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Microbiota comparative analysis of *Apis mellifera* populations from Tunisia and Algeria targeting 16 S rRNA gene amplicon

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Abstract
Bee populations are major contributors to ecosystem stability, providing vital services such as pollination in both in natural ecosystems and agricultural crops and thus to food security. Honeybees, the main pollinator, are currently undergoing severe global decline threatened by several factors. The study of host-microbiota interactions could partly explain this decline. In this study, we sequenced the V3-V4 hypervariable regions of the 16 S rRNA to assess the microbiota of two *Apis mellifera intermissa* and *Apis mellifera iberiensis* populations sampled in Tunisia and Algeria, respectively. Analysis of the targeted metagenomics data obtained by Illumina identified 59 and 114 ASVs at Tunisian and Algerian populations, respectively. At the genus level, *Providencia* pathogenic bacteria dominate the *A. mellifera intermissa* microbiota. While *Snodgrassella* and *Gilliamela* bacteria essential for pathogen resistance are the most abundant in *A. mellifera iberiensis*. These findings underscore the importance of host-microbiome interactions in honeybee resilience and provide a basis for targeted strategies to support colony health amid global declines.

Keywords Apis mellifera · Health · Host-microbiota interactions · V3–V4 amplicon sequencing

1 Introduction

Over recent decades, numerous studies have reported a sharp decline in bee diversity and abundance, a trend that also affects major pollinators such as the honeybee. This alarming decline poses a serious threat to ecosystems and agricultural productivity, calling for urgent and effective conservation measures targeting this key domestic species (Pasquali et al. 2025). Multiple factors contribute to this decline, including habitat destruction particularly the loss of flower-rich environments, excessive use of insecticides, pollution, invasive species, pathogens, and climate change (Neumann and Blacquière 2016).

The honeybee, *Apis mellifera*, is the primary domestic pollinator species for a wide variety of agricultural crops and contributes significantly to the human food supply (Engel et al. 2013). In last decades, however, like other pollinators, honeybee populations have declined substantially, and large-scale losses of managed and wild honeybee colonies have been reported worldwide (Dukas 2008; Neumann and Carreck 2010; Gregorc 2020). These declines are driven by a combination of biotic and abiotic stressors, such as pathogens, exposure to pesticides,

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loss of habitat and nutritional stress (Goulson et al. 2015; Potts et al. 2010). While, the direct effects of these stressors on bee physiology and behavior are well documented, several studies suggest that the gut microbiome plays a crucial part in determining honeybee health (Hristov et al. 2020; Naccache et al. 2023; Liberti et al. 2024). This community influences digestion, immunity and resistance to pathogens, and each species harbours a distinct and stable gut microbiota that can affect its resilience to environmental challenges (Engel and Moran 2013; Kwong and Moran 2016; Liberti et al. 2024). Therefore, understanding the composition, stability and functional roles of these microbial communities is essential for elucidating the mechanisms underlying colony health, as well as for guiding conservation and management strategies.

The investigation of insect-associated microbiota has increasingly relied on high-throughput DNA metabarcoding, a central approach that enables the simultaneous profiling of complex microbial communities with high sensitivity, reproducibility, and scalability (Caporaso et al. 2012; Pollock et al. 2018; Piper et al. 2019). Amplicon-based sequencing of conserved genetic markers, notably the bacterial 16 S rRNA gene, has been successfully applied to investigate gut microbiota composition, host microbe specificity, ecological adaptation, and responses to environmental stressors, including diet changes, pesticide exposure, pathogen infection temperature variation, and dietary shifts (Moran et al. 2005; Douglas 2015; Saraiva et al. 2015).

The bacterial microbiota is characterized by a stable core dominated by *Gilliamella*, *Snodgrassella*, *Lactobacillus*, and Bifidobacterium, alongside less prevalent non-core taxa such as *Frischella*, *Commensalibacter*, *Acinetobacter*, *Bombella*, and the opportunistic pathogen *Serratia* (Ge et al. 2021; Raymann et al. 2017; Saraiva et al. 2015). While, the honeybee gut microbiome is largely conserved, studies have shown that geography, forage diversity, and seasonal factors can significantly influence gut bacterial communities (Raymann et al. 2017; Su et al. 2022). However, these influences remain poorly explored in North African populations. This study addresses this gap by comparing the gut microbiota of Tunisian and Algerian honeybee populations based on next generation sequencing analysis targeting 16 S rRNA genes (V3-V4). This comparative analysis aims to explore potential geographic, seasonal and environmental influences on microbiota composition. The underlying hypothesis is that variations in microbial communities may reflect local adaptations and could significantly influence the health, immunity, and development of their bee hosts.

