Türk Tarım ve Doğa Bilimleri Dergisi 11(4): 904–914, 2024

https://doi.org/10.30910/turkjans.1455870

TÜRK
TARIM ve DOĞA BİLİMLERİ
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TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

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Araştırma Makalesi

Metagenomic Analysis of Bacterial Communities in Bee Bread in Türkiye

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Geliş Tarihi: 20.03.2024 Düzeltme Geliş Tarihi: 13.09.2024 Kabul Tarihi: 13.09.2024

ABSTRACT

This study aims to investigate the bacterial community structure in bee bread samples collected from 10 provinces of Türkiye using next-generation sequencing (NGS) and metagenomic analysis. Bacterial genomic DNA was extracted and sequenced using Illumina MiSeq. Bioinformatic analysis involved quality assessment, OTU classification, principal coordinate analysis (PCoA), and diversity index calculations. Heatmap and PCoA were utilized to explore the impact of locality and ecological zones on microbial diversity. Metagenomic analysis of 12 bee bread samples revealed 276,583 high-quality sequencing reads. The dominant bacterial phyla identified were *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, and *Firmicutes*. At the genus level, *Streptomyces*, *Streptococcus*, *Bacillus*, and *Synechococcus* were the most abundant, with *Streptomyces* and *Bacillus* playing key roles in the fermentation process of bee bread. The Shannon diversity index (ranging from 2.92 to 4.26) and Simpson's index (0.83 to 0.95) indicated high species diversity and relative abundance in bee bread. The study underscores the need for locality-specific approaches in beekeeping management and highlights the potential significance of beneficial bacterial taxa, particularly those involved in fermentation, in contributing to the nutritional and health properties of bee bread. These findings provide a foundation for further research on the microbial dynamics that support bee colony health.

Key words: Bee bread, bacterial community structure, metagenomic analysis, microbial diversity, Türkiye.

Türkiye'de Arı Ekmeğinde Bulunan Bakteriyel Toplulukların Metagenomik Analizi

ÖZ

Bu çalışma, Türkiye'nin 10 ilinden toplanan 12 adet arı ekmeği örneğindeki bakteriyel topluluk yapısını next-generation sequencing (NGS) ve metagenomik analiz kullanarak araştırmayı amaçlamaktadır. Bakteriyel genomik DNA, Illumina MiSeq kullanılarak ekstrakte edilmiş ve dizilenmiştir. Biyoinformatik analiz, kalite değerlendirmesi, OTU sınıflandırması, principal coordinate analysis (PCoA) ve çeşitlilik indeksi hesaplamalarını içermektedir. Isı haritası ve PCoA, lokasyon ve ekolojik bölgelerin mikrobiyal çeşitlilik üzerindeki etkisini belirlemek amacı ile kullanılmıştır. 12 arı ekmeği örneği analiz edilerek 276,583 yüksek kaliteli DNA dizilimi elde edilmiştir. En baskın bakteri şubeleri Proteobacteria, Actinobacteria, Cyanobacteria ve Firmicutes olarak belirlenmiştir. *Streptomyces*, *Streptococcus*, *Bacillus* ve *Synechococcus* en bol bulunan cinsler olarak belirlenmiştir. *Streptomyces* ve *Bacillus*, arı ekmeğinin fermantasyon sürecinde kilit rol oynamaktadır. Shannon çeşitlilik indeksi (2.92 ile 4.26 arasında değişmekte) ve Simpson indeksi (0.83 ile 0.95 arasında değişmekte) arı ekmeğinde yüksek tür çeşitliliği ve göreceli bolluğu göstermiştir. Çalışma, arıcılıkta lokasyona özgü yaklaşımların gerekliliğini vurgulamakta ve özellikle fermantasyon sürecine katılan faydalı bakteriyel taksonların arı ekmeğinin besin ve sağlık özelliklerine katkıda bulunmadaki potansiyel önemine dikkat çekmektedir. Bu bulgular, koloni sağlığını destekleyen mikrobiyal dinamikler üzerine gelecekte yapılacak araştırmalar için bir temel sağlamaktadır.

Anahtar kelimeler: Arı ekmeği, bakteri topluluk yapısı, metagenomik analiz mikrobiyal çeşitlilik, Türkiye.

INTRODUCTION

Situated at the crossroads of the Mediterranean, Euro-Siberian, and Irano-Turanian phytogeographic regions, Türkiye boasts a mosaic of diverse vegetation types and a rich flora, shaped by its unique geographical position (Davis, 1965-1985; Davis et al.,1988; Güner et al., 2000; Kösoğlu et al., 2019). A comprehensive compilation of floristic studies conducted until 2012 revealed a staggering 11,707 taxa, with an endemism rate of 31.82% in Türkiye (Güner et al., 2012). This botanical wealth, coupled with favorable ecological conditions, has not only positioned Türkiye as a global hotspot for biodiversity but has also laid the foundation for a thriving beekeeping industry.

