

16S rRNA metagenomic analysis of the Asiatic worker honeybee (*Apis cerana*) gut microbiome from an apiary in Bandung, Indonesia

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Abstract. Putri RD, Dikson, Victor H, Sanjaya A, Pinontoan R. 2025. 16S rRNA metagenomic analysis of the Asiatic worker honeybee (*Apis cerana*) gut microbiome from an apiary in Bandung, Indonesia. *Biodiversitas* 26: 1666-1674. Honeybees play a crucial role in crop pollination, and consequently, in maintaining global food security. Therefore, understanding their survivability and resilience to natural and human-induced factors is crucial. One critical aspect is the gut microbiome of the honeybees, which is comprised of diverse endosymbiotic bacteria that enhance nutrient uptake and pathogen resistance. Asiatic honeybees (*Apis cerana*) are commonly managed in Indonesian apiaries for honey production. Therefore, this study aimed to investigate the gut microbiome of *A. cerana* from a Bandung apiary using 16S rRNA metagenomic sequencing and analytical tools, such as Kraken2, Bracken, SILVA 16S database, and Vegan R library for genus-level identification and Alpha Diversity analysis. The analysis identified 81 genera, including the dominant genera (97.9%) *Gilliamella*, *Lactobacillus*, *Snodgrassella*, *Apibacter*, and *Bifidobacterium*. Given that *Gilliamella* constituted more than 50% of the microbiome, the gut microbiome had high genus richness, but potentially low genus diversity, as indicated by the five Alpha Diversity indices Chao1, ACE, Simpson, Inverse Simpson and Shannon indices. The core genera revealed in this study are consistent with *A. cerana* samples from Korea and Japan, with *Apibacter* uniquely present in *A. cerana* but absent in Western *A. mellifera*. This study provides insights into the diversity of *A. cerana* gut microbiome from an apiary in Bandung, Indonesia.

Keywords: 16S rRNA, *Apis cerana*, gut microbiome, honeybee, metagenomics

Abbreviations: ACE: Abundance-based Coverage Estimator; gDNA: Genomic DNA

INTRODUCTION

Honeybees, classified within the *Apis* genus, are natural pollinators that contribute significantly to plant pollination and honey production. These activities are essential for ensuring the availability of crops for human consumption (Khalifa et al. 2021). Some honeybees are managed by beekeepers at various scales not only to pollinate the surrounding crops but also to produce honey. These managed honeybees may occasionally be deployed elsewhere to provide pollination services for crops, using portable man-made hive frames transported over long distances (Melicher et al. 2019; Rocha et al. 2023). Given the large revenue generated by pollinating honeybees and their importance in maintaining global food security, honeybees have become the subject of intense scrutiny in research and conservation (Khalifa et al. 2021; Bixby et al. 2023; Warrit et al. 2023; Kortsch et al. 2024). Honeybees serve as valuable model organisms for studying the symbiotic relationships between their gut microbial community and the host, as well as the evolutionary transition of these microbes to endosymbionts (Zheng et al. 2018; Zhang et al. 2022).

Approximately 75% of the highest-produced global food crops benefit from pollinators such as honeybees, many of which are important to human health, including vegetables, fruits, nuts, and legumes. In the absence of these pollinators, it is estimated that their supplies would suffer significant losses. In addition to improving the quantity

of these crops, pollination also improves the quality and nutritional value (Garibaldi et al. 2022). Beekeepers have encountered a continuous decline in honeybee populations since 1990 (Theisen-Jones and Bienefeld 2017). This decline has been attributed to several factors, including climate change, land or farm conversion, pathogen infection, and the use of agrochemicals to deter pests and weeds. This negative trend not only affects honey production but also agricultural crop production, which consequently poses a threat to food security and exacerbates the risk of global-scale famine (Hristov et al. 2020; Cook et al. 2022; Ilgun and Schmickl 2022).

Western honeybees (*Apis mellifera* (Linnaeus, 1758)) and Asiatic honeybees (*A. cerana* (Fabricius, 1793)) are widely used and maintained in apiaries for pollination and honey production, each with its own strengths. *Apis cerana* have better self-hygiene behavior, which makes their colonies more resistant to *Varroa* mite infestations and improves their overall survivability. Their increased resilience to diseases and pathogen infections may also be attributed to their gut microbiome, which plays an important role in nutrient processing and absorption, immune system modulation, and defense against various pathogens. Accordingly, their gut microbiome has been extensively studied for composition, diversity, and relationship with the host. A prime example of a honeybees gut microbiome study is metagenomic sequencing, taxonomic classification and diversity analysis (Park et al. 2015; Engel

et al. 2016; Lanh et al. 2022; Katuwal and Pokhrel 2023).