2 Materials and methods

2.1 *Apis mellifera* sampling

The study involved the collection of *Apis mellifera* samples from two distinct sites in North Africa: Rades, Tunisia (Am_TN) ($n=30$), and Tizi Ouzou, Algeria (Am_DZ) ($n=30$). Both are situated within the Mediterranean biome. These regions are characterized by a seasonal climate with hot, dry summers and mild, wet winters. The Tunisian site is located at an altitude of 50 m near the coastal region of Rades (36°46’5.02"N; 10°16’31.01"E), characterized by the presence of orange shrubs and no record of pesticide use and exhibited an estimated annual colony mortality rate of approximately 30%, with colonies generally in normal health. Sampling was conducted in December 2019. On the other hand, the Algerian site, situated in land at 418 m of altitude (36°42’4.33"N; 4°1’30.34"E), features a diverse vegetation landscape, including 20% cultivated plants (thyme, lavender, eucalyptus), 40% trees and shrubs (olive, orange), and 40% olive farming, along with some wild plants such as wild carrots. Hives in Tizi Ouzou were moved during pesticide treatments of nearby trees, and colony health assessments revealed infections including varroosis, European foulbrood, and American foulbrood (*Paenibacillus larvae*), with estimated annual mortality rates ranging from 5% to 10%.

Honey bee specimens were collected directly from active colonies using sterilized metal tweezers to minimise contamination and preserve sample integrity. Immediately after collection, samples were preserved in 95–100% ethanol to maintain both structural and genetic integrity, enabling subsequent analyses of gut microbial communities and pathogen detection (Hammer et al. 2015).

2.2 DNA barcoding

Total DNA was extracted from the guts of honeybees collected using the CTAB protocol (Naccache et al. 2023). A PCR amplification of the intergenic mitochondrial DNA region COI-COII was performed to determine the subspecies of *Apis mellifera*, using the two primers H2 (5’-CAA TAT CAT TGA TGA CC-3’) and E2 (5’-GGC AGA ATA AGT GCA TTG-3’) as described by Garnery et al. (1993). The PCR reaction was carried out in a final volume of 25 µL, containing 1X Taq polymerase buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 10 µM of each primer, Taq DNA polymerase (5 U/µL), and 50 ng of extracted DNA. Amplification was conducted using an Applied Biosystems 2720

thermal cycler with the following conditions: initial denaturation at 92 °C for 3 min, followed by 30 cycles of denaturation at 92 °C for 1 min, primer annealing at 48 °C for 45 s, and extension at 62 °C for 2 min. A final elongation step was performed at 62 °C for 10 min after the completion of the cycles. The resulting sequences were sequenced using ABI3500 and sequence quality was assessed and manually edited using BioEdit v7.2.5, and multiple sequence alignments were performed using CLC Sequence Viewer v8.0 (https://digitalinsights.qiagen.com/). Treated sequences were compared to existing data using BLASTN (Altschul et al. 1990) against the GenBank database of the National Center for Biotechnology Information (NCBI). To ensure data quality and account for potential contamination blank controls were included throughout the DNA extraction and sequencing workflow.

2.3 16 S rRNA gene sequencing

For each species, gut DNA extracted from 30 individus was pooled into three independent DNA pools, each comprising DNA from ten individuals to account for biological redundancy (Ray et al. 2019). These DNAs were subjected to 16 S rRNA gene amplification targeting the V3–V4 hypervariable regions using the primers 341 F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Najjari et al. 2025). The resulting PCR products from the three pools were then combined and used for amplicon library preparation and sequencing analysis. The amplicons from the 16 S rRNA gene libraries were obtained using the Herculase II Fusion DNA Polymerase and Nextera XT Index Kit v2. High-throughput paired-end sequencing (2 × 300 bp) was performed on the Illumina MiSeq platform.

