Isolation and identification of Antibiotic Resistant Bacteria from the Gut of Western Honey Bee (*Apis Mellifera)* - A Case Study from North Pakistan

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Abstract- The western honey bee (*Apis mellifera*) has been domesticated mainly for the production of honey, crop pollination, and wax nest construction. The function of the gut microbiota (GM) in controlling the health of *A. mellifera* has become more evident in recent years. Antibiotic resistance of bacteria is increasing worldwide in beekeeping and may result in public health problems. Studies on the identification of GM of honey bees and its resistance towards antibiotics in the selected region are needed. In the literature, no such recorded data is found on antibiotic resistance of honey bee gut bacteria. Therefore, this study aimed to isolate and identify bacterial strains from the gut of *A.mellifera* to examine antibiotic resistance of the isolated bacterial strains. Bees were collected, and dissected, guts were extracted, and bacterial isolates were cultured on nutrient agar (NA) medium. Morphology of the isolated bacterial strains was observed on subculture plates by staining and microscopy, and the bacterial strains were checked for antibiotic resistance by applying various antibiotic discs particularly Oxytetracycline (OX), by disc diffusion method. Two bacterial strains *E.coli* and *Klebsiella* were identified through staining and microscopy. Higher resistance was observed in *E.coli* against Oxytetracycline (OX), Nalidixic acid (NA), Tetracycline (TEC), and Cephaprazone (SCF) having zones of inhibition 10mm, 12mm, 11mm, and 10mm, respectively (compared with CLSI;2013 table), while no significant resistance observed in *Klebsiella* against Cephalexin (CE), Oxytetracycline (OX), Nalidixic acid (NA), Tetracycline (TEC) and Cephaprazone (SCF) having Zones of inhibition 25mm, 30mm, 21mm, 19mm and 20mm, respectively. Experimental results reported that *E.coli* from the gut of honey bees is resistant while *Klebsiella* shows susceptibility towards these antibiotics.

Index Terms- Antibiotic resistance, *Apis mellifera*, Gut microbiota, *E.coli, Klebsiella*

I. INTRODUCTION

Honey bee, especially *Apis mellifera (A.mellifera)* is a beneficial insect for pollination as well as in the production of other honey products including wax, royal jelly, propolis, pollen, and bee venom (El-Seedi, Eid et al. 2022). *A.mellifera* pollinates a wide range of food crops around the world as a key plant-pollinator and because of the broad history of domestication of *A. mellifera*, it is now found in every region of the world (Hung, Kingston et al. 2018), and is the main pollinator of western food, pollinates about one-third of agricultural items, including nuts, vegetables and fruits (Brutscher, McMenamin et al. 2016). Studies proved that animals play a very important role in crop pollination and almost 35% of crop pollination is dependent on the animals, out of which honey bees provide about 90% of pollination (Genersch 2010).

Like all insects, honey bees also have three main body parts, the head, thorax, and abdomen. The digestive system in the abdominal part of the honey bee is divided into three parts, foregut, midgut, and hindgut which is the main reservoir of bacteria. Insect gut microbiota including a honey bee has been found to help in immunity, digestion, detoxification, pathogen resistance, health development, and physiology (Jing, Qi et al. 2020). The three classes of bacteria including Proteobacteria, Firmicutes, and Actinobacteria found in the gut of *A.mellifera* have been sequenced previously by 16S rDNA sequencing (Anjum, Shah et al. 2018). Gut microbiota (GM) plays a significant role in the fermentation of pollen e.g. vitamins (Engel, Martinson et al. 2012) and host immunity by producing bioactive substances against infectious agents (Olofsson, Butler et al. 2016).

