Composition and diversity of bacterial communities associated with honey bee foragers from two contrasting environments

Stela Lazarova[1], Lyudmila Lozanova[2], Boyko Neov[1], Rositsa Shumkova[3], Ralitsa Balkanska[4], Nadezhda Palova[5], Delka Salkova[6], Georgi Radoslavov[1] and Peter Hristov[1]

[1]Department of Animal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria; [2]Department of Ecosystem Research, Environmental Risk Assessment and Conservation Biology, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria; [3]Research Centre of Stockbreeding and Agriculture, Agricultural Academy, 4700 Smolyan, Bulgaria; [4]Department 'Special Branches', Institute of Animal Science, Agricultural Academy, 2230 Kostinbrod, Bulgaria; [5]Scientific Center of Agriculture, Agricultural Academy, Sredets 8300, Bulgaria and [6]Department of Experimental Parasitology, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Abstract

The honey bee is associated with a diverse community of microbes (viruses, bacteria, fungi, and protists), commonly known as the microbiome. Here, we present data on honey bee microbiota from two localities having different surrounding landscapes – mountain (the Rhodope Mountains) and lowland (the Danube plain). The bacterial communities of abdomen of adult bees were studied using amplicon sequencing of the 16S rRNA gene. The composition and dominance structure and their variability within and between localities, alpha and beta diversity, and core and differential taxa were compared at different hierarchical levels (operational taxonomic units to phylum). Seven genera (*Lactobacillus*, *Gilliamella*, *Bifidobacterium*, *Commensalibacter*, *Bartonella*, *Snodgrassella*, and *Frischella*), known to include core gut-associated phylotypes or species clusters, dominated (92–100%) the bacterial assemblages. Significant variations were found in taxa distribution across both geographical regions and within each apiary. *Lactobacillus* (Firmicutes) prevailed significantly in the mountain locality followed by *Gilliamella* and *Bartonella* (Proteobacteria). Bacteria of four genera, core (*Bartonella* and *Lactobacillus*) and non-core (*Pseudomonas* and *Morganella*), dominated the bee-associated assemblages of the Danube plain locality. Several ubiquitous bacterial genera (e.g., *Klebsiella*, *Serratia*, and *Providencia*), some species known also as potential and opportunistic bee pathogens, had been found in the lowland locality. Beta diversity analyses confirmed the observed differences in the bacterial communities from both localities. The occurrence of non-core taxa contributes substantially to higher microbial richness and diversity in bees from the Danube plain locality. We assume that the observed differences in the microbiota of honey bees from both apiaries are due to a combination of factors specific for each region. The surrounding landscape features of both localities and related vegetation, anthropogenic impact and land use intensity, the beekeeping management practices, and bee health status might all contribute to observed differences in bee microbiota traits.

Introduction

The western honey bee (*Apis mellifera*) is a key pollinator of many agricultural crops and wild plants (Hung *et al*., 2018). The ongoing trend of honey bee decline throughout the world has brought the attention to the great loss of ecological services provided by this species, its health status, and numerous threats it faces (Paudel *et al*., 2015; Boncristiani *et al*., 2020). Bees are associated with a diverse community of microbes (viruses, bacteria, fungi, and protists), commonly known as the microbiome. As a superorganism, the honey bee has complex interactions with its own microorganisms, the microbial communities of food stores, hive surfaces, and surrounding environments; therefore, four major groups of bee-associated microbiomes have been distinguished (Smutin *et al*., 2022). These include gut and body surface microbiota, microbiota of the in-hive environment (honeycombs, brood combs, propolis, royal jelly, etc.), and microbiota of the surrounding environment. Recent advances in DNA technologies and biomolecular analyses enabled new insights into the understanding of microbiota composition, dynamics, and impact on host biology and health. Except for a few bacterial pathogens that are widespread in apiaries and causing high mortality (Forsgren, 2010; Genersch, 2010), the functional role of most members of bee-associated microbiota on host and colony health is not

completely understood (Engel *et al.*, 2016; Khan *et al.*, 2020). The gut microbiome is the most extensively studied part of the bee-associated microbiota (Romero *et al.*, 2019) used as a model for gut microbiota research (Zheng *et al.*, 2018). It is relatively simple and specialized, consisting of 8–10 recurring phylotypes or species clusters called Alpha-1 (*Bartonella apis*), Alpha-2.1 (unclassified species of Acetobacteriaceae family), Alpha-2.2 (*Saccharibacter floricola*), Beta (*Snodgrassella alvi*), Bifido (*Bifidobacterium asteroides*), Gamma-1 (*Gilliamella apicola*), Gamma-2 (*Frischella perrara*), Firm-4 (*Lactobacillus mellis* and *L. mellifer*), Firm-5 (other *Lactobacillus* spp. including (*Apilactobacillus*) *kunkeei*) (Sabree *et al.*, 2012; Kwong and Moran, 2016). Despite the conserved bacterial phylotype composition, it was found that each honey bee harbors a distinct community at the functional level (Ellegaard and Engel, 2019).

It is well known that *S. alvi*, *G. apicola*, two species of *Lactobacillus* (*L. apis* and *L. helsingborgensis*), and *Bifidobacterium* spp. are ubiquitous and can be found in every adult honey bee worker worldwide, often considered as part of the core gut microbiome composition (Erban *et al.*, 2017; Nowak *et al.*, 2021). Other microbial species, e.g., *B. apis*, *Apibacter adventoris*, *F. perrara*, and species of the family Acetobacteraceae, have been sometimes found in the gut of many workers, but exceptions exist (Jones *et al.*, 2018; Raymann and Moran, 2018). The gut bacteria showed specific spatial distribution related to different sections of the honey bee digestive system (Martinson *et al.*, 2012; Callegari *et al.*, 2021). *Snodgrassella alvi* and *G. apicola* dominate in the ileum region of the hindgut, *F. perrara* occupy the pylorus near the beginning of the ileum, while the *Lactobacillus* strains and *Bifidobacterium* spp. are most abundant in the rectum region of the hindgut (Raymann and Moran, 2018).

The functional role of the gut microbiome was mainly related to host nutrition and metabolism, regulation of the immune system, and pathogen resistance (Emery *et al.*, 2017; Kwong *et al.*, 2017; Bonilla-Rosso and Engel, 2018; Daisley *et al.*, 2020; Castelli *et al.*, 2021), and alterations in gut microbiota (such as dysbiosis) may affect host metabolic homeostasis and immunity (Yuan *et al.*, 2019; Zhu *et al.*, 2020). Multiple factors, such as bee age, caste and social activity, presence of pathogens and parasites, exposure to agrochemicals and anthropogenic toxins, the microbiota of in-hive environment and stored food, as well as the landscape heterogeneity and related local environmental conditions (including vegetation diversity, land-use type, season), are known to affect honey bee gut microbiome composition and dynamics (Anderson *et al.*, 2013; Graystock *et al.*, 2015; Engel *et al.*, 2016; Kwong and Moran, 2016; Jones *et al.*, 2018; Bosmans *et al.*, 2018; Ribière *et al.*, 2019; Kešnerová *et al.*, 2020; Muñoz-Colmenero *et al.*, 2020). The versatility and potential utility of the western honey bee, including its associated microbiota, as biomonitors of environmental pollutants, pesticides use, presence of pathogens, climate change, and ecosystem health, has been recognized and recently reviewed (Cunningham *et al.*, 2022).

Traditionally, the microorganisms associated with honey bees and their food are determined by examination, and subsequent isolation and identification, using appropriate tests and taxonomic keys (see Snowdon and Cliver, 1996; Gilliam, 1997 for reviews). Very often taxonomic identification is difficult to implement in species with similar morphological features. This requires an application of high-sensitivity methods such as DNA sequencing including DNA barcoding (Porter and Hajibabaei, 2018; Eberle *et al.*, 2020). Recently, a novel method (environmental DNA metabarcoding) comprising DNA isolation from mixed or bulk environmental samples, amplification, high-throughput sequencing (HTS), and bioinformatics was widely applied for studying and monitoring biological diversity including microbiome diversity (Deiner *et al.*, 2017; Piper *et al.*, 2019; Kennedy *et al.*, 2020). HTS of 16S and internal transcribed spacer ribosomal RNA (rRNA) markers is now one of the most effective and extensively used approaches in microbiome studies that also provides quantitative estimates of the microbial community composition (Bukin *et al.*, 2019).

