Natural diversity of honey bee (*Apis mellifera*) gut bacteriome in various climatic and seasonal states

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ABSTRACT

As pollinators and producers of numerous human consumed products, honey bees have great ecological, economic and health importance. The composition of their bacteriota, for which the available knowledge is limited, is essential for their body's functioning. Based on our survey we performed a metagenomic analysis of samples collected by repeated sampling from representative geolocations regarding the study area's climatic types over two nutritionally extreme periods (March and May) of the collection season. In bacteriome composition, significant (p=0.002) difference was found between the samples from March and May. The samples' bacteriome from March showed a significant (p=0.02) composition difference between the cooler and the warmer regions. In samples of May, there were no significant bacteriome composition differences among the climatic classes. Based on our results, one may conclude that the composition of healthy core bacteriome in honey bees varies depending on the climatic and seasonal conditions. This is probably since climatic characteristics, and the vegetation states determine the availability and nutrient content of flowering plants. Our study results prove that to gain a thorough understanding of a microbiome's natural diversity, we need to obtain the necessary information from extreme ranges within the host's healthy state.

Introduction

Honey bees are important pollinators with high economic value and importance considering the ecosystem[1–3]. Their economic significance is based on their role in crop pollination and the different bee products they make[2,3]. Honey, their most well-known product, is an important component of human nutrition. There are shreds of evidence that honey consumption can improve the human health state and might have a role in disease management[4]. However, honey bees are exposed to their closer environment, and several factors threaten their health, including different pathogens, parasites and chemicals used as pesticides in the agriculture[5–7]. The global decline of this key pollinator poses threats to food security and the maintenance of biodiversity[8]. There is growing attention on the effect of different herbicides and pathogens on the bee gut microbiota[9–11]. However, there is no detailed knowledge about the natural variability of the honey bee gut bacteriota. Nevertheless, this could form the basis of studies considering the effect of different harmful agents on honey bees' gut bacteriota. Without this knowledge, one cannot decide if any suspected factor places the bacteriota composition into an adverse state. Although there are studies on the honey bee gut microbiota, microbiome[12–14], none was designed to describe the effect of feed on its composition. As is known for many other animals, feed affects the composite of gut bacteriota. Subsequently, it can be assumed that bee gut bacteria also may be changed by feed what can be affected by seasonal and environmental conditions. During the collection season, the different flowering plants provide diverse feed for the bees from time to time. The vegetation cycles, flowering, and pollen quantity and quality of plants are mostly influenced by meteorological conditions, especially precipitation and temperature. Our study aimed to get a deeper insight into the natural variation of gut bacteriome in healthy worker honey bees based on a country-wide, repeated measure survey. To achieve the research goal, we were guided by considering that it is advisable to work based on samples representing extremes states of bee gut bacteriota. Hence, samples were taken during the two most distinctive periods of the honey collection season and sampling sites were selected from markedly distinct areas based on their climatic characteristics.

Materials and Methods

Sampling design and sample collection

As the study's main goal was to understand the natural variability of honey bee (*Apis mellifera*) gut bacteriome of healthy individuals, we have designed the sampling to be representative to Hungary. For this purpose, a feasible way was to identify sampling points being a representative on climatic conditions. In Hungary, there are 175 local administrative units (LAU 1), for each of these units, we have calculated the 10-year average of the yearly growing degree days (GDD) with base 10°C[15,16] and the yearly total precipitation. Meteorology data for period 2008-2017 was gathered from the ERA-Interim reanalysis data repository[17] by the spatial resolution of 0.125°. For both environmental variables, two-two categories were defined: cooler-warmer and less-more for GDD and precipitation, respectively. Regarding GDD the lower two quartiles were classified as cooler, the upper two quartiles as warmer. For precipitation, the yearly mean below the country-wide median was assumed as less, above the median as more. Each LAU 1 was categorised by its own climatic variables (Fig 1). By stratified spatial random sampling[18,19], twenty local administrative units were chosen as sampling areas. The strata's sample size was proportional to the stratifying GDD and precipitation categories' country-wide frequency to reach representativeness. All data management and analysis were performed in the R environment[20].

From each involved LAU, one well-managed apiary was selected following personal conversation. Two sample collection campaigns were performed. The first was done between 20/03/2019 and 25/03/2019 (Fig 1/a) and the second in the range of 23/05/2019-01/06/2019 (Fig 1/b) will be referred to as sampling March and May, respectively. During the sampling period, March the honey bees were kept on additive feed mostly, with less or no flowers. In May, all apiaries were in acacia (*Robinia pseudoacacia*) forest. For both samplings, the same three-three colonies of the selected apiaries were planed to be involved. At each farm, 20-20 workers of the three colonies were collected and frozen immediately by dry ice. Parallel to the samplings, a query was applied to gather metadata on apiaries and their animal health management and status. According to the questionnaires, there was no disease or poisoning in any of the sampled colonies during the study. Since in Hungary mainly Carniolan honey bees (*Apis mellifera carnica*) are in operation, the samples were drawn from that subspecies.

Sample preparation

The collected samples were prepared for next-generation sequencing (NGS) in the Department of Parasitology and Zoology, University of Veterinary Medicine Budapest. From the deep-frozen workers there was chosen 10-10 by colonies. The bees' entire gastrointestinal tracts were removed and pooled on the apiary-sampling level. The gut preparation forceps were never used before, and one forceps was applied only for one pool (3x10 guts) processing.

DNA extraction and metagenomics library preparation

The Quick-DNA Fecal/Soil Microbe Kit from Zymo Research was used for the simple and rapid isolation of inhibitor-free, high-quality host cell and microbial DNA from the bee gut samples. Isolated total metagenome DNA was used for library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina).

