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Identification of Mesophylic Bacterial Flora in Deceased Worker Adults of *Apis mellifera caucasia* (Pollmann, 1889)

Elif Çil[1], Ömer Ertürk[2], Kamil Işık[3]

1 - Department of Math and Science, Ordu University, Ordu, Turkey
2 - Department of Molecular Biology and Genetics, Ordu University, Ordu, Turkey
3 - Department of Biology, Ondokuz Mayıs University, 55200 Samsun, Turkey

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Corresponding author
Elif Çil
Cumhuriyet mah. Ordu University, Faculty of Education, Department of Math and Science
52200 Altınoru/Ordu, Turkey.
E-Mail: elifdemir80@gmail.com

Abstract

Apis mellifera, widely farmed around the world, is the most economically important species within the genus *Apis*. While the microbiota of live honey bees have been extensively examined, bacteria found in deceased honey bees (which might indicate infection or opportunistic pathogens) is in contrast poorly studied. Therefore, we decided to investigate the mesophilic bacterial flora of dead honey bees. So, in September 2013, dead adult worker honey bees were collected from 12 different cities, most of which were in the border provinces of Turkey. We identified bacterial isolates at the species level by using different morphological, biochemical, physical and molecular methods, in conjunction with molecular phylogenetic analysis. We constructed phylogenetic trees for isolated bacteria with the MEGA 6.0 program and neighbor-joining trees were reconstructed based on 16S rDNA gene sequences. The phylogenetic trees indicated that isolates DE003, DE007, DE011, DE001, DE019 and DE016, DE029 could be new members of the genera *Erwinia, Acidovorax, Hydrogenophaga* and *Bacillus* genus, respectively. In the bioassay study results, we observed that DE019 *Hydrogenophaga* sp. (64.7%) and DE004 *Klebsiella grimontii* (73.3%) had lethal effects on the honey bees. The other mortalities ranged from 10% to 25% ($p>0.05$), and according to a One-Way ANOVA analysis DE004 and DE019 significantly affect the *A. mellifera caucasia* in adult worker honey bees. This study is the first report of *Hydrogenophaga* as honey bee pathogen.

Introduction

European honey bees (*Apis mellifera* L.) are among the most investigated insects in Turkey and globally, primarily because of their significant role in agriculture and the ecosystem and their high economic value. Honey bees are important not only for the honey, propolis, pollen, and wax they produce, but also as vital pollinators of agricultural and horticultural crops. A highly adaptable species, their range stretches from the southern parts of Scandinavia to Central Asia and throughout Africa (Sheppard & Meixner, 2003). A broad diversity of microorganisms is associated with honey bees (Engel et al., 2016). In recent years, severe losses of bees from beehives and a decline in bee populations have been reported by scientists (Stathers, 2017; Potts et al., 2016). It has been hyphothesised that the pollinators face numerous threats, including pathogenic microorganisms. Honey bees proceed through embryonic, larval, pupal, and adult development stages that each comprises a distinct ecological niche for microbes. How and if microbes transition successfully through this phase is mostly unknown, even though most microbes have transcribed to infect only the adult stage of the honey bee (Kwong & Moran, 2016).

Honey bee gut microbiota is well known nowadays, but it'sthis notdoes thenot sameapply forto its opportunistic pathogens. For bacterial community analyses, scientists

generally useduse full-length 16S rDNA sequences, single-cell genomic sequencing, or metagenomic datasets (Kwong & Moran, 2016). The full-length 16S rDNA sequence analyses can be used for culture-based bacteria identification with the combination of phenotypic, biochemical characterization (Engel et al., 2016).

Here we aim to debate the present-day information on bacteria found in bees, so we isolated, cultivated, and characterised the isolates from dead adult honey bee workers from different border locations in Turkey.

Materıal and Methods

The sample of honey bees was collected from twelve locations throughout the border provinces in Turkey from October to November 2013. They were immediatelly transported to the laboratory in plastic boxes (20cm×20 cm) with punched lids by Ordu Apiculture Research Institute staff. We used small sugar cakes to feed the honey bees during transport. Then we separated the dead adult bees and stored them at -20 °C (the locations were mentioned in Fig 1).

We obtained bacterial isolates from dead *A. mellifera caucasia* L. adults by homogenisation. They were individually surface sterilised by using 70% ethanol for 3 min and washed three times with sterile water, according to Boğ et al. (2020). The deceased honey bee bodies were homogenised in a feeder medium using a glass tissue mill, immersed in to the nutrient agar, and incubated at 30 °C for a week. The residuary mixtures were incubated at 30 °C to increase the number of bacteria. First, bacterial isolates were distinguished based on colony colour and morphology. Pure cultures of bacterial isolates were preserved in 20% glycerol (v/v) at -20 °C at Ordu University, Department of Molecular Biology and Genetics, Laboratory of Microbiology.

We counducted Gram staining first. We then tried to collect data from VITEK®2 and Microbial Identification System (MIS, whole-cell fatty acid analysis by gas chromatography). For identification by VITEK®2, the isolates were inoculated into the VITEK GNI (for gram-negative bacteria), GPI (for gram-positive bacteria), and CAP (for rod-shaped bacillus bacteria) cards, incubated at 35 °C in the reader incubator module for 18 h, and automatically read hourly by the optical scanner according to the manufacturer recommendations by using bioliaison software. For the determination of fatty acid profiles of bacterial isolates, we preferred to use MIS as described by Kireçci and Aktaş (2004). For bacillus-like organisms, we added crystal staining as described by Sharif and Alaeddinoğlu (1988). Except for Gram and crystal stainings, VITEK®2, and MIS tests, 37 phenotypic tests and growth temperature tests from 10°C to 60 °C at five-degree intervals were performed manually in the laboratory. For details of the tests in Table 1, Demir's master thesis can be viewed (Demir, 2005).