2.4 Metataxonomic analysis of bacterial community

DADA2 (Divisive Amplicon Denoising Algorithm) microbiome pipeline v1.22 embedded in R v4.5.2 was used for the paired-end reads analysis. Sequences were first screened for quality. Trimming was processed using an expected error threshold of 1 combined with the filtering of low-quality reads (>=150 bp were retained). Filtered reads were further dereplicated into unique sequences, denoised based on the DADA2 error estimation model and merged to get high-quality inferred sequences recognized as ASV (Amplicon Sequence Variants). Next, chimeras were removed using the consensus method. Finally, taxonomic assignment of ASVs was conducted using a SILVA nonredundant v138 training set (Quast et al. 2013). The global richness and the α-diversity indices (i.e. Observed_ASV, Shannon and Chao1) were calculated from phyloseq v1.38 and abdiv v0.2. The sample rarefaction curves were performed using the R package Ranacapa v0.1.

The obtained raw sequences were submitted to the National Center for Biotechnology Information (NCBI) as Sequence Read Archive (SRA) under accession numbers as follows: Am_Tn (SRR28547106) and Am_Dz (SSR28547105).

3 Results

3.1 DNA barcoding

The intergenic COI-COII regions of the mitochondrial DNA from two *Apis mellifera* lineages were amplified and sequenced, then analyzed using BLAST. The result showed that Am_Tn showed 100% sequence identity with *Apis mellifera intermissa* A1o (accession no. KX463746.1), while the Am_Dz showed 99.83% identity with *Apis mellifera iberiensis* A1h (accession no. KX463739.1).

3.2 Bioinformatic analysis of Illumina sequencing results

3.2.1 Diversity and richness estimates

A total of 99,149 and 90,531 raw reads were obtained for *A. mellifera iberiensis* (Am_DZ) and *A. mellifera intermissa* (Am_TN), respectively, with an average read length of approximately 300 bp for both populations. After sequence quality filtering, denoising, merging, and chimera removal, the number of high-quality reads retained for downstream analyses was 58,153 for Am_DZ and 64,137 for Am_TN (Table 1). The obtained sequence data represented a diverse bacterial community of 59 and 114 ASVs for *A.mellifera intermissa* (Am_Tn) and *A.mellifera iberiensis* (Am_Dz) species' respectively. Rarefaction curves and good coverage with values>99% for two species indicate an overall excellent ASVs coverage afforded by the deep sequencing (Fig. S1). Results showed that the species richness of Am_DZ was higher than Am_TN as measured by Chao1 (114 vs. 59 for Am_TN). The diversity index, as calculated using the Shannon showed also a difference between the two species (3.66 vs. 2.06 Am_Tn) (Table 1).

Table 1 Alpha-diversity of the bacterial communities associated with Am_Tn and Am_DZ species

Sample	Raw_reads	Filtered_reads	Non_chimeric	Observed_ASVs	Shannon	Chao1	Good's coverage (%)
Am_Dz	99,149	91,800	58,153	114	3.66	114	99.96
Am_Tn	90,531	82,975	64,137	59	2.06	59	99.97

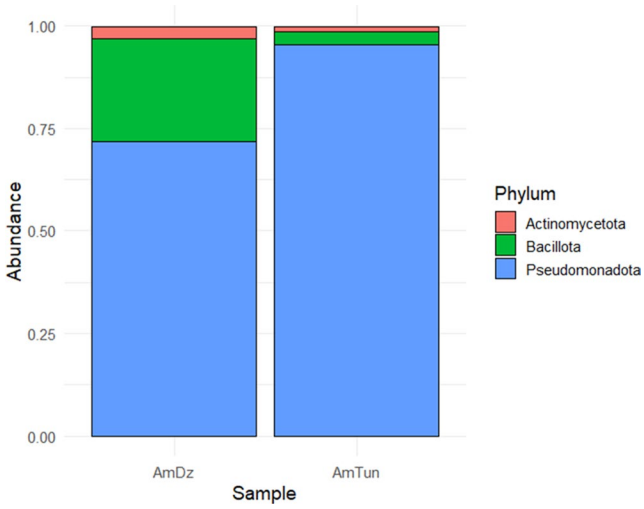
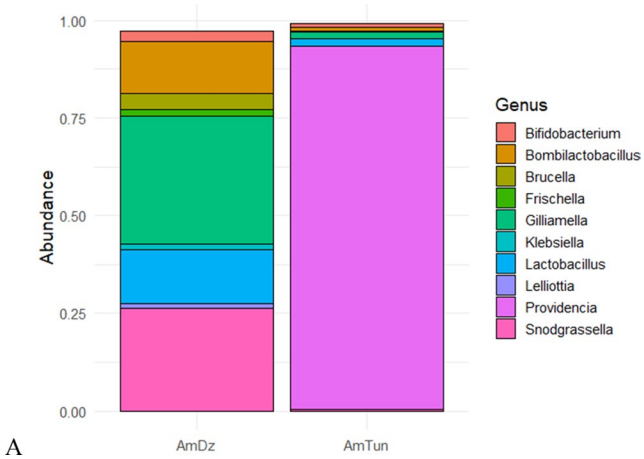


