Dynamics of the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) throughout the overwintering period (#121293)

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Dynamics of the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) throughout the overwintering period

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Honey bees play crucial roles as pollinators in both natural agricultural and ecological systems. The role of gut microbiota in the overwinter survival of honey bees is attracting attention. Compared with Western honey bees (*Apis mellifera*), Eastern honey bees (*Apis cerana*) are more tolerant to low-temperature stress. This study compared the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*), a subspecies of *A. cerana*, during the overwintering period (December) with that before overwintering (October) and after overwintering (March) to estimate beneficial hindgut bacteria contributing to survival during the overwintering period. Overall, the hindgut microbiota of *A. c. japonica* was occupied by Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria at the phylum level and *Apibacter*, *Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella* at the genus level. The hindgut microbiota composition of *A. c. japonica* was similar to that of *A. cerana* in other regions, suggesting that phylogeny influenced the composition. Many sequences assigned to the six core genera showed low homology (<98.7%) to type strains of honey bee gut bacteria, suggesting that *A. c. japonica* harbors novel candidate bacterial species. Comparison of the microbiota composition over the three periods showed that the relative abundance of *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus* was higher during overwintering than before overwintering. Our findings highlight changes in the core bacteria of the hindgut microbiota of *A. c. japonica* during overwintering and also suggest the presence of novel candidate bacterial species. The roles of the bacteria that were increased during the overwintering period require further elucidation.

Açıklama [V1]: Honey bees play crucial roles as pollinators in natural, agricultural, and ecological systems.

Açıklama [V2]: The role of gut microbiota in the overwinter survival of honey bees is gaining attention

Açıklama [V3]: Many sequences assigned to these six core genera showed <98.7% similarity to type strains, indicating potential novel bacterial species.

Açıklama [V4]: The relative abundance of *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus* was higher during overwintering than in other periods.

Article type: Research Article

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Keywords: hindgut microbiota, honey bees, *Apis cerana japonica*, overwintering

ABSTRACT

Honey bees play crucial roles as pollinators in both natural agricultural and ecological systems. The role of gut microbiota in the overwinter survival of honey bees is attracting attention. Compared with Western honey bees (*Apis mellifera*), Eastern honey bees (*Apis cerana*) are more tolerant to low-temperature stress. This study compared the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*), a subspecies of *A. cerana*, during the overwintering period (December) with that before overwintering (October) and after overwintering (March) to estimate beneficial hindgut bacteria contributing to survival during the overwintering period. Overall, the hindgut microbiota of *A. c. japonica* was occupied by Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria at the phylum level and *Apibacter*, *Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella* at the genus level. The hindgut microbiota composition of *A. c. japonica* was similar to that of *A. cerana* in other regions, suggesting that phylogeny influenced the composition. Many sequences assigned to the six core genera showed low homology (<98.7%) to type strains of honey bee gut bacteria, suggesting that *A. c. japonica* harbors novel candidate bacterial species. Comparison of the microbiota composition over the three periods showed that the relative abundance of *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus* was higher during overwintering than

before overwintering. Our findings highlight changes in the core bacteria of the hindgut microbiota of *A. c. japonica* during overwintering and also suggest the presence of novel candidate bacterial species. The roles of the bacteria that were increased during the overwintering period require further elucidation.

Introduction

The gut microbiota of honey bees plays a critical role in their health (Raymann & Moran 2018), including decomposing dietary compounds (Engel, Martinson & Moran 2012), producing short-chain fatty acids (SCFAs) as an energy source (Zheng et al. 2017), degrading potentially toxic plant metabolites (Motta et al. 2022), inhibiting the growth of honey bee pathogens (Wu et al. 2014), and stimulating the immune system (Kwong, Mancenido & Moran 2017; Motta & Moran 2024). Disruption of the gut microbiota composition due to antibiotic treatment and pesticide exposure causes dysbiosis, leading to host mortality (Raymann, Shaffer & Moran 2017; Motta & Moran 2024).

During winter, honey bees survive the severe cold environment in a metabolically and physically active state that is essential for ensuring the colony's survival until the following spring (Moeller 1977; Doeke, Frazier & Grozinger 2015). Cold stress is a major cause of individual and colony mortality in honey bees and also increases the risk of disease and infection outbreaks (Xu et al. 2017). Therefore, the health status of overwintering honey bees is crucial to the health of the entire colony (Doeke, Frazier & Grozinger 2015). During overwintering, feeding is essentially limited to food stored within the colony (pollen, bee bread, and honey). To cope with the surrounding cold stress, the honey bees must maintain the temperature of the

Açıklama [V5]: Therefore, the health status of overwintering honey bees is critical to the health of the entire colony

colony's outer edge and core by vigorously vibrating their flight muscles to generate heat (Doeke, Frazier & Grozinger 2015). The gut microbiota of overwintering honey bees has been increasingly recognized for its beneficial role in survival during the overwintering period. Most studies have focused on the Western honey bees (*Apis mellifera*), reporting an increase in gut bacteria abundance during the overwintering period (Kešnerová et al. 2020) along with changes in gut microbiota composition (Bleau et al. 2020; Kešnerová et al. 2020; Liu et al. 2021; Castelli et al. 2022; Li et al. 2022; Brar et al. 2025). These findings suggest that gut bacteria may play crucial roles in energy absorption and immune function, thereby substantially contributing to survival during the overwintering period.