In this study, we investigated the bacterial communities associated with honey bees from two regions in Bulgaria (mountain and lowland) by applying DNA metabarcoding approach based on 16S rRNA gene. The objectives were: (i) to detect 'core' bee-associated bacteria and differential taxa specific to each locality and (ii) to compare the bacterial community structure and diversity within and between localities.

Materials and methods

Sites description and sampling

Bee specimens were collected during honey production season (June 2018) from two different localities: the 'Experimental Apiary of the Research Center of Stockbreeding and Agriculture', located on the outskirts of Smolyan city (41°35′40″N, 24°40′39″E), the Rhodope Mountains (RM), Southern Bulgaria and from a private apiary in a lowland village near to Byala city (43°25′6″N, 25°49′14″E), Danube plain (DP) region, Northeastern Bulgaria. Both sites differed considerably by their topography and landscape characteristics, altitude, dominant land use type, and vegetation. The locality in the Rhodope Mtn is mountainous with an average altitude of about 1160 m, a very heterogeneous environment, steep slopes, more diverse landscape structures, numerous grasslands and pastures, and coniferous tree vegetation. In contrast, the landscape of the lowland locality in the Danube plain region is slightly hilly (altitude ranges 100–300 m a.s.l.), surrounded by agroecosystems with annual and perennial crops, broadleaved vegetation, small forest patches.

To identify bee-associated microbiota, 15 hives from each apiary were randomly selected. From each hive, one bee forager returning to the hive with pollen on their legs was collected with sterile tweezers from each hive near to hive entrance. Each bee specimen was placed in a sterile 50 μl falcon and immediately frozen on dry ice in the field. The samples were brought to the laboratory of the Institute of Biodiversity and Ecosystem Research and stored at −20°C before processing.

DNA extraction, PCR amplification, and sequencing

Prior to DNA extraction, the posterior body segment of bee specimens (known also as metasoma or abdomen) was cut-off with sterile scissors. For pragmatic reasons, we will use the term abdomen in this text. After that, the bees were mechanically homogenized by sterile pestle in a total of 350 μl Lyse T buffer (GeneMATRIX Tissue DNA Purification kit, Cat.no. E3550, EURx Sp. z o.o.; Gdansk, Poland) to complete the destruction of the abdomen. After removal of all tissue fragments, total DNA isolation was performed according to the manufacturer's instructions. DNA concentration and quality were measured using an Analytikjena spectrophotometer (Analytik Jena GmbH, Germany). The extracted DNA was then sent to the AIM

laboratory (https://www.aimethods-lab.com/) for DNA metabarcoding using NGS (sequencing and bioinformatics processing).

From each sample, 5 µl of extracted genomic DNA was used, along with Plant MyTAQ (Bioline, Luckenwalde, Germany). The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the adapted primers 16s-barcode primers (341f – TACACGACGCTCTTCCGATCTTCATCCTACGG GNGGCWGCAG – and 785r – CAGACGTGTGCTCTTCCGA TCCGCTCAGACTACHVGGGTATCTAATCC) (Morinière *et al.*, 2016; Thijs *et al.*, 2017; Morinière *et al.*, 2019). Amplification success and fragment length were assessed using gel electrophoresis. Amplified DNA was cleaned up and resuspended in 50 µl molecular water for each sample before proceeding. Illumina Nextera XT (Illumina Inc., San Diego, USA) indices were ligated to the samples in a second PCR reaction applying the same annealing temperature as for the first PCR reaction but with only seven cycles, and ligation success was confirmed by gel electrophoresis. DNA concentrations were measured using a Qubit fluorometer (Life Technologies, Carlsbad, USA), and samples were combined into 40 µl pools containing equimolar concentrations of 100 ng each. Pools were purified using MagSi-NGSprep Plus (Steinbrenner Laborsysteme GmbH) beads. A final elution volume of 20 µl was used. HTS was performed on an Illumina MiSeq using v3 (2×300 bp, 600 cycles, maximum of 25 mio paired-end reads) chemistry.

Bioinformatic processing

Paired-end merging was done using the -fastq_mergepairs utility of the USEARCH suite v11.0.667_i86linux32 (Edgar *et al.*, 2011). Adapter sequences were removed using CUTADAPT (Martin, 2011) (default parameters). The remaining pre-processing steps, namely quality filtering, dereplication, chimera filtering, and clustering were carried out using the VSEARCH suite v2.9.1 (Rognes *et al.*, 2016). Sequences were trimmed to a length of 400 bp and were only included in the analyses that matched perfectly to the barcode. 16S rRNA genes were classified using the SILVA database (Quast *et al.*, 2012) and NCBI database (O'Leary *et al.*, 2016) both with a minimum similarity threshold of 97%. The resulting file could then be used to map the reads to the operational taxonomic units (OTUs) to create the OTU table. To reduce likely false positives, a cleaning step was employed which excluded read counts in the OTU table that constituted less than 0.01% of the total number of reads in the sample. OTUs were blasted against a custom database downloaded from GENBANK using Geneious (v.10.2.5 – Biomatters, Auckland – New Zealand), and following methods described in Morinière *et al.* (2016). OTUs were additionally removed from the results based on negative control samples, i.e., if the combined number of reads in the negative controls constituted more than 20% of the total number of reads in the OTU. OTUs were also annotated with the taxonomic information from NCBI (downloaded from https://ftp.ncbi.nlm.nih.gov/pub/taxonomy/), followed by a creation of a taxonomic consensus between BOLD, NCBI, and RDP.

A total of 1,667,218 raw reads of the V3–V4 region of the bacterial 16S rRNA were obtained and assembled in 833,609 clean sequences with an average sequence length of 411 bp, and with a maximum of one expected error. These sequences were obtained from the 20 assayed samples (13 from the Rhodope Mountains and 7 from the Danube plain). Before chimera detection, 419 bacterial OTUs were identified at the 97% sequence similarity cut-off. After chimera filtering and removing data with low-quality scores for three bee specimens from Danube plain locality and OTUs

identified as organelle DNA (chloroplast and mitochondria), 66 OTUs and 17 samples remained for further analyses (Supplementary table S1).

Data analyses

The microbiome data analyses were performed using the web-based platform MicrobiomeAnalyst (Dhariwal *et al.*, 2017; Chong *et al.*, 2020) based on the OTUs abundance table for 17 samples containing raw counts of 66 OTUs. Taxonomy data in text table format and the metadata file were uploaded and further analyzed in the Marker-gene Data Profiling (MDP) module. Taxa having less than a 10% prevalence with a read number ≤ 2 were filtered out. The 39 most frequent and abundant OTUs were further involved in the analyses after data filtering. The OTU abundances were rarefied to the minimum library size (Supplementary fig. S1a) (Kandlikar *et al.*, 2018) and normalized. Rarefaction curves per each sample are presented (Supplementary fig. S1b). Core bacterial taxa were distinguished and visualized by setting up a 20% prevalence value and 0.01 for the relative abundance (RA) value. Three alpha-diversity measures (Shannon diversity, Simpson diversity, and Chao1 indices) were calculated at OTUs and generic levels and visualized (McMurdie and Holmes, 2013). Chao1 index estimates taxa richness by accounting for taxa (e.g., OTUs, species, or genera) that are undetected because of low abundance. Shannon and Simpson's indices are based on taxa richness and their abundances (or evenness). Pairwise comparisons and the significant differences between both groups were calculated using the non-parametric Mann–Whitney U test ($P \leq 0.05$). To observe the average dissimilarity in the composition of microbial communities in both regions of the country, beta diversity was estimated based on Bray–Curtis similarity distances and visualized with non-metric multidimensional scaling (NMDS) ordination (McMurdie and Holmes, 2013). The statistical significance of the clustering pattern in ordination plots was evaluated using the permutational multivariate analysis of variance (PERMANOVA). Differences in taxonomic classification between both geographical regions were illustrated using heat tree analysis (Foster *et al.*, 2017). It leverages the hierarchical structure of taxonomic classifications to quantitatively (using the median abundance) and statistically (using the non-parametric Wilcoxon rank-sum test, $P < 0.05$) depict taxonomic differences between microbial communities (view mode: comparison) or abundance profiles (view mode: abundance). Linear discriminant analysis of effect size (LEfSe) was applied to identify bacterial taxa specific to each locality using a significance level of $P < 0.05$ (Segata *et al.*, 2011).

