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Screening of Bacterial Microflora from the Gastrointestinal Tract of Apis Mellifera (Apidae; Hymenoptera), its Brood and Soil Samples from Prayagraj District: A Brief Study

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Abstract
Honeybees are economically important insects where studies on their gut microbiota has interestingly emphasized on the symbiotic association existing between them and honeybees. Previous studies have revealed very scarce reports on the studies of gut microflora of honeybees especially Uttar Pradesh, India. With this idea research was carried out to screen the microflora in the gut of live and dead worker honeybees (Apis mellifera) and their associated brood and soil samples, from the popular local apiaries located at two different regions of Prayagraj district India. Bacteriae identified from the gut of live and dead bees and brood and soil samples from both the sites belonged to the genus Staphylococcus, Enterobacter, Enterococcus, Bacillus and Pseudomonas. Microflora obtained from all the samples of site IFFCO Cordet showed significant results. It was found that the samples from live bees were significantly different from the bacterial counts obtained from the brood samples, while the bacteria isolated from the sample of dead bee was significantly different from both the samples of brood and soil respectively. Results of different samples from Jhunsi Chatnag site were found to be significantly different from each other. Highest bacterial count was obtained from live honey bee gut sample of IFFCO Cordet. The findings showed that there is a firm relationship between the gastrointestinal microflora balance and the health status of the host. It was concluded that Bacillus, Enterobacter and Enterococcus are reported to have positive symbiotic relationship with Apis mellifera and at the same time, present findings reports for the absence of Enterobacter and Bacillus in the gut of bee. Pseudomonas are reported as to be involved in xenobiotic degradation. At the same time species of *Pseudomonas* and *Staphylococcus aureus* can be so harmful that they may lead to colony collapse. Accordingly, our findings reports on the presence of both Pseudomonas and Staphylococcus in the dead bee samples. Thus, bacteria isolated in the findings can be further identified for their strains for better understanding the significance and relationship with its host and its specific utility can be further exploited at industrial levels as well.

Keywords: Honeybees, gut microflora, bacteria

1. Introduction

Pollinators play key role for the sustenance of life on the earth. As a pollinator, honey bee has a significant role in maintaining renewable agricultural environment along with production of honey and other natural products (Klein et al., 2007; Potts et al., 2010). Honeybee is a highly valued insect throughout the world, not only for honey production but also for its great importance to humans and ecosystems as pollinator, of many economically important crops and wild flora (Engelsdorp and Meixner, 2010). Unfortunately, honey bee populations has turned out to be declining at a disturbing rate from past few years and the causes of which are found to be in association with several biotic and abiotic factors, like the utilization of pesticides, habitat loss, spread of pathogens and parasites, impact of climatic changes (Potts et al., 2010), etc. However, gut microbiota has been recognized to be in association with the bees in several ways. Honey bees harbor a specialized gut community (Kwong and Moran, 2016) and these gut bacteria play significant roles in health and vitality (Dillon and Dillon, 2004), contribute enormously to host immunity (Mazmanian et al., 2005), boost nutrient deficient diets, degrade difficult food ingredients, and defend the host from parasites, and pathogens (Engel and Moran, 2013a). A well-balanced association of microbial species with many symbiotic and competitive interactions, referred to as an indigenous gastro-intestinal microflora, forms an integral part of any well-functioning healthy organism. (Máchová et al. 1997). Few researchers have performed studies on organisms cultured from bee guts and the hive, documenting a variety of metabolic and functional activities of these microbes (Gilliam and Prest, 1972, 1987; Gilliam and Valentine, 1974; Gilliam et al., 1974; Gilliam, 1978; Evans and Armstrong, 2006). The normal bacterial microflora is acquired by the consumption of pollens, nectar, other food, bee brood and through contacts with older bees in the colony. Bee brood is a product that has been used by man since ancient times for its pharmaceutical properties (Walker and Crane, 1987). It is still used as a remedy in folk medicine (Kujungjev et al., 1999) as a constituent of 'bio-cosmetics', 'health foods' and for numerous other purposes (Wollenweber and Buchmann, 1997). Although, the literature on the gut bacterium of honey bees are increasing endlessly, there is no elaborate information concerning the bacterial communities related with the gastrointestinal tract of the native honey bees present in India. Therefore, keeping in view the above facts, research was carried out on winter honeybees to isolate, compare and study the symbiotic association of the bacteria from the gastrointestinal tract of live and dead honeybees (*Apis mellifera)*, and its brood and soil samples, from the two local popular apiaries located at Jhunsi Chatnag and Iffco Cordet of Uttar Pradesh Prayagraj region.