The *Apis* genus is naturally distributed across Asia, Europe, and Africa (Ji 2021). The Western honey bees are widely distributed worldwide, including their native regions of Europe, Africa, and the Middle East, while Eastern honey bees (*Apis cerana*) are found throughout South, Southeast, and East Asia (Ji 2021). Compared with Western honey bees, Eastern honey bees exhibit superior cold tolerance and are more capable of surviving the harsh overwintering period (Li et al. 2012; Xu et al. 2017). Considering the beneficial involvement of the gut microbiota in overwintering honey bees, we hypothesized that the gut microbiota plays a significant role in enabling Eastern honey bees to tolerate cold stress and successfully overwinter. Characterizing the hindgut microbiota of overwintering honey bees will help narrow

Açıklama [V6]: The gut microbiota of overwintering honey bees has been increasingly recognized for its beneficial role in survival during this period.

Açıklama [V7]: Compared to Western honey bees, Eastern honey bees exhibit superior cold tolerance and are better adapted to surviving the harsh overwintering period (Li et al., 2012; Xu et al., 2017)

Açıklama [V8]: In consideration of the advantageous role of the gut microbiota in the overwintering process of honey bees, a hypothesis was formulated proposing its involvement in this phenomenon.
It has been demonstrated that this factor plays a significant role in enabling Eastern honey bees to tolerate cold stress and successfully overwinter.

down the candidate bacteria beneficial for survival under cold and harsh environments, providing novel insights into the symbiotic relationships between honey bees and their hindgut microbiota.

This study focused on the Japanese honey bees (*Apis cerana japonica*), a subspecies of the Eastern honey bee that is native to Japan. We aimed to develop a comprehensive inventory of the hindgut microbiota using high-throughput sequencing targeting the V3–V4 region of the bacterial 16S rRNA gene. We also compared the hindgut microbiota during the overwintering period, before overwintering, and after overwintering to elucidate the distinctive features of the hindgut microbiota associated with successful overwintering.

Açıklama [V9]: The aim was to develop a comprehensive inventory of the hindgut microbiota using high-throughput sequencing of the V3–V4 region of the bacterial 16S rRNA gene.

Açıklama [V10]: In addition, we compared the composition of the hindgut microbiota before, during, and after the overwintering period to identify distinctive microbial features associated with successful overwintering.

Materials & Methods

Sample collection

The study samples were collected from four *A. c. japonica* colonies in Ibaraki, Japan. Two were kept by our laboratory at the National Institute of Environmental Studies in Tsukuba City, and two by beekeepers in Tsukuba City and Inashiki District, respectively. We sampled 30 foragers from each colony using a net with clean plastic cups over three periods: October 2022 (before overwintering, BO group), December 2022 (during overwintering, OW group), and March 2023 (after overwintering, AO group) (Table 1). The total number of samples was 360 (30 honey bees

Açıklama [V11]: Two colonies were maintained by our laboratory at the National Institute of Environmental Studies in Tsukuba City, while the other two were managed by local beekeepers in Tsukuba City and Inashiki District, respectively.

per colony × four colonies × three periods). All honey bees were immediately placed on ice after collection and stored at 280# until DNA extraction.

Açıklama [V12]: All samples were immediately placed on ice after collection and stored at −80 °C until DNA extraction.

DNA extraction

After thawing the honey bees on ice, they were sterilized by soaking in 70% ethanol for 30 s and washing with ultrapure water for 30 s. The hindguts, including the pylorus, ileum, and rectum, were carefully removed on ice using sterile forceps. Ten hindguts were pooled in 2.0 mL sterile tubes containing TE buffer (10 mmol L^{21} Tris-HCl and 1 mmol L^{21} EDTA-2Na, pH 8.0) with 5% (v/v) Triton X-100 (MP Biomedicals, Irvine, CA, USA) and glass beads (1.0 mm diameter). The hindguts were disrupted by three cycles of crushing at 3,200 rpm for 30 s using Beads Crusher µT-12 (Taitec, Saitama, Japan) and 30 s of cooling on ice. The homogenates were centrifuged at 6,000 ×*g* for 10 min to sediment debris. Total bacterial DNA was purified from 180 ¿l of the resultant supernatant using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. The DNA concentration of the 36 samples (three replicates per colony × four colonies × three sampling periods) was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, MA, USA).

Açıklama [V13]: After thawing the honey bees on ice, they were surface-sterilized by immersing them in 70% ethanol for 30 seconds, followed by rinsing with ultrapure water for 30 seconds.

Açıklama [V14]: Ten hindguts were pooled into 2.0 mL sterile tubes containing TE buffer (10 mmol L^{-1} Tris-HCl and 1 mmol L^{-1} EDTA-2Na, pH 8.0) supplemented with 5% (v/v) Triton X-100 (MP Biomedicals, Irvine, CA, USA) and glass beads (1.0 mm diameter).

Açıklama [V15]: Total bacterial DNA was extracted from 180 µL of the resulting supernatant using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

High-throughput sequencing of the V3–V4 region of the bacterial 16S rRNA gene

The bacterial V3 and V4 regions of the 16S rRNA gene were amplified using a universal primer set; 341f: 5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG-3' and 805r: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC-3', which contain the adapter sequences for the Illumina library preparation kit and the primers for amplification of V3–V4 regions of the 16S rRNA gene. Polymerase chain reaction (PCR) was performed using template DNA (5 ng μl^{-1}) with Blend Taq Plus polymerase (Toyobo, Osaka, Japan) following the manufacturer's instructions. The PCR cycling conditions were initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products underwent 1.5% agarose gel electrophoresis at 100 V for 25 min and were stained with ethidium bromide and visualized under UV light to check for the presence of PCR amplicons. The PCR amplicons were sent to Bioengineering Lab. Co., Ltd. (Kanagawa, Japan) for sequencing. The amplicons were purified using AMPure XP Beads (Beckman Coulter, Brea, CA, USA), and the DNA concentrations were measured using a Synergy H1 multimode microplate reader (Agilent Technologies, Santa Clara, CA, USA) and a QuantiFluor dsDNA System (Promega, Madison, WI, USA). The libraries were constructed using purified amplicons with sample-specific dual indices. After determining the library concentrations as described

Açıklama [V16]: The bacterial V3–V4 hypervariable regions of the 16S rRNA gene were amplified using a universal primer set comprising 341F (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC-3′).These primers include adapter sequences compatible with the Illumina library preparation workflow and are specific to the amplification of the bacterial V3–V4 regions.

