveal a complex, symbiotic community in the gut of genus *Xylocopa* and provide a molecular basis for understanding the function of the gut microbiota.

Specifically, we determined the bacterial composition of the top 10 most abundant phyla and families of bacteria in two carpenter bees. The results showed that the dominant gut microbiota at the phylum level in two carpenter bees was Firmicutes, Actinobacteria, Proteobacteria, and Bacteroidetes, which were consistent with the previous study in honey bees (Cox-Foster et al., 2007; Khan et al., 2020). Many studies reported that Firmicutes and Proteobacteria were the foremost phyla of the microbiome in the insect gut microbiome, particularly in Hymenoptera (Jeyaprakash et al., 2003). They play a crucial role in processes, such as pectin digestion and mannose degradation, as well as in immune defense against the parasite such as *Nosema bombi* in bumblebees (Martinson et al., 2012; Hammer et al., 2021). The dominant families of the gut microbiome in honey bees are Lactobacillaceae, Bifidobacteriaceae, and Orbaceae. These families perform different functions, such as food digestion and nutrient absorption, which can benefit their host (Powell et al., 2014). In this study, three families dominated almost every sample we tested. As a result, our findings reveal a community composition of two carpenter bees at the phylum and family level that is similar to eusocial bees such as honey bees and bumblebees.

Eusocial bees perform vertically transmitted core gut microorganisms by the fecal–oral route, oral trophallaxis, and contact with hive materials (Powell et al., 2014). Seven bacterial species form the core microbiota of the bumblebee gut: *Snodgrassella*, *Gilliamella*, *Schmidhempelia*, *Bifidobacterium*, *Bombiscardovia*, *Bombilactobacillus* (Firm-4), and *Lactobacillus* (Firm-5). These species have been widely studied for their

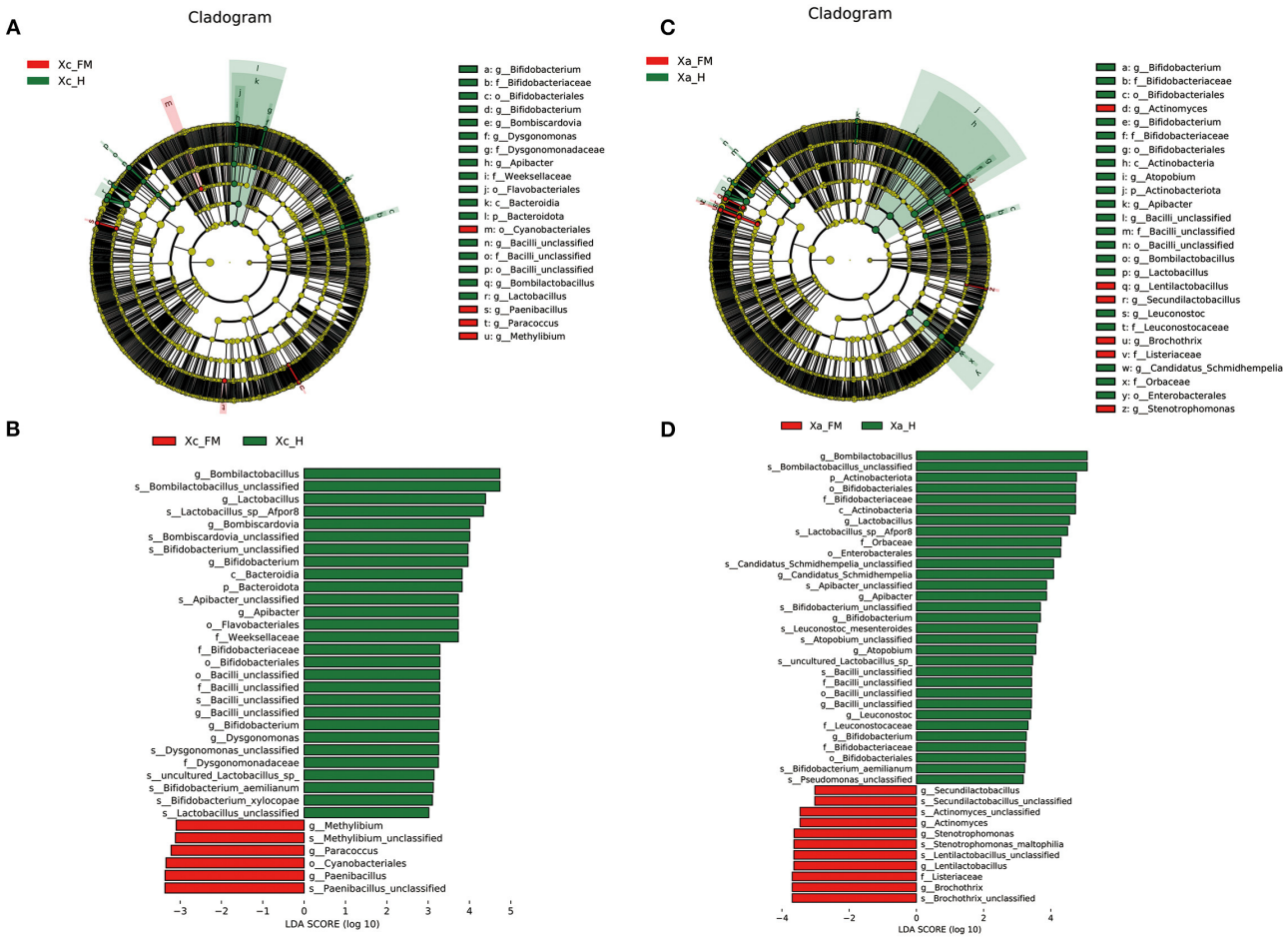

FIGURE 6
LEfSe analysis showing composition differences in the gut bacteria of fore-midguts and hindguts in two carpenter bees, **(A, B)** *X. caerulea* and **(C, D)** *X. auripennis*. Biomarker taxa are highlighted by colored circles and shaded areas. Each circle's diameter reflects the abundance of taxa in the community. Differential bacterial taxa are marked by lowercase letters. The threshold value of the LDA score was set to 3, and an LDA score of >3 was considered significant.

role in food digestion and nutrient absorption by their hosts (Hammer et al., 2021). In contrast, in non-social bee species, even those closely related to social corbiculates, individuals generally harbor microbiomes that are more variable and less distinct. These differences are likely due to the acquisition of microbes being driven by the environment, rather than social factors (McFrederick et al., 2012; Rothman et al., 2020). Moreover, some bee species with incomplete social structures, such as *Megalopta*, also possess gut core bacteria. *Lactobacillus* and *Saccharibacter* were found to be prevalent in 90% of tested adults (Graystock et al., 2017). The previous research has raised the point that environmental transmission appears to be more important than social transmission for *Megalopta* bees (McFrederick et al., 2014). These results reveal that some other factors, rather than social behavior, may be more critical in shaping microbiota structure and specialization. For example, *Xylocopa* species are considered non-classical sociality bees, and their microbiome composition has rarely been studied. However, a recent study has revealed that some incipiently social *Xylocopa* species also have core bacteria in their microbiomes, similar to social bees. In fact, two *Xylocopa* species share a set of core taxa, including *Bombilactobacillus*, *Bombiscardovia*, and *Lactobacillus*, which were found in most of the individual bees sampled (Handy et al., 2023). Four *Xylocopa* species in central Texas were found to have microbiomes dominated by bacterial taxa that were previously known only in social bees (Holley et al., 2022). In this study, several core gut bacteria of bumblebees were detected in two carpenter bees, which included *Lactobacillus*, *Bombilactobacillus*, and *Bifidobacterium*, and their abundance in hindguts was significantly higher than in the foregut and midgut. Lactobacillaceae, such as *Lactobacillus* and *Bombilactobacillus*, contain many genes encoding cell membrane proteins and phosphotransferase systems to assist hosts in the absorption and degradation of plant pollen (Kwong et al., 2014). In contrast, Bifidobacteriaceae degrade hexoses via a specific pathway, where the key enzymes are fructose-6-phosphate phosphoketolase (F6PPK) and xylulose phosphoketolase (Bottacini et al., 2012). The symbionts from the Lactobacillaceae and Bifidobacteriaceae were crucial for the health of honey bees, and the findings in carpenter bees were consistent with previous research (Genersch, 2010). Unexpectedly, two *Bombus*-specific core microbes, *Candidatus*