2. Materials and Methods

2.1. Place of study

All the experiments included in the study of microflora associated with honey bee gastrointestinal tract, brood and nearby soil samples were conducted in the Department of Zoology and Centre for Microbiology, Ewing Christian College, Gau Ghat, Prayagraj, Uttar Pradesh, India.

2.2. Collection of samples

The honey bee, brood and soil samples were collected from IFFCO Phulpur Cordet apiary and a local apiary situated in Chatnag, Prayagraj. Worker bees were collected from hives before evacuation of faeces outside the hive. Bees were dissected with sterilised tools for the collection of gut samples. The collected gut samples were then weighed separately with the help of electronic balance and 1 gm. mass was obtained for the study (Kačániová et al., 2004; Rada et al.,1997).

Soil samples were collected from within a range of one metre distance of apiary box from where the bees were collected.1 gm. soil was measured from each of the collected sample. Similarly, brood samples were also collected and measured 1 gram from each location separately. The brood sample was thoroughly washed using distilled water, dried in open air and then broken down into minute pieces and were soaked in 70 percent (%) ethanol for 48 hours at 37 degrees Celsius (^{0}C). Each sample was filtered through Whatman no. 1 filter paper. The wax was dried without any further contamination. All the samples were collected twice and were analysed microbiologically in triplicate manner.

2.3. Culture media:

Nutrient Agar media was used in the study for isolation and culturing the bacteria.

2.4. Isolation of bacteria

Bacteria were isolated through "Serial Dilution Pour Plate Technique" (Anjum et al., 2018) with a slight modification. For this 1 gram (gm.) of sample was suspended in 9 millilitre (ml.) sterilized dilution blanks and subsequent dilutions were made up to 10^6 level. From the final dilution 1 ml. suspension was measured via micropipette and then transferred to sterilized petriplates followed by 15-20 ml. of Nutrient Agar media, separately. After this the plates were covered immediately and kept undisturbed for about half an hour for proper solidification of media. Then Nutrient Agar plates were incubated in inverted positions at 37 degrees Celsius (^{0}C) for 24- 48 hours (hrs.) in the incubator. Finally, the plates were observed for microbial growth and the colonies obtained were counted and studied for their cultural, morphological and biochemical characteristics.

2.5. Identification of Bacteria

2.5.1. Morphological Characteristics

The bacteria isolated were primarily identified on the basis of morphological characteristics by Gram staining technique, where single colony of every isolated bacteria was taken and stained according to the standard protocol and observed under oil immersion microscope.

2.5.2. Biochemical tests

Certain biochemical tests were performed to identify and confirm the biochemical activity of the bacteria. (https://microbiologyinfo.com/).

1. **Carbohydrate Fermentation tests-**
   Phenol red broth base media was prepared, poured into tubes and Durhum tube was inserted in each tube for gas detection followed by the autoclaving at 15 pounds per inch square (lbs/inch^2) for 15-20 minutes (min). Different sugar substrates – Lactose, Glucose, Mannitol, Galactose, Arabinose and Maltose were prepared 1% and autoclaved at 10lbs/inch^2 (pounds per square inch) for 10 mins. After autoclaving, 1ml of respective sugar and isolated bacteria was transferred in the tubes and kept inside the incubator for 48 hours at 37˚C.
2. **Catalase test-** One drop of hydrogen peroxide was taken on a clean glass slide and very little amount of isolated colony was transferred on it with a clean glass rod. Immediate production of effervescence indicated positive result i.e. the culture can produce catalase enzyme.
3. **Motility test-** A straight needle was touched to a colony of isolated bacteria and stabbed once to a depth of half inch in the middle of the tube and incubated at 35-37˚C and examined daily for upto 7 days.
4. **Citrate Utilization test-** The isolated bacteria were inoculated in Simmons Citrate Agar and incubated at 37˚C for 24 hours. After 24 hrs, the change of media from green to blue was recorded accordingly

for the isolated bacteria.

