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RESEARCH ARTICLE

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Determination of Bacterial Diversity of Propolis Microbiota

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Propolis is a natural resinous mixture produced by the excretions of honeybees. PCR amplification of the 16S rRNA gene region was achieved using DNA of pre-enriched propolis samples collected from *Apis mellifera* production hives (n = 37) in Eastern Türkiye (Bingöl and its regions). Next-generation sequencing and metabarcoding techniques were used to identify bacterial communities in propolis samples. Firmicutes dominated the phylum structure, with Proteobacteria, Actinobacteria, Tenericutes, and Spirochaetes following. The top three bacterial families were *Bacillaceae*, *Enterobacteriaceae*, and *Enterococcaceae*. *Bacillus* (dominantly *B. badius* and *B. thermolactis* at the species level) was recognized at the genus level, followed by *Enterococcus* and *Clostridium sensu stricto*. Our study comprehensively identified the bacterial diversity of propolis samples. Further investigations targeting to enlighten the microbiota of propolis and its potential application fields are required to gain better insight into ecological, nutritional, and medicinal perspectives.

Keywords: *Apis mellifera*, metabarcoding, microbiota, next generation sequencing, propolis.

Introduction

Honeybees (*Apis mellifera*) are glamorous eusocial insects because of both honey production and pollination activities. The Greek roots of the word propolis (bee glue), which alludes to hive defense, are pro, which means "at the entrance of", and polis, which means "community". Propolis is a naturally occurring resinous combination made from plant fragments, buds, and honeybee salivary gland excretions. It is utilized for the construction and upkeep of hives, the defense against multiple macro- and micro-invaders, and even the immune system and overall health of the colony. Honey, bee pollen, royal jelly, propolis, beeswax, bee bread, and bee venom are among the honeybee products used by humans to treat a number of maladies, including cancer and infectious diseases caused by germs and viruses, since antiquity.[1–3] Propolis is of tremendous interest due to its possible anti-proliferative effect against several forms of cancer. Several research has been done to analyze its possible pro-apoptotic effects in cell culture models in prepara-

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tion for future assessments. In these studies, a variety of cell lines, including human tongue squamous cell carcinoma line,[4,5] Jurkat (T lymphocyte leukemia)-HepG2, human liver carcinoma, human lung carcinoma,[6] human monocytic cell lines (THP-1), human colorectal carcinoma cell lines (HCT-116), breast cancer cell lines (MCF-7),[7] human gastric carcinoma cell lines (HGC-27), and human non-small cell lung cancer cell lines[8] were used to assess apoptotic activities of propolis. Researchers focused primarily on the chemical components of propolis in inhibiting cancer cell proliferation and highlighted probable biological pathways.

Different chemicals with varying biological activity have been found in raw propolis, including resins and balms, fatty acids and waxes, essential oils, vitamins, minerals, flavonoids, phenolic acids, stilbene derivatives, and terpenoids.[9] Its antibacterial properties against human and food pathogens make it most well-known.[10,11] Because of its resinous content, unlike honey, it must be extracted via some organic solvents such as methanol or ethanol to be tested in pharmacological evaluations.[3,12] Propolis has a wide spectrum of bio-pharmacological characteristics in addition to its antimicrobial capabilities, including antioxidant, anti-inflammatory, immunomodulatory, anticancer, antiulcer, hepatoprotective, cardioprotective, and neuroprotective effects. Furthermore, it is essential to note that its composition is subject to alter depending on a variety of conditions, including climate, flora, and geographic region.[13,14]

A few studies targeted to investigate the microbial community structure of propolis since it was considered relatively aseptic for a long time. The first study group evaluated honeybee hive-component-based communities via phospholipid fatty-acid and fatty-acid methyl ester analyses and offered an approximate microbial community structure.[15] Recently, two other groups enlightened the diverse microbial community structure of propolis from *A. mellifera* hives from four locations in Mexico[2] and a single hive of *A. mellifera* in Italy.[16] New data also suggested that propolis affects the mouthpart microbiota of honeybees by reducing potential pathogens and promoting commensal and beneficial bacteria.[17] Saelao et al.[18] investigated the microbiota of honeybee colonies in propolis-rich and propolis-poor habitats. According to the findings of their sequencing research, *Bifidobacterium* and *Enterobacteriaceae* predominated in propolis-rich colonies, while *Bifidobacterium* and *Lactobacillus* were prominent in honeybees from propolis-poor colonies. These findings collectively suggested that propolis had a direct effect on bee health.

This study examined 37 Anatolian propolis samples from various hives of *A. mellifera* via a metabarcoding approach to identify bacterial communities from the kingdom to the species level by Next Generation Sequencing (NGS). In addition, the relationships of propolis samples with identified bacterial genera were determined by Detrended Correspondence Analysis (DCA). This is the first report on propolis microbiota community structure in Türkiye. It also represents the most comprehensive metabarcoding analysis conducted up to now on propolis in literature due to the satisfyingly high sample numbers included.

Results

Metabarcoding

This study aimed to evaluate the bacterial microbiota of 37 raw propolis samples from *A. mellifera* hives in Bingöl and its surrounding areas using a metabarcoding approach. At the beginning of this study, DNA extractions were performed directly from propolis samples using DNeasy® PowerFood® Microbial kit (Qiagen, Germany) and after a pre-enrichment step with Brain Heart Infusion Broth (BHI) using the phenol/chloroform/isoamyl alcohol method. PCR amplification could not be achieved with the DNA samples directly extracted from propolis samples, most probably due to low bacterial content. Hence, they were excluded from the study. Therefore, the results presented in this report refer to the metabarcoding analysis of pre-enriched propolis samples.

The amount of Operational Taxonomy Unit (OTU) allocated to the phylum, family, genus, and species levels are depicted in *Figures 1* through 4, respectively. All samples of propolis belonged to the kingdom Bacteria. Firmicutes was the most prevalent phylum, followed by Proteobacteria, Actinobacteria, Tenericutes, and Spirochaetes (*Figure 1*). *Figure 2* displays the percentages of bacterial OTUs ascribed to their respective families. *Bacillaceae* was the most prevalent family, followed by *Enterobacteriaceae*, *Enterococcaceae*, *Clostridiaceae*, and *Rhodobacteraceae*. *Bacillus* was the most prevalent genus in the propolis samples, followed by *Enterococcus*, *Clostridium sensu stricto*, *Enterobacter*, and *Micromonospora* (*Figure 3*). Additionally, several bacterial species were identified (*Figure 4*). *Bacillus badius* and *B. thermolactis* were identified as the predominant species in 20 propolis samples. In addition, *Micromonospora rifamycinica* predominated

