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Gut Bacterial Microbiota of The Honeybee, Apis mellifera (Hymenoptera: Apidae) with a New Host Record

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Highlights:
- Alimentary trac
- First to report
- Bacterial community

Keywords:
- Microbiota
- Southeast Turkey
- *Paenibacillus glucanolyticus*
- New host
- Honeybee

ABSTRACT:

Gut microbial communities of the honeybee, *Apis mellifera* L. have vital functions in food digestion, providing essential nutrients, improving immune system against pathogens, and detoxifying harmful molecules. The objective of this study was to characterize the core gut bacterial community of newly emerged *A. mellifera* queens and workers from southeastern Turkey. A total of newly emerged 15 queens and 30 workers were collected from the same apiary. The alimentary tract of collected samples was dissected under sterile conditions. Culture based isolation was performed from the digestive tract of the collected samples to obtain a pure microbe culture. The chemical, morphological and molecular description of each bacteria were accurately done via employment of pure culture. Five bacterial isolates were successfully isolated from the *A. mellifera* alimentary tract and purified. Molecular and biochemical identification showed that the isolates were *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus cereus* (Firmicutes: Bacillaceae) (MZ540443, MZ540444, MZ540445), *Staphylococcus haemolyticus* (Firmicutes: Staphylococcaceae) (MZ540447), and *Paenibacillus glucanolyticus* (Firmicutes: Paenibacillaceae) (MZ540446). This study is the first to report the presence of *P. glucanolyticus* in gut bacterial community of *A. mellifera.*

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INTRODUCTION

Bacteria have significant functions in the life of arthropod (Duron et al., 2008; Hilgenboecker et al., 2008). *Apis mellifera* L. (Hymenoptera: Apidae) is an extremely important insect thanks to its products and its contribution to the diversity and reproduction of flora via pollination (Klein et al., 2007). The knowledge of host bacteria is extremely important to understand their biological and potential benefits for their hosts. Gut microbiota have many functions such as food digestion, immunity at colony and individual levels, and food preservation of *A. mellifera* (Dillon and Dillon, 2004; Evans and Armstrong, 2006; Engel and Moran, 2013). Microbiota of a healthy *A. mellifera* is dominated by a conserved phylotype composition, and each phylotype may host a functionally distinct community (Ellegaard and Engel, 2019). The composition of microbiota varies depending on temporal and spatial changes, growth stage, diet, behavior and cast (Corby-Harris et al., 2014; Kapheim et al., 2015; Ludvigsen et al., 2015; Ludvigsen et al., 2017; Jones et al., 2018; Zheng et al., 2018; Tola et al., 2020; Dong et al., 2020).

In addition to being the primary location for digestion and food processing, honey bee guts are the habitat of many microorganisms. In some metagenomic studies, healthy bees have a simple, specific microbiota in their gut that does not change much. This microbiota is dominated by nine taxa species clusters, each corresponding to a species or a cluster of closely related species. This is a cluster of Lactobacillus strains collectively referred to as Firm-5 and Firm-4 Lactobacillus, followed by Bifidobacterium spp., *Gilliamella apicola*, and *Snodgrassella alvi*, *Frischella perrara*, *Bartonella apis*, *Apibacter adventoris*, and *Parasaccharibacter apium* (Corby-Harris et al. 2014; Khan et al., 2020). In addition to this, there may be new members in addition to this core microbiota in changing geographical locations (Ellegaard and Engel, 2019).

The 16S rRNA in Bacteria, the primary housekeeping gene used for verifying taxonomic identities, is present in all bacteria and provides a label for a particular species or species cluster. It's also useful to compare against previously identified and stored in publically available databases. After the DNA isolation from colonies, 16S rRNA can be amplified by selective PCR and then can be sequenced (McDonald et al., 2012).

This study was carried out to investigate gut microbial communities of newly emerged adult queens and workers of *A. mellifera* collected from Southeast-Turkey (Şırnak province). For this purpose, initially, each microbe was purified from the dissected alimentary tract by culture-based isolation, and reliable identification of microbes of interest, chemical, and molecular studies were performed.