5. **Indole Production test-** The suspected bacteria was inoculated in peptone broth and incubated at 37˚C for 48 hours. After incubation, Kovac's reagent (1ml. in each tube) was added and looked for the formation of red precipitate. `
6. **Gelatin Hydrolysis test-** In a set of test tubes Gelatin media was poured and autoclaved. After autoclaving a straight needle was touched to a colony of isolated bacteria and stabbed once to a depth of the tube and removed the needle through the same line as it went inside. The tubes were then incubated at 37˚C for 48 hours. After incubation, the tubes were kept in refrigerator to check the solidification of gelatin (in case gelatin was liquefied) and the gelatin hydrolase enzyme activity of the isolates.
7. **Oxidase Test:** For this test a small piece of filter paper was soaked in 1% Kovac's oxidase reagent and allowed to dry. After this with the help of inoculation loop a small amount of suspected bacteria was taken and was rubbed on the surface of the paper. If a dark purple color appeared on the paper the organism was positive for oxidase activity.

Further, results obtained were compared with Bergey's Manual and organisms were identified upto genus level.

2.6. Statistical Analysis: The bacterial counts obtained from the samples of both the sites were statistically analysed and compared using Two-way Anlaysis of Variance.

3. Results and Discussion

Dead and live honeybees were taken from the apiaries of two sites along with soil and brood sample from same site to study the bacterial microflora. Microflora obtained from both the sites, IFFCO Cordet and Jhunsi Chatnag showed statistically significant results. Where, bacteria isolated from the sample of live bees of the site IFFCO Cordet, was found to be significantly different only from the bacteria of brood samples, while the bacteria isolated from the sample of dead bee was significantly different from both the samples of brood and soil respectively (Table 1). On the other hand, all the samples obtained from the site Jhunsi Chatnag for the isolation of bacteria were found to be significantly different from each other. (Table 2). However, when all the samples of the two sites were compared with each other, the results showed non-significant difference., which might be due to insignificant difference in the climatic conditions prevailing at the apiaries of the two sites. (Table 3).

3.1. Isolation and enumeration of bacteria

Results obtained showed that the total number of bacterial isolates obtained from the sites Jhunsi Chatnag and IFFCO Cordet ranged from 38.66×10^6 to 175.66×10^6 Colony forming unit per millilitre (CFU/ml) in live bees while 72.33×10^6 to 173×10^6 CFU/ml in dead bees and 97.66×10^6 to 151.33×10^6 CFU/ml in the soil samples. Whereas, bacterial counts obtained in the brood samples from IFFCO Cordet and Jhunsi Chatnag ranged from 34.66×10^6 to 88.33×10^6 CFU/ml respectively. (Table 1&2; Figure (Fig.) 1&2).

Table 1. Bacterial count x10^6 CFU/ml.) in samples collected for experiment from the site IFFCO Cordet

	Samples	Dilution Blank	Minimum	Maximum	Average	Total count
1	Live Honey Bee Gut	10^6	163	186	175.66	175.66×10^6
2	Dead Honey Bee Gut	10^6	122	221	173	173×10^6

3	Brood	10^6	22	48	34.66	34.66×10^6
4	Soil	10^6	125	174	151.33	151.33×10^{-6}
F- test		S				
S. Ed. (±)		12.479				
C. D. (P = 0.05)		30.536				