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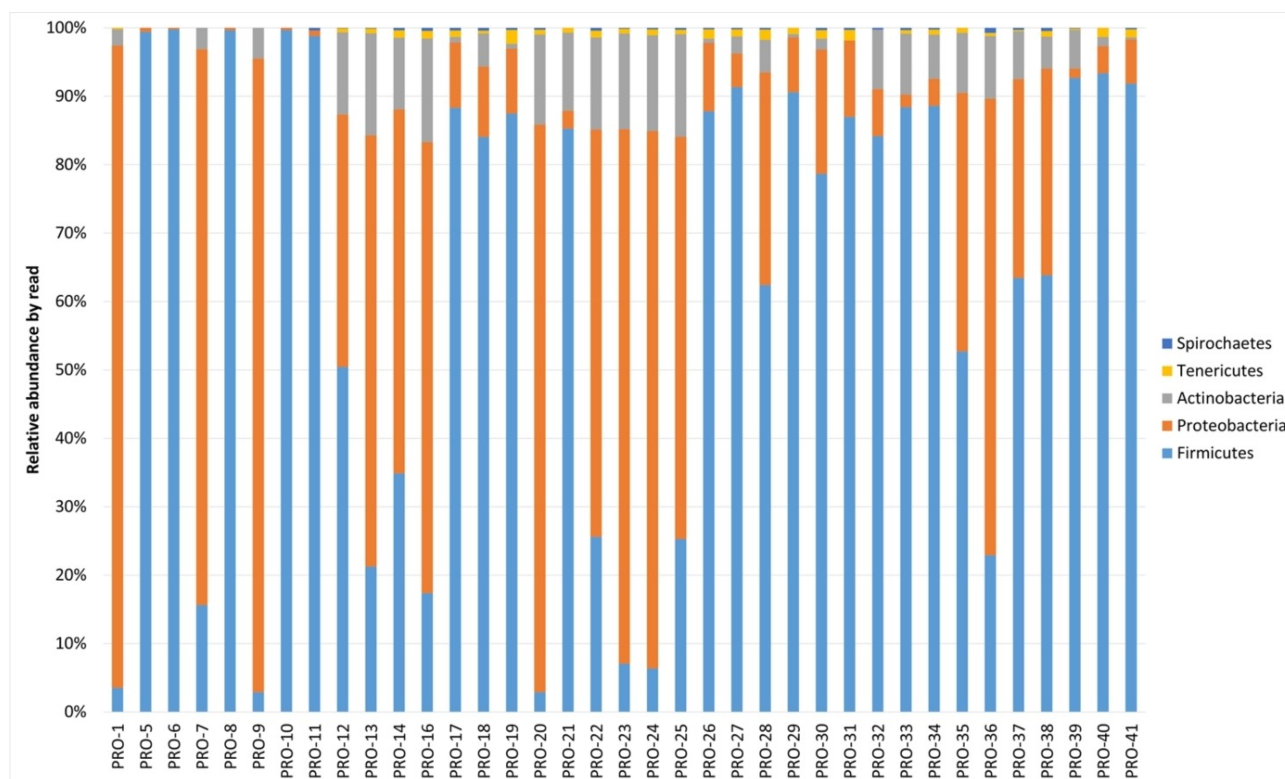

Figure 1. Composition of bacterial microbiota in the pre-enriched propolis samples at the phylum level. Top 5 identified bacterial phyla were shown.

in 15 samples. *Enterococcus faecalis* was detected with high read numbers in PRO-6 and PRO-17 samples, whereas *Pantoea ananatis* was identified with high read numbers in PRO-9 and PRO-24 samples.

Bacterial Diversities of Propolis Samples

In order to determine the relative abundance of species variety within the samples, the Shannon species diversity index values are shown in *Table 1*. Shannon species diversity index values and the identified number of species within each sample generated the evenness values. Lower values of evenness indicate a greater number of diversities. The PRO-10 sample from Karlıova obtained during the November 2019 season exhibited the most diversity. The Karlıova PRO-12 sample contained the greatest number of recognized species, whilst the Bingöl PRO-5 sample contained the least.

DCA demonstrated that most of the samples were associated with *Bacillus*, *Halobacillus*, *Lysinibacillus*, and *Thermoanaerobacter*. PRO-1 was associated with *Anaerovorax*, *Citrobacter*, and *Enterobacter*, and PRO-18 was related to *Clostridium sensu stricto*. On the other hand, *Paenibacillus*, *Salmonella*, *Siccibacter*, and *Amycolatopsis* were separated from other identified genera (*Figure 5*).

Discussion

Honey, pollen and propolis have long been used in traditional and complementary medicine. In addition to its antimicrobial properties, propolis has promising therapeutic potential to improve human health. Considering that propolis is consumed directly by humans, to identify microbiota of propolis is important. In this study, mainly Firmicutes and Proteobacteria constitute the bacterial microbiota of propolis. Recently, Proteobacteria (1–69.5 %), Bacteroidetes (3.3–35.8 %), Firmicutes (9.9–53.7 %), and Actinobacteria (9.3–31.4 %) have been reported in the first study that sought to elucidate the microbial composition of propolis samples taken from *A. mellifera* hives at four sites in Mexico.[2] These results are very similar to our results, as Firmicutes, Proteobacteria, and Actinobacteria were also included in the top three bacterial phyla in our analysis, however, there were few reads for Bacter-

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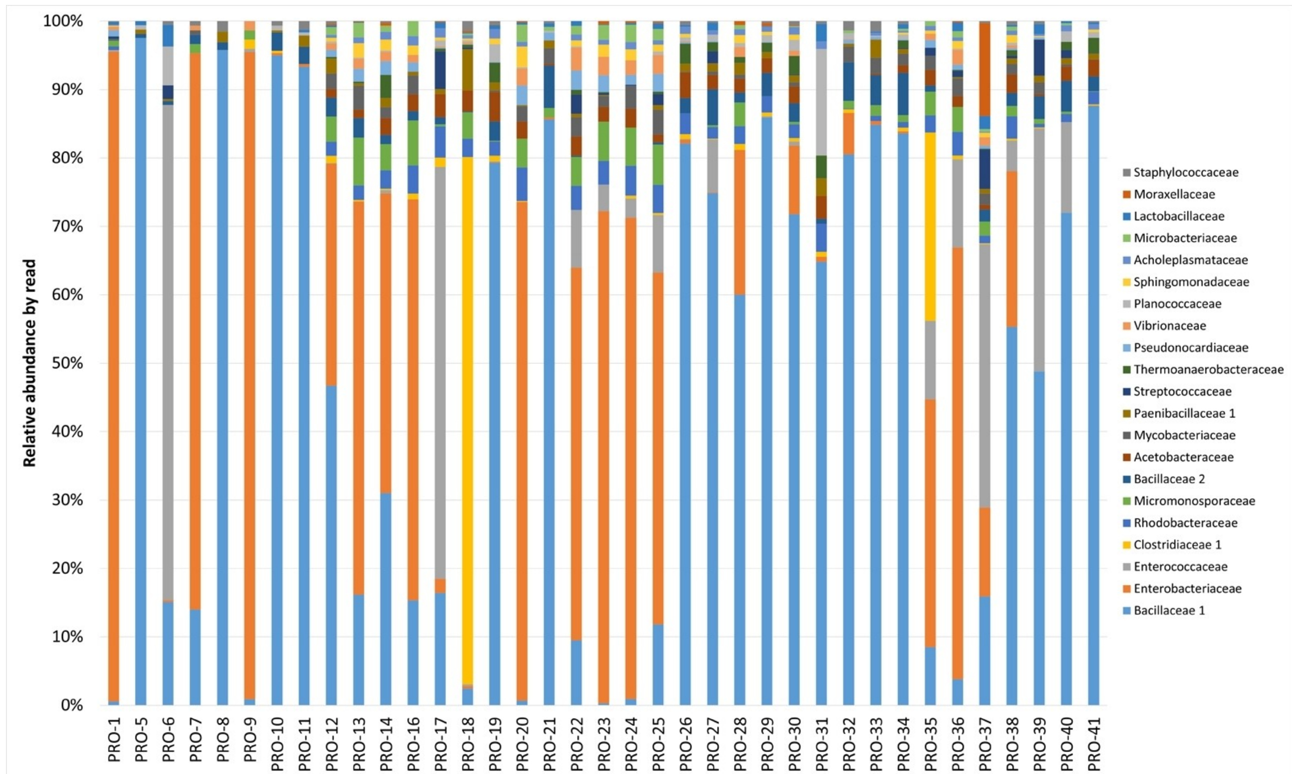

