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Composition and diversity of bacteria from giant Asian honeybee *Apis dorsata* gut

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Abstract. *Niode NJ, Adji A, Rimbing J, Tulung M, Tallei TE. 2021. Composition and diversity of bacteria from giant Asian honeybee* Apis dorsata *gut. Biodiversitas 22: 906-912.* As a social insect, honeybee possesses a unique gut bacteria community. Therefore, we need to understand the composition and diversity of bacteria in *Apis dorsata* gut, the largest honeybee species that live in the forest, especially in Southeast Asia. The present study aimed to investigate the gut bacteria of *A. dorsata* using a metabarcoding approach. The DNA barcode region used in this approach was the V3-V4 region of 16S rRNA. Honeybees were caught in a forest area located in Tareran, South Minahasa, North Sulawesi, Indonesia. There were 11 phyla identified from the gut of *A. dorsata*. The most abundant phyla were Firmicutes (95.8%), Proteobacteria (3.7%), and Actinobacteria (0.4%). The class Bacilli was responsible for 94.5% of the total bacterial population, the dominant family was Bacillaceae (87.2%), and the dominant genus was *Bacillus* (87%). Simpson (1-D) 0.24 and Shannon diversity index 0.98 indicated that the diversity of the genera was low. A nonsignificant number of species belong to the lactic acid bacteria was also detected, which may have certain benefits that need to be investigated further.

Keywords: 16S rRNA, *Apis dorsata*, bacteria, gut, honeybee, V3-V4 region

INTRODUCTION

Pollination is essential for every plant's survival, including vegetables and fruits. The honeybee is one of the insects that play a significant role in pollination. Besides acting as a pollinator, honeybees also produce honey that contains many benefits including rich in energy and nutritional properties. There are nine honeybee species known to this day, including *Apis dorsata* (Fabricius 1793). Based on its body size, this insect is called the giant honeybee. Apart from their aggressive behavior, this honeybee could make enormous nests with a single comb that hangs from tree branches (Weihmann et al. 2014). The honeybee inhabits Southeast Asia's forests and has never been reported to be seen in urban areas. Out of three, there are two subspecies of *A. dorsata* in Indonesia: *A. dorsata dorsata* and *A. d. binghami*. According to early studies, *A. d. binghami* was found only on the island of Sulawesi and its surroundings (Hadisoesilo 2001; Lo et al. 2010). Meanwhile, other subspecies, *A.d. breviligula*, was found only in the Philippines (Hadisoesilo 2001).

Honeybees are social insects. Their guts possess unique gut microbiota community, caused among others by the high number of individuals within a colony, the food sharing, and the close relationship between colony members. Therefore, these microbiomes are easily propagated from one another among colony members (Engel et al. 2016). Some bacterial species associated with honeybees, are clearly pathogenic, but most species have never been associated with any disease symptoms and their impact on the honeybee is largely unknown (Lamei 2018). Hence, studying the interaction between the microbiome in their gut with its host is very interesting. In previous research, the bacterial community in the midgut of *A. dorsata* has been studied insect development stages (Saraithong et al. 2017). It was reported that Proteobacteria and Firmicutes dominated the midgut, which consisted of four classes: Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Bacilli. However, the study was not based on a metagenomic approach. So, it is expected that it does not represent the true nature of the microbiome diversity in the gut of *A. dorsata*. By conducting cultivation, Kwong et al. (2018) reported that they found *Apibacter* spp. in the gut of *A. dorsata*. The bacterial species belongs to the phylum Bacteroidetes, reported as endemic to *A. dorsata* and *A. cerana*. Furthermore, it is also described that each honeybee species has a different *Apibacter* strain symbiont, thus this bacterium is considered to have adapted to its host (Kwong et al. 2017).

A direct method without microbial culture was best to apply to study the gut bacterial diversity and community structure of *A. dorsata*. Not all of these bacteria could be cultured, as reported by several previous researchers (Tajabadi et al. 2011; Evans and Armstrong 2006). Lombogia et al. (2020) has reported that the dominant bacteria in the gut of *A. nigrocinta* found in densely populated urban areas were Proteobacteria (58%), Firmicutes (29%), and Actinobacteria (8%). To our

knowledge, based on the analysis using metagenomics approach, the bacterial composition and diversity in *A. dorsata* gut have not previously been reported. Thus, this study was conducted to further established the topic.

