

Variation of gut microbiota composition in a honey bee breeding population: exploring potential links with docility and honey production



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ABSTRACT

The current global decline of bee populations is of great concern due to their crucial role as pollinators and for the conservation of biodiversity. Today the survival of bees is increasingly dependent on beekeeping practices. In this context, the present study explores the composition of honey bee gut microbiota, its changes in time and its potential relationship with two key traits of interest to beekeepers: docility and honey yield. In this study, 77 colonies, belonging to a breeding population selected for these phenotypes, were sampled three times over a 5-month period, leading to a total of 190 samples. Results showed that *Apis mellifera*, differently from other insects, hosts a specialised gut microbial community composed of five ever-present bacterial taxa. However, the proportional abundance of these bacterial taxa undergoes significant seasonal shifts, reflecting seasonal changes in diet. Moreover, the association between the composition of the honey bee microbiota and honey production was identified. In conclusion, this study offers insights into the composition and the seasonal dynamics of honey bee gut microbiota.

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Implications

Apis mellifera hosts a specialised gut microbial community that plays an important role in digestion, sugar fermentation, and immune defence. This study explores the honey bee gut microbiota composition, its seasonal variations, and its potential association with two key traits: docility and honey yield. Sampling 77 colonies over 5 months, our study unveils seasonal dynamics in microbiota, reflecting dietary changes. Moreover, associations between microbiota composition and honey yield, driven by Rhizobiaceae abundance, were observed, offering insights for beekeeping. However, no association was identified between microbiota and docility behaviour.

Introduction

The gastrointestinal tract hosts a complex composition of microbes. This microbial community shows significant variability in composition between different animal species as well as among individuals within the same species (Schloissnig et al., 2013). The microbial communities (microbiota) and their collective genomes (microbiome) play a role in digestion, influence several production

traits, and provide protection against pathogens in a wide range of animal species (Flint et al., 2012; Maltecca et al., 2020; Fan and Pedersen, 2021).

Unlike mammals, insects typically have less complex gut microbial communities, which tend to be unstable and predominantly populated by opportunistic environmental microorganisms rather than specialised symbiotic organisms that have adapted to the host environment (Dillon and Dillon 2004; Colman et al., 2012; Engel and Moran, 2013). However, honey bees (*Apis mellifera*) have a specialised core gut microbiota transmitted through social interactions adapted to a carbohydrate-rich diet (Lee et al., 2015; Powell et al., 2014; Zheng et al., 2018) composed by bacteria of the genera *Bifidobacterium*, *Lactobacillus*, *Bombilactobacillus*, *Gilliamella* and *Snodgrassella* (Kwong and Moran, 2016).

In detail, *Bifidobacterium*, *Lactobacillus* and *Bombilactobacillus* are Gram-positive bacteria that constitute the main part of honey bee gut microbiota, known as Lactic Acid Bacteria (LAB). LAB utilise carbohydrates for energy production, by lactic acid and short-chain fatty acid production (Hatti-Kaul et al., 2018; Nowak et al., 2021). Moreover, LAB play a key role in maintaining gut health and aiding in the preservation of pollen and nectar stores through their metabolic activities (Corby-Harris et al., 2014; Bradford et al., 2022). *Gilliamella* and *Snodgrassella* are two Gram-negative bacteria. *Gilliamella* is a fermenting sugar bacterium of Orbaceae family, while *Snodgrassella* is unable to ferment sugar and belongs to the Neisseriaceae family (Nowak et al., 2021).

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In honey bees, the gut microbiome has been demonstrated to play a critical role in host health, disease resistance, nutrition and growth (Nowak et al., 2021; Raymann and Moran, 2018; Almeida et al., 2023). In bumblebees, research has highlighted a potential gut-brain connection, where the microbiota influences neurophysiological processes that can shape behaviour (Leger and McFrederick, 2020). This suggests that similar mechanisms might be present in honey bees, where the gut microbiota appears to affect the levels of biogenic amines, which are linked to behavioural traits (Zheng et al., 2017).

Honey bees play a vital role in ecosystem conservation and biodiversity by serving as essential pollinators (Klein et al., 2007; Hung et al., 2018); however, their survival depends on beekeeping, where they are farmed for apicultural products as well as for their crucial role in crop pollination (Le Conte and Navajas, 2008; Le Conte et al., 2010; Henry et al., 2012).

This study explores the composition of the honey bee gut microbiota and its seasonal changes within colonies over a 5-month period. Moreover, it investigates the relationship between the gut microbiota and two traits, honey production and docility, crucial in the bee breeding context. To this end, differences in microbial richness (α -diversity) and microbial composition (β -diversity) were considered. The α -diversity reflects the diversity within each sample, indicating microbial richness and evenness, while β -diversity measures the variation in composition between samples (Lozupone and Knight, 2008). These metrics are essential for understanding the microbiota's role in influencing bee traits.

Honey is the primary apicultural product in terms of economic yield in beekeeping and for this reason, increasing its production is important. Docility is a desirable trait in bee management as calm and gentle bees are easier to manage, reducing the risk of getting stung or injuring bees and the queen during apiary inspection. Docility can be divided into two components: gentleness and calmness. Gentleness, also known as low defensive behaviour, measures the aggressiveness of bees towards humans. On the other hand, calmness measures the stillness and immobility of worker bees on the comb during inspections (Büchler et al., 2013; Guichard et al., 2021). Therefore, these traits are the most selected phenotypes in breeding programmes, along with swarming tendency and varroa resistance (Hoppe et al., 2020).

To this end, honey bees belonging to a breeding population, composed of 77 colonies were sampled three times between June and October 2021.

Material and methods

Data collection

Seventy-seven honey bee colonies were sampled three times in 2021: on June 24 during the honey production period (**T1**); on July 29 after honey production and before anti-varroa treatment (**T2**); on October 10, before the colonies entered in winter broodless condition (**T3**). All the colonies belonged to an apiary located in Cremona (LC), in Lombardy, Italy, situated in a predominantly rural and agricultural landscape. From each beehive, ten worker bees were collected and stored at -80°C in 50 ml empty Falcon tubes. The colonies were part of a selection programme in which virgin queens and drone were selected from the same population and mated at isolated mating stations. The selection was based on their estimated breeding values for three different traits: docility, honey production, and hygienic behaviour. These estimated breeding values are calculated using BLUP model adapted to the honey bee population structure as in Bienefeld et al. (2007).

The initial cohort was composed of 77 colonies. However, whenever a queen died for various reasons in a colony, the same colony was excluded from the selection scheme. Additionally,

two samples were lost during T1 due to data collection errors; however, these colonies were sampled at T2 and T3. As a result, the total number of sampling across the three different timepoints was 190, distributed as follows: 75 colonies in T1; 69 colonies in T2 and in 46 colonies in T3, with 10 worker bees sampled per colony at each timepoint.

During the study, a subset ($n = 31$) of colonies encountered the loss of their queens, due to various reasons, including accidental causes. To account for the queens that died between timepoints, a 'vitality' variable was created and colonies sampled at T1 were classified as follows: 45 colonies that survived throughout the entire experiment; eight colonies that experienced queen death between sampling at T1 and T2 and 23 colonies that were alive at T2 but experienced queen death between T2 and T3. All the queens were 1 year old at the beginning of the study. This variable was not the primary focus of the study but was included to ensure that queen mortality did not impact the results of the microbiota analysis (e.g. by introducing survival bias).

The phenotypes examined in this study include total honey yield (**HYT**), honey yield from the first harvest (**HY1**), honey yield from the second harvest (**HY2**), honey production class (**HYC**), and docility (**DOC**).

Total honey yield measured in kilograms combines honey yield obtained in May (HY1) and July (HY2). To calculate the net of honey yield, the super of each hive was weighed before being placed in its respective hive, and then reweighed at the end of the production period. The net honey yield was determined by the difference between the initial and final weights. Furthermore, the colonies were categorised based on their HYT phenotype into three HYC, defined according to the distribution of honey production observed in our colonies: low with honey yield ranging from 14.8 to 26.8 kg ($n = 15$), medium from 26.9 to 38.9 kg ($n = 40$), and high from 39 to 50.9 kg ($n = 20$). This classification was established by dividing the total production range into three approximately equal intervals to reflect low, medium, and high honey yield groups.