Figure 2. Composition of bacterial microbiota in the pre-enriched propolis samples at the family level. Top 21 identified bacterial families were shown.

oidetes. We identified Firmicutes as the most abundant phylum, followed by Proteobacteria, Actinobacteria, Tenericutes, and Spirochaetes (*Figure 1*). At the genus level, Garcia-Mazcorro et al.[2] reported the most abundant genus as *Rhodopila* followed by *Corynebacterium*, *Sphingomonas*, *Bacillus*, and *Prevotella*. *Rhodopila* including bacteria with one of the highest redox potentials among microorganisms, were not defined in our study. *Corynebacterium*, *Sphingomonas*, and *Prevotella* genera were not detected in our study, in contrast to the work of Garcia-Mazcorro et al.[2] Our study identified *Bacillus* most frequently, followed by *Enterococcus*, *Clostridium sensu stricto*, *Enterobacter*, and *Micromonospora* (*Figure 3*).

Garcia-Mazcorro et al.[2] defined the genus *Sphingomonas* as part of the core microbiota in propolis. Besides, the authors reported that two genera (*Bacillus* and *Prevotella*) comprised a very high percentage of all bacterial microbiota in a location and drew attention to the wide variation of the bacterial composition of propolis samples according to the sampling location. This significant finding must be considered while interpreting the possible similarities/dissimilarities. We subscribe to the quite beneficial suggestions of the authors for some restrictions of these kinds of bee-centered studies and possible amelioration suggestions for future improvements. The lack of control for the surrounding vegetation and specific subspecies of *A. mellifera*, which can be considered factors promoting varying microbial communities in bees, was also our study's restriction. A recent study included the bacterial composition in the gut and bee bread of three common species of stingless bees (*Lepidotrigona terminata*, *L. ventralis*, and *Tetragonula pagdeni*) and bee bread samples of *T. pagdeni*, the likely function of these restricting variables was also explored. The scientists discovered that the architecture of the gut microbiota of three stingless bees were distinct, and that bacterial abundances in the stomach varied even among communities of the same bee species. However, the gut of stingless bees was dominated by a variety of bacteria, including Acetobacter-like, *Snodgrassella*, *Lactobacillus*, *Psychrobacter*, *Pseudomonas*, and *Bifidobacterium*.[19] Rothman et al.[20] also revealed the surprising longitudinal fluctuation of the gut microbial communities from sister honeybees and the effects of floral fodder supplementation on the modification of the honeybee gut microbiome.

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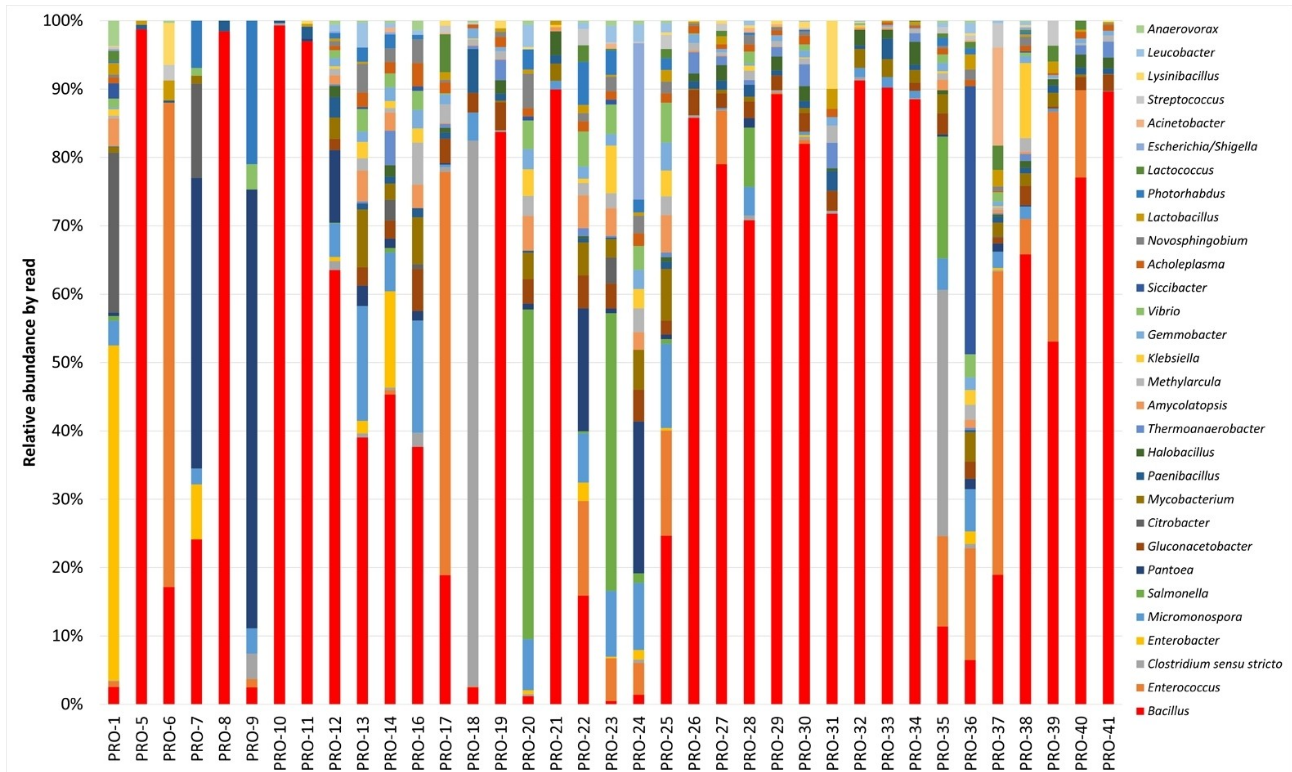

Figure 3. Composition of bacterial microbiota in the pre-enriched propolis samples at the genus level. Top 30 identified bacterial genera were shown.

The second report described the microbial community diversity of propolis by culture-dependent and culture-independent methods in Italy.[16] 16S rRNA analysis identified 352 different OTUs. Three dominating phyla was observed: Proteobacteria (85.4%), Firmicutes (8.8%), and Actinobacteria (4.9%). The highest diversity was obtained for Proteobacteria with 5 different genera with the dominating order of *Erwinia*, *Dickeya*, *Pseudomonas*, *Methylobacterium*, and *Sphingomonas*, while the main components of Firmicutes were *Lactobacillus*, *Bacillus* genera, and *Actinobacteria* for Microbacteriaceae family.[16] Detected phyla in our samples slightly differed from Casalone's study in which the top phylum in our study was Firmicutes. The above-mentioned dominating genera in Casalone's study showed incongruity with ours since the dominating genera in our propolis samples were *Bacillus*.

