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CHARACTERISATION OF THE GUT BACTERIOME OF HILL AND PLAIN RACE OF INDIAN HONEY BEE *APIS CERANA* FABRICIUS

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ABSTRACT

Microbial communities are virtually present in every site of the body of the animals, but the ones associated with the gastrointestinal tract are home to a vast majority of microorganisms and are of importance due to their diverse impacts on animal health. The guts of the honey bees also consist of a distinctive microbiome and traditionally culturing technique was used for the exploration of their gut bacteriome. However, this did not give us a complete picture of the bacterial community. Recently, a more complete and precise picture of the potential bacteria can be explored using next-generation sequencing. The honey bee gut bacteriome is an essential aspect of bee health and this study is aimed at the microbiome of the bee gut by targeting the V1-V9 hyper variable region of the 16S rRNA gene with Nanopore sequencing using adult worker bees was performed from the plain and hill regions of Coimbatore to understand the bacteriome variations. A total of 3, 88,947 reads were obtained revealing five phyla and the gut of the bees was found to be dominated by the Proteobacteria. In addition to the metagenomics approach, the traditional method of isolating bacterial species using the culturing techniques was also done and a BLAST search was performed for the identification of the cultural isolates using the universal bacterial primers. The number of unique OTUs for hill and plain races were 30 and 9, respectively, and 9 OTUs were common to both the races. In hill race, Actinobacteria was unique and in plain race, Bacteriodota abundance was more. Bacteriome profile variation was also observed in plain and hill honey bee races mostly at the species and genus level.

Key words: Indian honey bees, *Apis* spp. colour morphs, gut bacteriome, 16S rRNA gene, V1-V9 metagenomic sequencing, Operational Taxonomical Units (OTUs),

In India, crop production is an important aspect of the economic sector with agricultural yield contributing significantly to achieve food security, reducing the poverty in the country, the generation of employment, economic growth and environmental sustainability. Honey bees are the primary source of pollination (Klein et al., 2007) and majority of the food consumption for humans is dependent on bee pollination. Honey bees, the flagship pollinator species, are social insects (Gilliam, 1997) and live in a community (Miroslava, 2019). The taxonomic classification of honey bees is Arthropod phylum, Insecta class, order Hymenoptera and family Apidae. Honey bees yield honey and a number of other by-products including beeswax, propolis, royal jelly and honeycomb.

In Tamil Nadu, four honey bee species of *Apis* genera are well recognized. These include-*Apis cerana* (the Indian honey bee); *Apis dorsata* (the giant honey bee); *Apis florea* (the dwarf honey bee); and *Apis mellifera* (the western honey bee). Besides, many species of stingless bees are also utilized for gathering honey on a smaller scale. Among these, *A. cerana* is economically reared to get honey and other products. *A. cerana* is found at varying altitudes with appropriate flora and climate. In South India, based on morphological features, two "races" of *A. cerana* are identified: a black 'Hill' morph, that is often said to live at a higher elevation and a yellow "plain" morph found at lower elevations (Shashidhar et al., 2013; Fakrudin et al., 2013). The black and yellow morphs of *A. cerana* are reported from Kerala, Karnataka, Tamil Nadu and Andhra Pradesh (Shashidhar et al., 2013). The population from Karnataka also showed closer

relatedness with the bees from Assam and Jammu. Honey bees from mountainous zones were darker in color than those collected from sub mountainous zone. It was reported that the bees from higher altitudes and cold temperate regions were markedly darker than bees from lower altitudes and warm subtropical regions. Various studies confirmed that the morphometric features of *A. cerana* honey bees showed a wide range of variation in measurements of size, and colour pattern but they are not reproductively isolated/ genetically distinct (Shashidhar et al., 2013; Fakrudin et al., 2013).

The importance of gut-dwelling microbial communities in the health of animals, from humans to insects, has become widely appreciated (Moran, 2015). Honey bees also share a diverse microbiome with different bacterial taxa, ranging from gram-positive bacteria to alpha-, beta-, and gamma-proteobacteria (Gilliam, 1997; Jeyaprakash et al., 2003). Initially studying the microbes was possible only in laboratory environments, where the traditional culture-based methods gave an incomplete picture of the microbial communities. Recently the non-laboratory techniques have now become much more powerful and these approaches based on DNA sequencing have enabled more reliable results. The new methods have revolutionized our understanding of microbial ecology and provide insights on the dynamics of the communities and their potential effects to the organism (Moran, 2015; Zheng et al., 2018).