Açıklama [V17]: "The authors may consider including a final extension step (e.g., 72 °C for 5 minutes) in the PCR cycling protocol. This step is widely applied in 16S rRNA gene amplification to allow complete elongation of PCR products, which could be beneficial for ensuring high-quality amplicons in downstream library preparation and sequencing."

Açıklama [V18]: The resulting PCR amplicons were verified by 1.5% agarose gel electrophoresis at 100 V for 25 min, stained with ethidium bromide, and visualized under UV illumination.

Açıklama [V19]: Library preparation was performed using the purified amplicons with dual-index barcoding to enable sample multiplexing.

above, quality checking was performed using a dsDNA 915 Reagent kit (Agilent Technologies) on a Fragment Analyzer System (Agilent Technologies). Each library was pooled at an equimolar concentration and underwent 300 bp paired-end sequencing using MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) on a MiSeq benchtop sequencer (Illumina).

Data analysis

To generate amplicon sequence variants (ASVs), DADA2 ver. 1.16 (Callahan et al. 2016) in RStudio software ver. 2023.12.0+369 was used for trimming and filtering the forward and reverse fastq read data obtained following MiSeq high-throughput sequencing. Low-quality distributions and primers from each forward and reverse sequence were trimmed using parameters set to truncLen = c(290, 230) and trimLeft = c(17,21), respectively, and then the sequences were filtered using maxN = 0, maxEE = c(2,2), and truncQ = 2 parameters. Next, the forward and reverse reads were merged, after which chimeras and short reads (<400 bp) were discarded. The taxonomic classification of representative ASVs from phylum to genus level was assigned using the SILVA ver. 138.1 prokaryotic SSU database (Quast et al. 2013) as a reference dataset. Before downstream analysis, ASVs that were unclassified at the phylum level and were assigned as nonbacteria (e.g., Chloroplast and Mitochondria) were manually removed.

To standardize sequencing depth across samples, abundance-based read resampling was

Açıklama [V20]: paired-end FASTQ reads

Açıklama [V21]: non-bacterial groups

Açıklama [V22]: chloroplasts and mitochondria

151 performed using the *rrarefy* function of the vegan package ver. 2.6.4 (Oksanen et al. 2023),
152 based on the minimum read count among samples. Rarefaction curves were generated using the
153 *rarecurve* function of the vegan package and the ggplot2 package ver. 3.4.2 (Wickham 2016).
154 The *Coverage* function in the entropart package ver. 1.6.12 (Marcon & Hérault 2015) was used
155 to calculate coverage and evaluate whether the sequencing depth was sufficient to fully represent
156 the bacterial communities in each sample before and after standardization. After pooling ASVs at
15V the lowest taxonomic level (bacterial genus), the bacterial community composition at the phylum
158 and genus levels was visualized for each sample as bar plots using ggplot2. Taxa with a relative
159 abundance of <1% across all samples were grouped as "Others."
160 Bacterial genera with a relative abundance >1% across all samples were defined as the
161 core hindgut bacteria of *A. c. japonica*. All ASVs assigned to these core genera underwent
162 similarity searches against the bacterial 16S rRNA gene sequence database in EzBioCloud (Yoon
163 et al. 2017) to identify the closest related species.
164 We generated a non-metric multidimensional scaling (NMDS) plots based on the Bray–
165 Curtis dissimilarity index using the *metaMDS* function in vegan and ggplot2 to visualize the β-
166 diversity of hindgut microbiota at the ASV level for the three periods.
16V

168 **Statistical analysis**

Açıklama [V23]: among all samples

Açıklama [V24]: before and after rarefaction

Açıklama [V25]: and represented as "Others."

Açıklama [V26]: Apis cerana japonica

Açıklama [V27]: Non-metric multidimensional scaling (NMDS) plots were generated based on Bray–Curtis dissimilarity using the metaMDS function from the **vegan** package and visualized using **ggplot2**, in order to assess the β-diversity of the hindgut microbiota at the ASV level across the three time periods.

To detect differences in the hindgut microbial compositions among the three periods, we performed pairwise comparisons using permutational multivariate analysis of variance based on the Bray–Curtis dissimilarity index with 9,999 permutations using the *pairwise.adonis* function of the pairwiseAdonis package ver. 0.4.1 (Martinez 2020).

To investigate the effect of the three sampling periods on the abundance of the core bacterial genera, we performed a generalized linear mixed model (GLMM) analysis assuming a Poisson distribution and log link function using the *glmer* function of the lme4 package ver. 1.1.32 (Bates et al. 2015). The read count of each core bacterial genus was set as the response variable, with sampling period as a fixed effect and colony as a random effect. P-values < 0.05 were considered statistically significant for all comparisons.