Docility includes two different traits: gentleness and calmness. Both these traits following the standard protocol were scored on a scale from 1 to 4, where 1 corresponds to aggressive behaviour or restless, and 4 represents the non-aggressiveness and immobility on the comb (Büchler et al., 2013; Uzunov et al., 2015). In our population, we have evaluated these two traits together by assigning a single "docility" score that looks into account the behaviour in both aspects. Moreover, docility was tested in each colony four times between April and May, and the final scores were the average of these values, resulting in a linear variable. Moreover, the colonies were categorised based on their docility final score into three groups: aggressive, medium and gentle. The descriptive statistics and correlation of the phenotypes are presented in [Supplementary Tables S1 and S2](#), while [Fig. 1](#) illustrates the timeline of sample collection and the assessment of these phenotypes.

DNA extraction, library preparation and sequencing

The digestive tract, from the crop to the midgut, was extracted by pulling it from the stinger of ten worker bees per colony. The ten digestive tracts were then pooled together, and DNA was extracted using the 'Blood & Tissue Genomic DNA Extraction kit' of Fisher Molecular Biology (Rome, Italy). The V3-V4 region of the 16S rRNA gene was amplified following the Illumina protocol '16S Metagenomic Sequencing Library Preparation' and utilising the following primers from Eurofins Scientific (Luxembourg City, Luxembourg): "16S Amplicon PCR Forward Primer" TCGTCGGCAGCGT CAGATGTG TATAAGAGACAGCCTACGGGNGGCWGCAG and "16S Amplicon PCR Reverse Primer" GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGG ACTACHVGGGTATCTAATCC.

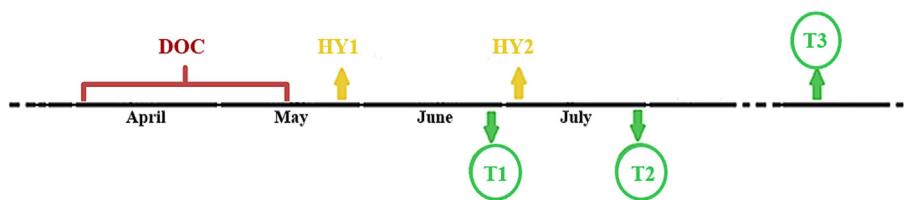


Fig. 1. Timeline of data collection. In green are the three timepoints of *Apis mellifera* sample collection (T1, T2 and T3). In red is the sampling period of phenotype docility (DOC) and in yellow are the first harvest honey yield (HY1) and second harvest honey yield (HY2). HYT and HYC are not shown as the first is given by the sum of HY1 and HY2, and the second is a categorisation based on HYT phenotype.

Quality control of the library was performed using the Fragment Analyzer (Agilent Technologies, Santa Clara, California, USA), and sequencing was carried out on an Illumina Novaseq sequencer (Illumina, San Diego, California, USA), producing paired-end reads of 250-bp. All steps, including DNA extraction, quality control, and sequencing, were performed by an external company, Nuova Genetica Italiana (Villa Guardia, Italy).

Bioinformatic analysis

Divisive Amplicon Denoising Algorithm 2 pipeline 1.16 from R package 'DADA2' was used to process the sequencing data (Callahan et al., 2016) with default settings, except for truncation or length filtering $\text{truncLen} = c(245, 240)$ using the 'filterAndTrim' function. The SILVA reference database (v132) was used for taxonomy assignments using a confidence cutoff of 2. For the subsequent analysis, a core microbiome was identified by retaining the Amplicon Sequence Variants (ASVs) that were present in at least 50% of the samples, to minimise sequencing errors and focus on the bacteria that are ecologically more important (Fridley and Wang, 2020; Neu et al., 2021). Moreover, for an accurate comparison between samples, the core was normalised through rarefaction, ensuring that the total sum of reads of each sample was 10 000. This value was used as the sample size parameter in the 'rarefy' function from the 'Phyloseq' package. To verify the representativeness of the bacterial communities within the core microbiome after normalisation to 10 000 reads, rarefaction curves were generated using the 'Vegan' package (Oksanen et al., 2015). The number of observed ASVs and α -diversity were estimated using Chao1, Shannon, Simpson, abundance-based coverage estimator (ACE) and Fisher diversity indices, using Phyloseq and Vegan packages (Oksanen et al., 2015; McMurdie and Holmes, 2013). The phenotypic traits and the α -diversity measures were centred and scaled using the z-score transformation with the 'scale' function in R.

Association between microbial composition and queen survival

The potential link between microbial composition and the survival of queens was tested by Kruskal-Wallis tests using the 'vitality' variable and the six α -diversity measures, using the 'kruskal.test' function in R. The relative taxa abundance at the family and genus levels was estimated and visualised using the 'plot.bar' function in R. Bray-Curtis dissimilarity metrics in R using the 'distance' function of Phyloseq package with the 'bray' method was used to assess diversity between 'vitality' groups.

Seasonal variation in microbial composition

To explore the seasonal variation in microbial composition, the entire dataset consisting of 190 samples was analysed. ANOVA was carried out using the 'aov' function in R, with the six α -diversity indices as dependent variables, and timepoint as the independent variable. Post-hoc Tukey tests were performed using the 'glht'

function from the 'Multcomp' package. The permutational multivariate analysis of variance (PERMANOVA) was conducted using the 'adonis2' function in the Vegan package (Oksanen et al., 2015), with the β -diversity of microbiota compositions (calculated using the Bray-Curtis distance) as the dependent variable, while timepoint was employed as the independent variable, with three levels. Non-metric Multidimensional Scaling was used for visualising the results of the β -diversity analysis. To identify ASVs that were significantly differentially present across the three timepoints, Differential abundance analysis was performed using the 'DESeq2' package in R, and the results were visualised with volcano plots (Neu et al., 2021). This analysis was conducted on both the rarefied core microbiome ASV dataset and the non-rarefied ASV dataset to comprehensively assess differential abundance patterns.

Association between microbial composition and phenotypic traits

The other aim of the study was to investigate the relationship between microbial composition and two phenotypic traits: honey yield and docility. Honey yield was examined across four distinct phenotypes: first honey yield, second honey yield, total honey yield and total honey yield categorised into three groups. In the analysis docility, the first, the second and total honey yield were treated as quantitative variables. Linear regression was conducted at each timepoint between these phenotypes and the α -diversity indexes. The 'lm' function in R was used to perform linear regressions. In the regressions performed, the dependent variable was structured so that it was temporally subsequent to the independent variable, although it is not possible to establish the causal effect a priori. PERMANOVA analysis was carried out with the "adonis2" function in Vegan package (Oksanen et al., 2015), using β -diversity based on Bray-Curtis distance as the dependent variable. The Independent variables were HYC (categorical variable) and docility (continuous variable). Differentially abundant ASVs between honey yield groups (HYC) were identified using a differential abundance analysis with the 'DESeq2' package in R. This analysis focused on the ASV counts across samples within each honey yield group, applied to both the rarefied core microbiome and non-rarefied datasets. The outcomes were visually represented through volcano plots (Anders and Huber, 2010). Moreover, the estimation and visualisation of taxonomic abundance at the family and genus levels were conducted using the 'plot.bar' function in R.

Results

Core microbiome and rarefaction data

The number of reads obtained at each step of the DADA2 pipeline for each sample is reported in Supplementary Table S3, with a summary provided in Supplementary Table S4. The core microbiome was selected from the total ASVs found to identify which taxa represent the most ecologically and functionally important microbial associates of the bees in each timepoint (Neu et al., 2021). The core microbiome selection was performed on the entire

dataset of 190 samples and on each of the three datasets for each timepoint. This led to a reduction in the number of ASVs. In detail, in the entire dataset of 190 samples, the total number of different ASVs found was 17 823, and the core microbiome, with 50% sharing, consisted of 2 036 ASVs. In the dataset of T1, initially composed of 8 232 ASVs, the core microbiome comprised 2 148 ASVs. In T2, the dataset initially comprised 8 788 ASVs, with a core microbiota of 2 225 ASVs. In T3, 7 926 ASVs were identified, and the core microbiota was of 1 912 ASVs.