MATERIALS AND METHODS

Sampling location

One honeybee worker of *A. dorsata* was captured in the forest near Rumoong Atas Village, Tareran Sub-district, South Minahasa District (1.2241455, 124.7356518). The identification result of the honeybee based on partial COI gene sequence has been submitted to GenBank (https://www.ncbi.nlm.nih.gov/nuccore/MT233417).

Sample preparation

Sample preparation was carried out accordingly with the protocol performed by Lombogia et al. (2020). The surface of the bees was sterilized by immersing the entire body of the bee into 1.0% hypochlorite solution for 10 minutes, then immersed into 70% ethanol for 2 minutes. After that, the bee's body was rinsed using sterile distilled water three times, then dried using clean tissue paper. The bee's body was dissected under a sterile condition using a sterile scalpel. The gut was put into a sterile Eppendorf tube.

gDNA extraction and metabarcoding process

The honeybee gut was homogenized using FastPrep-24 Instrument at 4 m/s for 25 seconds, then extracted using ZymoBiomics DNA Mini Kit (Zymo Research) obtain the gut bacterial gDNA. Furthermore, NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) was used to analyze the gDNA to ensure that it was sufficient for the next stage. The V3-V4 hypervariable regions were selected from the 16S rRNA gene for this metabarcoding process. The area was amplified using MyTaq ™ HS Red Mix (Bioline, BIO-25044) in Agilent SureCycler 8800 Thermal Cycler. The polymerase chain reaction (PCR) conditions were run as follow: the denaturation began at 95˚C for 3 min, then followed by 35 cycles of denaturation at 95˚C for 15 sec, annealing at 52˚C for 30 sec, extension at 72˚C for 45 sec, and a final extension to complete the process at 72˚C for 3 min.

16S rRNA library preparation

The next stage of the metabarcoding process was preparing the 16S rRNA amplicon library using the Illumina two-step PCR protocol. Firstly, the V3-V4 regions were generated using Nextera-style tag sequences (overhang sequences) with the following sequences: forward overhang P5-tag: 5'TCGTCGGCAGCGTCA GATGTGTATAAGAGACAG- [locus-specific sequence] and reverse overhang P7-tag: 5'GTCTCGTGAGGACCGG - [locus-specific sequence]. Then, the process was continued using specific Illumina Nextera XT dual indices (Nextera XT i7 index and Nextera XT i5 index) with the following sequences: P5-PCR primary index: 5'AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGTC and P7-PCRG primary index: 5'CAAGCAGAAG i7] GTCTCGTGGGCTCGG. Amplicon sequencing was conducted using Illumina paired-end platform to generate 250 bp paired-end raw reads (Raw PE), merged, and pretreated to obtain clean tags. In order to get effective tags, which was for subsequent analysis, the chimeric sequences in clean tags were removed using UCHIME.

Bioinformatics analysis

The Effective Tags with the identity of 97% were clustered and identified to obtain operational taxonomic units (OTUs), which were useful to study the microbial community composition in each sample (Tallei et al. 2019). Necessary information of different samples such as effective tags, low-frequency tags, and tags annotation data was collected while constructing OTUs.

Sequencing data processing

The sequenced data processing was carried out accordingly following the previous procedure conducted by Kusumawaty et al. (2020) and Lombogia et al. (2020). The primary sequence was separated from the 16S rRNA barcode region on paired-end reads. This area was then merged using FLASH V1.2.7 (http://ccb.jhu.edu/software/FLASH/) to generate raw tags, which then filtered to obtain clean tags according to the Qiime (V1.7.0). The gold database was used as a reference for clean tags comparison. Chimera sequences in clean tags were detected using the UCHIME algorithm before removal to finally obtain the effective tags.

OTU clustering and taxonomic annotation

This process was carried out using Uparse Software v7.0.1001. Sequences were clustered to the same OTUs if the similarity index reached ≥97%. Annotations were then obtained based on the screening of for each OTU. Species annotations for each representative sequence were obtained from the SSU rRNA database (SILVA Database (http://www.arb-silva.de/) using Mothur software, so that species annotations were obtained for each taxonomic rank (Threshold: 0.8 ~ 1) commenced from kingdom till species. Phylogenetic relationships of all OTUs were constructed using MUSCLE v3.8.31.

Microbial community analysis

Relative abundance was obtained from taxonomic annotations. This could be illustrated by making pie charts for phylum and genus/species, so the most abundant taxa and their proportion in the sample classification level could be obtained visually.