Results

Bacterial community structure, composition, and variability

The taxonomic assignment of the 39 most common and abundant OTUs revealed five phyla, six classes, 12 orders, 15 families, and 22 genera (fig. 1, Supplementary fig. S2). Among bacterial phyla, Proteobacteria, Firmicutes, and Actinobacteria dominated the bee-associated communities at both locations (99–100%, fig. 1a). Proteobacteria prevailed significantly in all bee specimens from the lowland locality (DP, 68–87%) and in five bee specimens from the Rhodope Mtn (RM, 52–79%), followed by Firmicutes (RM: 17–63%, DP: 10–27%) and Actinobacteria (0.4–14%). OTUs assigned to Bacteroidetes and Tenericutes were present in
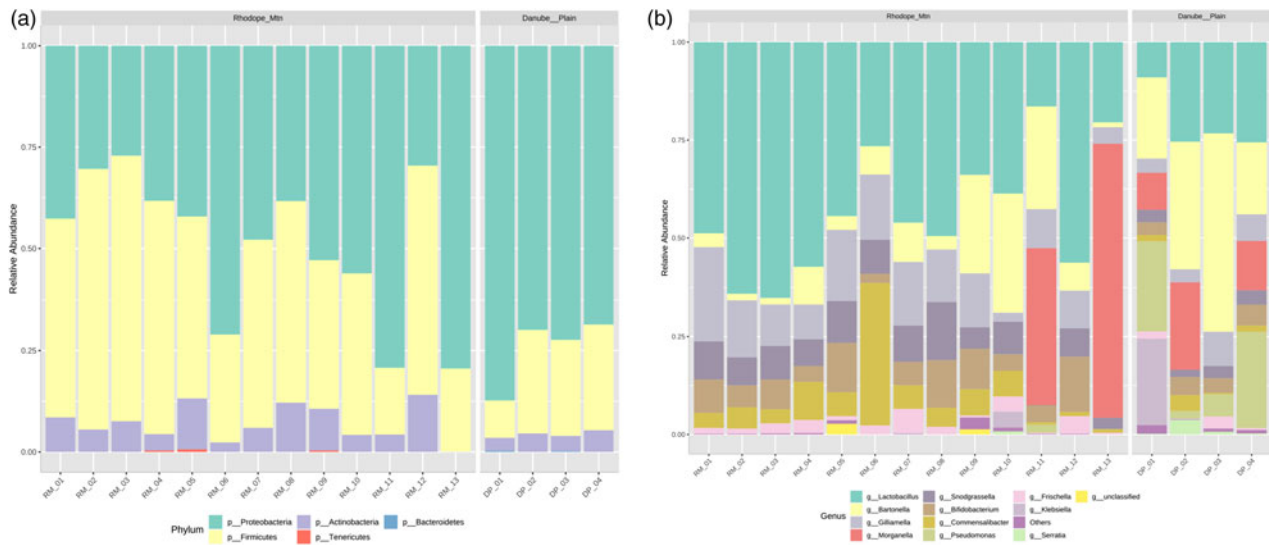
Figure 1. Bar charts illustrating the distribution and relative abundances of bacterial phyla (a) and genera (b). Data are represented for each honey bee specimen and locality. RM, Rhodope Mountains; DP, Danube plain.

a very low RA (0.3–0.9%) and occurrence in both localities. A clear variability within and between localities was also observed in the RAs for bacterial families (Supplementary fig. S2a) and genera (fig. 1b). In the bee specimens from the Rhodope Mtn, on average 43% of reads belonged to the family Lactobacillaceae (mainly *Lactobacillus*, 43%); Orbaceae make up 14% of Bacteria (*Gilliamella*, 12% and *Frischella*, 2%), followed by 10% RA of Rhizobiaceae (*Bartonella*), 9% – Enterobacteriaceae (mainly *Morganella*, 8%), 8% – Neisseriaceae (*Snodgrassella*), and about 7% of Bifidobacteriaceae (*Bifidobacterium*) and Acetobacteraceae (*Commensalibacter*). Other families, such as Leuconostocaceae (*Fructobacillus*), Pseudomonadaceae (*Pseudomonas*), Spiroplasmataceae (*Spiroplasma*), and Sphingomonadaceae (*Sphingomonas*), were found in less than 1% of the total OTU abundance (Supplementary fig. S2a).

In bee specimens from the Danube plain locality, on average 30% of OTUs belonged to the family Rhizobiaceae (*Bartonella*), 21% – Lactobacillaceae (mainly *Lactobacillus* – 20%), 18% – Enterobacteriaceae (*Morganella* – 10%, *Klebsiella* – 6%, *Serratia* – 1%), 14% – Pseudomonadaceae (*Pseudomonas* spp.), and 7% to Orbaceae (*Gilliamella* – 6% and *Frischella* – 1%). The average RA of bacteria belonging to families Neisseriaceae (*Snodgrassella*), Bifidobacteriaceae (*Bifidobacterium*), and Acetobacteraceae (*Commensalibacter*, *Bombella*) was lower than 3% (2–4%). Bacteria from five families (Carnobacteriaceae, Flavobacteriaceae, Oxalobacteraceae, Sphingobacteriaceae, and Sphingomonadaceae) were found in low frequency and RAs (>0.3%) (Supplementary fig. S2a). Heatmaps visualizing bacterial taxa composition and their RAs at various taxonomical levels (species, genera, families, and orders) for each locality and individual bee specimens are presented in the Supplementary fig. S3a–d.

Bacterial diversity, core, and differential taxa

The total number of OTUs found in all bees from both localities was 66 (Supplementary table 1) with one-third of them (or 27 OTUs) revealed in a very low number of reads (≤10 per bee specimen) mainly from the Danube plain locality. *Lactobacillus* and *Gilliamella* showed the highest OTU richness. Because of the

limited number of successfully sequenced specimens from this locality, we could not be sure if these are indeed rare bacterial phylotypes. Despite this bias in bee specimens' quantity, the OTU richness of microbial communities in bees from the Danube plain was higher as compared to those from the Rhodope Mtn (29 vs. 35, in total) and per bee specimen (fig. 2). Twenty-five OTUs and 15 genera were common for microbial communities of both localities, of them ten OTUs and five genera were unique for the Danube plain area (Supplementary fig. S4).

