



OPEN High abundance of lactobacilli in the gut microbiome of honey bees during winter

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Honey bee gut microbiota play specific roles in promoting host growth and physiology by regulating the immune system, behavior, metabolism, and neurological processes. While the gut microbiota of honey bee queens, workers, and larvae has been extensively studied, less is known about the composition of gut microbiota in the winter worker bees. This study investigates the dynamics of the gut microbiota in overwintering adult worker bees, focusing on two commercial bee strains: Bolton™ bees and Mann Lake™ bees. These *Apis mellifera* strains were investigated under different storage conditions (indoor storage at 6 °C and outdoor storage in natural conditions) during the winter months (October, November, and December). Utilizing 16S rRNA gene amplicon sequencing, we characterized the microbial composition of the whole gut. We observed the *Lactobacilli* dominated in all the overwintering honey bee guts with a significantly higher abundance of unclassified *Lactobacillus* species in November, while *Lactobacillus apis* showed significantly higher abundance in October. Bolton bees exhibited significantly higher abundance levels of *Bartonella* (denoted as uncultured) and *Bifidobacterium*, along with an unexpected presence of *Wolbachia*. In contrast, Mann Lake bees demonstrated an increased abundance of *Commensalibacter*. Our results suggest that Shannon diversity is influenced by the month rather than by the bee strain or storage conditions. We also found significant differences in Bray Curtis diversity index by month. Overall, taxonomical abundance was not affected by whether the hives were stored outside or in constant temperature indoor storage. However, various bacterial species showed differences in abundance across different months, with slight variations observed between bee strains. Given the potential benefits of the honey bee gut microbiome for health and nutrition, our data suggests that the genus *Lactobacillus* may play a significant role in bee health during winter and overwintering storage.

Keywords Honey bees, Gut microbiota, Cold climate, Winter bees, Climate change, *Lactobacillus*, *Bombilactobacillus*, *Apilactobacillus*, *Bartonella*, *Commensalibacter*, *Bifidobacterium*, *Wolbachia*, Overwintering storage

Winter presents a significant challenge for honey bee colonies in temperate regions. Over the last two decades, beekeepers have reported high colony losses during winters. These losses have been associated with low temperature stress, geographic relocation due to climate change, genotype, diseases, pesticides, and poor nutrition^{1–4}. In preparation for overwintering, a honey bee colony undergoes notable alterations in both the behavior and physiology of the worker bees. These changes include decreased individual activity, alterations in behavior regulated by endocrine systems, increased nutrient reserves, extended longevity, and thermoregulating clustering at the colony level¹. Previous research has shown that honey bees live longer and grow larger when exposed to low temperatures⁵. However, other studies indicate that low temperatures negatively impact the honey bee immune system⁶ and survival⁷ through higher larval mortality and reduced life expectancy in adulthood⁸. To mitigate these deleterious effects, honey bees have evolved life history strategies like cessation of brood rearing during the winters, increased immunity, and increased antioxidant expression⁹. Additionally, honey bees form a dense winter cluster to withstand harsh winter conditions. The thermoregulation of this winter cluster relies heavily on the insulation provided by the tightly packed outer layer of bees, known as the mantle bees, which is crucial for survival at low temperatures¹⁰. Moreover, to reduce the cold temperature stress on the bee colonies, and to improve overwintering survival, beekeepers often store the hives indoors. Storing

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hives under controlled cold climate conditions of 5 °C—7 °C and 25% relative humidity has been shown to improve health and survival^{11–13}.

The symbiotic gut microbiota of honey bees is vulnerable to temperature stress¹⁴. Winter losses of honey bee workers are primarily caused by harsh weather conditions, resulting in starvation and increased vulnerability to diseases¹⁵. Additionally, the honey bees experiencing cold stress have an increased risk of diseases and infections, which in turn raises the likelihood of colony losses. A recent transcriptomic study provides insight into the molecular mechanisms of cold resistance in *Apis cerana*. This study identified various temperature-sensitive proteins, such as heat shock proteins and zinc finger proteins, which were upregulated when bees were exposed to 0 °C and are considered candidate genes for honey bees' tolerance to cold stress¹⁶. Apart from host physiology, the role played by the honey bee gut microbiome has also emerged as one of the most important physiological aspects to investigate in relation to honey bee cold tolerance. The gut bacterial species within honey bees undergo alterations due to various environmental and developmental stresses, with temperature being a crucial one¹⁷.

The honey bee gut microbiome contains five core host-specific bacterial species that are highly conserved and comprise 95% of the total microbes^{18,19}. The hindgut of every adult worker contains *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus Firm-5*, *Bombilactobacillus* (formerly *Lactobacillus Firm-4*), and *Bifidobacterium* species¹⁷. Along with these core bacteria, there are non-core species including *Bartonella apis*, *Commensalibacter* spp., and some other identified and several unidentified species that have been collected from the surrounding environment like hives and plants^{20,21}. These bacterial species promote honey bee growth and physiology²², facilitate breakdown of toxic dietary compounds²³, and modulate immune functions²⁴. Dynamic changes to the microbiota composition have been observed throughout the seasons^{25–27}. The gut microbiota has been reported to differ between winter and summer honey bees, with reduced α -diversity and higher levels of *Bartonella* and *Commensalibacter* during winter²⁸. Moreover, temperature during different months affects the composition of the gut microbiota by gradually changing the bacterial diversity between the seasons. In subtropical conditions, precipitation affects the composition of the honey bee gut microbiota²⁷. Numerous studies have been conducted to understand the seasonal dynamics of honey bee gut microbiota. However, very few studies have explored the effects of prolonged exposure to harsh climatic conditions on honey bee gut microbiome. For instance, direct exposure to cold temperatures has been found to influence the gut microbiota in both honey bees and bumble bees²⁹. Additionally, elevated temperature has shown to affect nectar microbes where the abundance of bacteria increased in the warmer temperature, influencing bumble bee forager preference³⁰.

Beyond seasonality and temperature, vast differences in gut microbiota at the strain level has also been observed in two closely related honey bee species, *Apis mellifera* and *Apis cerana*^{31,32}. Additionally, significant variation in both composition and function among diverse Asian honey bee populations has been reported, showing distinct patterns of their gut microbiota³³. A previous study showed genetic divergence and functional convergence of gut bacteria in the eastern honey bee, *Apis cerana* and the western honey bee, *Apis mellifera*³⁴ and between *Apis cerana* and *Apis florea*³⁵. Differences in gut microbes between strains of *Apis mellifera* may influence the performance of those honey bee strains under different climate conditions.

Given that differences in gut microbes between honey bee strains may affect their performance under varying climate conditions, beekeepers will need to adopt different strategies to manage overwintering colonies effectively and prevent colony losses. Our study is important for beekeepers in two key ways. First, winter bees feed on pollen that has been stored in the hive for several weeks or months. Previous research has shown that consuming aged pollen affects the gut microbiota composition of nurse bees, leading to impaired development, increased mortality, and a higher risk of *Nosema* infection³⁶. Second, the winter bees may retain feces in their gut for extended periods, which can likely affect nutrient availability and physicochemical conditions in the gut. Without defecation, bacteria may accumulate over time in the guts of winter bees, whereas more frequent defecation in nurses or foragers may result in faster microbiota turnover²⁸. Hence, the goal of this study was to assess whether overwintering storage conditions disrupt the stability and diversity of the gut microbes in overwintering honey bees. To understand long term storage effects on gut microbiota, we compared the whole gut microbiota of two commercial strains of *Apis mellifera* i.e., claimed cold hardy bees Bolton Bees, MN (<http://boltonbees.com/pages/mn-hardy-hives>) that are bred and reared in Minnesota and Italian bees (Mann lake Bees, MN) of unknown rearing. Hives were stored either at a constant 6 °C indoors or kept outside in natural conditions during winter. We predicted that the hives stored outside in natural fluctuating temperatures will have different gut bacterial communities due to cold stress compared to hives stored at a constant temperatures. We also predicted that bacterial communities would change during the three-month sampling period of falling temperatures, viz. October, November, and December.

Material and methods

Bee samples

Honey bee hives were managed outside in the field (Fargo, North Dakota) from the first week of May to the last week of October and then 3 hives from each bee strain were shifted to refrigerated cargo containers for overwintering storage (6 °C) in November while 3 hives from each bee strain were left outdoors in the field. The first sampling was done on 10/17/2022 (base sampling of outdoor bees), followed by moving some hives inside storage on 10/21/2022. The second sampling was done on 11/15/2022 from hives both inside and outside. The third sampling was done 12/14/2022, again for both inside and outside hives (Fig. 1, Table 1). Hives were routinely inspected for signs of disease and mite infestations. While no diseases were detected, some hives with mite infestations were treated on 09/28/2022 using Mite Away Quickstrips (Apistan Anti-Varroa Mite Strips, Mann Lake Bee & Ag Supply). During the dearth period (when hives were moved to inside storage) in winter, the hives were supplemented with winter patties and Pro-Sweet liquid feed (Bee-Pro Patties, Mann Lake Bee & Ag Supply). Moreover, air temperatures were recorded from the North Dakota Agricultural Weather Network

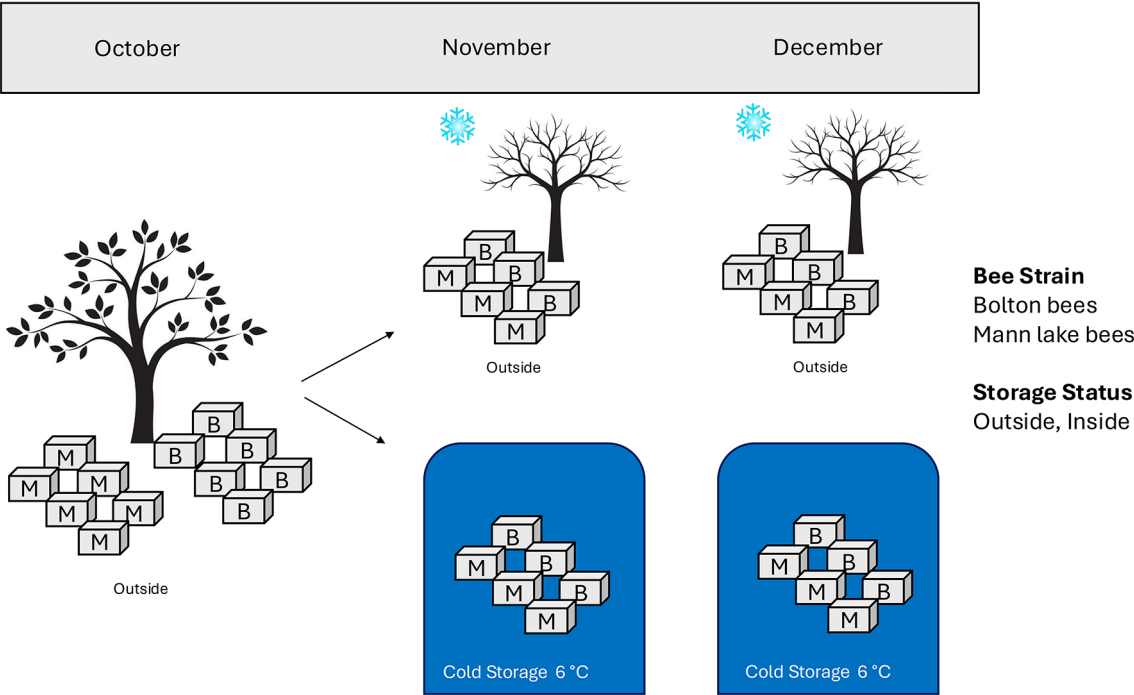


Fig. 1. Illustration of the management of honey bee hives from October to December. In October, hives were managed outside in the field. In November, three hives from each bee strain were shifted to refrigerated cargo containers (6 °C) for overwintering storage, while another three hives from each strain remained outdoors in the field. The same management was repeated in December. Two genetic strains of bees were used in the experiment, Bolton Bees (B) and Mann Lake bees (M).

