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# GUT BACTERIAL DIVERSITY AND THEIR ANTIBIOTIC RESISTANCE IN HONEY BEE COMMUNITIES OF DHAKA CITY

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**Abstract:** The study was undertaken to isolate and identify the bacteria from the gut of honey bees and to investigate their antibiotic sensitivity. A total 35 honey bees were collected from Sher-e-Bangla Agricultural University and Curzon Hall area of Dhaka city. This study was conducted to identify the gram-positive and gram-negative bacteria based on the morphological features using microscope. In this study, four species of honey bees were identified, of which, *Apis dorsata* was the most dominant (31.43%) species and followed by 17.14% for *Apis cerana* and 2.86% for *Apis mellifera* and *Apis florea*, respectively. In total, five gram-positive bacteria genera including *Lactobacillus* sp. (37.5%), *Streptococcus* sp. (9.38%), *Staphylococcus* sp. (21.88%), *Enterococcus* sp. (6.25%), and *Clostridium* sp. (9.38%); and two gram-negative bacteria i.e., *Pseudomonas* sp. (3.13%), and *Enterobacter* sp. (12.5%) were detected. Gram-positive bacteria were the most dominant at the honey bee gut in two sites, while bacteria did not detect in *A. dorsata* gut which was collected from Sher-e-Bangla Agricultural University. However, five types of bacteria were isolated from the gut of *A. dorsata* collected from Curzon Hall area. In total, the highest varieties (6 out of 7) and number of isolates (15) were found from *A. florea*. Whereas, the highest number of bacteria was isolated from *A. florea* (12) for Curzon Hall area, and *A. cerana* (6) for Sher-e-Bangla Agricultural University. In antibiotic susceptibility test, the isolated *Streptococcus* sp., *Staphylococcus* sp. and *Enterococcus* sp. were sensitive to the majority of the antibiotics. However, the isolated *Lactobacilllus* sp., *Clostridium* sp., *Enterobacter* sp. and *Pseudomonas* sp. were resistant to the majority of antibiotics. The results show that the presence or absence of bacterium in honey bees gut was an indicator for their overall health.

***Key words:*** Antibiotic sensitivity, Bacteria isolation, Probiotic bacteria, *Apis dorsata*, *Apis cerana*, *Apis mellifera*, *Apis florea*.

## INTRODUCTION

Honey bees are flying insects within the genus *Apis* under the largest

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family Apidae of order Hymenoptera (Danforth *et al.* 2006). In Bangladesh, only one genus of honey bee *Apis* is being found which includes four species: *Apis mellifera, Apis dorsata, Apis cerana,* and *Apis florea* (Akter *et al.*, 2019). For the past few decades, their number has been declined throughout the world alarmingly (Potts *et al.*, 2010).

Various types of microbes are associated with the animal kingdom and some of them are beneficial and some only cause nuisance and diseases (Hacquard *et al.*, 2015). The honey bees gut bacteria can be a pathogenic and or symbiotic. The presence of pathogenic bacteria has been significantly affected honey bee overall health while the symbiotic association with some microbes are obligatory for their survival (Engel *et al.,* 2016; Anjum *et al.,* 2006). The microbiota associated with the honey bee is mainly composed of yeasts, gram-positive bacteria (such as *Lactobacillus rigidus apis*, *L. constellatus, Bacillus* spp., *Streptococcus* and *Clostridium*), and gram-negative or gram variable bacteria (*Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus* and *Pseudomonas*) (Gilliam, 1978; Gilliam and Prest, 1987; Rada *et al.,* 1997; Mohr and Tebbe, 2006). Some gram-positive bacteria in honey bee gut i.e., *Lactobacillus* and *Streptococcus* are considered to be beneficial bacterial species which are immensely used as the important probiotics in the dairy products (Isolauri *et al.,* 2002).

Some gram-positive bacteria i.e., *Clostridium* and *Bacillus* and gram-negative bacteria i.e., *Enterobacter* and *Pseudomonas* are considered as pathogen in honey bee population. These are responsible for diseases and honey bee colony collapsing (Evans and Schwarz, 2011). According to Anjum *et al.* (2021) most of these pathogenic bacteria are also pathogenic to human which has substantially caused honey bee mortality thus honey bee population has been declined gradually. The study on honey bee gut microbiota and their antibiotic resistance in Bangladesh is not available. Therefore, the present study was conducted to identify the gut bacterial diversity and their antibiotic resistance in honey bee community. Additionally, this study was help to understand the overall health status of honey bee population in the Dhaka city.

