

## PROFILE OF THE GUT MICROBIAL COMPOSITION IN *Apis mellifera* LARVAE COLLECTED IN HA NOI

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### ABSTRACT

The gut microbiota plays a crucial role in food digestion, enhances the host's immune system, and against pathogens. Numerous studies have been conducted on the microbiota of insects in general and honeybees in particular. However, studies have primarily focused on adult honeybees, with fewer studies dedicated to larvae. Despite being within the hive, honeybee larvae still possess their distinct microbiota. To gain a deeper understanding of the microbiota in the larvae of *Apis mellifera* honeybees, the larva from honeybee colonies collected in Ha Noi, Vietnam was investigated. Next-generation sequencing (NGS) targeting the 16S rRNA gene was employed for microbiome analysis. Results revealed the presence of 5 phyla including Proteobacteria (70.43%), Actinobacteria (1.16%), Firmicutes (20.87%), Bacteroidetes (2.72%), and Chloroflexi (2%). Representative genera included *Bombella* (29.97%), *Lactobacillus* (14.91%), *Gilliamella* (9.59%), *Frischella* (4.69%), *Snodgrassella* (3.85%), and *Marinobacter* (1.21%). Further characterized species composition in the sample we identified the prevalence of *Bifidobacterium intestini* (29.96%), *Gilliamella apicola* (8.08%), *Frischella perrara* (4.55%), *Lactobacillus kimbladii* (2.85%), *Lactobacillus plantarum* (2.80%), *Snodgrassella alvi* (2.77%), *Lactobacillus mellis* (2.59%), *Lactobacillus\_uc* (unclassified or not yet classified to species, 2.19%), *Lactobacillus kunkeei* (1.43%), and *Lactobacillus melliventris* (1.31%). Understanding these microbial dynamics is crucial for developing strategies to support honeybee health and mitigate the challenges posed by factors, such as pesticides, environmental pollution, and honeybee diseases.

**Keywords:** *Apis mellifera*, honeybees, *Lactobacillus*, larvae, microbiome.

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## INTRODUCTION

Honeybees have been extensively studied due to their importance in agriculture and the characteristic social traits of insects (Hoover & Ovinge, 2018). Current research on honeybees indicates that the microbial community significantly influences the nutrition, body weight, endocrine signals, immune system function, and disease resistance of the host (Zheng et al., 2018). Disruptions in the microbial community can impact the overall health of the host (Motta et al., 2022). Like the human gut microbiota, the gut microbiota of bees may consist of a mixture of beneficial bacteria, pathogenic bacteria, or commensals. The relative and absolute richness of microbial components in the community and their interactions determine the collective contribution of the microbiota to the host's health. Microorganisms associated with bees include various types of viruses, bacteria, fungi, and protozoa, some of which pose threats to bees (Nanetti et al., 2021). The rapid decline in bee populations worldwide in recent years is attributed to stressors such as pesticides, environmental pollution, nutrient deficiencies, parasites, bacteria, viruses, and reduced genetic diversity (El-Seedi et al., 2022). This not only has a significant impact on agricultural economies but also affects biodiversity and food security globally. Dealing with diseases is challenging and costly, often requiring the removal of entire bee colonies or beekeeping materials. These factors have asked human beings to implement measures for maintaining bee populations. There are various approaches to protect honeybees from pathogens, of these, the application of beneficial bacteria is being considered as an effective strategy. Adult honeybees have been extensively studied and exhibited a relatively simple, conservative microbial community, comprising 8–10 core bacterial species. *Gilliamella*, *Snodgrassella*, *Bifidobacterium*, *Lactobacillus* Firm-4 and Firm-5, and *Bartonella* are genera with notable proportions (Zhang et al., 2022). The microbial community in bees undergoes development stages from larvae to pupae and finally to adult

bees. Moreover, microbial communities in bees show a differentiation percentage between host sources, suggesting that distinct hosts may be influenced by geographic factors, the origin of bees, food sources, or physiological conditions (Koch & Schmid-Hempel, 2011). In this study, we provide an overview of the presence of the microbial community in honeybee larvae (*Apis mellifera*) collected in Ha Noi, Vietnam. The results will contribute to a deeper understanding of the microbial composition in honeycombs and will be used as a foundation for establishing research measures and protecting honeybee colonies from diseases.