MATERIALS AND METHODS

Collection of Samples and Dissection of *Apis Mellifera*

Fifteen newly emerged queens and 30 newly emerged workers were collected from a bee farm located in Şırnak province in southeastern Turkey (37°20'24.91"N; 41°55'55.32" E). Brood frames of workers and queens cells were collected from four hives in Sirnak University. *Apis mellifera* hatched from the cells in laboratory conditions were removed from the frames and stored at -80 ^{o}C until processing. *Apis mellifera* samples were rinsed with 96% ethanol and then all alimentary canals were aseptically dissected. The removed alimentary canals were macerated in 0.8% NaCl solution under sterile conditions and stored at -20 ºC (Anjum et al., 2018).

Bacteria Culture

Different dilutions (i.e. 1/10, 1/100, and 1/1000) were prepared using the stored *A. mellifera* gut samples, and 100 µl aliquots of the diluted samples were inoculated into Nutrient Agar and Brain-Heart Infusion Agar plates. The samples were incubated at 37 ^{o}C for 24-48 hours. The bacteria grown in these medium were purified on the Tryptic Soy Agar plates taking different colony morphologies into account and incubated at 37 ^{o}C (Ganeshprasad et al., 2022).

Biochemical Tests

Various biochemical tests were used to identify the bacterial isolates using the Systematic Bacteriology Manual of Bergey. In this study casein, starch, gelatin, citrate, L-xylose, trahelose, galactose, glucose, sucrose, nitrate reduction, catalase, oxidase, voges proskauer (VP), β-galactosidase and hemolysis were determined to identify the bacterial isolates (Garrity et al., 2005).

DNA Extraction

The culture was incubated in Tryptic Soy Both (TSB) for 24 h at 30ºC and centrifuged for 2 min. Bacterial lysis was carried out by adding 10% SDS and 20 mg/ml proteinase K in TE buffer and incubated at 37 ºC for 1 hour. Then, 5 M NaCl and CTAB/NaCl were added and then incubated at 65 ºC for 10 min. The DNA was extracted using phenol/chloroform/isoamyl alcohol(Gomaa et al., 2007; William et al., 2012).

Colony PCR and 16S rRNA Gene Analysis

The polymerase chain reaction of 16S rRNA was carried out in 0.2 ml PCR tubes using a Thermal Cycler (MyGenie-96 Gradient Thermal Cycler, Korea). Two universal primers (27F: 5' TAGAGTTTGATCMTGGCTCAG-3') and 1492R: 5' TACGGYTACCTTGTTACGACTT-3') were used to amplify the DNA region encoding the 16S rRNA gene of the DNA samples obtained from the test organisms. The ab1 files were taken following the 16S rRNA region sequencing. The chromatograms were examined for each strain and contigs were generated using Codon CodeAligner V.6.0.2 (Codon-Code, Dedham, MA, USA) software. Molecular identification was performed using BLASTn software to compare the results of sequencing with those obtained from Genebank. Sequences of the samples were registered in the gene bank and access numbers MZ540443, MZ540444, MZ540445, MZ540446, and MZ540447 were obtained, respectively. Evolutionary analysis was carried out with MEGA X software (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2016). The HKY + G model was chosen based on the Jmodel test results and the maximum likelihood method was used to assess the evolutionary distance. The tree topology was evaluated by bootstrap resampling (1000 times).