Bacillus was identified in most of our samples after pre-enrichment process. At the species level, *B. badius*, *B. thermolactis*, *M. rifamycinica*, *B. isabeliae*, *Halobacillus aidingensis*, and *P. ananatis* were the most abundant bacteria in the propolis samples in this study (*Figure 4*). Indeed, *B. badius* is a Gram-positive mesophilic bacterium, and the common habitats of *B. badius* are dust, soil, and coastal waters. In a previous study, *B. badius* isolated from soil was identified as a potential penicillin G acylase (PGA) producer.[21] *B. thermolactis* is a thermotolerant bacterium that was first isolated from milk and dairy farm environment.[22] *M. rifamycinica* was first isolated from mangrove sediment samples and it was able to degrade starch and cellulose, but not gelatin or casein. It can produce rifamycin S and its isomer.[23] Moreover, *B. isabeliae* is a halophilic bacterium and it was isolated from a sea salt evaporation pond.[24] Due to its halotolerant nature, it can survive in extreme environments. *B. isabeliae* has also been isolated from cow dung and curd and it has potential for food preservation and as a probiotic, especially on fruit.[25] *H. aidingensis* was first isolated and identified in Aiding Salt Lake. The growth temperature ranges from 15 to 40 °C and the optimum salt concentration is 10% (w/v) NaCl.[26] *P. ananatis* is known as a broad-spectrum plant pathogen. *P. ananatis* can infect wheat and other monocotyledonous crops such as maize, southern grass, or rice.[27] Most probably bees, soil, and air or dust can contaminate propolis samples and they shape the microbiota.

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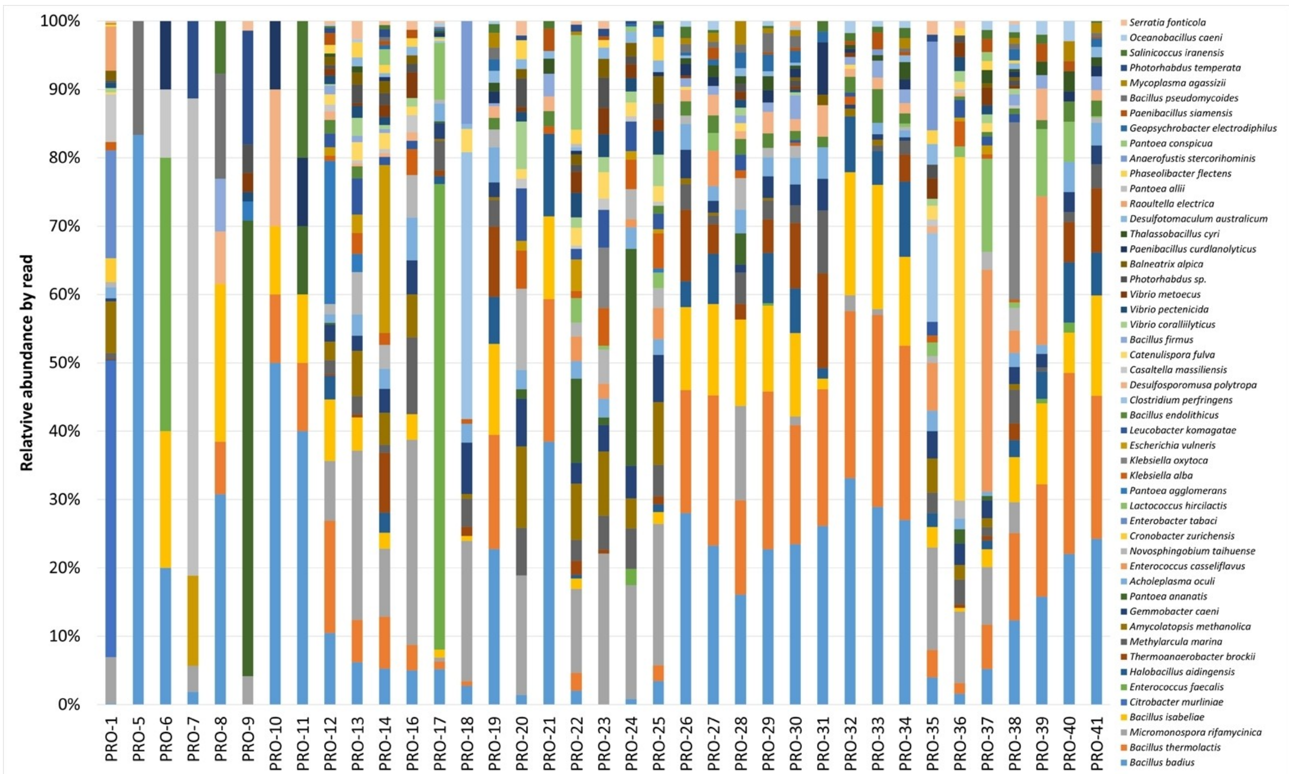

Figure 4. Composition of bacterial microbiota in the pre-enriched propolis samples at the species level. Abundance of top 50 identified bacterial species were shown.

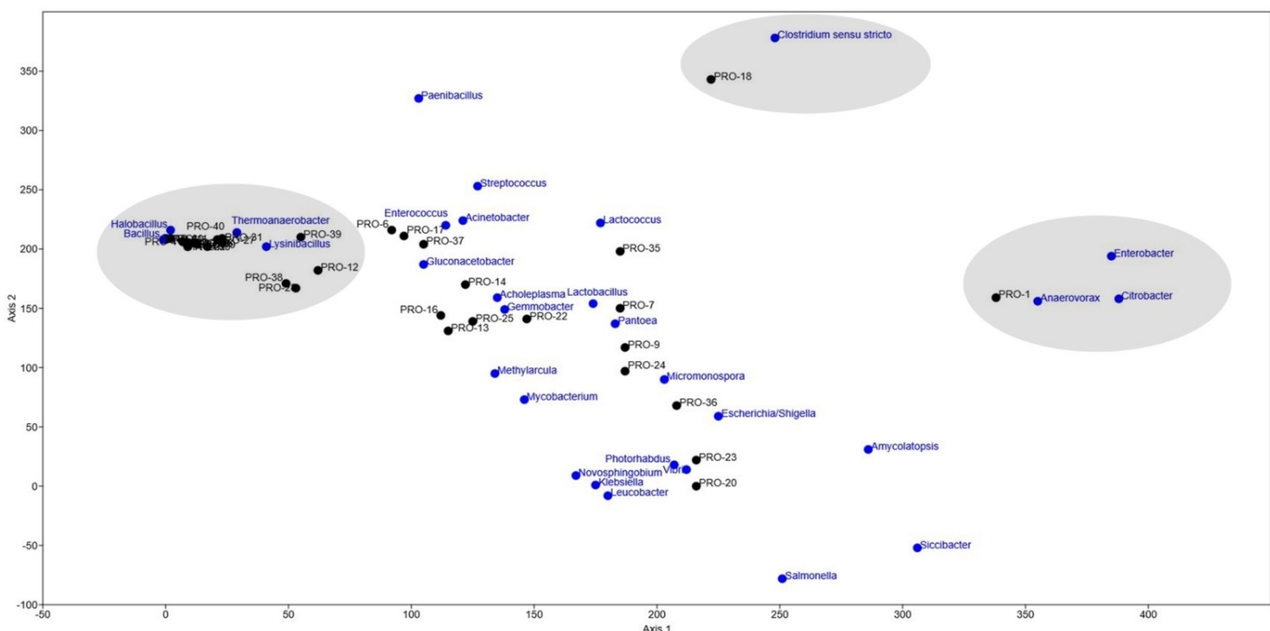

Figure 5. DCA results of the pre-enriched propolis samples based on identified genera interactions with the samples.