Bioinformatic analysis

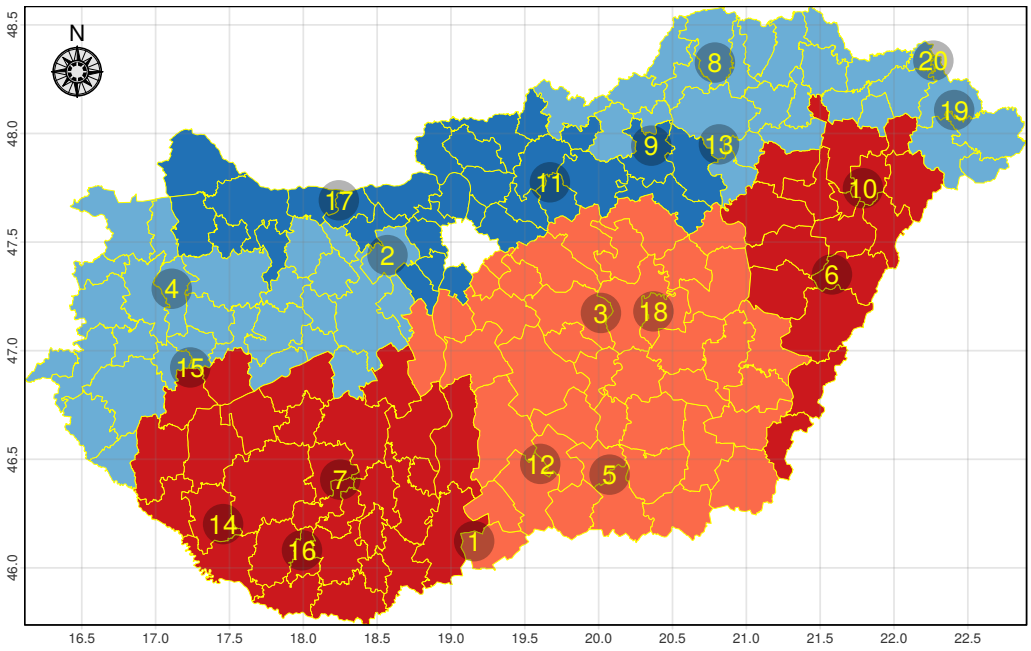
After merging the paired-end reads by PEAR[21] quality-based filtering and trimming was performed by Adapterremoval[22], using 15 as quality threshold and reads longer than 50 bp were retained only. The *Apis mellifera* genome (Amel_HAv3.1) sequences as host contaminants were filtered out by Bowtie2[23] with the very-sensitive-local setting minimising the false positive match level[24] in further metagenome classification. The remaining reads after deduplication by VSEARCH[25] were taxonomically classified using Kraken2 (k=35)[26] with the NCBI non-redundant nucleotide database[27]. Core bacteria was defined as the relative abundance of agglomerated counts on species-level above 0.1% at least in half of the samples. The taxon classification data was managed in R[20] using functions of package phyloseq[28] and microbiome[29].

Statistical analysis

The within-subject diversity (α-diversity) was assessed using the numbers of observed species (richness) and the Inverse Simpson's Index (evenness). These indices were calculated in 1000 iterations of rarefied OTU tables with a sequencing depth of 6129. The average over the iterations was taken for each apiary. The α-diversity expressed by Inverse Simpson's Index was compared between the conditions using linear models. Comparing the samples collected in March and May, a mixed-effect model was applied to handle the repeated measure by apiary as a random factor.

The between-subject diversity (β-diversity) was assessed by Bray-Curtis distance[30] based on the relative abundances of bacteria species. Using this measure, non-metric multidimensional scaling (NMDS) ordination was applied to visualise the samples' dissimilarity. To examine statistically whether the bacterial species composition differed by climate or seasonal

a)



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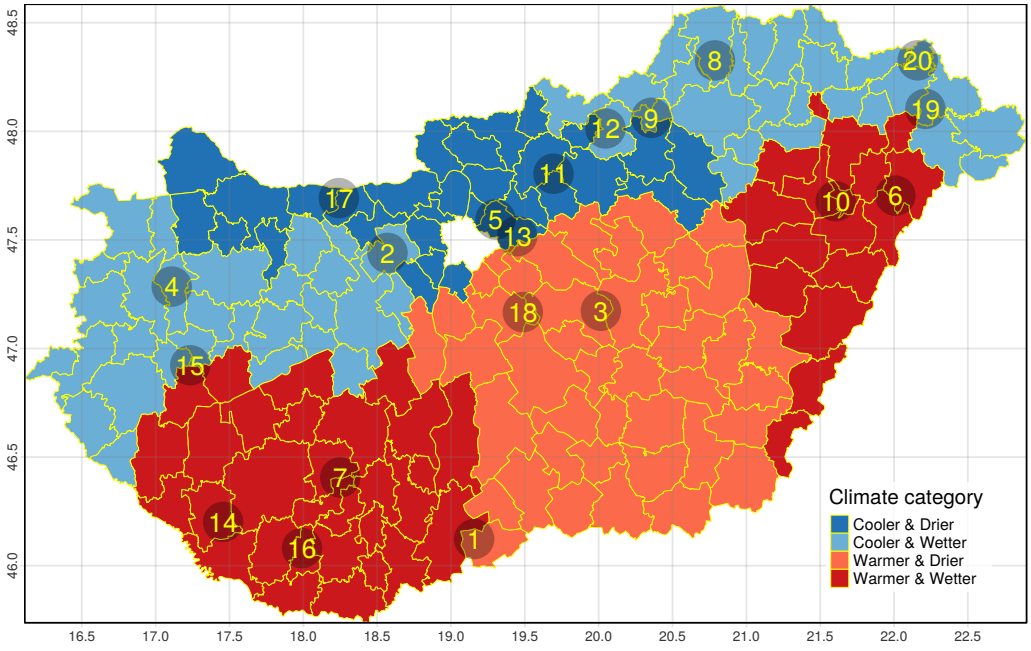

Figure 1. Climate category spatial pattern and sampling points The Hungarian local administrative units (LAU 1) coloured by climatic categories based on growing degree days (GDD) and precipitation of period 2008-2017. The numbers represent the identification number of the sampled apiaries in March (a) and May (b).

conditions PERMANOVA (Permutational Multivariate Analysis of Variance[31]) was performed using vegan package[32] in R[20]. The abundance differences in core bacteriome between the seasonal or climate conditions were analysed by a negative binomial generalised model of DESeq2 package[33] in R[20]. This approach was applied following the recommendation of Weiss et al.[34]. None of the compared groups had more than 20 samples, and their average library size ratio was less than 10. Since the apiaries were sampled repeatedly for capturing the seasonal effect, the samples were paired in the model. Considering the multiple comparisons, FDR-adjusted p value (q value) was considered less than 0.10 as significant. The statistical tests were two-sided.

Results

From the twenty apiaries, three-three families were sampled on the first collection period in March. In May there were two apiaries (ID: 6, 13) where one of the original three sampled colonies were not available (e.g. death of the queen, kept at different geographical location). For those apiaries, the pool contained only the two colonies that remained. Eight of the apiaries were migrated (ID: 5, 6, 9, 10, 12, 13, 18, 20), keeping the honey bees in different LAU in March and May. In four apiaries (ID: 5, 9, 12, 13) from these eight, the environmental classification was changed from March to May, because of migration. The apiary ID 5 and ID 12 migrated from a warmer LAU to a cooler one. The apiary ID 9 and ID 12 migrated from an LAU with less precipitation to an LAU with more precipitation. Apiary ID 13 moved from an LAU with more precipitation to an LAU with less precipitation.

The shotgun sequencing generated paired-end read counts of samples ranged between 311,931 and 546,924 with a mean of 413,629. The OTU table created by Kraken2 taxonomic classification contained counts of samples ranging between 11,646 and 114,573 with the mean of 44,280. The minimum, maximum and median read counts of the samples assigned as bacteria species were 6,129, 62,836 and 270,774, respectively.