RESULTS AND DISCUSSION

The sequence analysis and biochemical tests of gut samples of *A. mellifera* adult 95 queens and workers collected from Şırnak provinces of Southeastern Turkey yielded in five bacterial isolates. The sequences of HBO1, HBO2, HBO3, HBO4 and HBO5 were registered in the gene bank with access numbers MZ540443, MZ540444, MZ540445, MZ540446, and MZ540447 were obtained, respectively(Figure 1.). Three of these sequences (HBO1, HBO2 and HBO3) belonged to the genera *Bacillus*, HBO5 to genera *Staphylococcus* and HBO4 to genera *Paenibacillus* all of which belonged to a single phylum i.e. Firmicutes. The five partial 16S rRNA gene sequences obtained from this study and ten most similar gene sequences stored in NCBI were employed to create the phylogenetic tree (Figure 2.). The 16s rRNA sequencing and biochemical tests of isolates indicated that HBO1, HBO2,

HBO3, HBO4 and HBO5 strains were belonged to *Bacillus pumilus*, *B. subtilis*, *B. cereus, Paenibacillus glucanolyticus,* and *Staphylococcus haemolyticus* species, respectively (Table 1.).

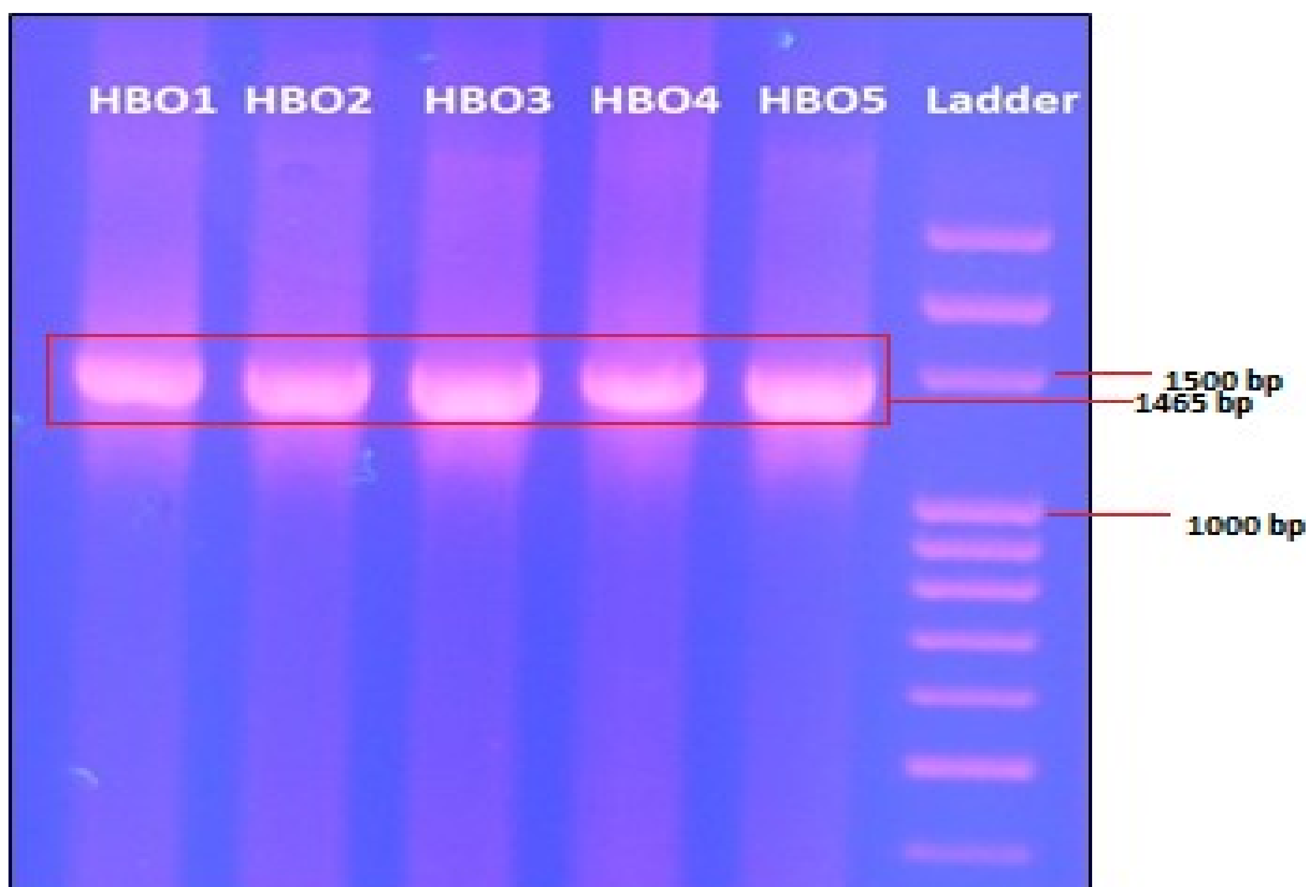

Figure 1. Imaging PCR products of 16S rRNA of HBO1, HBO2, HBO3, HBO4 and HBO5 strains of queen and worker *A. mellifera*, respectively (1465 bp products). ladder: 100bp plus (thermo)

Table 1. Phenotypic Reactions of the HBO1, HBO2, HBO 3, HBO4 and HBO5 Strains

Characteristic	HB0 1	HB0 2	HB0 3	HB0 4	HB0 5
Casein	+	+	+	-	-
Starch	-	+	+	+	-
Gelatin	+	+	-	-	-
Citrate	+	+	+	-	-
L-Xylose	-	+	-	+	-
Trahelose	+	+	+	+	+
Galactose	-	+	-	+	+
Glucose	+	+	+	+	+
Sucrose	+	+	-	+	+
Nitrate reduction	-	+	+	+	+
Catalase	+	+	+	+	-
Oxidase	+	+	-	-	-
Voges Proskauer (VP)	+	+	+	-	+
β-Galactosidase	+	+	-	-	-
Hemolysis	-	-	+	-	+