Results

Sequence dataset overview

The high-throughput sequencing yielded 1,381,026 raw reads (mean ± SD: 38,362 ± 5,199) from all samples. After trimming and filtering, 1,098,689 high-quality reads (mean ± SD: 30,519 ± 3,678) remained (Table S1), clustering into 260 ASVs (mean ± SD: 59 ± 10). Next, all nonbacterial reads were removed from all samples, and a read count-based cutoff was applied to match the minimum read count (21,808), resulting in a final set of 241 ASVs (mean ± SD: 54 ±

Açıklama [V28]: permutational multivariate analysis of variance (PERMANOVA)

Açıklama [V29]: To investigate the effect of sampling period on the abundance of core bacterial genera, a **generalized linear mixed model (GLMM)** analysis was conducted, assuming a Poisson distribution with a log link function, using the glmer function from the **lme4** package (v1.1-32; Bates et al., 2015).

Açıklama [V30]: generated

Açıklama [V31]: "resulting in a final dataset of..

8). The rarefaction curves for each sample plateaued at the minimum read depth (Figure S1), and the estimated coverage was >99% for all samples (Table S2), indicating that the sequencing depth was sufficient to identify most of the hindgut bacteria in the study samples.

Hindgut microbiota composition

The composition at the phylum and genus levels for each period were described for those taxa with a relative abundance >1% across all samples, while those with a relative abundance <1% were grouped as "Others" (Figure 1). At the phylum level, the hindgut microbiota of *A. c. japonica* was dominated by Actinobacteriota (0.4%–13.1%), Bacteroidota (11.7%–36.3%), Firmicutes (9.2%–33.9%), and Proteobacteria (35.2%–68.6%), collectively accounting for >99.9% of the relative abundance. At the genus level, six bacterial genera, namely *Apibacter* (11.6%–36.3%), *Bifidobacterium* (0.2%–13.1%), *Bombilactobacillus* (0.3%–13.6%), *Gilliamella* (26.6%–59.8%), *Lactobacillus* (6.9%–28.2%), and *Snodgrassella* (0.7%–22.4%) predominated, accounting for >96% of the hindgut microbiota in all three periods and all samples. The relative abundance of "Unclassified" and "Others" was 0.0%–5.4% and 0.0%–12.1%, respectively. The details of relative abundance at the phylum and genus levels are listed in Tables S3–S6.

Further examination of the six major bacterial genera revealed that, except for *Bifidobacterium*, many ASVs of the core genera exhibited sequence similarities with type strains

Açıklama [V32]: exceeded 99%"

Açıklama [V33]: Since three periods are mentioned, it is not specified whether there is a statistical difference (e.g. ANOVA, Kruskal–Wallis or PERMANOVA). This analysis may be in another section, but it would be useful to provide a brief reference here.

Açıklama [V34]: •The distinction between 'Unclassified' and 'Others' is scientific, but it can be expressed more clearly for readers: Unclassified" indicates sequences not assigned at the genus level; "Others" includes genera with <1% abundance across all samples.

Açıklama [V35]: "Were these values computed after removing chloroplast/mitochondrial reads and unclassified ASVs?"

Açıklama [V36]: Further examination of the six major bacterial genera revealed that, except for **Bifidobacterium**, many ASVs assigned to the core genera exhibited sequence similarities with type strains in the **EzBioCloud** database (Yoon et al., 2017) **below the 98.7% threshold**, which is commonly used to distinguish closely related species (Chun et al., 2018; Table 2).

in the EzBioCloud database (Yoon et al. 2017) below the threshold of 98.7% suggested for distinguishing closely related species from type strains (Chun et al. 2018) (Table 2). Notably, 90% (27/30) of the ASVs assigned to *Gilliamella* showed this pattern, followed by *Snodgrassella* (65%, 13/20), *Bombilactobacillus* (60%, 3/5), *Apibacter* (53.8%, 7/13), and *Lactobacillus* (41.6%, 5/12), suggesting the presence of potential novel species.

The result of the NMDS plot of β-diversity of the hindgut microbiota at the ASVs level based on Bray-Curtis dissimilarity is shown in Figure 2. Pairwise comparisons among three periods revealed that the hindgut microbiota composition only differed significantly only between BO group and OW group (F = 3.037, R^2 = 0.121, p = 0.029, Table S7).

Açıklama [V37]: potentially novel bacterial species within the hindgut microbiota of *A. cerana japonica*.

Açıklama [V38]: Pairwise comparisons among the three sampling periods revealed a significant difference in hindgut microbiota composition only between the BO and OW groups (F = 3.037, R^2 = 0.121, p = 0.029; Table S7).

Comparison of the core genera among the three periods

The GLMM analysis revealed that the OW group had a significant positive effect on the read counts of *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus* (coefficient: 0.977, 1.036, and 0.320; 95% CI: 0.237–1.716, 0.138–1.933, and 0.131–0.509; p = 0.009, 0.024, and 0.001, respectively; Table S8).

Açıklama [V39]: coefficients

Data availability

The raw amplicon sequence datasets generated in this study are available in the DDBJ Sequence

Read Archive (accession numbers: DRR685263–DRR685298 for DRA Run and PRJDB20791 for BioProject). All scripts and datasets are deposited to figshare under DOI: 10.6084/m9.figshare.29396408.