Bee strain				
Months	Mann lake bees: Outside (3 hives)		Bolton bees: Outside (3 hives)	
October	3 bees/hive	3 bees/hive	3 bees/hive	3 bees/hive
	Mann lake bees		Bolton bees	
	Storage—constant 6 °C (3 hives)	Outside (3 hives)	Storage—constant 6 °C (3 hives)	Outside (3 hives)
November	3 bees/hive	3 bees/hive	3 bees/hive	3 bees/hive
December	3 bees/hive	3 bees/hive	3 bees/hive	3 bees/hive

Table 1. Representation of honey bee sample collected from various storage environments across different months.

(NDAWN) weather station in north Fargo (latitude: 46.9003°, longitude: −96.8323°), near the filed site (Fig. 2). In addition, the temperature-managed cargo containers were frequently checked to ensure setpoint consistency.

DNA extraction

Bolton bees and Mann Lake bees were kept separate at two different locations in the field. The hives were rocked gently to prompt the bees to exit. The exiting bees were then captured at the entrance for sampling. Three bees from each treatment were used for whole gut dissection after surface sterilizing bees using 1% sodium hypochlorite followed by three washes using sterilized water in sterile conditions. During dissection, we ensured that the guts were intact and had not been perturbed or defecated prior to dissection. This served as a visual marker to maintain consistency in the sample collection process. Whole gut samples were prepared by bead-beating the samples on a Qiagen TissueLyser for 6 min at 30 Hz to disrupt recalcitrant cells. Samples for cell lysis were prepared by combining two 3-mm chromium steel beads and approximately 50 µl of 0.1 & 0.5 mm ZR BashingBeads™ inside lysis tube (Biospec, Bartlesville, OK) with 750 µl of ZymoBIOMICS™ lysis solution and 20 µl of proteinase K. A subsequent round of bead beating involved rotating the samples for 6 min at 30 Hz, followed by an incubation period at 56 °C for one hour. DNA was extracted using ZymoBIOMICS™ DNA microprep kit collection (Zymo research, Irvine, California) including 2 blank extractions as a no template control for further downstream analysis.

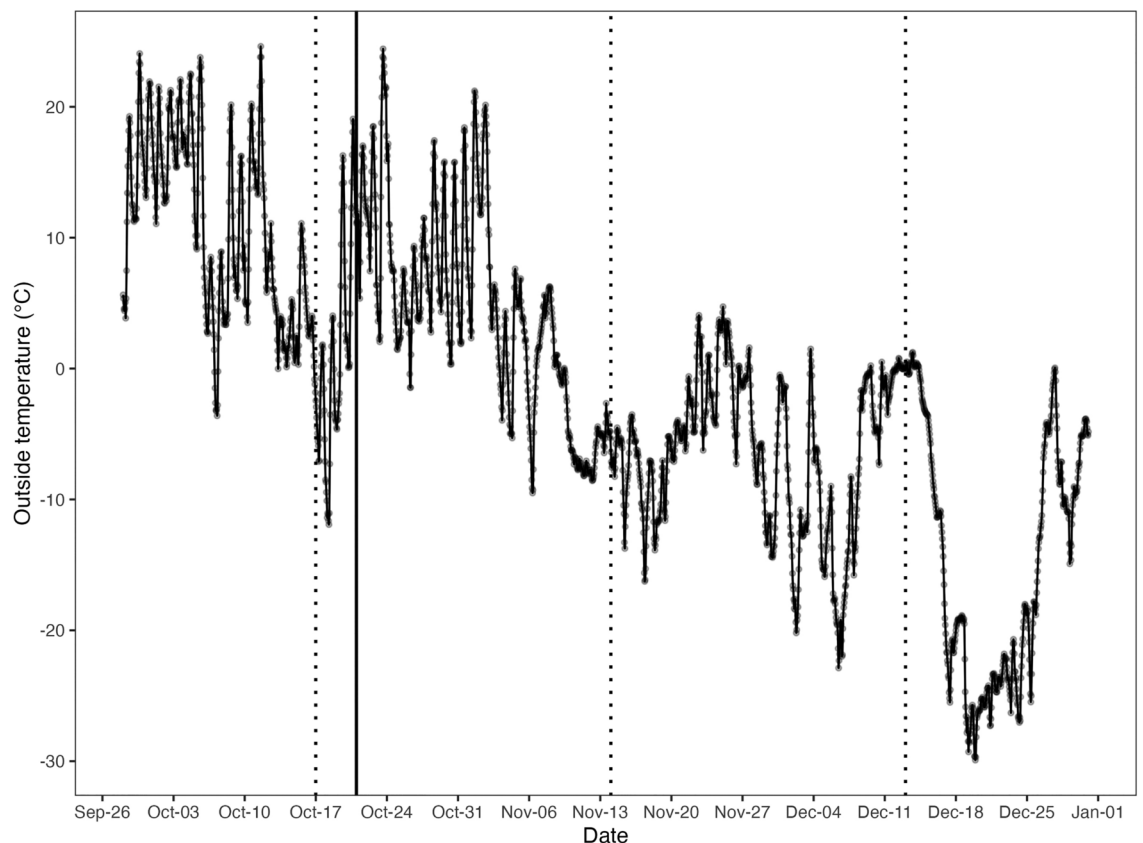


Fig. 2. Hourly air temperature at the hive location.. All hives experienced outside air temperatures in October. Hives that remained outside and were not moved to indoor storage experienced these temperatures through the end of December. Dotted lines represent sampling dates, while the solid line marks the date on which half of the hives were moved indoors. Grey dots represents the average temperature for each day.

PCR amplification and illumina sequencing

16S rRNA gene libraries for paired-end reads were generated following a previously published protocol^{37,38}. The V5-V6 region of the 16S rRNA gene was amplified using 16S rRNA gene primers (799F mod3: CMGGATTAGATACCKGG and 1115R: AGGGTTGCGCTCGTTG), each incorporating a unique barcode sequence. PCR1 reactions were conducted with 2 µl of DNA, 10 µl of 2 × Pfuusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 µl of ultrapure water, and 0.5 µl of 10 µM 799F-mod3 and 0.5 µl of 10 µM 1115R primers. The reaction condition for PCR1 was 94 °C for 3 min, 94 °C for 45 s, 52 °C for 1 min, 70 °C for 1:30 min, repeated step2 29X, and 72 °C for 10 min to amplify this region. We ran gel electrophoresis to confirmed if 16S rRNA primers were successfully attached to our samples. To complete the Illumina adapter sequence, we initiated the process by cleaning the PCR product with exonuclease and shrimp alkaline phosphatase. Exonuclease was applied to remove excess primers, while shrimp alkaline phosphatase was used to eliminate residual deoxynucleoside triphosphates (dNTPs). Four µL of the 1 × ExoSAP was mixed with each 7 µL PCR1 sample. This reaction was incubated at 37 °C for 30 min and then 95 °C for 5 min. Following this initial cleanup, the purified PCR products were employed as templates for a second PCR. This subsequent PCR (PCR2) utilized 1 µl of the cleaned PCR product as a template, using the same primers (PCR2F and PCR2R), and was conducted under conditions identical to the initial PCR. For PCR2, a 1 µl aliquot of Exo-Sap (Thermo Fisher Scientific) PCR1 product was utilized to conduct the second step of Illumina library preparation. In PCR2, the Exo-Sap PCR1 products were further amplified, incorporating linker poly-A primers for recognition on the sequencing platform. The PCR2 reaction conditions included an initial denaturation at 94 °C for 3 min, followed by cycles of 94 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min and 30 s, with step 2 repeated 14 times, and a final extension at 72 °C for 10 min, with final volume of 25 µl. To ensure uniform DNA input quantities, Invitrogen DynaMag TM-96 Side Skirted kit was employed for the normalization step across all samples prior to submitting a final volume of 10 µl for sequencing. The 16S rRNA gene sequencing was performed on NextSeq2000 P2 600 cycle kit (2 × 300xN/A) that produced total of 382.53 M reads at Q30 of 88.96%.

Bioinformatics

QIIME 2–2019 was used for the visualization and trimming of low-quality ends in reads from raw 16S rRNA sequence libraries. Subsequently, DADA2 was utilized for the assignment of sequences to amplicon sequence variants (ASVs), which represent 16S rRNA gene sequences with 100% matches. Taxonomy was assigned to

the ASVs through the sklearn classifier, trained specifically for the 799–1,115 region of the 16S rRNA gene, utilizing the SILVA 138.2 database (accessed November 2023). Additionally, local BLASTn searches against the NCBI 16S microbial database (accessed October 2023) were conducted. Features were filtered from the resultant ASV table, corresponding to contaminants identified in the blanks using the R package ‘decontam’ (version 1.10.0) at a conservative threshold of 0.5. This process aimed to identify contaminants while also eliminating ASVs identified as chloroplast and mitochondria. To standardize the number of sequences per library, alpha rarefaction in QIIME2 was employed, and 4000 reads per sample were selected. This approach ensured the retention of 107 out of 108 samples while capturing most of the diversity.

Generalized Linear Mixed-effects Models (GLMMs) were used to model the Shannon diversity index. All GLMMs were fitted using the lme4 package³⁹ and lmer Test package⁴⁰ in R⁴¹, using R studio version 4.2.2. Bee strain, storage treatment (exposure to natural vs. storage at 6 °C temperature), and sampling month were incorporated as fixed effects. Hive ID was incorporated as a random effect to account for the repeated measuring of each hive⁴². Hive ID corresponds to the hives, while the sample ID refers to the samples taken from those hives. A stepwise backward selection process was employed for model selection. Initially, a full model was run with all fixed effects and random effects. Then all possible interactions among fixed effects were examined, followed by the systematic removal of one fixed effect at a time. The process continued until no further model improvement (e.g., lower AIC) could be observed. A difference of ≤ 2 in the AIC indicated that models were similar, in which case the simpler model (i.e., with fewer parameters) was selected as the best-fit model⁴³. The best fit model was diagnosed for model assumptions through residual diagnostic plots using the ‘DHARMA’ package⁴⁴. Finally, package ‘ggeffects’ version 0.16.0⁴⁵ was used to extract means, standard deviations, and 95% confidence intervals for the predicted values of Shannon diversity index for different sampling times. Tukey HSD tests for multiple comparisons between treatments was employed with estimated marginal means comparisons (EMMs) using the ‘emmeans’ function⁴⁶. Other matrices like observed species richness, shannon diversity, and simpson diversity are provided in supplementary data (Data available; alpha amp.csv, Supplementary Fig. 1).

