Animal Health Prod and Hyg (2024) 13 (1) : 47 - 54

Animal Health, Production and Hygiene

www.dergipark.or.tr/tr/pub/aduveterinary

Research Article

Isolation and Antimicrobial Susceptibility of Some Bacteria from The Gut of Honey Bees in Siirt Province of Türkiye

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ABSTRACT

In this study, the presence of some aerobic bacteria were investigated from the gut samples of honey bees collected from the Siirt province of Türkiye. The bacteria was isolated by conventional bacteriological methods and identified by the bacteria identification test kit. The antimicrobial susceptibility of the isolates was determined by the disc diffusion method. The most isolated bacteria species in the research were *Staphylococcus* spp. and *Klebsiella* spp., followed by *Bacillus* spp. Extended spectrum beta-lactamase (ESBL) and plasmid-mediated AmpC resistance were determined in 6 (50%) of 12 Gram-negative bacteria. Additionally, imipenem resistance was high in *Enterobacteriaceae* isolates. On the other hand, almost all *Staphylococcus* spp. isolates were susceptible to antimicrobials used in the study. It was thought that the data obtained from this study would contribute to research on honey bee health.

***Keywords**: Honey bee, bacteria, antimicrobial susceptibility.*

Siirt İli ve Yöresindeki Bal Arılarının Bağırsak İçeriklerinden Bazı Bakteriyel Etkenlerin İzolasyonu ve Antimikrobiyal Duyarlılıkları

ÖZET

Bu çalışmada, Siirt ili ve yöresinde bulunan bal arılarının bağırsak içeriklerinden bazı aerobik bakterilerin varlığı araştırıldı. Bakteriyel etkenler konvansiyonel bakteriyolojik yöntemlerle izole edildi ve ticari identifikasyon test kiti ile identifiye edildi. İzolatların antimikrobiyal duyarlılığı disk difüzyon testi ile belirlendi. Çalışmada en yüksek oranda izole edilen etkenlerin *Staphylococcus* spp. ve *Klebsiella* spp. olduğu ve bunu sırasıyla *Bacillus* spp. izolatlarının izlediği belirlendi. Genişlemiş spektrumlu beta laktamaz (GSBL) ve plasmidik AmpC direnci 12 adet Gram negatif etkenin 6 (%50)'sında tespit edildi. Ayrıca *Enterobacteriaceae* izolatlarında imipenem direncinin yüksek olduğu belirlendi. Buna karşın *Staphylococcus* spp. izolatlarının çalışmada kullanılan antimikrobiyal maddelerin çoğuna duyarlı olduğu görüldü. Çalışmadan elde edilen verilerin bal arıları ile ilgili yapılan çalışmalara katkı sağlayacağı düşünüldü.

***Anahtar kelimeler**: Bal arısı, bakteri, antimikrobiyal duyarlılık.*

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Received Date: 02.01.2024 - Accepted Date: 25.03.2024
DOI: 10.53913/aduveterinary.1413768

Introduction

Antimicrobial resistance develops in bacterial agents can cause serious problems in human and animal health. An estimated 670.000 infections occur due to resistant bacteria and thus 33.000 fatalities are observed in these cases in a year (Resci and Cilia, 2023). Antimicrobial resistance can develop in bacterial agents in two ways. Firstly, intrinsic resistance is associated with the phenotypic features of the bacteria such as the lack of a cell wall (Berry et al., 2013; Kok et al., 2022; Rizvi and Ahammad, 2022). Secondly, acquired resistance is related to acquiring resistance genes from other bacteria and/or mutation (Christaki et al., 2019; Murugaiyan et al., 2022; Rizvi and Ahammad, 2022).