Discussion

This study revealed that the hindgut microbiota of *A. c. japonica* was dominated by four phyla: Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria, and six core bacterial genera: *Apibacter*, *Bifidobacterium*, *Bombilactobacillus*, *Gilliamella*, *Lactobacillus*, and *Snodgrassella*. This is consistent with the results of previous studies on the gut microbiota of honey bees (Kwong et al. 2017; Dong et al. 2020). In contrast, compared with the core gut microbiota of *A. mellifera*, *Apibacter* is more abundant in Asian honey bee species, such as *A. cerana*, *A. dorsata*, and *A. andreniformis* (Kwong & Moran 2016; Kwong et al. 2017; Duong et al. 2020; Ellegaard et al. 2020; Khan et al. 2023). The hindgut microbiota of *A. c. japonica* showed a similar trend at the genus level, suggesting that host phylogeny influenced microbial community structure. However, the identification of ASVs with similarities lower than the threshold for distinguishing closely related species suggests the presence of many potentially novel bacterial species, despite

Açıklama [V40]: Apis cerana japonica

Açıklama [V41]: This finding is consistent with previous studies

Açıklama [V42]: Apis cerana japonica

Açıklama [V43]: amplicon sequence variants (ASVs)

239 their genus-level similarity. Further studies involving bacterial isolation, biochemical
240 characterization, and genome analysis are warranted to elucidate the taxonomy and function of
241 these candidate novel bacteria.

242 The hindgut microbiota composition of *A. c. japonica* in the OW group differed
243 significantly from that of the BO group. Notably, the mean relative abundance of
244 *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus* in OW group was higher than that in BO
245 group. These three core bacterial genera are known to produce SCFAs from pollen-derived
246 polysaccharides and nectar-derived glucose (Zheng et al. 2017; Zheng et al. 2019). Among the
24V SCFAs derived from honey bee gut bacteria, butyrate is absorbed into the hemolymph via the
248 ileum or rectum and is therefore considered an important energy source for thermogenesis to
249 maintain hive temperature during the overwintering period (Den Besten et al. 2013; Zheng et al.
250 2017). Moreover, genera *Bifidobacterium* and *Lactobacillus* contribute substantially to infection
251 control and immune regulation in honey bees through mechanisms such as antimicrobial activity
252 against pathogens (Wu et al. 2013) and upregulating antimicrobial peptide expression (Daisley et
253 al. 2020). Therefore, the genus-level increase in *Bifidobacterium*, *Bombilactobacillus*, and
254 *Lactobacillus* in the OW group may play a beneficial role in the overwintering of honey bees in
255 terms of thermogenesis and immune activation. Further studies quantifying SCFA levels in the
256 gut and the expression of genes related to the immune system during the overwintering period

are necessary to clarify the functional roles of these gut bacteria in successful overwintering.

The observed compositional changes in the hindgut microbiota of *A. c. japonica* in the overwintering period are intriguing. A possible contributing factor is the difference in pollen and nectar sources consumed by honey bees before and during overwintering period. Honey bees forage across a wide temperature range (10°C–40°C) (Abou-Shaara et al. 2017), but during the overwintering period, when temperatures fall below 10°C, they rarely leave the hive to forage (Joshi & Joshi 2010). Consequently, honey bees are more likely to consume stored pollen and honey during overwintering period. Furthermore, the consumption of aged or stored pollen and honey influences gut microbiota composition (Maes et al. 2016). In our study, although daily maximum temperatures exceeded 10°C on all sampling days before and after overwintering period, only one-third of the days during the overwintering period reached this threshold (Japan Meteorological Agency 2025). Another factor that may influence hindgut microbiota is variations in hive temperature. Typically, the hive temperature is maintained at 33°C–35.5°C (Abou-Shaara et al. 2017). In *A. c. japonica*, the average winter hive temperature is 30.7°C, while the average temperature before and after winter is 34.3°C (Akimoto 2000). This temperature fluctuation may affect bacterial growth rates, thereby altering microbiota composition (Ludvigsen et al. 2015; Kešnerová et al. 2017).

Açıklama [V44]: Apis cerana japonica

Conclusions

This study on the hindgut microbiota of *A. c. japonica* revealed the influence of phylogeny on microbiota composition, the presence of potentially novel species, and distinctive compositional changes during the overwintering period. The biochemical properties of the genera that increased during overwintering period (i.e., genera *Bifidobacterium*, *Bombilactobacillus*, and *Lactobacillus*) suggest that these changes supply energy for thermogenesis and activate the host immune system. Further surveys in other regions with different dietary environments and studies focusing on elucidating the functional roles of hindgut microbiota during overwintering and their symbiotic relationship with host health are warranted.

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Author Contributions

Akihiko Suzuki: Conceived and designed the experiments; performed the experiments; analyzed the data; acquired funding; prepared figures and/or tables; authored or reviewed drafts of the paper; approved the final draft.

Shumpei Hisamoto: Analyzed the data; reviewed and edited the manuscript; approved the final draft.

Yoshiko Sakamoto: Conceived and designed the experiments; supervised the project; reviewed and edited the manuscript; approved the final draft.

Table legends

Table 1. Detailed information on sampling in this study.

Table 2. List of BLAST results against the EzBioCloud 16S rRNA database of ASVs assigned to the six core bacteria genera in the hindgut of the Japanese honey bees (*Apis cerana japonica*)

Figure legends

Figure 1. Hindgut microbiota composition of the Japanese honey bees (*Apis cerana japonica*) sampled for each period at the (A) phylum and (B) genus levels. BO: before overwintering, OW: during overwintering, AO: after overwintering.

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination plots of hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) at three sampling periods. The plot was generated with the Bray–Curtis dissimilarity index based on the ASVs obtained from each sample. BO: before overwintering, OW: during overwintering, and AO: after overwintering.

Supporting information

Table S1. Number of reads obtained after filtering and trimming each sample using DADA2.

Table S2. Number of coverages after rarefaction at the minimum lead (21,808) for each sample.