Globally ranked as the third-largest beekeeping country, boasting around 8,179,085 colonies and 72,325 professional beekeepers, Türkiye holds significant potential in apiculture, following closely behind China and India (Anonymous, 2022). Beyond its pivotal role in ecosystem pollination, the honey bee contributes to both wholesome nutrition and economically valuable products, encompassing honey, bee pollen, bee bread, royal jelly, beeswax, propolis, bee venom, and apilarnil. The honey bee, diligently foraging for nectar and pollen, utilizes nectar as a carbohydrate source, while pollen serves as the primary reservoir of protein, lipids, sterols, and micronutrients (minerals and vitamins) (Vaudo et al., 2020; Crone et al., 2022). Pollen, containing the male reproductive cells of flowering plants, constitutes a crucial component in the diet of both adult and larval honey bees, collected meticulously by foragers from flowering plants.

In recent years there has been an increasing research interest in bee pollen and bee bread due to their nutritional and health-related properties (Kieliszek et al., 2018). Bee pollen, harvested from flowering plants by foragers, undergoes a transformative process within the hive, culminating in bee bread. This final product is a fermented amalgamation characterized by a high content of carbohydrates, essential amino acids, fatty acids, and organic acids, as well as vitamins and minerals. This intricate transformation involves the collaborative efforts of various microbial entities, including bacteria, yeast, and LAB (lactic acid bacteria). Notably, the bacterial diversity within bee bread surpasses that of bee pollen, influenced by factors such as storage duration, processing, fermentation, and the high-sugar environment (Vásquez and Olofsson, 2009; Ghosh et al., 2022), highlighting the need for detailed exploration.

Advancements in next-generation technologies have significantly contributed to the exploration of microbial communities associated with bee products. Metagenomic and metabarcoding studies, prominently applied in apiculture research, have identified the core honeybee gut bacterial microbiota (Martinson et al.; 2011; Corby-Harris et al., 2014; Moran, 2015, Kwong and Moran, 2016; Jones et al., 2018; Yun et al., 2018; Papp et al., 2022) and microbiota linked to bee products (Arserim Uçar, et al. 2022; Ghosh et al., 2022). These technologies have also been pivotal in determining the botanical origins of honey. Analysis of the gut community through 16S rDNA revealed primarily nine bacterial types in the workers' intestines (Martinson et al., 2011; Corby-Harris et al., 2014; Moran, 2015; Kwong and Moran, 2016; Jones et al., 2018; Yun et al., 2018; Papp et al., 2022). The gut microbiome plays a crucial role in carbohydrate metabolism on the pollen cell wall and fermentation as probiotics. For instance, certain strains of *Gilliamella apicola* possess genes for pectin degradation, a component of the pollen grain cell wall. This degradative activity aids in pollen digestion, as bees cannot produce pectinases themselves (Kwong and Moran, 2016). Moreover, microbiota may contribute to detoxification processes by degrading xenobiotics, including pesticides (Pang et al., 2020). Beyond the gut microbiota of honeybees, the hive microbiota also contributes to the microbiota of bee bread (Donkersley et al., 2018; Anderson et al., 2013; Lozo et al., 2015).

Despite the growing interest in bee pollen and bee bread, there remains a scarcity of data on the bacterial communities associated with these essential beekeeping products in the Turkish context. This study aims to fill this knowledge gap by employing next-generation sequencing (NGS) and metagenomic analysis to investigate the bacterial community structure and identify dominant bacterial populations present in bee bread samples collected from Türkiye. In doing so, we aspire to deepen our understanding of the intricate microbial dynamics within bee bread, unveiling the unique interplay between Türkiye's diverse flora and the microbial communities contributing to the formation and properties of this crucial beekeeping product.

MATERIALS and METHOD

Sampling locations

Twelve bee bread samples were collected from 10 different provinces of Türkiye during in June 2022 (Figure 1). The sampling locations were selected based on their climatic conditions, landscape, and beekeeping type (Table 1).

The bee bread samples were collected from fresh honeycombs using a sterile steel comb to avoid contamination. To minimize external microbial influence, all tools used in the sampling process were sterilized

with alcohol before each use. Additionally, the samples were taken directly from newly formed bee bread that had been freshly stored by the bees to ensure the accuracy of the microbial analysis. Each sample was placed into sterile, labeled containers and immediately frozen at -20°C to preserve the microbial community and prevent any post-sampling contamination.

Figure 1. Map of bee bread sampling areas

Bacterial genomic DNA extraction and sequencing

In adherence to the guidelines provided by the manufacturer, bacterial DNA extracted from bee bread using the QIAGEN DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The quantification of nucleic acid content within each DNA sample was accomplished utilizing the Qubit dsDNA BR Assay Kit. Extracted DNA samples were kept at −20 °C prior to library preparation. Amplification of the V3–V4 region of the 16S rRNA gene was conducted using the primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), followed by sequencing on an Illumina MiSeq (Macrogen, Seoul, Korea).

Sequence analysis

The purification of 16S rRNA gene V3-V4 amplicon products was carried out using the Column-Pure Gel and PCR Clean-Up Kit. Subsequently, library construction for the 16S rRNA V3-V4 amplicon products was achieved using the Nextera XT DNA Library Prep Kit. Indexing was conducted with the TG Nextera XT Index Kit v2 Set A, which comprises 96 indices for 384 samples, also from Illumina. The sequencing process adopted a paired-end (PE) approach with 2 × 150 bp read lengths, executed on the Illumina MiSeq platform.