Figure 1. Bacterial count of Samples from the site IFFCO Cordet

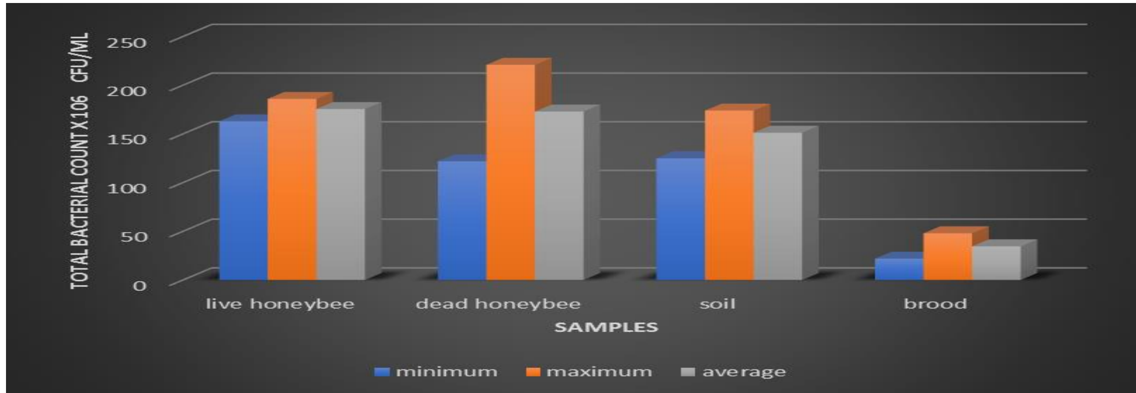


Table 2. Bacterial count x10^6 CFU/ml in samples collected for experiment from the site Jhunsi Chatnag

	Samples	Dilution Blank	Minimum	Maximum	Average	Total count
1	Live Honey Bee Gut	10^{-6}	18	59	39	$38.66\text{x}10^6$
2	Dead Honey Bee Gut	10^{-6}	57	72	72.33	$72.33\text{ x}10^6$
3	Brood	10^{-6}	63	113	88.33	$88\text{ x}10^6$
4	Soil	10^{-6}	85	110	97.66	$97.66\text{ x}10^6$
F- test		S				
S. Ed. (±)		3.903				
C. D. (P = 0.05)		9.551				

Figure 2. Bacterial Counts of Samples from the site Jhunsi Chatnag

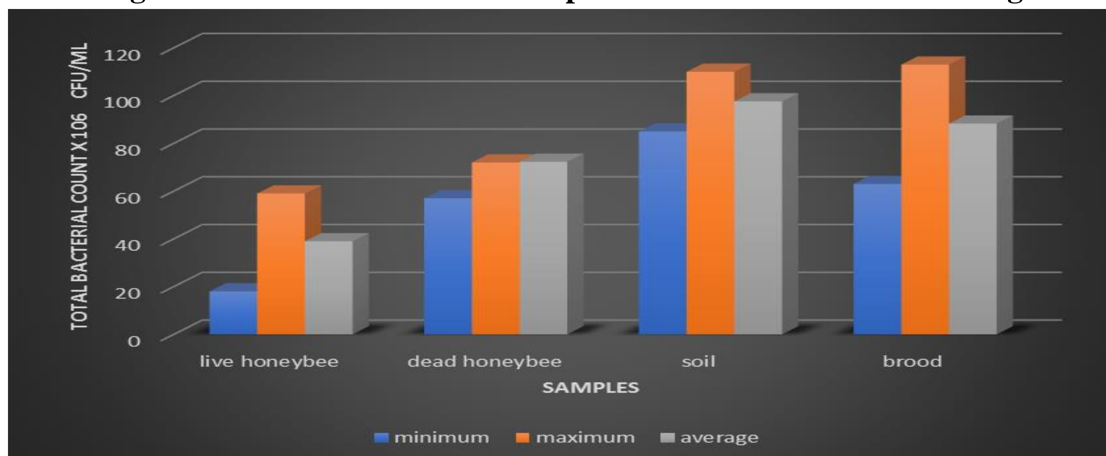
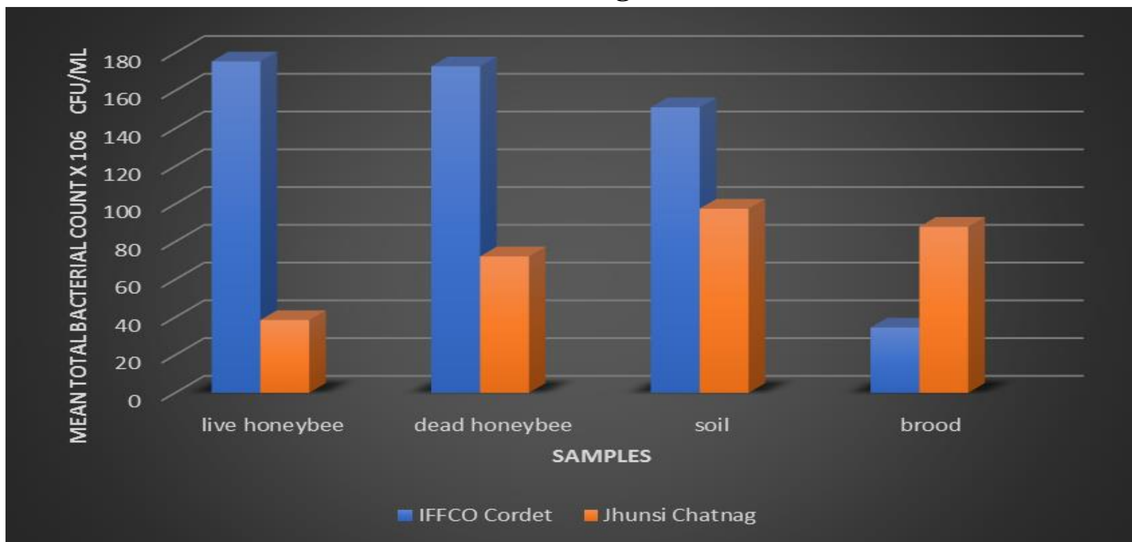