Moreover, as known, disparities in library sizes frequently lead to variations in the number of reads, potentially introducing errors in the analysis. These observed differences may not always accurately reflect true biological distinctions but could be attributed to discrepancies in sequencing efficiency (Kim, 2023). For this reason, rarefaction was conducted, ensuring that the total sum of reads in each sample was set to 10 000. This normalisation step adjusts the number of reads per sample but does not affect the selection of the core microbiome or the number of ASVs identified.

Association between microbial composition and queen survival

To analyse the association between microbial composition and the survival of queens, Kruskal-Wallis tests were performed at T1 between vitality and each α -diversity index. The results (Supplementary Table S5) indicate that there are no statistically significant differences among the groups for any of these measures. The β -diversity index, calculated using the Bray-Curtis distance and visualised through non-metric multidimensional scaling, with a stress value of 0.22, confirms that the three groups based on vitality do not exhibit distinct clustering (Fig. 2c). Additionally, the comparison of taxa abundance among three vitality groups shows a remarkable similarity in the distribution of taxa abundance across these groups at both family and genus levels (Fig. 2a, b).

Seasonal variation

In all the timepoints, the five core microbiome genera of honey bees were identified as the most representative components: *Bifidobacterium*, *Lactobacillus*, *Bombilactobacillus* (formerly referred to as *Lactobacillus Firm-4*), *Gilliamella* and *Snodgrassella*. Additionally, *Frischella* (Orbaceae family), *Commensalibacter* (Acetobacteriaceae family), and Rhizobiaceae family (unknown genus) were detected in all three timepoints, although they are not part of the core microbiome and are less abundant. However, the relative abundance of these taxa shows seasonal variation as reported in Table 1 and illustrated in Fig. 3.

When grouping taxa by family, a decrease in Acetobacteraceae and an increase in Bifidobacteriaceae were observed over time. Additionally, at family level, the Rhizobiaceae family showed to be more represented in T2. Grouping taxa by genus revealed a decrease in *Snodgrassella* and an increase in *Bifidobacterium* proportion. Finally, *Lactobacillus* showed a higher representation at T2.

Non-metric multidimensional scaling based on Bray-Curtis dissimilarities was used to assess β -diversity and is displayed in Fig. 4b. The Bray-Curtis distance shows a partial separation of samples based on timepoint, with T3 exhibiting the greatest distinction particularly when compared to timepoint 1. In contrast, T1 and T2 appear closer to each other.

To ascertain the significance of the observed differences in the microbiome across the three timepoints, both ANOVA and PERMANOVA were conducted. In the ANOVA, timepoint was used as the independent variable, while the 6 α -diversity indexes were examined as dependent variables (Table 2). All six diversity measures show statistically significant differences (P -value < 0.05) among the three timepoints. Fig. 4a shows graphical representation of Shannon and Simpson indices, highlighting a general slightly declining trend across the three timepoints, with T3 displaying

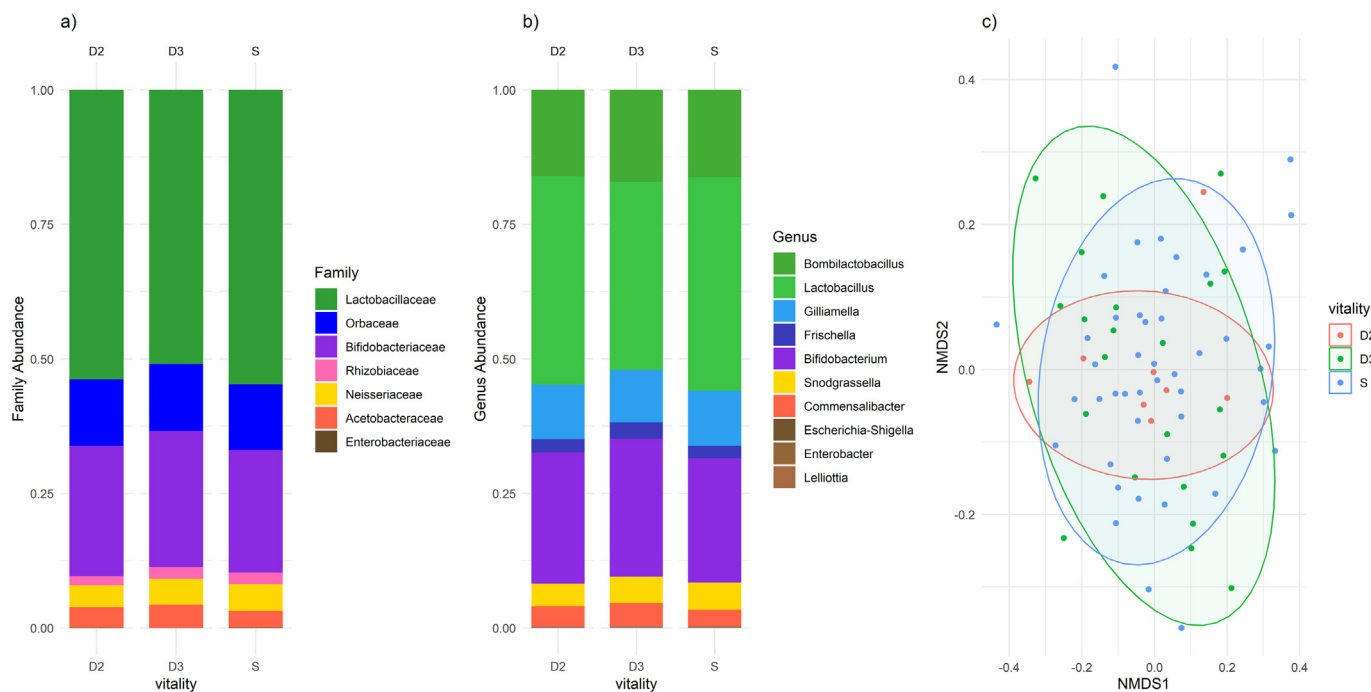


Fig. 2. Bar chart of the taxa abundance by family (A) and genus (B) across vitality groups of *Apis mellifera*. On the right (C), β -diversity based on Bray-Curtis distance is visualised through non-metric multidimensional scaling (NMDS), with a stress value of 0.22. Vitality groups are shown as follows: S (colonies that survived throughout the entire experiment) in blue, D2 (colonies that experienced queen death between T1 and T2) in red, and D3 (colonies that experienced queen death between T2 and T3) in green.