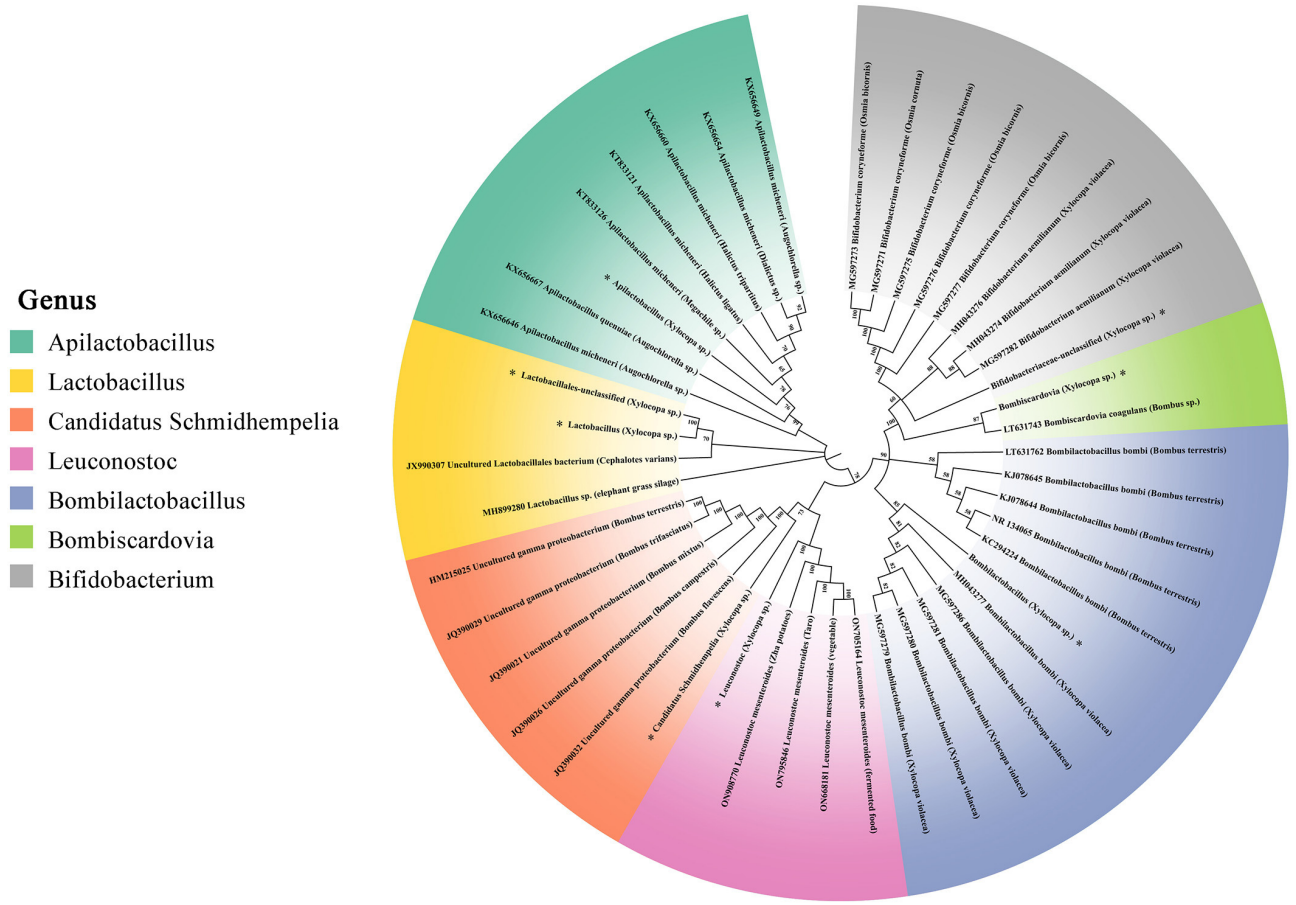

FIGURE 7
16S rRNA gene phylogenies of bacteria from *Apilactobacillus*, *Lactobacillus*, *Candidatus Schmidhempelia*, *Leuconostoc*, *Bombilactobacillus*, *Bombiscardovia*, and *Bifidobacterium*. Phylogenies were inferred by maximum likelihood. ASVs sequenced from *X. caerulea* and *X. auripennis* are represented by asterisks. Host insects are in parentheses.

Schmidhempelia and *Bombiscardovia,* were abundant in the hindguts of two carpenter bees. *Candidatus Schmidhempelia* is a *Bombus*-specific gamma-proteobacteria, which has been found in 90% of bumblebee guts and has a simplified genome unique to symbiotic bacteria (Martinson et al., 2014). *Bombiscardovia* is a genus of Bifidobacteriaceae originally isolated from *Bombus lapidaries* (Killer et al., 2010). Although the functions of the two genera have not been studied extensively, they are considered beneficial to their hosts. Our results, when compared to the reported gut microbiota of carpenter bees and eusocial bees, show that the genus *Lactobacillus* and *Bifidobacterium* are commonly found in the gut of all studied genus *Xylocopa*. In contrast, the genus *Bombilactobacillus*, *Gilliamella*, *Bombiscardovia*, and *Schmidhempelia* are inconsistently distributed in the guts of carpenter bees, and the genus *Snodgrassella* is only found in the guts of eusocial bees. In addition to the core bacteria, the genera *Leuconostoc* and *Apilactobacillus* were found in significantly higher abundance compared to other bacteria. The bacterial taxon known as *Apilactobacillus* was found to be common in the gut and provisions of solitary bees, as well as in the crop of bees (McFrederick et al., 2017). Hypotheses suggest that *Apilactobacillus* may have the ability to inhibit the growth of pathogens or prevent the spoilage of stored pollen (McFrederick et al., 2018).

The specific composition and spatial distribution of the gut microbiota in eusocial bees depend on their functional differentiation (Jeyaprakash et al., 2003). In this study, the differences in bacteria species between the fore-midguts and hindguts of the two carpenter bees were tested by LEfSe analysis. The members of Lactobacillaceae and Bifidobacteriaceae, such as the genera *Bombilactobacillus*, *Lactobacillus,* and *Bifidobacterium*, were significantly enriched in hindguts. This finding is consistent with the results observed in social bees. In honey bees *A. mellifera*, the hindguts function in fecal storage, reabsorption of nutrients, and enabling the colonization of core bacteria, such as *Bombilactobacillus*, *Lactobacillus*, and *Bifidobacterium*, which have enriched genes participating in the carbohydrate uptake and metabolism pathways (Kwong and Moran, 2016). The predicted functional pathways, tested by PICRUSt2, in the hindguts of *X. caerulea* and *X. auripennis* were concentrated in carbohydrate and lipid metabolic processes, which corresponded with bacterial species distributed in the hindguts. These results suggest that Lactobacillaceae and Bifidobacteriaceae are dominant in the hindguts and may play a vital role in carbohydrate and lipid

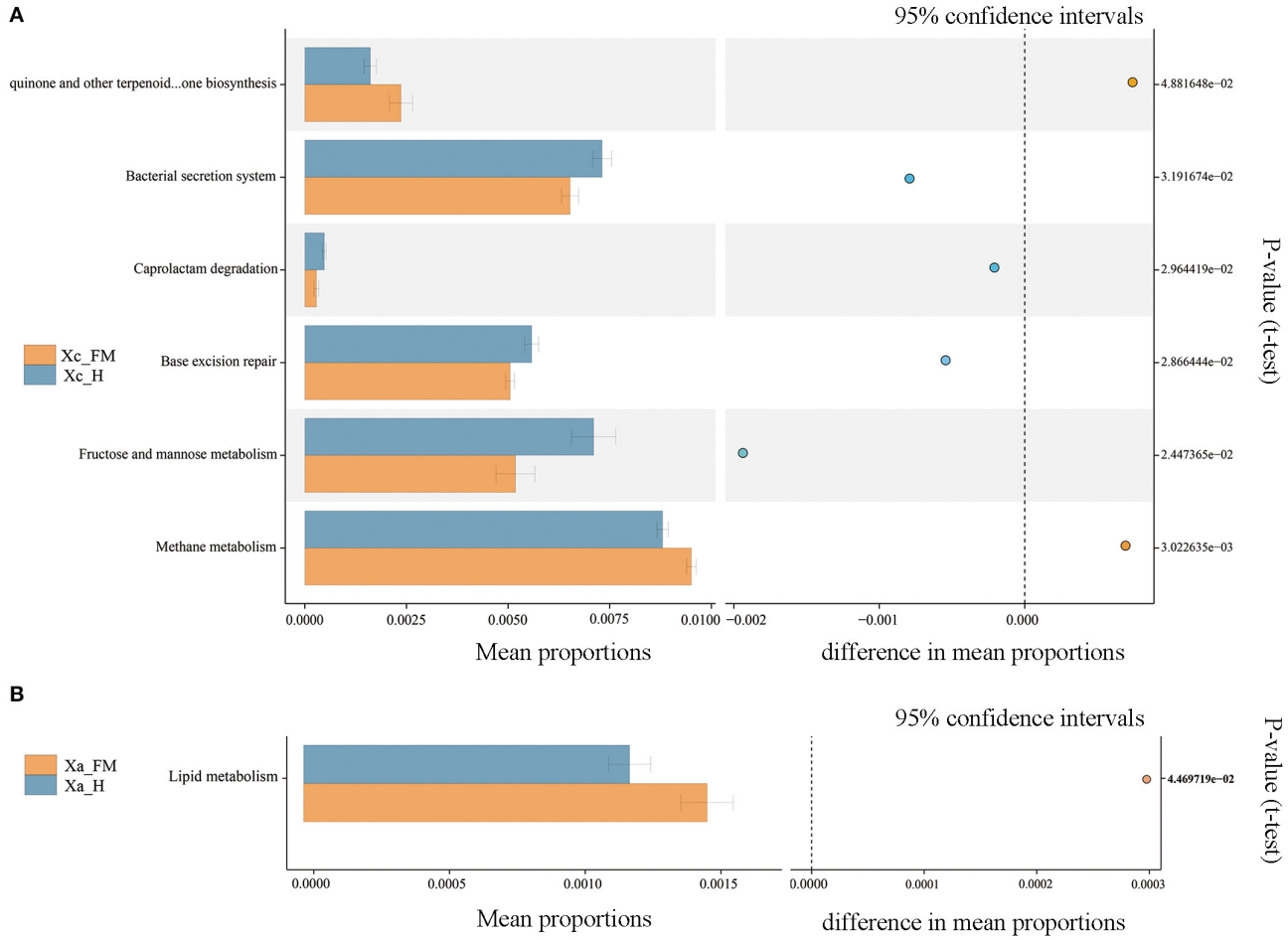

FIGURE 8
Functions of gut bacterial community predicted by PICRUSt. Significantly different KEGG pathways (level 3) were detected between the fore-midguts and hindguts of *X. caerulea* **(A)** and *X. auripennis* **(B)**.

metabolism. PICRUSt2 contains an updated and large database of gene families and reference genomes and provides interoperability with any operational taxonomic unit (OTU)-picking or denoising algorithm (Douglas et al., 2020). A large number of studies have used this method to predict the functions of gut microbiota. For example, in the oriental fruit moth *Grapholita molesta*, feeding on different plants can significantly change the functions of gut microbiota (Yuan et al., 2021). However, there are still some limitations when using PICRUSt2 to predict based on a fragment of the V3–V4 region. Future experiments should be designed to use metagenomic or single bacterial genome approaches to conduct more intensive studies.