Table 3. Comparative table showing bacterial counts (CFU/ml) in samples collected from the sites IFFCO Cordet and Jhunsi Chatnag

Mean Total Bacterial Count x10^6 CFU/ml.			
Samples		**IFFCO Cordet**	**Jhunsi Chatnag**
1	Live Honey Bee Gut	175.66×10^6	38.66x10^6
2	Dead Honey Bee Gut	173×10^6	72.33 x10^6
3	Brood	34.66×10^6	88 x10^6
4	Soil	151.33×10^6	97.66 x10^6
F- test		NS	
S. Ed. (±)		41.300	
C. D. (P = 0.05)		131.434	

Figure 3. Bacterial counts (CFU/ml) of samples collected from the sites IFFCO Cordet and Jhunsi Chatnag



3.2. Identification of isolated bacteria

The bacteria were identified and confirmed on the basis of various morphological and biochemical tests referring to Bergey's Manual (1994) respectively (Table 4).

Table 4. Biochemical tests performed for the identification of bacteria

Morphological Tests	*Staphylococcus sp.*	*Enterococcus sp.*	*Pseudomonas sp.*	*Bacillus sp.*	*Enterobacter sp.*
Cell shape	Spherical	Spherical	Small Rods	Cylindrical	Small rods
Cell arrangement	In bunch	In Chain	Mostly Single	Mostly Single	Mostly Single
Gram Reaction	Positive	Positive	Negative	Positive	Negative

Physiological Tests					
Catalase	Positive	Negative	Positive	Positive	Positive
Motility	Negative	Negative	Positive	Positive	Positive
Citrate Utilization	Positive	Negative	Positive	Positive	Positive
Indole	Negative	Negative	Negative	Negative	Negative
Gelatin Hydrolysis	Positive	Variable	Positive	Negative	Negative
Oxidase	Negative	Negative	Positive	Negative	Negative
Fermentation of					
Arabinose	Negative	Negative	-	Negative	Positive
Galactose	Positive	-	-	Negative	-
Glucose	Positive	Positive	Negative	Positive	Positive
Lactose	Positive	Positive	Negative	Negative	Negative
Maltose	Positive	Positive	Negative	Positive	Positive
Mannitol	Positive	Positive	Positive	Negative	Positive

3.3. Bacteria isolated from the Live bee and Dead bee samples of IFFCO Cordet and Jhunsi Chatnag site

The bacteria obtained from the gut of Live bee sample from IFFCO Cordet site were *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Enterococcus* and *Bacillus* species. While *Enterobacter* and *Bacillus* was not found in the gut samples obtained from the site Jhunsi Chatnag (Plates 1, 2, 3 & 4).

