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Research Article

Comparative characterization of gut microbiota of *Apis mellifera* and *Apis dorsata* using next-generation sequencing

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Abstract

The present study explores the gut microbiota of *Apis mellifera* and *Apis dorsata* using Next-Generation Sequencing (NGS) to understand microbial diversity and relative abundance. A total of 05 specimens of each honeybee species were collected from District Kasur, Pakistan, and processed under sterile conditions for gut dissection. DNA was successfully extracted, and by V3-V4 region of the 16S rRNA gene was successfully amplified. The analysis revealed significant bacterial phyla, with Proteobacteria dominating both species, accounting for 83% in *Apis mellifera* and 80% in *Apis dorsata.* Furthermore, prevalent phyla, including Bacteroidota, Firmicutes, and Actinobacteriota, were documented. Prominent genera identified in both species were *Commensalibacter, Dysgonomonas, Lactobacillus*, and *Gilliamella*, which contribute to gut health and fermentation processes. Differences were observed in the dominance of families with Enterobacteriaceae prevalent in *Apis mellifera* and Acetobacteraceae in *Apis dorsata*, potentially linked to diet and habitat. The findings suggest that variations in gut microbiota are shaped by environmental conditions, foraging behaviors, and evolutionary differences between these two species. These results provide baseline information for future research into the functional roles of microbiota in honeybee health and ecological adaptation.
Keywords: Honeybee, *E.coli*, NGS, *S. areus, Klebsiella*

Introduction

Insects are basic components of agroecosystems and play their role in ecosystem services such as pest control, crop production, and pollination of the crops. These services have monetary and non-monetary worth for humans. Honeybees are known as the social insects that are found in the community and are considered a leading pollinator species (Gasper & Terentjeva, 2019). In animal-pollinated crops, honey bees have been reported to increase the yield by about 35%

of worldwide food production (Guo et al., 2015). From the genus *Apis* four species are found in Pakistan from which three species are endemic (*A. cerana, A. dorsata* and *A. florea)* and one exotic species *A. mellifera* (Khan et al., 2020; Sajid et al., 2020; Shakeel et al., 2020). *Apis dorsata* and *Apis florea* species have variations according to behavior, phylogenetic location, and morphological appearance (Arias & Sheppard, 2005; Abrol, 2011; Streinzer et al., 2013; Meemongkolkiat et al., 2019; Oppenheim et al., 2020). The recorded length of the *Apis dorsata* (17 mm) is larger than *Apis florea* (7–10 mm) (Koeniger et al., 2010; Khademi, 2014).

Several investigations have been done to improve honeybee's health. According to the latest research, gut microbiota plays a significant role in improving the health of honeybees (Evans & Schwarz, 2011; Wu et al., 2013; Moran, 2015; Kakumanu et al., 2016; Yanez et al., 2016; Kwong et al., 2017). Several researchers are working on the application and isolation of beneficial microorganisms in order to protect the hosts from diseases. Similarly, the role of the microbiome in immunity and health is also gaining attention. The bacteria of the intestine are involved in synthesizing amino acids and vitamins and also improving metabolism for the betterment of immunity and health of the host (Khan et al., 2020). As many researchers represent, symbiotic microbiota present in the intestine tract are not only involved in acting as a barrier against pathogens but are also responsible for supplementary nutrition (Anderson et al., 2011, 2013). Numerous factors are involved in affecting the diversity and structure of gut microbiota among the *Apis* species (Kwong & Moran, 2016). For example, changes occur in the diversity of the gut microbiota as developmental stages take place (Hroncova et al., 2015). In the worker, honeybees engaged in different chores have differentially abundant gut microbial taxa (Jones et al., 2018). Season is another factor that is involved in affecting the microbiota during compositional shifts in bacterial communities, which were examined during summer and winter bees (Kesnerova et al., 2020).