In eusocial bees, such as honey bees, the core microbiota can be vertically transmitted between workers and larvae through trophallaxis and the fecal–oral pathway (Powell et al., 2014). In contrast, the gut microbes of solitary bees are believed to be acquired from the hive environment and food due to the absence of close social contact (Gilliam et al., 1984). There is mounting evidence that some bacteria taxa, previously known only in social bees, exist in the guts of solitary bees and may have recently transmitted from mother bees to larvae. Previous studies have shown that *Bifidobacterium* isolates from the guts of European *X. violacea* were closely related to those of honey bees and bumblebees (Alberoni et al., 2019). In this study, we found that *X. caerulea* and *X. auripennis* shared similar bee-associated bacterial community profiles despite inhabiting different ecological environments. Lactobacillaceae, Orbaceae, and Bifidobacteriaceae were the three main families of gut microbiota in bees, including the genera *Lactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Candidatus Schmidhempelia*, *Bifidobacterium*, and *Bombiscardovia*. Most of the ASVs found in carpenter bees of the three families in this study were closely related to previously identified bacterial taxa, which are widespread in social bees, particularly *Bombus*-specific genera *Candidatus Schmidhempelia* and *Bombiscardovia*. These results suggest that the vertical transmission of bacteria in *Xylocopa* may occur through certain mechanisms.

It is commonly believed that solitary bees exhibit no caring behavior, with mother bees only collecting food for the larvae and leaving the hive before the offspring mature. The gut microorganisms of their offspring are mainly acquired from the environment and from food sources, such as the genera

Megachile and *Osmia* (Keller et al., 2018; Voulgari-Kokota et al., 2019). However, a previous study of the bee species in the genera *Megalopta* (which contains solitary and social species) found a limited influence of sociality on bacterial composition (McFrederick et al., 2014). This demonstrated that microbiota was not only transmitted by direct social contacts, such as trophallaxis and fecal–oral contact, between concurrent members but also by some non-social behaviors. Furthermore, the vertical transmission of core gut bacteria in two carpenter bee species may be linked to other observed behaviors within the *Xylocopa* genus. In *X. sulcatipes*, different generations of mother bees fight for nest chambers due to competition in a resource-limited environment (Stark, 2010). The usurper drives the host out of the hive or defense against the enemy instead of reproducing and laying new eggs in the used hive, which probably drives microbe–host specificity by contact with old hive materials. In the social bee *A. mellifera*, newly emerged young honey bees chew their way out of cells and consume gut core microbiota that remained on hive surfaces (Martinson et al., 2012). Recent research has confirmed that the transmission of honey bee core hindgut microbiome is facultative and horizontal, with five out of six core hindgut species readily acquired from the built hive structure and natural diet (Anderson et al., 2022). The same route of transmission may exist in the genus *Xylocopa* and result in an accumulation of bacterial species in the guts of young bees from old hive materials. In this study, we collected samples of two carpenter bees in a relatively high population density region where nests are concentrated. Previous studies have predominantly collected solitary bees randomly in the wild, where there is minimal competition pressure and unstable gut microbiota. Thus, our findings indicate that the pressures of nesting and reproduction for *Xylocopa* seem to drive the reuse of old nests and the vertical transmission of gut bacteria, although the life habits of most *Xylocopa* species are poorly studied. The social transmission routes of *Xylocopa* species merit further investigation.

In conclusion, we characterized the gut microbial communities of two carpenter bees and found that some gut bacterial taxa exist in the guts of *X. caerulea* and *X. auripennis*, such as *Candidatus Schmidhempelia* and *Bombiscardovia*, which were closely related to those found in eusocial bees, especially bumblebees. Based on our results, we hypothesize that the gut bacteria of carpenter bees are transmitted from mother bees to larvae by reusing old nests. This study offers novel insight into the structure, distribution, and function of gut symbiotic bacteria in *Xylocopa* species. However, there were still some limitations in our study. Future experiments should be designed to compare the gut microbiota of these two carpenter bees with that of other species in the genus *Xylocopa* and eusocial bees. Moreover, isolating *Candidatus Schmidhempelia* and *Bombiscardovia* from the two *Xylocopa* species and elucidating their important functions using multi-omics will contribute to finding new probiotics that people can use.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI—PRJNA915489.

Author contributions

WH and YG designed the research and wrote the manuscript. YG, YW, SZ, and DL collected the samples. WH, YG, SW, and YZ performed the experiments and analyzed the data. WH and JG funded this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Gut microbiota variation of a tropical oil-collecting bee species far exceeds that of the honeybee

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Introduction: Interest for bee microbiota has recently been rising, alleviating the gap in knowledge in regard to drivers of solitary bee gut microbiota. However, no study has addressed the microbial acquisition routes of tropical solitary bees. For both social and solitary bees, the gut microbiota has several essential roles such as food processing and immune responses. While social bees such as honeybees maintain a constant gut microbiota by direct transmission from individuals of the same hive, solitary bees do not have direct contact between generations. They thus acquire their gut microbiota from the environment and/or the provision of their brood cell. To establish the role of life history in structuring the gut microbiota of solitary bees, we characterized the gut microbiota of *Centris decolorata* from a beach population in Mayagüez, Puerto Rico. Females provide the initial brood cell provision for the larvae, while males patrol the nest without any contact with it. We hypothesized that this behavior influences their gut microbiota, and that the origin of larval microbiota is from brood cell provisions.

Methods: We collected samples from adult females and males of *C. decolorata* (n=10 each, n=20), larvae (n=4), and brood cell provisions (n=10). For comparison purposes, we also sampled co-occurring female foragers of social *Apis mellifera* (n=6). The samples were dissected, their DNA extracted, and gut microbiota sequenced using 16S rRNA genes. Pollen loads of *A. mellifera* and *C. decolorata* were analyzed and interactions between bee species and their plant resources were visualized using a pollination network.

Results: While we found the gut of *A. mellifera* contained the same phylotypes previously reported in the literature, we noted that the variability in the gut microbiota of solitary *C. decolorata* was significantly higher than that of social *A. mellifera*. Furthermore, the microbiota of adult *C. decolorata* mostly consisted of acetic acid bacteria whereas that of *A. mellifera* mostly had lactic acid bacteria. Among *C. decolorata*, we found significant differences in alpha and beta diversity between adults and their brood cell provisions (Shannon and Chao1 p<0.05), due to the higher abundance of families such as Rhizobiaceae and Chitinophagaceae in the brood cells, and of Acetobacteraceae in adults. In addition, the pollination network analysis indicated that *A. mellifera* had a stronger interaction with *Byrsonima* sp. and a weaker interaction with *Combretaceae* while interactions between *C. decolorata* and its plant resources were constant with the null model.

Conclusion: Our data are consistent with the hypothesis that behavioral differences in brood provisioning between solitary and social bees is a factor leading to relatively high variation in the microbiota of the solitary bee.

KEYWORDS

gut microbiota, sociality, mother bee, pollen provision, oil-collecting bee

Introduction

Interest for bee microbiota has recently been rising, alleviating the gap in knowledge in regard to drivers of solitary bee gut microbiota. However, no study has addressed the microbial acquisition routes of tropical solitary bees. For both social and solitary bees, the gut microbiota has several essential roles including biosynthesis of nutrients, degradation of pectin and lignocellulose, and dietary carbohydrate metabolism (Onchuru et al., 2018). These symbionts are also important for the host's immune response to infections by pathogens, parasites, and parasitoids (Kwong et al., 2017; Onchuru et al., 2018; Steele et al., 2021). These critical immune roles have significant consequences for bee conservation (LeBuhn and Vargas Luna, 2021) as demonstrated by studies with the honeybee, *Apis mellifera*, the most important commercial honey producer and a highly valued species for the pollination services provided to crops (Hung et al., 2018). This social bee has been the most widely studied model organism in the field of bee gut microbiota. Regardless of the geography, environment, and subspecies, the microbiota of *A. mellifera* is highly conserved (Martinson et al., 2011), and is sometimes referred to as the global honeybee microbiome (Almeida et al., 2022). The composition of the honeybee core microbiota (a persistent set of low diversity bacterial phylotypes/OTUs) includes the following taxa: *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Bifidobacterium*, *Gilliamella*, *Snodgrasella*, *Bartonella apis,* and *Frischella* and other Alphaproteobacteria (termed 2.1 group; Martinson et al., 2011; Kwong et al., 2017). The recurrence of the microbiota in these social bees results from (1) the transmission from the mother colony to daughter queens (vertical transmission), and (2) by social interactions between individuals of the same nest, including food exchange (trophallaxis; Michener, 1974). In other words, sociality plays an important role in the vertical transmission of the microbiota (Koch et al., 2013).