For beta diversity analysis, Adonis Bray–Curtis distance dissimilarities and nonmetric multidimensional scaling (NMDS) ordination were conducted in R v4.3.0 with ‘vegan’ package. The ‘phyloseq’ (ver 1.34.0) R package was used for beta diversity analysis. Permutation analysis of variance (PERMANOVA) was applied using the ‘adonis’ function on distance matrices with 999 permutations. The differences between bee gut microbiomes including distance as a dependent variable and months (October, November, and December), bee strain (Bolton bees and Mann lake bees) and Storage Status (Inside and Outside) as independent variable were assessed using separate Adonis models. In all models, bee hives were accounted as a block- strata, where permutations were allowed among levels of all the variable, but within each level of bee hives (random variable), no permutations occurred.

To understand the effect of different variables on the abundance of candidate bacterial species, Generalized Linear Mixed-effects Models (GLMMs) was used to model the abundance of particular bacterial species. Bee strain, storage treatment (exposure to natural vs. storage at 6 °C), and sampling month were incorporated as fixed effects. Hive ID was incorporated as a random effect to account for the repeated measures of each hive⁴². The fitted GLMM and LM was diagnosed for issues with heterogeneity, overdispersion or missing co-variables using the residual plots. To evaluate the significance of fixed effect, analysis of variance tables for the GLMM was calculated using Wald chi-square tests. Tukey HSD tests was employed for multiple comparisons between treatments with estimated marginal means comparisons (EMMs) using the ‘emmeans’ function⁴⁶.

Results

Alpha diversity

To investigate alpha diversity patterns, generalized linear mixed models (LMM) that included Shannon diversity as a response variable was applied (Table 2). The most parsimonious, best-fit model included month as a fixed effect, and random effects for hive ID (different hives in each treatment) (Table 3). In contrast to the predictions, alternative models incorporating bee strain and storage treatment did not significantly improve the best-fit model. The random effects structure in the best fit model showed relatively little variation among hives ($SD = 0.1604$), when compared to the residual error of the model ($SD = 0.3560$). This indicates variation between hives and the replications do not account for the majority of the variation in Shannon diversity. Furthermore, the Shannon diversity index in October differed significantly from November (p value = 0.0482), while December showed no significant difference ($p = 0.1891$) (Data available; alpha diversity R file). Month explained only 8% of the variation in Shannon diversity, whereas the full model explained 23% of the variation in Shannon diversity. Overall, results indicate a temporal variation in Shannon diversity, but the strain of bee and whether the hives were stored inside or outside had no effect (Fig. 3). Non modelled matrices like observed species richness, Shannon diversity, and Simpson diversity were not effected by any of the predictor variable ($p > 0.05$).

Beta diversity

To investigate beta diversity patterns, we conducted PERMANOVA using the adonis2 package in R, testing various models with Bray–Curtis distance matrices (phyloseq) as a response variable. The simplest and best-fitting model included storage status and month in interaction with bee strain as a fixed effect, and block (strata) as bee hive ID; $Distance \sim Storage\ status + Month * Bee\ strain$, and strata = hive ID (Table 4). The overall composition of bee gut microbiomes differed by bee type (adonis $F = 3.19$, $df = 1$, $R^2 = 0.0272$, p value = 0.001), month (adonis $F = 5.09$, $df = 2$, $R^2 = 0.0872$, p value = 0.001), but not storage status (adonis $F = 0.57$, $df = 1$, $R^2 = 0.0048$, p value = 0.887). The interaction between month and bee strain was significant (adonis $F = 1.57$, $df = 2$, $R^2 = 0.0269$, p value = 0.021). Month explained only 8.7% of the variation, whereas the bee type, and interaction between month and bee strain explained 2.7%, and 2.6% of the variation, respectively. Overall, these findings suggest that the microbial dynamics in overwintering honey bee workers are influenced by bee strain

Model	AIC
Model: Shannon diversity index~	
Storage status + month + bee strain + 1 hive ID	110.0
Storage status * month * bee strain + 1 hive ID	112.8
Storage status * month + bee strain + 1 hive ID	106.8
Storage status + month * bee strain + 1 hive ID	114.6
Storage status * bee type + strain + 1 hive ID	113.7
Storage status + month + 1 hive ID	108.0
Month + bee strain + 1 hive ID	108.1
Storage status + bee strain + 1 hive ID	116.2
Month * bee strain + 1 hive ID	110.9
Storage status * bee strain + 1 hive	118.2
Storage status + 1 hive ID	114.3
Bee strain + 1 hive ID	116.4
Month + 1 hive ID	106.1
1 hive ID	112.5

Table 2. Comparisons of Akaike Information Criterion (AIC) value for different Generalized Linear Mixed Models (GLMMs) with relevant predictor and random variables. Best fit GLMM with the lowest AIC value is highlighted in bold. Δ AIC≤2 indicated similar models, which leads to the selection of simpler model (i.e. with less parameters).

Model	β Estimate ± SE	df	t value	p value	Variance	SD	
Shannon diversity index ~ Month + 1 hive ID							
Fixed Effects	(Intercept)	3.33555 ± 0.07521	17.62	42.31	< 2 × 10 ⁻¹⁶ a		
	Month (Reference: October						
	November	- 0.16872 ± 0.08512	92.53	-2.002	0.0482		
	December)	0.11101 ± 0.08447	92.37	1.323	0.1891		
Random Effects	Hive (Intercept)					0.025	0.1603
	Residual					0.126	0.3560
Number of observations = 107; groups: Hive, 12;							

Table 3. Results of best fit Generalized Linear Mixed Model from Shannon diversity index. ^aStatistically significant at *p* ≤ 0.05. SE = standard error; SD = standard deviation.

over the months, with stability observed in storage conditions (Fig. 4). Pairwise comparison showed significant differences in gut microbiota when compared October – November (*p* value=0.002), November – December (*p* value=0.001), and October—December (*p* value=0.001) where a significant decline in beta diversity in November was observed.

Taxonomical abundance

Following the removal of barcodes and primer sequences through QIIME2 (Demultiplexing), we obtained a collective sum of 34,879,494 paired-end 16S rRNA gene amplicon reads. The average read count per sample was 317,086, with an overall average quality score of 34. Across the 108 samples analyzed, the range of reads per sample varied from a minimum of 56,533 to a maximum of 1,314,819. Analyzing the taxonomic profile revealed a predominance of *Firmicutes* (70.96%), *Proteobacteria* (20.18%), and *Actinobacteriota* (5.83%) at the phylum level. We investigated the presence of the core gut microbiota and identified only a limited number of reads for *Snodgrassella*.

In contrast to *Snodgrassella*, we identified various species within the genus *Lactobacillus*, including unclassified *Lactobacillus*, *Bombilactobacillus mellis* (formerly Firm-4 group), *Lactobacillus apis* (Firm-5 group), *Apilactobacillus micheneri*, and *Lactobacillus* uncultured *Firmicutes*, which dominated across all bee samples. In this study we focused on examining the relative abundance of all the species under *Lactobacillus* genus and two non-core (*Bartonella* and *Commensalibacter*) and two core (*Gilliamella* and *Bifidobacterium*) microbes, along with the endosymbiont *Wolbachia*. Moreover, considering the importance of genus *Bartonella* and *Commensalibacter* in a previous study²⁸, we analyzed the abundance of two *Bartonella* species: *Bartonella apis* and another denoted as *uncultured Bartonella* and two *Commensalibacter* species: *Acetobacteraceae_bacterium* and another denoted as *Unclassified Commensalibacter*. The bacterial species-specific analysis for relative abundance was performed using mixed model with formula: *Abundance* ~ *Storage status* + *Month* + *Bee strain* + (1 | *hive ID*).

Our analysis revealed that the month significantly influenced the abundance of unclassified *Lactobacillus* (df = 99.29, F-value = 31.8402, *p*-value<0.005). Specifically, October showed a significantly higher abundance

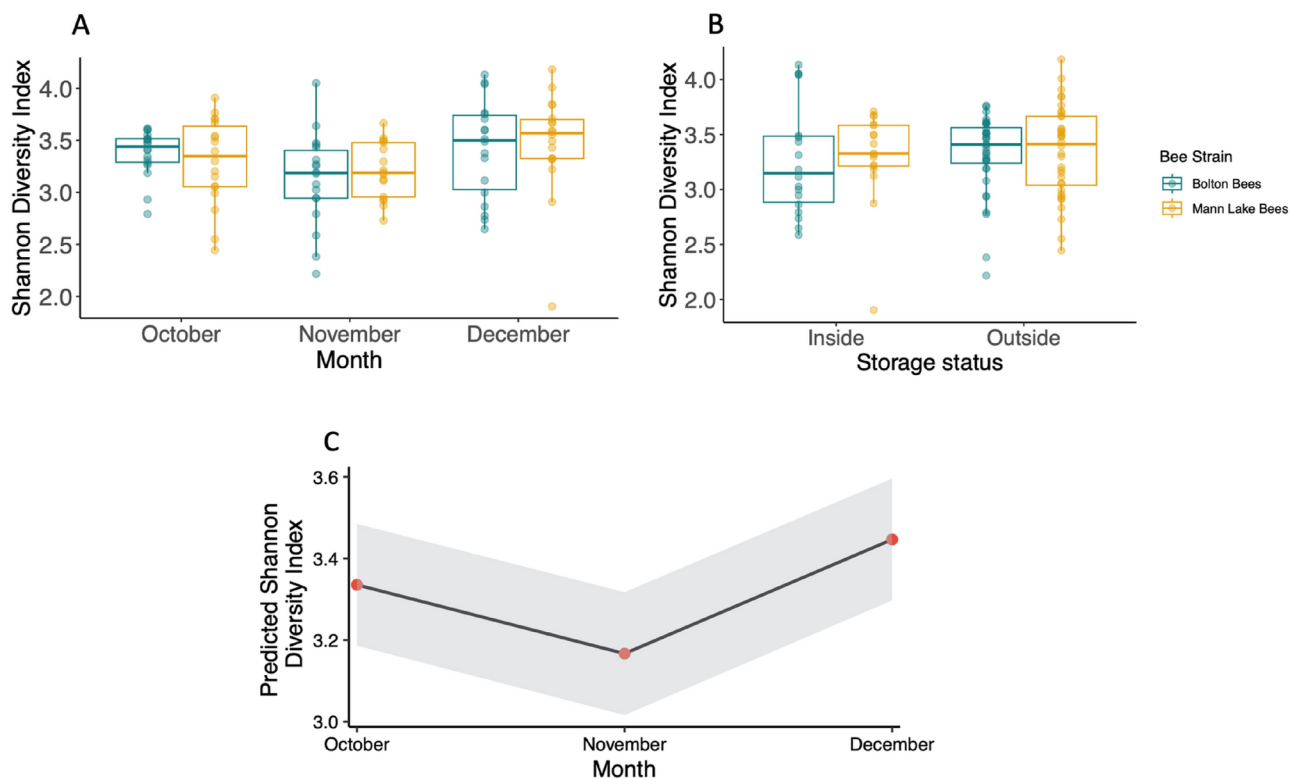


Fig. 3. Shannon diversity index by month, bee strain and storage status. Shannon diversity was significantly different in November ($p=0.0482$) as compared to October. October and December were not significantly different ($p=0.1891$). Neither bee strain nor storage condition explained Shannon Diversity (see Table 2). Model predictions for effect of sampling time on Shannon diversity index (see Table 3). In A and B, the line inside the box represents the median, while the whiskers represent the lowest and highest values within the 1.5 interquartile range. Individual sample values are shown as points. In C, grey shading represents the 95% confidence interval.