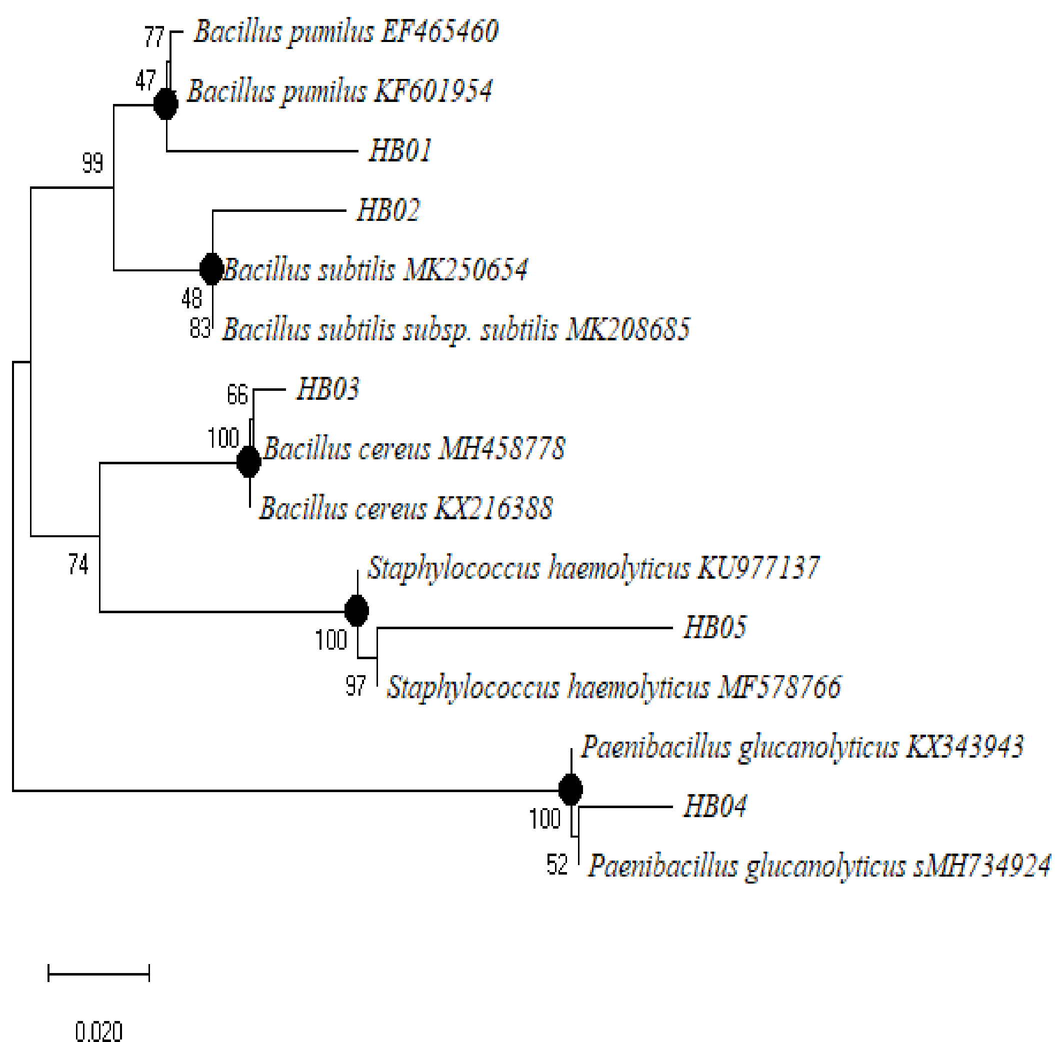

Figure 2. Phylogenetic tree of the 16S rRNA sequences isolated from the bacteria in gut of *Apis mellifera* collected from Şırnak Province, southeastern Turkey was created by MEGA X using the maximum likelihood method. The values of bootstrap (1000 replicates) were shown in the phylogenetic tree

The sequence analysis and biochemical experiments revealed presence of five bacterial species namely, *P. glucanolyticus, B. pumilus, B. subtilis, B. cereus,* and *S. haemolyticus* of gut samples of both newly hatched adult queens and workers of *A. mellifera* collected from Şırnak Provinces of Southeastern Turkey. The microbial flora of *A. mellifera* has been widely studied and the presence of *B. pumilus, B. subtilis, B. cereus,* and *S. haemolyticus* have already been reported in previous studies (Pridal et al., 1997; Moran et al., 2012; Hamdy et al., 2017; Ellegaard and Engel, 2019; Ellegaard et al., 2020; Khan et al., 2020). However, the efforts in the study did not yield in identification of all previously reported bacterial species inhabiting *A. mellifera* gut. A possible explanation of absence of such bacteria could be that previous works generally investigated gut microbial flora of foraging adults collected from fields while this study targeted the microbial flora of newly hatched (naïve) adults. To my best knowledge, this study is the first to report presence of*P. glucanolyticus* in the gut microbial community of *A. mellifera*. This suggest that research regarding characterization of the microbial community of *A. mellifera* should continue. In present study, the bacterial species were isolated from queen and worker *A. mellifera* adults newly hatched from pupal stage. Previous reports have indicated that gut microbiota composition of larvae and newly emerged adult *A. mellifera* did not differ (Gilliam, 1997; Vojvodic et al., 2013). The roles of gut microbiota might differ depending on feeding process

and landscape(Jones et al., 2018). For example, the gut microbiota of *A. mellifera* may contribute to digestion process during adult stage while they may play be important for immune system due to lack of feeding activity in larvae as the more fragile growth stage. The bacterial composition of *A. mellifera* were similar to those reported for some other insects. For example, (Pridal et al., 1997) isolated *B. pumilus, B. subtilis, B. cersus,* and *P. glucanolyticus* from the gut microbial community of laboratory reared larvae of worker and *P. glucanolyticus* from the guts of field collected queen adults of *Bombus terrestris* L. (Cano et al., 1994) also confirmed the presence of *B. pumilus, B. subtilis* and *B. cersus* the abdominal tissues of extinct stingless bees (*Plebeia, Nogueirapis*, and *Melipona*) in Dominican amber. It is clear that the bacterial composition of insect is highly dependent on host species and their biological stages(Chouaia et al., 2019).