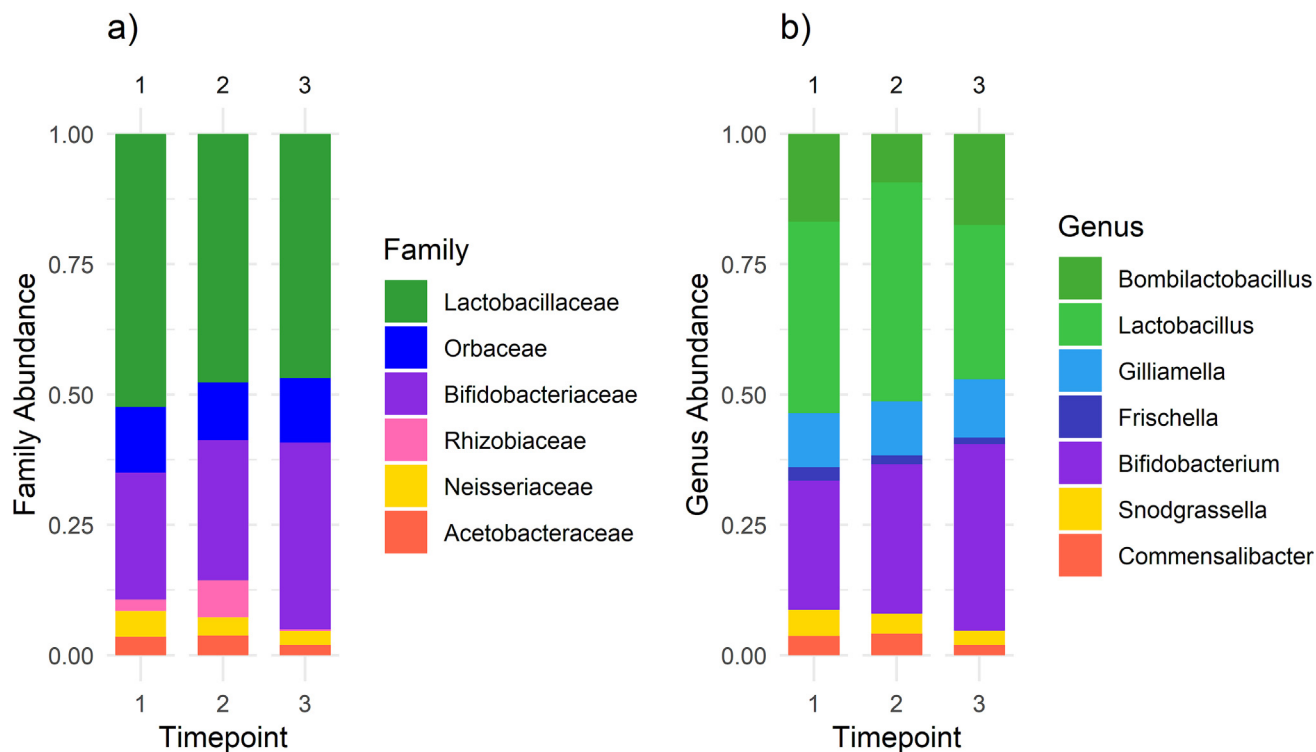


Fig. 3. Bar chart of the taxa abundance, by family (A) and genus (B), grouping samples of *Apis mellifera* by timepoints: T1 (June), T2 (July) and T3 (October).

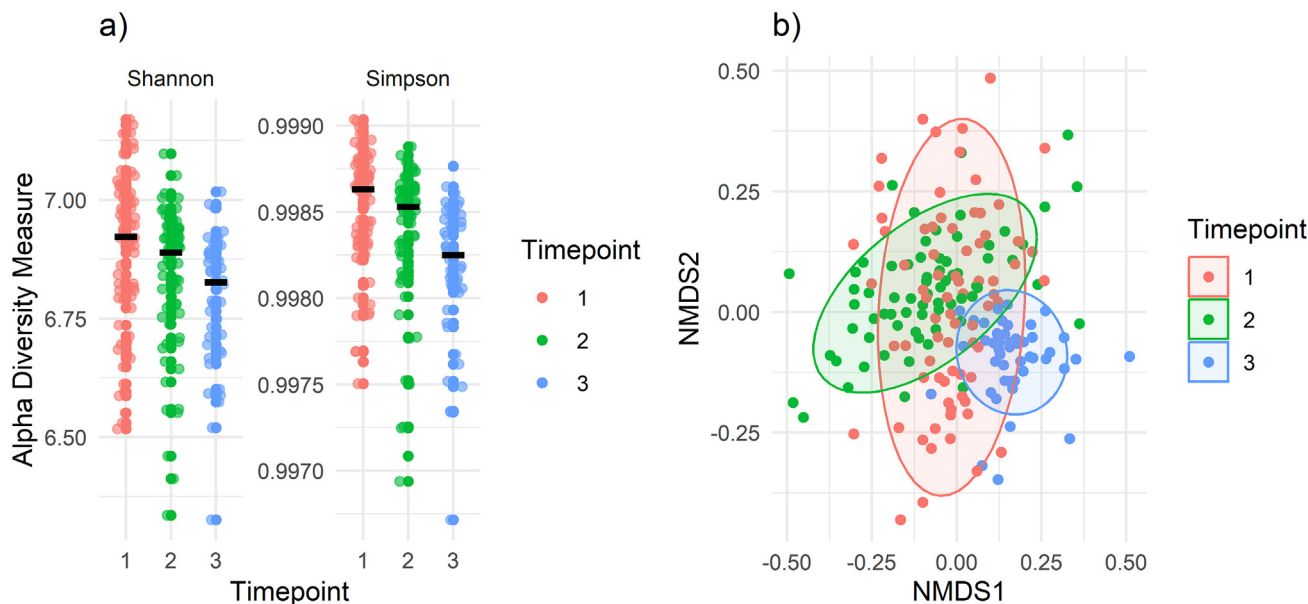


Fig. 4. α and β diversity graphics grouping samples of *Apis mellifera* by timepoint. Samples from timepoint 1 are shown in red, samples from timepoint 2 in green, and samples from timepoint 3 in blue. Part (a) displays Shannon and Simpson indexes, while Part (b) shows Bray-Curtis distance visualised through non-metric multidimensional scaling (NMDS).

the lowest levels for both indices. Post-hoc Tukey tests were then performed to identify which timepoints differ significantly. The results shown in Table 3 indicate that, while no significant differences were found for Observed ASVs, for all other indices, the comparison between T1 and T3 was consistently significant. Additionally, for Chao1 and Simpson indices, significant differences were also observed between T2 and T3.

The PERMANOVA analysis, which utilised Bray-Curtis dissimilarity to measure differences between timepoints, indicated that approximately 13% of the total variation in microbiome composi-

tion can be attributed to temporal changes. This difference was highly significant, with a P -value of 0.001, as reported in Table 4.

The concordance between the ANOVA results with α -diversity and the PERMANOVA results with β -diversity highlights consistent temporal shifts in both microbial richness and community structure over the observed time period.

Differential Abundance Analysis was conducted to identify ASVs associated with the observed microbiota changes across the three timepoints. For an accurate comparison between samples, the analysis was performed on both the rarefied and the non-rarefied

Table 1

Percentage of the relative abundances of bacterial taxa at both Family and Genus levels in the gut microbial community of *Apis mellifera* at each timepoint (T1, T2 and T3).

Taxa	T1	T2	T3
Family			
Acetobacteraceae	3.58	3.76	1.93
Bifidobacteriaceae	24.31	26.76	35.9
Lactobacillaceae	52.33	47.64	46.75
Neisseriaceae	4.92	3.56	2.67
Orbaceae	12.68	11.18	12.46
Rhizobiaceae	2.18	7.1	0.29
Genus			
<i>Bifidobacterium</i>	24.74	28.62	35.86
<i>Bombilactobacillus</i>	16.84	9.31	17.43
<i>Commensalibacter</i>	3.68	4.13	1.95
<i>Frischella</i>	2.64	1.65	1.28
<i>Gilliamella</i>	10.38	10.42	11.24
<i>Lactobacillus</i>	36.7	42	29.56
<i>Snodgrassella</i>	5.02	3.87	2.68

Abbreviations: T1 = Timepoint 1; T2 = Timepoint 2; T3 = Timepoint 3.

Table 2

Results of the ANOVA for the effect of timepoint on the six α -diversity measures of the gut microbiome of *Apis mellifera*.

Index	Sum Sq	Mean Sq	F-value	P-value
Shannon	13.79	6.90	7.36	8.37e-04***
Observed ASV	10.46	5.23	5.47	4.89e-03**
Chao1	19.74	9.87	10.90	3.32e-05***
Simpson	19.12	9.56	10.53	4.66e-05***
ACE	24.1	12.05	13.67	2.88e-06***
Fisher	10.73	5.36	5.63	4.23e-03**

Abbreviations: ASV = Amplicon sequence variants; ACE = Abundance-based coverage estimator; SumSq = Sum of squares; Mean SQ = Mean square. Significance levels: ** P-value \leq 0.01, *** P-value \leq 0.001.

Table 3

Results of Tukey's post-hoc test for pairwise comparisons of timepoints (T1, T2, T3) across the six α -diversity measures of the gut microbiota of *Apis mellifera*.

α -index	Comparison	Estimate	SE	T-value	P-value
Shannon	T1 vs T2	-0.36	0.16	-2.21	0.07
	T1 vs T3	-0.69	0.18	-3.78	6.0e-4***
	T2 vs T3	-0.33	0.18	-1.78	0.18
Observed ASV	T1 vs T2	-0.10	0.42	-0.24	0.97
	T1 vs T3	-0.46	0.42	-1.09	0.52
	T2 vs T3	-0.36	0.43	-0.84	0.68
Chao1	T1 vs T2	0.13	0.16	0.80	0.70
	T1 vs T3	-0.68	0.18	-3.82	5.0e-4***
	T2 vs T3	-0.81	0.18	-4.46	1.0e-4***
Simpson	T1 vs T2	-0.34	0.16	-2.13	0.08
	T1 vs T3	-0.82	0.18	-4.58	1.0e-3***
	T2 vs T3	-0.48	0.18	-2.64	0.02*
Fisher	T1 vs T2	-0.17	0.16	-1.05	0.55
	T1 vs T3	-0.61	0.18	-3.33	3.0e-3**
	T2 vs T3	-0.44	0.19	-2.36	0.05

Abbreviations: ASV = Amplicon sequence variants; T1 = Timepoint 1; T2 = Timepoint 2; T3 = Timepoint 3. Significance levels: * P-value \leq 0.05, ** P-value \leq 0.01, *** P-value \leq 0.001.