The quality assessment of raw NGS reads (FASTQ format) was conducted through FASTQC (Andrews, 2010), followed by trimming using Trimmomatic v0.32 (Bolger et al., 2014). Demultiplexing and filtering of low-quality reads were performed using CLC Genomics Workbench from Qiagen. The Kraken2 metagenomics system was employed to apply operational taxonomic unit (OTU) criteria for the classification of the resulting clean reads (Wood & Salzberg, 2014). Principal coordinate analysis (PCoA) plots based on Bray-Curtis distances were generated using OmicsBox software from BioBam Bioinformatics in Spain. Heatmaps were created using the PermutMatrix v1.9.3 package, utilizing Euclidean distance.

Shannon and Simpson diversity index

The Shannon and Simpson's diversity indices were computed at the species level by analyzing read count values in Excel. These indices were employed to assess species richness, evenness, and overall bacterial diversity in bee bread samples. The Shannon Diversity Index (H′) was computed utilizing the formula: $(H') = -\sum_{i=1}^{s}(p_i \ln p_i)$ where p_i represents the proportion of read counts of a sequence corresponding to a bacterial genus (i-th) divided by the total read counts of all bacterial genera in a sample. Regarding the Simpson Index (1−D), the formula used was $(1-D) = 1-[\sum n_i(n_i-1)/N(N-1)]$ where n_i denotes the read counts of a sequence representing a bacterial genus (i-th), and N is the total read counts of all bacterial genera in a sample. Typically, the Shannon

diversity index ranges between 1.5-3.5, increasing with evenness, while the Simpson diversity index (1-D) is a value between 0 and 1, with 1 indicating complete evenness.

Table 1. List of the location, flora, and beekeeping types of the collected bee bread samples used in the study

Code	Location	Landscape	Flora	Beekeeping type
1	Sirnak	Alpine meadow	Irano-Turanian floristic *region*	Stationary
2	Hakkari-2	Alpine meadow-above 1800 m	Irano-Turanian floristic *region*	Migratory
3	Hakkari-1	Alpine meadow-above 1800 m	Irano-Turanian floristic *region*	Stationary
4	Hakkari-3	Alpine meadow-above 1800 m	Irano-Turanian floristic *region*	Migratory
5	Van	Alpine meadow-above 1800 m	Irano-Turanian floristic *region*	Stationary
6	Gaziantep	Mesopotamian steppes between 400-1000 m	Irano-Turanian floristic *region* at the border of the Mediterranean phytogeographic region -and shows east Mediterranean climate	Stationary
7	Bitlis	Subalpine areas of east Anatolian steps 1000 m - 1800 m	Irano-Turanian floristic *region*	Migratory
8	Sanliurfa	Mesopotamian steppes between 400-1000 m	Irano-Turanian floristic *region* at the border of the Mediterranean phytogeographic region -and shows east Mediterranean climate	Migratory
9	Mardin	Mesopotamian steppes between 400-1000 m	Irano-Turanian floristic *region* at the border of the Mediterranean phytogeographic region -and shows east Mediterranean climate	Migratory
10	Bingöl	Subalpine areas of east Anatolian steps between 1000 m – 1800 m	The Irano-Turanian floristic *region* at the border of the Euro-Siberian phytogeographic region	Migratory
11	Erzincan	Subalpine areas of east Anatolian steps between 1000 m - 1800 m	The Irano-Turanian floristic *region* at the border of the Euro-Siberian phytogeographic region	Stationary
12	Tunceli	Subalpine areas of east Anatolian steps between 1000 m - 1800 m	The Irano-Turanian floristic *region* at the border of the Euro-Siberian phytogeographic region	Stationary

RESULTS

In this study, 12 bee bread samples of *A. mellifera* collected from different provinces of Türkiye were used in metagenomic analysis. Next-generation sequencing from samples resulted in a total of 276,583 high-quality sequencing reads (Table 2). In terms of quantitative assessment, the bacterial diversity was quantified using the Shannon diversity index and Simpson's index, as represented by Table 2. The Shannon diversity index exhibited values ranging from 2.92 to 4.26, while Simpson's index ranged from 0.83 to 0.95. These indices collectively indicate a notable species diversity and relative abundance observed in bee bread samples.

Table 2. Sequencing statistics, Shannon and Simpson indices of species-level diversity

Sample code	Number of the reads	Average reading length (base)	Number of the classified reads	Shannon Index (H) / (H / LN (N))	Simpson Index (1-D)
1	18644	137.7	18644 / 100.00%	3.657 / 0.5599	0.9433
2	32756	139.2	32756 / 100.00%	2.927 / 0.4264	0.8428
3	11829	139.8	11829 / 100.00%	2.97 / 0.4684	0.8355
4	9400	141.2	9400 / 100.00%	4.346 / 0.6696	0.9531
5	13772	138.0	13772 / 100.00%	3.243 / 0.5014	0.8476
6	24812	140.4	24812 / 100.00%	3.977 / 0.5415	0.9273
7	13345	139.6	13345 / 100.00%	3.929 / 0.5739	0.9269
8	14523	140.4	14523 / 100.00%	4.137 / 0.5899	0.9403
9	20530	140.1	20530 / 100.00%	4.022 / 0.5565	0.93
10	39717	122.3	39717 / 100.00%	3.521 / 0.4882	0.8989
11	19247	139.8	19247 / 100.00%	3.96 / 0.5661	0.9078
12	58008	140.8	58008 / 100.00%	4.262 / 0.5631	0.9417