These low diversity and recurring phylotypes appear not only in honeybees but also bumblebees (Kwong et al., 2017) as well as other primitively social apids such as *Xylocopa* spp. (Handy et al., 2022; Holley et al., 2022). *Lactobacillus* Firm-4 and Firm-5 can also be found in low abundance in solitary bees (McFrederick et al., 2012, 2017; Graystock et al., 2017; Cohen et al., 2020). These trace levels could represent occasional horizontal transfers from social bees. Essentially, solitary bees do not share the core phylotypes of social bees and are still able to process food and respond to pathogens. The solitary bee microbiota seems to be species-specific with diverse bacteria likely playing similar roles of protection and nutrition. Indeed, these host-microbiota associations are important, as they contribute to the survival and the growth of larvae (Dharampal et al., 2019). How these larvae acquire their symbiotic bacteria and what role the mother bee plays in the microbial establishment remains underexplored.

Solitary *Centris decolorata* is an oil-collecting bee of the tribe Centridini, a sister clade to the corbiculates (Michener, 2000). It is a common bee species in coastal tropical environments (Alves-dos-Santos et al., 2009; Starr and Vélez, 2009), nesting in typical coastal vegetation. In Puerto Rico, they form large nest patches during the wet season (April to November; pers. obs.). Centridini is widely distributed and typically have high host plant species richness, large body sizes, and important interactions with many plant groups (Sigrist and Sazima, 2004; Gaglianone et al., 2010). They constitute the most ancient lineage of floral oil-collecting bees (Buchmann, 1987; Renner and Schaefer, 2010; Martins et al., 2014; Powell et al., 2014). Compared to large nests of honeybees (hives), the nests of *C. decolorata* are quite simple. They are constructed by individual females (mother bees) and consist of 15 cm-long tunnels dug diagonally into sandy soils, and generally have one brood cell per tunnel. The brood cell walls are composed of oils, leaf materials, resins and secretions from the Dufour's glands (mostly aliphatic hydrocarbons; Roubik, 1989), which provide a hydrophobic barrier for the larva (Danforth et al., 2019). The source of oils is mainly from flowers of Malpighiaceae (Thiele and Inouye, 2007), which may be kilometers from the nest (pers. obs.). Oil collecting females provision each cell with pollen, mixed with oil, glandular secretions from Dufour's glands, and an egg (Roubik, 1989; Danforth et al., 2019). The absence of evaporated nectar in brood cell provisions has yet to be chemically tested across a wider range of oil collecting bee species (Neff and Simpson, 2017). The completed brood cell has a coating or lining that confers humidity homeostasis, serving as the first-line defense to foreign microbes (Danforth et al., 2019), whereas the mixture of the provisions includes antimicrobials from mandibular gland secretions serving as the second-line defense (Cane et al., 1983).

Females sometimes forage far from the nest but always return to it, while males patrol the immediate vicinity of the nest without ever entering it. Where these nests occur along beaches in Puerto Rico, the vegetation typically consists of *Canavalia rosea, Ipomoea pes-caprae, Vigna luteola, Bidens Alba*, and *B. pilosa* (Martinez-Llaurador, 2021). At nest sites, territorial males form aggregation patches and exhibit perching behavior (Alves-dos-Santos et al., 2009; Starr and Vélez, 2009). The foraging niche of *C. decolorata* along coastal environments has been partially characterized by utilizing observation-based pollination networks (Martinez-Llaurador, 2021). Although such networks provide useful information on plant-pollinator relationships, some important interactions may be missed that a study of pollen load composition could provide (Forup and Memmott, 2005; Greenleaf et al., 2007; Jędrzejewska-Szmek and Zych, 2013; Fisogni et al., 2018). Characterizing pollen loads also offers a better understanding of how pollen use may influence microbial acquisition (Dew et al., 2020). In this study, we aim to characterize and compare the pollen load composition of *C. decolorata* and *A. mellifera* and relate it to microbiota diversity and composition. If there is no difference in pollen load composition between the two species yet their microbiota differ, then other acquisition routes may be involved, e.g., by soil, mother bee, or in this case other plant materials such as floral oils.

We asked whether the microbiota of a solitary bee in Puerto Rico is similar to that of co-occurring social *A. mellifera*, a variant known as "gentle Africanized honeybees" (*gAHB*). *Apis mellifera* also served as a positive control in the sense that its microbiota has been widely discussed and reported in the literature (*cf.* phylotypes cited above) as the global honeybee microbiome. Even though the honeybees of Puerto Rico are somewhat unique in having a mosaic of traits between European and Africanized honeybees (Rivera-Marchand et al., 2012), we expect that their microbiota should be similar to that reported in the literature since the global honeybee microbiome is consistent even across subspecies of *A. mellifera* (Almeida et al., 2022). These bees have a core gut microbiota that changes with developmental stages (Ortiz-Alvarado, 2019). Authors described a microbiota clustered into two well-defined groups: *Fructobacillus* genus (Phylum Firmicutes), Rhodospirillales and Acetobacteraceae (Phylum Proteobacteria) in

early development stages, and Lactobacillaceae (Phylum Firmicutes), and Neisseriaceae (Phylum Proteobacteria) in late development stages (Ortiz-Alvarado, 2019).

As the solitary bee-microbiota is impacted by environmental acquisition routes (Voulgari-Kokota et al., 2019b), we expected higher microbial variation in *C. decolorata* compared to *A. mellifera*. We also hypothesized that more bacterial taxa would be shared between *C. decolorata* females and larvae, than that between males and larvae, due to female rearing and providing resources to the offspring. To our knowledge, this is the first study describing the differences in the gut microbiota between social and solitary bees in a tropical environment, while discussing the role of the solitary oil-collecting mother bees on the original gut microbiota of larvae.

Materials and methods

Bee collections and dissections

On 15 May and 22 May 2022, *Apis mellifera* foragers and adult *Centris decolorata* were collected with an insect net (Departamento Recursos Naturales, permit ID 2022-IC-019). *Apis mellifera* (honeybees) were collected in three sites from two different towns, to make sure they came from different hives: Coamo (18.036814, −66.374096) and Mayagüez (2 plots, 18.250797, −67.177461 and 18.251412, −67.178063), Puerto Rico, United States. Mayagüez is a coastal town and in these exact coordinates, *Centris decolorata* specimens were also collected (Figure 1A). *Centris decolorata* nests were excavated in two Mayagüez plots following the method by Marinho et al. (2018). A total of 46 individuals were collected for this study. These individuals include, 9 *A. mellifera* foragers—6 collected from Coamo and 3 from Mayagüez–; and 24 *C. decolorata* bees (12 females, 12 males and 13 brood cell contents), all from Mayaguez (Figure 1B). The adult digestive tract (foregut to hindgut) of each species were dissected using sterilized tools under the stereomicroscope. The brood cells were also dissected to retrieve the whole individual larvae and the associated brood cell provision (Figure 1C). Because some brood cells were empty and solely contained the starting/remaining brood cell provisions and some *A. mellifera* had very small sizes and had to be pooled for extractions, a selection of 39 samples was done for analyses: 6 *A. mellifera* workers (female foragers); 10 female (mother bees) and 10 male *C. decolorata* adults, 4 of their larvae, and 10 of their brood cell provisions. Even though reproducing female solitary bees are further referred as "solitary mother bees," their sampling has been done independently from their larvae. The female solitary bees we collected were considered as to be mother bees based on their behavior: returning to the nest at the end of the afternoon or carrying plant materials into the nest.

Microbiota analysis

DNA extraction

The DNA of the adult guts and of the entire larval body was extracted using the PowerSoil Pro Kit (QIAGEN LLC, Germantown Road, Maryland, United States) following the manufacturer's instructions, preceded by the addition of 20 µL of Proteinase K for 5 min. A Qubit® dsDNA HS assay kit (High Sensitivity; Waltham, Massachusetts, United States), was used to assess DNA concentrations of purified extracts (average DNA yield = 138 ng/µL).

The DNA obtained from all samples was normalized to 4 nM during 16S rRNA gene library preparation. We employed the Earth Microbiome Project standard protocols,[1] using the universal bacterial primers: 515F (5′GTGCCAGCMGCCGCGGTAA3′) and 806R (5′GGACTACHVGGGTWTCTAAT3′) to amplify the hypervariable region V4 of the 16S ribosomal RNA gene (~291 bp) with region-specific primers that include sequencer adapter sequences used in the Illumina flowcell (Caporaso et al., 2012). Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader (Infinite® 200 PRO, Tecan). Once quantified, volumes of each of the products were pooled into a single tube so that each amplicon is represented in equimolar amounts. This Pool is then cleaned up using AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen). Customized sequencing was outsourced at Argonne National Laboratory (Illinois, United States) using llumina MiSeq with the 2 × 250 bp paired-end sequencing kit. The reads obtained from the sequencer and its corresponding metadata were uploaded in QIITA study ID 14679. The raw data was made available at the European Nucleotide Archive Project (ENA) under the access number ERP141576.

Sequence processing and statistical analyses

The initial processing of the resulting Fastq files was done using QIITA (version 2022.07). This included demultiplexing and trimming to 200 bp, followed by deblurring against the SILVA database. Deblur methods to join, denoise, and duplicate sequences, including the removal of chimeric sequences, singleton reads, quality filtering, and joining of paired ends. The resulting *.biom* files (without taxonomy) were processed locally in QIIME2 (version 2022.02) and R (version 2021.09 build 351) after removing singleton reads and chloroplast/mitochondrial and plant related sequences. The bacterial sequences were classified using the pre-formatted SILVA 16S rRNA reference database and taxonomy files (138 release; Quast et al., 2012) trained with scikit-learn 0.24.1 (Pedregosa et al., 2012). The downstream processes with the *biom* table were followed as in previous studies (Rodríguez-Barreras et al., 2021; Ortiz et al., 2022; Ruiz Barrionuevo et al., 2022).

