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Pathogenicity of aerobic bacteria isolated from honeybees (*Apis mellifera*) in Ordu Province

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Abstract: The honeybee (*Apis mellifera*) is an important pollinator insect. Any pathogenic infection in this beneficial insect is undesirable. In this study, bacterial diversity in beehives was investigated to determine the potential of pathogenic bacteria in honeybees. To do this, bacterial isolations were carried out from dead and diseased adult bees collected from 9 districts in Ordu Province in Turkey. Twenty species of pathogenic bacteria, 18 of which were nonsporeforming *Staphylococcus lentus*, *Klebsiella oxytoca*, *Citrobacter freundii*, *Leucanostoc mesenteroides* ssp. *cremoris*, *Kocuria rosea*, *Kocuria kristinae*, *Sphingomonas paucimobilis* *slashline*, *Burkholderia cepacia*, *Leucanostoc mesenteroides* ssp. *dextranicum*, *Hafnia alvei*, *Escherichia coli*, *Aeromonas salmonicida*, *Citrobacter braakii*, *Pantoeca agglomerans*, *Streptococcus equi* spp. *zoopneumoniae*, *Staphylococcus pseudintermedius*, *Staphylococcus lugdunensis* and *Staphylococcus vitulinus* and 2 sporeforming *Bacillus licheniformis* and *Paenibacillus polymyxa*, were isolated and identified from the honeybees. The infectivity of these bacteria were also documented with bioassay experiments on the healthy bees. The highest insecticidal effect was determined with *Bacillus licheniformis* (84%), *Escherichia coli* (84%), and *Streptococcus equi* spp. *zoopneumoniae* (80%) on the adult honeybees. This result confirms that the honeybee contains a very large number of bacterial species and that the majority of them are pathogenic for the species in Turkey. In addition, some of the entomopathogenic bacteria used for biological control can have negative impact on this economically important insect.

Key words: *Apis mellifera*, pathogenic bacteria, biological control agent, infection

1. Introduction
Honeybees as animal pollinators play an important role in flowering plant reproduction[1]. The majority of plants require honeybee pollination to produce seeds and fruit, and all bees need a lot of energy in order to survive and pollinate plants. Honeybees produce very important foods such as honey and propolis. Therefore, bee health has a significant economic impact. In recent years, there has been a serious drop in the number of bees in beehives and a decrease in overall bee populations. There are various causes for the appearance of pathogenic microorganisms, but these cannot be prevented [2]. Bees are infected by pathogenic organisms such as bacteria, viruses, protozoa, fungi, and parasitic mites [3–6]. Honey also has several sources of microbiological contamination such as bacteria, fungi, and parasites such as microsporidia. The primary sources of this contamination include plant materials such as pollen and nectar and abiotic environmental material such as dust and soil [7]. In addition, entomopathogenic bacteria used for biological control of plant pests can also infect honeybees when they are applied to fields. In the present study, we aimed to determine the bacterial species of the honeybee and test their insecticidal effects on adult honeybees.

2. Materials and methods

2.1. Insect samples
Samples of honeybees were collected from bee farms in the vicinity of Ordu, Turkey from May to September. The adult bees collected from the hives were placed in plastic boxes (20 × 20 cm) with punched lids for ventilation, and sugar cake was given in small pieces until the bees were transported to the laboratory for examination. Following this, healthy, diseased, and dead adult bees were separated for observation and used to isolate bacteria that could cause disease.

2.2. Isolation of bacteria
Isolation and purification of bacteria was carried out individually from dead and live honeybees. The live and dead adults were individually surface sterilized by using 70% ethanol for 3 min [8] and washed 3 times with sterile water. The dead honeybee carcasses were homogenized in feeder medium using a glass tissue mill, and 2 layers

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714

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of cheesecloth were used to filter the homogenate from thresidues and placed in sterile tubes. Approximately 25, 50, and 75 μL of honeybee homogenate was coated on the nutrient agar and incubated at 30 °C for 1 week. The remaining mixtures were incubated at 30 °C to increase the number of bacteria. Isolates were selected individually by considering colony color and morphology. Pure bacterial colony cultures were prepared and stored in petri dishes on nutrient agar in the Department of Molecular and Genetics laboratory at Ordu University. Bacterial cultures were defined according to their morphology, nutrition, biochemical, and physiological characteristics.