The relative abundance of OTUs belonging to the phylum, the family, the genus and the species were shown in Figure 2-5. The main bacterial taxonomic units (94%) identified in the samples were belonging to five phyla Proteobacteria, Actinobacteria, Cyanobacteria, Firmicutes, and Bacteroidetes respectively (Figure 2). The relative abundance of Protobacteria, Actinobacteria, Cyanobacteria, and Firmucutes were the highest accounting for 59.59%, 50.14%, 45.12%, and 40.47% of Bingöl, Hakkari-3, Van, and Erzincan, respectively. The others belonged to the phyla Fusobacteriales, Acidobacteria, Planctomycetes, Spirochaetes, Tenericutes, and Verrucomicrobia.

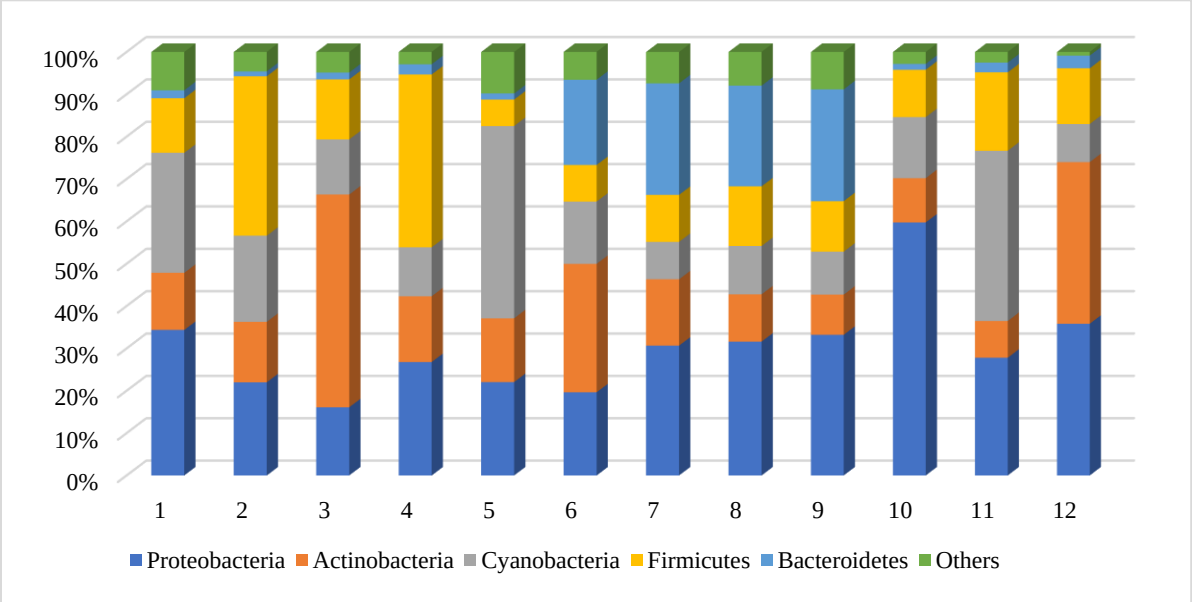

Figure 2. The phylum-level taxonomic distribution of bee bread samples

We also mapped the OTUs to the representative bacterial families (Figure 3). Out of the top 15 dominant families (>2% relative abundance), the relative abundance of Enterobacteriaceae (Bingöl), Streptomycetaceae (Hakkari-1), Bacillaceae (Hakkari-2) Synechococcaceae (Van-5) were the highest with 38.23%, 35.91%, 33.64%, and 31.30%, respectively.