A set of microbiota analyses were done comparing (1) social and solitary bees (at their adult stage), and (2) solitary bees (adult males and females) and their brood cells (brood cell provisions and larvae), referred hereafter as "comparison group 1" and "comparison group 2." For each comparison group, we computed analyses of beta and alpha diversity, taxonomic profiles, and putative biomarker taxa. Beta diversity analyses were done using the Bray–Curtis dissimilarity index and plotted using non-metric multidimensional scaling (NMDS) with samples colored according to the metadata categories, with 95% confidence ellipses. Beta diversity statistical tests including Permanova (Anderson, 2001), Permdisp (McArdle and Anderson, 2001), and Anosim (Clarke, 1993) were applied to quantify dissimilarity between both comparison groups. Permanova and Anosim were both applied to compare the dispersion of Bray–Curtis dissimilarity index in the

1 https://earthmicrobiome.org/protocols-and-standards/16s/

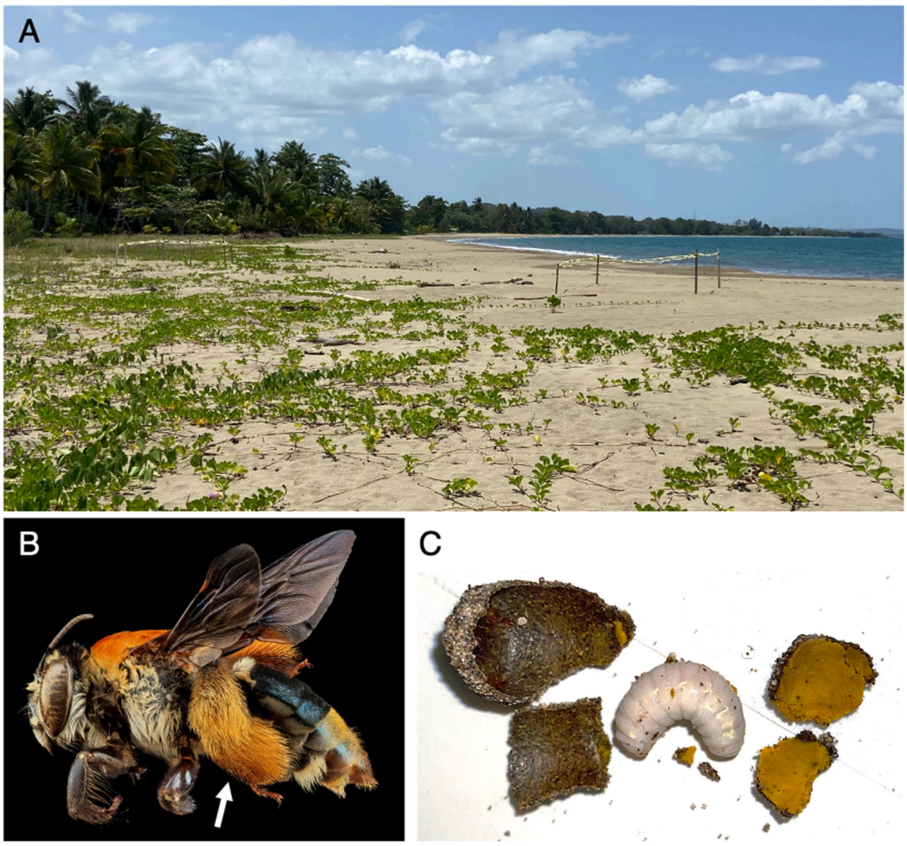

FIGURE 1
(A) Picture of the coastal environment where samples were collected. **(B)** Female *Centris decolorata*, arrow shows the hairy hindleg for pollen and floral oils collection. Credit: U.S. Geological Survey/photo by Wayne BooCanon. **(C)** Brood cell wall, larva of *C. decolorata* and brood cell provision.

Non-Metric Multidimensional Scaling. A Permdisp was used as an assumption of Permanova to test the null hypothesis of homogeneity of multivariate variances. For alpha diversity analyses, Chao 1 index (richness; Chao and Chiu, 2016) and Shannon (diversity index; Shannon, 1948) were visualized as boxplots using R (version 2021.09 build 351). Significant differences according to richness and diversity were assessed using Kruskal and Wallis (1952). Taxonomic profiles were visualized as standard QIIME2 barplots, and putative biomarker taxa differentially significant in multivariable associations with metadata variables were calculated in the package *maaslin* (Mallick et al., 2021). In addition, a core microbiota was identified for each variable of comparison group 2, using MicrobiomeAnalyst (Chong et al., 2020). The core microbiota considers taxa that are present in at least 50% of the samples for a given *C. decolorata* category (either female, male, larva, or brood cell provisions) and prevalence across samples for a given sample group is shown as heat colors.

Pollen analysis

Pollen slide preparation

Pollen loads from *C. decolorata* and *A. mellifera* were stained with Calberla's staining solution and analyzed with light microscopy. *Apis mellifera* legs as well as the body of *C. decolorata* were removed and placed over individual microscope slides (Wood et al., 2018). The contents of each microscope slide were bathed in 1–2 drops of ethyl acetate to wash off the pollen grains (Bezerra et al., 2020). Excess pollen grains still adhered to their legs and body were removed with the use of an entomological pin before staining with 2 drops of Calberla's solution. Cover slip borders were sealed over each sample with clear nail Polish.

Pollen species identification

Pollen slides of *C. decolorata* and *A. mellifera* were observed in their entirety and pictures of the pollen grains were taken using an Olympus EP50 digital camera (Supplementary Figure 1). Pollen grains from each sample were counted manually, categorized based on their morphology and identified to the lowest taxonomic level possible, using available resources (PalDat, 2000; Halbritter et al., 2018). Pollen types that were not identified to the lowest taxonomic level were assigned a unique ID based on their morphological characteristics. In addition, a pollen reference catalog was created with pollen collected directly from plant species located at the study site.

Pollen statistical analysis

We compared pollen load composition between *C. decolorata* and *A. mellifera*, by using the Shannon diversity index and constructing a pollination network. Pollen grain types with a count of less than 5 grains were excluded from the analysis as they could have been accidentally collected or a result of contamination (Bosch et al., 2009;

Fisogni et al., 2018). To calculate the proportion of the pollen volume of each pollen type, we measured the length of the polar and equatorial axes of 5 randomly encountered grains of each pollen type in each sample (da Silveira, 1991; O'Rourke and Buchmann, 1991; Stoner et al., 2022). Measurements were made using a calibrated EP50 digital camera at 400X. The volume of each pollen type was calculated using the average polar and equatorial lengths following the formulas for different shapes (O'Rourke and Buchmann, 1991). The proportion of the pollen volume of each pollen type was then calculated as follows:

$$Pollen\,volume\,proportion = \frac{Count\,of\ pollen\,grains\,x\,Volume\,of\ pollen\,grains}{Sum\,of\ total\,volume\ for\,all\,pollen\,types\,in\,the\,sample}$$

To account for the size and counts of each pollen type in each sample, the Shannon diversity index was calculated using the number of pollen grains multiplied by the volume. The interactions between *A. mellifera, C. decolorata,* and plant species were visualized using a pollination network plot based on the pollen volume proportion of pollen types found in individual samples. The network was constructed with the function "plot bipartite" of the package *econullnetr* (Vaughan et al., 2018). Plant resource selection was analyzed by running 1,000 simulations of null models. In addition, the function "plot preferences" of the *econullnetr* package was applied to better visualize and summarize the interaction strength between bee species and plant species. Plant species richness and diversity were compared using boxplots and a Kruskal-Wallis test was applied to assess differences between *C. decolorata* and *A. mellifera*. All indices and figures were produced using the R 4.2.2 version (PositTeam, 2022), and the *vegan* package was used to calculate plant diversity and richness (Oksanen et al., 2022).

Results

After sequence filtration and rarefaction (rarefaction value of 1,045 reads per sample), only two of four samples of larvae yielded enough reads, suggesting a *nearly* sterile individual at early stages (for larval body length < 16 mm, 34 and 44 reads). These two low read samples of larvae were thus not included in the analysis. Because 25 nests had already been excavated to obtain 13 complete brood cells, including 4 with larvae, we decided not to excavate more solitary bee nests at this location for conservation reasons (Table 1). The microbial analysis including larvae ($n = 2$) is shown but should be considered with caution, given the very low sample size. Because minimum sample sizes for Kruskal-Wallis test is five, any analysis with less than that does not approximate the chi-square distribution accurately. Our best data in terms of sample size are *A. mellifera* foragers, female *C. decolorata* and brood cell provisions (Table 1).

We found significant differences between the bacterial community structure of *A. mellifera* and *C. decolorata*. Beta diversity analyses revealed greater distances between *C. decolorata* individuals than between those of *A. mellifera* (PERMANOVA $p = 0.001$ and ANOSIM $p = 0.001$, Figure 2A; Supplementary Table 1). On the other hand, we found no difference in richness between *Apis mellifera* and *Centris decolorata* adults (Chao1 $p = 0.914$, Figure 2B; Supplementary Table 2). However, the gut microbiota of *A. mellifera* had a higher diversity than that of *C. decolorata* (Shannon $p = 0.0048$, Figure 2B; Supplementary Table 2).