The numbers of observed species and the Inverse Simpson's Index α-diversity metrics by environmental and seasonal strata are shown in Fig 2. The Inverse Simpson's Index outliers in the samples collected in March from districts with less and more precipitation are the apiary ID 9 and ID 13, respectively. The apiary ID 12 sampled in March had an outlying high number of observed species too. From the same sampling period among the samples gathered from districts with more precipitation, apiary ID 8 appears as an outlier.

In samples from the cooler environment collected in March, the α-diversity was significantly (p=0.0215) higher than in samples from warmer districts. There was no significant difference in α-diversity between the precipitation categories of samples collected in March (p=0.178). In samples collected in May, there was no significant difference between GDD or precipitation categories (p=0.463 and p=0.456, respectively).

The dissimilarity of the samples' bacteria species profiles (β-diversity) is visualised by NMDS ordination (Fig 3-4), based on Bray-Curtis distance. The ordination stress was 0.144, 0.062 and 0.116 for all samples, samples of March and samples of May, respectively. By PERMANOVA analysis of bacteria species composition significant (p=0.002) difference was found between the samples from March and May. The samples' bacteriome from March showed similar significant (p=0.02) distance between the cooler and the warmer districts. From the same period, the precipitation levels did not differ significantly (p=0.155). In the samples gathered in May, there was no significant distance between GDD and precipitation categories (p=0.277 and p=0.849, respectively).

The core bacteriome members having relative abundance above 0.1% in at least half of the samples are *Bartonella apis*, *Bifidobacterium asteroides*, and *Bifidobacterium coryneforme*, *Bifidobacterium indicum*, *Commensalibacter sp. AMU001*, *Frischella perrara*, *Gilliamella apicola*, *Lactobacillus apis*, *Lactobacillus bombi*, *Lactobacillus helsingborgensis*, *Lactobacillus kullabergensis*, L*actobacillus kunkeei*, *Lactobacillus mellis*, *Lactobacillus sp. wkB8*, *Snodgrassella alvi*. The relative abundances of each apiary's core bacteriome species are plotted by sampling periods and environmental strata in Fig 5. Table 1 shows the overall and grouped mean and standard deviation of core bacteriome species' relative abundances.

Associations between seasonal, climatic condition levels and the abundance of core bacteriome species were examined using negative binomial generalized linear models[33] (Table 2). The abundance of *Bartonella apis* (FC: 15.41, q<0.00001), *Bifidobacterium asteroides* (FC: 1.61, q=0.0084), *Commensalibacter sp. AMU001* (FC: 2.46, q=0.00001), *Lactobacillus helsingborgensis* (FC: 1.7, q=0.008), *Snodgrassella alvi* (FC: 1.49, q=0.011) is significantly increased from March to May. In the same comparison the abundance of *Lactobacillus apis* (FC: 0.64, q=0.0066), *Lactobacillus bombi* (FC: 0.64, q=0.0052), *Lactobacillus kullabergensis* (FC: 0.57, q=0.00056), *Lactobacillus mellis* (FC: 0.64, q=0.0052) was decareased significantly. In samples collected in March, the abundance of *Lactobacillus kunkeei* (FC: 3.86, q=0.094) was significantly higher in warmer regions than in cooler ones. In the same period the abundance of *Bartonella apis* (FC: 0.02, q<0.00001) and *Bifidobacterium asteroides* (FC: 0.47, q=0.0027) was significantly lower in warmer LMUs than in cooler ones. In March samples, the abundance of *Bartonella apis* (FC: 11.52, q=0.0066) was significantly higher in districts with more precipitation than in LMUs with less precipitation. The opposite of this was found in the case of *Lactobacillus kunkeei* (FC: 0.13, q=0.00087). In samples collected in May, none of the core bacteriome species showed significant alterations in abundance neither by GDD categories nor by the precipitation levels.