Table 4

Results of PERMANOVA analysis using Bray-Curtis dissimilarity to assess the variation in microbiome composition across the three timepoints in *Apis mellifera*.

Item	Df	SumSq	R2	F-value	P-value
Timepoint	2	2.04	0.12	12.74	0.001***

Abbreviations: SumSq = Sum of squares. Significance level: *** P-value \leq 0.001.

core datasets, comprising 2 036 ASV. This approach helps to avoid potential limitations and artefacts introduced by the normalisation, ensuring that biologically relevant variations in ASV abundances are clearly represented. However, the rarefaction, set at 10 000 ASVs, aims to normalise the data and mitigate biases from sequencing or PCR differences. For these reasons, analysing both datasets provides a more comprehensive understanding of the data. Moreover, separate comparisons were made between T1 and T2 (144 samples), T1 and T3 (121 samples), and T2 and T3 (115 samples).

Results of the analysis on the rarefied microbiota showed that in the comparison between T1 and T2, 825 ASVs on 2 036 ASVs were identified as highly differentially abundant (P-value < 0.05), but only 42 ASVs demonstrated a substantial change in expression, with three ASVs of Rhizobiaceae family (unknown genus) having a log₂ fold change smaller than -2 indicating an increased presence in T2 compared to T1. Additionally, 39 ASVs from the *Snodgrassella* genus exhibited a log₂ fold change larger than 2, signifying their increased abundance in T1 (Fig. 5a).

In the non-rarefied dataset, 989 ASVs on 2 036 ASVs were identified as significantly differentially abundant (P-value < 0.05) however, none of ASVs demonstrated a log₂ fold change higher than 2 or lower than -2 (Fig. 5d). However, all 39 ASVs of *Snodgrassella* that showed a log₂ fold change higher than 2 in the rarefied dataset, demonstrated a log₂ fold change between 1.41 and 1.95 in the non-rarefied dataset. Similarly, the three ASVs of Rhizobiaceae with log₂ fold change lower than -2 in the rarefied dataset showed log₂ fold change between -1.81 and -1.98 in the non-rarefied dataset.

In the comparison between T1 and T3 using the rarefied dataset, 896 ASVs on 2 036 ASVs were found to be significantly different (P-value < 0.05). Of these, 240 ASVs exhibited a log₂ fold change higher than 2: 101 belonged to the *Snodgrassella* genus, 65 to *Lactobacillus*, 64 to Rhizobiaceae, and 10 to *Commensalibacter* (Fig. 5b). In the non-rarefied dataset, 1 084 significant ASVs were identified, including 101 from *Snodgrassella*, 61 from *Lactobacillus*, and 64 from Rhizobiaceae, with log₂ fold change larger than 2 (Fig. 5e).

Finally, in the comparison between T2 and T3 using the rarefied dataset, 1 116 ASVs on 2 036 were found to be significant, with 129 of them exhibiting a log₂ fold change larger than 2. This subset included 64 ASVs from the Rhizobiaceae family and 65 from the *Lactobacillus* genus (Fig. 5c). Similarly, in the non-rarefied dataset, 1 421 ASVs on 2 036 were identified as significant, with 128 of them demonstrating a log₂ fold change larger than 2, comprising 64 ASVs from Rhizobiaceae and 64 from *Lactobacillus* (Fig. 5f). These ASVs are identical to those observed in the rarefied dataset and were also identified as significant with log₂ fold change larger than 2 in the comparison between T1 and T3, both in the rarefied and non-rarefied analyses.

Interestingly, the greatest difference was observed when comparing T1 and T3, followed by T2 and T3, while fewer differences were observed in the comparisons between T1 and T2, in agreement with the Tukey posthoc results and the clustering of timepoints in the Bray Curtis distance plot (Fig. 4b), which reflects the greater temporal separation of timepoint 3 from timepoints 1 and 2.

Association between microbial composition and phenotypic traits

The comparison of the relative taxa abundance at both the genus and family levels, among the honey production (HYC) groups shows a difference at T1, particularly notable when comparing groups with the lowest and highest honey production (Supplementary Fig. S1 and Supplementary Table S6). At the family level, the "low" group exhibits higher Lactobacillaceae (58 vs 50%) and lower Rhizobiaceae, which are nearly absent (0.5 vs 4%). At the genus

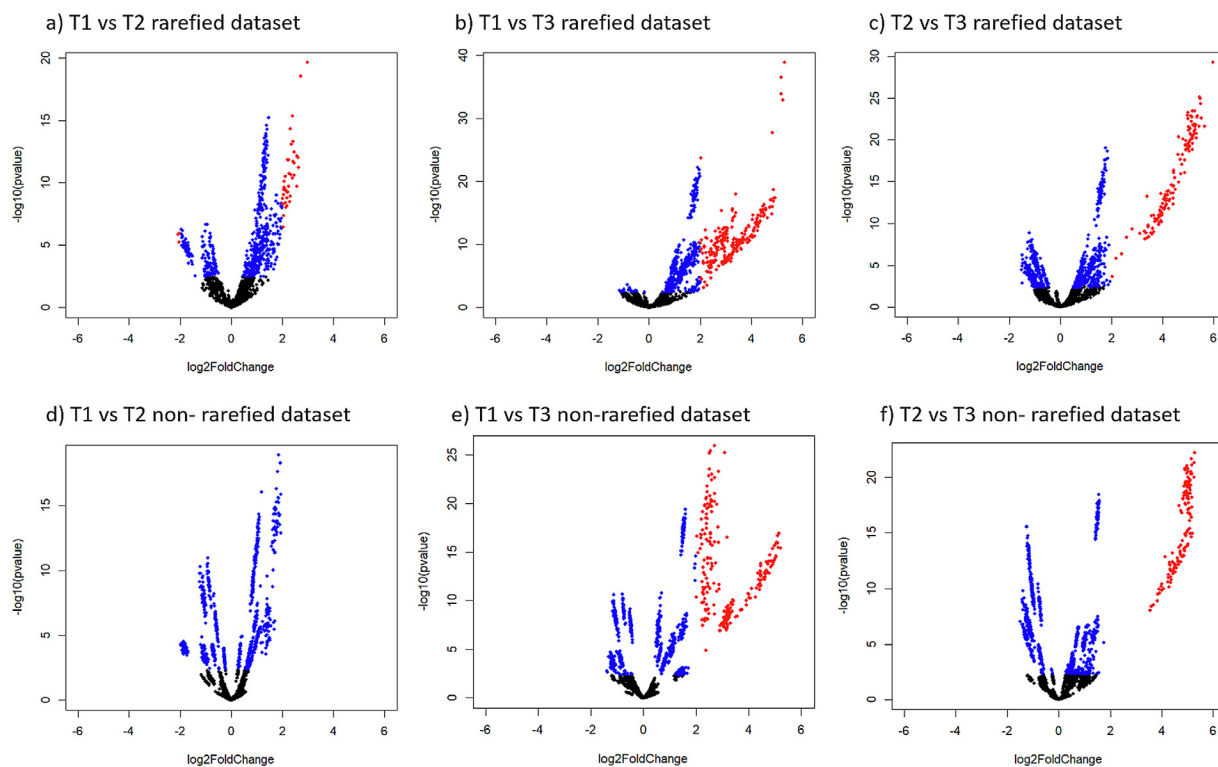


Fig. 5. Volcano plots showing the amplicon sequence variants (ASVs) differentially expressed in the gut microbiota of *Apis mellifera*, using rarefied datasets (a, b, c) and non-rarefied datasets (d, e, f) between timepoints 1 and 2 (a, d), timepoints 1 and 3 (b, e), and timepoints 2 and 3 (c, f). ASVs with P -values smaller than 0.01 are illustrated in blue, while ASVs with P -values smaller than 0.01 and \log_2 fold change smaller than -2 or \log_2 fold change larger than 2 are shown in red. The y-axis represents “ $-\log_{10}(P\text{-value})$ ” and the x-axis represents \log_2 of the fold change.

level, the observed difference in Lactobacillaceae appears to be driven by *Lactobacillus*, ranging from 43% in the “low” group to 35% in the “high” group. Additionally, a disparity in *Gilliamella* (belonging to Orbaceae family) proportion is evident, with the low group showing a lower percentage (8%) compared to the high group (12%).