Core taxa in social vs. solitary bees

In feral foragers of Puerto Rico honeybees, the simple and recurrent phylotypes of the gut microbiota remain as previously described in other honeybees (Martinson et al., 2011; Thompson et al., 2017). The gut microbiota of both bee species has the same phyla (Figures 2C, 3); however, the families are different. The core taxa of *Apis mellifera* comprises the families of Lactobacillaceae, Bifidobacteriaceae, Bartonellaceae, Neisseriaceae, Orbaceae, Rhizobiaceae, and Acetobacteraceae (i.e., *Commensalibacter* spp.; Figure 2D; Supplementary Figure 2). In contrast, the core microbiota of *Centris decolorata* is composed by bacteria from the Acetobacteraceae (i.e., undescribed Acetobacteraceae) and Moraxellaceae (Figures 2D, 4C,D). Some *C. decolorata* females displayed trace levels of undescribed species of Lactobacillaceae and Bifidobacteriaceae (Figure 2D; Supplementary Figure 2).

The microbiota of brood cell provisions and adults of *Centris decolorata* are distinct

Microbiota composition and structure of brood cell provisions are different from all other samples (Figures 4A,B). Brood cell provisions had significantly higher diversity than any gut microbiota of adult solitary bees, but not strongly different than larvae (alpha-diversity differences using Chao1, adjusted-$p = 0.286$ and using Shannon, adjusted-$p = 0.081$; Supplementary Table 1). While no significant differences in diversity

TABLE 1 Summary of study variables, samples, reads and OTUs.

Species	Sample type	Details	*n*	Ave. reads	Ave. OTUs
Apis mellifera	Gut *A. mellifera*	Worker	6	17,995.17 ± 3543.57	47.5 ± 20.80
Centris decolorata	Gut *C. decolorata* female	Female	6	4,999.83 ± 3,807.87	42 ± 32.22
	Gut *C. decolorata* male	Male	4	2,673.75 ± 2824.71	80 ± 22.63
	larva	Larva	2	9,703.50 ± 7350.37	168.25 ± 112.15
	brood cell provision	pollen provision, and possibly nectar and oils	8	19,887.75 ± 3004.27	42.17 ± 10.23
Total			26	12583.62 ± 8083.15	85.08 ± 84.07

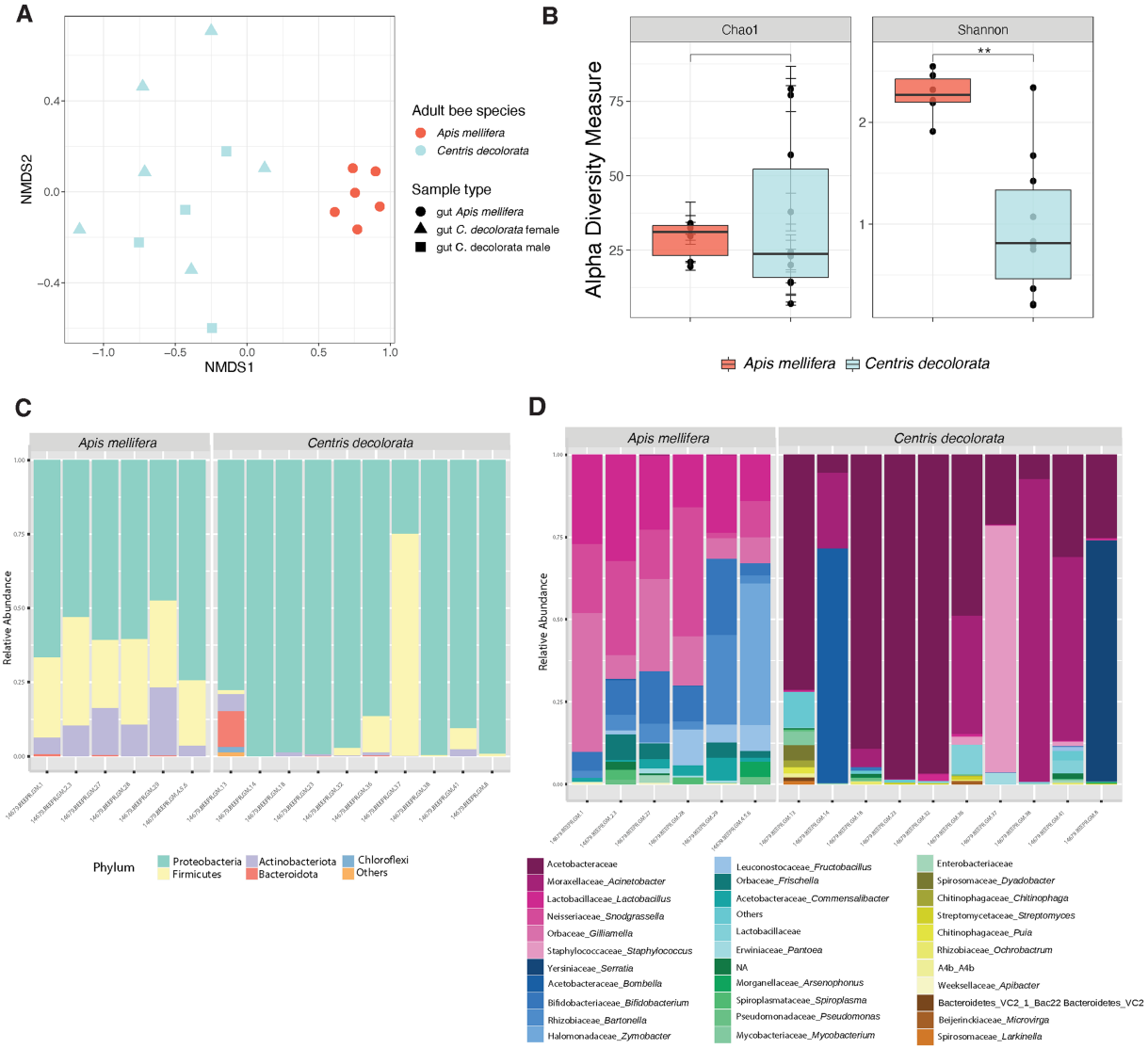

FIGURE 2
Diversity analyses comparing the microbiota of the two species of bees *Apis mellifera* (social) and *Centris decolorata* (solitary). **(A)** Beta diversity analysis, represented in a 2D NMDS with Bray-Curtis distances for species and sample types, depicts distinct clustering between the brood cell content and the adult bee with PERMANOVA value of p=0.001; ANOSIM value of p=0.001. **(B)** Alpha-diversity among species using Chao1 and Shannon indices. Asterisks depict significant values (*, **, *** representing 0.05, 0.01, 0.001, respectively). Bar Plots show the relative abundance (minimum 5%) of bacteria at the phyla **(C)**, and genus levels **(D)**.

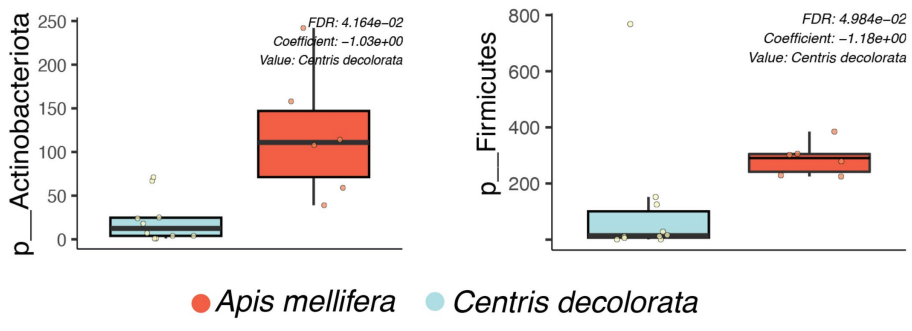

FIGURE 3
Bacterial phyla-level boxplots that discriminate among the two bee species with a q-value cut-off = 0.05. The corrected value of p for each taxon is shown in the upper right of the boxplots using MaAsLin.