By using high throughput sequencing like next-generation sequencing, various investigations help to examine the microbiota of bees (Engel et al., 2012; Kwong et al., 2014; Lim et al., 2015). In 1987, when the technology of first-generation sequencing was developed at the same time, molecular biology techniques were also developed speedily, which resulted not only in the discovery of the earlier unknown microbiota but also made gut microbiota more systematic. This sequencing technology has been extensively used because of its precise and fast analysis of the composition and structure of gut microbiota. With this technology, isolating the pathogens among bees will be fast and appropriate and will be very beneficial to see the link between colony health and pathogens (Keller et al., 2018). Several pathogenic bacteria of animals and plants exist in water. Similarly, various pathogens can be transmitted from animal feces to plants because of insects, fertilizers, and irrigation water. Pathogenic bacteria of plants like *Pseudomonas syringae*

and *Erwinia amylovora* can be transmitted through the honeybees during pollination (Pattemore et al., 2014). Thus, this study was conducted to record the gut microbiota of honeybees from *Apis* species.

Material and Methods

Sample collection

A total of 05 specimens of each species were captured from the study area to examine the gut bacteria microbiota. Figure 1 shows the GIS-based map of the study area. Live bees were transferred to the Postgraduate Laboratory Department of Wildlife and Ecology, University of Veterinary and Animals Sciences, Lahore, on the same day. Sterilization was done with 7% sodium hypochlorite and 70% ethanol (Inglis et al., 2012). Samples were washed with sterile 1x phosphate-buffered saline (PBS), followed by dissection. By using (0.9%) normal saline, the bee's alimentary canal was aseptically removed. The dissected guts were shifted to 1 ml of PBS and kept instantly at -20°C for further processing.

DNA extraction

For the extraction of the DNA Phenol chloroform method was used. The honey bee gut was crushed in a mortar and pestle in 500µl of high salt buffer. After homogenization, the lysate was transferred to the Eppendorf tubes, and 20µl of PK and 20% SDS were added. Then, samples were incubated at 56ºC for 24 hours. For cell digestion, the samples were further preceded with treatment with 500 µl of phenol, isoamyl alcohol, and chloroform. For mixing, the solutions were centrifuged (13000 rpm) for 10 min then for the purification and separation of DNA aqueous phase was shifted to another tube. In this aqueous layer, 500 µl of chloroform and isoamyl alcohol was added (C: I, 24:1), then centrifuged again at 13000 rpm for 10 min, and the aqueous layer was shifted into the centrifuge tube. 500 µl of isopropanol and 55 µl of sodium acetate were added. These samples were centrifuged for 10 min at 13000 rpm. Later on, the supernatant was discarded and then treated with 500 µl of 70% ethanol. Then again centrifuged for 5 min at 7500 rpm in order to purify the pellet and the air dried. The DNA pellet was stored at 4ºC in TE buffer.

Agarose gel electrophoresis

Gel electrophoresis was done with 1% agarose gel (100 ml 1X TAE buffer). 7 µl ethidium bromide was added to it and then poured into the gel casting tray. After solidification, the gel caster was shifted to the gel tank, and the combs were removed carefully. Bromophenol blue dye was added in 5 µl of DNA, then loaded in the wells, and the gel was visualized under a UV Trans-Illuminator bio Doc analyzer.

PCR and amplicon sequencing

For the analysis of honeybee gut bacterial diversity, the NGS library was prepared preparation by V3-V4 region of 16S rRNA gene using primer F515: 5'-GTGCCAGCMGCCGCGGTAA-3')

and 806R 5'GGACTACHVGGGTWTCTAAT-3'. The resulting library was purified, pooled in equimolar concentration, and sequenced using 2x 250bp v2 Chemistry on the Illumina MiSeq platform (Caporaso et al., 2010; Kumbhare et al., 2015).