3.4. Bacteria isolated from the Brood and Soil samples of IFFCO Cordet and Jhunsi Chatnag sites

The bacteria identified from the brood and soil samples from both IFFCO Cordet and Jhunsi Chatnag were *Staphylococcus* and *Pseudomonas* while the soil samples from both the sites showed the presence of *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Enterococcus* and *Bacillus* species.

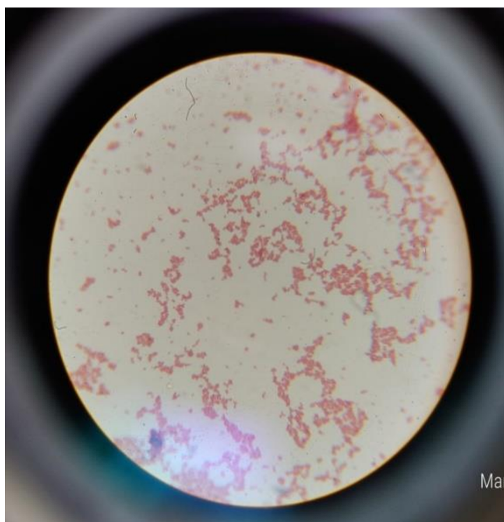


Plate 1: *Staphylococcus* sps.

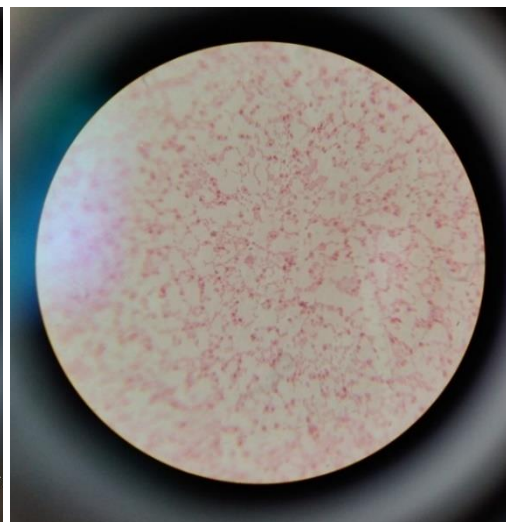


Plate 2: *Staphylococcus* sps.