Honey bees (*Apis mellifera*) are social insects that live in perennial colonies. Colonies consist of queens, drones, and worker bees (Gilliam, 1997; Resci and Cilia, 2023). Worker bees are the predominant members of the colonies (Koeniger et al., 2015). Worker bees can fly many kilometers, collecting pollen from different sources in a day (Seeley, 1995). They have a body surface adorned with covered with bristles and hairs and thus environmental material such as pollen, pesticides, heavy metals, and pathogenic and resistant bacteria can adhere to their body. Additionally, they might play an important role in antimicrobial resistance by carrying resistant bacteria (Porrini et al., 2014; Negri et al., 2015; van der Steen et al., 2016). Because of this, honey bees have been known to be bioindicators of environmental pollution (Porrini et al., 2002).

Different bacterial species (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4, *Lactobacillus* Firm-5, *Bifidobacterium asteroids*, *Frischella perrara*, *Bartonella apis*, and *Gluconobacter* Alpha2.1) can be present in varying proportions in the gut microbiota of honey bees (Resci and Cilia, 2023). The abundance of these bacteria, which assist digestion with their enzymatic systems, has been shown to vary depending on factors such as the developmental stage of bees, geographical location, pollen sources, and the use of medicinal treatments (Cilia et al., 2020).

Literature reviews had revealed that the gut microbiota of honey bees had been investigated by bacteriological and molecular methods. In these studies, it had been revealed that members of *Enterobacteriaceae*, *Staphylococcus* spp., *Bacillus* spp, Gram-positive pleomorphic bacteria, yeast, and mold were identified from the content of honey bee gut (Gilliam, 1997; Ebrahimi and Lotfalian, 2005; Cenci-Goga et al., 2020; Piva et al., 2020; Dang et al., 2022). Also, antimicrobial resistance profile and resistance genes had been investigated in isolated bacteria or direct samples of the gut (Cenci-Goga et al., 2020; Baffoni et al., 2021; Laconi et al., 2022; Zaghloul and El Halfawy, 2022).

The aim of this study was to determine the presence of aerobic bacteria in gut samples collected from honey bees in the Siirt province of Türkiye. Furthermore, the antimicrobial susceptibility of these bacteria was examined.

Materials and Methods

Dead honey bee (*Apis mellifera*) samples were collected from 24 different apiaries in the Siirt province between June 2022 and June 2023 in this study. Approximately, 30-50 bee samples were randomly collected from each apiary. The samples were placed in sterile tubes and transported immediately to the microbiology laboratory under cold chain conditions.

Isolation and Identification of Bacteria

The abdomen of the dead honey bee samples was dissected, and the guts were removed. The gut samples were crushed in a sterile mortar and suspended in 3-5 ml of sterile saline solution. The suspension was streaked onto blood agar base (Oxoid, CM0271, England) supplemented with 5% defibrinated sheep blood, MacConkey agar (Merck, 1.05465, Germany), and mannitol salt agar (Oxoid, CM85, England) plates. The plates were incubated aerobically at 37*°C for* 24-48 hours. Colonies obtained from pure cultures were examined using Gram staining, catalase, and oxidase tests. The isolates were identified using Microgen™ STAPH-ID, Microgen™ GnA + GnB-ID, and MicrogenTM Bacillus-ID kits. The tests were carried out according to the manufacturer's recommendations. Results were manually evaluated and analyzed using the firm's proposed MID Ver 1.2 program for identification.

Determination of Antimicrobial Susceptibility

Phenotypic Determination of Extended-Spectrum Beta-Lactamases (ESBL) and Plasmidic AmpC Beta-Lactamases in Enterobacteriaceae

The presence of extended spectrum beta-lactamases (ESBL) in members of *Enterobacteriaceae* was determined by a disk diffusion test. For this purpose, cephalosporin group antibiotic disks [cefpodoxime (CPD, 10 µg, Himedia, India), ceftazidime (CAZ, 30 µg, Himedia, India), aztreonam (AT, 30 µg, Himedia, India), cefotaxime (CTX, 30 µg, Himedia, India), and ceftriaxone (CI, 30 µg, Himedia, India)] were used (Clinical and Laboratory Standards Institute (CLSI), 2018). For confirmation of ESBL positivity, a cefotaxime-clavulanate (CEC, 30/10 µg, Himedia, India) disk was applied. A difference of ≥5 mm between the zone diameters with and without clavulanate was considered ESBL positive.