Model		Df	Sum of squares	R ²	F	Pr (> F)
Distance ~ Storage status + Month * Bee strain, strata = hive ID						
Fixed Effects	Storage Status	1	0.0894	0.00487	0.5701	0.887
	Month	2	1.5975	0.08702	5.0950	0.001
	Bee stain	1	0.5001	0.02724	3.1903	0.001
	Month * bee strain	2	0.4945	0.02694	1.5771	0.021
	Residual	100	15.6767	0.85394		
Total		106	18.3581	1.00000		

Table 4. Best fit permutation analysis of variance (PERMANOVA) model applied using the adonis function with distance matrices with 999 permutations and bee hives accounted as a blocks – strata. a Statistically significant at $p \leq 0.05$.

compared to December ($SE=3.56$, $df=112.7$, $p\text{ value}=0.0261$), while October had a significantly lower abundance compared to November ($SE=3.56$, $df=112.7$, $p\text{ value}=0.0002$). Additionally, December had a significantly lower abundance compared to November ($SE=3.10$, $df=97.5$, $p\text{ value}<0.0001$) (Fig. 5). Neither storage status ($df=28.17$, $F\text{-value}=0.1432$, $p\text{ value}=0.7079$) nor bee strain ($df=12.15$, $F\text{-value}=1.0835$, $p\text{ value}=0.3182$) significantly affected the abundance of unclassified *Lactobacillus*. The abundance of *Lactobacillus apis* did not vary by storage status ($df=107$, $F\text{-value}=0.885$, $p\text{ value}=0.3489$) or bee strain ($df=107$, $F\text{-value}=1.4531$, $p\text{ value}=0.2306$), but month had a significant effect ($df=107$, $F\text{-value}=4.7334$, $p\text{ value}=0.0107$). We found *Lactobacillus apis* had significantly higher abundance in October compared to December ($SE=1.50$, $df=110$, $p\text{ value}=0.0125$), whereas no significant difference was observed between October and November ($SE=1.50$, $df=110$, $p\text{ value}=0.0673$), nor between November and December ($SE=1.34$, $df=102$, $p\text{ value}=0.7473$). None of the predictors (storage status, month, or bee strain) significantly affected the abundance of *Bombilactobacillus mellis* (storage status: $df=107$, $F\text{-value}=0.2015$, $p=0.654$; month: $df=107$, $F\text{-value}=2.530$,

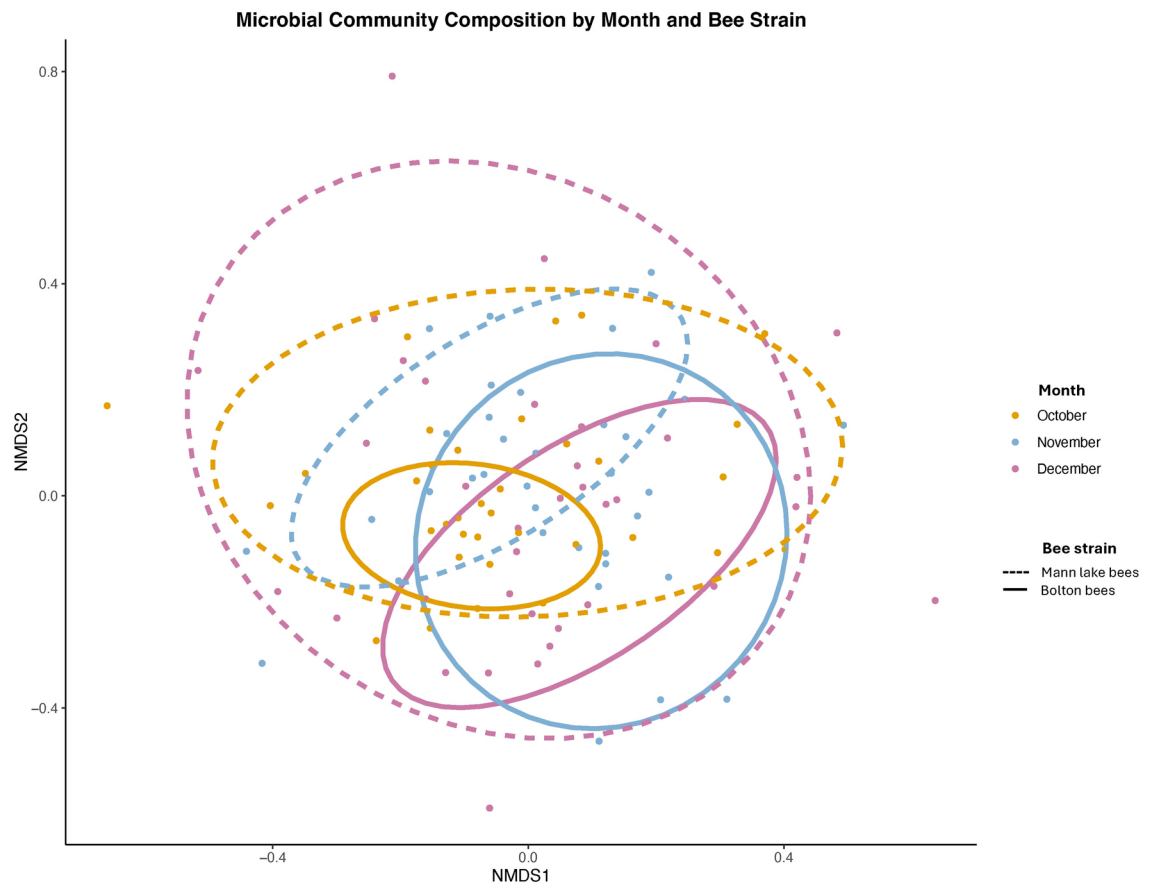


Fig. 4. Non-metric multidimensional scaling (NMDS) performed with Bray–Curtis dissimilarity. Month ($p=0.001$) and bee strain ($p=0.001$) significantly affected beta diversity, and their interaction was also significant ($p=0.021$). Each point on the plot represents a honey bee gut sample and samples that share greater similarity are ordinated closer together. Ellipses on the plot indicate 95% confidence intervals.

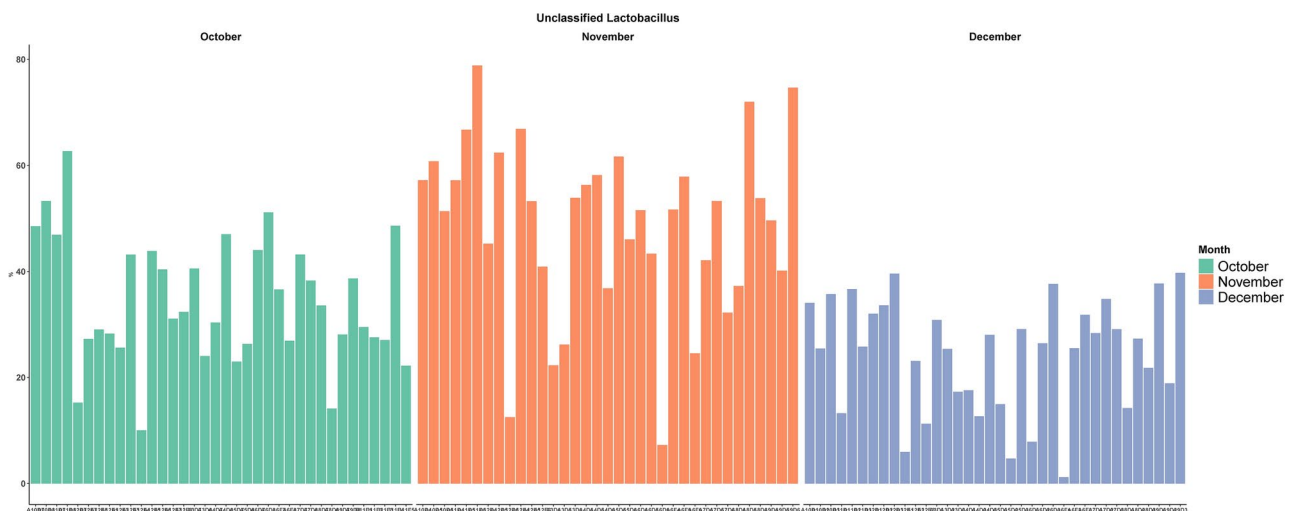


Fig. 5. Relative abundances of Unclassified *Lactobacillus* present in honey bee gut across different months. Each column represents an individual bee. The relative abundance, represented in percentages, is shown on the y-axis.

p value = 0.0844; bee strain: $df=107$, F -value = 0.6398, p value = 0.4256), *Apilactobacillus micheneri* (storage status: $df=107$, F -value = 2.0463, p value = 0.1555; month: $df=107$, F -value = 0.5612, p value = 0.5722; bee strain: $df=107$, F -value = 2.1700, p value = 0.1437), and *Lactobacillus* uncultured *Firmicutes* (storage status: $df=107$, F -value = 1.2865, p value = 0.2592; month: $df=107$, F -value = 1.680, p = 0.1896; bee strain: $df=107$, F -value = 3.987, p value = 0.05).

Bartonella apis abundance did not differ by month ($df=99.11$, F value = 2.27, p value = 0.1085), bee strain ($df=38.26$, F value = 0.1824, p value = 0.6717), or storage status ($df=38.26$, F value = 0.1824, p value = 0.6717). However, the *Bartonella* uncultured ASV significantly differed by month ($df=98.72$, F value = 4.74, p value = 0.0107) while storage status ($df=34.58$, F value = 0.0528, p value = 0.81954) and bee strain ($df=11.559$, F value = 0.1322, p value = 0.72274) were not significant. We found a significant difference in abundance between December and November ($SE=1.03$, $df=97$, p value = 0.0085), but not between October and December ($SE=1.21$, $df=112$, p value = 0.358) or October and November ($SE=1.21$, $df=112$, p value = 0.456). For *Commensalibacter* (*Commensalibacter Acetobacteraceae bacterium*), bee strain ($SE=1.48$, $df=102$, p value < 0.0001) was the only factor significantly influencing abundance with Mann Lake bees having a higher abundance than Bolton bees. The effect of storage status ($df=107$, F value = 0.261, p value = 0.6097) and month ($df=107$, F value = 1.6663, p value = 0.1938) was not statistically significant. Moreover, none of the predictors significantly influenced the abundance of unclassified *Commensalibacter* (storage status: $df=107$, F -value = 0.4946, p value = 0.4834; month: $df=107$, F -value = 0.1538, p value = 0.8577; bee strain: $df=107$, F -value = 1.0855, p value = 0.2998).