## MATERIALS AND METHODS

### Sample collection

The *A. mellifera* larvae (3-6 days old) were collected from three randomly bee colonies in a beekeeping household in Gia Lam, Ha Noi, Vietnam in November 2022 (20°0'04"N, 105°55'60"E).

### DNA extraction

Genomic DNA from the larvae was extracted using the PCI method as described in our previous study (Duong et al., 2020). In brief, 30 individual honeybee larvae from 3 colonies (10 individuals per colony) were grinded and homogenized in 3 mL sterile lysis buffer (100 mM Tris HCl, 50 mM EDTA, 50 mM NaCl, 1% SDS, pH 7.0) within a sterile falcon tube. Subsequently, 50 µL of protease K (20 mg/mL) and 20 µL of lysozyme (100 mg/mL) were added, and the mixture was incubated at 65 °C for 2 hours, followed by centrifugation at 5,000 rpm for 5 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube, and phenol/chloroform/isoamyl alcohol (25:24:1) in a 1:1 (v/v) ratio was added, mixed, and then centrifuged at 12,000 rpm for 30 minutes. The supernatant phase was transferred to a new microcentrifuge tube. Isopropanol was added in a 1:1 (v/v) ratio, followed by incubation at room temperature for 30 minutes and centrifugation at 12,000 rpm for 30 minutes at 4 °C. The pellet was washed twice with 70% ethanol, dried using a Speedvac (Thermo Scientific,

Waltham, MA, USA) for 10 minutes, and subsequently resuspended in nuclease-free water. DNA concentration and purity were assessed using a Nanodrop 2000 UV spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA quality was verified through 1% agarose gel electrophoresis.

### Illumina Miseq Sequencing

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 341F (5'-TCGTCGGCAGCGTC-AGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The thermal profile for the PCR was a cycle at 95 °C for 3 min and 25 cycles of 95 °C for the 30 sec, followed by 55 °C for 30s and 72 °C for 30 s, and a final cycle at 72 °C for 5 min. Secondary amplification for attaching the Illumina NexTera barcode was performed with i5 forward primer (5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXXX-TCGTCGGCAGCGTC-3'; X indicates the barcode region) and i7 reverse primer (5'-CAAGCAGAAGACGGCATACGAGAT-XXXXXXX-GTCTCGTGGGCTCGG-3'). The condition of secondary amplification is the same as the former, except for the amplification cycle set to 8. The PCR product was purified with the CleanPCR (CleanNA, Waddinxveen, The Netherlands) and its quality and size were assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) using a DNA 7500 chip. The sequencing was carried out at Chunlab, Inc. (Seoul, Korea) with Illumina MiSeq Sequencing System (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.

### 16S rRNA gut community analysis

The raw data obtained from Illumina MiSeq were started quality check (QC) and removed low-quality reads (< Q30) using Trimmomatic v0.32 (Bolger et al., 2014). Quality-assured reads are subsequently organized through UCLUST clustering (Edgar, 2010). The sequences are then taxonomically assigned using USEARCH

with the EzBioCloud database (Myers & Miller, 1988). Chimeras are checked using UCHIME (Edgar et al., 2011) against the non-chimeric 16S rRNA database from EzBioCloud. Operational Taxonomic Units (OTUs) are formed from sequenced reads using CL\_OPEN\_REF\_UCLUST\_MC2. Each read is identified at the species level against the reference database with a similarity cutoff of 97% for 16S rRNA gene sequences. *De novo* clustering is performed using CD-HIT (Fu et al., 2012) and UCLUST to generate additional OTUs. Diversity indices, such as Ace and Chao1 for species richness estimation, and Shannon and Simpson for species evenness estimation, are calculated based on the number and pattern of observed OTUs in the sample. Taxonomic composition is determined at the bacterial phylum, genus, and species levels, using a cut-off of 1%.