Plasmidic AmpC β-lactamases were detected in members of *Enterobacteriaceae* using the method outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2019). The isolates found to be resistant to cefoxitin (CX, 30 µg, Himedia, India) were suspected as producers of plasmidic AmpC β-lactamases. To phenotypically confirm the presence of plasmidic AmpC β-lactamases, the combined disk method as described by Tan et al. (2009) was employed.

Determination of Antimicrobial Susceptibility of Enterobacteriaceae and Staphylococcus spp.

For the investigation of antimicrobial susceptibility of the members of *Enterobacteriaceae,* aminoglycosides [gentamicin (GEN, 10 µg, Himedia, India) and streptomycin (S, 10 µg, Himedia, India)], fluoroquinolones [enrofloxa-

cin (EX, 5 µg, Himedia, India) and ciprofloxacin (CIP, 5 µg, Himedia, India)], carbapenems [ertapenem (ERT, 10 µg, Himedia, India) and imipenem (IMP, 10 µg, Himedia, India)], piperacillin-tazobactam (PIT, 100/10 µg, Himedia, India), trimethoprim+sulfamethoxazole (COT, 1.25/23.7 µg, Himedia, India), chloramphenicol (C, 30 µg, Himedia, India), and tetracycline (TE, 30 µg, Himedia, India) disks were used. The criteria reported by CLSI (2018) were taken into account in the evaluation of the test. *E. coli* ATCC® 25922 was used as the control strain.

For the determination of antimicrobial susceptibility of *Staphylococcus* spp. isolates, penicillin (P, 10 IU, Liofilchem, Italy), cephalosporins [cefoxitin (CX, 30 µg, Himedia, India) and cefpodoxime (CPD, 10 µg, Himedia, India)], fluoroquinolones [enrofloxacin (EX, 5 µg, Himedia, India) and ciprofloxacin (CIP, 5 µg, Himedia, India)], lincosomid [clindamycin (CD, 2 µg, Liofilchem, Italy)] and macrolid [erythromycin (E, 15 µg, Liofilchem, Italy)], tetracyclines [tetracycline (TE, 30 µg, Himedia, India), and doxycycline (DXT, 30 µg, Liofilchem, Italy)], aminoglycoside [gentamicin (GEN, 10 µg, Himedia, India)], rifampicin (RD, 5 µg, Liofilchem, Italy), trimethoprim+sulfamethoxazole (COT, 1.25/23.7 µg, Himedia, India), and chloramphenicol (C, 30 µg, Himedia, India) disks were used. The results were evaluated according to CLSI (2018) and EUCAST (2019). *S. aureus* ATCC® 25923 was used as the control strain.

The isolates were classified as susceptible (S), intermediate (I), and resistant (R) according to inhibition zone diameters. Isolates that showed resistance to at least one or more antibiotics of three different groups were considered multi-drug resistant (Maluta et al., 2012).

Statistical Analysis

The relationship between the antimicrobial susceptibility rate of *Enterobacteriaceae* and *Staphylococcus* spp. isolates were analysed by using Fisher's exact test (SAS proc freq v.8.2. Zo). The value of P≤0.05 was accepted as statistically significant (Tikofsky et al., 2003).

Results

Bacterial agents were isolated from 18 (75%) of the 24 sampled apiaries, while no bacteria were isolated from the remaining 6 (25%) apiaries. Twenty-four isolates were obtained from the samples. Six (33.33%) of the culture-positive samples yielded two different bacterial species while pure cultures were obtained from 12 (66.66%) of the positive samples.