Bacterial genomic DNA was extracted from the isolates (DE001, DE003, DE004, DE017, DE019, DE023, DE024, DE029) by the employment of ExiPrep plus Bacteria Genomic DNA Kit (the automated DNA extraction) supplemented by the manufacturing company (Bioneer/Korea) and by using the ExiPrep 16 plus instrument (Bioneer/Korea). The others were extracted by using a method by modifying the "guanidine thiocyanate DNA isolation method" of Pitcher et al. (1989). The taxonomic positions of the bacterial isolates were determined by 16S rDNA analysis. The 16S rRNA genes (rDNA) were amplified by using universal primers 27f (5-AGA GTT TGA TCM TGG CTC AG-3) and 1525r (5-AAG GAG GTG WTC CAR CC-3) (Lane, 1991). Amplification was carried out in a thermocycler (Eppendorf, Mastercycler Gradient, Hamburg, Germany) for 36 reaction cycles.

Fig 1. The locality of dead honey bees collected from different provinces of Turkey Province name, isolate number(s).

Table 1. The microbiological analyses applied to the test microorganisms manually.

Nitrojen sources tests (0.1 w/v)	Carbon sources tests (1% w/v)	Degredation tests
L-Proline (L-Pro)	D(-) Arabinoz	DNA (10 % w/v)
L-Iso-leucine (L-Ile)	paraffine	L-Tyrosine (0.4% w/v)
L-Valine (L-Val)	D-Mannitole	hypoxanthine (0.4% w/v)
L-Methionine (L-Met)	Rhamnose	starch (0.1% w/v)
L-cysteine (L-Cys)	Glicose	Tween 20 (1% v/v)
L- Alanine (L-Ala)	(+) Galactose	Tween 40 (1% v/v)
	Lactose	Tween 80 (1% v/v)
Other tests	Fructose	Casein*
Catalase activity§	D(+) Xylose	Tween 20√
Aerobic growth#		Tween 40√
Salt concentration tolerance (5–10%, w/v)		Tween 80√
Hemolysis on blood agar surface		
Kligler Iron Agar (KIA)		
Methyl red		
Indol		
Citrat		
Voges-Proskauer		
H_2S		

* Crosley Skim Milk Agar (Oxoid) was used for casein degredation.
√ Their capacity to metabolize Tween 20, 40 and 80 were examined on Sierra medium.
§ Catalase activity was determined by measurements of bubble production after the application of a 3% (v/v) hydrogen peroxide solution.
# Aerobic growth were conducted using tryptone-glucose-yeast extract (TGY) media.

The 16S rRNA gene (rDNA) products were run on an ABI 3730 XL genetic analyzer (Applied Biosystems) with cycle sequencing kits (GEN Plaza Biotechnology Center, Turkey). Oligonucleotide primers 27f (previously described), 518f (5-CCAGCAGCCGCGGTAATAC-3) (Muyzer, 1993), 800r (5-TACCAGGGTATCTAATCC-3) (Chun, 1995), 1492r (5-TACGGYTACCTTGTTACGACTT-3) (Gyobu & Miyadoh, 2001).

DNA sequences were edited visually using MEGA 6.0 and aligned manually. Almost-complete 16S rDNA gene sequences of the isolates were deposited in the GenBank. We preferred to use the current version of the EZBioCloud (http://ezbiocloud.net/; Yoon et al., 2017) server. The sequence alignments were used for constructing a phylogenetic tree, which was generated for the neighbor-joining as described by Jukes and Cantor (1969) using the MEGA server 6 (Tamura et al., 2013) package.

The insecticidal potential of the bacterial isolates was tested under laboratory conditions. After macroscopic examination, we selected the healthy adult worker honey bees used in bioassay analyses at random. The obtained single colonies were inoculated into nutrient broth medium and incubated at 30°C overnight. Some of the isolates were incubated at 30°C for two days due to their slow growth. After incubation, the bacterial density was measured with a BioSan densitometer, which provided the opportunity to measure solution turbidity in wide range McFarland units (Ben-Dov et al., 1995; Moar et al., 1995). One mL 6.0 McFarland bacterial suspension of each isolate was saturated into 50% glucose syrup and placed in square shape singular cages (10cm×10cm×10cm). Each cage contained 40 healthy adult worker honey bees and glucose syrup suspension with a single bacterial isolate. The bacterial inocula were expected to enter the honey bee body via feeding. The control group was fed sterilised glucose syrup. We did not observe the feeding behaviour of honey bees during the experiment. Each bioassay experiment was separately carried out at least twice in duplicate. Mortality data were corrected by Abbott's formula (Abbott, 1925). The final results were presented as mean and standard deviations. The bioassay experiment data were analysed by One-Way ANOVA, followed by Tukey's *post-hoc* test using PAWS. A value of $p<0.05$ was considered significant.

Results

In the present study, we found few bacteria isolated from dead *A. mellifera caucasia* adult workers and divided them into three groups by constructing three neighbour-joining trees (*Bacillus-Brevibacillus* group, non-*Bacillus* Gram-positive group, and Gram-negative group). On the other hand, bacterial isolates obtained from *A. mellifera caucasia* adults produced different mortality values compared to each other

and the control group. The insecticidal activity of isolates at 1.8×10^9 bacteria.mL^{-1} doses within seven days of application to *A. mellifera*. There was a statistically significant difference in the lethal toxicity of selected bacterial strains. Homogeneity of variance 0.244 $p>0.05$ so the data is homogeneous. Means for groups in homogeneous subsets were displayed in the same letter (Tukey test, $p<0.05$). Also the provinces from which the bacteria were isolated are given in parentheses. We discussed these results together by grouping organisms below.

Thermophilic rod-shaped, aerobic or facultatively anaerobic, endospore-forming organisms like *Bacillus* that grow optimally over the temperature range of 45-60°C have been isolated from both thermophilic and mesophilic environments. Currently, thermophilic bacilli are classified into seven genera which including *Bacillus* and *Brevibacillus* (Bae et al., 2005). In this study, all *Bacillus* isolates were Gram-stain and catalase positive, motile, rod-shaped cell that produced endospor and also all strains was found to negative results for indole test. However, in the *Bacillus* genus, some characters are variable such as degradation of tyrosine, nitrate reduction, hydrolysis of aesculin, etc. We selected some descriptive test characteristics for differentiation based on previously reported and proposed tests (Table 2).

In Fig 2, the nearly complete 16S rRNA gene sequences of eleven *Bacillus* isolates were clustered together with those of closely related twelve type species. The phenotypic characters of the group (*Bacillus* and *Brevibacillus* isolates) were characterized following the minimal standards for aerobic, endospore-forming bacteria, recommended by Logan et al. (2009) and a phylogenetic tree was constructed by neighbor-joining (Fig 2).