## RESULTS

### Summary of NGS

After quality filtering, a total of 64,304 valid reads were obtained with an average sequence length of 416 bp. 85.8% of the reads were utilized for species-level identification, revealing 1982 distinct species. The microbial abundance and diversity were analyzed utilizing alpha diversity parameters, species richness (ACE, Chao 1) and species evenness (Shannon, Simpson) based on OTUs (Thukral, 2017). The values for ACE Chao 1, Shannon and Simpson were 4235.1, 3863.8, 4.383, and 0.103, respectively. The results indicate high species diversity and the data sufficiently covered the bacterial components in the sample.

### Bacterial composition

The results showed that the bacterial species in the larvae sample belong to five major phyla, including Proteobacteria (70.43%), Actinobacteria (1.16%), Firmicutes (20.87%), Bacteroidetes (2.72%) and Chloroflexi (2%) (Fig. 1a). At the genus level, we identified six genera with a proportion exceeding 1% in the sample comprising *Bombella* (29.97%), *Lactobacillus* (14.91%), *Gilliamella* (9.59%), *Frischella* (4.69%),

*Snodgrassella* (3.85%), *Marinobacter* (1.21%). In addition, 35.71% corresponds to bacterial genera with proportions lower than 1% was also detected (Fig. 1b).

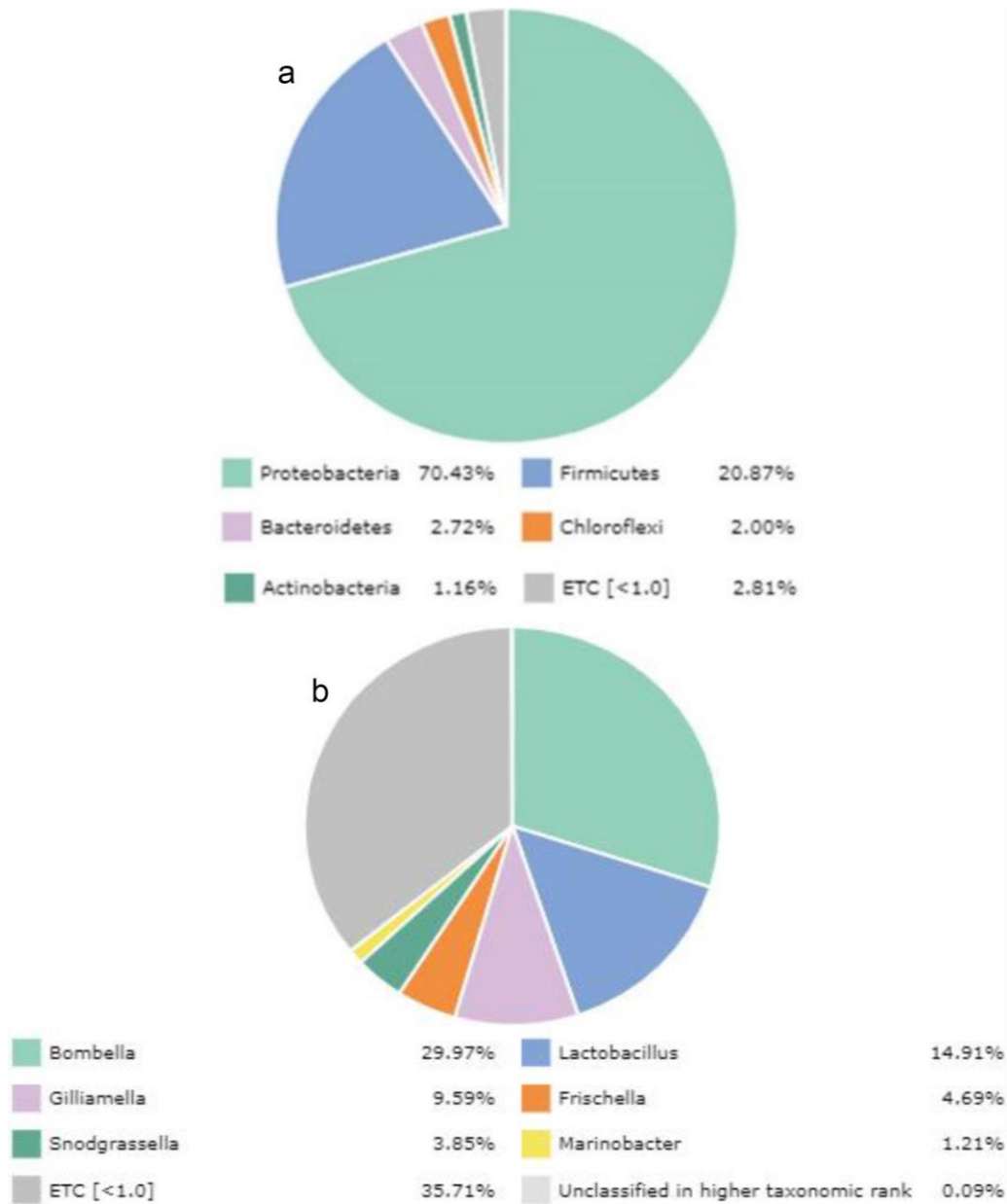