Despite playing a crucial role in pollination, the population of *A. mellifera* in Northern America and Europe is in a critical situation and has death rates of between 30 and 40 percent every year (López-Uribe and Simone-Finstrom 2019). This kind of critical situation, pollinator decrease has very serious effects on global food safety and economic stability. The pathogens that lead to various diseases and illnesses in bees include bacteria, fungi, viruses, Varroa mites, and Acarapis mites (Amiri, Strand et al. 2017). Hence, identification and characterization of the gut microbiome of bees are important for the perception of various bacterial and parasitic pathogens (Anjum, Shah et al. 2018). Currently, Oxytetracycline (OTC) is one of the approved antibiotics used to treat EFB disease in Canada and is applied to each hive in three doses, spaced 4-5 days apart, at a dosage of 200 mg per 20 g of icing sugar (Richards, Tell et al. 2021). In the United States, Oxytetracycline has been used for the treatment of bacterial diseases of honey bees, such as European foulbrood (EFB) and American Foulbrood (AFB), and is accessible in the form of powdered sugar mixed with antibiotic at a fixed concentration. For the treatment of AFB-infected hives Oxytetracycline, lincomycin, and tylosin, are the antibiotics approved by the FDA (Applegate and Petritz 2020).

Antibiotic resistance of bacteria is increasing worldwide in beekeeping and may result in public health problems (Ebrahimi

and Lotfalian 2005). Some studies revealed that specific fluctuations occur with time in the composition of gut microbiota through antibiotic-mediated dysbiosis and recovery. For the treatment of *C. difficile*, two antibiotics metronidazole and vancomycin, have been compared and demonstrated to exhibit various effects. Gram-positive bacteria were selectively killed by Vancomycin, while metronidazole mainly targets anaerobic bacteria (Kim, Covington et al. 2017). Antibiotics used for the control of infections by pathogenic bacteria have also an effect on other non-pathogenic useful bacteria present in healthy host bees. Resistant factors, frequently encoded on mobile genetic components can easily move among the members of a community and might accumulate as a result of the selective pressure enforced by an antibiotic (Tian, Fadhil et al. 2012). The effect of antibiotics on the gut microflora of the animals is a definite concern, as gut microbiota act as pools for resistance genes that can be stimulated to destructive pathogens and also since perturbation of gut microflora by antibiotic treatments might interrupt beneficial functions of the healthy hosts (Kim, Covington et al. 2017). Since the 1950s, antibiotic Oxytetracycline has been frequently applied to bee hives in the United States to treat larval foulbrood infections caused by the bacteria *Melissococcus pluton* and *Paenibacillus larvae*; until 2005, Oxytetracycline was the only antibiotic approved for the use in beekeeping (Jernberg, Lofmark et al. 2010). Oxytetracycline antibiotics were first introduced in the 1950s and till now prescribed for the control of pathogens in apiculture. Additionally, the Tylosin and Lincomycin were approved by the FDA in 2005 and 2012 respectively but these are rarely prescribed for apiculture pathogens control (Reybroeck, Daeseleire et al. 2012). All three of these antibiotics are broad-spectrum bacteriostats that use similar mechanisms of action where they bind to the 50S ribosomal subunits of bacteria to inhibit protein synthesis and stop bacterial growth (Adzitey 2015). Oxytetracycline has historically been used to treat and control the spread of *Paenibacillus larvae* and *Melissococcus plutonius* which are the causative agents of AFB and EFB, respectively. The use of OTC in the treatment of AFB disease increases the chance of *M. plutonius* developing resistance to it. Currently, Canada has approved other treatments for AFB disease that include lincomycin (LMC) and tylosin (TYL). A macrolide antibiotic particularly, TYL is extensively used for the treatment of livestock animals in western Canada that are commonly raised in neighboring apiaries in the region. Similar to macrolides, Lincosamide antibiotics like LMC, attach to the ribosome's 23S subunit to prevent protein synthesis. Bacteria can acquire cross-resistance to TYL and LMC through macrolides, Lincosamide, and Streptogramin B (MLSB) resistance due to physiological similarities, including shared drug binding sites. Typically, methyl transferase genes that alter an antibiotic's common target site are linked to MLSB resistance. (Masood, Thebeau et al. 2022).

Due to increased antibiotic resistance in the region, studies on the identification of the gut microflora of honey bees and its resistance towards antibiotics in the province of Khyber Pakhtunkhwa, Pakistan are needed. As per the literature, no recorded data is found on antibiotic resistance of honey bee gut bacteria, particularly in the study region. This study aims to isolate and examine the gut bacteria of western honey bees (*A. mellifera*) and to find out the antibiotic resistance potential of the strains isolated from the gut of honey bees. We hypothesized that antibiotics used for the treatment of pathogenic bacteria have also adverse effects on beneficial and probiotic flora of honey bee gut, and gut bacteria have a prominent role in developing antibiotic resistance towards medicines used for bacterial pathogens treatment. In this study, we isolate and morphologically identify bacterial strains from the gut of *A. mellifera* and examine antibiotic resistance potential of the isolated bacterial strains by disc diffusion method.