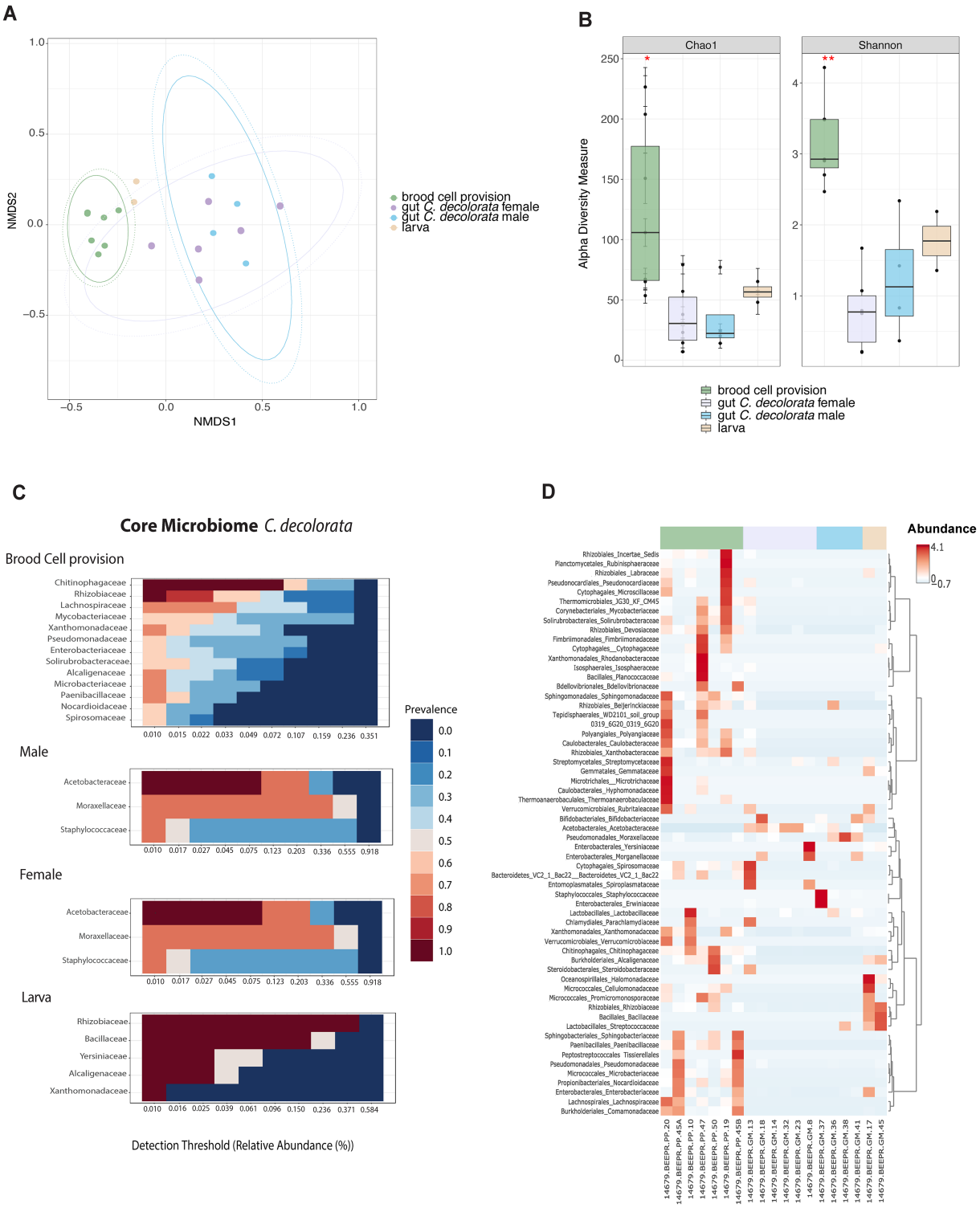

FIGURE 4
Overview of microbiota analyses for *Centris decolorata* samples (n=19) considering larva and brood cell provisions. **(A)** Beta diversity analysis, represented in a 2D NMDS with Bray-Curtis distances for *Centris decolorata* samples, depicts distinct clustering between the brood cell content and the adult bee with PERMANOVA value of p=0.001; ANOSIM value of p=0.001. **(B)** Alpha-diversity among *C. decolorata* samples using Shannon index, stars are showing significative values, Shannon value of p between brood cell content and female=0.001, and between brood cell content and male=0.05. Heatmaps showing the relative abundance of the bacterial phylum-level (assigned per phylum) **(C)** Core bacterial biota at the family-level per each *C. decolorata* sample groupings, corresponding to taxa detected in a fraction of at least 50% of individuals with greater than 0.01% of relative abundance. Prevalence is show as heat colors **(D)** Taxonomic heatmap at the order and family-level for each sample groups.

metrics were found between larvae and brood cell provisions, both beta and alpha diversities of brood cell provisions are significantly different from that of adult females and males (PERMANOVA, $p=0.001$; ANOSIM, $p=0.001$; Chao1 and Shannon index, $p<0.05$, Supplementary Tables 1, 2; Figures 4A,B). Brood cell provisions are composed by diverse families of bacteria having a higher number of taxa as part of the core microbiome as compared to other sample types. Females and males were mostly composed in Acetobacteraceae and in Moraxellaceae (Figure 4C), in fact only Acetobacteraceae are part of the female core microbiome. Furthermore, brood cell contents and adults did not display the same core microbiota at 50% sample prevalence. Rhizobiaceae, Chitinophagaceae, Lachnospiraceae, Enterobacteriaceae, Xanthomonadaceae, Alcaligenaceae, and Microbacteriaceae constituted the core microbiota of brood cells, while Acetobacteraceae constituted that of adults (Figures 4C,D). Undescribed Acetobacteraceae explained most of the differences between adults and brood cells (mostly females) using MaAsLin statistical analysis. Only males had abundant *Staphylococcus* sp. (Supplementary Figure 3).

Pollen diet in *Apis mellifera* and *Centris decolorata*

A total of 28 pollen types were identified from *A. mellifera* and *C. decolorata* pollen loads (*cf.* "Plant Pollen and Bee Pollen Grain Catalog" in Supplementary Presentations 1, 2). Of these, 16 pollen types were excluded after filtering for pollen types with less than five grains per slide. Seven of the remaining pollen types were found in *C. decolorata*. Three pollen types, including *Byrsonima* sp. (an oil flower) and *Combretaceae*, were found shared by both bee species (Supplementary Figure 4A). The pollen types associated with *C. decolorata* did not reflect the plant species near their nests, such as *Canavalia rosea*, *Ipomoea pes-caprae,* and *Bidens Alba* (Martinez-Llaurador, 2021). *Apis mellifera* had a weaker interaction with *Combretaceae* sp.1 and a stronger interaction with *Byrsonima* sp. than expected compared to the null model (Supplementary Figure 4B). The remaining interactions between both bee species and plant resources were described as consistent with the null model (Supplementary Figures 4B,C). Although *A. mellifera* had a higher plant resource species richness and diversity than *C. decolorata*, there were no significant differences (Kruskal-Wallis test $p=0.20$ and $p=0.28$ respectively).

Discussion

Our first attempt to compare the gut microbiota of social (*A. mellifera*) and solitary (*C. decolorata*) bees has revealed that (1) microbial variability is higher in *C. decolorata* compared to *A. mellifera* and (2) for the solitary bee, the microbiota of their brood cell contents is significantly different from the gut microbiota of adults.

Life history influences the gut microbiota of bees, as well as their nest microbiota

The lower physical contact between solitary bee individuals, compared to social ones (Wittwer et al., 2017) is one of the factors that lead to variability in microbial communities among individuals. With social interactions, including trophallaxis, social bees directly share their gut bacteria, reducing probability of interindividual variation. This participates in the maintenance of a consistent core gut microbiota. Compared to social bees, environmental transmission pathways of solitary bees play a stronger role in the acquisition of bacteria, probably due to differences in nesting habits and materials (Voulgari-Kokota et al., 2019b). Solitary bees such as *Centris* use pollen, nectar, secretions from mandibular and Dufour's glands, and floral oils to build their nest. After the brood cell is completed, provisioned, provided with an egg, and sealed, the female has no contact with its brood. Through various strategies, the brood is protected from parasites, microbes, predators, and external environment variation, which is especially important in warm and humid environments (Danforth et al., 2019). As a first-line defense, female solitary bees coat their brood cells with glandular secretions which may be combined with other collected materials. Secretion from their Dufour's gland is the primary source for lining brood cells. It consists mostly of large polar molecules, providing waxy, hydrophobic coating to the brood cell (Danforth et al., 2019). Their exact composition varies among *Centris* species (Cane and Brooks, 1983), though *Centris* from the Antilles have yet to be analyzed. Further studies should evaluate if female *Centris* use these secretions only to coat the brood cells or also to mix them with provisions, as do some other solitary bees, e.g., Megachilids (Williams et al., 1986).

Some solitary bees also use mandibular gland secretions as antimicrobials (Cane et al., 1983), sometimes to first disinfect the brood cell prior to lining (Cane and Tengö, 1981). For instance, linalool, citral, geraniol, nerol or citronellol, all mandibular secretions, are effective inhibitors of fungal and bacterial growth in multiple species of solitary bees (Cane et al., 1983). These molecules and their specific targets are yet to be described for Centridini. Floral oils may also serve as protective coating materials (mostly stearic acid and elaiophore lipids; Danforth et al., 2019) which females collect from multiple plant families such as Malpighiaceae, Calceolariaceae, Iridaceae, Orchidaceae, Krameriaceae, Plantaginaceae, and Solanaceae (Martins et al., 2015).

In addition to brood cell lining, females may also mix the floral oils with provisions as an energy source (Buchmann, 1987), but the generality of this incorporation is not well understood, due to the paucity of species for which nest provisions and linings have been chemically analyzed (Neff and Simpson, 2017). Whether floral oils replace nectar is not absolute: brood cell provisions may contain trace to appreciable amounts of sugar and oils (Neff and Simpson, 1981a). Alternatively, Neff and Simpson proposed that mixing floral oils with provisions is advantageous to oil-collecting bees nesting in environments susceptible to flooding. This argument is based on the absence of oil-collecting habits for *Centris* species in xeric habitats. Indeed, incorporating floral oils to the provisioning could inhibit hygroscopic effects of provisions from bees nesting in extremely moist environments, but also control mold or bacterial infection, or act as a deterrent against nest parasites (Neff and Simpson, 1981b). For instance, levulinic acid, an oil collected for brood cells, acts as an anti-fungal agent (Neff and Simpson, 1981a).

Given this wide variety of materials used for brood cell construction and provisioning, it is not surprising to see such diverse microbiota present in the guts of adult solitary bees, and even more in

the brood cell provisions. How these bacteria survive in this antimicrobial, yet nutrient-rich brood cell microcosm is unknown, yet. We assume that the presence of these bacteria is important, as they will determine the digestibility of the raw pollen clump by larvae. Further research should describe the chemical composition of the brood cell provisions of Centridini bees, as well as the microbial targets of glandular secretions and floral oils.