B. aryabhattai and *B. aerius* are aerobic endospore-forming bacteria, isolated first time from cryotubes used for collecting air from the upper atmosphere by Shivaji et al. (2009). Then Khaled et al. (2018) isolated *B. aryabhattai, B. laterosporus, B. techuilensis*, and *B. sonorensis* strains from the digestive tract of healthy honey bees in Saudi Arabia. DE014 (Kırklareli), DE023 (Hakkari), DE024 (Hakkari), and DE026 (Kırklareli) colonies were white/cream opaque colour onto nutrient agar, and their endospore positions within the sporangia appeared to be generally central. All isolates in this clade grew well at 44°C and up to pH 10 but weak at 50°C and pH 5. The isolates could hydrolyze aesculin and could not hydrolysis tween 80. All four isolates were positive for the utilization of paraffin. None of the isolates produced acid from glucose, sucrose, and lactose and used L-tyrosine

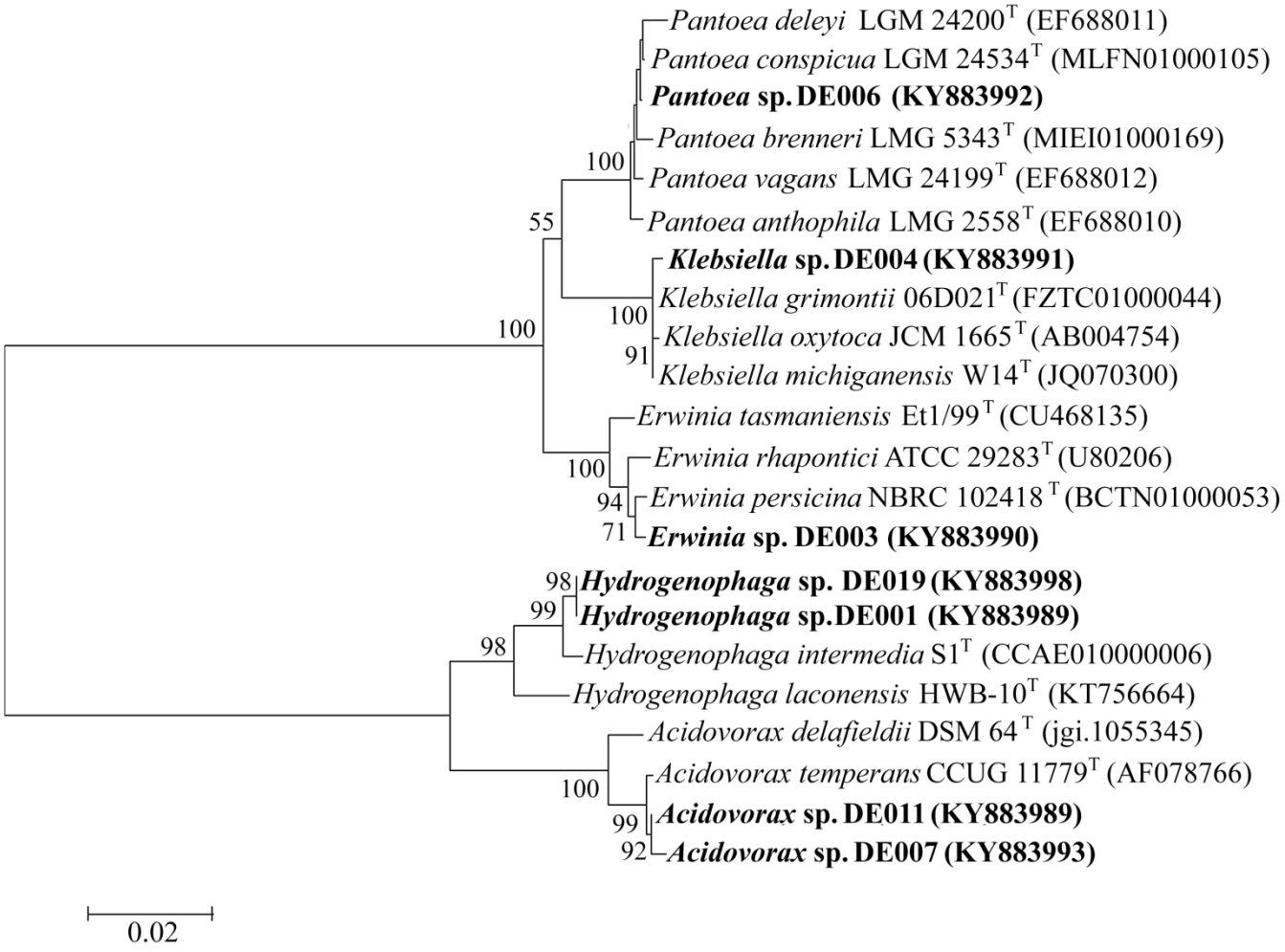

Fig 2. Neighbor-joining tree reconstructed based on 16S rDNA gene sequences showing the phylogenetic relationship between *Bacillus* isolates and closely related type strains. Bootstrap values (500 replication) >50% are given at nodes.

also DE031's insecticidal activity was 10±1[f]%. These results were the same as *B. megaterium* and *B. aryabhattai.* DE014 displayed ß hemolysis, and the others in the clade displayed γ hemolysis on blood agar and also 16S rDNA gene sequence similarity were given on Table 2. We could say DE026's insecticidal activity was low (10±2[f]%). *B. megaterium* and *B. aryabhattai* are closely related to bacterial species, so this causes mismatched identifications only by commercial identification systems or 16S rDNA analyses (Strejcek et al., 2018). When all the results were evaluated, we concluded that DE014 was *B. aryabhattai*, DE026 was *B. megaterium*, and DE023 and DE024 could be new relatives of *B. aryabhattai*.