The 16S rRNA sequence has been immensely exploited to distinguish the strains based on the polymorphism within the gene. Recently, with the availability of the next-generation sequencing approaches, full-length gene sequencing is possible. Although the hyper variable region V3-V4 of the genome is widely being sequenced, the full-length gene sequencing from the first to the last hyper variable region would provide better coverage of more bacterial populations (Johnson et al., 2019). Although representative genomes of *A. cerana* micro-biota have been sequenced, considerable variation between strains, indicating the existence of large pan genomes within each microbial species that encode a diversity of genes and functionalities was documented (Engel et al., 2015; Zheng et al., 2018). The aim of this study is to document the bacteriome of the *A. cerana* honey bees isolated from the gut of indigenous yellow and black races using culturable and non- culturable approaches (metagenomics sequencing).

MATERIALS AND METHODS

Healthy, live foraging worker honey bee samples of *Apis cerana* were collected from two locations of the Tamil Nadu state of India during the period of January, 2022 to March, 2022. One set of hill race bee samples were collected from the mountain ranges in Burliar (in Coonoor Ghat Road at an altitude of 830 metres) and the other set of bee samples were from the plains of Coimbatore (411m altitude) district of Tamil Nadu from the apiary situated at the Tamil Nadu Agricultural University. The whole alimentary canal of bee samples was aseptically dissected at the Molecular Ecology lab at the Centre for Plant Molecular Biology and Biotechnology, TNAU. The bee thorax was stabbed with a sterile needle tip and was pinned on wax. With the use of sterile micro forceps, the complete alimentary canal was gently pulled off in a single motion (Coleman et al., 2007). The dissected guts were transferred to PBS and immediately stored at −20°C for experimental studies (Anjum et al., 2018).

The bee gut samples (n = 5) were homogenised in 100 µl PBS using a micro pestle. Different dilutions (i.e., 1/10, 1/100, and 1/1,000) of this composite homogenate were made, and 10 µl aliquots each of the diluted samples were inoculated aerobically into three different media, namely, Nutrient agar (NA), De Man, Rogosa and Sharpe (MRS) agar and Luria Bertani (LB) agar and incubated for at 37°C for 18-24 hours. The bacterial colonies grown on the plates were enumerated and selected based on different colony morphologies. The distinctive colonies in master plates were repetitively sub-cultured to obtain pure bacterial colonies.

For the isolation of DNA, the CTAB method of extraction was utilised (Ellegard and Engel, 2019). The dissected guts of *A. cerana* bees were placed in 1.5 ml tubes containing 200 µl CTAB (lysis buffer) to homogenise the tissues using the micro pestle. The homogenised tissue was then placed in a water bath at 65°C for 45 min to 1 hour. The incubated samples are then centrifuged at 10,000 rpm for 10 min and the supernatant is placed in a new 1.5 ml tube. Next, chloroform: isoamyl alcohol (24:1) was added to the tube, thoroughly mixed by inversions and the next round of centrifugation was done at 12000 rpm for 12 min. The upper aqueous layer (supernatant) was removed and placed in a new 1.5 ml tube, with the addition of 300 µl isopropanol and 15 µl sodium acetate to it. The resultant mixture was stored overnight at -20°C. Next

day, the samples were centrifuged at 12000 rpm for 12 min and then the supernatant was removed carefully, leaving the pellet behind in the Eppendorf tube. The tubes were air dried until all traces of isopropanol are dried and the pellet was then dissolved in sterile water (50 µl) and stored at -20 °C for further use.

The 16S rRNA gene of all the isolates was amplified using the universal bacterial primers 8F and 1492R, which covered almost the full length of the gene. The PCR cycle consisted of an initial denaturation step at 94 °C for 2 min followed by 35 cycles of denaturation, annealing and extension at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, respectively. The final extension was kept for 10 min at 72°C. The PCR products were then separated on 1.5% agarose gel. The PCR bands were visualised under the UV-Trans illuminator and documented using documentation unit (UVITEC CAMBRIDGE). Amplicon size of 1.5 kb was obtained and the products were sent for Sanger sequencing to Biokart Pvt. Ltd., Chennai. The resultant sequences in the form of FASTA files for both the forward and reverse primers were used to perform homology search using the BLASTn programme at the National Centre for Biotechnology Information.