Bee strain was found to be a significant predictor of *Bifidobacterium* (*Bifidobacterium uncultured_Bifidobacterium*) abundance where *Bifidobacterium* showed higher abundance in Bolton bees compared to Mann Lake bees ($SE=0.077$, $df=102$, p value = 0.0014). The storage status ($df=107$, F value = 0.0682, p value = 0.7944) and month ($df=107$, F value = 0.8069, p value = 0.4489) variables did not contribute significantly to the variation in this model. The storage status ($df=37.34$, F value = 6.67, p value = 0.013) and month ($df=99.15$, F value = 14.18, p value = 3.808×10^{-6}) significantly affected the abundance of *Gilliamella* (*Unclassified Gilliamella*), while Bee strain did not show a significant effect ($df=12.10$, F value = 0.675, p value = 0.426). Pairwise comparison showed significant differences in abundance during October and December ($SE=0.197$, $df=112$, p value < 0.0001), November and December ($SE=0.166$, $df=97.5$, p value = 0.002), but no significant difference during November and October ($SE=0.197$, $df=112$, p value = 0.5361). A significant effect of the storage status on the abundance of *Gilliamella* was seen between the Inside and Outside bees ($SE=0.215$, $df=45$, p value = 0.0272). storage status ($df=48.66$, F value = 0.0377, p value = 0.846) and Month ($df=99.42$, F value = 0.97, p value = 0.38) is not a significant predictor of abundance in *Wolbachia* (*Unclassified Wolbachia*). *Wolbachia* abundance was significantly different between the bee strains ($df=11.78$, F value = 5.352, p value = 0.03959). This results suggest a potential difference in gut microbial abundance between Bolton bees and Mann lake bees, but the significance was marginal ($SE=0.243$, $df=14$, p value = 0.0570). Overall, this taxonomic data emphasizes the marginal differences in gut microbiome composition between bee types, revealing a temporal changes in microbial composition that are not influenced by storage conditions in overwintering worker honey bee (Fig. 6A, 6B, 6C).

Discussion

We used a 16S rRNA gene amplicon sequencing approach to study/characterize the gut microbiota of overwintering adult worker bees between (a) two commercial bees (Bolton Bees and Mann Lake bees), (b) two storage conditions and, (c) three months during a season. In this study, we hypothesized that temperature would be one of the most important environmental factors affecting the gut microbiota in overwintering honey bees. Surprisingly, we found no changes in the diversity, composition, or abundance of the microbiota when the bees were stored at constant 6 °C or outside during the winter. Interestingly, we found that the overwintering bees' gut microbiome was dominated by the genus *Lactobacillus*, contributing to an increased relative bacterial abundance across all samples, regardless of storage conditions. An increase in *Lactobacillus* had been previously detected at relatively lower abundances in earlier studies on winter bees^{47,48}.

The dominant *Lactobacilli* in our study included unclassified *Lactobacillus*, *Bombilactobacillus mellis*, *Lactobacillus apis*, *Lactobacillus* uncultured *Firmicutes*, and *Apilactobacillus micheneri*. Recently, Zheng et al., 2020 reclassified the genus *Lactobacillus* into 25 distinct genera, 23 of which are newly established based on phenotypic and genotypic characteristics⁴⁹. Three previously described species, along with *Bombilactobacillus mellis* (formerly *Lactobacillus mellis*⁵⁰), have been assigned to the newly defined genus *Bombilactobacillus*, which belongs to the Firm-4 group^{51–54}. Another major group within the reclassified bacterial genus is the former *Lactobacillus* Firm-5, which includes *Lactobacillus apis* as a member⁵⁵. Additionally, *Lactobacillus kosoi* has been recently reclassified as a heterotypic synonym for *Apilactobacillus micheneri*⁵⁶, a species found abundantly in solitary bees. Each of these species has been studied for their distinct functions. For example, *Lactobacillus apis* (Firm-5) has been shown to convert tryptophan to an indolic aryl hydrocarbon receptor agonist, improving learning and memory behaviors in honey bees⁵⁵. Honey bees rely on the bacterial metabolite succinate, primarily derived from *Lactobacillus apis*, to maintain energy metabolic homeostasis⁵⁷. Both Firm-4 and Firm-5 are closely related *Firmicutes*⁵⁸, with *Lactobacillus apis* having well-documented distinct functions, while *Bombilactobacillus mellis* remains relatively understudied. *Apilactobacillus*, the most abundant microbe in the solitary bee microbiome, is environmentally acquired⁵⁹ and exhibits antibiotic resistance⁶⁰. An unbalanced microbial community can regulate the abundance of *Apilactobacillus micheneri*, potentially influencing solitary bee biology and compromising their growth and survival⁵⁹. Moreover, many studies have found genus *Lactobacillus* serves as the most important genus in the guts of honey bees^{61,62} that plays an important role in the health of bees by showing probiotic characteristics^{63–65}. *Lactobacillus* has shown numerous function in regulating honey bee health like stimulating immune system⁶⁶, nutrient metabolism⁶⁴, ability to digest flavonoids and other compounds present in the pollen wall⁶⁷, and to inhibit pathogens^{62,68,69}. Moreover *Lactobacilli* have been shown to release short chain fatty acids and vitamins which are utilized by midgut cells as an energy

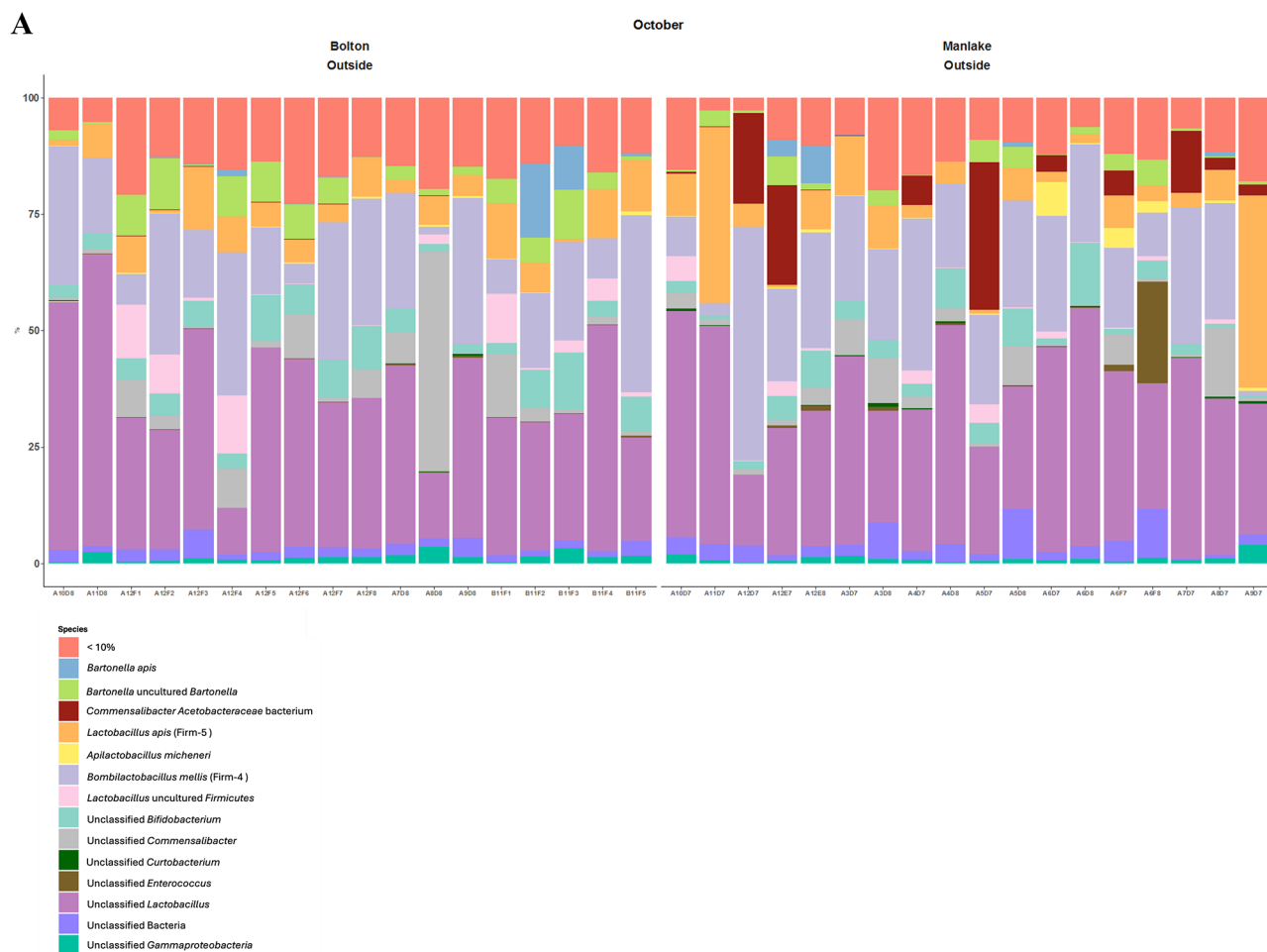


Fig. 6. Relative abundances of the top fifteen bacterial species present in honey bee gut in different storage conditions in October (A), November (B), and December (C). Less than 10% is a category of low abundance species that made up less than 10% of the median number of reads in each gut samples. Each column represents an individual bee. The relative abundance, represented in percentages, is shown on the y-axis.

source^{70,71} and play a role in regulating metabolism⁶⁷. This suggests that the higher abundance of *Lactobacillus* during the overwintering phase may regulate host energy metabolism and support heat production by worker bees, thereby stabilizing colony temperature. Despite the physiological stress induced by cold temperatures, this study demonstrates that honey bees have the ability to acclimatize their gut microbiota in cold conditions. One possible explanation for these findings is that honey bees rely on specific bacterial species to maintain their health, utilizing the functions performed by the gut microbiota. Thus, bees may retain these bacterial species, particularly those belonging to *Lactobacillus*, to extract sufficient resources at low temperatures, regardless of bee strain or storage conditions.

We observed differences in the abundance of several bacterial species between the two bee types. Bolton bees exhibited significantly higher abundance of *Bartonella* (in some instances), *Bifidobacterium*, and *Wolbachia*, whereas Mann lake bees showed significantly higher abundance of *Commensalibacter Acetobacteraceae bacterium* when compared to Bolton bees. In particular, the bees obtained from Bolton Bees represent specific genetic lines known for their resilience in Minnesota's harsh climate. This genetic line, named after the location, where the parent Queen overwintered (Bolton, Minnesota), is derived from the base stock called MN-Hardy. The queens from the MN-Hardy line, used as grafts for queen production, have successfully endured the challenges of a prolonged and frigid Minnesota winter. Typically, such severe winters could affect honey stores, cleansing flights, and brood-laying, but these MN-Hardy Queens are claimed to have demonstrated ability to thrive under these cold winter conditions (<https://boltonbees.com/pages/mn-hardy-hives>). On the other hand, "OHB Italian" Mann Lake bees, sourced from Olivarez Honey Bees (OHB) in Northern California, are accompanied by claims for their hive performance, disease resistance, and overall robust health. The Italian Queens from OHB have reportedly undergone extensive breeding and careful genetic selection. The incorporation of traits such as "Minnesota Hygienic" and "VSH" (Varroa Sensitive Hygiene) further strengthens their innate ability to resist diseases and combat mite infestations. These two distinct types of bees have been specifically employed for their resilience to withstand the challenging winters typical of the Midwest, making them interesting subjects to