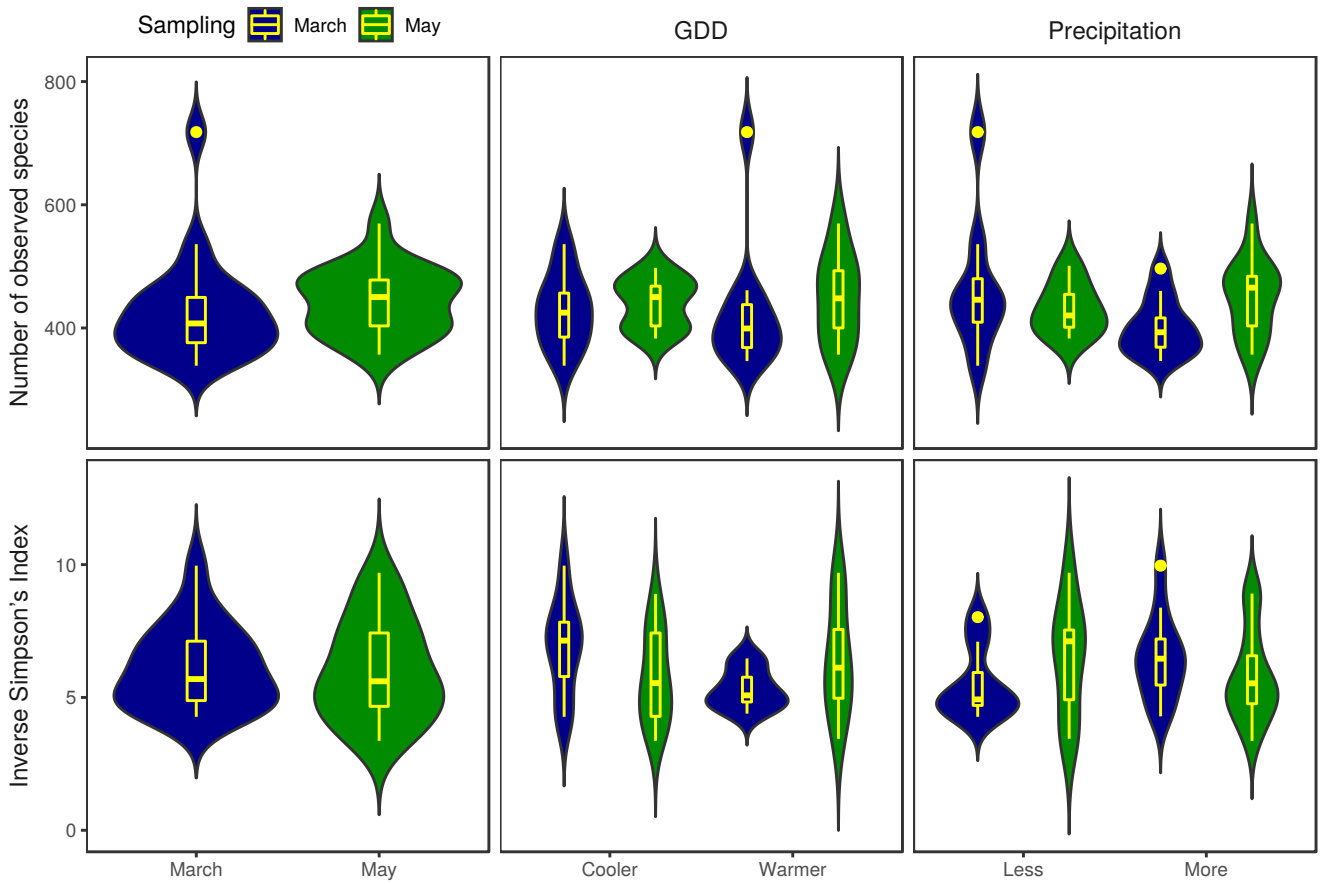

Figure 2. Richness and evenness of honey bee gut bacteriome by sample groups. The numbers of observed species (richness) and the Inverse Simpson's Index (evenness) as α-diversity metrics are presented as a violin and box plot combination. These indices were calculated in 1000 iterations of rarefied OTU tables with a sequencing depth of 6129. The average over the iterations was taken for each apiary. The violin plot shows the probability density, while the box plot marks the outliers, median and the IQR. For Inverse Simpson's Index, the comparison of samples from cooler and warmer districts collected in March showed significant (p=0.0215) differences only.

Discussion

We have examined the bee gut microbiome's population-level dynamics in different climatic conditions and at different time points in the honey-producing season by determining its α- and β-diversity. Besides the uniformity of the microbiome's richness and evenness in almost every examined condition, a significant difference was found between warmer and cooler regions (Figure 2). Considering the differences in the beta-diversity, only the sampling times and the different precipitation levels among the March samples showed significant distinctions (Figures 3 and 4). In concordance with previous findings, where only slight seasonal variations were observable[35,36], these results indicate a very conserved, although not unchangeable nature of the honey bee gut microbiome.

Bees not only possess a very constant, but a rather simple microbiome consisting of only a small subset of phylotypes and associated species, regardless of geographic locations[35–44]. Unsurprisingly, our analysis yielded a similar result to the above-cited articles (Figure 5).

However, on the species level, we found the abundance of several bacterial residents of the honey bee gut to exhibit temporal and environmental differences. On the temporal level, for example, *Snodgrassella alvi*, a microaerophilic Gram-negative bacterium[45] showed an elevated proportion in the samples collected in May (Figure 5, Table 2). *S. alvi* possibly colonises the gut through an oral-faecal way in the first days of the adult bee's life[46]. This bacterium forms a biofilm-like layer on the intima of the ileum of the bee gut, along with *Gilliamella apicola*, another characteristic member of the honey bee gut microbiome[38,40,41,47]. *G. apicola* was the most abundant member of the core-bacteriome in our study (Table 1). They do not have only a strong relationship in the biofilm formation, but possibly complement each other in metabolism as well. It

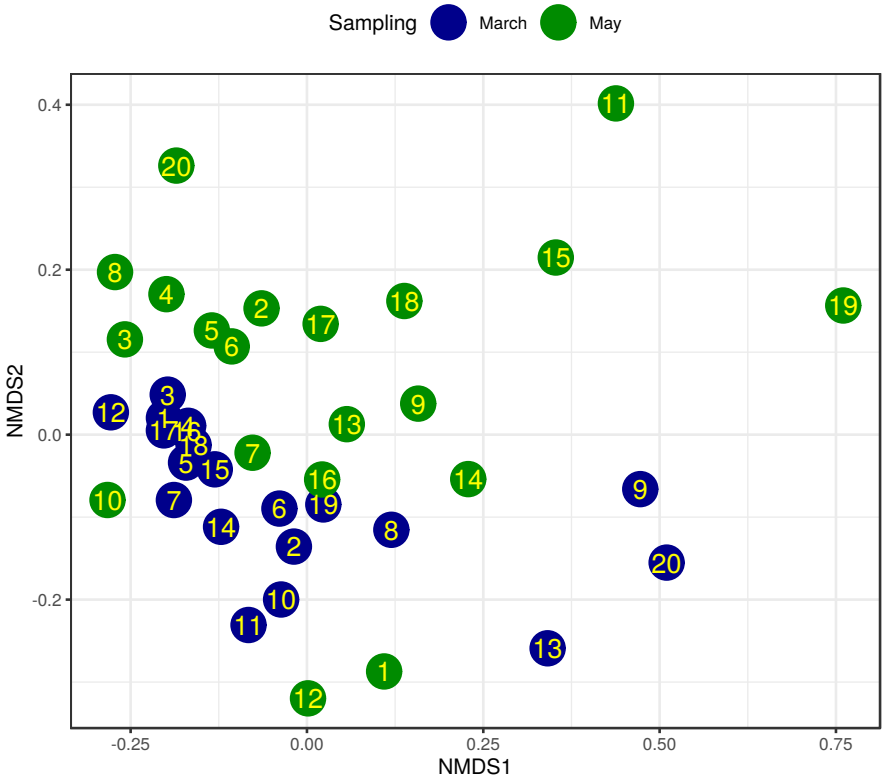

Figure 3. NMDS ordination of bacteriome for sampling March and May. Bray-Curtis dissimilarity was calculated using the species-level abundance of core bacteria. The samples from apiaries (IDs in dots) collected in March (blue) and May (green) are plotted using these dissimilarities. Based on the same measures, PERMANOVA analysis showed significant differences between the sampling time periods (p=0.002, stress=0.144).

was previously shown that *S. alvi*, despite lacking the enzymes of carbohydrate degradation, gain energy from oxidative phosphorylation, which is made possible by utilising the required substrates produced by other microorganisms, for example, *G. apicola*[48]. The role of these microbes in the honey bee gut is very diverse, including the defence against different pathogens by the biofilm formation[49]. Also, *G. apicola* was shown to exhibit pectinase activity, a major constituent of the pollen wall[42,50]. Their relevance is further stressed out by the fact that an *S. alvi* strain was found to possess streptomycin resistance genes associated with transposons previously found in *E. coli*[51] as well and also *Snodgrassella* is a candidate microorganism for a bioengineered microbe against bee pathogens[52].

Besides *S. alvi*, several Lactic Acid Bacteria (LAB), constituting the core-bacteriome in our experiment, showed temporal differences as well (Figure 5, Table 2). They are Gram-positive, catalase-negative, lactic acid fermenting bacteria[53] and regularly encountered members of the honey bee gut microbiota[37,43,54,55]. Their biofilm-forming capacity was also revealed[54,56,57]. LAB species associated with honey bees and bumblebees constitute a highly specialised flora in these insects' gut, unique in the insect world[56,58]. They can metabolise different types of sugars, making them a suitable inhabitant of these species' intestinal tract, considering that honey bee diet contains a large amount of carbohydrates. They also can degrade carbohydrates that are indigestible for the host[42,50,56]. Nevertheless, it was shown that they even exhibit an ability to metabolise sugars toxic to honey bees[42,50,56,59]. Besides the above mentioned nutritional role in the bee gut, they also have a protective function against different pathogenic threats on honey bees[60,61]. As a reason of the above-mentioned attributes of the LAB species, it is believed, that at least partly, they are the reason of the beneficial effects of the fresh honey[62].