Table S3. Relative abundance of bacterial phyla in the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) in all samples.

Table S4. Relative abundance of bacterial phyla in the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) at each of the three sampling periods.

Table S5. Relative abundance of bacterial genera in the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) in all samples.

Table S6. Relative abundance of bacterial genera in the hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) at each of the three sampling periods.

Table S7. Results of pairwise permutational multivariate analysis of variance of the hindgut microbiota in the Japanese honey bees (*Apis cerana japonica*) from four colonies during three sampling periods.

Table S8. Generalized linear mixed model analysis. The number of reads of the six core bacterial genera in the hindgut microbiota of the Japanese honey bees (Apis cerana japonica) was used as the objective variable, the three time periods as explanatory variables, and the colony as a random effect. BO group is set as a reference group.

Figure S1. Rarefaction curves of the microbiota in the hindgut of the Japanese honey bees (*Apis cerana japonica*) before (A) and after (B) setting the minimal sequence read (21,808) from the raw read dataset. The black dotted line in panel (B) shows 21,808 leads as the rarefaction point. The letter in each sample indicates the colony ID, and the number indicates the month of sample collection.

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Figure 1

Hindgut microbiota composition of the Japanese honey bees (*Apis cerana japonica*) sampled for each period at the (A) phylum and (B) genus levels.

BO: before overwintering, OW: during overwintering, AO: after overwintering.

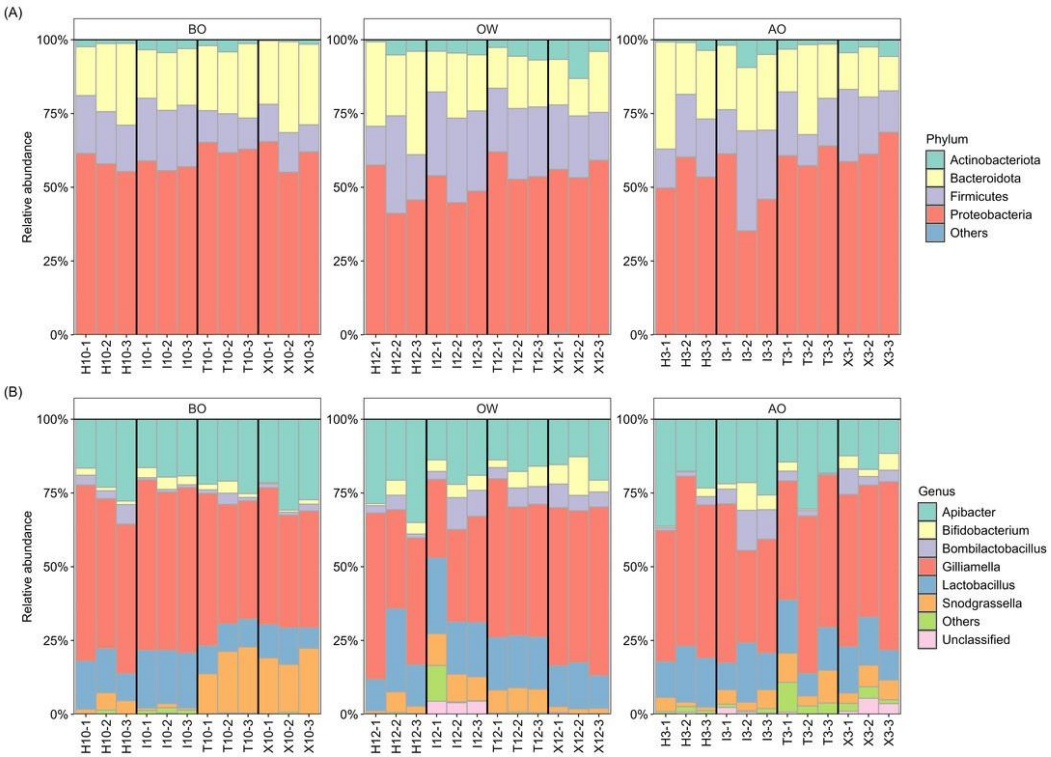

Figure 2

Nonmetric multidimensional scaling (NMDS) ordination plots of hindgut microbiota of the Japanese honey bees (*Apis cerana japonica*) at three sampling periods.

The plot was generated with the Bray3Curtis dissimilarity index based on the ASVs obtained from each sample. BO: before overwintering, OW: during overwintering, and AO: after overwintering.