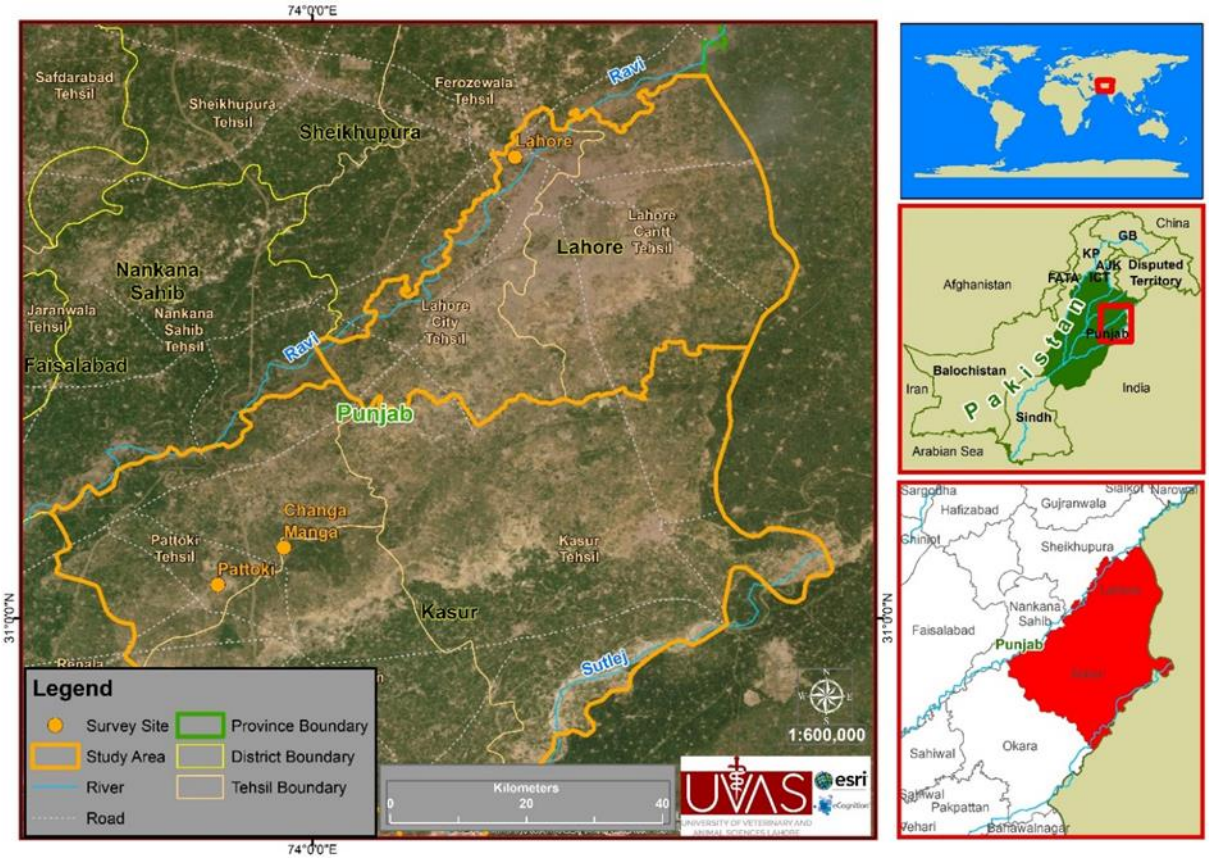

Figure 1: GIS-based map of district Kasur, Punjab, Pakistan

Data analysis

The paired-end method was used to construct a fragment library for paired-end sequencing. Raw sequencing data was in the form of FASTQ files. Raw paired-end reads (FASTQ file format) from the DNA fragments were imported into the QIIME2 v 2022.8 environment (Hall & Beiko, 2018). Quality filtering, denoising, and chimeric sequence removal were done using the Divisive Amplicon Denoising Algorithm (DADA2) (Barnes et al., 2020). SILVA database (v138) for 16S rRNA was used as a reference database (Callahan et al., 2016). Fit-classifier-naive Bayes and q2-feature classifier plugin were used to train the classifier for V3-V4 regions of 16S rRNA for taxonomy assignment to the representative sequences. This classifier was trained on the 99% similarity OTU sequences from a reference database. The relative abundance of the sample was also calculated through the relative frequency feature in QIIME2. Alpha was calculated through phyloseq in R language (McMurdie & Holmes, 2013). Alpha diversity was calculated using different diversity measures like Chao1, ACE, Shannon, and Simpson. The phylogenetic tree was

constructed in the QIIME2 environment. MAFFT (Katoh & Standley, 2013) alignment method was used to align the sequences, while the FastTree tool was used to construct the maximum likelihood tree from the aligned sequences. The constructed Phylogenetic tree was visualized in iTOL, a web-based tool.

Results

Sample collection and preparation

A total of 10 worker native and farm honeybee specimens were captured to examine the gut bacteria from selected sites in district Kasur. All samples were successfully sterilized and dissected under aseptic conditions. The dissected guts were stored at -20°C for further analysis.

DNA extraction

After DNA extraction, the quality of the DNA was checked with 1% agarose gel. Agarose gel electrophoresis showed distinct DNA bands with no significant smearing, confirming the high integrity of the extracted DNA (Figure 2). The purity of the DNA was checked through NanoDrop (Table 1).