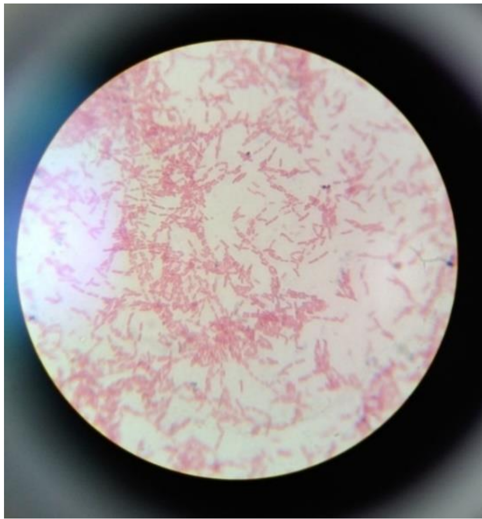


Plate 3: ***Bacillus* sps.**

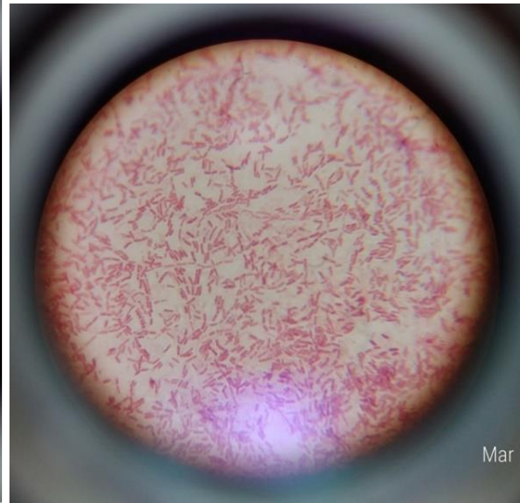


Plate 4: ***Enterobacter* sps.**

3.5. Significance of bacteria in the gut of honeybee

Results that showed the presence of *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Enterococcus* and *Bacillus* in the gut of honeybee are in concurrence with the findings of Rada et al., (1997). However, microbial counts were found to be significantly higher in the IFFCO sample when compared to the counts of Chatnag samples. Researchers have reported that there is a firm relationship between the gastrointestinal microflora balance and the health status of the host. (Douglas, 2011). Metabolic activities of microbiota are key for symbiotic interactions in the gastrointestinal tract and lays impact on health and disease of the host in various ways. Gut bacteria facilitate the breakdown of refractory and toxic dietary compounds (Brune, 2014; Engel and Moran 2013), produce metabolites that promote host growth, physiology and modulate immune functions in the gut (Smith et al., 2013) and other tissues (Trompette et al., 2014; Rooks and Garrett, 2016). Gut bacteria play significant roles in health, such as participating in metabolic activities, prevention of colonization by pathogens, and immunologic effects to defense against pathogenic bacteria (Wang et al, 2018).

Present findings report on the presence of *Bacillus* species in the guts of live bees and absence from the guts of dead honeybees. At the same time, reports have revealed that the occurrence of *Bacillus* in bee guts, can be directly associated with the increase in amylase that occurs in nectar in the foregut of bees. While, *Bacillus cereus* has been reported to have high potential to inhibit *Paenibacillus larvae*, the causative agent of American foulbrood (Evan and Armstrong, 2005; Yoshiyama and Kimura, 2009). It is also reported that *Bacillus* spp. administered to bee colonies increased the number of bees and honey storage, and reduced *Nosema* sp. and parasitic *Varroa* mites (Sabate et al., 2012).

Higher counts of these bacteria in gut might be due to its abundance in brood and soil. Further, various researches have mentioned that *Enterobacter* produce protease (Feder et al., 1998; Tondo et al., 2004) and lipase (Zhang et al., 2009) that may play roles in food digestion such as pollen grains for the honey bee, by using intine as a nutrient source. Moreover, it is also reported that bees survive exposure to pathogens by innate immune response of honey larva stimulated by non-pathogenic bacteria (Evans and Lopez, 2004).

It has been reported that *Enterococcus* is commonly found in honey bee colonies (Feizabadi et al., 2021; Elzeini et al., 2021; Audisio et al., 2005), isolated from the adults of *A. mellifera* (Carina Audisio et al., 2011) and gastrointestinal tract of *A. dorsata* (Tajabadi et al., 2011). This confirms the ability

of *Enterococcus* strains to persist in the intestinal tracts of different honeybee species. It has been reported that *E. faecium* is resistant to bile salts and the harsh conditions of the gastrointestinal tract. It shows auto-aggregation and adhesion ability, and produces a wide variety of bacteriocins called enterocins (Zommiti et al., 2018; Izquierdo et al., 2009). Results on the presence of *Enterococcus* in the soil samples are in concurrence with the reports that have already discussed, *Enterococcus* as being very resilient, can stay viable in different environments, and thus can be found in soil, sand, water, as well as on plants (Gaspar et al., 2009; Paulsen et al., 2003).