Bacillus frigoritolerans, a strictly aerobic, chemo-organotrophic member of the genus *Bacillus* (formerly *Brevibacterium*), was originally isolated from the arid soils of Morocco and known as an entomopathogenic species (Selvakumar et al., 2011). *B. megaterium, B. simplex,* and *B. frigotolerans* are closely related species. All isolates and type strains in this clade can not grow at 44°C but degraded DNA and hypoxanthine. The isolates and type strains exhibited positive results for L-tyrosine degradation, hydrolysis of tween 80, but negative results for hydrolysis of arbutin, aesculin. Nevertheless, we also had some results different from type strains in this clade (Table 2). DE030 (Antalya) and DE031 (İzmir) could not grow on MacConkey agar as *B. frigotolerans*, but *B. simplex* could grow (Beesley et al., 2010). DE031 gave positive results to α-glucosidase but also gave negative results for *ß* glucosidase and *N*-acetyl-*ß*-glucosaminidase test like *B. simplex* and also could tolerate pH10 on agar medium like *B. frigotolerans* and also DE031's insecticidal activity was 10±1[f]%. DE030 could be a member of *B. simplex* clade and DE031 could be a strain of *B. frigoritolerans*.

To better understand the phenotype differences between DE022 (Ordu) and DE029 (Niğde), we reviewed the literature of previously reported phenotypes (Fritze 2004; Aramideh et al., 2010; Jiménez et al., 2013) and performed some additional tests. All isolates and related type strains' aesculin, arbutin were positive and grew well at 10-40°C but not 4% NaCl added medium. DE022 and DE029 displayed ß hemolysis on blood agar like *B. cereus* group as described before (Fritze 2004). DE022 could utilize D-mannose like *B. thuringiensis* ATCC 10792[T]. However, some phenotypical characters were different from the nearest neighbors; for example, citrate and casein test results (Table 2). Also, DE029 did not motile and gave a negative result for the L-tyrosine test. DE022's insecticidal activity was 15±1[e]%. We know that classifying the *B. cereus* group based on the synthesis of the parasporal crystals is so popular nowadays. Nevertheless, these crystals generally coded by plasmids (*cry* genes), and *B. toyonensis* has lack of that plasmids. According to coomassie brilliant blue staining results, DE022 and DE029 could not synthesize of the parasporal crystal. The results are in good agreement with Ohba et al. (2000) and Jiménez et al. (2013) observations that *B. cereus* genomospecies and *B. toyonensis* with non-insecticidal parasporal inclusions are far more widely distributed in nature than those with insecticidal inclusions. Our findings suggest that DE022 was a strain of *B. thuringiensis* and DE029 could be a novel species of *Bacillus* genus. So, DE029 needs to be evaluated taxonomically in more depth.

DE013 (Edirne), DE016 (Niğde), and DE027 (Mersin) were grouped into the genus *Bacillus* and also in the same clade, which also included *B. aerius*, *B. paralicheniformis,* and *B. licheniformis*. *B. aerius, B. paralicheniformis*, and *B. sonorensis* are very similar to *B. licheniformis*, but could not be distinguished from each other only phenotypic characteristics, MIS or 16S rDNA analyses (Fig 2). The isolates could not be distinguished from the close relatives by fatty acid profiles, and the same results were reported previously (Dunlap et al., 2015; Palmisano et al., 2001). The isolates spore position within the sporangia appeared to be generally subterminal. All isolates in this clade were able to grow tryptone glucose yeast extract agar (TGY), tyrosine agar (ISP 7) and Mannitol salt agar (MSA). The colonies grew well on ISP 7 and TYG mediums. It appeared creamy white on ISP 7 agar medium, mucoid on TGY at 37°C. None of the isolates and related type strains grew at pH 4. Hydrolysis of aesculin and urea tests were positive. All isolates gave positive results for Tween 20, Tween 40, Tween 80 degradation, and could tolerate 10% NaCl in medium, like *B. licheniformis* ATCC 14580[T]. However, L-alanine utilization test results were different from *B. licheniformis*, and these three isolates gave positive results like *B. paralicheniformis* KJ-16[T], *B. sonorensis* NBRC 101234[T] and *B. aerius* JCM 13348[T]. The isolates and type strains except *for B. aerius* JCM 13348T in this clade could tolerate 55 °C. DE013, DE016, and DE027 had nitrate reductase enzyme, but DE027 also had nitrite reductase enzyme too. DE013 and DE016 displayed ß hemolysis, but DE027 displayed γ hemolysis on blood agar also DE013's insecticidal activity was 10.7±1.5[f]%. To further distinguish the isolates from its closely related *Bacillus* species, 16S rDNA gen sequences were analyzed (Fig 2). According to the obtained results, We conclude that DE013 and DE027 were appeared to be closely related to the type strains *B. paralicheniformis* KJ-16[T]. But DE016 could be a novel species of the genus.

In Fig 3, the nearly complete 16S rRNA gene sequences of three non-*Bacillus* Gram-positive isolates were clustered together with those of closely related nine type species. All isolates were non-spore forming, H_2S and indole negative, methyl red positive bacteria. We selected some descriptive test characteristics for differentiation based on previously reported and proposed tests for non-spore forming Gram-positive bacteria (Table 3).

DE009 (Artvin) had a nearly identical 16S rDNA gene sequence with *Staphylococcus edaphicus and S. saprophyticus* type strains. DE009 was aerobic cocci. β-hemolytic activity on blood agar. Good growth at 10°C, 45C°, pH 10 and week growth in the presence of 15% NaCl. Mannitol fermentation, allantoin and hydrolysis of Tween 20 tests were positive.

Table 2. Comparisons of the isolatesand closely related *Bacillus* type strains. Characteristics are scored as: -, negative reaction; +, positive reaction; nd, not detected.