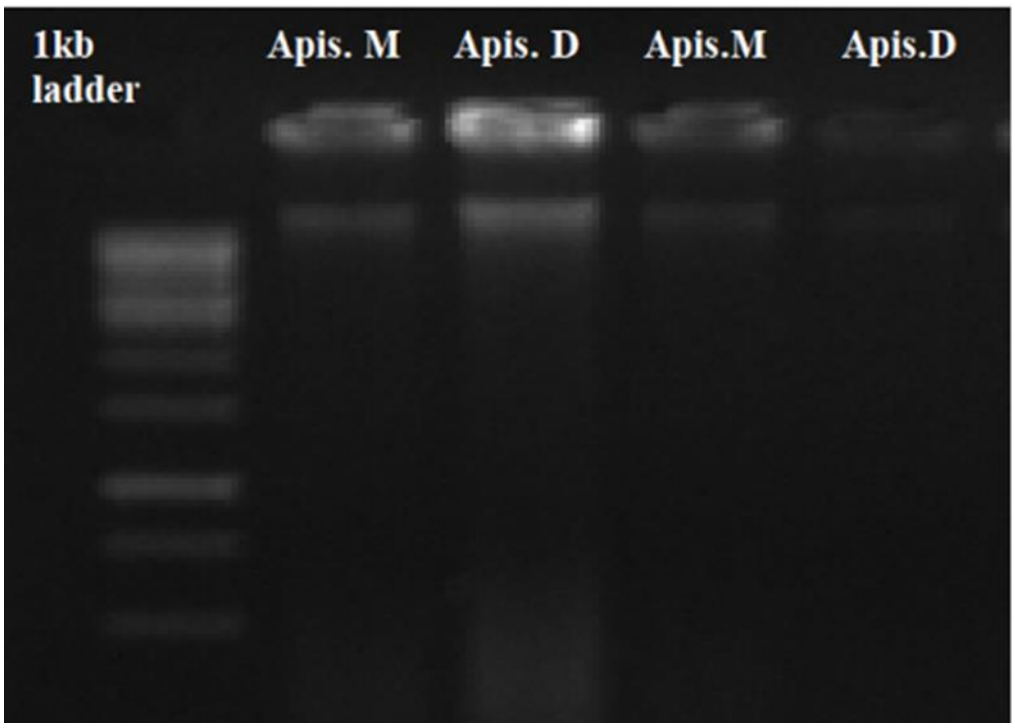

Figure 2: Gel electrophoresis of successfully extracted DNA samples

PCR and amplicon sequencing

High-quality amplicon libraries of 16S rRNA gene were prepared. The PKSSU4.0 database of 16S rRNA gene of prokaryotic was used for pre-process analysis. The valid DNA reads were used for Operational Taxonomic Unit (OTU) picking. Using OTU data, the relative abundance of different bacterial groups was estimated.

Relative abundance of bacterial groups

The relative abundance of bacterial groups at the phylum level in the gut of *A. mellifera* and *A. dorsata* is shown in Figure 3. The bar plot was created to represent the relative abundance of different bacterial groups in Operational Taxonomic Units (OTUs).

Gut microbiota of *Apis mellifera*

The KRONA plot was constructed on the relative abundance of bacterial groups of *A. mellifera* shown in Figure 4. Circles from inward to outward showed diverse classification levels, and the area of division means a specific concentration of different OTU annotation results. Identified Phylum and their percentages were as follows Proteobacteria 83% > Bacteroidota 12% > Firmicutes 3% > Actinobacteriota 1%. The order of abundance of bacterial classes was Gammaproteobacteria 44% > Alphaproteobacteria 39% > Bacteroidia 12% > Bacilli 2% > Actinobacteria 1% > Clostridia 0.3%. Similarly, Enterobacterales, Acetobacterales, Bacteroidales, Pseuomonadales, Lactobacillales, Burkholderiales, and Bifidobacteriales were different bacterial order Taxa identified in the gut of *A. mellifera*. The order of relative abundance was as follows 32% > 20% > 11% > 4% > 2% > 2% >1% respectively. The order of identified families were Enterobacteriaceae 31% > Rhizobiaceae 19% > Dysgonomonaaceae 11% > Orbaceae 5% > Comamonaaceae and Lactobacillaceae both were 2% > Bifidobacteriaceae and Weeksellaceae both were 1% while the identified genera were include *Commensalibacter* 20% > *Ochrobactrum* 12% > *Dysgonomonas* 11% > *Klebsiella* 8% > *Bartonella* 7% > *Gilliamella* 5% > *Pseudomonas* 4% > *Lactobacillus* 2% > *Bifidobacterium* 1% respectively.