The ~1500 bp 16S rRNA gene comprises nine variable regions interspersed throughout the highly conserved 16S sequence. Using an *in-silico* dataset of sequences taken from public databases we show that commonly targeted 16S sub-regions, such as V3-V4, are unable to match the taxonomic accuracy achieved when sequencing the full 16S gene. Using long-read sequencing (V1-V9) of mock and in-vivo communities, it was demonstrated that it was possible to accurately resolve the divergent copies of the 16S gene that exist within the same genome (Johnson et al., 2019). In the present study, metagenomic sequencing (V1-V9) was done by Syngenome (OPC) Private Limited, Coimbatore. The DNA samples from the worker honey bees of both plain and hill races were used for metagenomic sequencing of 16S rRNA. Samples were subjected to both quantitative and qualitative analysis by nano drop method and agarose gel electrophoresis (1% agarose/ TAE), respectively. A total of 50 ng of good quality DNA was then used for nano pore library preparation for 16S rRNA gene amplification using V1-V9 specific primers and LongAmp (NEB) Taq 2X master mix. The expected amplicon length was ~1.5 kb which was confirmed by agarose (1%)/ EtBr gel electrophoresis. The PCR products were purified using 1.6x Ampure XP beads (Beckmann Coulter, USA).

A total of ~50 ng from each amplicon DNA was end-repaired (NEBnext ultra II end repair kit, New England Biolabs, MA, USA); cleaned up with 1x AmPure beads (Beckmann Coulter, USA). Barcoding adapter ligation (BCA) was performed with NEB blunt/TA ligase (New England Biolabs, MA, USA) and cleaned with 1x AmPure beads. Qubit quantified adapter ligated DNA samples were barcoded using PCR reaction (LongAmpTaq 2x New England Biolabs, MA, USA) and cleaned up with 1.6x AmPure beads (Beckmann-Coulter, USA). Qubit quantified barcode ligated DNA samples were pooled at equimolar concentration and end-repair was performed using NEBnext ultra II end repair kit (New England Biolabs, MA, USA). End-repaired DNA was cleaned up with 1x AmPure beads. Adapter ligation (AMX) was performed for 15 minutes using NEB blunt/ TA ligase (New England Biolabs, MA, USA). Library mix was cleaned up using Ampure beads and finally eluted in 15 µl of elution buffer. Sequencing was performed on MinION platform (Oxford Nano pore Technologies, Oxford, UK located at Syngenome Technologies, Chennai) using SpotON flow cell R9.4 (FLO-MIN106) in 8h sequencing protocol on MinKNOW (version 22.03.2, ONT) with live base calling enabled with default parameters. Nanopore raw reads ('fast5' format) were base-called ('fastq' format) and de-multiplexed using Guppy v6.0.6.

RESULTS AND DISCUSSION

Due to the importance of the bacteriome in the host health and development (Gilliam, 1997; Vojvodic et al., 2013; Moran, 2015; Zheng et al., 2018; Wu et al., 2021), this study analysed the gut bacteriome of plain and hill races of the Indian honey bees (foraging worker bees). One set of samples were the domesticated bees in the multiflora vegetation and the other set of samples were collected from the mountain ranges located near Coimbatore. Based on the colony morphology and characteristics, bacterial isolates underwent 16S rRNA gene amplification followed by sequencing. Consequently, the obtained sequences were analysed through BLAST for identification of the species (Wu et al., 2014; Anjum et al., 2018; Miroslava, 2019). In agreement with the previous studies (Disayathanoowat et al., 2012; Mathialagan et al., 2018; Anjum et al., 2018), the major phyla contributed was Proteobacteria followed by Firmicutes. The most probable genera isolated by a culturing approach were *Apibacter, Citrobacter, Lactobacillus* and *Enterobacter* in the honey bees. Apart from these other genera reported

were *Pseudomonas, Streptococcus, Lactococcus* and *Enterococcus* (Table 1). Prevalence of *Pseudomonas* has been observed in the Hill race honey bees gut microflora, which was found to have an important role in the spread of plant pathogens which causes plant diseases (Pattemore et al., 2014; Anjum et al., 2021). In addition, reports on the whole microbiome also dictate the same observation, where *Pseudomonas* SPS is being harbored by the carpenter bees (Subotic et al., 2019) and wild bees (Shell and Rehan, 2022).

