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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, Pantoea agglomerans, Streptococcus equi* ssp. *zooepidemicus, Staphylococcus pseudintermedius, Staphylococcus lugdunesis* 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 effectwas determinedwith *Bacillus licheniformis* (84%), *Escherichia coli* (84%), and *Streptococcus equi* ssp. *zooepidemicus* (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 ofenergyin 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 thesecannot 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 nectarand abiotic environmental material such as dust and soil [7]. In addition, entomopathogenic bacteria used forbiological 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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of cheesecloth were used to filter the homogenate from theresidues 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 tonutrient broth and allowed to grow at 30 °C overnight. Some isolates were left to growat 30 °C for 2 days due to their slow growth. After incubation, bacterial density was adjusted to 1.89 at OD_{600} (about 1.8×10^9 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). Fourty 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. Only2 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, Leucanostoc 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 1.8×10^9 bacteria/mL doses within 7 days of application to healthy honeybee adults were tested with several bioassay experiments. Bacterial isolates produced differentmortality 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. Thehighest 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. oxytoca*was 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]

Table. The VITEK2 bacterial identification system defined bacterial types by their name and the identification percentage of organisms.

Isolates	Identified bacterial species	Similarity (%)	Locality
AY2-1	*Staphylococcus lentus*	85	Aybastı
AY4-1	*Staphylococcus lentus*	89	Aybastı
GU6-1	*Staphylococcus lentus*	87	Gürgentepe
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* ssp. *cremoris*	97	Gölköy
PE2-1	*Leuconostoc mesenteroides* ssp. *cremoris*	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* ssp. *dextranicum*	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	Gürgentepe
PE4-1	*Streptococcus equi* ssp. *zooepidemicus*	87	Perşembe
FA1-1	*Staphylococcus pseudintermedius*	93	Fatsa
FA0-1	*Staphylococcus lugdunesis*	90	Fatsa
KA1-1	*Staphylococcus vitulinus*	97	Kabataş

isolated this bacterium from a poplar pest, *Nycteolaasiatica* (Lepidoptera: Nolidae).

Two members of the genus *Citrobacter, C. freundii* and *C. braaki*, 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 paucimobilis* 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, *Lecounostoc 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 wasalso 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

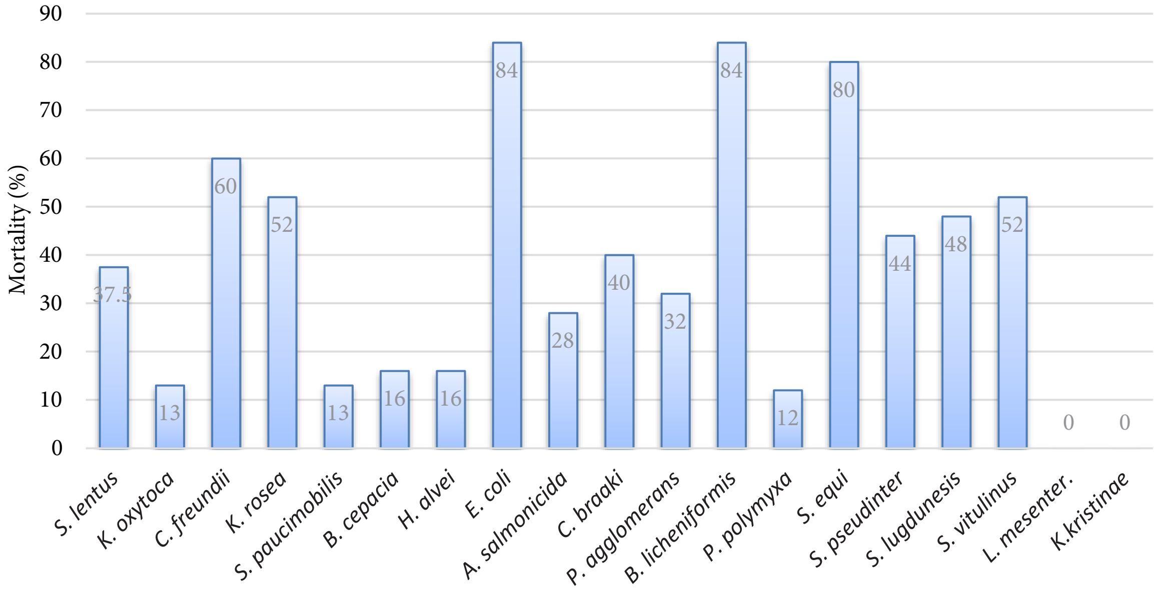

Figure. Pathogenicity of the bacterial isolates for honey bee adults. Mortality data were corrected by Abbott's formula.

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 bepathogenic 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* ssp. *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 entomopatogenic 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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