Figure 1. The bacterial composition at the phylum level (a) and the genus level (b) in the larvae of *Apis mellifera* honeybees

The species composition in the *A. mellifera* honeybee larvae sample was further characterized, and the results *Bombella intestini* group (29.96%), *Gilliamella apicola* group (8.08%), *Frischella perrara* (4.55%),

*Snodgrassella alvi* (2.77%) were detected. The bacterial species in the investigated sample was less than 37.26%, and 4.19% of the bacteria were not classified into a higher taxonomic level (Fig. 2).

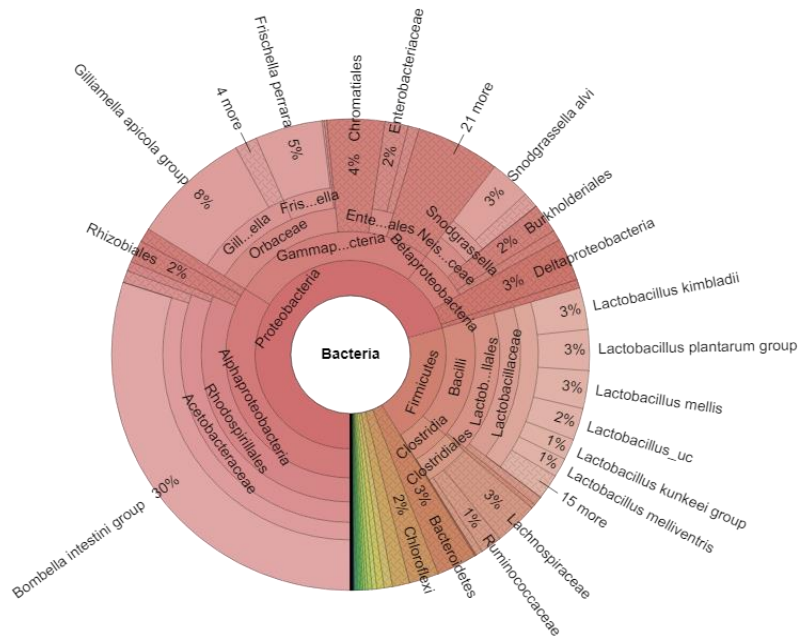


Figure 2. The bacterial composition of species in the larvae of *Apis mellifera* honeybees

There are many bacterial species in different genera that belong to Lactic Acid Bacteria (LAB), the most well-known LAB genera include *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Lactococcus* and *Streptococcus* (Ramos et al., 2020). However, in this study we only detected the presence of bacteria from 2

genera *Bifidobacterium* and *Lactobacillus* from the investigated *Apis mellifera* larvae samples. Among them, *Bifidobacterium asteroides* (0.27%), *Lactobacillus kimbladii* (2.85%), *Lactobacillus plantarum* group (2.80%), *Lactobacillus mellis* (2.59%), *Lactobacillus\_uc* (2.19%), *Lactobacillus kunkei* group (1.43%), *Lactobacillus melliventris* (1.31%) (Fig. 3).