Alpha diversity analysis

QIIME (Version 1.7.0) software was used to calculate the alpha bacterial diversity obtained from the *A. dorsata* gut, which included Simpson (1-D) and Shannon-Wiener (H') indices (Fatimawali et al. 2020).

Construction of genus evolutionary tree

The tree was constructed by using NJ methods based on 100 top genera and provided by vendor based on amplicon sequences (region V3-V4). The sequence alignment was conducted using PyNAST software (Version 1.2) and compared with the "core set" dataset in the GreenGene database.

RESULTS AND DISCUSSION

The bacterial community that inhabits the insect digestive system serves a significant function for its host's health. Therefore, a slight disturbance in the balance of the bacterial community will affect the insect's health and survival (Cariveau et al. 2014). As one of the social insects, honeybee has a microbiome community that is mostly limited to the gut. It is mainly transmitted through social interactions and helps metabolize dietary carbohydrates and protect against pathogens (Raymann and Moran 2018). This microbiota is very dynamic and adaptive because it is influenced by several factors, such as nutrition, the hive environment, social interactions, and the age of the honeybees, while the composition follows seasonal patterns (Lamei 2018).

The presence of symbiont bacteria communities in the gut of *A. dorsata* was analyzed using the metabarcoding approach. The DNA barcode region used in this approach was V3-V4 which is a hypervariable region of 16S rRNA. The result of this metabarcoding shows a total of 11 phyla that can be identified from the gut of *A. dorsata* (Table 1). The most abundant phyla are originated from Firmicutes (95.8%), followed by Proteobacteria (3.7%), and Actinobacteria (0.4%), as well as other phyla with very insignificant numbers (Figure 1). This study has generated substantially different results from those found by Lombogia et al. (2020) in the gut of *A. nigrocinta*, in which the dominant phyla are Proteobacteria (58%), Firmicutes (29%), Actinobacteria (8%), and Bacteroidetes (4%). A similar result has also been listed by Yun et al. (2018), which the dominant phyla are Proteobacteria (62.1%) and Firmicutes (20.7%). Other researchers have also agreed that the dominating phyla in the honeybee gut consist of Firmicutes, Actinobacteria, and Proteobacteria (Jain et al. 2018; Martinson et al. 2011; and Moran et al. 2012). The relative and absolute abundance of bee communities and their interactions with each other will determine the overall contribution of the microbiome to the health of its hosts (Engel et al. 2016).

Simpson's (1-D) value of 0.23 indicates low phylum diversity in the gut of *A. dorsata*, and there is a dominating taxon in the community (Dominance index: 0.77). Several studies have shown that these results vary, depending on the honeybee caste itself, whether foraging bees or queen (Lombogia et al. 2020).

The class Bacilli is responsible for 94.5% of the total bacterial population, followed by Gammaproteobacteria (1.8%), Alphaproteobacteria (1.7%), and Clostridia (1.1%). The dominant family is Bacillaceae (87.2%), later Planococcaceae (6.5%). Other families are also detected with insignificant numbers, namely Rhizobiaceae (1.6%), Orbaceae (0.9%), Staphylococcaceae (0.6%), Lachnospiraceae 0.5%), and Moraxellaceae (0.5%). The dominant genus is *Bacillus* (87%) and several genera are detected in small numbers, such as *Lysinibacillus* (6%), *Gilliamella* (0.9%), *Staphylococcus* (0.6%), *Acinetobacter* (0.5%), *Bifidobacterium* (0.4%), *Faecalibacterium* (0.2%), and *Blautia* (0.2%) (Figure 2). This result is very different from the bacteria in the gut of *A. nigrocincta* found in densely populated areas in the city of Manado (Lombogia *et al.*, 2020). *Bifidobacterium* has been found with a percentage of 8% in *A. nigrocincta* (Lombogia et al. 2020) and 3.34% in *A. mellifera* (Wang et al. 2020).