Two members of core-bacteriome in this study showed temporal shifts were *Bartonella apis* and *Commensalibacter sp. AMU001* (both of them was more abundant in the samples collected in May, Figure 5, Table 1). Both of them are non-core members of the honey bee gut microbiome, and members of the *Alphaproteobacteria* class[37,38,40,41,43,63,64]. *Bartonella apis* ferments carbohydrates under microaerophilic conditions[64]. It might also have a role in recycling nitrogen end products of the honey bee nitrogen metabolism and the degradation of plant secondary metabolites found in the nectar or pollen[65]. *Commensalibacter* genus is a member of the Acetobacteraceae family, which is prevalent in the intestinal tract of many insects,

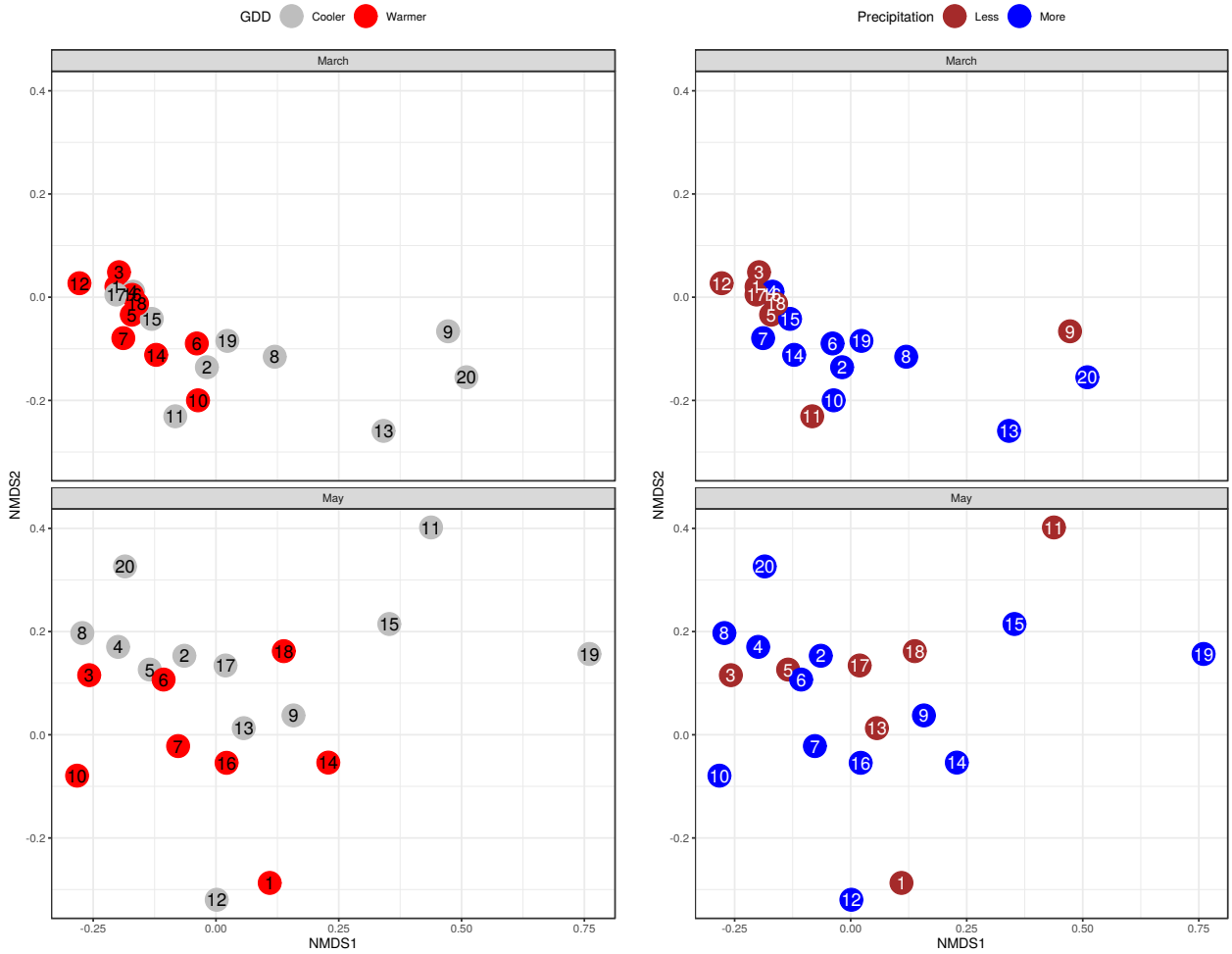

Figure 4. NMDS ordination of bacteriome for environmental condition categories by sampling period. The colours represent the environmental condition categories, and the numbers are corresponding to the apiary IDs. The stress was 0.062 and 0.116 for March and May, respectively. The samples' bacteriome from March showed significant (p=0.02) distance between the cooler and the warmer districts. From the same period, the precipitation levels did not differ significantly (p=0.155). In the samples gathered in May, there was no significant distance neither between GDD- nor between precipitation categories (p=0.277 and p=0.849, respectively).

mostly those that consume a sugar-rich diet[66], for example, butterflies[67]. They were also detected in flowers, where they possibly originated from visiting insects[68]. However, they were also found in the gut of the red carpenter ant (*Camponotus chromaiodes*), which species were phylogenetically close to those originated from different bees[69].

The observed temporal changes in the abundance of the above-mentioned species could be due to several reasons. For example, the bees' foraging behaviour can change by time to match the colonies' nutritional needs[70]. The relocation of the hives during the season and the seasonal variation of the visited flowers by the foragers[71,72] can account for such changes. Others also noted similar changes in the abundance of the honey bee gut microbial species. Subotic and colleagues[35] also found an increased abundance of *Snodgrassella alvi* over time and variable changes considering different LAB species. Although, they found an increase of *Bartonella apis* on the contrary to our observations. However, it is important to note that they collected the samples from June to September and only from one location.

Besides the temporal changes, we also observed alternations in the abundance of some particular species between various environmental conditions. For example, the Fructophilic Lactic Acid Bacterium (FLAB), *Lactobacillus kunkeei* showed significantly increased abundance in the warmer and drier regions in samples collected in May (Table 2).