Of the 24 isolated strains, 12 (50%) were Gram-positive, while the remaining were Gram-negative. The most isolated bacteria species were *Staphylococcus* spp. (29.16%) and *Klebsiella* spp. (29.16%) followed by *Bacillus* spp. (20.83%) (Table 1).

In this study, it was determined that all *Enterobacteriaceae* isolates (100%) were susceptible to aminoglycoside (gentamicin), fluoroquinolones (enrofloxacin and ciprofloxacin) and piperacillin-tazobactam. Resistance to trimethoprim+sulfamethoxazole, chloramphenicol, and ertapenem were determined in 8.33% of the isolates. The study revealed that 66.66% of *Enterobacteriaceae* isolates exhibited resistance to imipenem (Figure 1).

Table 1. Distribution of the bacteria isolated from 24 apiaries.

Bacteria	n	%
Gram positive		
Staphylococcus xylosus	5	20.83
Staphylococcus haemolyticus	1	4.16
Staphylococcus capitis	1	4.16
Bacillus licheniformis	2	8.33
Bacillus pumilus	2	8.33
Bacillus lentus	1	4.16
Total	**12**	**50**
Gram negative		
Klebsiella ozaenae	5	20.83
Klebsiella oxytoca	2	8.33
Escherichia coli	2	8.33
Ewingella americane	2	8.33
Enterobacter gergoviae	1	4.16
Total	**12**	**50**