II. MATERIALS AND METHODS

2.1. Case study

Due to increased antibiotic resistance, studies on the identification of the gut microflora (GM) of honey bees and its resistance towards antibiotics, especially in Khyber Pakhtunkhwa, Pakistan are needed. No specific recorded data is found on antibiotic resistance of honey bee gut bacteria in the study region. Therefore, this study aimed to isolate and identify bacterial strains from the gut of *A. mellifera* and to examine antibiotic resistance of the isolated bacterial strains. The scope of the study was KUST bee farm, Kohat, dissected, guts were extracted, and bacterial isolates were cultured on nutrient agar (NA) medium.

2.2. Collection and dissection of bees

To investigate cultivable bacteria from the gut of honey bees, around 30 worker adult bees (*A. mellifera*) were collected from the KUST bee farm, Kohat by the use of insect net and transported to the Bee lab, Department of Zoology, KUST, Kohat in small cages of size (20 × 20 × 20) cm^3 having the feed of sugar powder and were stored at −20°C until further processing (Anjum, Shah et al. 2018). Before dissection, a surface was washed with 90% ethanol to remove any surface contamination for better results.

2.3. Culturing of gut bacteria

After washing whole bees with 90% ethanol, whole guts were extracted with sterile forceps and by using sterilized dissection tools, extracted guts were macerated in 0.8% NaCl solution. Different dilutions of 1/10, 1/100, and 1/1000 concentrations were made using 0.8% NaCl solution made earlier. An aliquot of 100 µl sample from the diluted solution was taken by micropipette and inoculated onto the surface of nutrient agar plates at 37°C for the time duration of 24-48 hours. To get pure isolated colonies of bacteria, mixed and impure colonies from the Master plate were recultured in respective agar plates at 37°C for 24 hours. For morphological identification of bacteria, morphological parameters such as colony structure, Gram staining reaction, and microscopy were performed.

2.4. Gram staining and microscopy

Bacterial isolates were smeared on a clean glass slide and fixed heat under a sterile environment. For staining, crystal violet was applied for 30 seconds, rinsed with distilled water followed by applying Gram's iodine (Sigma-Aldrich, USA) for the time duration of 32 seconds, and then washed again with slow tap water smoothly. For decolorization, 95% acetone alcohol was poured on a slide for 5 to 7 seconds. After washing, the counter strain safranin was poured on the slide for 45 seconds, then washed with tap water for a last time, air dried, and observed by microscope at 1000X magnification using 100X oil immersion objective and 10X eyepiece.

2.5. Susceptibility analysis

Antimicrobial susceptibility tests for the isolated bacteria were determined by the process of disc diffusion assay (Bauer 1966)

following the Clinical and Laboratory Standards Institute (CLSI; CLSI 2013) for which MHA (Oxoid) media were prepared and poured into sterile petri dishes. For inoculum preparation nutrient broth (HiMedia) was prepared and bacterial isolates were cultured at 35 ℃ for 24 hours.

By using the McFarland turbidity standard, the turbidity of the broth culture was checked. By using sterile cotton swabs, the inoculum of evenly mixed bacterial suspension was then spread uniformly to the entire surface of the MHA plate. After 15 minutes, when plates were dried, the antimicrobial discs (Oxoid, Basingstoke, UK) as given in **Table 1**, were placed at a uniform distance by using sterilized forceps. The petri plates were then set for incubation for the time duration of 18 to 20 hours at 35°C and then zones of inhibition were measured. The drug resistance profile was then checked with EUCAST guidelines.