## MATERIAL AND METHODS

This research work was conducted in Entomology Laboratory, Department of Zoology and Microbiology Laboratory, Department of Microbiology, University of Dhaka. Curzon Hall area of University of Dhaka. The Sher-E-Bangla Agricultural University campus was selected as the collection site of honey bees. Honey bees were collected by sterilizing insect net and hand picking. After catching by insect net, they were transferred to air tight jar. Inside the jar there

was some cotton which was mixed with chloroform for anesthetizing. Collected bees were placed into the sterile tube individually inflicting minimal injury to them. Identification was made by examining the external morphology of the bees individually and following standard taxonomic keys (Engel, 1999). After identification, fresh honey bee samples were soaked in 70% ethanol for five minutes to decontaminate their external surface and dried, followed by washing with sterile saline to remove traces of ethanol. The alimentary tract of honey bees was aseptically dissected out using autoclaved sterilized entomological dissecting needles under a dissecting microscope. The instrument was dipped in ethanol and flamed between dissections. The excised gut was homogenized in 9 ml of sterile normal saline water. Then it was diluted. In total, 35 honey bees were collected for the experiment. All collected homogenate were cultured using spread plate technique into Nutrient Agar (NA) media and M-FC agar media followed by incubation at 37°C for 24 hours. Growth on all plates was observed and the numbers of bacteria colonies were counted. Isolated colonies were cultured using NA media to obtain pure culture. Moreover, the pure isolated colonies were then cultured using Mac Conkey media and Mannitol salt agar and M-FC agar for further identification. These were then transferred in the incubation chamber to developed colonies, which were identified based on morphological features of each colony (size, shape, elevation, color, consistency, opacity, pigmentation).

Steps for gram staining were followed by Pelczar *et al.* (1993) to determine the size, shape, arrangement and gram reaction to isolated organisms. The presumptive identification of isolates was done by performing various biochemical tests i.e., motility test, indole test, citrate utilization test, lactose fermentation test and glucose fermentation test, gas formation test. $H_2S$ in Kingler's Iron Agar (KIA) were performed for gram-negative bacteria and catalase test and oxidase test were performed for gram-positive bacteria. All the tests were accomplished by following the standard protocol as described in Bergey's Manual of Systematic Bacteriology (Garrity, 2001). Antibiotic susceptibility testing of bacterial isolates was done by Kirby Bauer disk diffusion method using Mueller Hinton Agar (MHA) plate. Commercial antimicrobial discs were used which include: Ampicillin (AMP 25), Penicillin-G (P), Chloramphenicol (C), Tetracycline (TE), Amoxicillin (AML), Imipenem (IMI), Erythromycin (E).

## RESULTS AND DISCUSSION

A total of 35 honey bees of four different species were identified from Sher-e-Bangla Agricultural University and Curzon Hall, which were then used to examine the bacteriological study. In this study, in total, four species of honey bees were identified i.e., *A. dorsata*, *A. florea*, *A. cerana* and *A. mellifera*; these

**Table1: Honey bee species distribution in two study areas**

| Honey bee species | Sher-E-Bangla Agricultural University | Curzon hall | Total |
|---|---|---|---|
| *Apis dorsata* | 2 (5.71%) | 9 (25.71%) | 11(31.42%) |
| *Apis florea* | 7(20%) | 10 (28.57%) | 17 (48.57%) |
| *Apis cerana* | 6 (17.14%) | 0 | 6 (17.14%) |
| *Apis mellifera* | 1(2.86%) | 0 | 1(2.86%) |
| **Total** | **16(45.71%)** | **19(54.28%)** | **35** |