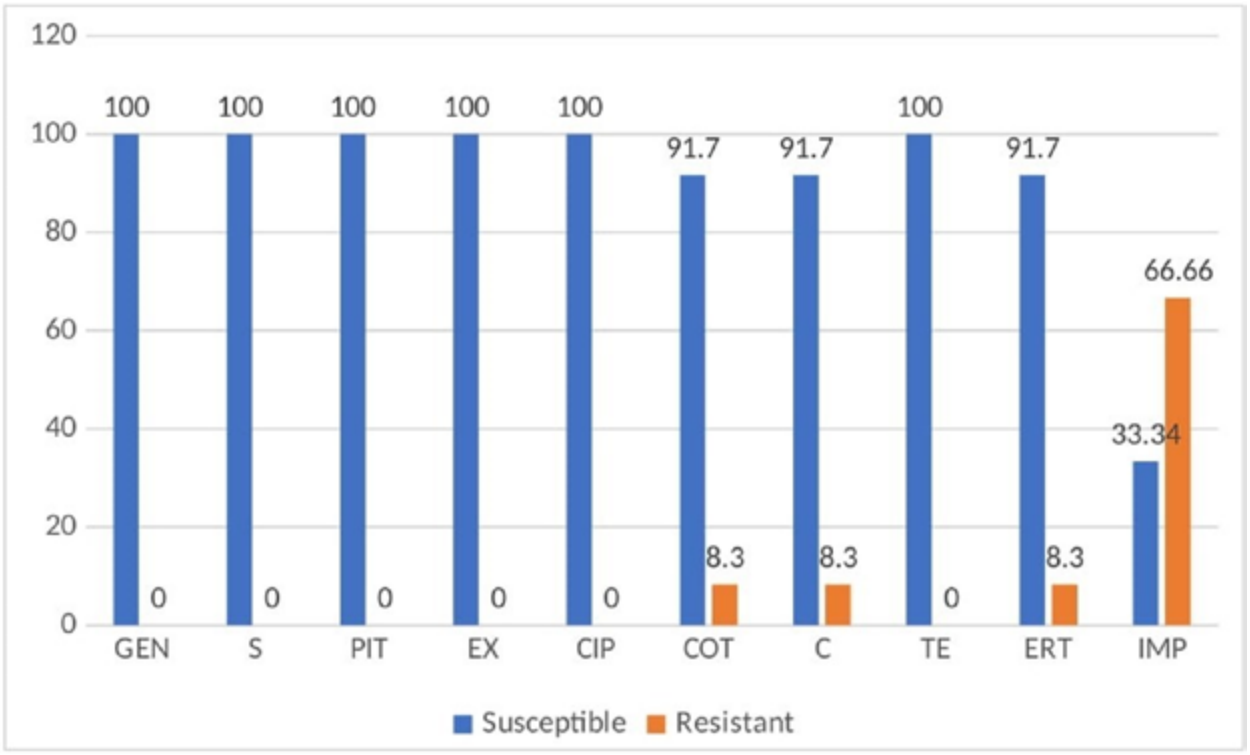

Figure 1. Distribution (%) of antimicrobial susceptibility of *Enterobacteriaceae* isolates (n:12) (GEN: Gentamicin, S: Streptomycin, PIT: Piperacillin-tazobactam, EX: Enrofloxacin, CIP: Ciprofloxacin, COT: Trimethoprim+sulfamethoxazole, C: Chloramphenicol, TE: Tetracycline, ERT: Ertapenem, IMP: Imipenem)

It was determined that 62.50% of imipenem resistant isolates were also resistant to cephalosporin groups. Furthermore, one isolate (*Ewingella americane*) was found to be resistant to carbapenems, cephalosporin groups and sulfamethoxazole+trimethoprim. Five *Enterobacteriaceae* isolates (41.66%), comprising 3 *Klebsiella* spp. and 2 *E. coli* isolates, were suspected of producing ESBL. Additionally, 3 other isolates (25%), consisting of 1 *Klebsiella* spp., 1 *Enterobacter gergoviae*, and 1 *Ewingella americana*, were suspected to produce both ESBL and plasmidic AmpC β-lactamases. Upon examination, three isolates (2 *Klebsiella* spp. and 1 *E. coli*) were confirmed to