Fig. 1 Relative abundance of phyla distribution of Am_TN *Apis mellifera intermissa* and Am_DZ *Apis mellifera*. Bacterial composition and their relative abundance at the phylum level. Only Phyla upper than 1% in relative abundance are displayed



A

Fig. 2 Relative abundance of genera distribution of Am_TN *Apis mellifera intermissa* and Am_DZ *Apis mellifera.* Only genera upper than 1% in relative abundance are displayed

3.2.2 Honeybee-associated microbiota analysis

At the phylum level, the bacterial community composition in both *Apis mellifera* populations was dominated by three major phyla: Pseudomonadota, Bacillota, and Actinomycetota, although their distribution varied between specimens (Fig. 1). Pseudomonadota was the most abundant phylum, accounting for 95.45% in Am_TN and 72.34% in Am_DZ. This was followed by Bacillota, which showed a relative abundance of 24.32% in Am_DZ and only 2.98% in Am_TN. The third phylum, Actinomycetota, was present at low levels in both samples, with relative abundances of 3.27% in Am_DZ and 1.55% in Am_TN.

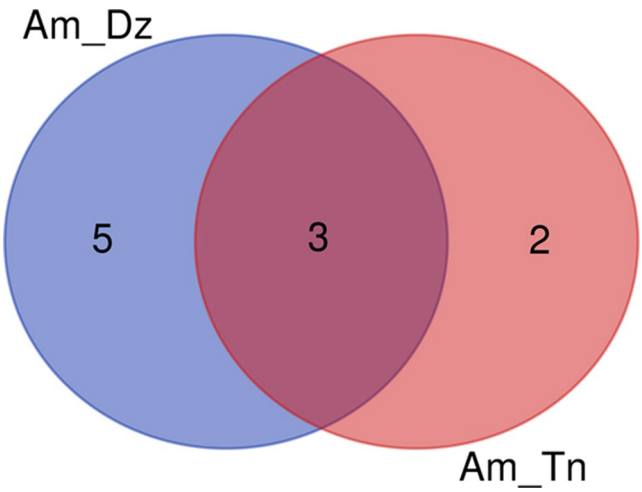


Fig. 3 Venn diagram showing core and unique bacterial genera identified in Am_Tn and Am_DZ samples. The core microbiota consisted of three shared genera: Gilliamella, Lactobacillus, and Bombilactobacillus. Providencia and Bifidobacterium were detected exclusively in Am_Tn, whereas Am_DZ harbored five unique genera, including Snodgrassella, Brucella, Frischella, Lelliottia, and Klebsiella

At the genus level, significant differences were observed between the gut microbiota profiles of the Tunisian (Am_Tn) and Algerian (Am_Dz) *Apis mellifera* samples (Fig. 2). In Am_Tn, the microbiota was overwhelmingly dominated by *Providencia*, which accounted for 92.22% of the total relative abundance. All other detected genera were present at very low levels, including *Lactobacillus* (2.01%), *Bifidobacterium* (1.55%), *Gilliamella* (1.52%), and *Bombilactobacillus* (1%). In contrast, Am_DZ exhibited a more diverse and balanced bacterial profile. The dominant genera were *Gilliamella* (29.28%) and *Snodgrassella* (24.21%), followed by *Lactobacillus* (12.81%) and *Bombilactobacillus* (12.24%). Other genera, including *Brucella* (3.77%), *Frischella* (1.46%), *Lelliottia* (1.05%), and *Klebsiella* (1.31%), were detected at lower relative abundances. The main core genera is composed of three species including *Gilliamella*, *Lactobacillus* and *Bombilactobacillus*. Several genera were found to be unique to each group. *Providencia* was detected exclusively in Am_Tn, accounting for 92.22% of the relative abundance. In contrast, Am_DZ harbored other unique genera including *Snodgrassella* (24.21%), *Brucella* (3.77%), *Frischella* (1.46%), *Lelliottia* (1.05%) and *Klebsiella* (1.31%) (Fig. 3).