Microbiome metagenomic sequencing is a widely used method for investigating the gut microbiome for composition, diversity, and even relationship with the host by first extracting, amplifying, and sequencing collective gut microbiome DNA using next-generation sequencing technologies. Subsequently, DNA sequences can be identified and analyzed for taxonomic identity, diversity, abundance, and potential relationship with the host. This technique is particularly valuable for studying the diversity of gut microbes that cannot always be cultured in a laboratory setting, offering benefits, such as speed, cost-effectiveness, and comprehensive coverage of microbial diversity compared to traditional bacterial cultures (Lagier et al. 2015).

By examining the honeybees gut microbiome through metagenomic analysis, researchers can gain insights into its evolution and the relationships it maintains, which in turn influence honeybees health. The honeybees gut microbiome serves as a tractable model system because it is predictable, cultivatable, spatially structured, taxonomically simple, and amenable to manipulation (Anderson and Ricigliano 2017; Lazarova et al. 2023). Despite the growing global interest in metagenomic sequencing and analysis of the honeybees gut microbiome, studies on the gut microbiome of *A. cerana* maintained in equatorial tropical regions particularly Indonesia are limited, notwithstanding the honeybees' importance in contributing to defense of the Indonesian food security. Therefore, this study aimed to offer insights into the gut microbiome composition and diversity of *A. cerana* maintained in an apiary in Bandung, Indonesia by utilizing V3-V4 16S rRNA metagenomic sequencing, taxonomic identification, and diversity analysis.

MATERIALS AND METHODS

Worker honeybees collection area

Apis cerana honeybees utilized in this study were worker honeybees sourced from the Syifa Madu apiary, situated in the vicinity of Dago Village, Bandung District, West Java, Indonesia (Latitude: -6.853996346666752, Longitude: 107.64365357558391). The apiary provides *Calliandra* plants as a foraging food source for the bees.

Gut microbiome sample collection

80 worker honeybees body surfaces were sterilized by immersion in 70% ethanol solution, followed by sterile water for 60s each. Portions of the honeybees digestive tract, particularly the midgut and hindgut, were aseptically extracted by gently pulling the tail with sterile tweezers (Figure 1.B-E). The extracted midgut and hindgut were suspended in 1 mL 7.2 pH phosphate-buffered saline solution and subsequently homogenized using sterilized toothpicks and a vortexing device.

Gut microbiome DNA extraction and quality control

The genomic DNA (gDNA) from the worker honeybees midgut and hindgut was extracted using a Wizard® Genomic DNA Purification Kit (Promega). The results of the spectrophotometric absorbance measurements indicated a gDNA concentration of 427 µg/mL, with a purity ratio of

A260/A280 equal to 1.87, which confirmed the viability of the gDNA for metagenomic sequencing and analysis. The gDNA samples were subsequently delivered to NovoGene (Hong Kong) for hypervariable 16S rRNA region amplification, sequencing, and preprocessing.

Hypervariable 16S rRNA region amplification and sequencing

The hypervariable V3-V4 region of the 16S rDNA genes were amplified using a specific primer pair 341F (5'-CCT AYG GGR BGC ASC AG-3') and 806R (5'-GGA CTA CNN GGG TAT CTA AT-3'), which flanks the region. The Phusion® High-Fidelity PCR Master Mix (New England Biolabs) was used for PCR tests. Subsequently, equal parts of the amplicon and 1X loading buffer containing SYBR Green dye were loaded onto a 2% agarose gel for visualization by electrophoresis. The 400-450 bp amplicon bands were extracted and purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). Prior to sequencing, amplicons were prepared as libraries using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina and quantified using Qubit fluorometer and Q-PCR. Finally, the libraries were sequenced using Ion Torrent Ion S5 XL and analyzed using the Illumina platform.

Sequencing data processing

Paired-end reads were allocated to samples based on a unique barcode and truncated by removing the barcode and primer sequences. Paired-end reads were combined using FLASH (V1.2.7, <http://ccb.jhu.edu/software/FLASH/>) to produce Raw Tags, which were subsequently filtered for low-quality and/or chimeric sequences using the QIIME quality control process (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html). The Tags were compared with a reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) to detect the chimeric sequences (<https://drive5.com/usearch/manual/chimeras.html>). Cleaned Effective Tags were obtained as Clean Sequences and subsequently used for taxonomic classification and diversity analysis.