**Table 2: Number of isolates among different species of honey bees**

| Study area | Honey bee species | No. of Isolates | Percentage of isolates |
|---|---|---|---|
| Sher-E-Bangla Agricultural University | *Apis dorsata* | 0 | 0% |
| | *Apis florea* | 3 | 9.38% |
| | *Apis cerana* | 6 | 18.75% |
| | *Apis mellifera* | 1 | 3.12% |
| Curzon hall | *Apis florea* | 12 | 37.5% |
| | *Apis dorsata* | 10 | 31.25% |
| **Total** | | **32** | **100%** |

were recorded in Sher-e-Bangla Agricultural University while two honey bee species *A. florea* and *A. dorsata* were observed in Curzon Hall area. On the basis of percentage composition, honey bees constituted of 54.28% and 45.71% total population in Curzon Hall and Sher-e-Bangla Agricultural University, respectively. Among the four species of Honey bees, *A. florea* (48.57%) was the most dominant species and followed by *A. dorsata* (31.43%) and *A. cerana* (17.14%), while *A. mellifera* (2.86%) was least dominant species (Table 1). The bacteriological characteristics of 35 honey bees were investigated. A total of 32 bacterial isolates were prepared from the bees. Among 12(37.5%) were collected from *A. florea* and 10(31.25%) from *A. dorsata* of Curzon Hall area. Additionally, 3(9.38%) isolates were collected from *A. florea,* 1(3.12%) from *A. mellifera* and 6(18.75%) from *A. cerana* of Sher-E-Bangla Agricultural University campus. Not any type of microorganism was found from the gut of *A. dorsata* collected from Sher-e-Bangla Agricultural University. (Table-2). Based on biochemical tests and growth on selective media, the isolates were presumptively identified as *Lactobacillus* sp. (37.5%), *Streptococcus* sp. (9.38%), *Staphylococcus* sp. (21.88%), *Enterococcus* sp. (6.25%), *Clostridium* sp. (9.38%), *Pseudomonas* sp. (3.13%), and *Enterobacter* sp. (12.5%) (Fig. 1). Seven types of bacteria species were isolated, of which. five (*Lactobacillus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Enterococcus* sp., and *Clostridium* sp.) were gram-positive while the two (*Pseudomonas* sp. and *Enterobacter* sp.) were gram-negative bacteria. Among the isolated gram-positive bacteria, *Lactobacillus* sp. was more frequent and among the gram-negative, *Enterobacter* sp. was the most dominant. *Pseudomonas* sp. and *Enterococcus* sp. did not find from the samples of Curzon Hall. Similarly,

**Table 3: Biochemical test result of Gram-negative isolates collected from 2 selected areas**

| Study area | Honey bee species | Sample no. | Isolate no. | Growth on | | Gram stain | Biochemical test | | | | | | | Presumptive Identification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | MacConkey | MFC | | Kingler's Iron Agar test | | | | Citrate use | Motility test | Indole production | |
| | | | | | | | Lact. | Gluc. | Gas | H2S | | | | |
| Sher-E-Bangla Agricultural University | *Apis florea* | S- 8 | I | + | + | GN-rod | + | + | - | + | - | - | + | *Enterobacter* sp. |
| | | S- 9 | K | + | + | GN-rod | - | + | + | - | + | + | - | *Pseudomonas* spp |
| Curzon hall | *Apis dorsata* | C17 | H, J | + | + | GN-rod | + | + | - | + | - | - | + | *Enterobacter* sp. |
| | *Apis florea* | C10 | N2 | + | + | GN-rod | + | + | - | + | - | - | + | *Enterobacter* sp. |