High microbial diversity in brood cells related to mass provisioning by mother solitary bees

While other studies showed that bacteria from the honeybee gut is transferred to their corbicula pollen during the process of pollen packing (Prado et al., 2022), the bacteria isolated from brood cells of *C. decolorata* clearly have a plant origin. Some of these bacteria are known to induce plant growth, e.g., Rhizobiaceae, Chitinophagaceae, or Lachnospiraceae, or to inhibit it, e.g., Xanthomonadaceae, or Alcaligenaceae (Gnanamanickam, 2006). It would be interesting to test if bees are able to modulate the abundance of plant-inhibiting bacteria in later stages of brood cell provisions, as pollen from brood cells is no longer available for pollination. Another constituent of the core microbiome from *C. decolorata*'s brood cell, Enterobacteriaceae, has been previously found in pollen (Madmony et al., 2005; Ambika Manirajan et al., 2016; Straumite et al., 2022) and larvae (Parmentier et al., 2018). This latter family also has a plant origin, especially flowers (Gnanamanickam, 2006; Junker et al., 2011; Junker and Keller, 2015). Other studies reported on the presence of some *Lactobacillus*, namely *L. micheneri*, *L. timberlakei*, and *L. quenuiae*, in the pollen provisions, bee guts and flowers. In addition to being tolerant of osmotic stress, these lactic acid bacteria are able to degrade the outer pollen wall (Lipiński, 2018), which makes pollen digestible for early larval feeding (Gurevitch and Padilla, 2004; Gilliam, 2006; Vuong et al., 2019; Voulgari-Kokota et al., 2019a). Given *Lactobacillus* presence in trace amounts, future description of the brood cell core microbiome combined to pollen wall degradation analyses at brood cell age should help identifying bacteria involved in pollen pre-digestion process.

All these brood cell constituents (pollen, possibly evaporated nectar and floral oils, and the associated bacteria) are definitely brought by *Centris decolorata* females to the larvae. But even though females are the ones provisioning the brood cells for the larvae, their gut microbiota is significantly different from the microbiota of the brood cell provisions, at least at early stages. These results contrast with previous findings of another solitary bee from a semi-arid region (although in dense aggregations of millions of bees), where the gut microbiota of females and larvae were similar (Kapheim et al., 2021). In *C. decolorata*, females provision the brood cell for the larva independently to what she ingests. In our study, the gut microbiota of both male and female solitary bees are more similar to each other than to that of brood cell contents, at least initially. Indeed, the collected brood cells were at early stages of larval development. The provisions were thus essentially composed in flower-specific bacteria, which is coherent considering that provisions are mainly constituted by pollen. Later, these flower-specific bacteria shift to bacteria able to grow on nutrient rich mixture, i.e., the proteins from pollen, and possibly sugar from nectar and floral oils. Microbial composition of the larvae and provisions therefore changes along with larval development (Voulgari-Kokota et al., 2018). To confirm the differences in microbial composition between females and nest provisions, future microbial assessment should consider a larger brood cell sampling, with early and later larval stages (using larval development as a proxy for bacterial shift). Indeed, assessing the brood cell provisions at the middle/end of the *C. decolorata* season would probably lead to higher probability to encounter brood cells of later larval stages. These brood cells would thus be composed in bacteria able to grow on nutrient rich mixture, probably similar to microbiota of females and larvae.

Dominance of acetic acid bacteria in nesting solitary bees

Gut acidification of solitary and social bees seems to be driven by different phylotypes: Lactic Acid Bacteria in honeybees vs. Acetic Acid Bacteria in solitary *C. decolorata*. The trace amounts of Lactobacillaceae in *C. decolorata* guts could represent horizontal acquisition from *A. mellifera*, or environmental pools of related strains to *C. decolorata*. Acetic Acid Bacteria (AAB) are strict aerobic bacteria and ubiquitous. They occur in a wide variety of substrates such as in plants and flowers (Crotti et al., 2010). They are widespread in carbohydrate-rich, acidic, and alcoholic niches, such as nectar, which has been proposed as an origin for these bacteria (Morris et al., 2019; Ravenscraft et al., 2019). In addition to being considered environmental and ubiquitous bacteria, AAB are also important insect symbionts, as for food uptake and host survival. Insect associations are stable and follow several transmission routes for their propagation (Crotti et al., 2010). In our samples, two families of AAB were found: Moraxellaceae and Acetobacteraceae. Bacteria from the Moraxellaceae family, especially *Acinetobacter* have been isolated from the solitary male guts, but not from the brood cell provisions of our study, even though reported in the literature as present in pollen provisions coming from Mediterranean plants (Álvarez-Pérez et al., 2013). Although not found in pollen provision of our study, the bacteria from Acetobacteraceae were present in the guts of adult *Centris decolorata* (both males and females) and constitute their core microbiome. It was scarcely present in foraging honeybees that typically do not interact with larvae in hives (Winston, 1987), as reported in domestic local honeybees (gentle Africanized Honeybee, *gAHB*) by Ortiz-Alvarado (2019). Acetobacteraceae has been isolated from the gut of adult honeybees (Sabree et al., 2012; Anderson et al., 2013; Corby-Harris et al., 2014; Thompson et al., 2017; Ortiz-Alvarado, 2019), and different Acetobacteraceae (i.e., Alpha 2.2 Acetobacteraceae) from honeybee larvae (Corby-Harris et al., 2014; Ortiz-Alvarado, 2019). Interestingly, Acetobacteraceae was found in larvae, nymphs, young nest bees, and royal jelly in the same study, but it was almost absent in honeybee foragers. Acetobacteraceae could thus be a family of bacteria related to nursing bees, i.e., larvae, nymphs, young nest bees, and royal jelly in honeybees, and in female solitary bees, who play a nursing role. The absence of Acetobacteraceae in the brood

cell of this study could suggest that the transfer of these symbionts could mostly be vertical for *Centris decolorata,* but this remains to be identified in a larger sample size of solitary bee larvae. If AAB such as Acetobacteraceae are present in females and larvae but not in the brood cell provisions, then vertical transmission of these symbionts would be preferred by *Centris decolorata*.

Pollen acquisition routes do not explain microbial differences between solitary and social bees

Some Megachilids show a significant association between the composition of their foraged pollen and the pollen bacterial communities and larval bacterial communities. In these bees, where bacterial transmission pathways through eusociality are impossible, pollen foraging appears to be very important to obtain their bacterial symbionts (Voulgari-Kokota et al., 2019a). In our limited pollen study, we found that pollen resources foraged by *Apis mellifera* and *Centris decolorata* were not significantly different whereas their gut microbiota were composed of different bacterial phylotypes. Therefore, pollen acquisition routes cannot explain differences in the gut microbiota between the studied social and solitary bees. *Apis mellifera* has been previously shown to be weakly impacted by microbiota from pollen (Donkersley et al., 2018; Jones et al., 2018). Pollen samples of *C. decolorata* evidence the presence of additional plant resources along their foraging range, suggesting they forage for pollen over long distances from their nest locations, in addition to use pollen resources from plant species found near their nests (Pers. Obs.). Interactions between *C. decolorata* and *Byrsonima* sp. reflected a lower pollen abundance, suggesting that individuals visit *Byrsonima* sp. to primarily collect oils and incidentally collect pollen along their bodies. Although *C. decolorata* transports fewer pollen grains between individuals of *Byrsonima* sp., these could be sufficient to pollinate the flowers. However, *A. mellifera* appears to be an effective pollen forager having a stronger interaction, influenced by a greater abundance of pollen on its corbicula, with *Byrsonima* sp. For a broader overview of plant species visited by *C. decolorata*, bee sampling and pollen analyses over the season and on different daily periods should be considered for future studies.

Conclusion

Bee population decline is a global threat with possible losses of important ecosystem services which they provide, most importantly pollination. While most bee species are solitary, these have been understudied compared to social bees (e.g., honeybees and bumblebees). Unfortunately, conservation strategies to reverse population declines may not be the same for solitary bees as they may be for social bees. To our knowledge, the present study is the first microbiota inventory from a tropical solitary bee. We collected solitary bees in Puerto Rico and characterized the gut microbiota in adults and brood cells. A higher microbial variability in *Centris decolorata* was observed compared to co-occurring, feral *Apis mellifera*, and unexpectedly there was a low number of shared bacteria between females and brood cell contents. Even though female solitary bees are the ones rearing and providing resources to the offspring, larvae and their brood cell provision differ significantly from adult males and females. Females thus provide an independent provisioning of materials to the brood cells affecting their microbiota. These results highlight diversity in wild solitary bees, i.e., remarkable diversity in morphological traits, nesting habits and host-plant associations (Danforth et al., 2019), and their differences from wild social bees, e.g., *Bombus terrestris* which has relatively long period of activity, a tolerance for temperate extremes, and a broad diet (Ghisbain, 2021). As such, this study points to the need for further research on microbiota, pollen sources, and metabolism of this and other solitary bees for developing conservation strategies and securing pollination services. The coastal oil-collecting bee *Centris decolorata* is indeed an important ecosystem service provider, as it nests in the dunes and pollinates its vegetation. Indirectly, pollination by *C. decolorata* acts as a barrier to erosion, especially in case of extreme climatic events such as hurricanes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, ERP141576.

Author contributions

EK and FG-V: conceptualization. EK: data curation. EK, AG-R, and FG-V: formal analysis. FG-V: funding acquisition, project administration, and supervision. EK, FG-V, AG-R, and JA: investigation and writing—original draft. EK, AG-R, and JA: methodology. EK, AG-R, TG, JA, and FG-V: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1122489/full#supplementary-material

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