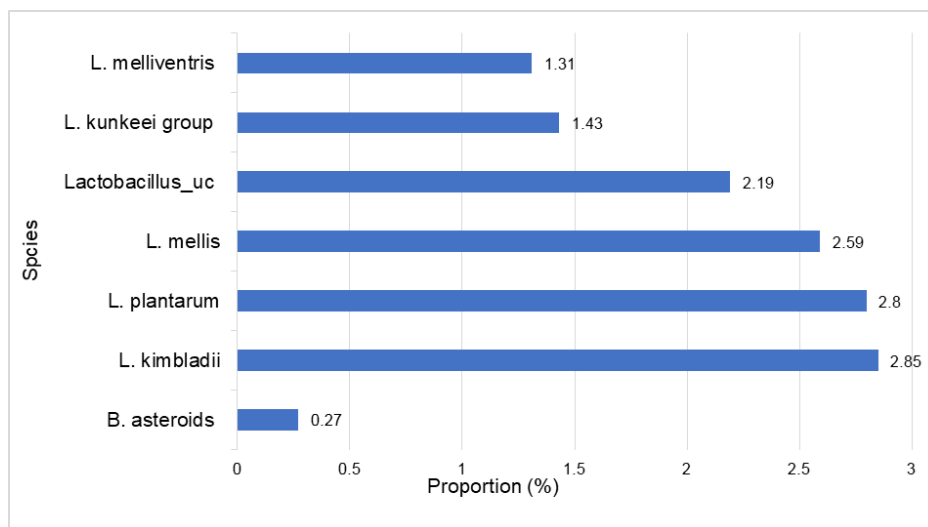


Figure 3. The composition of lactic acid bacteria at the genus level (%) in the *Apis mellifera* larvae

## DISCUSSION

Although the larvae fed by nurse bees and their intestinal structure are not fully developed, they still have a distinct microbiota. These bacteria are affected by horizontal transmission pathways through contact with nurse bees and other materials within the nest. In our study, by NGS targeting the 16S rRNA gene, we identified the presence of five phyla in *A. mellifera* honeybee larvae, with Proteobacteria and Firmicutes accounting for the highest proportions. These microbial phyla were also found in adult bees, including both *A. mellifera* and *Apis cerana* (Duong et al., 2020). In our recent report, Proteobacteria (70.7%), Actinobacteria (10.7%), Firmicutes (10.3%), and Bacteroidetes (8.4%) were found in adult *A. cerana* bees (Duong et al., 2020). In other study, Firmicutes (81.55%) and Proteobacteria (17.0%) were found as predominant phyla in *A. cerana* bee larvae (Lanh et al., 2022). Previously, we also identified the predominant bacterial groups in *A. cerana* bees isolated in Ha Noi in 2021, comprising the *L. kunkeei* group (30.11%), *Melissococcus plutonius* (25.03%), *Lactobacillus\_uc* (13.04%), *Commensalibacter AY370188\_s* (8.23%), *Enterococcus faecalis* (8.05%), and *B. intestinii* group (6.94%). This reveals distinctions from the bacterial groups found in *A. mellifera* bees utilized in current research, which could be attributed to the species-specific differences. In addition to geographical differences, dietary patterns also yield distinct in the microbiota. This hypothesis was supported by a study on honeybee *A. mellifera* larvae gut microbial in China (Yu et al., 2021) in which they revealed the presence of *Yersinia* (14.73%), *Geobacillus* (9.70%), *Pseudomonas* (7.73%), *Acinetobacter* (4.53%), *Escherichia* (1.72%), *Fructobacillus* (1.16%), *Streptococcus* (0.99%), *Burkholderia* (0.96%) in larvae (6 days old). Additionally, in their study two core bacteria, *Lactobacillus* and *Bifidobacterium*, were observed in the larvae. The differences in species composition within the larval samples could be explained that they utilized laboratory-cultured samples, while our study conducted with field samples.