2.3. Identification of bacteria
Stock culture strains of the isolated bacteria were subcultured on feeder medium plates to control their purity. All bacterial isolates were initially stained with gram stain to identify gram positive or gram negative bacteria and tested for biochemical reactions. VITEK bacteria identification systems (Prod. No 21341, bioMerieux, Craponne, France) for gram-negative bacteria and 21342 for gram-positive bacteria were then used to identify the isolated bacteria. In addition, since the VITEK system requires oxidase (gram negative) and catalase (gram positive) test results for identification, we also determined the properties of these tests [9].

2.4. Preparation of bacterial isolates and bioassays with honeybees
Adult honeybees were obtained from the Ordu Beekeeping Research Institute. After macroscopic examination, the healthy adult bees were randomly selected for bioassays. A total of 40 adult bees were used for each group in the initial bioassay experiments. Fresh prepared glucose syrup (50%) was used as a diet for feeding adults bee in the experiments.

Twenty bacterial isolates were tested for pathogenicity in bioassay experiments. The individual colonies obtained for each isolate were transferred to nutrient broth and allowed to grow at 30 °C overnight. Some isolates were left to grow at 30 °C for 2 days due to their slow growth. After incubation, bacterial density was adjusted to 1.89 at OD 600 (about 1.8 × 10⁸ cfu/mL) [10,11]. One mL of bacterial suspension for each isolate was saturated in 50% glucose syrup and placed in square-shaped, individual cardboard beehive boxes (10 cm × 10 cm × 10 cm). Forty healthy adult bees were placed in the cardboard beehives for each replicate. The control group was fed sterilized glucose syrup. Mortality data were corrected with Abbott’s formula [12].

3. Results
During the study, bacterial isolations were carried out from diseased and dead honeybee adults. A total of 20 bacterial species from 17 genera, including 9 gram negative and 11 gram positive bacteria, were isolated. Only 2 of them were sporeforming bacteria. Colonies of isolated bacteria were observed at different colors on feeder medium. The isolated bacteria were identified using VITEK bacterial identification systems (Prod. No. 21341 and 21342, bioMerieux, Craponne, France) (Table). Staphylococcus lentus, Klebsiella oxytoca, Leuconostoc mesenteroides ssp. cremoris, Sphingomonas paucimobilis slashline, and Bacillus licheniformis were the most isolated bacterial species found on the honeybees.

We also tested the pathogenicity of bacterial isolates on honeybees. For this, the insecticidal activity of isolates at 1.8 × 10⁸ bacteria/mL doses within 7 days of application to healthy honeybee adults were tested with several bioassay experiments. Bacterial isolates produced different mortality values in comparison to each other and the control group. Seventeen of 20 bacterial isolates were found to be pathogenic to honeybees. Only 3 isolates did not show any mortality. Seven bacteria showed more than 50% mortality on honeybees. The highest mortalities were obtained from E. coli, B. licheniformis, and S. equi ssp. zooepidemicus isolates with a 84, 84, and 80% mortality rate, respectively (Figure).

4. Discussion
Honeybee colonies can be affected by biotics such as pathogens, parasites, and abiotic factors such as environmental pollution and insecticide application for agricultural purposes. Honeybees are frequently exposed to pathogenic microorganisms due to different sources. These organisms cause major infections in honeybees and affect honeybee biology. However, several bacterial species are used to control pest insects ecologically in biological controls. Some of these bacteria are also pathogenic for honeybees. In this study we looked for bacterial species of honeybees and tested their pathogenity on the adult bees.