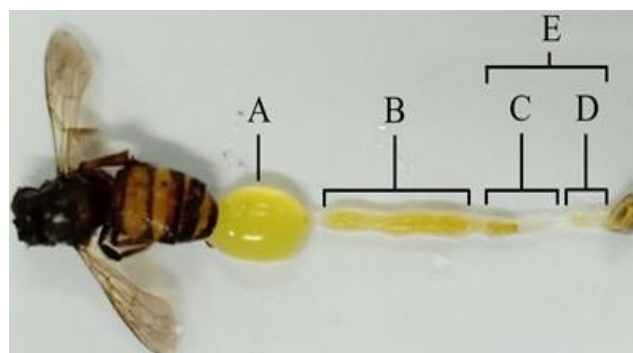


Figure 1. Anatomy of an *Apis cerana* worker honeybee digestive tract. A. Honey stomach; B. Midgut; C. Ileum; D. Rectum; and E. Hindgut

Taxonomic classification and diversity analysis

Following the metagenome analysis protocols outlined by Lu et al. (2022), Clean Sequences were classified using Kraken2 with a confidence threshold of 10% against a pre-made SILVA 16S rRNA release 138 database (Quast et al. 2013; Wood et al. 2019). The abundance of these sequences at the genus level was estimated using Bracken with a k-mer value of 250 and a threshold value of at least one read per bin (Lu et al. 2017). Genus abundance values were then visualized using a Sankey diagram created using the Pavian R library (Breitwieser and Salzberg 2020), as outlined by Guzmán-Solís et al. (2021). Finally, the Bracken-created Kraken2 taxonomic binning report was converted to a genus-frequency table using the kraken2OTU Python script (<https://github.com/sipost1/kraken2OTUtable>), as described by Lee et al. (2025), for Alpha Diversity index analysis. The five Alpha Diversity indices, ACE, Chao1, Shannon, Simpson Diversity, and Inverse Simpson, were calculated using the vegan v2.6-7 library in R v4.4.0, as described by a metagenomics study conducted by Jing et al. (2024).

RESULTS AND DISCUSSION

Metagenome sequencing raw reads processing

Ion Torrent sequencing of the *A. cerana* honeybees gut microbiome, spanning the V3-V4 region of the 16S rRNA gene, yielded raw reads that were subsequently processed using FLASH, a program for merging short reads. This was followed by filtering the chimeric sequences using QIIME, which resulted in the production of Clean Sequences (Table 1).

The 140,345 Clean Sequences obtained had a total base count of 60,162,099 bp, with a guanine/cytosine content of 50.92% and a quality score 20 portion size of 82.78%, indicating that the portion had been base called with an accuracy of 99% during metagenomic sequencing (Zhang et al. 2017). This high yield of Clean Sequences with high base calling accuracy allowed for subsequent taxonomic classification and diversity analysis.

Taxonomic classification and diversity analysis

Taxonomic classification of the Clean Sequences was conducted using Kraken2 with a 10% confidence threshold against the SILVA 138 16S rRNA database. The genus-level abundance of the Clean Sequences was estimated using Bracken with a threshold set at ≥ 1 sequence per taxonomic bin. Of the 140,345 Clean Sequences obtained from raw read processing, 140,248 were successfully classified with Kraken2 taxonomic classification and Bracken abundance estimation, and 140,206 were successfully classified at the genus level. The classification and abundance estimation results were presented as a

Sankey diagram visualizing the distribution of Clean Sequences across top 10 taxa at each taxonomic level (Figure 2) and a table listing the classified genera with their abundance in percentage values (Table 2).

The genus classification of Clean Sequences by Kraken2 and their abundance estimation by Bracken indicated a predominance of the five genera; *Gilliamella*, *Lactobacillus*, *Snodgrassella*, *Apibacter*, and *Bifidobacterium*, which accounted for 97.9% of the identified sequences. Smaller proportions of the sequences (<1%) were classified as belonging to the genera, *Stenotrophomonas*, *Enterobacter*, *Providencia* and *Rosenbergiella*. The remaining 0.59% were distributed across 72 other genera in small percentages, whereas <0.1% were either uncultured or had an uncertain taxonomic placement (*Incertae sedis*). These findings indicate limited diversity within the *A. cerana* worker honeybees gut microbiome maintained in the Bandung apiary, which is dominated by only a few abundant genera. Five Alpha Diversity indices were calculated using the vegan library in R to assess genus richness, evenness, and abundance in the gut microbiome. These Alpha Diversity indices included Chao abundance-based non-parametric richness estimator (Chao1) and Abundance-based Coverage Estimator (ACE) indices which are sensitive to rare taxa (singletons and doubletons), as well as Simpson Diversity, Inverse Simpson, and Shannon indices (Table 3).