Property	**Isolates**											*B. aryabhattai*	*B. megaterium*	*B. paralicheniformis*	*B. toyonensis*	*B. thuringiensis*	*B. frigoritolerans*	*B. simplex*
	DE014	**DE023**	**DE024**	**DE026**	**DE030**	**DE031**	**DE022**	**DE029**	**DE013**	**DE016**	**DE027**							
Motile	+	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
Hydrolysis of casein	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-
Hydrolysis of starch	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+
Gelatinase	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	-	-	-	-	+	+	+	-	+	+	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	+		+	+	+	+	-	+	+	+	+	-
Citrate	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-
Mannitole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
L-Rhamnose	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
ß galactosidase	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	+
VITEK®2 (similarity %)	nd	nd	nd	*B. megaterium* (91%)	nd	*B. smithii* (94%)	*B. laterasporus* (90%)	nd	*B. licheniformis* (87%)	nd	nd							
MIS (similarity %)	*B. megaterium* (49%)	*B. megaterium* (81%)	*B. megaterium* (76%)	*B. megaterium* (82%)	*B. viscosus* (83%)	*B. megaterium* (72%)	nd	nd	nd	nd	nd							
16S rDNA similarity with The closest relative type strain % (nt difference)	100.00% (0)	99.93% (1)	99.93% (1)	99.93% (1)	99.86% (2)	99.93% (1)	99.86% (2)	99.93% (1)	99.92% (1)	99.92% (1)	99.92% (1)							

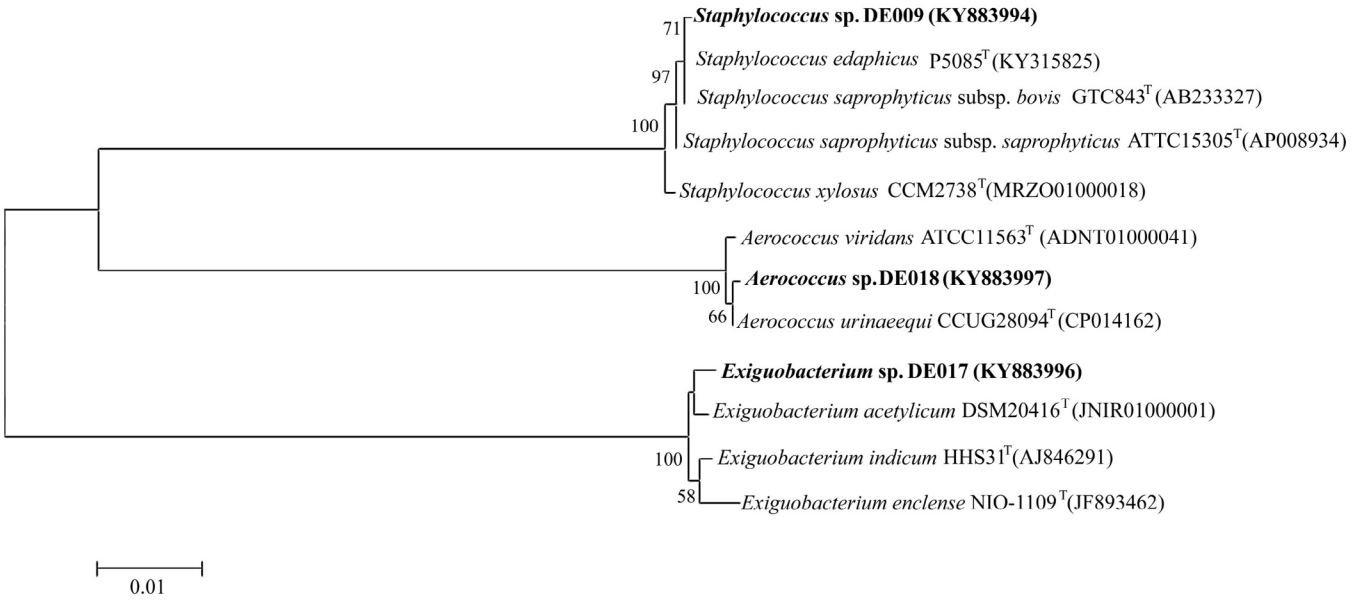

Fig 3. Neighbor-joining tree reconstructed based on 16S rDNA gene sequences showing the phylogenetic relationship between non-*Bacillus* Gram-positive isolates and closely related type strains. Bootstrap values (500 replication) >50% are given at nodes.

Hydrolysis casein, esculin, L-Tyrosine, DNA and gelatine tests were negative. Susceptible to polymyxin B (300 IU) but resistance to novobiocin (5µg). ß-Galactosidase and ß-Glucuronidase tests were essential to differentiate closely related *Staphylococcus* speciesand test results were showned in Table 3. DE009 also showed different test results opposite to *S. edaphicus* that pyrrolidonyl arylamidase, Tween 80 tests were negative and not resistance to bacitracin (0.2 IU). *S. edaphicus* showed δ-hemolytic activity on blood agar but DE009 was different (β-hemolytic activity). Data from the study demonstrate that although DE009 are in the same clade with *S. saprophyticus* subsp. *saprophyticus* and *S. edaphicus*. This isolate could be a strain of *S. saprophyticus* subsp. *saprophyticus.*

Aerococcus had been isolated from air, soil, human, and different animal samples. Cells of DE018 (Edirne) were coccoid. Colonies were non-pigmented, circular, and grey-white on nutrient agar. Also, cells produced a ß hemolytic reaction. D- glucose, D-fructose, D-mannose and gelatine hydrolysis test results were positive and D-sorbitol, D-tagatose test results were negative like closely related type strains *A. urinaeequi* and *A. viridans*. Aesculin (-) and Trehalose (+) test results are the same as *A. urinaeequi. A. urinaeequi* is a non-motile organism, but DE018 was motile. *A. viridans* caused alpha hemolysis on blood agar, but DE018 caused ß hemolysis. DE018's insecticidal activity was 15±1^{e}%. It is challenging to differentiate correctly *A. urinaeequi* from the closely related type strain *A. viridans* through only phenotypic methods or even using 16S rRNA sequencing (Zhou et al., 2016; Rasmussen, 2016). But these results indicate that DE018 was a strain of *A. urinaeequi.*

Exiguobacterium motile, rod-shaped, alkaliphilic bacterium. DE017 (Hakkari) yellowish-orange colony colour like *E. acetylicum, E. indicum,* and *E. enclense*. The colony

Table 3. Comparisons of the isolatesand closely related type strains. Characteristics are scored as: -, negative reaction; +, positive reaction; nd, not detected.