FLAB is a distinct and special group of LAB, whose main characteristics are the preference of fructose over glucose and that they can metabolise glucose only in the presence of external electron acceptors (e.g. pyruvate or oxygen)[73,74]. Considering

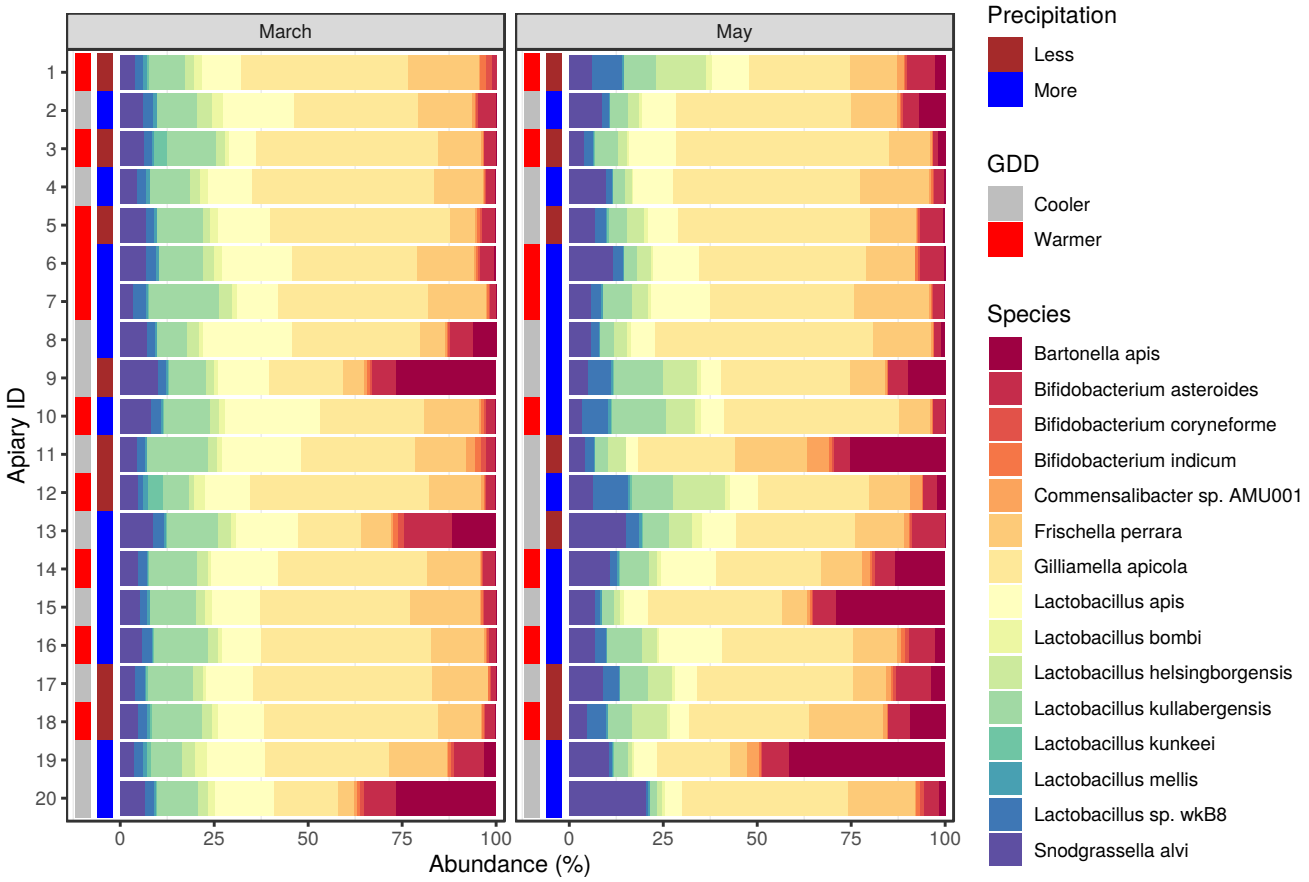

Figure 5. Core bacteriome composition of honey bee gut samples. The relative abundance is plotted for the first (March) and second (May) sampling. Besides the bacteria species of the core bacteriome, the environmental condition categories of sampling places are also marked. The growing degree-day (GDD) categories are coloured by grey and red on the plot's left sidebar. The next to the right from GDD shows the precipitation categories of apiaries.

the honey bee's fructose-rich diet and the high sugar tolerance of FLAB species makes them suitable for this environment[74,75].

L. kunkeei is the most important FLAB species found in the honey bee intestinal tract, most abundantly found in the honey stomach[36,55,57,73–76]. Although usually, *L. kunkeei* is one of the most abundant members of the honey bee intestinal tract[41], in this case, we found *Lactobacillus apis* and *Lactobacillus kullabergensis* to be that (Figure 5, Table 1). However it is an important member of the bee microbiome, *L. kunkeei* can be found in several bee products, such as honey or the bee bread[55,75,77]. It can also be found in flowers and fruits[73,78]; nevertheless, it can get into the products made of them. For example, *L. kunkeei* was originally isolated from wine[79]. This species' previous gene sequence analysis revealed a major gene loss[80] accompanied with an enrichment of genes involved in fructose metabolism and lipid- and amino acid synthesis[80–82]. The genetic composition of this species indicates its high adaptation to sugar (mainly fructose) rich environments. Besides the highly adapted nature to this environment, *L. kunkeei* was shown to have different techniques developed to protect its ecological niche. Phenotypical and genotypical experiments demonstrated the ability of *L. kunkeei* to form biofilms, which is a major advantage in antimicrobial activity. This potential makes it a good candidate probiotic either for humans and insects[81,83,84]. These attributes make *L. kunkeei* a protective barrier against environmental pathogens in the honey bees. It was found to be effective against different pathogens, including *Nosema ceraneae*, *Paenibacillus larvae* and *Melissococcus plutonius*[75,85]. Also MP2 strain of *L. kunkeei* was shown to possess genes for isoprenoid synthesis, indicating its possible role in honey bee nutrition[81]. Its effect was determined even in bee bread maturation[77].

Changes in the abundance in different environmental conditions were also found in the case *Bartonella apis* (more abundant in colder regions with more precipitation, Table 2) and *Bifidobacterium asteroides* (more abundant in colder regions, Table 2).

Differences observed in various environmental conditions can be originating from the fact that different environmental conditions, for example, temperature or exposure to sunlight can affect the nectar production and nectar composition of different

Table 1. Relative abundances by environmental and seasonal categories.