The Vegan R library calculated the five Alpha Diversity indices of the *A. cerana* honeybees gut microbiome, which revealed high genus richness based on the Chao1 (96.909, standard error ± 8.684) and ACE (96.885, standard error ± 4.890) indices, due to their sensitivity to rare taxa such as singletons (18 + 2 uncultured) and doubletons (10 + 2 uncultured). Despite the high genus richness of the *A. cerana* honeybees gut microbiome, a few genera dominate the microbiome, as indicated by the Simpson Diversity, Inverse Simpson and Shannon Indices. As such, although the microbiome has high genus richness, it may have low genus diversity because of the few dominant genera in the microbiome (Kim et al. 2017; Rai et al. 2022).

Discussion

The digestive system of the honeybees comprises the honey stomach, midgut, and hindgut, the latter of which includes the ileum and rectum. A significant number of essential endosymbionts have been identified in the midgut and hindgut (Kešnerová et al. 2017; Daisley et al. 2020). These two sections were the focus of the metagenomic analysis because of their roles in nutrient absorption, which leads to the expectation that the large and diverse bacterial community residing in these sections supports honeybees health (Khan et al. 2020; Smriti et al. 2024).

Table 1. Amplicon sequence processing results

Raw reads (Base pairs)	Clean sequences (Base pairs)	Mean read length (Base pairs)	GC content (%)	Q20 (%)	Effectiveness (%)
151,469	140,345	428	50.92	82.78	92.66

Note: GC: Guanine/Cytosine; Q20: Quality score 20. Effectiveness is a percentage of clean sequences in raw reads

Table 2. Kraken2 genus classification and Bracken abundance estimation of the *Apis cerana* honeybees gut microbiome using the SILVA 138 database

Genera	Abundance (%)
>0.1% genera	
<i>Gilliamella</i>	58.310
<i>Lactobacillus</i>	22.200
<i>Snodgrassella</i>	10.329
<i>Apibacter</i>	5.994
<i>Bifidobacterium</i>	1.082
<i>Stenotrophomonas</i>	0.758
<i>Enterobacter</i>	0.356
<i>Providencia</i>	0.227
<i>Rosenbergiella</i>	0.122
<0.1% genera	
<i>Pseudomonas</i>	0.046
<i>Peptoclostridium</i>	0.046
<i>Acinetobacter</i>	0.043
<i>Commensalibacter</i>	0.043
<i>Trichococcus</i>	0.028
<i>Bacteroides</i>	0.024
<i>Christensenellaceae R-7 group*</i>	0.023
<i>Lachnospiraceae NK4A136 group*</i>	0.016
<i>Proteiniborus</i>	0.016
<i>JGI-0000079-D21*</i>	0.016
<i>Neisseria</i>	0.015
<i>Clostridium sensu stricto 1*</i>	0.012
<i>Reyranella</i>	0.011
<i>Blautia</i>	0.011
<i>CL500-29 marine group*</i>	0.011
<i>Coprothermobacter</i>	0.011
<i>Mesotoga</i>	0.011
<i>Corynebacterium</i>	0.007
<i>Faecalibacterium</i>	0.006
<i>Clostridium sensu stricto 13*</i>	0.006
<i>Parasutterella</i>	0.005
<i>UCG-004*</i>	0.005
<i>Alcaligenes</i>	0.004
<i>Holdemanella</i>	0.004
<i>Acidaminobacter</i>	0.004
<i>Clostridium sensu stricto 2*</i>	0.004
<i>Candidatus Saccharimonas</i>	0.004
<i>OM27 clade*</i>	0.004
<i>Streptococcus</i>	0.003
<i>Anaerovorax</i>	0.003
<i>Thermacetogenium</i>	0.003
<i>Alistipes</i>	0.003
<i>Georgenia</i>	0.003
<i>Ahniella</i>	0.002
<i>Agitococcus lubricus group*</i>	0.002
<i>Ignatzschineria</i>	0.002
<i>Cellvibrio</i>	0.002
<i>Allobaculum</i>	0.002
<i>Faecalitalea</i>	0.002
<i>Caldanaerobacter</i>	0.002
<i>Syntrophomonas</i>	0.002
<i>Rikenellaceae RC9 gut group*</i>	0.002
<i>Actinomyces</i>	0.002
<i>Iamia</i>	0.002
<i>Legionella</i>	0.001
<i>Halomonas</i>	0.001
<i>Candidatus Alysiosphaera</i>	0.001
<i>Ileibacterium</i>	0.001
<i>Holdemania</i>	0.001
<i>Mycoplasma</i>	0.001