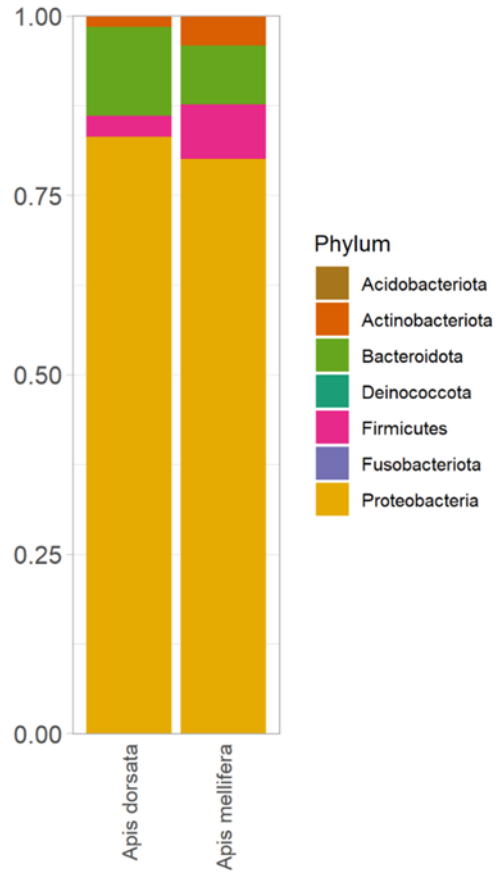

Figure 3. Bar plot showing the abundance of bacterial phyla in the gut of *A. mellifera* and *A. dorsata*

Gut microbiota of *Apis dorsata*

Similarly, the KRONA plot was constructed on the relative abundance of bacterial groups of *A. dorsata* shown in Figure 5. Identified Phylum and their percentages were as follows Proteobacteria 80% > Bacteroidota and Firmicutes both were 8% > Actinobacteriota 3%. The order of abundance of bacterial classes was Alphaproteobacteria 47% > Gammaproteobacteria 33% > Bacteroidia 8% > Bacilli 6% > Actinobacteria 4% > Clostridia 2%. In the same way, Enterobacterales 19% > Bacteroidales are Lactobacillales both were 6% > Burkholderiales and Pseuomonadales both were 4% > Rhizobiales 3% were different bacterial order Taxa identified in the gut of *A. dorsata*. The order of identified families was Acetobacteraceae 44% > Enterobacteriaceae 19% > Dysgonomonaaceae 6% > Comamonaaceae and Pseudomonadaceae both were 4% > Rhizobiaceae 3% > Weeksellaceae 2% while the identified genera were include *Dysgonomonas* and *Gilliamella* both were 6% > *Lactobacillus* 5% > *Pseudomonas* 4% > *Bartonella*, *Bifidobacterium* and *Klebsiella* were 3% respectively.

Table 1. DNA quantification using NanoDrop.

Species	Nucleic Acid (ng/uL)	A260/A 280	A260/A 230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance
Apis mellifera								
1	468.599	1.630	0.918	9.373	5.789	50	336	0.499
2	467.530	1.567	0.850	9.174	5.390	49	337	0.509
3	467.350	1.480	0.870	9.271	5.613	50	338	0.520
4	468.980	1.678	0.910	9.280	5.585	49	337	0.530
5	467.880	1.567	0.972	9.860	5.762	49	340	0.499
Apis dorsata								
1	207.50	1.547	0.89	3.98	2.670	49	339	0.165
2	207.30	1.480	0.85	4.16	2.585	50	340	0.175
3	207.40	1.529	0.87	4.15	2.650	49	339	0.166
4	207.11	1.482	0.89	3.98	2.650	49	338	0.165
5	207.30	1.545	0.85	4.16	2.670	50	339	0.175