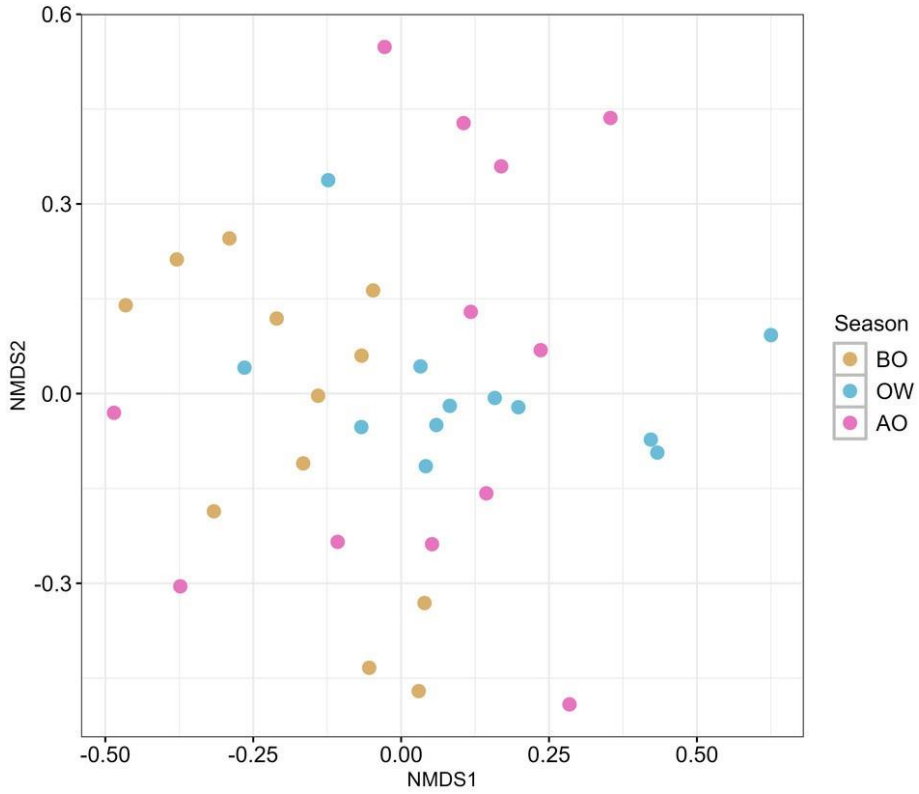

Table 1 (on next page)

Detailed information on sampling in this study.

Table 1. Detailed information on sampling in this study.

Period [1)]	Sampling date	Colony ID	Location [2)]	DNA sample ID
BO	10/19/2022	H	Tsukuba city, Ibaraki, Japan	H10-1, H10-2, H10-3
	10/27/2022	I	Inashiki district, Ibaraki, Japan	I10-1, I10-2, I10-3
	10/20/2022	T	NIES	T10-1, T10-2, T10-3
	10/20/2022	X	NIES	X10-1, X10-2, X10-3
OW	12/19/2022	H	Tsukuba city, Ibaraki, Japan	H12-1, H12-2. H12-3
	12/18/2022	I	Inashiki district, Ibaraki, Japan	I12-1, I12-2. I12-3
	12/19/2022	T	NIES	T12-1, T12-2. T12-3
	12/19/2022	X	NIES	X12-1, X12-2. X12-3
AO	3/15/2023	H	Tsukuba city, Ibaraki, Japan	H3-1, H3-2, H3-3
	3/16/2023	I	Inashiki district, Ibaraki, Japan	I3-1, I3-2, I3-3
	3/7/2023	T	NIES	T3-1, T3-2, T3-3
	3/7/2023	X	NIES	X3-1, X3-2, X3-3

[1)] Before overwintering: BO, during overwintering: OW, after overwintering: AO.

[2)] NIES: National Institute for Environmental Studies (Tsukuba, Ibaraki, Japan).

Table 2(on next page)

List of BLAST results against the EzBioCloud 16S rRNA database of ASVs assigned to the six core bacteria genera in the hindgut of the Japanese honey bees (*Apis cerana japonica*).

Table 2. List of BLAST results against the EzBioCloud 16S rRNA database of ASVs assigned to the six core bacteria genera in the hindgut of the Japanese honey bees (*Apis cerana japonica*)

Assigned genus	ASV ID [1)]	Length (bp)	Top-hit taxon (strain level) [2)]	Accession ID	Similarity (%)
Apibacter	ASV1	423	*Apibacter* sp. B3924	WINM01000002	100
	ASV16	423	*Apibacter mensalis* R-53146^T	LIVM01000008	99.5
			Apibacter sp. B3924	WINM01000002	99.5
	ASV9	423	*Apibacter mensalis* R-53146^T	LIVM01000008	99.8
			Apibacter sp. B3924	WINM01000002	99.8
	ASV32	423	*Apibacter* sp. B3924	WINM01000002	99.8
	ASV33	423	*Apibacter mensalis* R-53146^T	LIVM01000008	100
	ASV118	**423**	***Apibacter* sp. B3924**	**WINM01000002**	**96.2**
	ASV76	**426**	***Apibacter* sp. B3924**	**WINM01000002**	**97.9**
	ASV133	**409**	***Apibacter mensalis* R-53146^T**	**LIVM01000008**	**91.0**
			***Apibacter* sp. B3924**	**WINM01000002**	**91.0**
	ASV151	**425**	***Apibacter* sp. B3924**	**WINM01000002**	**97.6**
	ASV254	**423**	***Apibacter* sp. B3924**	**WINM01000002**	**90.6**
	ASV280	**423**	***Apibacter* sp. B3924**	**WINM01000002**	**91.0**
	ASV174	**431**	***Apibacter mensalis* R-53146^T**	**LIVM01000008**	**90.5**
	ASV279	422	*Apibacter* sp. B3924	WINM01000002	99.6
Bifidobacterium	ASV51	408	*Bifidobacterium indicum* JCM 1302^T	LC071807	100
	ASV11	410	*Bifidobacterium* sp. 7101	AWUN01000009	100
Bombilactobacillus	ASV34	432	*Bombilactobacillus mellifer* Bin4N^T	JX099543	99.8
	ASV47	**431**	**Uncultured Firmicutes bacterium D08062C1**	**HM215046**	**98.1**
	ASV169	**430**	**Uncultured Firmicutes bacterium D08062C1**	**HM215046**	**89.4**
	ASV12	429	*Bombilactobacillus mellis* Hon2^T	KQ033880	100
	ASV23	**431**	**Uncultured Firmicutes bacterium D08062C1**	**HM215046**	**98.4**
Lactobacillus	ASV6	428	*Lactobacillus panisapium* Bb 2-3^T	KX447147	100