<i>Acholeplasma</i>	0.001
<i>Family XIII UCG-001*</i>	0.001
<i>Guggenheimella</i>	0.001
<i>Caldicoprobacter</i>	0.001
<i>Syntrophaceticus</i>	0.001
<i>Capnocytophaga</i>	0.001
<i>Anaerocella</i>	0.001
<i>Butyricimonas</i>	0.001
<i>Lentimicrobium</i>	0.001
<i>Ignavibacterium</i>	0.001
<i>Helicobacter</i>	0.001
<i>Desulfomicrobium</i>	0.001
<i>Syntrophobacter</i>	0.001
<i>Defluviitoga</i>	0.001
<i>Candidatus Solibacter</i>	0.001
<i>Subgroup 10*</i>	0.001
<i>Halocella</i>	0.001
<i>Nitrospira</i>	0.001
<i>Nannocystis</i>	0.001
<i>RS25G*</i>	0.001
<i>Elusimicrobium</i>	0.001
<i>Fusobacterium</i>	0.001
Uncultured	0.064
<i>Incertae sedis</i>	0.026

Note: Genera marked with an asterisk (*) are provisional names provided by the Microbial Database for Activated Sludge, included in the SILVA database

Table 3. Alpha Diversity indices of *Apis cerana* honeybees gut microbiome calculated using the vegan R library

Metric coverage	Alpha Diversity Index	Value
Genus richness	Chao1	96.909
	ACE	96.885
Genus dominance	Simpson Diversity	0.596
Genus diversity	Inverse Simpson	2.472
	Shannon	1.227

The gut microbiome of *A. cerana* sampled across multiple geographic regions in Asia showed five abundant genera shared with the *A. cerana* gut microbiome sampled in Bandung, Indonesia. The genera were *Gilliamella*, *Lactobacillus*, *Snodgrassella*, *Bifidobacterium*, and *Apibacter* (Table 4) (Ellegaard et al. 2020; Wu et al. 2022; Yun et al. 2022).

Gilliamella has the highest abundance in the gut of *A. cerana* and plays a crucial role in the degradation of complex carbohydrates and in the metabolism of toxic sugars found in the nectar and pollen that honeybees forage prior to absorption, particularly in the ileum. Pollen is protected by a carbohydrate exine that is resistant to digestion by the honeybees gut. Toxic sugars cannot be metabolized by honeybees without modification, which may cause toxicity in younger worker honeybees. Studies have reported that *Gilliamella* is important for the digestion of pollen and metabolism of toxic sugars in the gut of *A. cerana* (Zheng et al. 2016; Anderson and Ricigliano 2017; Kešnerová et al. 2017; Ellegaard and Engel 2019; Papp et al. 2022).

The second most abundant genera are lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium*. They have been investigated for their potential roles in regulating the microbiota within the honeybees gut. These lactic acid bacteria produce antimicrobial compounds that assist in combating pathogens and improving the immune system (Moharrami et al. 2022). Additionally, through metabolic processes that result in lactic acid production, *Lactobacillus* and *Bifidobacterium*, which belong to the phylum *Firmicutes*, can inhibit the growth of the main pathogens that threaten honeybees, such as *Paenibacillus larvae* and *Melissococcus plutonius* (Engel et al. 2016).

The genera *Gilliamella*, *Lactobacillus*, and *Bifidobacterium* are often used to evaluate the health of honeybees (Forsgren et al. 2010). According to Anderson and Ricigliano (2017), a thriving honeybee colony is characterized by a higher relative abundance of *Lactobacillus* and *Bifidobacterium* than *Gilliamella* in the worker honeybees gut microbiome. However, an increased relative abundance of *Gilliamella* can lead to an imbalanced gut microbiota in honeybees, making them more susceptible to pathogens. This imbalance can occur as worker honeybees reach senescence, and if it occurs

prematurely, it may indicate damage to, or a shortage of, honeybee colony populations (Anderson et al. 2018; Ribière et al. 2019; Copeland et al. 2022).