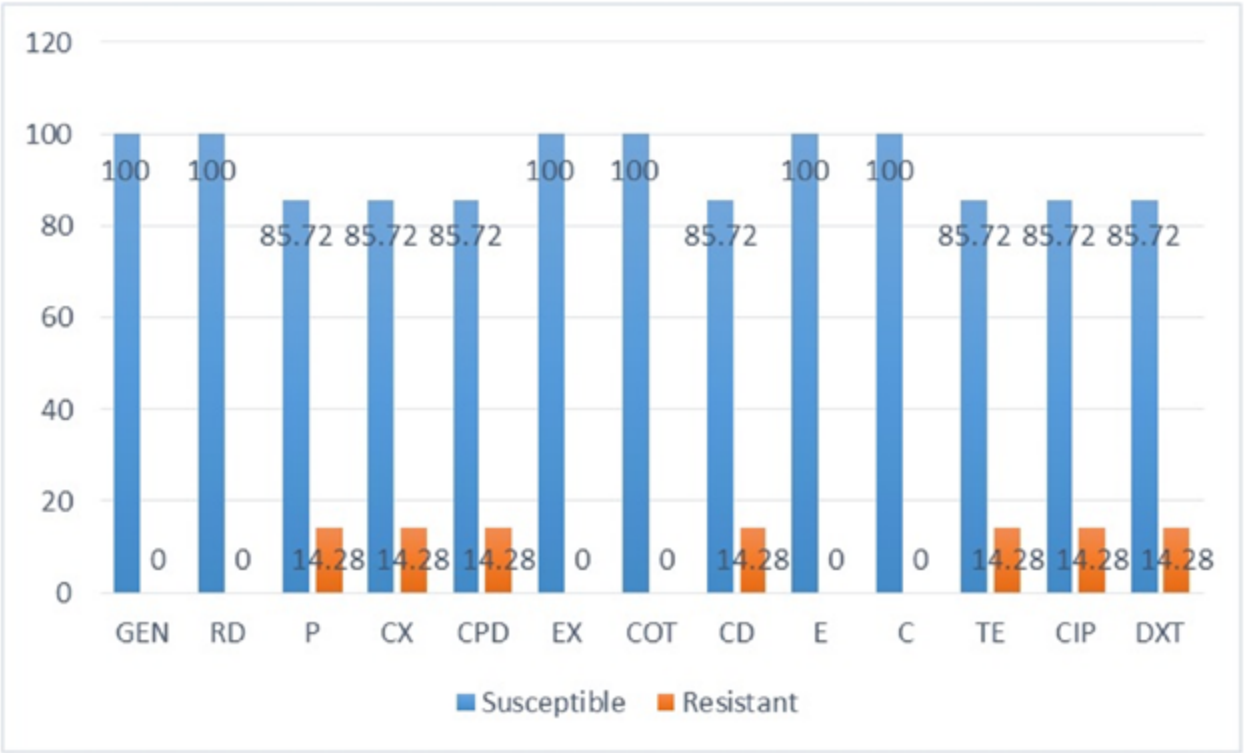

Figure 2. Distribution (%) of antimicrobial susceptibility of *Staphylococcus* spp. isolates (n:7) (GEN: Gentamicin, RD: Rifampin, P: Penicillin, CX: Cefoxitin, CPD: Cefpodoxime, EX: Enrofloxacin, COT: Trimethoprim+sulfamethoxazole, CD: Clindamicin, E: Erythromycin, C: Chloramphenicol, TE: Tetracycline, CIP: Ciprofloxacin, DXT: Doxycycline)

be ESBL producer, while three other isolates (1 *Klebsiella* spp., 1 *E. gergoviae*, and 1 *Ewingella americana*) were confirmed to produce both ESBL and plasmidic AmpC β-lactamases.

All *Staphylococcus* spp. isolates (100%) were susceptible to enrofloxacin, trimethoprim+sulfamethoxazole, erythromycin, and chloramphenicol. Resistance to penicillin, cephalosporin groups, clindamycin, tetracyclines (tetracycline and doxycycline) and ciprofloxacin was observed in 14.28% of the isolates (Figure 2). Methicillin, penicillin, lincosamid and fluoroquinolones resistance was determined in one (14.28%) *S. haemolyticus* isolate. Also, one *S. xylosus* isolate was found to be resistant to tetracyclines.

In this study, rate of the resistant bacteria was determined to be limited. Nearlly, all isolate was susceptible to antimicrobials that were used in this study. In the statistical examination with Fisher's exact test, there was not a significant relation between antimicrobial susceptibility rate of *Enterobacteriaceae* and *Staphylococcus* spp. isolates (P>0.05).

Discussion

Several studies have proposed that insects play a role in the dissemination of antimicrobial resistance (Piva et al., 2020; Zaghloul et al., 2020; Gwenzi et al., 2021). Due to their behavioral, biological, and social characteristics, honey bees can come into contact with resistant bacteria during their flights. They can uptake resistant bacteria or genes associated with antimicrobial resistance from various environmental sources. Consequently, they can disseminate this phenomenon through the pollination of plants consumed by both animals and humans (Zurek and Ghosh, 2014; Ignasiak and Maxwell, 2017; Gwenzi et al., 2021; Cilia et al., 2022).

This study aimed to investigate the presence of aerobic bacteria in the gut samples of honey bees and assess the antimicrobial susceptibility of these bacteria. *Staphylococcus* spp. isolates were the most frequently encountered bacteria, while *Klebsiella* spp. predominated among the members of *Enterobacteriaceae*. The majority of *Enterobacteriaceae* spp. isolates exhibited resistance to imipenem, whereas nearly all *Staphylococcus* spp. isolates were susceptible to the antimicrobials tested in this study.

Various bacterial species were identified in gut samples obtained from honey bees. *Gilliamella apicola*, *Snodgrassella alvi*, *Gilliamella apis*, *Bartonella apis*, *Bombilactibacillus* spp., *Lactibacillus* spp., *Bifidobacterium* spp., *Frischella* spp., and *Enterococcus faecium* were identified in gut samples using molecular methods (Tian et al., 2012; Ludvigsen et al., 2017; Ludvigsen et al., 2018; Baffoni et al., 2021; Zaghloul and El Halfway, 2022).