Alpha diversity assessed by Shannon, Simpson, and Chao1 indices revealed different trends when calculated at OTUs and genus levels (Supplementary fig. S5a–f). The bacterial communities of bees from the Danube plain were significantly more diverse than those of the Rhodope Mtn when genus data were compared (Mann–Whitney U test, Shannon ($P = 0.04$), Simpson ($P = 0.059$), and Chao1 ($P = 0.01$); Supplementary fig. S5d–f). Except for Chao1's diversity index ($P = 0.004$; Supplementary fig. S5c), no
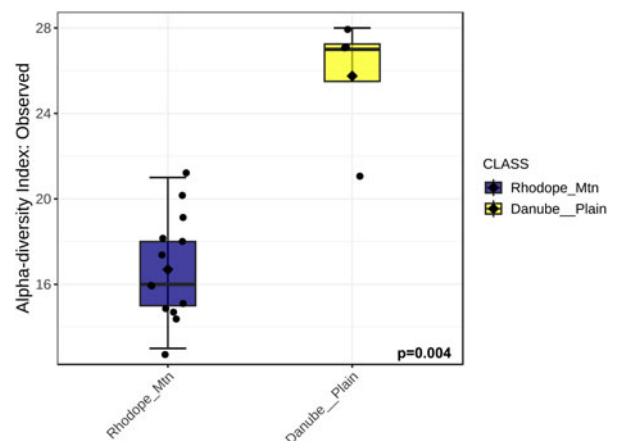

Figure 2. Alpha diversity measure showing the observed variation of OTU numbers (richness) in the bacterial communities. Data are represented as a box plot. RM, Rhodope Mountains; DP, Danube plain; Mann–Whitney U test statistic: 0.05, P value: 0.004.
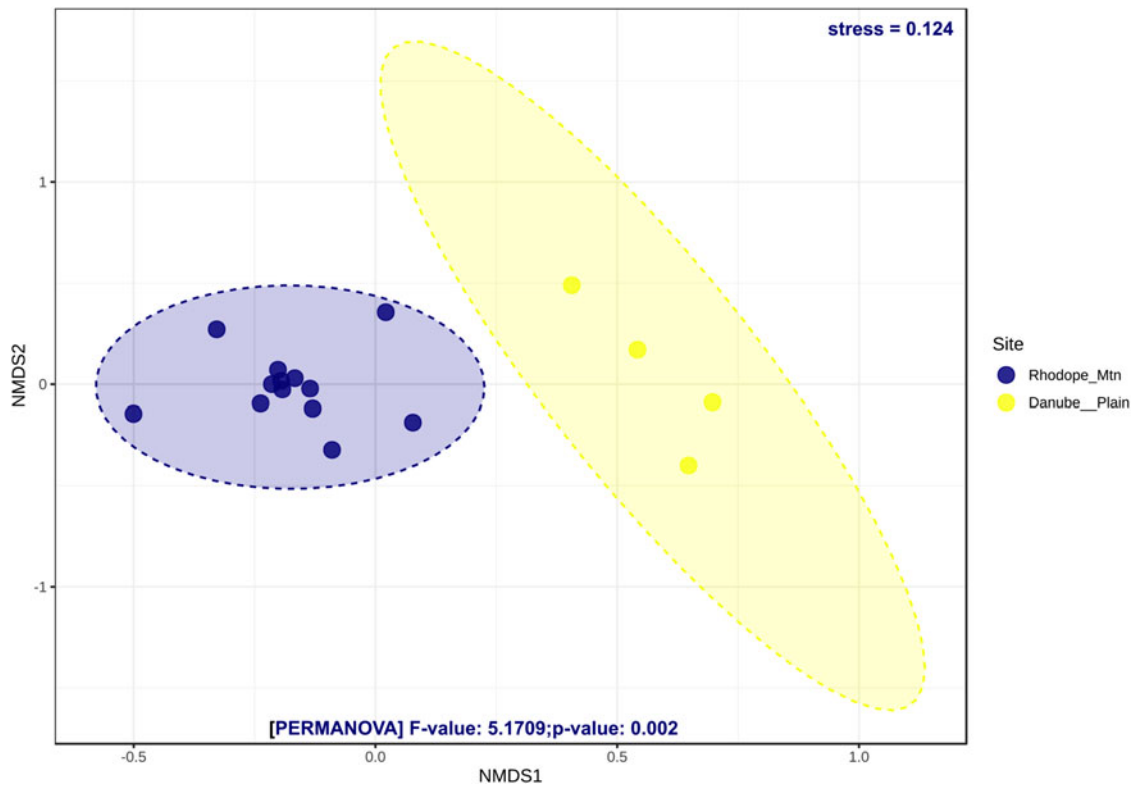
Figure 3. Non-metric multidimensional scaling (NMDS) ordination of honey bee communities at OTU level using Bray–Curtis as a dissimilarity measure. Results from the PERMANOVA test (*F*, stress, and *P* values) are represented for each taxonomic level. Individuals from the same colony and explained variances are represented by the same colors.

significant differences in OTU diversity between both localities were revealed ($P > 0.05$; Supplementary fig. S5a, b). Beta diversity analyses clearly distinguished the bacterial communities from both localities ($P = 0.002$) (NMDS; fig. 3). The microbial communities of bee specimens from the Rhodope Mtn were more similar to each other than those of the Danube plain region.

Several core bacterial taxa occurring with high frequency and abundance specific to each locality were revealed (fig. 4). *Bartonella* (*B. apis*), *Bifidobacterium* sp., *Snodgrassella* (*S. alvi*), *Frischella* (*F. perrara*), and phylotypes of *Gilliamella*, *Lactobacillus*, and *Commensalibacter*, known as honey bee gut-associated bacteria, dominated in almost all bee individuals (92–100%). However, the percentage of these specific core gut bacterial genera was less abundant (32–73%) in a few bee specimens. OTUs assigned to other four genera (*Pseudomonas*, *Serratia*, *Morganella*, and *Klebsiella*) were present with higher RA in bee specimens from the Danube plain and occasionally – in bees from the Rhodope Mtn (Supplementary fig. S3a, b).

The geographical differences in the bacterial microbiome composition and structure were also supported by the results of two additional analyses – the heat tree analysis (fig. 5) and the LEfSe (Supplementary fig. S6, table S3). Both analyses showed differences in the distribution of seven bacterial genera, i.e., *Lactobacillus* (LEfSe, $P = 0.02$), *Gilliamella* (LEfSe, $P = 0.03$), *Snodgrassella* (LEfSe, $P = 0.03$), *Bartonella* (LEfSe, $P = 0.02$), *Pseudomonas* (LEfSe, $P < 0.00$), *Serratia* (LEfSe, $P < 0.00$), and *Apibacter* (LEfSe, $P = 0.01$) with the first three genera being significantly more abundant in the Rhodope Mtn (Supplementary fig. S7). Bacteroidetes was found in honey bees from the Danube plain only (Wilcoxon's $P = 0.001$) with *Apibacter* sp.

occurring in three out of four specimens (LEfSe, $P = 0.01$, Supplementary table S3). The order Pseudomonadales, composed of four *Pseudomonas* spp., prevailed significantly in bee specimens of the Danube plain region (Wilcoxon's $P = 0.001$) (fig. S6). Other differential taxa distinguishing bee-associated bacterial communities of both localities are presented in the Supplementary materials (fig. S6 and table S3).

Discussion

In our study, we focused on the composition and diversity of bacterial communities associated with honey bees from two apiaries located in the Rhodope Mtn and Danube plain regions having different landscape topography – mountain and lowland, respectively. We characterized the bee-associated bacteria at different hierarchical levels (OTUs to phylum) by applying various community-based analyses such as composition and dominance structure, and their variability within and between localities, α and β diversity, analyses for revealing core and differential taxa.