Snodgrassella is a bacterial genus that resides in the gut of honeybees, where it plays an essential role in protecting against pathogens by forming a biofilm that lines the gut, serving as a barrier to infection. However, *Snodgrassella* is vulnerable to some herbicides, including glyphosate, which impair its growth and compromise the integrity of its biofilm. Consequently, honeybees are made more susceptible to diseases, such as infection by *Nosemia* spp. and gut tissue disorders (Engel et al. 2015; Maes et al. 2016; Anderson and Ricigliano 2017).

Furthermore, the genus *Apibacter* metabolizes some toxic monosaccharides, such as glucose, fructose, and mannose, via anaerobic respiration and fermentation. The *Apibacter* genome contains genes that encode enzymes necessary for sugar metabolism (Kwong et al. 2018). Moreover, the *Apibacter* genome gained host-specific genes and lost extraneous genes during its evolutionary transition to better adapt as an endosymbiont to the microaerophilic environment of the honeybees gut (Zhang et al. 2022).

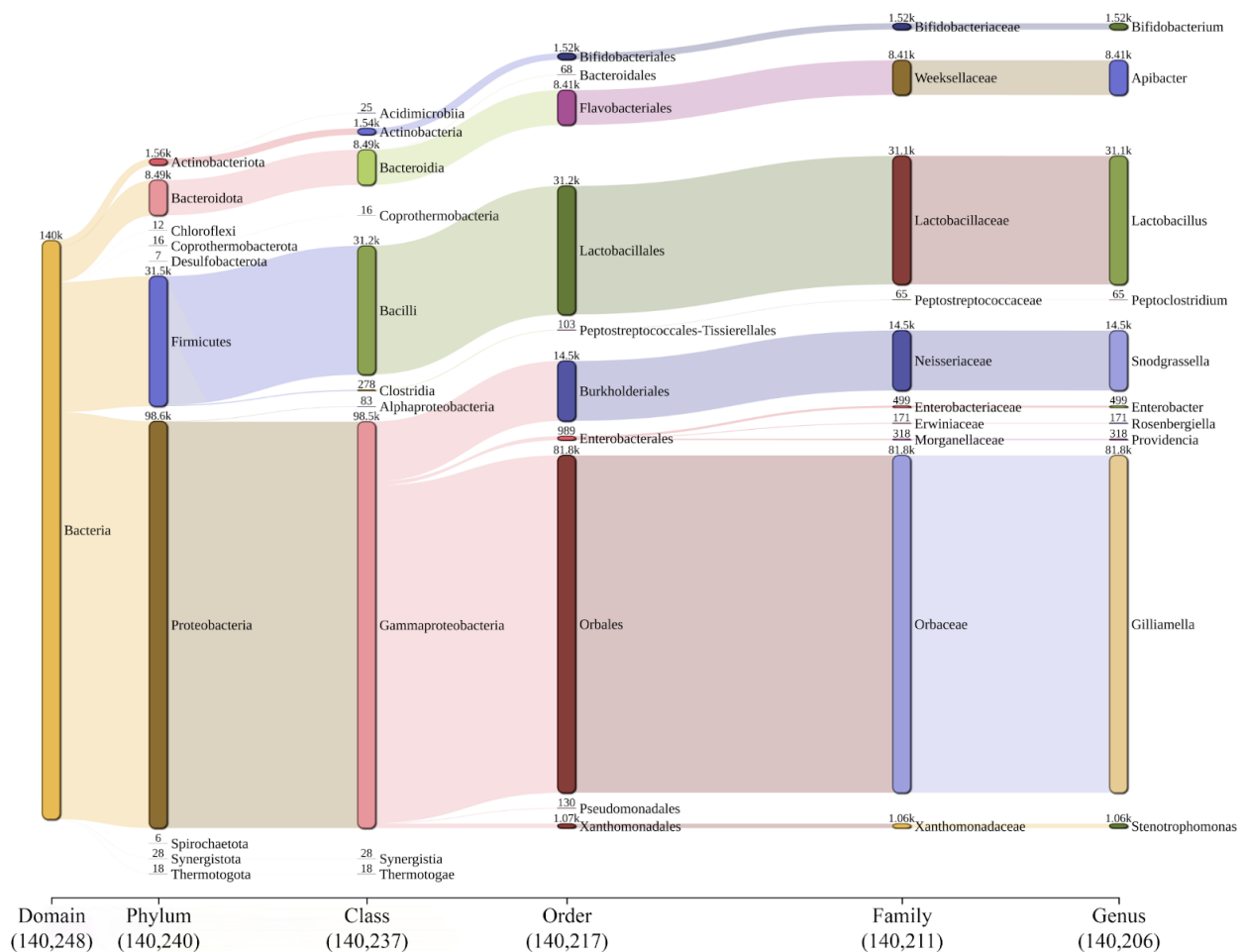


Figure 2. Sankey diagram of *A. cerana* worker honeybees gut microbiome genus classification and abundance estimation with Kraken2 and Bracken visualized using Pavian. Each taxonomic level consisted of the 10 most abundant taxa and the number of sequences classified at each level

Table 4. Comparison of *A. cerana* and *A. mellifera* honeybees gut microbiome genera

Indonesia Bandung*	<i>A. cerana</i>						<i>A. mellifera</i>		
	China (hindgut) ¹⁾				Korea (whole gut) ²⁾		Japan (hindgut) ³⁾	Japan (hindgut) ³⁾	Switzerland (hindgut) ³⁾
	Beijing	Guangzhou	Hangzhou	Kunming	SBV Individual Resistance	SBV Social Resistance			
<i>Gilliamella</i>	<i>Gilliamella</i>	<i>Bartonella</i>	<i>Gilliamella</i>	<i>Gilliamella</i>	<i>Lactobacillus</i>	<i>Gilliamella</i>	<i>Gilliamella</i>	<i>Bifidobacterium</i>	<i>Lactobacillus</i>
<i>Lactobacillus</i>	<i>Snodgrassella</i>	<i>Gilliamella</i>	<i>Snodgrassella</i>	<i>Bifidobacterium</i>	<i>Gilliamella</i>	<i>Lactobacillus</i>	<i>Lactobacillus</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>