We isolated and identified 20 bacterial species from 17 genera. S. lentus, K. oxytoca, L. mesenteroides ssp. cremoris, S. paucimobilis slashline, and B. licheniformis were isolated more than once from honeybees and were the most frequently isolated bacteria.

The members of the genus Kocuria were the most common bacteria in honeybees. Three species of this genus, K. oxytoca, K. rosea, and K. kristinae were isolated from the honeybees. Similarly, these bacteria were isolated from honeybees and other sources by several authors. K. oxytocawas isolated from a diseased bee family by Vassart et al. [13]. Disayathanoowat et al. [14] isolated this bacterium from A. cerana indica. Another species of the genus Kocuria, K. rosea, identified in this study was isolated from stigmas and hypanthia of apple blossoms [15]. Other species, such as K. kristinae, was isolated from the nectar of Nicotiana glauca [16]. K. kristinae is also known as entomopathogenic bacterium. Yaman et al. [17]
isolated this bacterium from a poplar pest, *Nycteolaasiatica* (Lepidoptera: Nolidae).

Two members of the genus *Citrobacter*, *C. freundii* and *C. braakii*, were also isolated from honeybees in our study. The presence of *Citrobacter* sp. was also reported in healthy bees in France [18,19]. Two isolates of *Sphingomonas pa ucimobilis* were isolated from honeybees in 2 different localities (Kabataş and Çatalpınar) in this study. Fukui [20] isolated and identified some strains of this bacterium from guttation fluids of anthuriums. *S. paucimobilis* in this study probably infects honeybees when they gather nectar from flowers. Another bacterium found in honeybees in our study, *Lecunostoc mesenteroides*, was also isolated from honeybees collecting pollen obtained in 3 different regions in Algeria [21]. Only one species of the genus *Hafnia*, *H. alvei*, was identified on honeybees in this study. Jonathan [22] isolated this bacterium from the digestive tracts of ground beetles. *Escherichia coli* identified in this study was also isolated from the intestinal contents of *A. mellifera* [23].

*Aeromonas salmonicida* was another bacterium isolated and identified from honeybees in this study. *A. salmonicida* ssp. *masoucida* was isolated from pupae of *Camerariaohridella*, the most dangerous horse-chestnut's pest [24]. *Pantoea agglomerators* isolated from honeybee are known as a possible biological control agent against *Erwinia amylovora* and the facultative pathogen of humans. The isolates of this bacterium were collected from