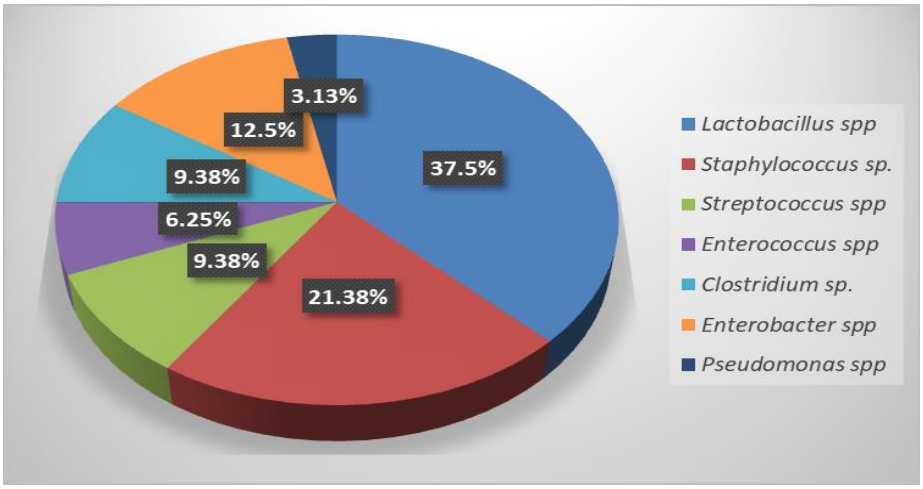


Fig.1. Percentage of different bacteria found in honey bee species

*Clostridium* sp. and *Staphylococcus* sp. were not detected among the samples of Sher-E-Bangla Agricultural University campus. (Table 3 and Table 4). It was investigated that *A. cerana* carried *Lactobacillus* sp. and *Enterococcus* sp. (Table 4). However, Disayathothonoowat *et al.* (2012) isolated bacteria from the gut of *Apis cerana* and found *Klebsiella* and *Enterobactor* sp. It was also found that *A. mellifera* contained only *Enterococcus* sp. (Table 4). This result match with the study investigated by Audisio *et al.* (2010), who found five strain *Enterococcus* from the gut of *A. mellifera*. *A. dorsata* contained *Lactobacillus* sp., *Streptococcus* sp., *Staphylococcus* sp., *Clostridium* sp., and *Enterobacter* sp. (Table 4). In case of *A. dorsata* the study result was consistent with the study of Tajabad *et al.* (2012) where *Lactobacilli* sp. was isolated from the honey bee stomach. Most of these bacteria are act as the causative agent of various diseases in human and the presence of human pathogenic bacteria is a reason for the reduction of bee community, as these pathogens affect host overall health (Ishii *et al.,* 2014). Malignant external otitis, endophthalmitis, endocarditis, meningitis, pneumonia, and septicemia are the results of *Pseudomonas* sp., while *Staphylococcus aureus* can cause skin infection and food poisoning (Bodey *et al*, 1983, Forster *et al.,* 2009). *Enterococcus* sp. is associated with urinary tract and biliary tract infections and *Streptococcus sp.* are responsible for endocarditis, respiratory and urinary tract infections (Patterson, 1996). Various bacteria such as *Lactobacillus* sp., *Enterococcus* sp. and *Streptococcus* sp. etc. are notably known as probiotic for honey bee (Ljungh and Wadström, 2006; Du, *et al.*, 2021; Hutkins and Goh, 2014). According to Poormuntaseri *et al.* (2014), *Clostridium* sp. do not cause

**Table 4: Biochemical test result of Gram-positive isolates collected from 2 selected areas**

| Study area | Honey bee species | Sample no | Isolate no | Growth on | | | Gram stain | Biochemical test | | Presumptive Identification |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | MacConkey | MSA | MFC | | Catalase | Oxidase | |
| Sher-E-Bangla Agricultural University | *Apis florea* | S-3 | D | - | + | - | Gram +ve Cocci | - | - | *Streptococcus* sp. |
| | *Apis cerana* | S- 10, 12, 13, 14, 15 | P, Q1, T, V, X | - | + | - | Gram +ve Rod | - | + | *Lactobacillus* sp. |
| | | S-12 | Q2 | - | + | - | Gram +ve Cocci | - | - | *Enterococcus* sp. |
| | *Apis mellifera* | S-16 | Z | - | + | - | Gram +ve Cocci | - | - | *Enterococcus* sp. |
| Curzon hall | *Apis florea* | C- 1 | A1 | - | + | - | Gram +ve Cocci | - | - | *Streptococcus* sp. |
| | | C- 4, 8, 9 | A2, F, L, M | - | + | - | Gram +ve Rod | - | + | *Lactobacillus* sp. |
| | | C- 2 | B1 | - | + | - | Gram +ve Rod | - | - | *Clostridium* sp. |
| | | C- 2, 3, 7, 10 | B2, E, G1, G2, N1 | - | + | - | Gram +ve Cocci | + | - | *Staphylococcus* sp. |
| | | C- 12, 13 | O, S | | | | Gram +ve Cocci | + | - | *Staphylococcus* sp. |
| | | C- 12, 16 | R, b | | | | Gram +ve Rod | - | - | *Clostridium* sp. |
| | *Apis dorsata* | C- 14 | U, W, Y | | | | Gram +ve Rod | - | + | *Lactobacillus* sp. |
| | | C- 16 | a | | | | Gram +ve Cocci | - | - | *Streptococcus* sp. |