Bacterial OTUs assigned to seven genera (*Bartonella* (*B. apis*), *Bifidobacterium* sp., *Snodgrassella* (*S. alvi*), *Frischella* (*F. perrara*), and *Gilliamella*, *Lactobacillus*, and *Commensalibacter*), known as specific gut-associated bacteria of *A. mellifera*, dominated (92–100%) the bee-associated assemblages in most specimens. Phylotypes or species clusters from these seven genera are known as specific gut microbiota that is well-adapted to honey bees worldwide, mainly transmitted through social interactions between individuals (Kwong and Moran, 2016). They occupy different sections of the bee digestive system and have evolved specific host–microbe interactions and functions (Martinson *et al.*,
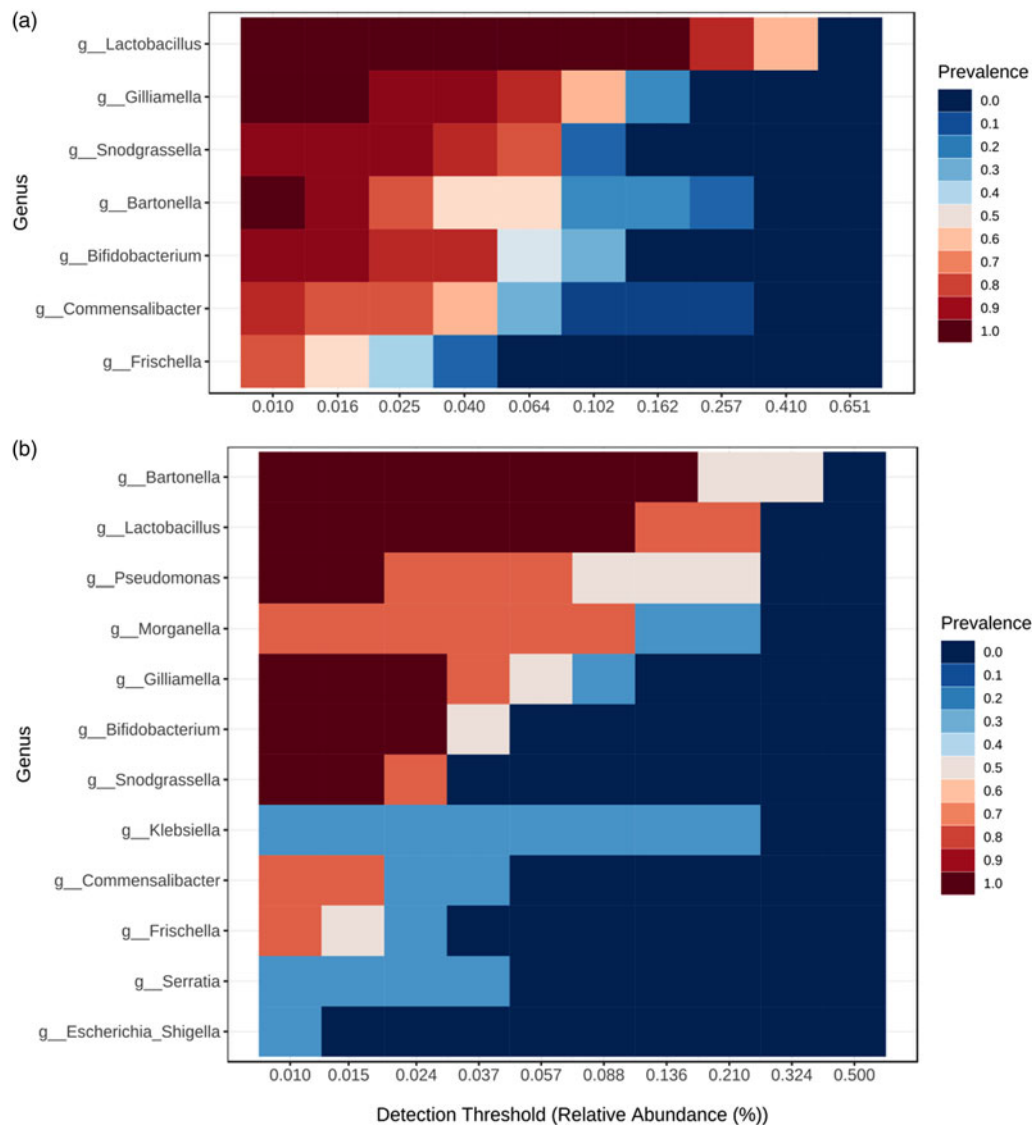
Figure 4. Heatmap representing the core bacterial genera dominating the honey bee communities from the Rhodope Mountains (a) and Danube plain (b). The *y*-axis represents the prevalence of core genera across the detection threshold range (relative abundance) on the *x*-axis. The prevalence of each core species is indicated by a color gradient from dark blue (low) to dark red (high).

2012). Two ubiquitous Gram-negative genera *Snodgrassella* (*S. alvi*) and *Gilliamella* (members of the Proteobacteria phylum) and several Gram-positive *Lactobacillus* bacteria (Firmicutes phylum) prevailed significantly in the Rhodope Mtn locality. One Proteobacteria species (*B. apis*), usually found with lower abundance and frequency, dominated the bacterial communities of bees from the Danube plain locality. These primary bacterial genera are part of the core gut microbiota of honey bees (Kwong and Moran, 2016). The *F. perrara* usually localized in a restricted niche of the bee gut (Kwong and Moran, 2016), and one unidentified *Bifidobacterium* species were present in all specimens from both localities with low and relatively high RA, respectively. Except for *F. perrara* (Engel *et al.*, 2015a, 2015b; Maes *et al.*, 2016), these bee-specific bacterial taxa are considered beneficial gut symbionts of social bees that have protective and nutritional functions (Kwong and Moran, 2016). For example, *Lactobacillus* spp. has essential roles in carbohydrate metabolism and in protecting hosts by producing antimicrobial metabolites (such as

organic acids, diacetyl, benzoate, and bacteriocins) and sugars (Zhang *et al.*, 2022). *Bifidobacterium* spp. are involved in the biosynthesis and assimilation of trehalose that is used for energy storage in insects and its presence and abundance are considered bacterial indicators for assessing the age of bee workers (Dong *et al.*, 2020).

The honey bee biology and colony behavior, as well as the microbiome composition of the in-hive environment, strongly affect the routes of acquisition and the initial composition of bacteria associated with worker bees (Powell *et al.*, 2014; Smutin *et al.*, 2022). Subsequently, the microbiomes of various in-hive structures (surfaces), stored foods (e.g., honeycombs, bee wax, propolis, pollen, royal jelly, honey, etc.), and out-hive environments (floral nectar and pollen) can contribute to the acquisition of non-core bee-associated microbiota (Snowdon and Cliver, 1996; Kačániová *et al.*, 2009; Anderson *et al.*, 2011; Rering *et al.*, 2018; Cullen *et al.*, 2021). Species of the genus *Lactobacillus*, the most recognized and diverse honey bee
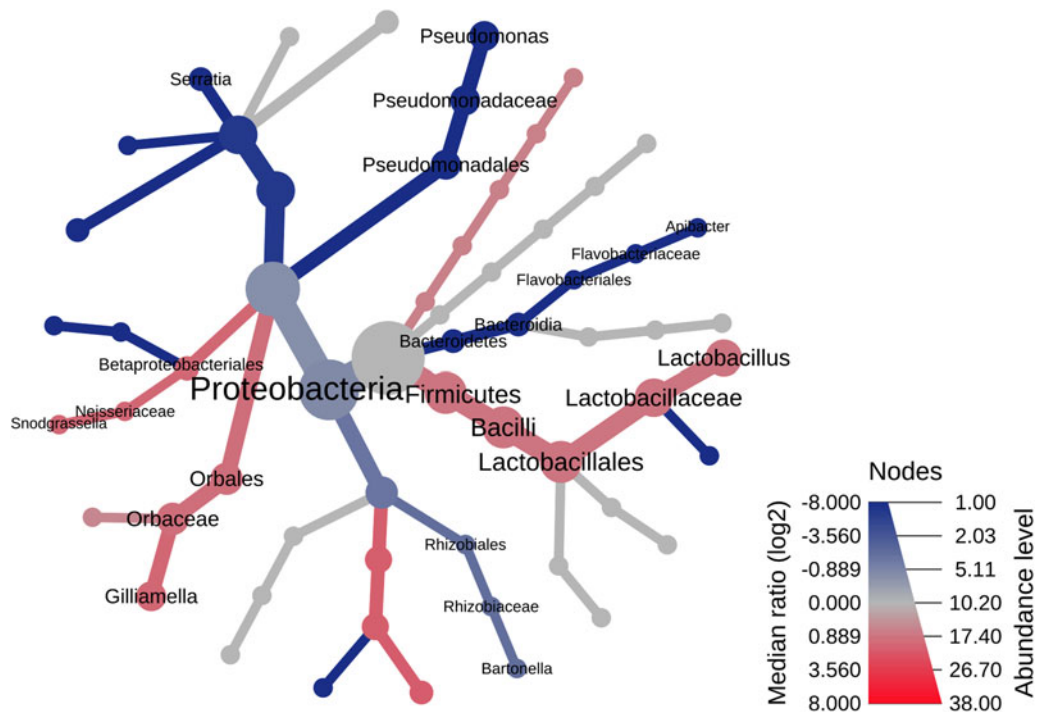
Figure 5. Heat tree graph representing the geographical differences in the bacterial communities associated with honey bees. Significantly different taxonomic ranks are shown on the tree as a light red and dark blue nuance for communities of the Rhodope Mountains and the Danube plain, respectively. Node diameter is proportional to the number of OTUs classified as that taxon.