Further characterized the larval bee samples, we identified the presence of core bacteria, similar to those found in adult *A. mellifera* bees, including *S. alvi*, *G. apicola*, *Lactobacillus Firm-4* (*L. mellis*), *Lactobacillus Firm-5* (*L. kimbladii*, *L. melliventris*), and *B. asteroides* (Smutin et al., 2022). The similarity in the microbial composition between larvae and adult bees may be attributed to their exposure to nurse bees and other hive materials. Despite variations in proportions, the occurrence of these bacteria in both larvae and adult bees could potentially reflect the concept of a “common stomach” in social honeybees (Schmickl & Karsai, 2017). This observation may contribute to a better understanding of disease transmission pathways within the bee colony.

While honeybee has a relatively simple microbiota composition, it serves specific functions related to metabolic exchanges. For instance, honeybee larvae exhibit a high proportion of bacteria belonging to the genus *Bombella*, represented by the *B. intestini* group (29.96%). This genus is not only found in the honeybee *A. mellifera* but also in the Bumble bee (Li et al., 2015) and other insects with sugar-based diets such as mosquitoes, fruit flies, and sugarcane mealybugs, as well as in environments rich in ethanol (Crotti et al., 2010). The genus *Bombella* belonging to the family Acetobacteraceae, represents a group of Acetic Acid Bacteria (AAB) that play a crucial role in food digestion and contribute to protecting honeybee larvae from pathogenic threats (Härer et al., 2022). The phylum Firmicutes is predominantly represented by the genus *Lactobacillus*. Species constituting more than 1% include *L. kimbladii* (2.85%), *L. plantarum* (2.8%), *L. mellis* (2.59%), *Lactobacillus\_uc* (2.19%), *L. kunkeei* group (1.43%), *L. melliventris* (1.31%), and over 15 bacterial species with proportions less than 1% (Fig. 2). *Lactobacillus* bacteria, characterized by lactic acid production, play a crucial role in protecting the host from harmful bacteria by competing for adhesion sites and nutrition (Sengupta et al., 2013). Some studies have indicated that certain strains of *Lactobacillus*

have the ability to inhibit the growth of pathogenic bacteria such as *Chalkbrood*, which is inhibited by *Lactobacillus helsingborgensis* and *L. melliventris*. Similarly, American Foulbrood caused by *Paenibacillus* larvae is inhibited by *Lactobacillus apis*, *L. helsingborgensis*, and *L. melliventris* (Iorizzo et al., 2022). This result emphasizes the potential for applying LAB strains to develop probiotics for the prevention and treatment of diseases caused by pathogenic bacteria on honeybees. In addition to *Lactobacillus*, we also found another lactic acid-producing bacterium, *B. asteroides*, representing a minor proportion (0.27%) within the phylum Actinobacteria. This species is typically a core member of the adult bee gut microbiota but can also be found in other parts of the hive, including larvae. Notably, the larval samples also exhibited the presence of *Melissococcus plutonius* (0.5%) belonging to the family Enterococcaceae. This bacterium is known to cause European Foulbrood (EFB), a larval disease (Biová et al., 2021). Its presence in the colony without causing disease in bees supports the hypothesis that the presence of harmful microorganisms in the host's body at a certain ratio helps train the immune system (Kogut et al., 2020).

*S. alvi* and *G. apicola* are two species exclusively found in the gut of honeybees and not in other hive materials. These two species tend to coexist, forming a symbiotic relationship within the bee gut, utilizing contrasting oxygen utilization mechanisms. While *G. apicola* is an anaerobic organism, *S. alvi* is facultative anaerobic. The combination of these two species establishes a physical barrier that reduces the attachment sites for harmful bacteria on the intestinal epithelial cells, thereby limiting the pathogenic microorganisms (Smutin et al., 2022). In the investigated larval samples, we identified the presence of *F. perrara* bacterium (4.55%). Beyond its metabolic functions, this bacterium is recognized for its role in melanization (scab phenotype) processes. *F. perrara* is prevalent in the worker bees of *A. mellifera* and functions as a crucial antibacterial mechanisms within the insect (Schmidt et al., 2023).

## CONCLUSION

Our study provides a comprehensive analysis of the microbial community in the larvae of *A. mellifera* honeybees, shedding light on a relatively understudied aspect of honeybee microbiota. The obtained results lay the foundation for future investigations into the intricate interactions between honeybee larvae and their microbiota, contributing to the development of measures aimed at preserving bee populations and ensuring global food security.

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