III. RESULTS AND DISCUSSIONS

Beekeeping is experienced in Pakistan, in districts Karak and Kohat districts of Khyber Pakhtunkhwa and the central and northern areas of the Punjab province. Honey produced in Pakistan is famous in the Middle East for its unique taste and superior quality. Pakistan annually sells about 4000 tons of honey about $ 23 million of cost, to Arab countries, (Usman, Hasnain et al. 2022). Due to its role in nutrition, development, and immunity, it is essential to know about the gut bacteria of worker bees and their role in resistance towards antibiotics used for the treatment of bacterial diseases which are used in different apiaries. We carried out morphological identification of two bacteria *E.coli* and *Klebsiella* from the gut of worker bees *Apis mellifera* through differential media, gram staining & microscopy and then performed susceptibility analysis for these two bacteria by using five different types of antibiotics through disc diffusion assay (Liasi, Azmi et al. 2009) following the Clinical and Laboratory Standards Institute (CLSI; CLSI 2013). *E. coli* (shown in **Fig. 1**) and *Klebsiella* (**Fig. 2**) were identified through culturing on differential media, staining, and microscopy (**Fig. 2a, b**).

Fig. 1 *E. coli* on MacConkey media gives bright pink colonies

Fig. 2 (a) *Klebsiella* appears large, mucoid, (b) pink to red in color on MacConkey agar

E. coli on MacConkey media appears as bright pink colonies (El-Mongy, Bayome et al. 2017) while *Klebsiella* on MacConkey agar appears large, mucoid, and pink to red in color as also reported by (Rahman, Begum et al. 2023).

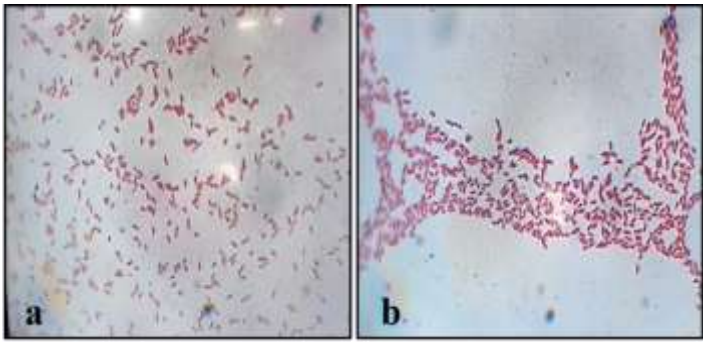

Fig. 3 (a) Microscopic images of *E. coli* (b) Visualization pattern

Fig. 3 (a, b) show the red/pink microscopic images of bacteria *E. coli* representing that the strain crystal violet did not adhere to the outer wall of bacteria labeled as gram-negative bacteria.

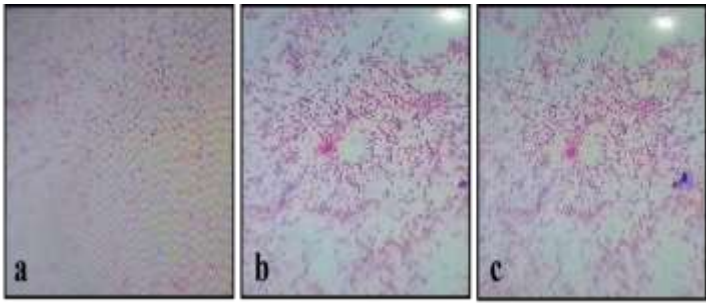

Fig. 4 (a, b & c) Microscopic analysis of *Klebsiella*

Fig 4 (a, b, and c) shows microscopic images of a gram-negative bacteria *Klebsiella* which has a thin peptidoglycan layer and crystal violet does not adhere to the outer layer, so it stains pink. And since it's a *bacillus*, it looks like a little pink rod under the microscope. The results of the antibiotic susceptibility for bacterial isolate (*E. coli*) are given in **Table 1**. Five different types of antibiotic discs CE, OX, NA, TEC, and SCF were used against *E. coli* and zones of inhibition were measured in mm against these antibiotics. These values were compared with standard resistant, intermediate and susceptible values of CSLI, 2013.