symbionts, were commonly found in the in-hive environment including bee bread, royal jelly, bee pollen, and nectar (Anderson et al., 2013, 2014). For example, the fructophilic lactic acid bacteria L. (Apilactobacillus) kunkeei isolated from all beehive substrates and crop sections of the bee alimentary tract (Corby-Harris et al., 2014) have been found worldwide on different plants, especially associated with floral nectar and pollen (McFrederick et al., 2012; Neveling et al., 2012; Anderson et al., 2013; Rering et al., 2018; Casalone et al., 2020; de Oliveira Scoaris et al., 2021; Rering et al., unpublished).

In some specimens, a substantial deviation from the core gut-associated taxa was revealed. Several genera (Pseudomonas, Morganella, Klebsiella, Serratia, and Providencia), belonging to families Pseudomonadaceae and Enterobacteriaceae, were present with high RAs mainly in bees from the Danube plain area and occasionally in a few individuals from the Rhodope Mtn. We relate the presence of these bacteria to body surface-associated microbiota. These genera have widespread distribution, found in various environments (water, soil, plants, insects, and other animals including humans). However, some species are also known as potential and opportunistic bee pathogens that can disrupt gut microbiota and increase host susceptibility to infection and reduced lifespan (Kwong and Moran, 2016; Raymann et al., 2017; Lakhman et al., 2021; Al Naggar et al., 2022). When foraging on flowers, honey bees can transfer numerous pollen-associated bacteria that differ significantly in plant species and pollination type (Manirajan et al., 2018), including some plant pathogenic species (e.g., Pseudomonas), thereby facilitating species-specific crop diseases (McArt et al., 2014; Pattemore et al., 2014).

Alpha diversity indices and beta diversity analyses confirmed the observed differences in honey bee-associated microbiota from both localities. One of the possible explanations for the

observed community shifts in bacterial composition and higher diversity in bees from the Danube plain locality might be related to the higher infectious rate with Nosema microsporidian parasites found in the same apiary (Radoslavov et al., 2017). Alterations in the community composition and diversity of bee gut microbiome were found to be positively correlated with the presence of eukaryotic parasites and pathogens (Cariveau et al., 2014; Hubert et al., 2017; Felden et al., 2021). Moreover, a higher microbial diversity was associated with a lower bumblebee resistance against infection by an intestinal parasite (Näpflin and Schmid-Hempel, 2018).

The presence and higher diversity of non-specific gut bacteria in bees from the lowland area may have been related to the landscape features of the location and the level of anthropogenic impact. This is a rural area with higher land use intensity. Increased exposure to agricultural chemicals due to the prevalence of agroecosystems may have had long-term consequences on bee health and its microbiome structure and function. As a main pollinator of various crops, often located in areas with high land use intensity, A. mellifera has diverse interactions with the local environment that can significantly affect bee health and colony performance. In general, each location is a complex of interacting local, global, and regional environmental factors, some of which acting as bee stressors. Land use intensity, agrochemicals, and nutrition quality have been found to contribute to alterations in the honey bee-associated microbiome (Kakumanu et al., 2016; Jones et al., 2018; Motta et al., 2018; Muñoz Colmenero et al., 2020; Cullen et al., 2023). In contrast to the lowland area, the topography of the Rhodope Mtn creates a heterogeneous environment with more diverse landscape structures and ecosystems owing to the variations in temperature, vegetation, lack of homogeneous monocultures, and lower pollution levels. This leads to a larger diversity of non-managed habitats, such as natural

grasslands, low-intensity pastures, forests, patches of shrubs, and single trees that provide higher plant diversity and nutritional resources for honey bees. Both sites differed in many aspects, including elevation, landscape heterogeneity, land use type and vegetation, access to floral resources, application of agrochemicals that may affect alone or in combination. However, the lower number of sampling locations, smaller sampling sizes, and the combined effect of multiple abiotic and biotic factors hinder the clear interpretation of our findings. Therefore, further studies with higher sampling rate, involving pathogen and fungal community analyses, would be required to disentangle the influence of local and regional factors.

References

Al Naggar Y, Singavarapu B, Paxton RJ and Wubet T (2022) Bees under interactive stressors: the novel insecticides flupyradifurone and sulfoxaflor along with the fungicide azoxystrobin disrupt the gut microbiota of honey bees and increase opportunistic bacterial pathogens. *Science of the Total Environment* 849, 1–14.

Anderson KE, Sheehan TH, Eckholm BJ, Mott M and DeGrandi-Hoffmanet G (2011) An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*). *Insectes Sociaux* 58, 431–444.

Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, Walton A, Jones BM and Corby-Harris V (2013) Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*). *PLoS ONE* 8, e83125.

Anderson KE, Carroll MJ, Sheehan T, Mott BM, Maes P and Corby-Harris V (2014) Hive-stored pollen of honey bees: many lines of evidence are consistent with pollen preservation, not nutrient conversion. *Molecular Ecology* 23, 5904–5917.

Boncristiani H, Ellis JD, Bustamante T, Graham J, Jack C, Kimmel CB, Mortensen A and Schmehl DR (2020) World honey bee health: the global distribution of western honey bee (*Apis mellifera* L.) pests and pathogens. *Bee World* 98, 2–6.

Bonilla-Rosso G and Engel P (2018) Functional roles and metabolic niches in the honey bee gut microbiota. *Current Opinion in Microbiology* 43, 69–76.

Bosmans L, Pozo MI, Verreth C, Crauwels S, Wilberts L, Sobhy IS, Wäckers F, Jacquemyn H and Lievens B (2018) Habitat-specific variation in gut microbial communities and pathogen prevalence in bumblebee queens (*Bombus terrestris*). *PLoS ONE* 13, e0204612.

Bukin YS, Galachyants YP, Morozov IV, Bukin SV, Zakharenko AS and Zemskaya TI (2019) The effect of 16S rRNA region choice on bacterial community metabarcoding results. *Scientific Data* 6, 190007.

Callegari M, Crotti E, Fusi M, Marasco R, Gonella E, De Noni I, Romano D, Borin S, Tsiamis G, Cherif A, Alma A and Daffonchio D (2021) Compartmentalization of bacterial and fungal microbiomes in the gut of adult honeybees. *NPJ Biofilms and Microbiomes* 7, 42.

Cariveau DP, Elijah Powell J, Koch H, Winfree R and Moran NA (2014) Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). *ISME Journal* 8, 2369–2379.

Casalone E, Cavalieri D, Daly G, Vitali F and Perito B (2020) Propolis hosts a diverse microbial community. *World Journal of Microbiology & Biotechnology* 36, 1–11.

Castelli L, Balbuena S, Branchiccela B, Zunino P, Liberti J, Engel P and Antúnez K (2021) Impact of chronic exposure to sublethal doses of glyphosate on honey bee immunity, gut microbiota and infection by pathogens. *Microorganisms* 9, 845.