The presence of halotolerant bacteria producing antimicrobial substances in propolis can be shown as

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Table 1. Diversity values based on Shannon species diversity indices, the number of identified species and evenness in the propolis samples.

Sample ID	Shannon Species Diversity Index	Number of Identified Species	Evenness
PRO-1	0.594	91	0.132
PRO-5	0.189	08	0.091
PRO-6	0.329	23	0.105
PRO-7	0.631	09	0.287
PRO-8	0.277	16	0.100
PRO-9	0.519	13	0.202
PRO-10	0.205	12	0.082
PRO-11	0.291	17	0.103
PRO-12	0.702	94	0.155
PRO-13	0.664	83	0.150
PRO-14	0.516	62	0.125
PRO-16	0.572	51	0.145
PRO-17	0.582	64	0.140
PRO-18	0.557	68	0.132
PRO-19	0.413	52	0.105
PRO-20	0.576	55	0.144
PRO-21	0.332	31	0.097
PRO-22	0.658	73	0.153
PRO-23	0.726	69	0.171
PRO-24	0.590	60	0.144
PRO-25	0.474	66	0.113
PRO-26	0.518	50	0.132
PRO-27	0.528	58	0.130
PRO-28	0.613	43	0.163
PRO-29	0.571	61	0.139
PRO-30	0.543	55	0.136
PRO-31	0.370	27	0.112
PRO-32	0.542	55	0.135
PRO-33	0.346	40	0.094
PRO-34	0.568	57	0.140
PRO-35	0.662	60	0.162
PRO-36	0.608	57	0.150
PRO-37	0.406	73	0.095
PRO-38	0.667	77	0.154
PRO-39	0.609	62	0.148
PRO-40	0.621	33	0.178
PRO-41	0.660	64	0.159

the reason why propolis samples are more resistant to extreme conditions in nature and show antimicrobial properties.

In directly studied propolis samples, we could not amplify bacterial DNA. Due to the antimicrobial compounds present in propolis, it could inhibit the growth of some bacterial species. Meanwhile, these detected bacterial communities were most probably resistant to antimicrobial compounds present in propolis. It has been shown to have important roles of polyphenols in propolis and other honeybee products, mainly responsible with their antibacterial properties.[28,29] In addition, 66 lipophilic and 37 volatile organic compounds were obtained from ethanolic extract of Anatolian propolis samples, and they showed antibacterial effects especially on *B. cereus*.[30] Organic solvents make the bioactive constituents of propolis available. The chemical components of propolis are complex and vary in quantity and type according to the extraction methods, process conditions, parameters, and solvents utilized.[31] The bioactive properties, including the antibacterial potential of propolis, varied depending on its chemical composition.[30,32] All propolis forms have low water solubility, which means they have low biological activity, including antibacterial activity. In the study of Kubiliene et al.[33] the propolis water extract had no antimicrobial action against *B. cereus* ATCC 8035, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 33499, and *Candida albicans* ATCC 60193. Caffeic acid, *trans-p*-coumaric acid, and ferulic acid are bioactive chemicals found in water-based propolis extract; ethanol-based propolis extract also contains naringenin, kaempferol, and galangin.[33]

A variation in the microbiota of directly studied and the pre-enriched propolis samples could be seen. However, the composition of propolis for the cultivated bacteria in Casalone's study revealed an abundance of *Bacillus*, *Paenibacillus*, and *Staphylococcus*.[16] Thus, the findings of these two studies must be carefully interpreted in the light of some advantages and restrictions; Casalone's study revealed the analysis of the microbiota community structure of propolis sample from a single hive in Italy via both metabarcoding analysis and culture-dependent method while our study consisted of as much as 37 propolis samples from different hives in East Anatolia.

Amplification of 16S rRNA gene region and subsequent sequencing for the characterization of bacteria in stingless bees *Heterotrigona itama* from the honey, bee bread, and propolis in bee farms in different regions of Malaysia, the most prevalent bacteria in nest products belonged to the phylum Firmicutes, followed by Proteobacteria and Actinobacteria. The majority of the isolates at the genus level are *Bacillus* species,[34] and these findings of the report are in complete agreement with our results. According to previous findings, the apparent similarity in bacterial composition between honeybees and stingless bees may indicate similar roles in bee colonies. *Bacillus* species isolated from honeybee products were previously mentioned as having a role in secreting compounds to deter competing microbes from inhibit-

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ing food storage and creating a wide variety of enzymes with a role in food digestion for storage.[35]

DCA is a multivariate method to arrange species and samples along environmental gradients and it could provide important insights in the distribution of dominant bacterial communities in the collected propolis samples. Community structure of PRO-1 and PRO-18 samples were not closely similar to other samples. *Bacillus* dominated in most of the samples as well as *Halobacillus* and *Lysinibacillus* genera. Taken together, these findings suggested that geographic locations, ecology, and plant flora affect propolis microbiota.

The top five genera found in our analyzed propolis samples were *Bacillus*, *Enterococcus*, *Clostridium sensu stricto*, *Enterobacter*, and *Micromonospora*. The predominant bacteria identified from floral nectar and the foreguts of bees were primarily *Bacillus*, and the amylase amounts in nectar highly increased when bacteria were added, confirming the role of bacteria in the transformation of floral nectar into honey in the foreguts of bees.[36] After *Bacillus*, the second leading genus was *Enterococcus*. *E. faecalis*, which was also recognized as one of the most prevalent species in our investigation (sixth species), is commonly found in gastrointestinal tracts, fermented foods, dairy products, and other habitats such as soil and water. It is capable of persisting in the digestive tracts of other *Apis* genera and conferring resistance to bile salts and harsh gastrointestinal conditions. *Clostridium sensu stricto* was identified as the only genus whose decrease was associated with the development of steatosis and fibrosis in a cohort of obese patients with metabolic dysfunction-associated fatty liver disease severity. Further research is required to determine the precise effect of *Clostridium sensu stricto* on honey bees, but its potential preventive effect in enhancing human health was identified in metabolic dysfunction-associated fatty liver disease severity in a cohort of obese patients.[37] *Enterobacter*, a member of the Enterobacteriaceae family, is a standard component of the human intestinal flora; however, not all species are known to cause human disease with multidrug-resistant phenotypes.[38] Soil-derived *Micromonospora* strains are found as endophytic microorganisms associated with plant tissue. *Micromonospora*-derived metabolites, especially macrolides, aminoglycosides, and ansamycins classes, are known for their antimicrobial and anticancer efficacies.[39]

Recent studies focused on propolis supplementation as adjuvant therapy in medicine. The growth inhibitory effects of propolis rendered it a good candidate for preventing periodontal diseases. In support of this idea, Namikawa et al.[40] reported that ethanol-extracted propolis (EEP) specifically inhibited the growth of the periodontal bacterium *Porphyromonas gingivalis* in a dose-dependent manner without damaging the bacterial flora in healthy individuals. Propolis supplementation as an adjuvant agent has become an intriguing topic in cancer chemotherapy/radiotherapy regimens management. Mucoadhesive gel containing 5% Brazilian green propolis was utilized to prevent radiation-induced oral muscositis in patients with oral cancer.[13] A subsequent investigation demonstrated that a gel containing 3% ethanolic extract of Brazilian green propolis was significantly effective on oral microbiota without affecting the oral ecology in patients with mandible fractures.[41] Oral care with propolis was effective for the oral health of head-and-neck cancer chemotherapy patients.[42]

Conclusions

Our study identified dominant bacterial communities present in pre-enriched propolis samples via a culture-independent method. Contrary to the recently reported pioneering studies, our propolis samples did not reflect a huge diversity based on diversity index indicators like Shannon and evenness. It is noteworthy to emphasize some restrictions and potential confounding factors faced with bee-centered studies: The possible effects of supplemental floral forage, which are the sources for visiting and collecting nectar/pollen for honeybees, may be an influencing factor and thus result in the alterations of the gut microbiome of honeybees. Also, the intra-species genetic variations between honeybee colonies must be considered. Hence, an increasing number of reports about the microbial community structure of propolis samples from different geographical regions would enable researchers to compare and stratify the results. In conclusion, our work provides a comprehensive metabarcoding analysis to characterize the bacterial microbiota composition of the propolis samples from various hives of *A. mellifera*. The study is the first report from Türkiye. It includes the highest sample number (n = 37) reported in propolis samples up to now. Thus, revealing the microbial community structure of propolis might lead to employing such data in improving both industrial and medicinal applications.