	ASV10	430	*Lactobacillus panisapium* Bb 2-3^{T}	KX447147	99.8
	ASV15	428	*Lactobacillus panisapium* Bb 2-3^{T}	KX447147	99.5
	ASV24	429	*Lactobacillus melliventris* Hma8N^{T}	JX099551	99.5
	ASV90	**429**	***Lactobacillus panisapium* Bb 2-3^{T}**	**KX447147**	**98.4**
	ASV135	427	*Lactobacillus panisapium* Bb 2-3^{T}	KX447147	99.5
	ASV164	**428**	***Lactobacillus panisapium* Bb 2-3^{T}**	**KX447147**	**95.6**
	ASV129	430	*Lactobacillus huangpiensis* F306-1^{T}	LC597580	99.8
	ASV267	429	*Lactobacillus panisapium* Bb 2-3^{T}	KX447147	99.3
	ASV271	**427**	***Lactobacillus panisapium* Bb 2-3^{T}**	**KX447147**	**96.7**
	ASV292	**428**	***Lactobacillus panisapium* Bb 2-3^{T}**	**KX447147**	**93.7**
	ASV185	**428**	***Lactobacillus panisapium* Bb 2-3^{T}**	**KX447147**	**97.9**
Gilliamella	**ASV2**	**428**	***Gilliamella apicola* wkB11**	**JFON01000004**	**97.4**
	ASV3	428	*Gilliamella apis* NO3^{T}	NASD01000045	100
	ASV4	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**98.6**
	ASV5	**428**	***Gilliamella apis* NO3^{T}**	**NASD01000045**	**96.7**
	ASV8	**428**	***Gilliamella apicola* wkB11**	**JFON01000004**	**97.7**
	ASV13	430	*Gilliamella apis* NO3^{T}	NASD01000045	99.8
	ASV14	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**98.4**
	ASV22	**429**	***Gilliamella apicola* wkB7**	**CM004509**	**98.4**
	ASV28	**429**	***Gilliamella apicola* wkB7**	**CM004509**	**98.4**
	ASV30	**431**	***Gilliamella apicola* wkB11**	**JFON01000004**	**97.5**
	ASV39	**429**	***Gilliamella apicola* wkB11**	**JFON01000004**	**97.4**
	ASV41	**427**	***Gilliamella apicola* wkB7**	**CM004509**	**98.4**
	ASV58	427	*Gilliamella apicola* App2-1	LZGR01000055	99.5
	ASV71	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**98.4**
	ASV73	**429**	***Gilliamella apicola* wkB11**	**JFON01000004**	**95.3**
	ASV96	**428**	***Gilliamella apicola* wkB11**	**JFON01000004**	**94.0**
	ASV100	**432**	***Gilliamella bombi* LMG 29879^{T}**	**FMWS01000047**	**95.4**
	ASV102	**431**	***Gilliamella apicola* wkB7**	**CM004509**	**96.5**
	ASV104	**432**	***Gilliamella apis* NO3^{T}**	**NASD01000045**	**97.7**
	ASV109	**428**	***Gilliamella apicola* wkB11**	**JFON01000004**	**96.5**

	ASV138	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**96.0**
	ASV149	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**96.0**
	ASV162	**427**	***Gilliamella apicola* wkB7**	**CM004509**	**98.1**
	ASV170	**432**	***Gilliamella apicola* wkB11**	**JFON01000004**	**95.4**
	ASV177	**429**	***Gilliamella apicola* wkB11**	**JFON01000004**	**97.2**
	ASV190	**428**	***Gilliamella apicola* wkB11**	**JFON01000004**	**93.7**
	ASV207	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**96.3**
	ASV231	**428**	***Gilliamella apicola* wkB7**	**CM004509**	**96.3**
	ASV275	**427**	***Gilliamella apicola* wkB7**	**CM004509**	**97.7**
	ASV298	**427**	***Gilliamella apicola* wkB7**	**CM004509**	**96.5**
Snodgrassella	ASV7	428	*Snodgrassella alvi* wkB298	MEIK01000026	100
	ASV17	428	*Snodgrassella alvi* wkB298	MEIK01000026	99.8
	ASV18	**428**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**98.6**
	ASV21	429	*Snodgrassella alvi* wkB298	MEIK01000026	99.0
	ASV25	429	*Snodgrassella alvi* wkB298	MEIK01000026	98.8
	ASV27	**430**	***Snodgrassella alvi* WF3-3**	**MEIO01000062**	**98.6**
	ASV31	428	*Snodgrassella alvi* wkB298	MEIK01000026	98.8
	ASV35	**428**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**98.4**
	ASV40	**428**	***Snodgrassella gandavensis* LMG 30236^{T}**	**OU943324**	**98.6**
	ASV45	428	*Snodgrassella gandavensis* LMG 30236^{T}	OU943324	98.8
	ASV60	**428**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**98.6**
	ASV66	430	*Snodgrassella alvi* WF3-3	MEIO01000062	98.8
	ASV111	**431**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**98.4**
	ASV143	**429**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**96.5**
	ASV234	**431**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**98.1**
	ASV235	**429**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**96.3**
	ASV276	**433**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**93.5**
	ASV278	**430**	***Snodgrassella alvi* wkB298**	**MEIK01000026**	**95.6**
	ASV307	**432**	***Snodgrassella alvi* wkB2^{T}**	**CP007446**	**93.1**

ASV310	**431**	***Snodgrassella alvi* wkB298**		**MEIK01000026**	**93.7**

[1)] The ASVs showing < 98.7% homology against the top-hit taxon are bold.

[2)] The superscript T means type strains of the bacteria species.