The role of *Pseudomonas* in the gut microbial communities of the solitary bees analyzed in this study is not well understood yet, but reports from Pampas region of Argentina suggests that the presence of *Pseudomonas* is correlated with the extensive use of glyphosate in the area from which bees were sampled. It is well known that *Pseudomonas* can catabolize this molecule and use it as additional carbon source (Zhao et al., 2015; Wang et al., 2020; Andriani et al., 2017) and, therefore, its abundance may be an adaptation to contaminated nectar. Indeed, a remarkable amount of honey samples resulted to be contaminated with glyphosate worldwide (Rubio et al., 2014; Medici et al., 2022). Recently, Motta (2020) in his findings have reported that glyphosate can perturb the gut microbiome of honey bees, but the perturbation might also be an adaptation to the xenobiotics. To confirm the role of insect gut bacteria in xenobiotic degradation, it was observed that in the wasp *Nasonia vitripennis* both the gut bacteria *Serratia* and *Pseudomonas* contributed to atrazine degradation, conferring resistance to wasp populations (Wang et al., 2020). These reports lead to the possibility that the bees from the apiaries might have acquired *Pseudomonas* while foraging.

At the same time researchers have also reported numerous causes of severe honey bee colony losses which includes pesticides toxicity, poor nutrition (Brodschneider and Crailsheim, 2010) and genetic diversity (Anjum et al., 2017). A high load of parasites and microbial pathogens, especially bacteria are strongly connected with the disappearance of bee populations at certain places (Core et al., 2012; Di Prisco et al., 2013; Olofsson and Vásquez, 2008). A wide range of bacteria including the species of *Pseudomonas aeruginosa* and *Staphylococcus aureus* may also affect honey bee colony, some are so harmful that they may lead to the colony collapse (Potts et al., 2010; Evans and Schwarz, 2011). *S. aureus* is also transported by infected bee which acts as a vector to other hive mate and 50% of the population was found dead within 24 hours (Ishii et al., 2014). Similar studies were carried out on *Drosophila melanogaster* infected with *P. aeruginosa* and same results were obtained (Linder et al., 2008). Therefore, presence of human pathogenic bacteria in bee gut can also be one of the main causes for bee mortality and decline in bee population. Moreover, reports claim that *Staphylococcus* bacteria are capable of producing skin infection and necrosis that may have profound health negative effect if comes in contact with humans as well (Kujumgiev et al., 1999; Kwong and Moran, 2016). The possible routes of bacterial contamination in honey bee and its by-products are human, hive tools, sugar feeders, wind and dust. Beekeepers skin infections, fecal contamination and sneezing can introduce pathogenic microbes into the hive environment (Anjum et al., 2018). Thus, on the basis of above findings, characterization of bee gut microbiome can provide valuable insight on various beneficial and harmful bacteria. These bacteria might have specific properties that can be extracted at industrial level and exploited for the betterment of the society.

4. Conclusion

The samples were collected from two sites, IFFCO and Chatnag and the common bacteria found in all the samples were *Staphylococcus, Enterobacter, Pseudomonas, Bacillus,* and *Enterococcus*, while the dead

bees did not report for the presence of *Enterobacter* and *Bacillus*. It was concluded that *Bacillus, Enterobacter,* and *Enterococcus* are reported to have positive symbiotic relationship with *Apis mellifera* and at the same time, present findings report for the absence of *Enterobacter* and *Bacillus* in the gut of dead bees. Moreover, the beneficial role of *Pseudomonas* has not clearly defined yet, though some reports do talk on their role in the gut as xenobiotic degradation process. At the same time, it was found that the species of *Pseudomonas aeruginosa* and *Staphylococcus aureus* can be so harmful that they may lead to the colony collapse. Our findings report on the presence of both *Pseudomonas* and *Staphylococcus* in the dead bee samples collected from both the sites respectively. More broadly, our findings also emphasizes that the possible route of bacterial contamination in honeybee gut are via its food, surrounding, wind and dust etc. therefore bee guts can act as a carrier of opportunistic bacterial pathogens. Significant difference in bacterial counts from both the sites might be due to difference in environment, its pollution and by human influence around the apiaries. The outcome reviewed here is the first to report and provide the possibility to understand the significance and relationship of gut bacteria with its host, *Apis mellifera* in the Prayagraj region of Uttar Pradesh India. Thus, the above findings promote for further specific studies on gut microbiota, molecular identification and characterization of species which will provide concrete information on the various microbes inhabiting the gut, where various negative and positive interactions can be explored and their properties can still be further exploited at industrial level for the benefit of the society.

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