	Species	All samples Mean (SD)	GDD Cooler	Warmer	Precipitation Less	More
March						
	Bartonella apis	3.78 (8.25)	7.41 (10.7)	0.14 (0.14)	3.41 (9.32)	4.02 (7.89)
	Bifidobacterium asteroides	4.08 (3.02)	5.64 (3.62)	2.52 (0.92)	2.83 (1.64)	4.92 (3.49)
	Bifidobacterium coryneforme	0.64 (0.43)	0.72 (0.48)	0.56 (0.38)	0.73 (0.54)	0.58 (0.35)
	Bifidobacterium indicum	0.62 (0.41)	0.69 (0.44)	0.55 (0.39)	0.71 (0.52)	0.56 (0.33)
	Commensalibacter sp. AMU001	0.56 (0.49)	0.66 (0.65)	0.45 (0.24)	0.7 (0.73)	0.46 (0.22)
	Frischella perrara	12.49 (4.11)	11.46 (4.85)	13.52 (3.13)	12 (4.25)	12.82 (4.17)
	Gilliamella apicola	37 (10.63)	31.92 (11.56)	42.08 (6.9)	41.6 (10.67)	33.93 (9.85)
	Lactobacillus apis	15.05 (4.73)	16.23 (4.03)	13.87 (5.29)	12.76 (3.93)	16.57 (4.75)
	Lactobacillus bombi	1.73 (0.71)	1.75 (0.78)	1.71 (0.68)	1.72 (0.75)	1.74 (0.72)
	Lactobacillus helsingborgensis	2.71 (0.61)	2.91 (0.57)	2.52 (0.61)	2.26 (0.49)	3.01 (0.49)
	Lactobacillus kullabergensis	11.78 (2.77)	11.17 (2.42)	12.4 (3.08)	11.49 (2.82)	11.98 (2.84)
	Lactobacillus kunkeei	0.6 (1.13)	0.27 (0.35)	0.94 (1.52)	1.22 (1.61)	0.2 (0.3)
	Lactobacillus mellis	0.75 (0.29)	0.72 (0.29)	0.77 (0.31)	0.77 (0.36)	0.73 (0.26)
	Lactobacillus sp. wkB8	2.34 (0.41)	2.37 (0.28)	2.3 (0.53)	2.07 (0.34)	2.52 (0.37)
	Snodgrassella alvi	5.88 (1.8)	6.09 (2.11)	5.66 (1.5)	5.74 (2.06)	5.97 (1.69)
May						
	Bartonella apis	7.6 (11.43)	10.13 (13.9)	3.81 (4.87)	6.19 (8.99)	8.36 (12.84)
	Bifidobacterium asteroides	5.12 (2.24)	5.29 (2.37)	4.86 (2.16)	6.11 (2.83)	4.59 (1.75)
	Bifidobacterium coryneforme	0.43 (0.29)	0.42 (0.28)	0.44 (0.33)	0.42 (0.2)	0.43 (0.33)
	Bifidobacterium indicum	0.44 (0.28)	0.43 (0.25)	0.45 (0.34)	0.41 (0.19)	0.45 (0.33)
	Commensalibacter sp. AMU001	1.33 (1.37)	1.58 (1.66)	0.96 (0.69)	1.72 (1.89)	1.13 (1.02)
	Frischella perrara	12.79 (4.43)	12.42 (4.67)	13.35 (4.28)	13.79 (4.18)	12.25 (4.62)
	Gilliamella apicola	38.71 (10.71)	38.92 (11.45)	38.4 (10.25)	37.91 (12.12)	39.15 (10.38)
	Lactobacillus apis	8.74 (3.92)	6.82 (2.18)	11.62 (4.29)	7.67 (3.26)	9.32 (4.24)
	Lactobacillus bombi	0.97 (0.5)	0.95 (0.56)	1 (0.43)	1.07 (0.73)	0.92 (0.34)
	Lactobacillus helsingborgensis	5.15 (3.81)	4.74 (3.79)	5.77 (4)	6.64 (3.69)	4.35 (3.76)
	Lactobacillus kullabergensis	6.52 (3.43)	5.56 (3.42)	7.96 (3.08)	6.26 (1.61)	6.66 (4.15)
	Lactobacillus kunkeei	0.18 (0.17)	0.18 (0.2)	0.18 (0.14)	0.12 (0.15)	0.21 (0.18)
	Lactobacillus mellis	0.42 (0.19)	0.41 (0.2)	0.42 (0.19)	0.43 (0.27)	0.4 (0.15)
	Lactobacillus sp. wkB8	3.44 (2.44)	3.07 (2.5)	4.01 (2.4)	4.06 (2.07)	3.11 (2.63)
	Snodgrassella alvi	8.15 (4.16)	9.09 (4.64)	6.74 (3.07)	7.18 (3.91)	8.67 (4.35)

plant species[86–88]. Nevertheless, it is important to note the differences in plant species composition of each ecological region. The different flowers honey bees foraging can affect, for example, the honey stomach microbiota[55].

Frischella perrara is also commonly found in the gut of honey bees[38,40,41,89], and it was found to be a member of the core-bacteriome in our study as well. Although it was present here, we could not reveal any variation in its abundance neither temporally, neither between different environmental conditions (Figure 5, Table 1, Table 2). *F. perrara* is a close relative of *G. apicola*, and has a similar metabolism[89]. Usually found in the ileum of the bee gut[90] and was shown to share a strong relationship with the honey bee immune system[91].

Although consisting only of a few species, honey bee microbiome possesses several functions affecting its host's health. It has a role in the degradation of different polysaccharides originating from the bees' diet, for example, the pollen walls. These are often undegradable for bees[42,92]. They might also have a role in recycling the nitrogen waste materials of the honey bee nitrogen metabolism[92] and can metabolise sugars potentially toxic for bees[42]. Besides these metabolic functions, the gut microbiome has a positive impact on the host immune system[93], and it protects the bees from pathogens[93,94].

Considering the honey bee gut microbiome's diverse role, everything affecting it can potentially threaten bee health. For example, it was shown that the widely used herbicide, glyphosate, impacts the microbiota[9,10], and other stressors can induce changes in its composition as well[93,95–97]. However, to understand and evaluate the potential impact of these effects on honey bees, we need to know the microbiota's normal composition and function.

Conclusion

Based on our results, one may conclude that the composition of healthy core bacteriome in honey bees varies depending on the climatic and seasonal conditions. This is probably since climatic characteristics, and the vegetation states determine the availability and nutrient content of flowering plants. Our study results prove that to gain a thorough understanding of a microbiome's natural diversity, we need to obtain the necessary information from extreme ranges of the host's healthy state.

Table 2. Abundance alterations of core bacteriome by seasonal and climatic conditions. A negative binomial model estimated the association between species abundance of core bacteriome and sampling seasons, GDD- and precipitation level.