The DNA was isolated from the guts of the 20 worker bees. The DNA isolates were subjected to 16S rRNA V1-V9 region sequencing. A total of 3,88,947 reads were generated from the nano pore sequencing after quality filtration with the average length of the reads for the plainer race was 1432bp and for the hill race was 1466 bp. The processed reads were analysed for taxonomic classification using the QIIME2 (version QIIME2-2021) and the number of OTUs revealed were 48 with 97% identity at species level.

The gut microbiota was relatively constant across the two geographical regions. Four major phyla of the bacteria, including Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were detected in both plainer and hill race worker bee samples. However, Gemmatinodota and Cyanobacteria phylum were also reported to a small extent. Amongst these, proteobacteria accounted for about 60% in the hill race and up to 80% of relative abundance in the plain race (Fig. 1). Proteobacteria was found to be the most dominant bacterial phylum reported in this study for both the culturable as well as the non culturable approaches. Actinobacteria was the second abundant with around 30% presence in the hill race but was not reported abundant in the plain samples, followed by Firmicutes

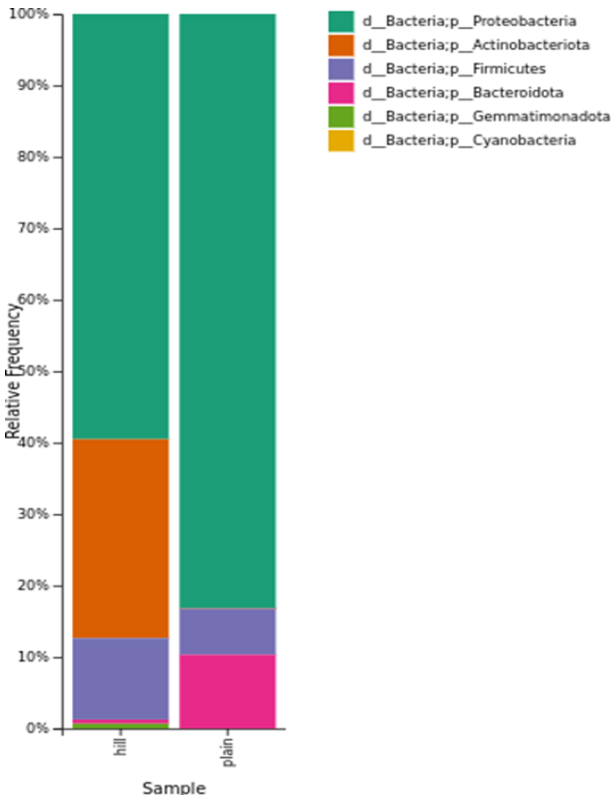


Fig. 1. The bacteriome classification at second level of taxonomy with Proteobacteria being the most dominant phyla reported in both hill and plain race followed by Actinobacteria, Firmicutes, Bacteroidota, Gemmatimonadota and Cyanobacteria phyla.

of around 10 to 15% and lastly Bacteroidetes with more active presence in the plain race as compared to hill race honey bees. Gemmatinodota and Cyanobacteria were reported very less. Though a change in proportion (was observed in the relative abundance of the various phyla between the present study and the previous research but the results were found in alignment with the former gut microbiome studies for honey bees (Disayathanoowat

Table 1. Characterisation of the culturable bacteria from the gut of the honey bees based on the Colony structure (elevation, texture, edge and colony colour) and 16S rRNA gene amplification.

Bee species	Caste and Pooled Compartment	Colony Morphology				16S rRNA gene
		Elevation	Colony texture	Edge	Colony Color	
A. cerana (Plain race)	Worker; Whole gut	Raised	Smooth, shinny	Round	Whitish	*Lactobacillus*
		Flat	Course	Rough	Creamy	*Enterococcus*
		Raised	Smooth	Round	Creamy	*Apibacter*
		Raised	Smooth	Round	Whitish	*Citrobacter*
A. cerana (Hill race)	Worker; Whole gut	Raised	Smooth	Round	Whitish	*Pseudomonas*
		Raised	Smooth	Round	Whitish	*Apibacter*
		Raised	Shiny	Round	Whitish	*Lactobacillus*

Table 2. Classification of the *A.cerana* gut bacteriome (using the metagenomic approach targeting the V1-V9 region of the 16S rRNA gene.)