The relative taxa abundance of aggressive, medium, and gentle bee groups shows similar distribution across all docility groups at each timepoint, at both the family and genus levels (Supplementary Fig. S2 and Supplementary Table S7).

Linear regression analyses were conducted at each timepoint to explore the relationships between phenotypes and α -diversity indexes of bacterial communities. Results identified a significant association between HY2 and Fisher, Shannon, Simpson, Observed, and ACE indices at T1, with estimated coefficients that fell within the range of 0.21–0.28 and P -values < 0.05 . Similarly, HYT exhibited associations within the same range and with Fisher, Shannon, Simpson, and Observed indices at T1. Moreover, both HY2 and HYT displayed an association with the Chao1 index at T3, suggesting a dynamic relationship over time. In contrast, no significant association was observed in either HY1 or docility (DOC) with the α -diversity of the microbiota across all three timepoints. All these results are shown in the lollipop chart (Fig. 6), and more details are given in Supplementary Table S8.

Additionally, a PERMANOVA analysis was conducted to evaluate differences in β -diversity among the different groups of docility and HYC. The results show a significant difference (P -value of 0.003) in β -diversity at T1 among the honey yield class, indicating that 4.8% of the total variation in microbiome composition is linked to HYC. No significant difference was found for docility behaviour. All results of the PERMANOVA analysis are reported in Supplementary Table S9. These results agree with regression analyses, rein-

forcing the relationship observed between honey yield and microbial composition of the gastrointestinal tract of honey bees in June across different diversity indexes.

Differential abundance analysis was performed on both rarefied and non-rarefied datasets to identify ASVs associated with significant abundance changes across HYC at T1. Separate comparisons were conducted between the low and high groups ($n = 35$), medium and high groups ($n = 58$), and between medium and low groups ($n = 53$). In both datasets, the comparison between low and high groups (Fig. 7a and Fig. 7d) identified significantly different abundant ASVs (P -value < 0.05).

In detail, in the non-rarefied dataset, 62 significant ASVs belonging to the Rhizobiaceae family (unknown genus) were identified, which ASVs exhibited a substantial change in expression (\log_2 fold change smaller than -2) (Fig. 7d). In the rarefied core dataset, 60 significant ASV were identified, of which 58 has \log_2 fold change smaller than -2 (57 Rhizobiaceae and 1 *Lactobacillus*), while the remaining 2 were *Bifidobacterium* (\log_2 fold change of -0.8 and -0.93) (Fig. 7a). A total of 56 ASVs belonging to the Rhizobiaceae Family showing a \log_2 fold change lower than -2 were in common between the ASV datasets.

Discussion

One of the main objectives of the study was to investigate the seasonal influence on the honey bee microbiota. To this end, the honey bee microbial composition of 77 colonies from an apiary in Italy was analysed, sampling 3 times over a 5-month period.

During the 5 months of sampling, 31 queens died for unknown reasons, leading to the exclusion of their colonies from subsequent sampling. A variable called ‘vitality’ was created to test the possibility of association of specific microbiota composition and vitality,

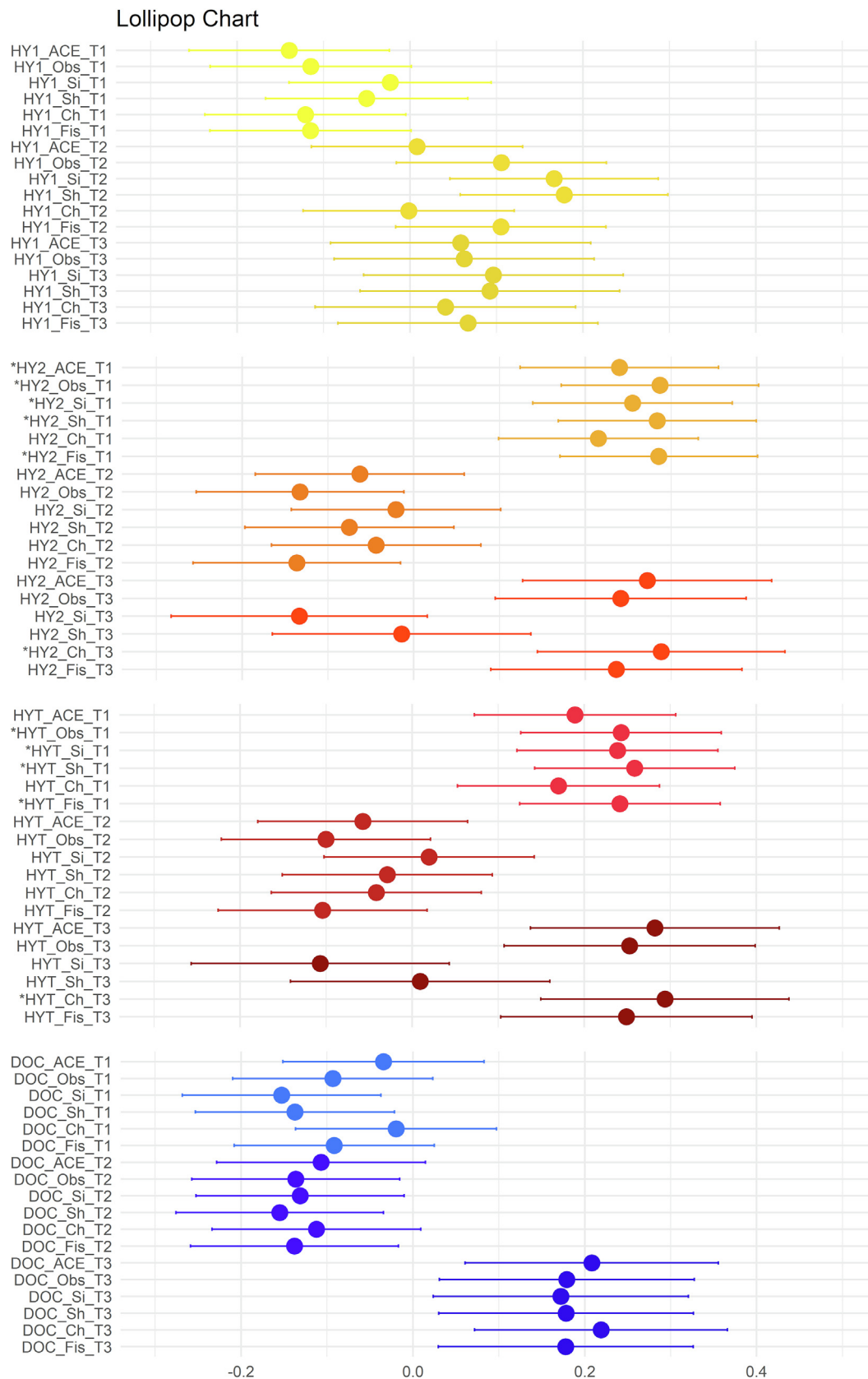


Fig. 6. Lollipop chart illustrating the results of linear regression analysis for *Apis mellifera* phenotypes. On the y-axis, the phenotypes are reported (DOC, HY1, HY2, HYT), followed by the α -diversity indexes: Abundance-based coverage estimator (ACE), Observed Amplicon Sequence Variants (Obs), Simpson (Si), Shannon (Sh), Chao1 (Ch), Fisher (Fi), and further specified by the timepoints (T1, T2, T3). The x-axis represents the estimates of the regression.