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Experimental Section

Samples

Propolis (n = 37) were sampled in Türkiye Bingöl city center and its surrounding neighborhoods throughout winter and spring seasons (between November and April) from November 2019 to November 2020. They were kept at +4 °C before extraction of DNA content. *Figure 6A–D* displayed the sampling process and samples, and *Table 2* listed the sampling locations and dates.

DNA Extraction

Direct and pre-enriched propolis samples were used to extract DNA. In direct DNA extraction procedure, the DNeasy® PowerFood® Microbial kit (Qiagen, Germany) was used. Propolis samples (5 g) were homogenized for 5 min at room temperature in buffered peptone water (45 ml) (Oxoid, UK) for pre-enrichment. BHI (Oxoid, UK) was then combined with 1 ml of homogenate and shaken at 200 rpm for 24 h while it was incubated at 35 °C (KS-4000ic control, Ika, Germany). The pellet was then resuspended in 1x TE buffer (500 μl) containing 4 mg/ml lysozyme (Applichem, Germany) after 1 ml of pre-enriched propolis culture was treated by centrifugation (10,000 rpm for 5 min). Total DNA from the growing culture was extracted using the phenol/chloroform/isoamyl alcohol method.[43]

NGS and Metabarcoding

The 16S metagenomic sequencing library was prepared by assembling DNA library for amplicon sequencing (Illumina, Inc., USA), using primers including overhang forward and reverse adapter sequences as given in *Table 3*.[44] Using KAPA HiFi HS Mix, PCR amplification of bacterial 16S rRNA V3-V4 regions were obtained (Roche, Germany), and labelling of samples with dual indexes using the Nextera XT Index Kit v2 Set-A kit (Illumina) was followed. The amplicons and indexed samples were cleaned with AMPure XP beads (Beckman Coulter, USA) by magnetic pull-down (Dyna-

Figure 6. Collection of propolis samples. A&B, Scraping propolis from a beehive. C&D, Propolis samples collected from different locations in Bingöl-Türkiye.

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Table 2. Sampling information of propolis samples.

Sample ID	Sampling Place	Sampling Date	Coordinates[a]
PRO-1	Bingöl	November 2019	38.94497 N, 40.26348 E
PRO-5	Bingöl	November 2019	38.97225 N, 40.42321 E
PRO-6	Kığı	November 2019	39.317612 N, 40.289421 E
PRO-7	Genç	November 2019	38.74901 N, 40.75238 E
PRO-8	Yedisu	November 2019	39.47223 N, 40.39224 E
PRO-9	Salhan	November 2019	39.00780 N, 40.89087 E
PRO-10	Karlıova	November 2019	39.09933 N, 40.82158 E
PRO-11	Yayladere	November 2019	39.22689 N, 40.07201 E
PRO-12	Karlıova	April 2020	39.13133 N, 40.88509 E
PRO-13	Karlıova	April 2020	39.23384 N, 40.97765 E
PRO-14	Karlıova	April 2020	39.39747 N, 40.85938 E
PRO-16	Karlıova	November 2020	39.19750 N, 41.12436 E
PRO-17	Karlıova	November 2020	39.42180 N, 41.04483 E
PRO-18	Yedisu	April 2020	39.41374 N, 40.53367 E
PRO-19	Yedisu	April 2020	39.38154 N, 40.75175 E
PRO-20	Yedisu	April 2020	39.39206 N, 40.85415 E
PRO-21	Yedisu	November 2020	39.39176 N, 40.85405 E
PRO-22	Solhan	April 2020	38.973815 N, 41.062791 E
PRO-23	Solhan	April 2020	38.90660 N, 41.07844 E
PRO-24	Solhan	April 2020	38.73478 N, 41.03612 E
PRO-25	Bingöl	April 2020	38.93409 N, 40.75497 E
PRO-26	Bingöl	April 2020	38.95459 N, 40.34219 E
PRO-27	Bingöl	April 2020	38.83607 N, 40.55401 E
PRO-28	Adaklı	April 2020	39.19563 N, 40.46313 E
PRO-29	Adaklı	April 2020	39.07979 N, 40.29186 E
PRO-30	Adaklı	April 2020	39.28088 N, 40.56733 E
PRO-31	Adaklı	November 2020	39.19235 N, 40.54394 E
PRO-32	Adaklı	November 2020	39.31273 N, 40.44753 E
PRO-33	Kığı	April 2020	39.26623 N, 40.21953 E
PRO-34	Kığı	April 2020	39.31005 N, 40.34989 E
PRO-35	Kığı	April 2020	39.29268 N, 40.29703 E
PRO-36	Yayladere	April 2020	39.21330 N, 40.07794 E
PRO-37	Yayladere	April 2020	39.16473 N, 40.07274 E
PRO-38	Genç	April 2020	38.74901 N, 40.75238 E
PRO-39	Genç	April 2020	38.76360 N, 40.77615 E
PRO-40	Genç	April 2020	38.78048 N, 40.86580 E
PRO-41	Yedisu	April 2020	38.83384 N, 40.49223 E

[a]Sampling coordinates were given based on Google Maps locations.

Table 3. The used primers in the bacterial 16S rRNA amplicon PCR.

Primers	Sequence
16S-Forward	5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′
16S-Reverse	5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′

MagTM-96 Side, Invitrogen, Norway) throughout the preparation of the library. The prepared equimolar amounts of samples were combined, and the resulting DNA library was diluted to 35 pM. The iSeq100 v1 cartridge was then loaded with a 20 μl library containing 5 % (v/v) PhiX control DNA (Illumina). The sequencing was performed using a pair-end read type and two 151 bp reads on an Illumina iSeq100 machine.[44] The collected sequencing raw data were analyzed using the 16S Metagenomics, Version: 1.1.0 software in BaseSpace Sequence Hub (Illumina), and an OTU approach was used to establish the bacterial sequence identity of clustered sequences from the kingdom to the species level. Using 16S Metagenomics software Version 1.1.0 (Illumina) with RefSeq RDP 16S v3 May 2018 DADA2 32 bp taxonomical interference and the

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Ribosomal Database Project (RDP) Classifier, Shannon species diversity index, the number of identified species, and the evenness of propolis samples were determined.[45] DCA was used to examine the associations between propolis samples and prevalent bacterial taxa using the PAleontological STatistic (PAST) Software version 4.06b package.[46]

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Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The bacterial 16S rRNA gene amplicon sequencing data generated in this study using NGS was submitted to NCBI Sequence Read Archive (SRA) database with BioProject accession number PRJNA780802. https://www.ncbi.nlm.nih.gov/sra/PRJNA780802

Author Contribution Statement

Esra Ersoy Omeroglu: Conceptualization, Methodology, Writing-Original Draft; Dılhun Keriman Arserim-Uçar: Methodology, Investigation, Writing-Original Draft; Zeynep Yegin: Methodology, Writing-Original Draft; Nevzat Çağlayan: Methodology, Sample collection; Mediha Nur Zafer Yurt: Methodology; Behiye Busra Tasbasi: Methodology; Elif Esma Acar: Methodology; Samet Ucak: Methodology, Veli Cengiz Ozalp: Supervision, Writing-Review&Editing, Mert Sudagidan: Conceptualization, Methodology, Investigation, Writing-Original Draft. All authors read and approved the final manuscript.