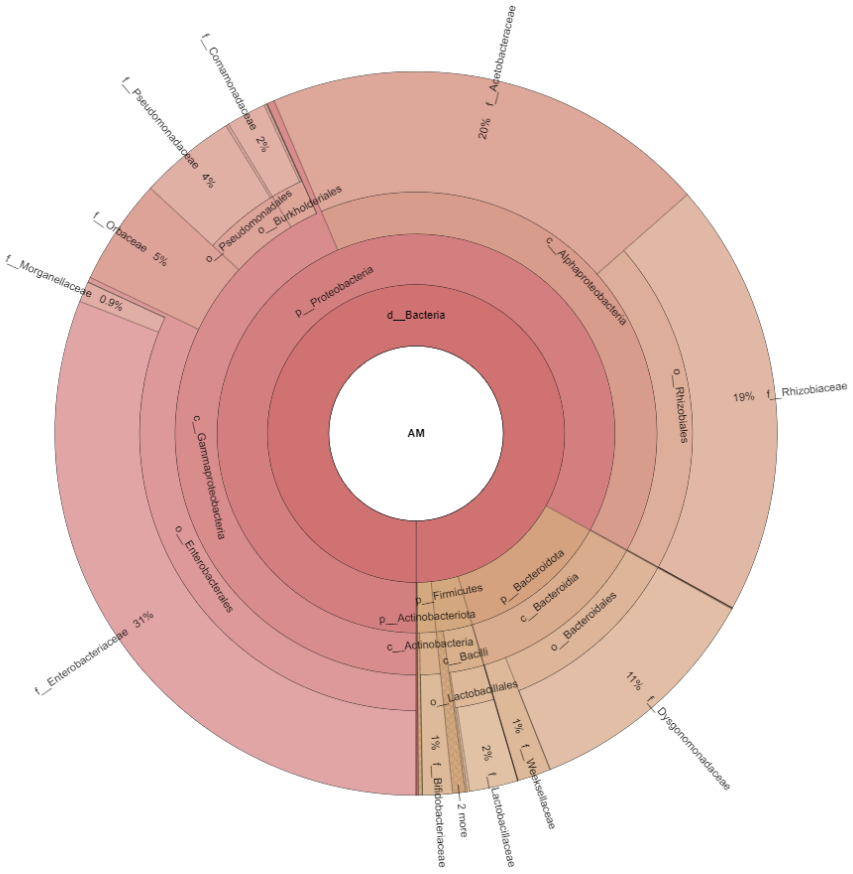

Figure 4. KRONA plot showing the relative abundance of identified bacterial taxa in the gut of *Apis mellifera*.

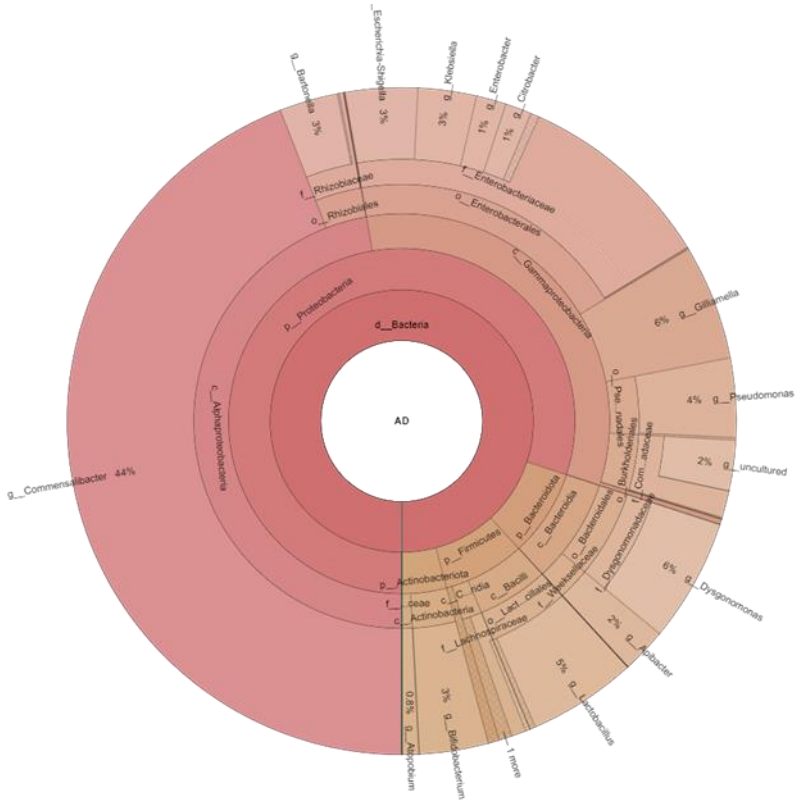

Figure 5. KRONA plot showing the relative abundance of identified bacterial taxa in the gut of *Apis dorsata*