At the species level, the gut microbiota of Am_Tn was strongly dominated by the *Providencia alcalifaciens* group, accounting for 91.53% of the total bacterial abundance. In contrast, in Am_Dz the majority of detected taxa could not be confidently assigned to classified species; however, the identified fraction was primarily affiliated with core honey bee gut symbionts, notably the *Gilliamella apicola* group (36.39%) and *Snodgrassella alvi* (17.73%).

4 Discussion

Social insects comprising honeybees, bumble bees, and stingless bees are associated with many microbes. Host-microbiome interactions are important for sustainable homeostasis and promoting bee health. These interactions make it possible to focus on the coevolution of social insects and their microbiomes as well as the interaction pattern between the insect and its environment (Kwong and Moran 2016; Mazel et al. (2025; Prasad et al. 2025). Here, we investigated the bacterial community diversity and host specificity of gut microbiota of two honeybee subspecies from North Africa (Tunisia and Algeria countries).

The intergenic COI–COII regions were used for species identification via DNA barcoding, revealing two species: *A. m. intermissa* (haplotype A1o, accession number KX463746.1) in the Am_Tn populations and *A. m. iberiensis* (haplotype A1h, accession number KX463739.1) in the Am_Dz populations. *A. m. intermissa* is a North African honey bee subspecies (Khalil and Elsayed 2016) and *A. m. iberiensis*, is a subspecies of the Western honey bee, primarily found in the Iberian Peninsula, which includes Spain and Portugal (González-Cabrera and González 2018.

The honey bee populations Am_TN from Rades, Tunisia, and Am_DZ from Tizi Ouzou, Algeria, highlight key differences in environmental conditions and health status. Am_TN is located at a lower altitude and is characterized by simple vegetation and natural management practices without phytosanitary treatments. In contrast, Am_DZ colonies were situated at a higher altitude and characterized by more diverse vegetation, providing a broader range of foraging resources. However, this population is challenged by hive movement during pesticide treatments and infections such as varroosis and foulbrood. These findings underscore the importance of sustainable management practices to improve the health and resilience of honey bee populations in the face of ecological and health challenges.

The metabarcode analysis targeting the V3-V4 regions of the 16 S rRNA gene revealed distinct bacterial community structures in the guts of two honey bee species with 59 ASVs for *A. m. intermissa* and 114 ASVs for *A. m. iberiensis*. The bacterial community composition in the two honeybee populations, was dominated by three major phyla Pseudomonadota, Bacillota and Actinomycetota. The relative abundance distribution of these phyla differed significantly between the samples. Pseudomonadota was the most abundant accounting for 95.45% of the bacterial community in Am_Tn and 72.34% in Am_Dz. This indicates a strong prevalence of this phylum in both populations, consistent with other studies highlighting the importance of Pseudomonadota in the gut microbiota of various insects, including bees (Kwong and Moran 2016; Engel and Moran 2013). Bacillota exhibited notable differences in relative abundance distribution with 24.32% in Am_Dz compared to 2.98% in Am_TN. This difference suggests that environmental factors or differences in diet may influence the presence of Bacillota. This variation may reflect differences in environmental conditions and local forage availability. The Am_TN site is dominated by orange shrubs, providing a relatively uniform foraging source, while the Am_Dz site features a more diverse landscape, including 20% cultivated plants (thyme, lavender, eucalyptus), 40% trees and shrubs (olive, orange), and 40% olive farming, along with some wild plants such as wild carrots. The greater diversity of floral resources at Am_Dz may support a richer and more balanced gut microbiota, influencing the higher prevalence of Bacillota in this population. Several studies have reported that local nutrition, environmental conditions, and geographical location strongly shape the gut microbial communities of bees. These microbiota, adapted to their local environment, can enhance host immunity and overall colony health, highlighting the importance of preserving diverse, natural landscapes to support resilient bee-microbiome interactions (Ge et al. 2021; Maes et al. 2016; Mee et al. 2023; Santorelli et al. 2023). Other research has shown that the gut microbiome can reflect the ecological niches and foraging behaviours of bee populations (Martinson et al. 2011). Lastly, Actinomycetota was present at low levels in both samples, at 3.27% and 1.55%, respectively. Although less dominant, the presence of Actinomycetota is consistent with studies indicating its role in degrading complex organic materials, which could benefit nutrient cycling within the gut (Rudra Gouda et al. 2024).