Furthermore, researchers have examined the gut microflora of *Apis mellifera* using bacteriological methods. In Iran, Ebrahimi and Lotfalian (2005) reported isolating *E. coli* and *S. aureus* from the guts of honey bees at rates of 75% and 36.66%, respectively. Piva et al. (2020) found that *Klebsiella* spp. were the most commonly isolated species among members of the *Enterobacteriaceae* family in Italy. Additionally, researchers identified *E. coli*, *Enterobacter* spp., *Pantoea agglomerans*, *Serratia marcescens*, and *Providencia rettgeri* in gut samples from honey bees. Boğ et al. (2020) reported that *S. lentus*, *K. oxytoca*, *Leucanostoc mesenteroides* ssp. *cremoris*, *Sphingomonas paucimobilis*, and *Bacillus licheniformis* were the most frequently isolated bacteria from honey bees in Ordu province. Lyapunov et al. (2008) emphasized that *Klebsiella* spp. were the predominant bacteria in the intestinal microflora of honey bees in the Western Urals. Another study reported that the most abundant species identified from the midgut of Asian honey bees in Thailand were *K. pneumoniae*, *E. cloacae*, and *K. oxytoca* (Disayathanoowat et al., 2012). Moreover, some researchers noted that bacterial counts are higher in the intestinal microbiota of honey bees during warmer seasons, and the season might influence the species of bacteria isolated from the gut microbiota (Ebrahimi and Lotfalian, 2005; Lyapunov et al., 2008). This study was conducted in Siirt province, characterized by hot climatic conditions. Similar to findings in other studies, *Staphylococcus* spp. and *Klebsiella* spp. strains were found to be the most commonly isolated microorganisms from the intestinal contents of honey bees. However, the utilization of only conventional bacteriological methods in this study might have resulted in the failure to isolate bacteria that could not grow well in standard media or require complex growth conditions (Gilliam, 1997).

Boğ et al. (2020) explored the pathogenicity of bacteria isolated from honey bees. They found that *E. coli* and *B. licheniformis* strains obtained from honey bees resulted in mortality rates exceeding 80% among *Apis mellifera* individuals. Conversely, several studies demonstrated that *Bacillus* and *Enterobacteriaceae* species isolated from the intestinal contents of honey bees might contribute to food digestion through their diverse enzymatic activities (Gilliam, 1997; Disayathanoowat et al., 2012; Ngalimat et al., 2019). Furthermore, *Klebsiella* and *Bacillus* spp. were found to inhibit the growth of the causative agent of American foulbrood (Disayathanoowat et al., 2012). In the present study, *E. coli* and *Enterobacter* spp., commonly associated with the intestinal contents of other animals, as well as with the environment, soil, and water, were isolated from the samples (Disayathanoowat et al., 2012). It was hypothesized that honey bees might acquire these bacteria from the environment during feeding and pollen collection. It was presumed that the causative agents of American and European foulbrood could not be detected in the samples examined in this study by PCR (unpublished data) due to the presence of *Klebsiella* and *Bacillus* spp.. However, the isolation of *Klebsiella*, *Enterobacter* species, and *E. coli*, which are known to cause nosocomial infections in humans (Santaniello et al., 2020), from the intestinal contents of bees, raises potential concerns for public health.

In various studies, the susceptibility of bacteria isolated from honey bees to different antimicrobial agents were investigated. Tetracycline resistance was reported to be high (21-100%) in various bacteria isolated from different

samples collected from honey bees (Evans, 2003; Tian et al., 2012; Krongdang et al., 2017; Ludvigsen et al., 2017). Ebrahimi and Lotfalian (2005) reported that resistance to tetracycline group antibiotics was determined as 14.30% and 33.30% in *Staphylococcus* spp. and *E. coli* isolates, respectively. In contrast to other studies, resistance to tetracycline groups was observed in only one *Staphylococcus* spp. strain in this study.