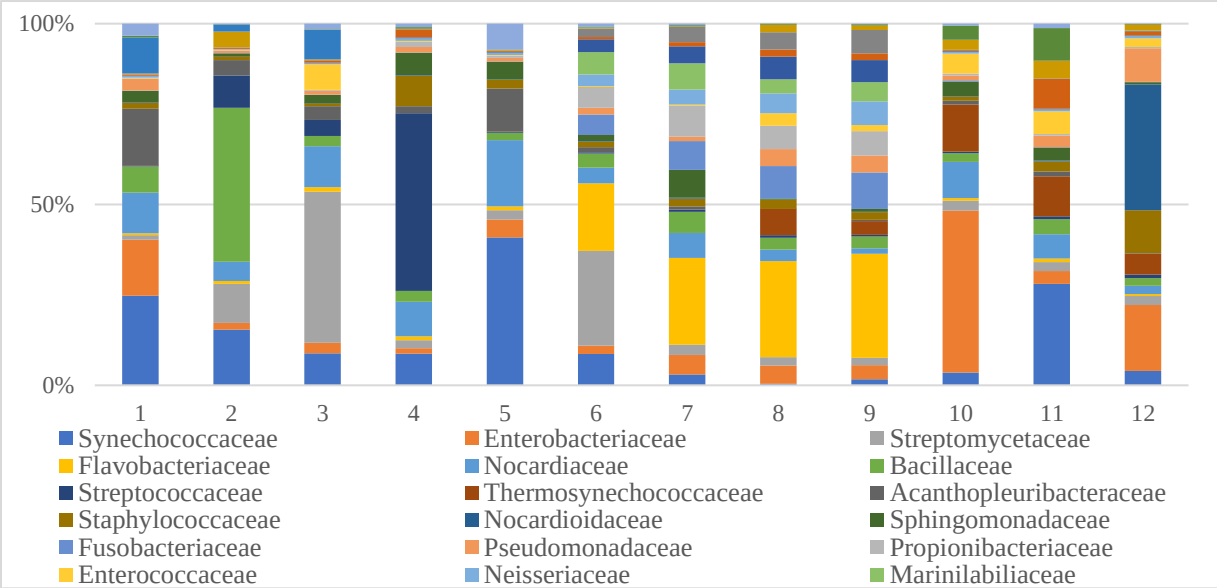

Figure 3. Family-level distribution of bacterial population in bee bread

Based on their readings, 22 of the 1412 bacterial taxa identified by the analysis were deemed to be the most abundant (Figure 4). *Streptomyces* (36.22 %), *Streptococcus* (35.71 %), *Bacillus* (34.56 %), and *Synechococcus* (31.33 %) had the highest relative abundances in Hakkari-1, Hakkari-3, Hakkari-2, and Van samples, respectively.

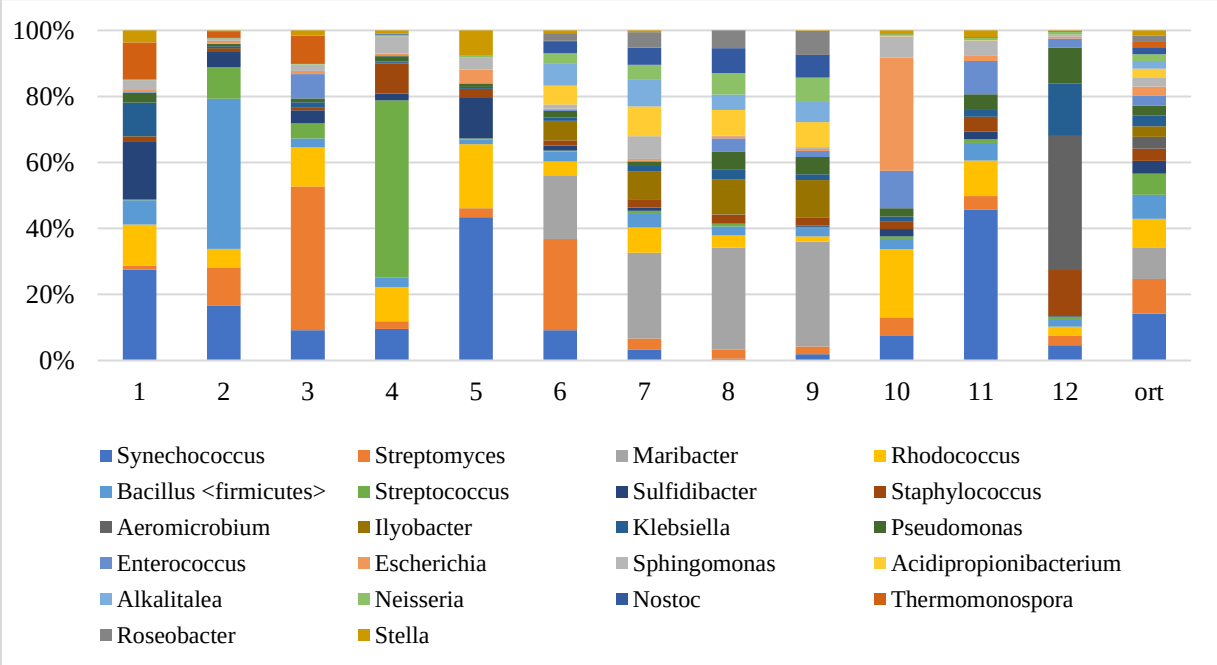

Figure 4. Genus-level distribution of bacterial population in bee bread

A total of 4914 species were identified in all bee bread samples. To determine the impact of the locality and ecological zones on the metagenomic diversity and richness heatmap clustering and PCoA analysis were performed. According to the heatmap, *Synechococcus* sp. PCC_7336, and *Rhodococcus coprophilus*, are two species with the highest abundance (> 1%) in 11 of the 12 samples. *Maribacter hydrothermalis* (Bitlis, Şanlıurfa, and Mardin), and *Synechococcus* sp. PCC_7336 (Şırnak, Van, and Erzincan) are meausered as the species with the highest abundance in three samples. *Bacillus thuringiensis* (Hakkari-2), *Streptomyces gilvosporeus* (Hakkari-1), *Rhodococcus coprophilus* (Hakkari), *Streptomyces lincolnensis* (Gaziantep), *Klebsiella pneumoniae* (Tunceli), and *Enterobacter bugandensis* (Bingöl) were the most abundant species in one sample (Figure 5).