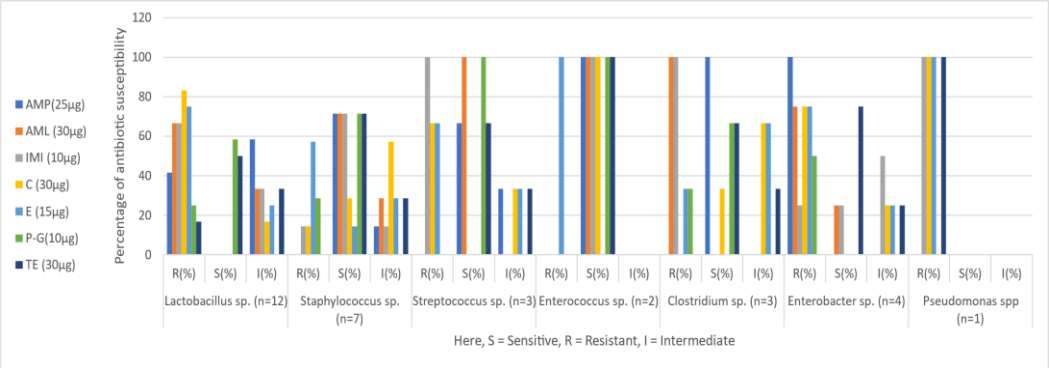


Fig. 2. Percentage of antibiotic susceptibility of different isolated bacteria species.

any disease in bee, but the reason behind botulism in humans, this gram-positive bacterium affects mainly children and those with weakened immune systems. *Enterobacter* sp. are responsible for causing many nosocomial infections, including urinary tract infections (UTI), respiratory infections, soft tissue infections in human and also cause bacterial disease in honey bee (Ramirez and Giron, 2021). Human depends on bee and bee products include honey, which could be contaminated by various sources, but the primary source of microbial contamination is the digestive tracts of honeybees (Peter *et al.,* 2007). Identified isolates from gut of bees were tested for their antibiotic susceptibility against seven commonly used antibiotics. Most of the *Lactobacillus* isolates were resistant to those antibiotics. The highest resistance pattern (83.33%) was found against Chloramphenicol and the lowest (16.67%) was found against Tetracycline antibiotic. It was found that 100% *Pseudomonas spp.* were resistant to Chloramphenicol, Imipenem, Erythromycin and Tetracycline, while most of the individual of other isolates found sensitive to Tetracycline. Among the four isolates of *Enterobacter* spp., highest (75%) susceptibility was observed to Tetracycline and highest (100%) resistance was found against Amplicin. By contrast, among three isolates of *Clostridium* spp. all of them were resistant (100%) against Amoxicillin and Imipenem and 100% sensitive to Amplicin (Fig. 2). It was observed that, all of the two isolates of *Enterococcus* sp. were resistant (100%) against theses antibiotics, except in Erythromycin, where the isolates found 100% sensitive. Most of the isolates of *Staphylococcus* spp. were sensitive. Among them, they were mostly sensitive (85.71%) to Amplicin and most resistant (57.14%) against Erythromycin antibiotic. But according to Akter *et al.*, (2021), most of the isolates of *Staphylococcus* spp. found resistant. were equally susceptible and resistant against these antibiotics. All of the three isolates (100%) *Streptococcus* sp. were resistant against Imipenem and sensitive to Amoxicillin and Penicillin-G antibiotic (Fig. 2).

As some bacteria in bee affects host nutrition, weight gain, endocrine signaling, immune function, and pathogen resistance (Zheng *et al.*, 2019), it is important to investigate whether these pathogens create any impact on other beneficial and obligatory microbes present in bee gut. Honey bee health could be improved by using probiotic (Kaznowski, 2005) and in this study we found three natural probiotics namely, *Lactobacillus* sp., *Enterococcus* sp. and *Streptococcus* sp. They assist in healthy replacement of good bacteria in the intestinal tract following antibiotic therapy (Sanders, *et al.,* 2000). Therefore, it can be said that the *Lactobacillus* sp. which originates from the honey stomach can be selected as a food probiotic and food preservatives supplement for human consumption.

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