References

[1] V. D. Wagh, 'Propolis: a wonder bees product and its pharmacological potentials', *Adv. Pharmacol.* **2013**, *2013*, 308249.

[2] J. F. Garcia-Mazcorro, J. R. Kawas, A. G. Marroquin-Cardona, 'Descriptive bacterial and fungal characterization of propolis using ultra-high-throughput marker gene sequencing', *Insects* **2019**, *10*, 402.

[3] F. Nainu, A. Masyita, M. A. Bahar, M. Raihan, S. R. Prova, S. Mitra, T. B. Emran, J. Simal-Gandara, 'Pharmaceutical prospects of bee products: Special focus on anticancer, antibacterial, antiviral, and antiparasitic properties', *Antibiotics* **2021**, *10*, 822.

[4] U. Czyżewska, K. Siemionow, I. Zaręba, W. Miltyk, 'Proapoptotic activity of propolis and their components on human tongue squamous cell carcinoma cell lines (CAL-27)', *PLoS One* **2016**, *11*, e0157091.

[5] K. Celińska-Janowicz, I. Zaręba, U. Lazarek, J. Teul, M. Tomczyk, J. Pałka, W. Miltyk, 'Constituents of propolis: chrysin, caffeic acid, *p*-coumaric acid, and ferulic acid induce PRODH/POX-dependent apoptosis in human tongue squamous cell carcinoma cell (CAL-27)', *Front. Pharmacol.* **2018**, *9*, 336.

[6] Y. A. Elnakady, A. I. Rushdi, R. Franke, N. Abutaha, H. Ebaid, M. Baabbad, M. O. Omar, A. A. Al Ghamdi, 'Characteristics, chemical compositions and biological activities of propolis from Al-Bahah, Saudi Arabia', *Sci. Rep.* **2017**, *7*, 41453.

[7] S. Touzani, W. Embaslat, H. Imtara, A. Kmail, S. Kadan, H. Zaid, I. ElArabi, L. Badiaa, B. Saad, '*In Vitro* evaluation of the potential use of propolis as a multitarget therapeutic product: physicochemical properties, chemical composition, and immunomodulatory, antibacterial, and anticancer properties', *BioMed Res. Int.* **2019**, 4836378.

[8] B. Aru, E. Güzelmeric, A. Akgül, G. Y. Demirel, H. Kırmızıbekmez, 'Antiproliferative activity of chemically characterized propolis from Turkey and its mechanisms of action', *Chem. Biodiversity* **2019**, *16*, e1900189.

[9] H. R. Hepburn, 'Composition and synthesis of beeswax', In Hepburn, H. R. (Eds.), Honeybees and Wax: An Experimental Natural History, **1986** Springer: Berlin/Heidelberg, Germany. p. 44–56.

[10] R. P. Dantas Silva, B. A. Machado, G. A. Barreto, S. S. Costa, L. N. Andrade, R. G. Amaral, A. A. Carvalho, F. F. Padilha, J. D. Barbosa, M. A. Umsza-Guez, 'Antioxidant, antimicrobial, antiparasitic, and cytotoxic properties of various Brazilian propolis extracts', *PLoS One* **2017**, *12*, e0172585.

[11] A. Burgut, 'Growth inhibition of foodborne pathogens by co-microencapsulation of lactobacilli cell free and propolis extracts', *J. Food Saf.* **2021**, *41*, e12863.

[12] S. Huang, C. P. Zhang, K. Wang, G. Q. Li, F. L. Hu, 'Recent advances in the chemical composition of propolis', *Molecules* **2014**, *19*, 19610–19632.

[13] V. R. Noronha, G. S. Araujo, R. T. Gomes, S. H. Iwanaga, M. C. Barbosa, E. N. Abdo, E. Ferreira e Ferreira, A. C. Viana Campos, A. A. Souza, S. R. Abreu, V. R. Santos, 'Mucoadhesive propolis gel for prevention of radiation-induced oral mucositis', *Curr. Clin. Pharmacol.* **2014**, *9*, 359–364.

[14] M. Silveira, F. Teles, A. A. Berretta, T. R. Sanches, C. E. Rodrigues, A. C. Seguro, L. Andrade, 'Effects of Brazilian