<i>Snodgrassella</i>	<i>Apibacter</i>	<i>Snodgrassella</i>	<i>Bartonella</i>	<i>Apibacter</i>	<i>Apibacter</i>	<i>Apibacter</i>	<i>Bifidobacterium</i>	<i>Snodgrassella</i>	<i>Gilliamella</i>
<i>Apibacter</i>	<i>Lactobacillus</i>	<i>Apibacter</i>	<i>Bifidobacterium</i>	<i>Snodgrassella</i>	<i>Hafnia</i>	<i>Bifidobacterium</i>	<i>Apibacter</i>	<i>Gilliamella</i>	<i>Snodgrassella</i>
<i>Bifidobacterium</i>	<i>Bifidobacterium</i>	<i>Lactobacillus</i>	<i>Apibacter</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>	<i>Hafnia</i>	<i>Snodgrassella</i>	<i>Bartonella</i>	<i>Bartonella</i>
<i>Stenotrophomonas</i>	<i>Bartonella</i>	<i>Bifidobacterium</i>	<i>Lactobacillus</i>	<i>Frischella</i>	<i>Snodgrassella</i>	<i>Snodgrassella</i>	-	<i>Frischella</i>	<i>Commensalibacter</i>
<i>Enterobacter</i>	<i>Frischella</i>	<i>Commensalibacter</i>	<i>Frischella</i>	-	-	-	-	<i>Commensalibacter</i>	<i>Frischella</i>
<i>Providencia</i>	<i>Commensalibacter</i>	<i>Frischella</i>	-	-	-	-	-	-	-

Note: Korean honeybees whole gut microbiome was studied for its influence on honeybees Sacbrood Virus (SBV) resistance with genus abundance percentage cut off at 1% (Yun et al. 2022). *Apis cerana* honeybees gut microbiome in this study is denoted with an asterisk (*). 1: Wu et al. (2022); 2: Yun et al. (2022); 3: Ellegaard et al. (2020)

Although *Apibacter* is reported to be endemic to Asiatic *A. cerana* and *A. dorsata* gut microbiomes, including the former honeybee species sampled in this study, it is scarce or even absent in the Western *A. mellifera* gut microbiome (Kwong et al. 2017; Ellegaard et al. 2020; Niode et al. 2021), despite its ability to colonize the gut of *A. cerana* and *A. mellifera*, as reported by Wu et al. (2022) and Zhang et al. (2022). This scarcity of *Apibacter* in the *A. mellifera* gut microbiome is yet to be fully understood beyond the underlying strong competition in the *A. mellifera* gut microbiome (Zhang et al. 2022).