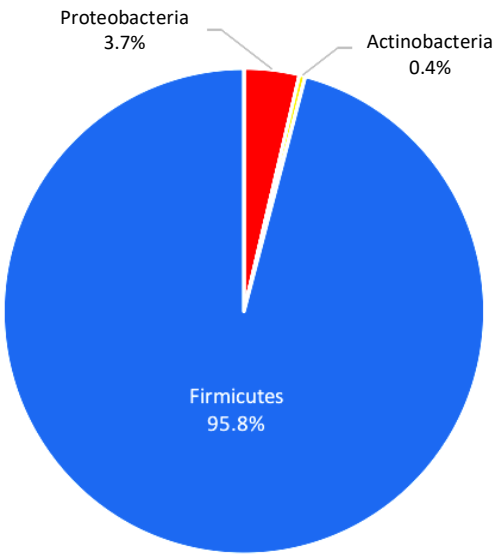

Figure 1. Relative abundance of phyla in the of *Apis dorsata dorsata* by using metabarcoding approach

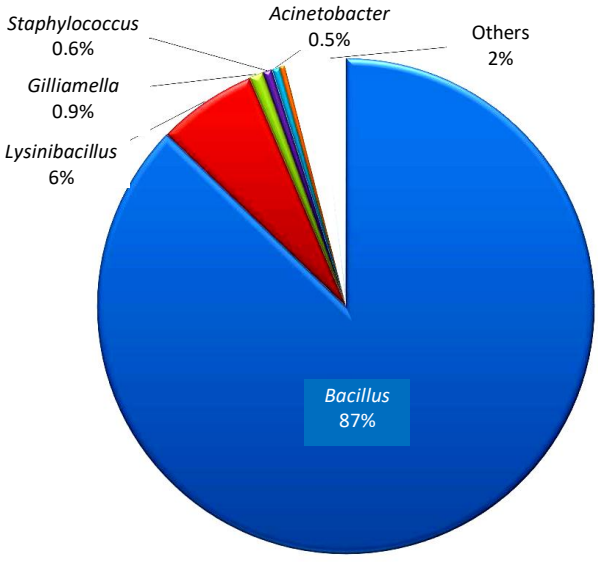

Figure 2. Relative abundance of genera in the gut of *Apis dorsata dorsata*

Table 1. Relative abundant of phyla in gut of *Apis dorsata dorsata*

Phylum	Relative abundance
Firmicutes	0.95766
Proteobacteria	0.03652
Actinobacteria	0.00438
Bacteroidetes	0.00073
Chloroflexi	0.00026
Acidobacteria	0.00021
Gemmatimonadetes	0.00009
Planctomycetes	0.00003
GAL15	0.00003
Cyanobacteria	0,00002
Verrucomicrobia	0.00001
Others	0,.00006

The abundance of *Bacillus* in the gut of *A. dorsata* is presumably related to the flowering season in the area where the specimen is found. The sampling area was located at an altitude of about 600 meters above sea level. The vegetation was dominated by *Arenga pinnata* (L.), *Piper aduncum* (L.), *Syzygium aromaticum*, and bushes. Wang et al. (2015) have reported that *Bacillus* dominated the gut of *A. mellifera* during the flowering season of the rape plant (Brassicaceae). They have also proposed a hypothesis that the presence and abundance of microbes in the gut of bees were closely related to the availability of feed around them, in which these microbes help process the feed. Functionally, too, these microbes secreted enzymes involved in the metabolization of bee feed. In their research, Wu et al. (2014) have also found abundant amount of *Bacillus* in Japanese honeybee's gut. Likewise, the European honeybee, *A. mellifera*, has been reported to contain many *Bacillus* species in their guts (Evans and Armstrong 2005). These results may have implied that members of the genus *Bacillus* have existed in the gut of *Apis* spp. (Wu et al. 2014). Most studies have found that *Bacillus* provides benefits for honeybees in pollen fermentation and food protection and disease prevention (Wu 2014). Research by Khan et al. (2020) has shown that *Bacillus* isolated from the intestine of *A. mellifera* can inhibit chalkbrood pathogens. Through gluconic acid production, *Bacillus senegalensis* can control the mushroom chalkbrood *Ascosphaera apis*. Research by Sabaté et al. (2009) has found that *B. subtilis* isolated from honeybees' gut can inhibit pathogens *Paenobacillus larvae* and *A. apis*. This is because the G2III strain of *B. subtilis* produces surfactin (bacterial cyclic lipopeptide), a very strong surfactant, which is usually used as an antibiotic. A similar case was also found in *B. licheniformis* isolated from *A. m. jemenitica*. This bacterium reduced the mortality rate of bee larvae infected with *P. larvae* (Al-Ghamdi et al. 2018). Ngalimat et al. (2019) found many *Bacillus* species isolated from the stingless bee (*Heterotrigona* sp.) had many activities such as proteolytic, lipolytic, and cellulolytic. Also, one of the strains, *B. amyloliquefaciens* PD9, showed broad-spectrum antimicrobial activity. Kacaniova et al. (2019) have reported *B. cereus*, *B. megatherium*, *B. olerenius*, and *B. thuringiensis* isolated from the gut of *A. mellifera* had antimicrobial activity against *P. larvae*. *Bacillus* is also a probiotic group member (Niode et al. 2020). Zulkhairi Amin et al. (2019) have suggested that the *Bacillus* strain isolated from the stingless bee has probiotic potential. The probiotic *Bacillus* can eliminate Methicillin-resistant *Staphylococcus aureus* (Piewngam et al. 2018), while *B. cereus* isolated from the gut of *A. nigrocincta* can inhibit *S. aureus* (Lombogia et al. 2020).