Samples	Species	Mean counts†	Fold change (95% CI)	q§	Mean counts	Fold change (95% CI)	q
		GDD Warmer vs. cooler			Precipitation More vs. less		
March	*Bartonella apis*	1068.87	0.02 (0, 0.08)	<0.00001	*321.94	11.52 (2.73, 48.63)	0.00660
	Bifidobacterium asteroides	1144.10	0.47 (0.31, 0.71)	0.00274	1144.10	1.52 (0.94, 2.47)	0.26310
	Bifidobacterium coryneforme	180.26	0.78 (0.47, 1.3)	0.55291	180.26	0.72 (0.43, 1.2)	0.38749
	Bifidobacterium indicum	176.76	0.81 (0.49, 1.33)	0.55291	176.76	0.72 (0.43, 1.19)	0.38749
	Commensalibacter sp. AMU001	162.07	0.68 (0.37, 1.25)	0.46158	162.07	0.58 (0.32, 1.05)	0.26310
	Frischella perrara	3662.30	1.18 (0.84, 1.65)	0.55291	3662.30	0.97 (0.68, 1.37)	0.84853
	Gilliamella apicola	11004.31	1.29 (0.94, 1.78)	0.42005	11004.31	0.75 (0.55, 1.04)	0.26310
	Lactobacillus apis	4366.20	0.84 (0.65, 1.09)	0.46158	4366.20	1.17 (0.89, 1.54)	0.42531
	Lactobacillus bombi	495.19	1.03 (0.73, 1.44)	0.86802	495.19	0.89 (0.63, 1.26)	0.65673
	Lactobacillus helsingborgensis	786.53	0.87 (0.71, 1.07)	0.46158	786.53	1.2 (0.96, 1.5)	0.26310
	Lactobacillus kullabergensis	3454.42	1.11 (0.87, 1.41)	0.55291	3454.42	0.96 (0.74, 1.24)	0.79727
	Lactobacillus kunkeei	187.49	3.86 (1.25, 11.89)	0.09368	187.49	0.13 (0.05, 0.36)	0.00087
	Lactobacillus mellis	213.99	1.13 (0.79, 1.61)	0.62292	213.99	0.86 (0.59, 1.25)	0.56927
	Lactobacillus sp. wkB8	681.21	0.97 (0.8, 1.18)	0.84023	681.21	1.1 (0.89, 1.36)	0.54445
	Snodgrassella alvi	1706.23	0.92 (0.71, 1.2)	0.62292	1706.23	0.94 (0.71, 1.24)	0.77490
May	*Bartonella apis*	1553.31	0.28 (0.08, 1.01)	0.22572	1553.31	1.46 (0.36, 5.85)	0.86244
	Bifidobacterium asteroides	942.33	0.83 (0.56, 1.22)	0.74756	942.33	0.9 (0.6, 1.37)	0.86244
	Bifidobacterium coryneforme	79.75	0.92 (0.49, 1.73)	0.98269	79.75	1.13 (0.59, 2.18)	0.86244
	Bifidobacterium indicum	81.39	0.9 (0.49, 1.63)	0.98269	81.39	1.23 (0.66, 2.28)	0.86244
	Commensalibacter sp. AMU001	255.76	0.51 (0.26, 1)	0.22572	*204.88	1.33 (0.7, 2.5)	0.86244
	Frischella perrara	2522.61	1.02 (0.66, 1.56)	0.98269	2522.61	0.98 (0.62, 1.54)	0.92388
	Gilliamella apicola	7869.43	1.01 (0.65, 1.57)	0.98269	7869.43	1.09 (0.69, 1.73)	0.86244
	Lactobacillus apis	1698.58	1.62 (1.07, 2.45)	0.22572	1698.58	1.23 (0.77, 1.97)	0.86244
	Lactobacillus bombi	183.30	1.06 (0.68, 1.66)	0.98269	183.30	1.03 (0.65, 1.65)	0.92388
	Lactobacillus helsingborgensis	952.28	1.2 (0.66, 2.17)	0.98269	952.28	0.79 (0.43, 1.45)	0.86244
	Lactobacillus kullabergensis	1258.90	1.5 (0.93, 2.41)	0.29310	1258.90	1.17 (0.7, 1.97)	0.86244
	Lactobacillus kunkeei	36.06	1.08 (0.39, 2.97)	0.98269	36.06	1.65 (0.59, 4.57)	0.86244
	Lactobacillus mellis	78.82	1 (0.65, 1.56)	0.98269	78.82	1.12 (0.7, 1.77)	0.86244
	Lactobacillus sp. wkB8	648.44	1.33 (0.75, 2.36)	0.74756	648.44	0.91 (0.49, 1.66)	0.86244
	Snodgrassella alvi	1567.32	0.66 (0.43, 1.02)	0.22572	1567.32	1.43 (0.9, 2.25)	0.86244
		May vs. March					
All	*Bartonella apis*	1538.45	15.41 (6.07, 39.17)	<0.00001			
	Bifidobacterium asteroides	1074.14	1.61 (1.16, 2.24)	0.00837			
	Bifidobacterium coryneforme	118.29	0.74 (0.52, 1.05)	0.12117			
	Bifidobacterium indicum	118.69	0.78 (0.55, 1.11)	0.18588			
	Commensalibacter sp. AMU001	232.29	2.46 (1.7, 3.57)	0.00001			
	Frischella perrara	3046.15	1.17 (0.85, 1.61)	0.34279			
	Gilliamella apicola	9304.64	1.22 (0.92, 1.62)	0.18588			
	Lactobacillus apis	2740.93	0.64 (0.48, 0.86)	0.00656			
	Lactobacillus bombi	309.86	0.64 (0.48, 0.84)	0.00519			
	Lactobacillus helsingborgensis	928.22	1.7 (1.19, 2.43)	0.00800			
	Lactobacillus kullabergensis	2115.13	0.57 (0.43, 0.76)	0.00056			
	Lactobacillus kunkeei	99.88	0.64 (0.36, 1.14)	0.16276			
	Lactobacillus mellis	133.69	0.64 (0.49, 0.85)	0.00519			
	Lactobacillus sp. wkB8	680.91	1.35 (0.98, 1.87)	0.10342			
	Snodgrassella alvi	1700.90	1.49 (1.12, 1.98)	0.01099			

†Sequence read counts were normalized by dividing raw counts by DESeq size factors
§FDR-adjusted p-value. FDR adjustment was conducted in each pairwise comparison separately
*Mean counts of two comparisons differ because of outlier replacement

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Author contributions statement

NS takes responsibility for the integrity of the data and the accuracy of the data analysis. LB, LM, NS and RF conceived the concept of the study. GM, LB, LM, NS and RF performed sample collection and procedures. MP and NS participated in the bioinformatic and statistical analysis. MP and NS participated in the drafting of the manuscript. DT, GM, LB, LM, MP, NS, and RF carried out the manuscript's critical revision for important intellectual content. All authors read and approved the final manuscript.

Additional information

Availability of data and material The short read data of samples are publicly available and accessible through the PRJNA685398 from the NCBI Sequence Read Archive (SRA). Reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA685398?reviewer=3if5rmnvevgdq3qskhos2dlj4r
Competing interests The authors declare that they have no competing interests.
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