In a study, there was a high prevalence of penicillin and macrolid resistance among *Staphylococcus* spp. (100% and 55%) and *E. coli* (71.4% and 92.1%) strains isolated from honey bees (Ebrahimi and Lotfalian, 2005). Conversely, the bacteria isolated from this study was susceptible to macrolid while only one *Staphylococcus* spp. strain was found to be resistant to penicillin. Furthermore, Ebrahimi and Lotfalian (2005) reported that aminoglycoside (4%-21%) and chloramphenicol (5.12%) resistance were found to be low in these strains. Paralel to Ebrahimi and Lotfalian (2005), in presented study aminoglycoside and chloramphenicol resistance were found to be low in both Gram positive and Gram negative bacteria. Although all *Klebsiella* spp. strains were susceptible to beta-lactamase inhibitör (piperacillin+tazobactam) in this study, Piva et al. (2020) reported that resistance to beta-lactamase inhibitor (amoxicillin-clavulanic acid) was determined as 37.5-100% in *Klebsiella* spp. isolates.

In the presented study, it was determined that antibiotic resistance was limited in the isolated bacteria. It was thought that the prohibition of antibiotic use in beekeeping, coupled with the practice of breeding bees in remote areas away from human settlements, might have contributed to this scenario. However, high imipenem resistance was detected in *Enterobacteriaceae* isolates in the study. Additionally, resistance to ertapenem was also observed in one *Ewingella americana* strain. In contrast to the present study, Piva et al. (2020) did not encounter imipenem resistance in *Enterobacteriaceae* isolates in their studies. Carbapenems (imipenem and ertapenem) are mainly used in human medicine to treat infections caused by Gram negative bacteria with multidrug resistance (Campanella and Gallagher, 2020). It was suggested that the development of resistance in strains obtained from honey bees to this antimicrobial agent, which is not widely used in veterinary medicine, might be environmentally mediated.

ESBL-producing *Enterobacteriaceae* can cause serious infections in both animals and humans (Gumus et al., 2017; Zogg et al., 2018; Paredes et al., 2019; Kaplan and Gulaydin, 2023). On the other hand, the prevalence of plasmidic AmpC β-lactamases was reported to be low in samples collected from animals (Aslantaş and Yılmaz, 2017; Gumus et al., 2017; Paredes et al., 2019). There were a limited number of studies investigating ESBL resistance in *Enterobacteriaceae* isolated from honey bees. Piva et al. (2020) found that only one *E. cancerogenus* isolate was resistant to cephalosporins (ceftazidime). However, the researchers reported that the bacteria were not ESBL producer. However 50% of *Enterobacteriaceae* isolates were ESBL and plasmidic AmpC β-lactamase producers, in this study. The mixing of sewage waters and various fertilizers used in agricultural activities into the environmental sources where honey bees meet their food and water needs was thought to have led to the contamination of honey bees with resistant enteric bacteria.

Conclusion

The presence of aerobic bacteria in the gut samples of honey bees was investigated in this study. *Staphylococcus* spp. were the most frequently isolated bacteria species, followed by *Klebsiella* spp.. The overall antimicrobial resistance profile of the isolates was generally low. However, high levels of imipenem and ESBL resistance were observed in *Enterobacteriaceae*, posing a potential risk to both public and animal health. It was concluded that the characterization of antimicrobial resistance profiles and resistance genes should be conducted both in bacteria isolated from honey bee samples and in those obtained from beekeepers in future studies. It was believed that the data obtained from the study would contribute to research on the health of honey bees and the broader 'One Health' approach.

Acknowledgements

This work was supported by the Siirt University Agriculture and Livestock Specialization Coordination Center with the project number of 2022-IHTVET-02.

Conflict of interest

There is no conflict of interest.

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