Property	Isolates						
	DE009	**DE018**	**DE017**	*S. edaphicus*	*S. saprophyticus* subsp. *saprophyticus*	*A. urinaeequi*	*E. acetylicum*
Motile	-	+	-	-	-	-	+
Catalase	+	-	+	+	+	-	+
Urease	+	-	-	+	+	-	-
Voges Proskauer	+	-	+	+	+	-	+
Mannitole	+	+	+	+	+	+	+
ß galactosidase	+	-	+	-	+	-	+
ß glucuronidase	-	-	-	+	-	-	-
Nitrate reduction	-	-	-	+	-	-	-
VITEK®2 (similarity %)	*S. saprophyticus* (99 %)	nd	nd				
MIS (similarity %)	*S. cohnii cohnii* (80%)	nd	nd				
16S rDNA similarity with The closest relative type strain % (nt difference)	99.86 % (1)	99.93% (1)	99.66% (5)				

shape of DE017, *E. indicum,* and *E. enclense* were round, but *E. acetylicum* colony shape was irregular. Hydrolyze casein, gelatine, esculin, and methyl red tests were positive for DE017 and *E. acetylicum* but negative for others. Starch utilization of DE017 was weakly positive. Also DNAse activity test was negative for DE017 and *E. indicum* but positive for *E. acetylicum.* Data from the study demonstrate that DE017 could be a member of *E. acetylicum*. Khan et al. (2017) reported that *E. acetylicum* is a gut microbiota species from Riyadh, Saudi Arabia, and our results supported this data.

In Fig 4, the nearly complete 16S rRNA gene sequences of seven rod-shaped, Gram-negative non-spore-forming bacteria which were clustered together with those of closely related 15 type species. All strains were catalase and mannitole positive and H_2S negative. We selected some descriptive test characteristics for differentiation based on previously reported and proposed tests for rod-shaped non-spore forming Gram-negative bacteria (Table 4).

Colonies of DE006 (Ardahan) were yellow, round, and convex like *Pantoea vagans*. Colour of *P. brenneri* and *P. conspicua* colonies were beige colour. All clade members utilized the following carbon sources at 37° C within 7 days: D-glucose, D-galactose, aesculin. However, 5-ketogluconate, lactose, and L–tyrosine test results were negative. All these test results are the same as *P. vagans* and *P. deleyi. P. anthophila* can use 5-ketogluconate weakly, and this test could be used for showing differentiation. Also, Tweens 40 test result was positive like closely related type organisms, but Tween 80 and maltose test results, which were different from the other type strains, were negative. DE006's insecticidal activity was 20.7^{d}±0.6. Our findings suggest that DE006 could be a strain of *P. vagans.*

Our first results revealed that DE004 (Balıkesir) was a member of *K. oxytoca* phylogroup. *K. grimontii* 's most closely related neighbors are *K. oxytoca* and *K. michiganensis* (Fig 4). *K. michiganensis* and *K. grimontii* do not exist in the VITEK® 2 databases. So, only 16S rDNA gene sequence or VITEK® 2 results were not enough to differentiate these isolates. So, we selected some phenotypic tests for differentiation. DE004 colonies on the nutrient agar are smooth, circular, white, capsulated and glistening and grown readily on ordinary media commonly used to isolate Enterobacteriaceae, e.g., nutrient agar, blood agar, and MacConkey agar like *K. grimontii*. DE004 is different from *K. michiganensis* because *K. michiganensis*' colonies are opaque. DE004 was capable of growing over a temperature

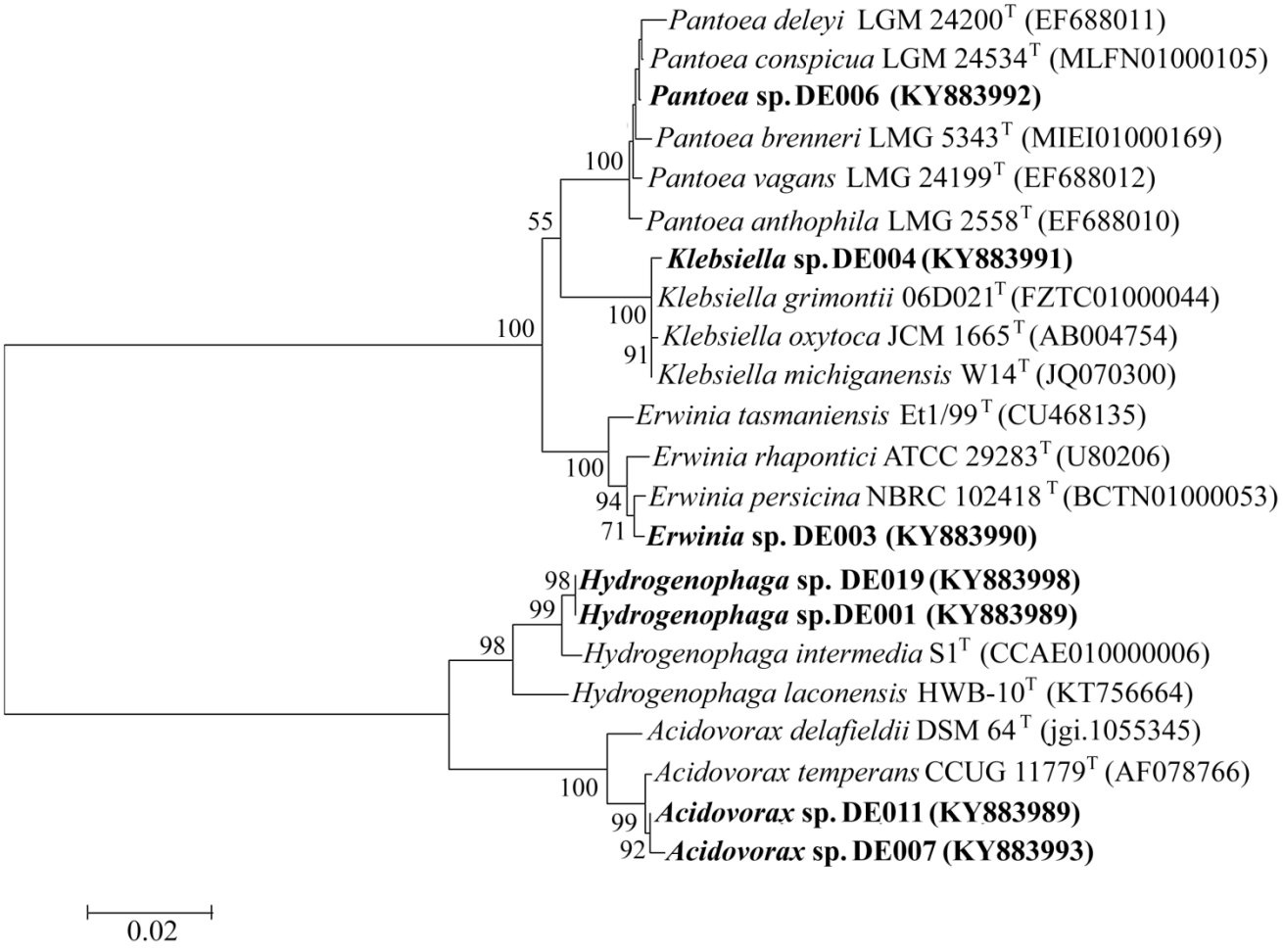

Fig 4. Neighbor-joining tree reconstructed based on 16S rDNA gene sequences showing the phylogenetic relationship between Gram-negative isolates and closely related type strains. Bootstrap values (500 replication) >50% are given at nodes.