Discussion

In *A. mellifera* and *A. dorsata,* the taxonomic classification of gut microbiota represents similarities and differences, which can lead to alteration in foraging activities, habitat, and evolutionary division. The present study was based on the KRONA plots and relative abundance of bacterial taxa, showed that Proteobacteria was dominant in both species as 83% was recorded in *Apis mellifera* and 80% in *Apis dorsata* followed by Bacteroidota and Firmicutes.

Previous studies showed that the gut microbiota of the honey bee is dominated by the bacterial phyla consisting of Proteobacteria, Bacteroidota, Firmicutes, and Actinobacteriota (Disayathanoowat et al., 2012; Powell et al., 2014; Kwong & Moran, 2016; Anjum et al., 2018; Mathialagan et al., 2018; Romero et al., 2019; Kim et al., 2023). Ganeshprasad et al. (2022) recorded honey bee gut microbial community in *A. florea* gut showed dominant Bacteroidetes at 51.3%, followed by Proteobacteria, Euryarchaeota, and Actinobacteria. Our study showed that in identified families of *Apis dorsata*, Acetobacteraceae was dominant (44%). At the same time, in *Apis mellifera,* Enterobacteriaceae was dominant (31%), indicating adaptation of *Apis dorsata* towards environmental conditions or might be a particular foraging niche. Genus *Commensalibacter* belongs to Acetobacteraceae is linked with sugar-rich diets and nectar.

Findings of this study support the hypothesis that variation in habitat and dietary behavior results in shape of gut microbiota (Kesnerova et al., 2017; Corby et al., 2014).

In our research Bacteroidota were recorded more abundant as 12% in *Apis mellifera* in contrast to *Apis dorsata* (8%). In both species *Dysgonomonas* was observed as a significant genus. Kikuchi et al. (2011) reported that *Dysgonomonas spp.* is well known as it can ferment the polysaccharides, which play an important role in bee feeding depending upon several floral sources. In both species, the existence of Firmicutes consisting of *Bifidobacterium* and *Lactobacillus* displays their part in gut health by protecting against pathogens because of their ability to produce lactic acid (Vasquez et al., 2012). The main microbiota was found in both *Apis mellifera* and *Apis dorsata,* including the genera *Lactobacillus*, *Snodgrassella*, and *Gilliamella,* was involved in maintaining the stability of gut microbiota among the species (Moran et al., 2012). These bacterial taxa are involved in various functions, like protection from pathogens and fermentation of carbohydrates obtained from plant sources (Engel et al., 2012).

In the present study, variations in relative abundance were observed as the percentage of *Klebsiella* (8%) was high in *Apis mellifera* as compared to *Apis dorsata* (3%), which suggests that certain environmental factors may regulate gut microbiome. Both species showed variations in the relative abundance of bacterial families like Rhizobiaceae, Acetobacteraceae, and Enterobacteriaceae, suggesting that diet and location are both factors involved in the shaping of gut microbiota. Previous research also reported that how gut microbiota of honeybees can vary by diet, contact with pesticides, and environmental stressors pesticides (Jones et al., 2018; Steffan et al., 2019). The relative abundance of bacterial families, including Bifidobacteriaceae, Lactobacillaceae, and Comamonaaceae involve in pathogen resistance, absorption of nutrients, and gut health as well (Kwong & Moran, 2016). *Pseudomonas spp*. Contributes in nitrogen cycling and also suggested that honeybee gut bacteria may lead to contribute to host health by nutrient absorption (Corby et al., 2014).

Conclusion

The findings of the present study suggest that variations in gut microbiota are shaped by environmental conditions, foraging behaviors, and evolutionary differences between these two species. These results provide baseline information for future research into the functional roles of microbiota in honeybee health and ecological adaptation. Further research is required in order to investigate the functional implications of these variations and how they affect the health of honeybees.

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