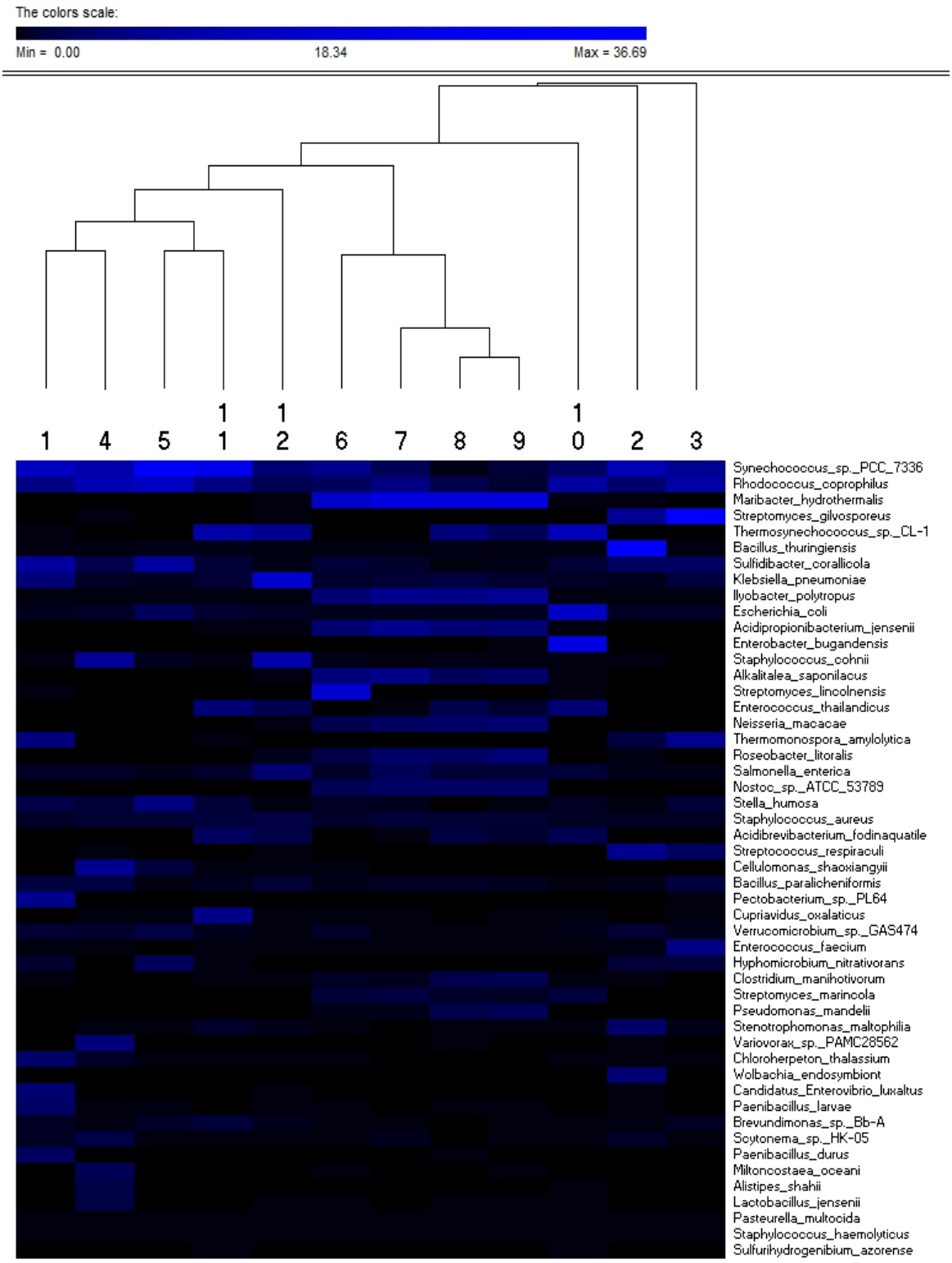

Figure 5. A Community Heatmap was constructed by assessing the abundance of the top 50 most prevalent species in the bee bread samples. The columns in the heatmap are aligned with individual samples, while each row represents the microbiota at the species level. Hierarchical clustering was performed using the Euclidean distance metric.

PCoA analysis for the bacterial species demonstrated the separation of clusters for alpine meadows, Mesopotamian steps, subalpine areas of Mesopotamian steps and except for sample 7 (Bitlis) (Figure 6).