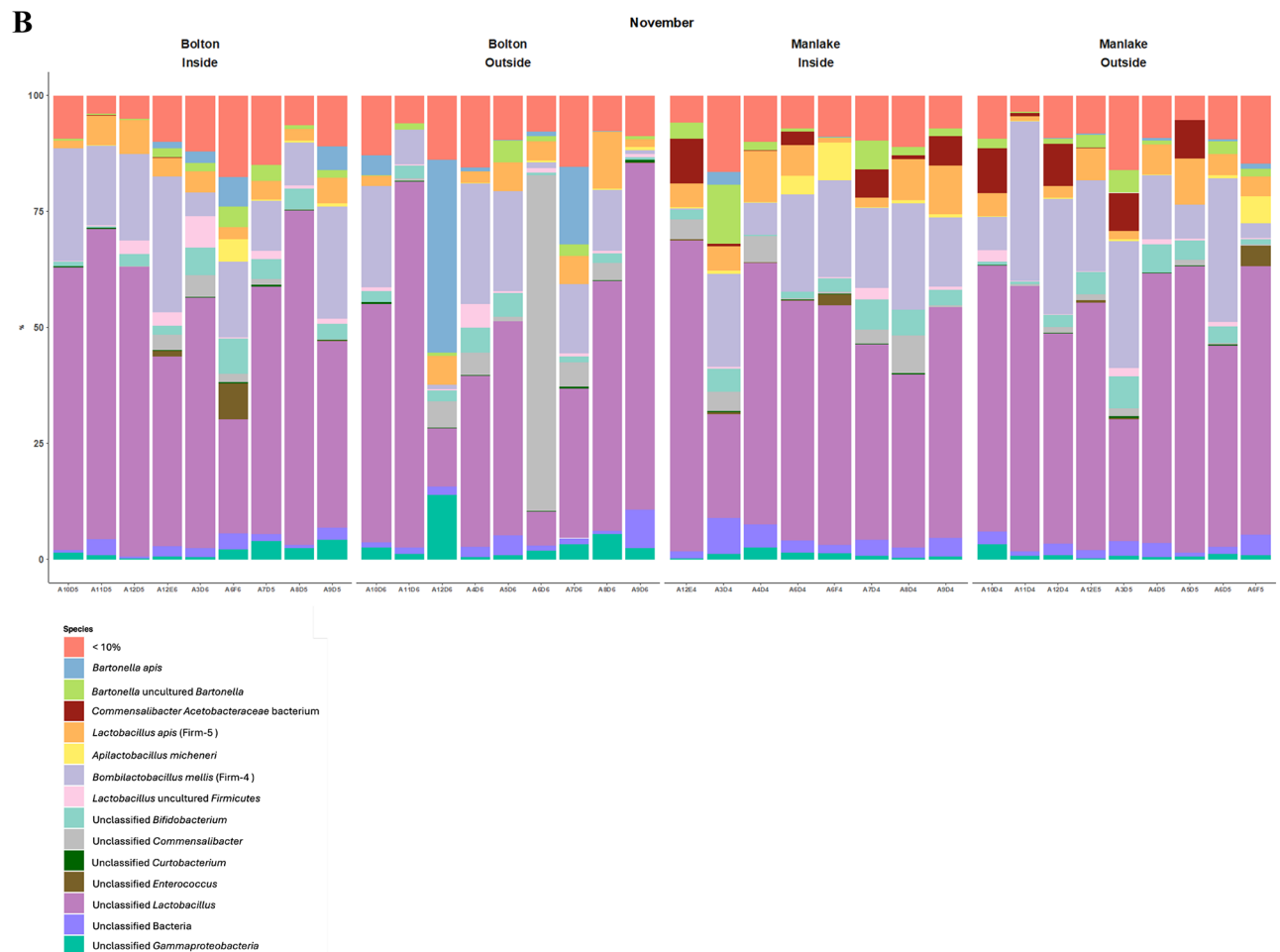


Figure 6. (continued)

study the gut microbiome during the overwintering period. In a previous study on honey bees, the colonization of specific sets of bacteria, such as *Lactobacillus* Firm5 and *Bifidobacterium* Bifido-1.2, in genetically varied hosts strongly suggests that the genotype significantly influences the microbiota structure³². Another study on honey bees has identified marked differences in the core gut microbial community when comparing different lineages, which include Maltese honey bees (lineage A) to the Italian honey bees (lineage C). Notably, Maltese honey bees exhibited an inverse proportion of *Lactobacillaceae* and *Bartonellaceae* when compared to Italian honey bees⁷². The functionality of these differences between bee strains have not been studied. This finding underscores the extent of strain-level diversity within the bacterial communities. However, our study showed extensive overlap of the gut microbial diversity between bee strains and with significant differences in only a few bacterial species specifically, *Bartonella* exhibited higher abundance levels in Bolton bees, while Mann Lake bees showed increased abundance of *Commensalibacter*.

Several studies have demonstrated differences in gut bacterial communities between bee adults based on geographical locations at the same point in time^{33,73,74}. However, very few studies have explored differences between bee genotypes based on their geographical origins. Given that most colony loss occurs during the winter months and that honey bee gut microbial communities fluctuate seasonally¹⁷, significant differences exist between the gut microbiota of summer and winter bees. Winter bees in previous studies have shown a dominance of *Bartonella* and *Commensalibacter*²⁸. Additionally, the variation in *Lactobacillus* species may be influenced by winter conditions in different geographical regions. For instance, the study conducted at the University of Lausanne, Switzerland²⁸ reported less extreme cold temperatures compared to Fargo, ND, where our study was conducted (average temperature in Oct- 13.33 °C, Nov- 8.8 °C and Dec- 3.3 °C, data collected from weatherspark.com). The geographical variation could account for the higher abundance of *Lactobacillus* observed in the Fargo, ND study compared to the Lausanne, Switzerland study. Lausanne (46.5197° N, 6.6323° E, elevation 1,624 ft), located on the northern shore of Lake Geneva, features a hilly landscape, whereas Fargo (46.8772° N, 96.7898° W, elevation 906 ft) is situated in a flat region predominantly covered by agricultural land. These differences in topography and land use may influence the microbial communities. Therefore, research on the gut bacteria of winter bees is still in its early stages, with only a few studies available^{47,75,76}. Additionally, a recent study found that *Lactobacillus* was one of the most abundant bacterial genera in hives that either survived or failed to survive the winter of 2022 in

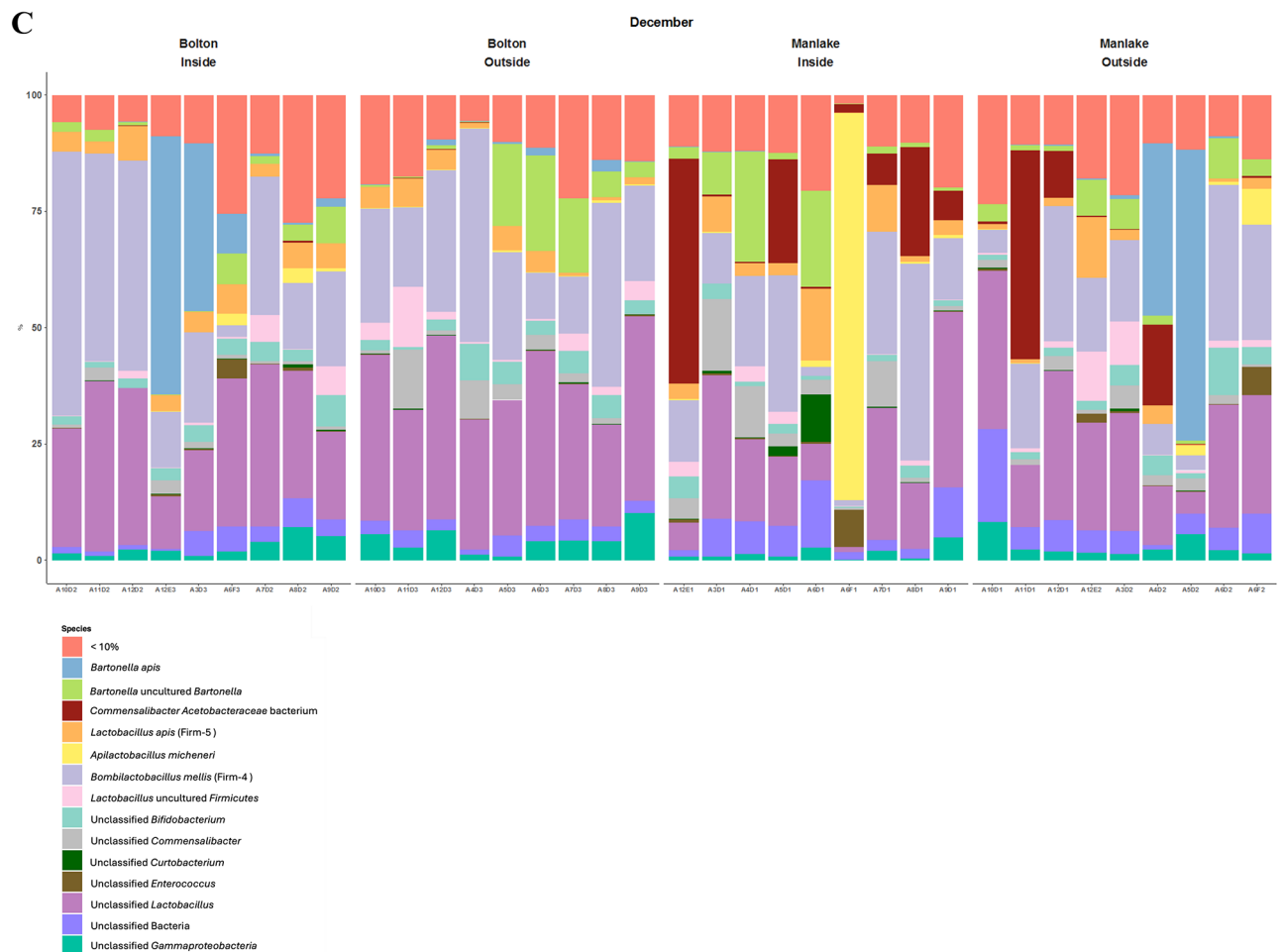


Figure 6. (continued)

northern Virginia, USA, hence supporting its role in winter survival. Previous studies suggested that the broad environment⁷⁷, hive location⁷⁸, artificial pollen supplements⁷⁹ have subtle influence on the relative abundance of the honey bee microbiota. These findings suggest that the higher abundance of *Lactobacilli* may be attributed to the significant impact of environmental acquisition routes like solitary bees, just like in solitary bees⁵⁹. Moreover, in-depth analysis of this study revealed that *Commensalibacter* and *Snodgrassella* were positively associated with winter hive survival, underscoring their importance for hive resilience⁷⁶. Similarly, in our study, we observed that *Commensalibacter* exhibited differential relative abundance between bee strains, with *Lactobacillus* dominating in all winter bees. However, we detected little to no *Snodgrassella* in our samples. Therefore, this study observed that the winter honey bee gut microbiota differs from that in other studies conducted across different months, particularly under natural winter conditions in the midwestern United States.