Chong J, Liu P, Zhou G and Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nature Protocols* 15, 799–821.

Corby-Harris V, Maes P and Anderson KE (2014) The bacterial communities associated with honey bee (*Apis mellifera*) foragers. *PLoS ONE* 9, e95056.

Cullen NP, Fetters AM and Ashman TL (2021) Integrating microbes into pollination. *Current Opinion in Insect Science* 44, 48–54.

Cullen MG, Bliss L, Stanley DA and Carolan JC (2023) Investigating the effects of glyphosate on the bumblebee proteome and microbiota. *Science of the Total Environment* 864, 161074.

Cunningham MM, Tran L, McKee CG, Ortega Polo R, Newman T, Lansing L, Griffiths JS, Bilodeau GJ, Rott M and Guarna MM (2022) Honey bees as biomonitors of environmental contaminants, pathogens, and climate change. *Ecological Indicators* 134, 108457.

Daisley BA, Chmiel JA, Pitek AP, Thompson GJ and Reid G (2020) Missing microbes in bees: how systematic depletion of key symbionts erodes immunity. *Trends in Microbiology* 28, 1010–1021.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N, Pfrender ME and Bernatchez L (2017) Environmental DNA metabarcoding: transforming how we survey animal and plant communities. *Molecular Ecology* 26, 5872–5895.

de Oliveira Scoaris D, Hughes FM, Silveira MA, Evans JD, Pettis JS, Bastos EMAF and Rosa CA (2021) Microbial communities associated with honey bees in Brazil and in the United States. *Brazilian Journal of Microbiology* 52, 2097–2115.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB and Xia J (2017) MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research* 45, W180–W188.

Dong ZX, Li HY, Chen YF, Wang F, Deng XY, Lin LB, Zhang QL, Li JL and Guo J (2020) Colonization of the gut microbiota of honey bee (*Apis mellifera*) workers at different developmental stages. *Microbiological Research* 231, 126370.

Eberle J, Ahrens D, Mayer C, Niehuis O and Misof B (2020) A plea for standardized nuclear markers in metazoan DNA taxonomy. *Trends in Ecology & Evolution* 35, 336–345.

Edgar RC, Haas BJ, Clemente JC, Quince C and Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200.

Ellegaard KM and Engel P (2019) Genomic diversity landscape of the honey bee gut microbiota. *Nature Communications* 10, 446.

Emery O, Schmidt K and Engel P (2017) Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*). *Molecular Ecology* 26, 2576–2590.

Engel P, Bartlett KD and Moran NA (2015a) The bacterium *Frischella perrara* causes scab formation in the gut of its honeybee host. *mBio* 6, e00193–15.

Engel P, Vizcaino MI and Crawford JM (2015b) Gut symbionts from distinct hosts exhibit genotoxic activity via divergent colibactin biosynthesis pathways. *Applied and Environmental Microbiology* 81, 1502–1512.

Engel P, Kwong WK, McFrederick Q, Anderson KE, Barribeau SM, Chandler JA, Cornman RS, Dainat J, de Miranda JR, Doublet V, Emery O, Evans JD, Farinelli L, Flenniken ML, Granberg F, Grasis JA, Gauthier L, Hayer J, Koch H, Kocher S, Martinson VG, Moran N, Munoz-Torres M, Newton I, Paxton RJ, Powell E, Sadd BM, Schmid-Hempel P, Schmid-Hempel R, Song SJ, Schwarz RS, vanEngelsdorp D and Dainat B (2016) The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions. *mBio* 7, e02164–15.

Erban T, Ledvinka O, Kamler M, Hortova B, Nesvorna M, Tyl J, Titera D, Markovic M and Hubert J (2017) Bacterial community associated with

worker honeybees (*Apis mellifera*) affected by European foulbrood. *PeerJ* **5**, e3816.

Felden A, Baty JW and Lester PJ (2021) Gut microbial communities and pathogens infection in New Zealand bumble bees (*Bombus terrestris*, Linnaeus, 1758). *New Zealand Entomologist* **44**, 71–80.

Forsgren E (2010) European foulbrood in honey bees. *Journal of Invertebrate Pathology* **103**, S5–S9.

Foster ZSL, Sharpton TJ and Grünwald NJ (2017) Metacoder: an R package for visualization and manipulation of community taxonomic diversity data. *PLoS Computational Biology* **13**, e1005404.

Gensersch E (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology* **103**, S10–S19.

Gilliam M (1997) Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiology Letters* **155**, 1–10.

Graystock P, Goulson D and Hughes WOH (2015) Parasites in bloom: flowers aid dispersal and transmission of pollinator parasites within and between bee species. *Proceedings of the Royal Society B: Biological Sciences* **282**, 20151371.

Hubert J, Bicianova M, Ledvinka O, Kamler M, Lester PJ, Nesvorna M, Kopecky J and Erban T (2017) Changes in the bacteriome of honey bees associated with the parasite *Varroa destructor*, and pathogens *Nosema* and *Lotmaria passim*. *Microbial Ecology* **73**, 685–698.

Hung K-LJ, Kingston JM, Albrecht M, Holway DA and Kohn JR (2018) The worldwide importance of honey bees as pollinators in natural habitats. *Proceedings of the Royal Society B: Biological Sciences* **285**, 20172140.

Jones JC, Fruciano C, Hildebrand F, Al Toufalilia H, Balfour NJ, Bork P, Engel P, Ratnieks FL and Hughes WO (2018) Gut microbiota composition is associated with environmental landscape in honey bees. *Ecology and Evolution* **8**, 441–451.

Kacániová M, Pavlicová S, Hascík P, Kociubinski G, Kňazovická V, Sudzina M, Sudzinová J and Fikselová M (2009) Microbial communities in bees, pollen and honey from Slovakia. *Acta microbiologica et immunologica Hungarica* **56**, 285–295.

Kakumanu ML, Reeves AM, Anderson TD, Rodrigues RR and Williams MA (2016) Honey bee gut microbiome is altered by in-hive pesticide exposures. *Frontiers in Microbiology* **7**, 1255.

Kandlikar GS, Gold ZJ, Cowen MC, Meyer RS, Freise AC, Kraft NJB, Moberg-Parker J, Sprague J, Kushner DJ and Curd EE (2018) An R package and Shiny web app to explore environmental DNA data with exploratory statistics and interactive visualizations. *F1000Research* **7**, 1734.

Kennedy SR, Prost S, Overcast I, Rominger AJ, Gillespie RG and Krehenwinkel H (2020) High-throughput sequencing for community analysis: the promise of DNA barcoding to uncover diversity, relatedness, abundances and interactions in spider communities. *Development Genes and Evolution* **230**, 185–201.

Kešnerová L, Emery O, Troilo M, Liberti J, Erkosar B and Engel P (2020) Gut microbiota structure differs between honeybees in winter and summer. *ISME Journal* **14**, 801–814.

Khan KA, Al-Ghamdi AA, Ghramh HA, Ansari MJ, Ali H, Alamri SA, Al-Kahtani SN, Adgaba N, Qasim M and Hafeez M (2020) Structural diversity and functional variability of gut microbial communities associated with honey bees. *Microbial Pathogenesis* **138**, 103793.

Kwong WK and Moran NA (2016) Gut microbial communities of social bees. *Nature Reviews Microbiology* **14**, 374–384.

Kwong WK, Mancenido AL and Moran NA (2017) Immune system stimulation by the native gut microbiota of honey bees. *Royal Society Open Science* **4**, 170003.

Lakhman A, Galatiuk O, Romanishina T, Behas A and Zastulka O (2021) Bees Klebsiellosis: key aspects of pathogenesis. *Advances in Animal and Veterinary Sciences* **9**, 1190–1193.

Maes PW, Rodrigues PAP, Oliver R, Mott BM and Anderson KE (2016) Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and Nosema disease in the honeybee (*Apis mellifera*). *Molecular Ecology* **25**, 5439–5450.