Conversely, several other genera found in the gut microbiome of *A. mellifera*, such as *Frischella* and *Bartonella*, were scarce or even absent in the *A. cerana* gut microbiomes sampled in Bandung, Korea, and Japan. These genera may or may not be present and may have varying abundances based on habitat conditions (Subotic et al. 2019), given their presence in the *A. cerana* gut microbiome sampled in China (Wu et al. 2022). *Frischella* enhances the honeybees immune system (Emery et al. 2017) while *Bartonella* metabolizes carbohydrates and secondary plant metabolites under microaerophilic conditions (Subotic et al. 2019).

Consequently, the dominance of some genera and the absence of others in the gut microbiome suggests a specialized adaptation of *A. cerana* to its local environment and food sources, suggesting bidirectional symbiosis with honeybees. However, according to Disayathanooat et al. (2012), honeybee colonies managed by beekeepers tend to lose several core bacterial communities that should be present in the honeybees gut. This loss is likely to be due to the influence of human activities on honeybee colonies, including habitat fragmentation, plant monocultures, and the administration of pollen or sugar syrup to sustain honeybee lives during the dry season (Engel et al. 2016; Graystock et al. 2016; Anderson and Ricigliano 2017; Motta et al. 2018; Regan et al. 2018; Wang et al. 2020; Powell et al. 2023).

A study conducted by Raymann et al. (2017) demonstrated that administration of antibiotics by beekeepers can perturb the equilibrium of honeybees gut microbiota. This was originally intended to prevent the spread of *P. larvae*, a pathogen that infects honeybee larvae. The typically used antibiotics, tetracycline and tylosin, are administered along with pollen or sugar syrup. Tetracycline is a broad-spectrum antibiotic capable of killing Gram-positive and Gram-negative bacteria. Treatment with tetracycline at a concentration of 450 µg/mL for seven days increased the relative abundance of *Gilliamella* in the intestines of honeybees, whereas the relative abundance of *Bifidobacterium*, *Lactobacillus*, and *Bartonella* genera decreased. *Gilliamella apicola* is thought to act as a vector for the tetracycline-resistance gene *tetL*, which is obtained from pathogenic *P. larvae*. *Gilliamella apicola* in the honeybees gut is resistant to tetracycline administration. This resistance has been demonstrated by an increase in the relative abundance of *Gilliamella* after tetracycline administration (Graystock et al. 2016; Anderson and Ricigliano 2017; Motta et al. 2018; Regan et al. 2018).

The honeybees gut microbiome, which comprises diverse bacterial communities, provides vital benefits to honeybees. These benefits include improving nutrient uptake, detoxifying toxic sugars, increasing body weight, conferring resistance to pathogens, and playing roles in endocrine signaling and immune functions (Zheng et al. 2018). Therefore, the microbiome and its diversity are paramount to the health and survival of honeybees, which this study aimed to elucidate by performing metagenomic analysis and Alpha Diversity estimation of the gut microbiome. Alpha Diversity indices are useful metrics that provide insights into the taxonomic richness, diversity, and dominance of a particular sample or community, based on the number of taxonomic groups and their abundance, as well as their proportions. Several examples of Alpha Diversity Indices include the Chao1, ACE, Simpson Diversity, Inverse Simpson, and Shannon Indices. However, there are numerous Alpha Diversity Indices available, and several can be used simultaneously in biodiversity studies to obtain a fuller picture of the Alpha Diversity within a sample or community (Qian et al. 2020).

In conclusion, metagenomic analyses of the gut microbiome of *A. cerana* honeybees sampled from a Bandung apiary identified 81 bacterial genera. Notably, several genera, such as *Gilliamella*, *Lactobacillus*, *Snodgrassella*, *Apibacter*, and *Bifidobacterium* dominated the gut microbiome. Among the dominant genera, *Gilliamella* accounted for more than 50% of the microbiome. This leads to high genus richness but potentially low genus diversity, as indicated by the Alpha Diversity indices, underlining the genus *Apibacter* as a distinctive genus in *A. cerana*. This study provides valuable insights into the diversity of the gut microbiome of *A. cerana* maintained in an apiary in Bandung District, West Java, Indonesia.

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