range of 10–45 °C with an optimum of 35 °C, Vogeus-Proskauer and lysine decarboxylase tests were positive and ornithine decarboxylase negative as *K. oxytoca, K. michiganensis, and K grimontii*. Melezitoze fermentation test has been used for differantiation *K. grimontii* from closely related type strains. DE004 could not fermantiate melezitose as *K. grimontii* (Passet & Brisse, 2018). Stephan et al. (2019) reported that some bacteria from *A. mellifera* microbiota are pathogenic and *Klebsiella* could be one of them. DE004's *A. mellifera caucasia* mortality rate was 73.3[a]±0.6%. According to One-Way ANOVA analyses DE004 which isolated from Balıkesir province, had significantly affected the *A. mellifera caucasia* adult worker honey bees. This was the highest mortality rate among the isolates. *K. grimontii* is a pathogenic microorganism so this is normal and expected result.

Erwinia is a member of the family Enterobacteriaceae like *Klebsiella*. DE003 (Kırıkkale) colonies on Nutrient agar were light-beige, circular, smooth. Strains grew well on nutrient agar at 36-37 °C, and no diffusible pigment is observed. *E. tasmaniensis* is not able to grow at 36°C, but *E. percinicus* can grow at 36°C like DE003. *E. percinicus* produce water-soluble pink pigment, but DE003 strains do not. The indole and Voges–Proskauer tests are negative, but the citrate test is positively opposed to *E. tasmaniensis* and *E. rhapontici*. Nitrate reduction test is an important test to distinguish the *Erwinia* strains. Nitrate reduction test result is positive, like *E. rhapontici.* But gelatine was liquefied, and the urease test was positively opposed to other *Erwinia* type strains. Thus, based on the phenotypic and molecular evaluation, we concluded that DE003, this isolate could be a novel strain of *the Erwinia* genus.

Table 4. Comparisons of the Gram-negative isolatesand closely related type strains. Characteristics are scored as: -, negative reaction; +, positive reaction; nd, not detected.

Property	Isolates													
	DE006	**DE004**	**DE003**	**DE019**	**DE001**	**DE011**	**DE007**	*P. vagans*	*K.michiganensis*	K. oxytoca	*K. grimontii*	*E. persicina*	*H. intermedia*	*A. temperans*
Motile	+	-	+	-	-	-	+	+	-	-	-	+	+	+
Gelatinase	-	+	+	+	+	-	-	-	-	-	-	-	-	-
Urease	-	-	+	-	+	-	+	-	-	+	-	-	+	-
Indole	-	+	-	-	-	-	-	-	+	+	+	-	-	-
Citrate	+	+	+	-	-	+	-	+	+	+	+	+	-	-
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	-	-
ß galactosidase	+	+	-	-	+	-	+	+	+	+	+	+	-	-
Nitrate reduction	-	+	+	-	-	+	+	-	+	+	+	+	+	+
VITEK®2 (similarity %)	*P. agglomerans* (81%)	*K. oxytoca* (98%)	*S. paucimobilis* (92%)	*S. paucimobilis* (86%)	*S. paucimobilis* (96%)	nd	nd							
MIS (similarity %)	*P. agglomerans* (91%)	*S. choleraesuis houtenae* (88%)	nd	*S. odorifera* (70%)	nd	nd	nd							
16S rDNA similarity with The closest relative type strain % (nt difference)	99.78% (3)	99.85% (2)	99.45 % (8)	99.02% (13)	99.07% (14)	98.64% (20)	98.25% (25)							

DE001 (Ordu) and DE019 (İzmir)'s partial 16S rDNA gene sequence pairwise analysis result exhibited that *Hydrogenophaga intermedia* $S1^T$ was the closest neighbor. We know that, in the VITEK® 2 databases, *Hydrogenophaga intermedia* does not exist. The genus *Hydrogenophaga* consists of yellow-pigmented that is generally considered capable of using hydrogen as an energy source. DE001 and DE019 were slightly convex and smooth, pale yellow colonies on YPG agar. Glucose fermentation, utilization of D-xylose, D-cellobiose, D-maltose, and L-Histidine are negative; utilization of D-mannitol, Gamma-glutamyl transpeptidase and lipase results were positive like *H. intermedia* (Contzen et al., 2000). On the other hand, some phenotypical characters were different from the nearest neighbor (Table 4). Isolate DE019's mortality rate was $64.7^b \pm 1.2$. %. According to One-Way ANOVA analyse, DE019 which isolated from İzmir province, had significantly affected the *A. mellifera caucasia* adult worker honey bees. Jin et al. (2018) raported that *H. intermedia* is a potential waterborne pathogen. Our findings suggest that DE001 (Ordu) and DE019 (İzmir) could be new members of this genus.

DE011 (Artvin) and DE007 (Ardahan) isolates were closely related with *Acidovorax temperans*. In the VITEK® 2 databases, *A. temperans* does not exist like *H. intermedia*. All *Acidovorax* strains can not utilize D-xylose but can degrade tween 80. DE007 and DE011 were grown well at 28-37°C on nutrient agar medium but not 40°C and up like *A. temperans*. *A. delafieldii* had not catalase activity but DE007 and DE011 had. All type strains should be gave negative results for urease and *ß* galactosidase tests but DE007 gave positive results for both. On the other hand DE011's some phenotypic characters were different from DE007 and the closest type strain. For example DE007 was motile like *A. temperans* but DE011 did not motile. It can be seen from the results in Table 4 that DE011 and DE007 could be new members of this genus.