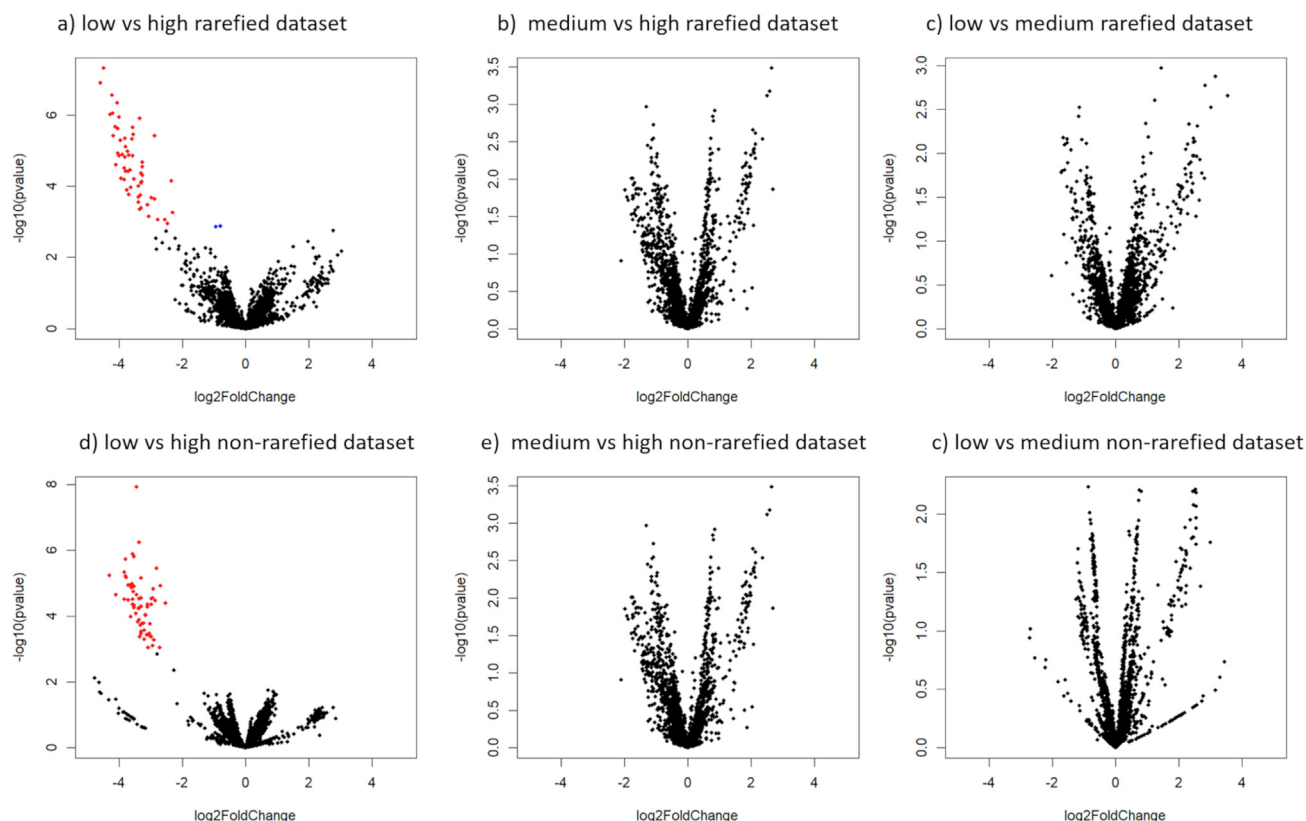


Fig. 7. Volcano plots showing the amplicon sequence variants (ASVs) differentially expressed in the gut microbiota of *Apis mellifera* at timepoint 1 using the rarefied dataset (a, b, c) and non-rarefied dataset (d, e, f). The plots illustrate the comparison between high and low honey yield classes (HYC) (a, d), medium and high HYC (b, e), and medium and low HYC (c, f). ASVs with P -values smaller than 0.05 are shown in blue, while ASVs with P -values smaller than 0.05 and \log_2 fold change smaller than -2 or \log_2 fold change larger than 2 are shown in red. The y-axis represents “ $-\log_{10}(P\text{-value})$ ” and the x-axis represents \log_2 of the Fold Change.

and also in order to check whether the experimental design would become unbalanced because of the misrepresentation of some families. Both α -diversity and β -diversity analyses did not show evidence of differences among the three classes (Supplementary Table S5 and Fig. 2c). This allowed us to proceed and consider our experimental design balanced, despite the loss of some colonies. It is worth highlighting that our study focused on the gut microbiota of worker bees, rather than queens. Previous studies demonstrated that there are substantial differences in their microbiota composition, due to the different diet and the caste-specific factors (Kapheim et al., 2015; Tarpy et al., 2015). Therefore, the lack of significant associations between ‘vitality’ classes and microbiota composition in worker bees may be influenced by the different microbial composition of queens, which was not a primary focus of our analysis.

Since no differences were found in ‘vitality’ classes, the entire dataset of 190 samples from 77 colonies was used to study the seasonal variations covering three different seasons: spring, summer and autumn. The first two timepoints are closely spaced in time and both fall within the period of honey production when the bees forage, while the third was chosen to be far apart after the honey production period.

In all three timepoints, the most prevalent bacteria belonged to the five genera identified by previous studies (Kwong and Moran, 2016; Nowak et al., 2021; Kešnerová et al., 2020) as the core honey bee microbiome: *Bifidobacterium*, *Lactobacillus*, *Bombilactobacillus*, *Gilliamella* and *Snodgrassella*. Other taxa not part of the core microbiome but already described in honey bees were found: *Frischella*, *Commensalibacter*, and Rhizobiaceae (Kwong and Moran, 2016). While the same taxa were found in all three timepoints, their pro-

portions varied (Fig. 3 and Table 1). In detail, *Bifidobacterium* (Bifidobacteriaceae Family) increases over time ranging from 25% in T1 to 36% in T3, becoming the most prevalent genus in autumn (T3). The Lactobacillaceae Family remained constant over time; however, a closer examination of genera showed that T2 had fewer *Bombilactobacillus*, accounting for only 9% compared to 17% in T1 and T3. Simultaneously *Lactobacillus* increased to 42% compared to T1 (37%) and T3 (30%). *Snodgrassella* of the Neisseriaceae family showed a slight decrease over time, declining from 5% in T1 to 3% in T3. *Commensalibacter* of the Acetobacteraceae Family exhibited comparable proportions between T1 and T2 (4%), but decreased to only 2% in T3. In contrast, the Rhizobiaceae family was the least stable taxon; in T2, it constitutes 7% of the microbiota community, while in T3, it is almost absent (0.3%).

The Orbaceae Family, represented by the genera *Frischella* and *Gilliamella*, exhibited a small decrease in the relative abundance of *Frischella* (from 3 to 1%) from June to October, while *Gilliamella* increased slightly over this 5-month period (from 10 to 11%). Our results are in partial agreement with a recent study conducted by Kešnerová et al. (2020) in Switzerland that analysed the seasonal shifts between summer (July and August) and winter worker bees (January) and reported lower levels of *Frischella* in winter compared to summer but no difference in the levels of *Gilliamella* (Kešnerová et al., 2020).

Differential abundance analysis performed on the rarefied dataset between T1 and T2 identified 39 ASVs belonging to the *Snodgrassella* genus as increasing and three ASVs of Rhizobiaceae Family as decreasing. The same genus and families showed variation even in Almeida et al. (2023) study, conducted across six apiaries located in southern Ireland, in 3 timepoints from late April to

early September 2019. Furthermore, differential abundance analysis between T1 and T3 showed *Snodgrassella*, *Lactobacillus* and the Rhizobiaceae to differ significantly (Log₂ fold change larger than 2). Moreover, only in the rarefied dataset, ASV changes of *Commen-salibacter* were identified to be significant and over 2 log₂ fold change. Finally, in the comparison between T2 and T3 were identified ASVs from the Rhizobiaceae family and from the *Lactobacillus* genus.