| Isolates | Identified bacterial species | Similarity (%) | Locality |
|----------|-----------------------------|----------------|----------|
| AY2-1    | *Staphylococcus lentus*      | 85             | Aybastı |
| AY4-1    | *Staphylococcus lentus*      | 89             | Aybastı |
| GU6-1    | *Staphylococcus lentus*      | 87             | Gürşentepe |
| AY1-1    | *Klebsiella oxytoca*         | 97             | Aybastı |
| AY3-1    | *Klebsiella oxytoca*         | 97             | Aybastı |
| GO4-1    | *Klebsiella oxytoca*         | 99             | Gölköy |
| GO1-1    | *Citrobacter freundii*       | 95             | Gölköy |
| GO2-1    | *Leuconostoc mesenteroides*  | 97             | Gölköy |
| PE2-1    | *Leuconostoc mesenteroides*  | 97             | Perşembe |
| GO3-1    | *Kocuria rosea*              | 95             | Gölköy |
| KA3-1    | *Kocuria kristinae*          | 88             | Kabataş |
| KA4-1    | *Sphingomonas paucimobilis*   | 95             | Kabataş |
| CA1-1    | *Sphingomonas paucimobilis*   | 91             | Çatalpınar |
| PE1-1    | *Burkholderia cepacia*       |              | Perşembe |
| UL2-1    | *Leuconostoc mesenteroides*  | 85             | Ulubey |
| UL4-1    | *Hafnia alvei*               | 97             | Ulubey |
| PE3-1    | *Escherichia coli*           | 96             | Perşembe |
| OM4-1    | *Aeromonas salmonicida*      | 93             | Ordu Merkez |
| UL5-1    | *Citrobacter braakii*        | 95             | Ulubey |
| KA2-1    | *Pantoea agglomerans*        | 93             | Kabataş |
| UL3-1    | *Bacillus licheniformis*     | 85             | Ulubey |
| OM2-1    | *Bacillus licheniformis*     | 87             | Ordu Merkez |
| GU5-1    | *Paenibacillus polymyxa*     | 86             | Gürşentepe |
| PE4-1    | *Streptococcus equi ssp.*    | 87             | Perşembe |
| FA1-1    | *Staphylococcus pseudintermedius* | 93 | Fatsa |
| FA0-1    | *Staphylococcus lagudensis*  | 90             | Fatsa |
| KA1-1    | *Staphylococcus vitilimus*   | 97             | Kabataş |
different sources such as flowers, honey sacs, and nectar [25]. However, this bacterium was also isolated from the predatory beetle Rhizophagus grandis [26] and pest insects belonging to Helicoverpa armigera [27], and it was found to be pathogenic for these insects. Furthermore, Yaman et al. [17] isolated this bacterium from a hazelnut pest, Gypsonoma dealbana (Lepidoptera: Tortricidae). This bacterium was also found to be pathogenic for D. micans at a rate of 86% on larvae and 44.4% on adults for possible potential biological control agents [26]. Çelebi et al. [28] isolated this bacterium from the sunn pest, Eurygaster integriceps (Hemiptera: Scutelleridae), and they tested the insecticidal potential on this pest. Paenibacillus polymyxa isolated from honeybees in this study was found in a variety of environments [29].

Streptococcus equi subspecies zooepidemicus (S. zooepidemicus) isolated from bees in this study causes severe infections in humans [30]. We observed that Staphylococcus was the most common isolated genus on honeybees, belonging to the 4 following species: S. lentus, S. vitulinus, S. pseudintermedius, and S. lugdunesis. One of the sporeforming bacteria isolated from honeybees was Bacillus licheniformis. The B. licheniformis identified on honeybees in this study is known to be an insect pathogen and was tested against pest insects for biological control [31–33]. Yaman et al. [17] isolated this bacterium from a poplar pest, Nycteola asiatica (Lepidoptera: Nolidae). Blinech et al. [34] tested it against both coleopteran and lepidopteran pests. Furthermore, this bacterium was also isolated from Vitis vinifera for the biological control of phytopathogens in plants [35].

Bioassay experiments to determine the insecticidal activity of the isolates in honeybee adults showed that 17 of the 20 bacterial isolates were pathogenic, with a mortality rate of 12%–84% against honeybees (Figure). The highest insecticidal effect was determined with Bacillus licheniformis (84%), Escherichia coli (84%), and Streptococcus equi subspecies zooepidemicus (80%) on honeybee adults. Six bacteria showed more than a 50% mortality rate on the honeybees. Among the bacteria found to be pathogenic to honeybees in this study, 4 species, B. licheniformis, E. coli, C. freundii, and P. agglomerans, were found as to be effective biological control agents and tested against several insect pests to find possible biological control agents [26,27,32,36]. Entomopathogenic bacteria cause infections in pest insects and, therefore, they are accepted as favorable organisms. Furthermore, diseases and mortality caused by entomopathogenic bacteria in harmful insects are always favorable. However, our study confirms that entomopathogenic bacteria cause undesirable infections in honeybees, and these infections cause the death of bees. Healthy and active honeybees are always desirable for pollination and honey production. Some of the entomopathogenic bacteria used for biological control can have a negative impact on economically important insects such as honeybees. The results indicated that honeybees have a very large number of bacterial species and that the majority of them are pathogenic for them in Turkey. We think that when the entomopathogenic bacteria listed here are used for biological control in the field, their negative impact on honeybees around the world should be considered.
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