Table 1. Antibiotic susceptibility test for bacteria *E. coli*

E. coli					*CSLI*			*Remarks*
S. No	**Antibiotic**	**Symbol**	**Disk(μg)**	**Zone of inhibition(mm)**	**Resistant (mm)**	**Intermediate (mm)**	**Susceptible (mm)**	**Conclusion**
1	Cephalexin	CE	30 μg	15	≤14	15-22	≥23	Intermediate
2	Oxytetracycline	OX	5 μg	10	≤11	12-14	≥15	Resistant
3	Nalidixic acid	NA	30 μg	12	≤13	14-18	≥19	Resistant
4	Tetracycline	TEC	30 μg	11	≤11	12-14	≥15	Resistant
5	Cefoperazone	SCF	105 μg	10	≤15	16-20	≥21	resistant

Increased resistance was observed in *E.coli* against TEC, OX, NA, and SCF having zones of inhibition 11, 10, 12, and 10 respectively when compared with standard resistant values of CSLI 2013. (**Fig. 5**).

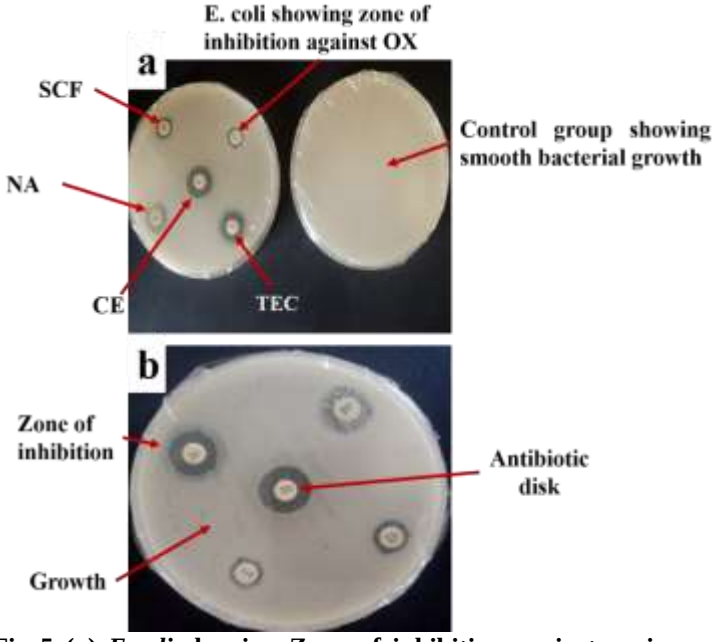

Fig 5 (a) *E.coli* showing Zone of inhibition against various antibiotics and control plate showing smooth bacterial growth (b) *E. coli* showing clear zones of inhibition against antibiotics

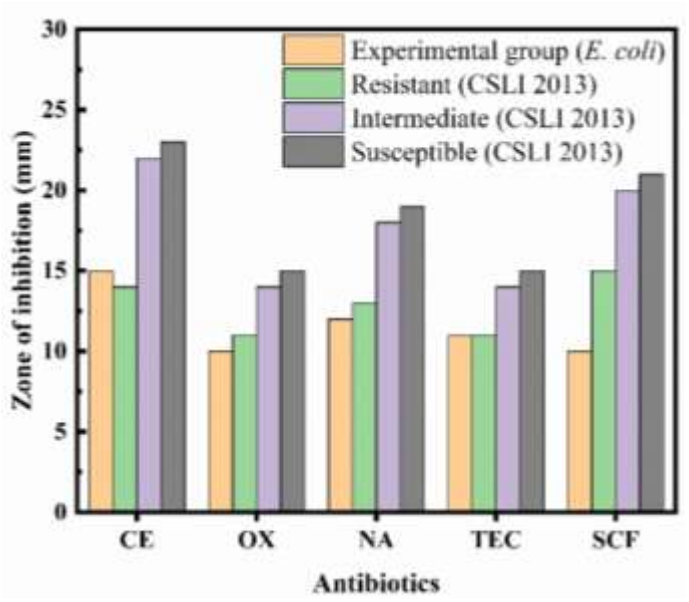

Fig. 6. Graphical representation of *E. coli* showing zones of inhibitions against various antibiotics

The results of the antibiotic susceptibility for bacterial isolate (*Klebsiella*) are provided in **Table 2**. No significant resistance was observed in *Klebsiella* against CE OX, NA, TEC, and SCF having Zones of inhibition 25mm, 30mm, 21mm, 19mm, and 20mm respectively compared with CSLI values as illustrated in (**Table 2**).