The asymmetry observed in the log₂ fold change of the volcano plots of the differential abundance analysis (Fig. 5), with a slope leaning towards one side, is attributed to the decrease in abundance of the majority of the ASVs across timepoints with time. The only exception is the Rhizobiaceae family that initially increases in abundance from T1 to T2 before experiencing a sharp decline in T3. Furthermore, the ASVs that show significant change between T1 and T3 are also consistently significant between T2 and T3, irrespective of the dataset used for the analysis. This can be attributed to the lowest presence of these ASVs in T3. Additionally, the results highlight that the differences between T1 and T2 are less marked than those observed between T1 vs T3 or T2 vs T3. This suggests that the honey bee microbiota during the honey production season (T1 and T2) are more similar to each other than in autumn (T3). This finding is consistent with the results of Almeida et al. (2023) which showed increasing dissimilarity from April to September, with T1 being more similar to T2 than to T3, suggesting that the difference can be caused by the different nectar, pollen and bee bread resources. This result is linked to differences in the diet of bees during the honey production season (T1 and T2) compared to autumn (T3), when honey bees primarily consume candy due to limited natural forage. It is also important to note that colonies were still producing brood at T3, indicating that the observed differences are not due to a broodless state, while another factor that may have impacted microbiota composition is temperature. Variations in the temperature of the colonies and bees can influence bacterial growth rates as hypothesised by Kešnerová et al. (2020) to explain the observed difference between winter and summer bee microbiota composition in Switzerland (Kešnerová et al., 2020). Indeed, the temperature has been shown to influence bacterial growth (Membré et al., 2005), and the body temperature of honey bees in winter is around 21 °C while in summer is approximately 35 °C (Fahrenholz et al., 1989).

The significant difference in microbiota composition is confirmed by PERMANOVA analysis using the Bray–Curtis distance, indicating that 12.74% of the honey bee gut microbiota undergoes seasonal changes. As shown in Fig. 4b, the samples formed a distinct cluster for timepoint 3, while there is a slight separation between timepoints 1 and 2. Our results agree with what was found by Bleau et al. (2020), Kešnerová et al. (2020) and Almeida et al. (2023) that showed a clear clustering of samples according to the season. Furthermore, also the α -diversity measures show significant differences between timepoints in agreement with Kešnerová et al. (2020) that reported a reduction in α -diversity in winter.

In conclusion, our findings align with previous studies, whereas some dissimilarities were still identified. These differences could be attributed to the difference in the climates under study, since we are comparing the microbiota of bees sampled in Italy with research conducted in Colorado (USA), Canada, Norway, Ireland and Switzerland (Almeida et al., 2023; Kešnerová et al., 2020; Subotic et al., 2019; Bleau et al., 2020; Ludvigsen et al., 2015). Moreover, the different timing of timepoints may contribute with climate change to the differences observed.

Among all the overmentioned studies, including ours, there appears to be the consistent representation of the core microbiota, comprising five key taxa (*Bifidobacterium*, *Lactobacillus*, *Bombilactobacillus*, *Gilliamella* and *Snodgrassella*) with significant seasonal vari-

ation in their relative proportions (Almeida et al., 2023; Kešnerová et al., 2020; Subotic et al., 2019; Bleau et al. 2020; Ludvigsen et al., 2015). This strengthens the findings, despite potential regional and temporal differences, and provides valuable insights into the dynamics of honey bee gut microbiota.

Associations between the bee microbial community and two significant traits relevant to beekeeping (honey yield and docility) were also analysed in the present study. These traits represented breeding goals for this specific population, which has been undergoing selection for years. Docility was evaluated four times in spring, to account for environmental influences, such as heat and food scarcity (Breed et al., 2004). However, it is considered a life-long trait of the colony, largely determined by genetic factors, with high heritability (0.37 for gentleness and 0.38 for calmness) (Brascamp et al., 2016). By averaging multiple assessments, we aimed to reduce the impact of short-term environmental conditions and better capture the behavioural tendency of the colony.

Previous studies have provided evidence for the existence of a gut microbiota–brain axis, demonstrating that gut microorganisms can influence over neurophysiological processes, consequently shaping the behaviour of insects (Leger and McFrederick, 2020; Vernier et al., 2020). Moreover, the gut microbiota can modulate hormonal signalling and regulate the levels of essential biogenic amines (Zheng et al., 2018). However, no studies have been conducted on the relationship between the docility behaviour and honey bee microbiota. In our finding, this specific behaviour did not show significant associations with either the α or β diversity indices. Also, the relative abundance (Supplementary Fig. S2), of the aggressive, medium, and gentle bees, show similarities in taxa proportion at all three timepoints, confirming no difference in microbiota composition related to this behaviour. While our results indicate no clear link, further studies are needed to explore this potential relationship in greater depth.

Honey yield is another trait of interest for beekeepers since it constitutes the primary source of income. The colonies analysed produced two distinct honey crops, related with the different blooming of flowers: the first in May (acacia honey), and the second in June (wildflower honey). Consequently, honey yield was evaluated as the combined sum of the two harvests and as the two individual harvest yields. The results of the linear regression analyses identified significant associations (P -value < 0.05) between the α -diversity indexes of the microbiota at T1 with HY2 and with HYT.

The stronger association between HY2 and T1 is likely due to the fact that T1 is temporally closer to the period of the second honey harvest (HY2). Moreover, the similarity of the results of the HY2 and HYT is probably due to the larger proportion of wildflower honey collected in July over the acacia honey collected in May, the average of HYT is 32.67, of HY1 IS 4.69 and of HY2 is 27.97 (Supplementary Table S1). Finally, no association with HY1 could be explained by the fact that HY1 was collected before the microbiota samplings began.

The results showed a significant difference in the Chao1 index at T3 for both HYT and HY2, where colonies with higher honey yields showed a higher Chao1 index. The Chao1 index, which estimates species richness by accounting for both abundant and rare species (Chao and Chiu, 2006), suggests that colonies with higher yields may harbour more diverse microbial communities. This outcome may be linked to their diet, as more productive colonies tend to have greater honey supplies accumulated in the season. As a result, these colonies might have access to a more varied diet, including both remaining honey and not only the sugar candy, which could promote a more diverse gut microbiota.

Furthermore, the total honey yield was examined as a categorical factor (HYC) by categorising colonies into three groups (low, medium and high honey yield). The results of the PERMANOVA,

which was performed using this categorical factor, agree with the regression analysis and indicated a significant association between honey yield categories and microbiome composition at T1. Although the variation explained by honey yield is relatively small (4.8%), this finding, combined with the other analyses presented in the study, supports the presence of an association between honey yield and microbiome composition in June. Almeida et al. (2023) showed a correlation between microbiome composition and honey production, measured as the number of honey frames. However, their study identified a positive correlation with *Apilactobacillus*, which in our case, has similar relative abundance among HYC groups.

Differential abundance analysis was performed on rarefied and non-rarefied core microbiota dataset. In both the analyses, only the comparison between the low and high groups, representing the two extremes in honey yield, exhibited significant differences, identifying mainly ASVs from the Rhizobiaceae family (57 ASV in rarefied dataset and 62 in non-rarefied ASV dataset). These ASVs within Rhizobiaceae family had log₂ fold change smaller than -2, indicating a higher abundance in the “high” yield group. Overall, the relative abundance of the Rhizobiaceae family is 4% in the high group compared to 0.5% in the low group, where it is nearly absent (Supplementary Fig. S1a). In summary, all of these findings highlight significant associations between honey yield and the composition of the gut microbial community. These associations may be explained by the fermentation properties of microorganisms present in the digestive tract, especially in the crop, which can play a role in the transformation of nectar into honey, as described by Silva et al. (2017).

In conclusion, our study offers valuable insights into the seasonal dynamics of honey bee microbiota and its potential associations with honey yield. Seasonal variations in microbiota composition were evident, with specific genera showing changes in their relative proportions over time. The core microbiome remained predominant over the seasons, underlining the peculiarity of bees to have a specialised microbiome, similar to mammals. Moreover, our investigation demonstrated a positive link between honey yield and the α -diversity and β -diversity indexes in T1, suggesting that higher microbiota diversity and increased presence of Rhizobiaceae in June correspond to increased honey production. The results of our study contribute to the understanding of the intricate relationship between honey bee microbiota and key beekeeping traits. Further investigation of the digestive microbiota could potentially lead to the identification of probiotics that not only improve colony health but also enhance productivity, offering new avenues for improving beekeeping practices.

Supplementary material

Supplementary Material for this article (<https://doi.org/10.1016/j.animal.2025.101474>) can be found at the foot of the online page, in the Appendix section.

Ethics approval

Not applicable.

Data and model availability statement

These data (sequences and phenotypes) are based on a breeding population used for selection by commercial breeders and have commercial value. Therefore, restrictions apply to the availability of these data, which are not publicly available. The authors can be contacted for a specific request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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CRediT authorship contribution statement

M.G. De Iorio: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **G. Minozzi:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualisation. **F. Tiezzi:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Conceptualisation.

Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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