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green propolis on proteinuria and renal function in patients with chronic kidney disease: a randomized, double-blind, placebo-controlled trial', *BMC Nephrol.* **2019**, *20*, 140.
[15] K. J. Grubbs, J. J. Scott, K. J. Budsberg, H. Read, T. C. Balser, C. R. Currie, 'Unique honeybee (*Apis mellifera*) hive component-based communities as detected by a hybrid of phospholipid fatty-acid and fatty-acid methyl ester analyses', *PLoS One* **2015**, *10*, e0121697.
[16] E. Casalone, D. Cavalieri, G. Daly, F. Vitali, B. Perito, 'Propolis hosts a diverse microbial community', *World J. Microbiol. Biotechnol.* **2020**, *36*, 50.
[17] H. Dalenberg, P. Maes, B. Mott, K. E. Anderson, M. Spivak, 'Propolis envelope promotes beneficial bacteria in the honey bee (*Apis mellifera*) mouthpart microbiome', *Insects* **2020**, *11*, 453.
[18] P. Saelao, R. S. Borba, V. Ricigliano, M. Spivak, M. Simone-Finstrom, 'Honeybee microbiome is stabilized in the presence of propolis', *Biol. Lett.* **2020**, *16*, 20200003.
[19] Q. H. Tang, C. H. Miao, Y. F. Chen, Z. X. Dong, Z. Cao, S. Q. Liao, J. X. Wang, Z. W. Wang, J. Guo, 'The composition of bacteria in gut and beebread of stingless bees (Apidae: Meliponini) from tropics Yunnan, China', *Antonie van Leeuwenhoek* **2021**, *114*, 1293–1305.
[20] J. A. Rothman, M. J. Carroll, W. G. Meikle, K. E. Anderson, Q. S. McFrederick, 'Longitudinal effects of supplemental forage on the honeybee (*Apis mellifera*) microbiota and inter-and intra-colony variability', *Microb. Ecol.* **2018**, *76*, 814–824.
[21] K. Rajendran, S. Mahadevan, R. Jeyaprakash, G. Paramasamy, A. B. Mandal, 'Strategies for enhancing the production of penicillin G acylase from *Bacillus badius*: influence of phenyl acetic acid dosage', *Appl. Biochem. Biotechnol.* **2013**, *171*, 1328–1338.
[22] A. Coorevits, N. A. Logan, A. E. Dinsdale, G. Halket, P. Scheldeman, M. Heyndrickx, P. Schumann, A. Van Landschoot, P. De Vos, '*Bacillus thermolactis* sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*', *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 1954–1961.
[23] H. Huang, J. Lv, Y. Hu, Z. Fang, K. Zhang, S. Bao, '*Micromonospora rifamycinica* sp. nov., a novel actinomycete from mangrove sediment', *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 17–20.
[24] L. Albuquerque, I. Tiago, M. Taborda, M. F. Nobre, A. Veríssimo, M. S. da Costa, '*Bacillus isabeliae* sp. nov., a halophilic bacterium isolated from a sea salt evaporation pond', *Int. J. Syst. Evol. Microbiol.* **2008**, *58*, 226–230.
[25] V. Dhundale, V. Hemke, D. Desai, P. Dhundale, 'Evaluation and exploration of lactic acid bacteria for preservation and extending the shelf life of fruit', *Int. J. Fruit Sci.* **2018**, *18*, 355–368.
[26] W. Y. Liu, J. Zeng, L. Wang, Y. T. Dou, S. S. Yang, '*Halobacillus dabanensis* sp. nov. and *Halobacillus aidingensis* sp. nov., isolated from salt lakes in Xinjiang, China', *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1991–1996.
[27] K. Krawczyk, B. Wielkopolan, A. Obrępalska-Stęplowska, '*Pantoea ananatis*, A new bacterial pathogen affecting wheat plants (*Triticum* L.) in Poland', *Pathogenesis* **2020**, *9*, 1079.
[28] C. Curuţiu, L. M. Diţu, A. M. Grumezescu, A. M. Holban, 'Polyphenols of honeybee origin with applications in dental medicine', *Antibiotics* **2020**, *9*, 856.
[29] A. Meto, B. Colombari, A. Meto, G. Boaretto, D. Pinetti, L. Marchetti, S. Benvenuti, F. Pellati, E. Blasi, 'Propolis affects *Pseudomonas aeruginosa* growth, biofilm formation, eDNA release and phenazine production: Potential involvement of polyphenols', *Microorganisms* **2020**, *8*, 243.
[30] D. K. Arserim Ucar, İ. H. Gecibesler, M. Sudagidan, İ. Dursun, V. Süzerer, R Solmaz, 'Determination of biological activity, lipophilic and volatile organic compounds of Bingöl propolis isolates', *Tr. J. Nature Sci.* **2020**, *9*, 92–102.
[31] V. Bankova, B. Trusheva, M. Popova, 'Propolis extraction methods: A review', *J. Apic. Res.* **2021**, *60*, 734–743.
[32] R. Hossain, C. Quispe, R. A. Khan, A. S. M. Saikat, P. Ray, D. Ongalbek, B. Yeskaliyeva, D. Jain, A. Smeriglio, D. Trombetta, R. Kiani, F. Kobarfard, N. Mojgani, P. Saffarian, S. A. Ayatollahi, C. Sarkar, M. T. Islam, D. K. Arserim Uçar, M. Martorell, A. Sureda, G. Pintus, M. Butnariu, J. Sharifi-Rad, W. C. Cho, 'Propolis: An update on its chemistry and pharmacological applications', *Chin. Med.* **2022**,*17*, 1–60.
[33] L. Kubiliene, V. Laugaliene, A. Pavilonis, A. Maruska, D. Majiene, K. Barcauskaite, R. Kubilius, G. Kasparaviciene, A. Savickas, 'Alternative preparation of propolis extracts: comparison of their composition and biological activities', *BMC Complementary Altern. Med.* **2015**, *15*, 156.
[34] M. S. Ngalimat, R. Raja Abd Rahman, M. T. Yusof, A. Syahir, S. Sabri, 'Characterisation of bacteria isolated from the stingless bee, *Heterotrigona itama*, honey, bee bread and propolis', *PeerJ* **2019**, *7*, e7478.
[35] M. Gilliam, D. W. Roubik, B. J. Lorenz, 'Microorganisms associated with pollen, honey, and brood provisions in the nest of a stingless bee, *Melipona fasciata*', *Apidologie* **1990**, *21*, 89–97.
[36] M. Wang, W. Zhao, H. Xu, Z. Wang, S. He, '*Bacillus* in the guts of honeybees (*Apis mellifera*; Hymenoptera: Apidae) mediate changes in amylase values', *Eur. J. Endocrinol.* **2015**, *112*, 619–624.
[37] N. Lanthier, J. Rodriguez, M. Y. Nachit, H. Sophie, P. Trefois, 'Lower abundance of *Clostridium sensu stricto* is associated with liver steatosis and fibrosis severity in a prospective cohort of obese patients with metabolic dysfunction-associated fatty liver disease', Belgian Week of Gastroenterology **2021** (du 03/03/2021 au 04/03/2021). In: Acta Gastro-Enterologica Belgica, Vol. 84, no.1, p. A20.
[38] A. Davin-Regli, J. P. Lavigne, J. M. Pagès, '*Enterobacter* spp.: Update on taxonomy, clinical aspects, and emerging antimicrobial resistance', *Clin. Microbiol. Rev.* **2019**, *32*, e00002–19.
[39] M. S. Hifnawy, M. M. Fouda, A. M. Sayed, R. Mohammed, H. M. Hassan, S. F. AbouZid, M. E. Rateb, A. Keller, M. Adamek, N. Ziemert, U. R. Abdelmohsen, 'The genus *Micromonospora* as a model microorganism for bioactive natural product discovery', *RSC Adv.* **2020**, *10*, 20939–20959.
[40] D. Namikawa, H. Maruyama, A. Masago, C. Mashimo, T. Nambu, T. Okinaga, K. Takahashi, 'Speciesspecific growth inhibitory effect of propolis', *J. Osaka Dent. Univ.* **2021**, *55*, 83–89.
[41] I. Niedzielska, Z. Puszczewicz, A. Mertas, D. Niedzielski, B. Różanowski, S. Baron, T. Konopka, A. Machorowska-Pieniążek, M. Skucha-Nowak, M. Tanasiewicz, J. Paluch, J.

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Markowski, B. Orzechowska-Wylęgała, W. Król, T. Morawiec, 'The influence of ethanolic extract of Brazilian green propolis gel on hygiene and oral microbiota in patients after mandible fractures', *Biomed Res. Int.* **2016**, 9190814.

[42] M. H. Akhavan-Karbassi, M. F. Yazdi, H. Ahadian, M. J. Sadr Abad, 'Randomized double blind placebo controlled trial of propolis for oral mucositis in patients receiving chemotherapy for head and neck cancer', *Asian Pac. J. Cancer Prev.* **2016**, *17*, 3611–3614.

[43] D. Liu, A. J. Ainsworth, F. W. Austin, M. L. Lawrence, 'Use of PCR primers derived from a putative transcriptional regulator gene for species-specific determination of *Listeria monocytogenes*', *Int. J. Food Microbiol.* **2004**, *91*, 297–304.

[44] Illumina, Metagenomic sequencing library preparation guide. Part # 15044223 Rev.B. https://support.illumina.com/documents/documentation/chemistry documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf.

[45] Q. Wang, G. M. Garrity, J. M. Tiedje, J. R. Cole, 'Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy', *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267.

[46] Ø. Hammer, D. A. T. Harper, P. D. Ryan, 'Past: Paleontological statistics software package for education and data analysis', *Palaeontol. Electron.* **2001**, *4*, 1–9.

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