Table 2. Zones of inhibition by *Klebsiella* against various antibiotics

Klebsiella					CSLI			Remarks
S.No	**Antibiotic**	**Symbol**	**Disk**	**Zone of inhibition (mm)**	**Resistant (mm)**	**Intermediate (mm)**	**Susceptible (mm)**	**Conclusion**
1	Cephalexin	CE	30 μg	25	≤14	15-22	≥23	Susceptible
2	Oxytetracycline	OX	5 μg	30	≤11	12-14	≥15	Susceptible
3	Nalidixic acid	NA	30 μg	21	≤13	14-18	≥19	Susceptible
4	Tetracycline	TEC	30 μg	19	≤11	12-14	≥15	Susceptible
5	Cefoperazone	SCF	105 μg	20	≤15	16-20	≥21	Susceptible

Fig. 6 shows a Graphical representation of *E. coli* showing zones of inhibitions against various antibiotics. The orange color bar represents our experimental group (*E. coli*) while green, purple, and grey represent approximate resistant, intermediate, and susceptible values when compared with CSLI;2013. **Fig. 7** shows clear and wide zones of inhibition by *Klebsiella* against various

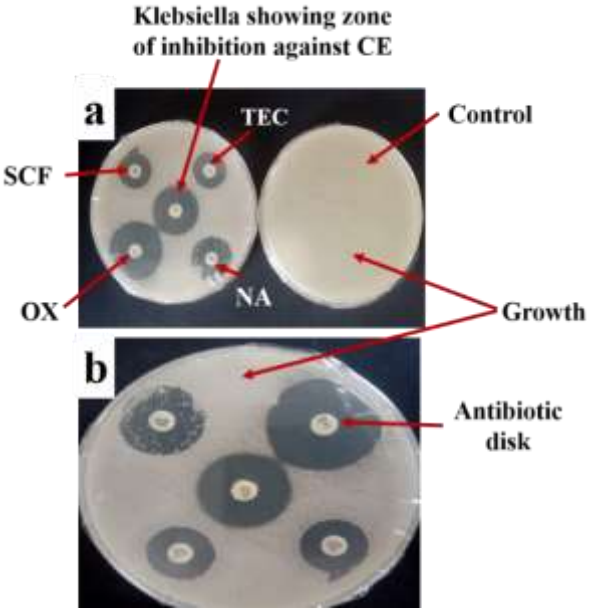

Fig 6. *Klebsiella* showing "zones of inhibition against various antibiotics

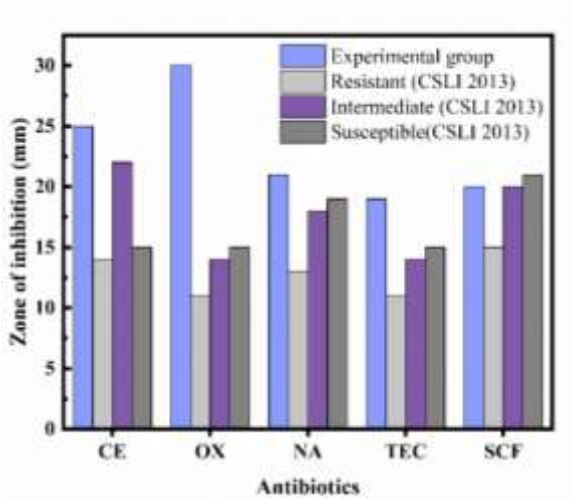

Fig. 8: Graphic representation of *Klebsiella* showing zones of inhibition against various antibiotics

Fig 8 is the graphical representation of zones of inhibition by bacteria Klebsiella against 5 types of antibiotics. Similar to the current study, not only honey bees but some other insect's gut also harbor diverse communities of bacteria found in different environments. Like extensive bacterial communities of *Bacillus* and *Pseudomonas* species were found in Gypsy moth caterpillars (*Lymantria dispar* L) and bacterial taxa of Proteobacteria were most frequently found in the intestinal bacterial communities of the caterpillars of the *Pieris rapae*, the cabbage white butterfly (Raffa, Iannuzzo et al. 2005). In termites

Bacillus sp. BMP01 and *Ochrobactrum oryzae* BMP03, two bacterial strains were identified by (Bahiru Tsegaye, Chandrajit antibiotics. No significant resistance was observed in *Klebsiella* against CE, OX, NA, TEC, and SCF having zones of inhibition 25mm, 30mm, 21mm,19mm, and 20mm, respectively compared with CSLI resistant values for Cephalexin, Oxytetracycline, Nalidixic acid, Tetracycline and Cephaprazone are ≤14 (less than or equal to 14mm), ≤ 11, ≤13, ≤11, ≤15 respectively.