At the genus level, a core of three genera *Gilliamella*, *Lactobacillus*, and *Bombilactobacillus* was identified with uneven distribution. *Gilliamella* and *Lactobacillus* were more abundant in Am_Dz compared to Am_TN. In fact, the Tunisian and Algerian samples were collected at different times of the year (December and October, respectively) and seasonal variation is known to strongly affect gut microbiota composition (Bleau et al. 2020; Brar et al. 2025) . For instance, *Lactobacillus* spp. tends to dominate during spring, whereas *Gilliamella* is more prevalent in summer and winter, which is consistent with the patterns observed in our study (Bleau et al. 2020; Brar et al. 2025). These genera function as a critical symbiotic community for host health, highlighting their specific and important roles in digestion and protection against pathogens (Lang et al. 2022).

On the other hand, unique genera was identified *Providencia* (92.22%) and *Bifidobacterium* (1%) within Am_Tn. The dominance of *Providencia* in raises concerns, as this genus is often associated with opportunistic pathogenicity in various hosts (Engel and Moran 2013; Kwong and Moran 2016). In contrast, the Am_Dz sample exhibited a more balanced

gut microbiota composition, dominated by core bacterial genera such as *Gilliamella* (29.28%) and *Snodgrassella* (24.21%). These genera are reported to be essential for gut health, nutrient digestion, and pathogen defense, exhibiting high species diversity and strong host adaptation (Zang et al. 2022). Two other abundant genera included *Lactobacillus* (12.81%) and *Bombilactobacillus* (12.24%). These bacteria are important for the health, nutrition, and immune defense of the host (Brar et al. 2025). Additional genera, including *Brucella* (3.77%), *Frischella* (1.46%), *Lelliottia* (1.05%), and *Klebsiella* (1.31%) exhibited lower relative abundances. This divergence suggests that the gut microbiota of Am_Tn may reflect altered microbial environment, which could have implications for the health and resilience of these bees. The findings from this study align with previous research indicating that environmental factors, management practices, and host genetics can significantly influence gut microbiota composition in honey bees (Martinson et al. 2011). Furthermore, research by Su et al. (2022) emphasises the role of diet and environmental factors in shaping gut microbiota diversity, which may explain the observed differences. Overall, metabarcoding can reveal community composition, biodiversity and potential ecological interactions. However, it may be limited by contamination, technical errors and reliance on relative abundance. To improve reliability and interpretability, future studies should incorporate larger sample sizes and functional assays. Taken together, these findings suggest the need for further investigation into the factors contributing to these microbial shifts and their implications for honeybee health and productivity.

5 Conclusion

This study provides an overview of the gut microbiota diversity of two honey bee subspecies from Tunisia and Algeria. DNA barcoding confirmed *A. m. intermissa* in Tunisia (Am_Tn) and *A. m. iberiensis* Algeria (Am_Dz). The two locations differ in vegetation and management: Am_Tn has simple orange shrub vegetation with no phytosanitary treatments, while Am_Dz features more diverse habitats, including cultivated plants, trees, and occasional wild flora, with hives moved during pesticide treatments. Metabarcoding analysis of the 16 S rRNA V3–V4 regions revealed significant differences in the structure of bacterial communities and their relative abundances in the two populations. In Am_Tn, the gut microbiota was largely dominated by *Providencia*, whereas the Am_Dz population exhibited a more diverse community, with core gut genera such as *Gilliamella* and *Snodgrassella* predominating. These differences likely reflect variation in environmental conditions and forage availability rather than a altered microbial environment. These findings emphasise the critical role of host-microbiome interactions in maintaining honey bee health and resilience, highlighting the importance of sustainable management strategies that are tailored to local environmental and ecological conditions. Further research is required to elucidate the functional implications of these microbial shifts and their effect on colony productivity and survival.

Author contributions Afef Najjari: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. Chahnez Naccache: Writing–Visualization, Software, Formal analysis. Selama Djebbi: review & editing, Resources. Imen Kharrat: review & editing, Methodology. Amira Souii: Formal analysis. Maha Mezghani Khemakhem: Review & editing, Resources, Supervision, Funding acquisition and Conceptualization.

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Data availability The obtained raw sequences were submitted to the National Center for Biotechnology Information (NCBI) as Sequence Read Archive (SRA) under accession numbers as follows: Am_Tn (SRR28547106) and Am_Dz (SSR28547105).

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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