Three of identified bacterial species belonged to the genera of *Bacillus*. *Bacillus* species are mostly known as the producers of a great range of peptide antibiotics and can be isolated from different arthropod taxa (Gebhardt et al., 2002). The ecological functionality of *Bacillus* species for their hosts were reported previously. *Bacillus subtilis* strains have been detected as the dominant bacteria in both plant nectars and *A. mellifera* stomach, especially correlated with an increase in the concentration of amylase enzyme (Wang et al., 2015). Furthermore, especially *B. subtilis* subsp *subtilis* is known to increase both honey production and the resistance of the *A. mellifera* against diseases and pests (Sabaté et al., 2009; Sabate et al., 2012). The previous studies showed that some strains of *B. subtilis* have levansucrase enzyme which can cleavage sucrose to fructose and glucose to form fructans (Levan) (Hamdy et al., 2017). The fructants are prebiotics and have several useful benefits to human health (Bello et al., 2001; Esawy et al., 2013; Hamdy et al., 2017). The results of a study conducted to investigate the inhibitory activities against *Paenibacillus larvae* isolated from *A. mellifera jemenitica* of Saudi Arabia reveled that *B. subtilis* can reduce mortality to some extent against American foulbrood(Al-Ghamdi et al., 2018). Four isolate members of the genus *Bacillus* (belonging to *B. cereus* and *B. subtilis*) isolated from *Apis cerana japonica* also showed a high inhibitory effect on the growth of *P. larvae* (Yoshiyama and Kimura, 2009).

Results of this study concerning the presence of *Staphylococcus haemolyticus* are concordant with previous studies that the bacteria was available in the gut of adult *A. mellifera* (Khan et al., 2017; Anjum et al., 2018), and other insects such as *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), a herbivorous insect species(Snyman et al., 2016).

Species belonging to the *Paenibacillus* genus isolated from a wide range of habitats have important enzymes such as pectinases, oxygenases, dehydrogenases, lignin- modifying enzymes, lipases, amylases, cellulases, hemicellulases, and mutanases (Grady et al., 2016). The *P. glucanolyticus* degrades a variety of ß-glucans, and the enzymes produced by *P. glucanolyticus* can hydrolyze carboxymethyl cellulose (ß, 1–4 linked glucose), curdlan (ß, 1–3 linked glucose), pustulan (ß, 1–6 linked glucose), and xylose (Alexander and Priest, 1989; Kanzawa et al., 1995). The growth experiments conducted by (Mathews et al., 2014; Mathews et al., 2016) confirmed that under both aerobic and anaerobic conditions, *P. glucanolyticus* strain grows on cellulose, hemicellulose and lignin which are the basic carbon sources. This bacterium degrades lignocellulose which is a component of plant cell walls and contains hemicellulose, cellulose and lignin (Pérez et al., 2002). In addition, the presence of pectin, lignin, cellulose and hemicellulose in the wall structure of pollen grains (Heslop-Harrison, 1968; Weng and Chapple, 2010) facilitates the role of *P. glucanolyticus* in *A. mellifera* gut. The *P. glucanolyticus* inhabited in intestines of *A. mellifera* may degrade lignin, cellulose and hemicellulose found in the cell walls of pollen grains, enables the *A. mellifera* to digest pollens as food and store pollen as a beebread.

CONCLUSION

The microbiota of *A. mellifera* from southeastern Turkey is introduced by the bacteria species from the core microbiota of newly emerged queens and workers. All bacterial species detected in the alimentary tract of *A. mellifera* have been reported in previous studies except *P. glucanolyticus.* Further studies are needed to investigate the strains for their antimicrobial, digestion, pathogen resistance and fermentation abilities. The results may provide greater insight into the specific role of each strain in the life history traits of *A. mellifera.* New members of the bee intestinal flora may be reported in future studies carried out in different parts of the world on microbiota of *A. mellifera*. Such studies will provide contributions to beekeeping by filling the information gaps in this area.

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Conflict of Interest

The article author declare that there is no conflict of interest.

Author's Contributions

The author declares that the whole article has been written by him.

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