Balomajumder et al. 2018), while the results of 16S rRNA sequencing of weevil showed that Proteobacteria and Bacteroidetes dominate the intestinal lumen of weevil (Jing, Qi et al. 2020). By using the cultural dependent method, the midgut bacterial microbiota of *Helicoverpa armigera*, a species that is also very common in South Asia, Australia, Southeast Asia, China, and Southern and Eastern Africa, was studied in India. Most of the genera identified were firmicutes, with Enterococcus and Enterobacter being among the most commonly occurring bacteria in all samples. Other major genera included Bacillus, Paenibacillus, Enterococcus, and Micrococcus (Actinobacteria), Sphingomonas (Alpha Proteobacteria), Ralstonia (Beta Proteobacteria), and Micrococcus (Gama Proteobacteria) (Gayatri Priya, Ojha et al. 2012).

Another study conducted in Tucson, Arizona reported the same bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Shigella sp.*, isolated from the gut contents of healthy adult worker forager honey bees, *A. mellifera*, (Gilliam and Valentine 1974) and in East Hararghe, Oromia Regional State, Ethiopia two species of *klebsiella* namely *Klebsiella pneumoniae* and *Klebsiella oxytoca* were reported by (Abdurehman Damissie and Abdurahman Musa 2022). Along with some other bacterial species, *E. coli* dominated the gut of honey bees. However, some strains of bacteria, *E. coli*, including LF82, the causative agent of inflammatory bowel syndrome, have been shown to affect the intestinal and cognitive functions of honeybees. For example, honey bees when exposed to LF82, exhibited the effect of increased gut permeability, impaired learning and memory, and reduced lifespan compared with bees exposure to the non-pathogenic strains of *E. coli* MG1655 (Chang, Chen et al. 2022).

A study was conducted for antibiotic resistance of *E.coli* against various antibiotics in the central area of Iran according to which *E.coli* was resistant against Erythromycin and Nalidixic acid (Ebrahimi and Lotfalian 2005). Higher resistance of *E. coli* against OX, TEC, NA, and SCF may be due to using of that antibiotic in the apiaries. A study performed in Bangladesh results that *Klebsiella pneumoniae* were susceptible to various antibiotics including Tetracycline, Gentamicin, Streptomycin, Neomycin, Azithromycin, and Levofloxacin (Rahman, Begum et al. 2023). For antibiotic susceptibility, we follow the same method documented in El Natroon Valley (Egypt) in which antibiotic susceptibility of the bacterial isolates was determined by using the disc diffusion assay according to the guidelines outlined by the Clinical and Laboratory Standards Institute (Hamdy, Elattal et al. 2017).

IV. CONCLUSION

In this study, isolation and identification of bacterial strains from the gut of *A. mellifera* to examine antibiotic resistance of the isolated bacterial strains were performed. Results showed that an increased resistance was observed in *E. coli* against OX, TEC, NA, and SCF. No such resistance was observed in *Klebsiella* against

CE, OX, NA, TEC, and SCF. So, it can be estimated that *E. coli* in bee gut *A. mellifera* is significant in developing antibiotic resistance towards medicines used for bacterial pathogens treatment. It is the first study on antibiotic resistance shown by the gut bacteria of *A. mellifera* in the country particularly in the study region.

Author Contributions

Conceptualization, Iram Nowsheen; Data curation, Iram Nowsheen and Syed Ishtiaq Anjum; Formal analysis, Muhammad Anees; Methodology, Iram Nowsheen and Sajida Afzal; Project administration, Syed Ishtiaq Anjum; Resources, Muhammad Anees and Syed Ishtiaq Anjum; Software, Muhammad Hanif; Supervision, Syed Ishtiaq Anjum; Visualization, Muhammad Hanif and Iram Nowsheen; Writing – original draft, Iram Nowsheen; Writing – review & editing, Muhammad Hanif, Muhammad Anees, and Sajida Afzal. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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