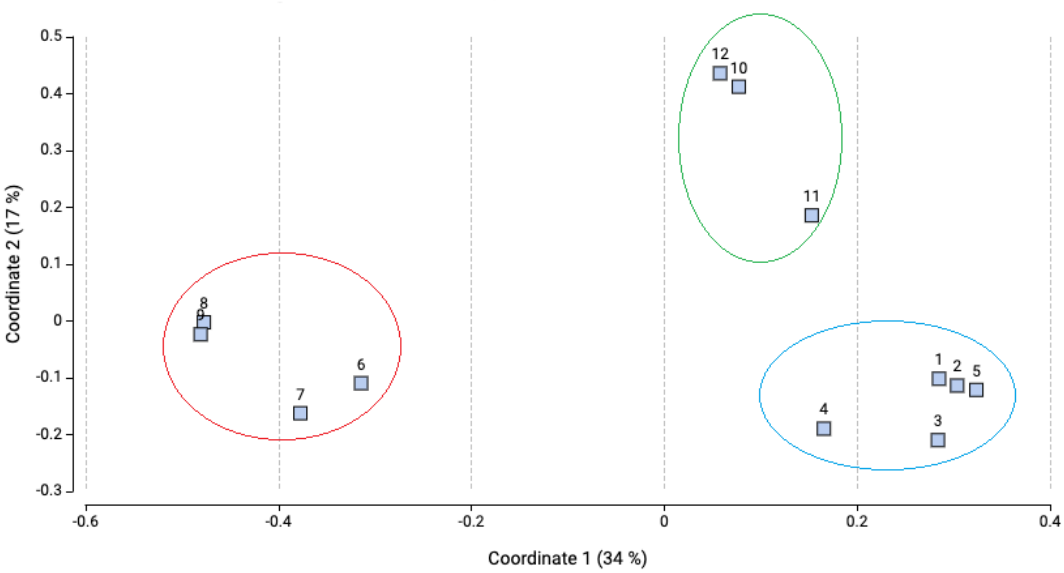

Figure 6. Principal Coordinate Analysis (PCoA) of microbiota structure in bee bread samples. Each data point on the plot represents an individual sample. The PCoA was calculated using Bray-Curtis distance with a multivariete t-distance.

DISCUSSION

The rich floral diversity of Türkiye, influenced by its unique geographical position at the convergence of diverse phytogeographic regions, has created an optimal environment for a flourishing beekeeping industry. The present study delves into the bacterial communities within bee bread, a product of complex interactions between bee-collected pollen, bacterilal symbionts, and environmental factors. Through metagenomic analysis, we aimed to unravel the intricate dynamics of bacterial communities within bee bread, shedding light on the interplay between Türkiye's diverse flora and the microbial populations that contribute to the formation of this vital beekeeping product.

The results reveal a high level of bacterial diversity within the bee bread samples, as indicated by the Shannon and Simpson diversity indices. The Shannon diversity index, ranging from 2.92 to 4.26, underscores the richness and evenness of bacterial species, while the Simpson index, within the range of 0.83 to 0.95, further supports the high species diversity and relative abundance in bee bread. This is consistent with previous studies, emphasizing the complex microbial networks associated with bee bread (Anderson et al., 2013; Anderson et al., 2014; Disayathanoowat et al., 2020; Saraiva et al., 2015; Ghosh & Jung, 2022; Uçar Arserim et al., 2022).

The dominant phyla identified in the bee bread samples—Proteobacteria, Actinobacteria, Cyanobacteria, Firmicutes, and Bacteroidetes—align with findings in other bee-related studies (Vásquez & Olofsson, 2009; Ghosh et al., 2022; Saraiva et al., 2015). The variations in the relative abundance of these phyla across different provinces, such as Bingöl, Hakkari-3, Van, and Erzincan, highlight the influence of regional factors on microbial composition.

Enterobacteriaceae, Streptomycetaceae, Bacillaceae, and Synechococcaceae emerged as dominant families, showcasing regional variations in bacterial populations. The relative abundance of these families, especially Enterobacteriaceae and Bacillaceae, has implications for the fermentation and preservation processes in bee bread (Stabler et al., 2021; Ghosh et al., 2022). The study reported by Anderson et al. (2013) encompasses the bacterial families found in bee bread samples obtained from the United States. The bacterial families Streptomycetaceace, Pseudonocardiaceace, Corynebacteriaceace, Staphylococcaceae, Bacillaceace, Leuconostocaceace, Lachnospiraceace, Enterobacteriaceae, Cornabacteriaceae, Enterococcaceae, and Lactobacillaceace were detected in these bee bread samples. The resemblance with this study was attributable to the diverse flora of plants and crops. Indeed, honeybees are natural pollinators of crops, contribute to the shaping of the microbiota in both plants, and subsequently influence the microbiota of bee products.

At the genus level, Streptomyces, Streptococcus, Bacillus, and Synechococcus exhibited the highest relative abundances in specific samples, suggesting their key roles in the microbial ecology of bee bread. The varying abundance of these genera across samples may be attributed to differences in floral sources and environmental conditions (Disayathanoowat et al., 2020; Ghosh & Jung, 2022, Ghosh et al., 2022).

The identification of 4914 bacterial species in all bee bread samples further emphasizes the complexity of microbial communities associated with this beekeeping product. Notably, species such as *Synechococcus* sp. PCC_7336 and *Rhodococcus coprophilus* displayed high abundances across multiple samples, indicating their potential significance in the microbial ecology of bee bread.

Heatmap clustering and PCoA analysis revealed distinct clustering patterns based on the geographical locations of sampling. Species-specific abundance patterns, such as *Maribacter hydrothermalis* in Bitlis, Şanlıurfa, and Mardin, and *Bacillus thuringiensis* in Hakkari-2, highlight the impact of regional factors on the composition of microbial communities in bee bread. PCoA analysis further delineates spatial separation, indicating a correlation between microbial diversity and regional characteristics.