Blot et al. (2019) reported that *Lysinibacillus* was also found in the gut of *A. mellifera*. In the gut of *A. dorsata*, the species found was *L. sphaericus*. This bacterium, formerly known as *B. sphaericus*, is usually found in the soil and has a larvicidal effect on mosquitoes (*Culex and Anopheles*) and is therefore suggested to be a pathogenic insect (Berry 2012). In addition, this species is reported to have antifungal activity (Naureen et al. 2017). They serve as a nutritional ingredient that increases the ability to fix nitrogen, nitrify, and solubilize phosphorus (Aquirre-Monroy et al. 2019). It produces bacteriocins against foodborne bacterial and fungal pathogens (Ahmad et al. 2014). Thus, it is thought that the presence of *L. sphaericus* in a sufficiently large amount does have an important role for *A. dorsata*, which is to digest nutrients and protect the host's gut.

Gilliamella apicola is taken 1% of the bacterial community. According to Zheng et al. (2016), this species is one of the core bacterial species in the gut of the *Apis* spp. and plays a role in carbohydrate metabolism. Genomic analysis has identified various genes in the *G. apicola* strain, which involve sugar absorption and fermentation (Alatawy et al. 2020). *G. apicola* strain contains many sugar transporters and sugar utilization pathways (Moran 2015). Engel et al. (2012) found an abundance of genes that process carbohydrates, especially in the *G. apicola* strain isolated from the gut of worker bee communities. In some *G. apicola* strains, pectate lyase that digests pectin in the pollen cell walls was present, while others were absent. This suggests that certain strains in individual worker bees or in colonies can affect bees' nutritional ecology or may function to neutralize food toxins (Moran 2015).

The relative abundance of the genus *Bacillus* and *Lysinibacillus* can also be seen in the phylogenetic diagram in Figure 3. Based on this diagram, it is confirmed that there are a considerable number of genera/species. With 194 genera/species observed, Simpson (1-D) 0.24 indicates that the diversity of genus/species in the gut is low, and with a Dominance index value of 0.76, there is a genus/species that dominates the community. The Shannon diversity index obtained was 0.98, indicating that the diversity of the genus is low. This is because although the richness of the taxa is relatively high, the evenness is low, and therefore the diversity of genus/species is low. Kirsten et al. (2020) have suggested that the high level of diversity of the microbiota in the gut of *A. mellifera* can facilitate foraging on a wider variety of pollen sources to adapt more quickly to environmental condition dynamics. However, this high diversity can lead to increased gut microbiota competition. On the other hand, the low gut microbiota diversity of bees may contain various strains adapted to more favorable local conditions.