Overall, the findings in this study reveal varying degrees of presence of bacterial species in overwintering honey bees across different months. There were significant differences in the richness and evenness (Shannon Diversity Index) between October and November, but no differences were noted in the bees sampled in November when compared to December samples. Additionally, beta diversity shows difference in species composition and relative abundance in different months. Workers showed significant differences in species composition across months, with a significant variation in beta diversity, consistent with previous studies^{47,80}. Several studies have examined the gut microbiota composition in various types of worker bees across different seasons, consistently demonstrating that the microbial community structure remains relatively stable over time^{79,81,82}. However, recent study have noted that the gut microbiota differs between winter and summer honey bees, with the long-lived winter bees exhibiting a stable microbiota with reduced α -diversity and higher levels of *Bartonella* and *Commensalibacter*²⁸. This lower community alpha diversity with *Bartonella* and *Commensalibacter* as dominant bacterial species may confer certain physiological benefits. Hence, these studies also collectively reveal minor variations in gut microbial communities in temperate honey bee colonies during winter, indicating a shift in dominance, with the non-core bacterium *Bartonella* surpassing the core bacterial species^{28,80,83}. Another recent study also highlighted the significance of *Bartonella*, highlighting its expanded capability to convert metabolic wastes such as lactate and ethanol into pyruvate, which potentially provides energy for the host as well as other symbionts⁷⁵. Considering the importance of *Bartonella* in previous studies, we analyzed two different species

of *Bartonella* for their taxonomic abundance. However, we found the *Lactobacillus* genus as dominant, present in higher abundance, contrasting with the findings of a previous study where the *Bartonella* genus showed dominance in winter bees.

The role of *Lactobacillus* in overwintering honey bee workers in this study is limited by the nature of 16S rRNA sequencing. Additionally, 16S rRNA sequencing leaves a gap in understanding the absolute abundances of bacterial species as it only reliably provides relative abundances. To improve bacterial species detection resolution and functional profiling at the gene level, future studies could utilize qPCR or shotgun sequencing instead of 16S amplicon sequencing. Additionally, our study was limited by its timeframe, as sampling was conducted only in the month of October, November, and December. We observed a significant decline in beta diversity in November, which may vary if bees are sampled in later months (e.g., January, February, and March) as temperatures continue to drop during these colder months.

Conclusion

In conclusion, our study shows that the gut microbiota of overwintering honey bees is dominated by one specific bacterial genus, *Lactobacillus*, which likely contribute to the bees' ability to withstand environmental stressors^{61,62,67–70}. The results of this study raise two key questions. (1) Why was the *Lactobacillus* genus predominant in winter bees and why was this pattern not observed in previous studies? Could this genus contribute to honey bee survival under extreme cold conditions, such as those found in North Dakota? (2) While the overall gut microbiota remained relatively stable, there were slight shifts in the composition of specific host-associated bacterial communities across genetically distinct honey bee colonies. Do these subtle compositional differences have functional implications that influence colony health or survival? Overall, our study adds to the growing body of research on honey bee gut microbiota and its role in bee health during winter. Future research should focus on specific *Lactobacillus* species to better understand the microbial dynamics that support their functional roles, and to explore the mechanisms underlying the resilience of honey bee gut bacteria and its implications for overall bee health. Lastly, but importantly, only a few studies have reported the presence of *Wolbachia* in honey bees, including findings detailed in^{84,85}. Interestingly, *Wolbachia*, which is exclusively maternally transmitted and known to alter host reproduction through various mechanisms, has been detected in the gut of these winter bees. Although its abundance is notably low, this observation is surprising and intriguing. This finding opens new avenues for research on the prevalence of *Wolbachia* in the honey bee gut and highlights the need for future studies to explore the potential relationship between *Wolbachia* and honey bee workers.

Data availability

Data is provided within the manuscript Data for the interactive files 16Staxabarplots_clean.qzv, alpha_rarefaction.qzv, jaccard_emperor.qzv, stats-dada2.qzv, unweighted_unifrac_emperor.qzv, weighted_unifrac_emperor.qzv, alpha_amp.csv, and alpha diversity R file can be downloaded from this link: <http://datadryad.org/stash/shar/e/ycQx06SicJFuCPsZ0XMkwLVX9F6DetlucISxvTQoQF0> and viewed on <https://view.qiime2.org/>.

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References

1. Genersch, E. Honey bee pathology: Current threats to honey bees and beekeeping. *Appl. Microbiol. Biotechnol.* **87**, 87–97 (2010).
2. Abou-Shaara, H. F., Owayss, A. A., Ibrahim, Y. Y. & Basuny, N. K. A review of impacts of temperature and relative humidity on various activities of honey bees. *Insect. Soc.* **64**, 455–463 (2017).
3. Giannini, T. C. et al. Crop pollinators in Brazil: A review of reported interactions. *Apidologie* **46**, 209–223 (2015).
4. Hristov, P., Shumkova, R., Palova, N. & Neov, B. Factors associated with honey bee colony losses: A mini-review. *Vet. Sci.* **7**, 166 (2020).
5. Szentgyörgyi, H., Czekeńska, K. & Tofilski, A. Honey bees are larger and live longer after developing at low temperature. *J. Therm. Biol.* **78**, 219–226 (2018).
6. Butolo, N. P., Azevedo, P., Alencar, L. D., Malaspina, O. & Nocelli, R. C. F. Impact of low temperatures on the immune system of honeybees. *J. Therm. Biol.* **101**, 103082 (2021).
7. Wang, Q. et al. Low-temperature stress during capped brood stage increases pupal mortality, misorientation and adult mortality in honey bees. *PLoS One* **11**, e0154547 (2016).
8. Steinhauer, N., vanEngelsdorp, D. & Saegerman, C. Prioritizing changes in management practices associated with reduced winter honey bee colony losses for US beekeepers. *Sci. Total. Environ.* **753**, 141629 (2021).
9. Aurori, C. M. et al. What is the main driver of ageing in long-lived winter honeybees: Antioxidant enzymes, innate immunity, or vitellogenin? *J. Gerontol. - Ser. A. Biol. Sci. Med. Sci.* **69**, 633–639 (2014).
10. Stabentheiner, A., Pressl, H., Papst, T., Hrassnigg, N. & Crailsheim, K. Endothermic heat production in honeybee winter clusters. *J. Exp. Biol.* **206**, 353–358 (2003).
11. Degrandi-Hoffman, G., Graham, H., Ahumada, F., Smart, M. & Ziolkowski, N. The economics of honey bee (Hymenoptera: Apidae) management and overwintering strategies for colonies used to pollinate almonds. *J. Econ. Entomol.* **112**, 2524–2533 (2019).
12. Owens, C. D., States, U. & Service, A. R. The thermology of wintering honey bee colonies. 171857 Preprint at (1971).
13. Meikle, W. G., Corby-Harris, V., Ricigliano, V., Snyder, L. & Weiss, M. Cold storage as part of a Varroa management strategy: Effects on honey bee colony performance, mite levels and stress biomarkers. *Sci. Rep.* **13**(1), 15 (2023).
14. Coulbaly, K. A. S., Majeed, M. Z., Sayed, S. & Yeo, K. Simulated climate warming influenced colony microclimatic conditions and gut bacterial abundance of honeybee subspecies *Apis mellifera ligustica* and a *mellifera sinixinyuan*. *J. Apic. Sci.* **66**(15), 27 (2022).
15. Zhang, Y. et al. Mediating a host cell signaling pathway linked to overwinter mortality offers a promising therapeutic approach for improving bee health. *J. Adv. Res.* **53**, 99–114 (2023).
16. Xu, K., Niu, Q., Zhao, H., Du, Y. & Jiang, Y. Transcriptomic analysis to uncover genes affecting cold resistance in the Chinese honey bee (*Apis cerana cerana*). *PLoS One* **12**, e0179922 (2017).
17. Raymann, K. & Moran, N. A. The role of the gut microbiome in health and disease of adult honey bee workers. *Curr. Opin. Insect Sci.* Preprint at <https://doi.org/10.1016/j.cois.2018.02.012> (2018).

18. Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S. & Moran, N. A. Honey bees as models for gut microbiota research. *Lab Anim. (NY)* **47**, 317–325 (2018).
19. Engel, P. et al. The bee microbiome: Impact on bee health and model for evolution and ecology of host-microbe interactions. *Am. Soc. Microbiol.* <https://doi.org/10.1128/mBio.02164-15> (2016).
20. Kwong, W. K. & Moran, N. A. Gut microbial communities of social bees. *Nat. Rev. Microbiol.* **14**, 374 (2016).
21. Martinson, V. G., Moy, J. & Moran, N. A. Establishment of characteristic gut bacteria during development of the honeybee worker. *Appl. Environ. Microbiol.* <https://doi.org/10.1128/AEM.07810-11> (2012).
22. Bonilla-Rosso, G. & Engel, P. Functional roles and metabolic niches in the honey bee gut microbiota. *Curr. Opin. Microbiol.* **43**, 69–76 (2018).
23. Engel, P. & Moran, N. A. Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. *Gut. Microb.* **4**, 60–65 (2013).
24. Engel, P. & Moran, N. A. The gut microbiota of insects – diversity in structure and function. *FEMS Microbiol. Rev.* **37**, 699–735 (2013).
25. Ludvigsen, J. et al. Shifts in the midgut/pyloric microbiota composition within a honey bee apiary throughout a season. *Microb. Environ.* **30**, 235–244 (2015).
26. Almeida, E. L. et al. Geographical and seasonal analysis of the honeybee microbiome. *Microb. Ecol.* **85**, 765–778 (2023).
27. Castelli, L., Branchicella, B., Romero, H., Zunino, P. & Antúnez, K. Seasonal dynamics of the honey bee gut microbiota in colonies under subtropical climate: Seasonal dynamics of honey bee gut microbiota. *Microb. Ecol.* **83**, 492–500 (2022).
28. Kešnerová, L. et al. Gut microbiota structure differs between honeybees in winter and summer. *ISME J.* **14**(801), 814 (2019).
29. Hammer, T. J., Le, E., Moran, N. A. & Hammer, T. J. Thermal niches of specialized gut symbionts: the case of social bees. *Proc. R. Soc. B.* **288**, 1944 (2021).
30. Russell, K. A. & McFrederick, Q. S. Elevated temperature may affect nectar microbes, nectar sugars, and bumble bee foraging preference. *Microb. Ecol.* **84**, 473–482 (2022).
31. Ellegaard, K. M., Suenami, S., Miyazaki, R. & Engel, P. Vast differences in strain-level diversity in the gut microbiota of two closely related honey bee species. *Curr. Biol.* **30**, 2520–2531.e7 (2020).
32. Wu, J. et al. Honey bee genetics shape the strain-level structure of gut microbiota in social transmission. *Microbiome* **9**, 1–19 (2021).
33. Su, Q. et al. Significant compositional and functional variation reveals the patterns of gut microbiota evolution among the widespread Asian honeybee populations. *Front. Microbiol.* **13**, 934459 (2022).
34. Wu, Y. et al. Genetic divergence and functional convergence of gut bacteria between the Eastern honey bee *Apis cerana* and the Western honey bee *Apis mellifera*. *J. Adv. Res.* **37**, 19–31 (2022).
35. Khan, K. A. et al. Gut microbial diversity in *Apis cerana indica* and *Apis florea* colonies: A comparative study. *Front. Vet. Sci.* **10**, 1149876 (2023).
36. Maes, P. W., Rodrigues, P. A. P., Oliver, R., Mott, B. M. & Anderson, K. E. Diet-related gut bacterial dysbiosis correlates with impaired development, increased mortality and *Nosema* disease in the honeybee (*Apis mellifera*). *Mol. Ecol.* **25**, 5439–5450 (2016).
37. McFrederick, Q. S. & Rehan, S. M. Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee. *Mol. Ecol.* **25**, 2302–2311 (2016).
38. Russell, K. A. & McFrederick, Q. S. Floral nectar microbial communities exhibit seasonal shifts associated with extreme heat: Potential implications for climate change and plant-pollinator interactions. *Front. Microbiol.* **13**, 3107 (2022).
39. Bates, D., Mächler, M., Zurich, E., Bolker, B. M. & Walker, S. C. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* <https://doi.org/10.18637/jss.v067.i01> (2015).
40. Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82**, 1–26 (2017).
41. R core team. R: The R Project for Statistical Computing. <https://www.r-project.org/> (2022).
42. Harrison, X. A. et al. A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ* <https://doi.org/10.7717/peerj.4794> (2018).
43. Meurisse, N., Pawson, S. M. & Somchit, C. Bark beetles on pine logs: forecasting winter colonisation dynamics based on trap catches and temperature records. *J. Pest. Sci.* **2004**(94), 1357–1373 (2021).
44. Hartig, D. DHARMA: residual diagnostics for hierarchical (multi-level/mixed) regression models. <https://cran.r-project.org/web/packages/DHARMA/vignettes/DHARMA.html> (2018).
45. Lüdtke, D. ggeffects: Tidy data frames of marginal effects from regression models. *J. Open Sour. Softw.* **3**, 772 (2018).
46. Russell, V. Lenth. Package 'emmeans' Type Package Title Estimated Marginal Means, aka Least-Squares Means. *Am. Stat.* <https://doi.org/10.1080/00031305.1980.10483031> (2024).
47. Bleau, N., Bouslama, S., Giovenazzo, P. & Derome, N. Dynamics of the honeybee (*Apis mellifera*) gut microbiota throughout the overwintering period in Canada. *Microorganisms* **8**, 1146 (2020).
48. Bustin, S. A. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J. Mol. Endocrinol.* **29** 23–39 Preprint at <https://doi.org/10.1677/jme.0.0290023> (2002).
49. Zheng, J. et al. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int. J. Syst. Evol. Microbiol.* **70**, 2782–2858 (2020).
50. Olofsson, T. C., Alsterfjord, M., Nilson, B., Butler, E. & Vásquez, A. *Lactobacillus apinorum* sp. nov., *Lactobacillus mellifer* sp. nov., *Lactobacillus mellis* sp. nov., *Lactobacillus melliventris* sp. nov., *Lactobacillus kimbladii* sp. nov., *Lactobacillus helsingborgensis* sp. nov. and *Lactobacillus kullabergensis* sp. nov., isolated from the honey stomach of the honeybee *Apis mellifera*. *Int. J. Syst. Evol. Microbiol.* **64**, 3109–3119 (2014).
51. Ahn, J. H. et al. Pyrosequencing analysis of the bacterial communities in the guts of honey bees *Apis cerana* and *Apis mellifera* in Korea. *J. Microbiol.* **50**, 735–745 (2012).
52. Heo, J., Kim, S. J., Kim, J. S., Hong, S. B. & Kwon, S. W. Comparative genomics of *Lactobacillus* species as bee symbionts and description of *Lactobacillus bombintestini* sp. nov., isolated from the gut of *Bombus ignitus*. *J. Microbiol.* **58**(445), 455 (2020).
53. Martinson, V. G. et al. A simple and distinctive microbiota associated with honey bees and bumble bees. *Mol. Ecol.* **20**, 619–628 (2011).
54. Kang, J. P. et al. *Bombilactobacillus apium* sp. nov., isolated from the gut of honeybee (*Apis cerana*) Arch. Microbiol. **203**(2193), 2198 (2021).
55. Zhang, Z. et al. (2022) Honeybee gut *Lactobacillus* modulates host learning and memory behaviors via regulating tryptophan metabolism. *Nat. Commun.* **13**(1), 13 (2022).
56. Li, T. T., Liu, D. D., Fu, M. L. & Gu, C. T. Proposal of *Lactobacillus kosoi* chiu et al. 2018 as a later heterotypic synonym of *Lactobacillus michenieri* McFrederick et al. 2018, elevation of *Lactobacillus plantarum* subsp. *argentoratensis* to the species level as *Lactobacillus argentoratensis* sp. nov., and *Lactobacillus zhaodongensis* sp. nov., isolated from traditional Chinese pickle and the intestinal tract of a honey bee (*Apis mellifera*). *Int. J. Syst. Evol. Microbiol.* **70**, 3123–3133 (2020).
57. Han, B. et al. *Lactobacillus* Firm-5-derived succinate prevents honeybees from having diabetes-like symptoms. *Proc. Natl. Acad. Sci. U S A* **121**, e2405410121 (2024).
58. Engel, P., Martinson, V. G. & Moran, N. A. Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. U S A* **109**, 11002–11007 (2012).

59. Brar, G. et al. Environmentally acquired gut-associated bacteria are not critical for growth and survival in a solitary bee *Megachile rotundata*. *Appl. Environ. Microbiol.* <https://doi.org/10.1128/aem.02076-23> (2024).
60. McFrederick, Q. S., Mueller, U. G. & James, R. R. Interactions between fungi and bacteria influence microbial community structure in the *Megachile rotundata* larval gut. *Proc. R. Soc. B: Biol. Sci.* <https://doi.org/10.1098/rspb.2013.2653> (2014).
61. Vásquez, A. et al. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS One* **7**, e33188 (2012).
62. Forsgren, E., Olofsson, T. C., Vásquez, A. & Fries, I. Novel lactic acid bacteria inhibiting *Paenibacillus* larvae in honey bee larvae. *Apidologie* **41**(1), 99–108 (2010).
63. Moharrami, M., Mojgani, N., Bagheri, M. & Toutiaee, S. role of honey bee gut microbiota in the control of American foulbrood and European foulbrood diseases. *Arch. Razi. Inst.* **77**, 1331–1339 (2022).
64. Daisley, B. A. et al. *Lactobacillus* spp. attenuate antibiotic-induced immune and microbiota dysregulation in honey bees. *Commun. Biol.* **3**, 1–13 (2020).
65. Forsgren, E., Locke, B., Sircoulomb, F. & Schäfer, M. O. Bacterial diseases in honeybees. *Curr. Clin. Microbiol. Rep.* **5**, 18–25 (2018).
66. Lang, H. et al. Specific strains of honeybee gut *Lactobacillus* stimulate host immune system to protect against pathogenic *Hafnia alvei*. *Microbiol. Spectr.* <https://doi.org/10.1128/spectrum.01896-21> (2022).
67. Kešnerová, L. et al. Disentangling metabolic functions of bacteria in the honey bee gut. *PLoS Biol.* **15**, e2003467 (2017).
68. Yoshiyama, M. & Kimura, K. Bacteria in the gut of Japanese honeybee, *Apis cerana japonica*, and their antagonistic effect against *Paenibacillus* larvae, the causal agent of American foulbrood. *J. Invertebr. Pathol.* **102**, 91–96 (2009).
69. Sabaté, D. C., Carrillo, L. & Carina Audisio, M. Inhibition of *Paenibacillus* larvae and *Ascosphaera apis* by *Bacillus subtilis* isolated from honeybee gut and honey samples. *Res. Microbiol.* **160**(193), 199 (2009).
70. Den Besten, G. et al. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid. Res.* **54**, 2325–2340 (2013).
71. Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S. & Moran, N. A. Honey bees as models for gut microbiota research. *Lab. Anim.* Preprint at <https://doi.org/10.1038/s41684-018-0173-x> (2018).
72. Gaggia, F. et al. Environment or genetic isolation? An atypical intestinal microbiota in the Maltese honey bee *Apis mellifera* spp. *Front. Microbiol.* **14**, 1127717 (2023).
73. Ge, Y., Jing, Z., Diao, Q., He, J. Z. & Liub, Y. J. Host species and geography differentiate honeybee gut bacterial communities by changing the relative contribution of community assembly processes. *mBio* <https://doi.org/10.1128/mBio.00751-21> (2021).
74. Hroncova, Z. et al. Variation in honey bee gut microbial diversity affected by ontogenetic stage age and geographic location. *PLoS One* **10**, e0118707 (2015).
75. Li, C., Tang, M., Li, X. & Zhou, X. Community Dynamics in Structure and Function of Honey Bee Gut Bacteria in Response to Winter Dietary Shift. *mBio* <https://doi.org/10.1128/mbio.01131-22> (2022).
76. Carlini, D. B., Winslow, S. K., Cloppenburg-Schmidt, K. & Baines, J. F. Quantitative microbiome profiling of honey bee (*Apis mellifera*) guts is predictive of winter colony loss in northern Virginia (USA). *Sci. Rep.* **14**(1), 12 (2024).
77. Jones, J. C. et al. Gut microbiota composition is associated with environmental landscape in honey bees. *Ecol. Evol.* **8**, 441–451 (2018).
78. Donkersley, P., Rhodes, G., Pickup, R. W., Jones, K. C. & Wilson, K. Bacterial communities associated with honeybee food stores are correlated with land use. *Ecol. Evol.* **8**, 4743–4756 (2018).
79. Rothman, J. A., Carroll, M. J., Meikle, W. G., Anderson, K. E. & McFrederick, Q. S. Longitudinal Effects of Supplemental Forage on the Honey Bee (*Apis mellifera*) Microbiota and Inter- and Intra-Colony Variability. *Microb. Ecol.* **76**, 814–824 (2018).
80. Liu, P. et al. Overwintering honeybees maintained dynamic and stable intestinal bacteria. *Sci. Rep.* **11**(1), 10 (2021).
81. D'Alvise, P. et al. The impact of winter feed type on intestinal microbiota and parasites in honey bees. *Apidologie* **49**, 252–264 (2018).
82. Kapheim, K. M. et al. Caste-specific differences in hindgut microbial communities of honey bees (*Apis mellifera*). *PLoS One* **10**, e0123911 (2015).
83. Papp, M. et al. Natural diversity of the honey bee (*Apis mellifera*) gut bacteriome in various climatic and seasonal states. *PLoS One* **17**, e0273844 (2022).
84. Pattabhiramaiah, M., Brueckner, D., Witzel, K. P., Junier, P. & Reddy, M. S. Prevalence of *Wolbachia* in the European honeybee, *Apis mellifera carnica*. *World Appl. Sci. J.* **15**, 1503–1506 (2011).
85. Hoy, M. A., Jeyaprakash, A., Alvarez, J. M. & Allsopp, M. H. *Wolbachia* is present in *Apis mellifera capensis*, *A. m. scutellata*, and their hybrid in Southern Africa. *Apidologie* **34**(53), 60 (2003).

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Author contributions

Gagandeep Brar (GB) is the first author, with Lyna Ngor (LN), Quinn McFrederick (QSM), Preetpal Singh (PS), Arun Rajamohan (AR), Alex Torson (AT), Joseph Rinehart (JR), and Julia H. Bowsher (JB) as co-authors. GB, JR, and JB conceived and designed the study. GB and LN processed samples, and GB and PS analyzed the data. GB wrote the first version of the manuscript. All authors contributed to reviewing the final manuscript.

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Upon approval, we give full consent for publication.

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