In terms of bacterial community structure, several factors such as climate, geographic locations, land use, plant, and honeybee types can affect and shape the microbiota of bee bread (Disayathanoowat et al., 2020; De Palma et al., 2016; Anderson et al., 2013; Anderson et al., 2014; Danner et al., 2017). While the limited number of the studies regarding the microbial diversity in bee bread samples have been reported throughout the world (Anderson et al., 2013; Anderson et al., 2014; Asama et al., 2015; Disayathanoowat et al., 2020; Saraiva et al., 2015) there is only one study had been conducted in Türkiye (Uçar Arserim et al., 2022). In this study, the bacterial community structure of 11 bee bread samples from Bingöl, Konya, and Hakkari were determined. This scarcity highlights the need for more comprehensive research in Türkiye. Understanding the microbial dynamics within bee bread is crucial for optimizing beekeeping practices, ensuring the production of high-quality bee products, and promoting the overall health of honeybee colonies. The variations observed in bacterial composition across regions underscore the need for locality-specific approaches in beekeeping management.

CONCLUSION

This study represents a significant step in elucidating the intricate microbial dynamics within bee bread in the context of Türkiye's diverse flora. The regional variations in bacterial diversity and abundance emphasize the need for tailored beekeeping practices, considering the unique ecological and climatic conditions of each locality. As the beekeeping industry continues to play a pivotal role in Türkiye's agricultural landscape, the insights gained from this study contribute to the sustainable management of honeybee colonies and the production of high-quality bee products. Further research into the functional roles of specific bacterial taxa and their contributions to the nutritional and health properties of bee bread will undoubtedly enhance our understanding of this symbiotic relationship between bees and their microbial partners.

The metagenomic analysis of 12 bee bread samples collected from various provinces of Türkiye provides valuable insights into the bacterial diversity associated with *Apis mellifera*. The next-generation sequencing approach generated a substantial dataset of 276,583 high-quality sequencing reads, facilitating a comprehensive exploration of microbial composition within the bee bread samples.

The quantification of bacterial diversity using the Shannon diversity index and Simpson's index revealed consistently high species diversity and relative abundance across all samples. The Shannon diversity index ranged from 2.92 to 4.26, while Simpson's index ranged from 0.83 to 0.95. These findings underscore the richness and evenness of microbial communities present in bee bread.

The taxonomic analysis identified Proteobacteria, Actinobacteria, Cyanobacteria, Firmicutes, and Bacteroidetes as the dominant phyla, collectively constituting 94% of the bacterial taxa. Notably, Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes exhibited the highest relative abundance in specific provinces, emphasizing regional variations in microbial composition. The identification of less dominant phyla, such as Fusobacteriales, Acidobacteria, Planctomycetes, Spirochaetes, Tenericutes, and Verrucomicrobia, contributes to a more nuanced understanding of bee bread microbial ecology.

At the family level, Enterobacteriaceae (Bingöl), Streptomycetaceae (Hakkari-1), Bacillaceae (Hakkari-2), and Synechococcaceae (Van-5) emerged as the top four dominant families, each with distinctive prevalence in specific locations. The 22 most abundant bacterial taxa, including *Streptomyces, Streptococcus, Bacillus*, and *Synechococcus*, exhibited varying relative abundances across samples, suggesting niche-specific preferences.

The examination of the most common genera and their biochemical properties further enriches our understanding of the functional roles these bacteria may play within the bee bread ecosystem. A total of 4914 species were identified, with a subset showing a minimum abundance of 0.5% in at least one sample. This detailed taxonomic profiling enhances our ability to discern key contributors to the bee bread microbiome.

The heatmap clustering and Principal Coordinates Analysis (PCoA) based on locality highlight distinct patterns in metagenomic diversity. *Synechococcus* sp. PCC_7336 and *Rhodococcus coprophilus* emerge as species with consistently high abundance (>1%) across multiple samples, indicating their ecological significance. Additionally, locality-specific species, such as *Maribacter hydrothermalis* and *Streptomyces gilvosporeus*, contribute to the regional diversity of bee bread microbiota.

The PCoA analysis reveals clustering patterns associated with different ecological zones, including alpine meadows, Mesopotamian steps, and subalpine areas of Mesopotamian steps. The observed separation of clusters aligns with the notion that environmental factors associated with distinct geographical regions contribute significantly to shaping the bee bread microbiome.

This study highlights the complex microbial dynamics of bee bread and its dependency on ecological and geographical factors. The findings suggest that bacterial communities in bee bread are influenced by the soil-plant-bee interaction, where bacteria may be introduced through pollen and nectar, bee gut microbiota, and the fermentation process within the hive. Particular attention should be paid to beneficial bacteria, such as those from the *Lactobacillaceae* family, which play a significant role in fermentation, preserving bee bread, and enhancing its nutritional value. Emphasizing the role of these beneficial bacteria could contribute to improving the overall health and resilience of bee colonies. Further research into how these bacteria are introduced and their precise roles in bee bread fermentation will help in developing strategies for sustainable beekeeping.

Acknowledgments: The author expresses gratitude to the Turkish Beekeepers Association and the beekeepers who willingly shared the bee breads, for their invaluable contribution throughout the sampling duration.

Authors Contribution: The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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