Manirajan BA, Maisinger C, Ratering S, Rusch V, Schwiertz A, Cardinale M and Schnell S (2018) Diversity, specificity, co-occurrence and hub taxa of the bacterial-fungal pollen microbiome. *FEMS Microbiology Ecology* **94**, 1–11.

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal* **17**, 10–12.

Martinson VG, Moy J and Moran NA (2012) Establishment of characteristic gut bacteria during development of the honeybee worker. *Applied and Environmental Microbiology* **78**, 2830–2840.

McArt SH, Koch H, Irwin RE and Adler LS (2014) Arranging the bouquet of disease: floral traits and the transmission of plant and animal pathogens. *Ecology Letters* **17**, 624–636.

McFrederick QS, Wcislo WT, Taylor DR, Ishak HD, Dowd SE and Mueller UG (2012) Environment or kin: whence do bees obtain acidophilic bacteria? *Molecular Ecology* **21**, 1754–1768.

McMurdie PJ and Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217.

Morinière J, Cancian de Araujo B, Lam AW, Hausmann A, Balke M, Schmidt S, Hendrich L, Doczkal D, Fartmann B, Arvidsson S and Haszprunar G (2016) Species identification in malaise trap samples by DNA barcoding based on NGS technologies and a scoring matrix. *PLoS ONE* **11**, e0155497.

Morinière J, Balke M, Doczkal D, Geiger MF, Hardulak LA, Haszprunar G, Hausmann A, Hendrich L, Regalado L, Rulik B, Schmidt S, Wägele JW and Hebert PDN (2019) A DNA barcode library for 5,200 German flies and midges (Insecta: Diptera) and its implications for metabarcoding-based biomonitoring. *Molecular Ecology Resources* **19**, 900–928.

Motta EVS, Raymann K and Moran NA (2018) Glyphosate perturbs the gut microbiota of honey bees. *Proceedings of the National Academy of Sciences of the USA* **115**, 10305–10310.

Muñoz-Colmenero M, Baroja-Careaga I, Kovačić M, Filipi J, Puškadija Z, Kezić N, Estonba A, Büchler R and Zarraonaindia I (2020) Differences in honey bee bacterial diversity and composition in agricultural and pristine environments – a field study. *Apidologie* **51**, 1018–1037.

Näpflin K and Schmid-Hempel P (2018) High gut microbiota diversity provides lower resistance against infection by an intestinal parasite in bumblebees. *American Naturalist* **192**, 131–141.

Neveling DP, Endo A and Dicks LMT (2012) Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hives. *Current Microbiology* **65**, 507–515.

Nowak A, Szczuka D, Górczyńska A, Motyl I and Kręgiel D (2021) Characterization of *Apis mellifera* gastrointestinal microbiota and lactic acid bacteria for honeybee protection – a review. *Cells* **10**, 701.

O'Leary NA, Wright MW, Brister JR, Ciufo S, Haddad D, McVeigh R, Rajput B, Robbertse B, Smith-White B, Ako-Adjei D, Astashyn A, Badretdin A, Bao Y, Blinkova O, Brover V, Chetvernin V, Choi J, Cox E, Ermolaeva O, Farrell CM, Goldfarb T, Gupta T, Haft D, Hatcher E, Hlavina W, Joardar VS, Kodali VK, Li W, Maglott D, Masterson P, McGarvey KM, Murphy MR, O'Neill K, Pujar S, Rangwala SH, Rausch D, Riddick LD, Schoch C, Shkeda A, Storz SS, Sun H, Thibaud-Nissen F, Tolstoy I, Tully RE, Vatsan AR, Wallin C, Webb D, Wu W, Landrum MJ, Kimchi A, Tatusova T, DiCuccio M, Kitts P, Murphy TD and Pruitt KD (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. *Nucleic Acids Research* **44**, D733–D745.

Pattemore DE, Goodwin RM, McBrydie HM, Hoyte SM and Vanneste JL (2014) Evidence of the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae*. *Australasian Plant Pathology* **43**, 571–575.

Paudel YP, Mackereth R, Hanley R and Qin W (2015) Honey bees (*Apis mellifera* L.) and pollination issues: current status, impacts, and potential drivers of decline. *Journal of Agricultural Science* **7**, 93–109.

Piper AM, Batovska J, Cogan NOI, Weiss J, Cunningham JP, Rodoni BC and Blacket MJ (2019) Prospects and challenges of implementing DNA metabarcoding for high-throughput insect surveillance. *GigaScience* **8**, giz092.

Porter TM and Hajibabaei M (2018) Scaling up: a guide to high-throughput genomic approaches for biodiversity analysis. *Molecular Ecology* **27**, 313–338.

Powell JE, Martinson VG, Urban-Mead K and Moran NA (2014) Routes of acquisition of the gut microbiota of the honey bee *Apis mellifera*. *Applied and Environmental Microbiology* **80**, 7378–7387.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J and Glöckner FO (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**, D590–D596.

Radoslavov G, Hristov P, Shumkova R, Mitkov I, Sirakova D and Bouga M (2017) A specific genetic marker for the discrimination of native Bulgarian honey bees (Apis mellifera rodopica): Duplication of coI gene fragment. *Journal of Apicultural Research* **56**, 196–202.

Raymann K and Moran NA (2018) The role of the gut microbiome in health and disease of adult honey bee workers. *Current Opinion in Insect Science* **26**, 97–104.

Raymann K, Shaffer Z and Moran NA (2017) Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biology* **15**, e2001861.

Rering CC, Beck JJ, Hall GW, McCartney MM and Vannette RL (2018) Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator. *New Phytologist* **220**, 750–759.

Rering C, Rudolph A, Li QB, Munoz P, Read Q and Hunter C (unpublished) Microbial communities in blueberry (*Vaccinium* spp.) nectar differ between managed and native species, organic and conventional management approaches, and locations.

Ribière C, Hegarty C, Stephenson H, Whelan P and O'Toole PW (2019) Gut and whole-body microbiota of the honey bee separate thriving and non-thriving hives. *Microbial Ecology* **78**, 195–205.

Rognes T, Flouri T, Nichols B, Quince C and Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**, e2584.

Romero S, Nastasa A, Chapman A, Kwong WK and Foster LJ (2019) The honey bee gut microbiota: strategies for study and characterization: studying the honey bee gut microbiota. *Insect Molecular Biology* **28**, 455–472.

Sabree ZL, Hansen AK and Moran NA (2012) Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees. *PLoS ONE* **7**, e41250.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS and Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biology* **12**, R60.

Smutin D, Lebedev E, Selitskiy M, Panyushev N and Adonin LS (2022) Micro"bee"ota: honey bee normal microbiota as a part of superorganism. *Microorganisms* **10**, 2359.

Snowdon JA and Cliver DO (1996) Microorganisms in honey. *International Journal of Food Microbiology* **31**, 1–26.

Thijs S, De Beeck MO, Beckers B, Truyens S, Stevens V, Van Hamme JD, Weyens N and Vangronsveld J (2017) Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys. *Frontiers in Microbiology* **8**, 494.

Yuan X, Pan Z, Jin C, Ni Y, Fu Z and Jin Y (2019) Gut microbiota: an under-estimated and unintended recipient for pesticide-induced toxicity. *Chemosphere* **227**, 425–434.

Zhang Z, Mu X, Cao Q, Shi Y, Hu X and Zheng H (2022) Honeybee gut *Lactobacillus* modulates host learning and memory behaviors via regulating tryptophan metabolism. *Nature Communications* **13**, 2037.

Zheng H, Steele MI, Leonard SP, Motta EVS and Moran NA (2018) Honey bees as models for gut microbiota research. *Lab Animal* **47**, 317–325.

Zhu L, Qi S, Xue X, Niu X and Wu L (2020) Nitenpyram disturbs gut microbiota and influences metabolic homeostasis and immunity in honey bee (*Apis mellifera* L. *Environmental Pollution* **258**, 113671.