Discussion

Turkey has a wide variety of climatic conditions and geological structures that show significant regional differences. This has played an essential role in the evolution of many living species as it forms a natural bridge between Africa, Europe, and Asia. Honey bees are one of these species, and Turkey is home to many honey bee subspecies, including *A. mellifera caucasia* (Kence, 2006). While the microbiota of live bees have been extensively examined, bacteria found in deceased bees (which may be indicative of infection or opportunistic pathogens) is in contrast poorly studied. So, we attempted to highlight mesophilic microorganisms found in deceased *A. mellifera caucasia* and describe their characteristic properties in this study. The presence of bacterial isolates was revealed by culture-dependent approaches in this project. In the future, the culture-independent methodology should be added to the study program. Most of the isolated bacteria were of environmental origin, which was expected.

In healthy adult *Apis mellifera*, five bacteria genus called "core" bacteria (most Gram-negative Proteobacteria and Gram-positive Firmiculates firmiculates) is found (Khan et al., 2020). It also has been reported to contain multiple gut symbionts, including about 1% yeast-like microbes, 29% Gram-positive bacteria such as *Bacillus, Lactobacillus, Bifidobacterium, Corynebacterium, Streptococcus*, and *Clostridium,* and 70% Gram-negative or Gram-variable bacteria such as *Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus*, and *Pseudomonas* (Raymann & Moran, 2018; Khan et al., 2020). In the present study, we found the same bacteria genus, which isolated from *Apis cerana japonica* larvae's gut (Yoshiyama & Kimura, 2009). Yoshiyama & Kimura reported that *Erwinia*, *Bacillus*, and *Staphylococcus saprophyticus* subsp. were in *A. c. japonica*'s gut which was the same as the European honey bee. Khan et al. also reported that *Bacillus* is one of the most frequently isolated bacteria genus from the honey bee gut for Australian chalkbrood-inhibiting (Khan et al., 2020). Thus, It is not the first *Bacillus* report in the honey bee studies; previously, it was reported from different parts of the World (Wang et al., 2015; Audisio, 2017; Kačániová et al., 2020; Anjum et al., 2018; Boğ et al., 2020).

On the other hand, *Bacillus* is a diverse genus, including over three hundred strains and its predominance can be explained by its prevalence in environmental samples and its ability to survive in harsh conditions by forming endospores (Lupan et al., 2014; Luis et. al., 2020). The species within the *B. subtilis* complex cannot be reliably differentiated based on morphological, physiological, biochemical, or other phenotypic properties (Rooney et al., 2009). According to Logan et al., although 16S rRNA gene sequence comparisons are valuable in the determination of approximate phylogenetic relationships at the subspecies and generic levels and higher levels for describing new taxa of aerobic, endospore-forming bacteria, they are not always appropriate for the classification of strains at the species level (Logan et al., 2009). We also agree with Strejcek et al. (2018) that the relationship between whole-cell mass spectra, the average nucleotide identity of orthologous genes, and biological reproducibility issues, need to be addressed in the future to maximise the benefits of similarity-based and reference-free approaches. We agree with Shivaji et al. and others that *Bacillus* is a heterogeneous genus, and 16S rRNA gene sequence analysis is not enough to clarify the strains to species level (Shivajii et al., 2009; Jeyaram et al., 2011; Owusu-Darko et al., 2020). Despite the high similarity in the 16S RNA gene sequence, many *Bacillus* species can differ in their phenotypic and chemotaxonomic characteristics. Unconditionally a polyphasic approach should be adopted in the definition of *Bacillus* isolates (Logan et al., 2009; Lana et al., 2020). According to previous studies, current prokaryotic taxonomy classifies phenotypically and genotypically diverse microorganisms by using a polyphasic approach, and nowadays, next-generation sequencing technologies and computational tools for analysis

of the genomes are very popular among bacterial taxonomists (Khan et al., 2017; Porrini et al., 2017). However, unlike homogeneous geniuses such as *Bacillus*, the correct identification of an isolate can be a bit difficult. Despite the high degree of genetic similarity among the different *Bacillus* strains, these strains show substantial phenotypic variability from mammal or entomopathogen strains from all over the world (Maughan & Van der Auwera, 2011; Patino-Navarrete & Sanchis, 2017). Since 1987, microbiologists have generally prefered to classify microorganisms by using only one type of DNA sequence (Mahato et al., 2017). But now we know that it is not enough to classify taxa alone, especially environmentally variable strains. In recent years, an increasein magnitude in the number of species predicted could lead to the conclusion that we are vastly underestimating the number of ecologically distinct species of *Bacillus* (Maughan & Van der Auwera, 2011). On the other hand, this brings us back to the fundamental flaw of defining species based on their ecological traits, which may not be congruent with their phylogenomic structures. We are probably missing out on our microbiological richness and the incredible molecular and ecological diversity of *Bacillus* by choosing incorrect or insufficient taxonomic criteria.

According to Munson and Carroll, *K. michiganensis* is a new taxon that could conceivably transcend human clinical disease, so its pathogenic potential should be noted (Munson & Carroll, 2017). In the Google Scholar database, we saw that, so many researchers prefer to use VITEK microbial identification systems. This system is so practiced, and also very useful for quick biochemical test results but its identity results are not reliable every time, especially for environmental isolates. That's because, the system should be updated and new pathogenic strains, like *K. michiganensis,* should be added to the database. Sung et al. (2000) reported that *S. paucimobilis* is the most misdiagnosed organism in the VITEK AutoMicrobic system, and this study has supported that data too. DE001, DE003, DE017, and DE019 were identified as *S. paucimobilis* by VITEK® 2, but none of these identifications was correct. New and quicker methods (reagents, instruments, software, etc.) need to be developed to characterise an isolate appropriately.

We conclude that the honey bee's bacterial biodiversity could have an essential role in its health and genomic heritage. Many studies show that Turkey is a resource of genetic diversity of *A. mellifera caucasia*, and it is microflora. This brings with it the need to conserve genetic resources (Kence, 2006; Solorzano et al., 2009).

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Authors Contribution

E. Ç. performed all the experiments with the help of Ö. E. and K. I. and wrote the manuscript. E. Ç and Ö. E. designed the experiment. All the authors contributed to the revision of the manuscript and approved the version to be published.

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