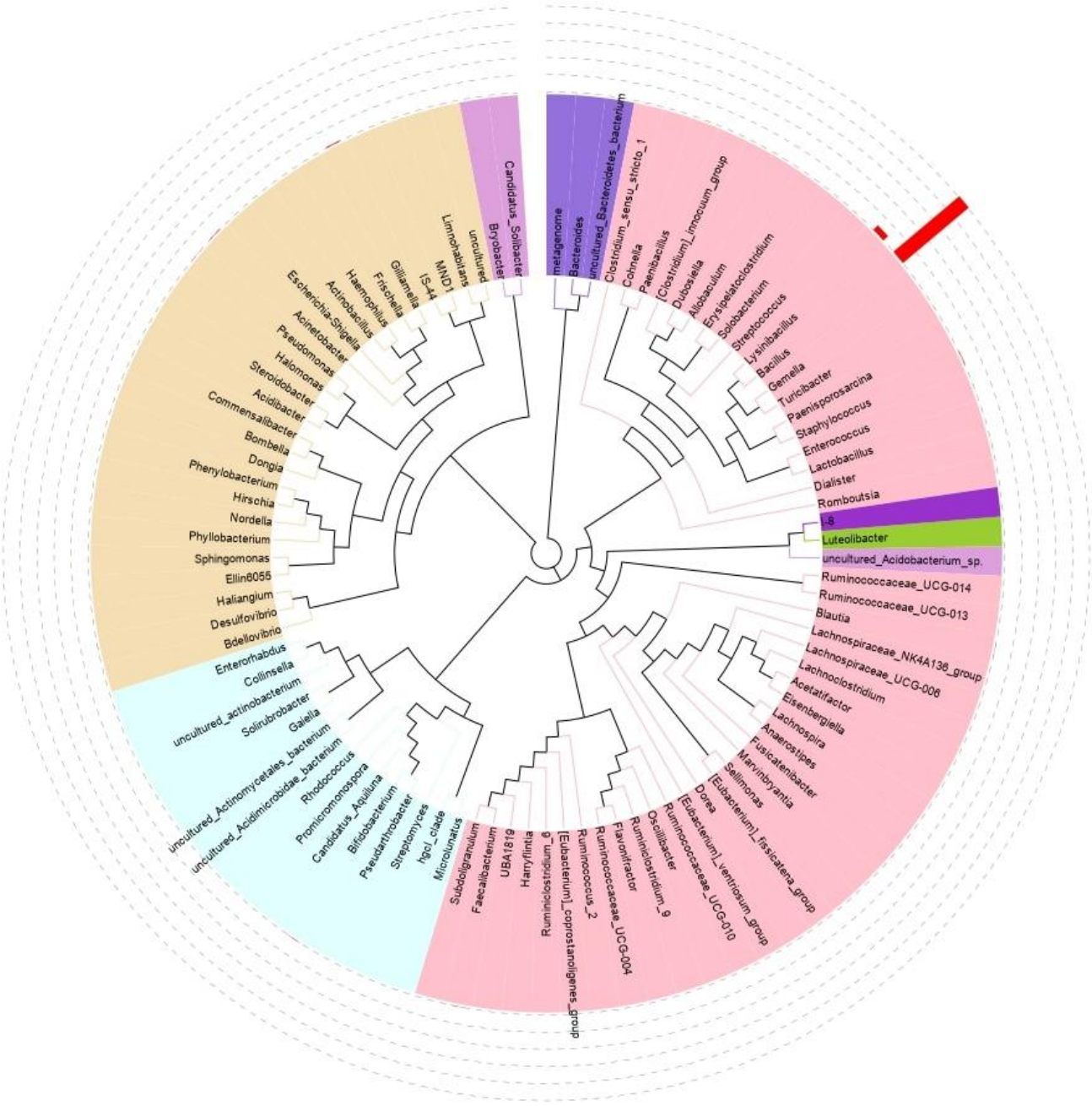

Figure 3. Genus evolutionary tree by using NJ methods. Red bars indicate the relative abundance of the taxa. Different colors of the branches represent different phyla. Relative abundance of each genus in each group is displayed outside the circle

Faecalibacterium and Bifidobacterium are 2 of 10 genera abundant in the gut of *Apis mellifera* (Wang et al. 2020). *Faecalibacterium prausnitzii*, which is the only species in the genus *Faecalibacterium*, is relatively abundant in the healthy human gut (Laursen et al. 2017) and they help in balancing the immune system (Miquel et al. 2013). This species can metabolize fiber which helps in energy conservation and anti-inflammatory (Hiippala et al. 2018).

Bifidobacterium can produce pectin-degrading enzymes, glycoside hydrolase, and Polysaccharide lyase which can degrade complex carbohydrates (Zheng et al. 2019; Wang et al. 2020). The striking differences in the composition of the bacteria inhabiting the *A. dorsata* gut compared to other honey bees can be attributed to the substrate needed by these bees. This is also closely related to the ecological niche in which these bees live. Hence the structure, composition, and abundance of bacteria in the gut are strongly influenced by food sources.

In conclusion, this study shows the bacterial community's composition and diversity in *A. dorsata* gut using the V3-V4 hypervariable region of 16S rRNA gene

marker. Firmicutes dominated most of the phyla, while Bacilli dominated the bacterial class. Relative abundance was found in the genera *Bacillus* and *Lysinibacillus*. Simpson (1-D) 0.24 and Shannon diversity index 0.98 indicated low genus diversity. This study can provide data on the microbiome's diversity in the gut of *A. dorsata*. However, further research will be required to determine the specific relationship between the gut microbiome of *A. dorsata*, its host, and its ecological niche.

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