Phylum	Class	Order	Family	Genus	Species
Proteobacteria	Gammaproteobacteria	Burkholderiales	Oxalobacteraceae	*Noviherbaspirillum*	*Gamma proteobacterium*
Actinobacteria	Alphaproteobacteria	Orbales	Neisseriaceae	*Massillia*	*Snodgrasella alvi*
Firmicutes	Actinobacteria	Pseudomonadales	Comamonadaceae	*Snodgrassela*	*Terrabacter tumescens*
Bacteroidota	Bacilli	Sphingomonadales	Burkholderiaceae	*Ramlibacter*	*Enterobacteriaceae*
Gemmatimonadota	Bacteroidota	Acetobacterales	Orbaceae	*Caenimonas*	*Spinghomonas spp.*
	Gemmatimonadetes	Caulobacterals	Moraxellaceae	*Variovorax*	*Phyciococcus ochagensis*
		Rhizobiales	Sphingomonadaceae	*Polynucleobacter*	*Ceanimones*
		Micrococcus	Acetobacteraceae	*Gilliamella*	*Massillia spp.*
		Lactobacillus	Caulobacteraceae	*Frischella*	*Bifidobacterium asteroids*
		Bacflavobacteriales	Beijerinckiaceaea	*Orbaceae*	*Arthrobacter spp.*
		Bacteroidetes	Intrasporangiaceae	*Acinetobacter*	*Acinetobacter spp.*
		Micrococceals	Micrococcaceae	*Sphingomonas*	*Enterobacter cloacae*
		Frentials	Bifidobacteriaceae	*Altererythrobacter*	*Apibacter mensalis*
		Proionibacterials	Microbacteriaceae	*Belnapia*	*Bifidobacterium indicum*
		Bifidobacterials	Lactobacillaceae	*Neokomagataea*	*Variovoraxparidoxus*
			Weeksellaceae	*Phenylobacterium*	*Klebsiella pneumoniae*
			Dysogononomedaceae	*Blastococcus*	*Gilliamella apicola*
			Gemmatinonadaceae	*Pseudoarthrobacter*	*Pseudoarthrobacter sulfonivorans*
				Physicoccus	*Lactobacillus hesingborgensis*
				Terrabacter	*Pedococcus dokdenensis*
				Arthrobacter	*Enterobacter rogenkampii*
				Lactobacillus	*Uncultured bacterium*
				Apibacter	
				Elizabethkingia	
				Dysgonomonas	
				Roseisolibacter	
				Gemmatimonas	

et al., 2012; Dong et al., 2020; Duong et al., 2020; Tola et al., 2020; Lombogia et al., 2020).

Amongst these, the most dominant family of bacterial diversification was reported to be the Oxalobacteraceae family in the hill race with around 30% of abundance and the plain samples were more dominated by the Orbaceae family with 80% abundance (Table 2; Fig. 2). The hill race samples showed more variation at family level as compared to plain race samples. The hill race also revealed Lactobacillaceae, Sphingomonadaceae, Intrasporagiaceae, Neisseriaceae families with good relative abundance. The plain race samples showed less abundance of families in the bacteriome with most dominance by Orbaceae and few relative abundances of Lactobacillaceae, Neisseriaceae and Weeksellaceae families. Eneterobaceae and Bifidobacteriaceae families were also in the list with more presence in the hill race honey bees. The presence of low Biforbacteriaceae and Lactobacillaceae was speculated to point towards the presence of pathogens in the gut (Lombogia et al., 2020).

The phylum Proteobacteria dominated both at the genus and the species level (Fig. 3). At the genus level within Proteobacteria, for the hill race of honey bees, a good number of genera are reported with no clear dominant members. The present study reported the *Noviherbaspirillum, Sphingomonas, Snodgressela,* and *Masillia*, *Gilliamella* dominated the most with about 80% abundance in the plain race. The phylum Firmicutes was dominated by the *Lactobacillus* genus, with these genera being the second highest at the genus level of diversification. The presence of lactic acid bacteria as *Lactobacillus* species has been reported to produce compounds that are antimicrobial in nature.

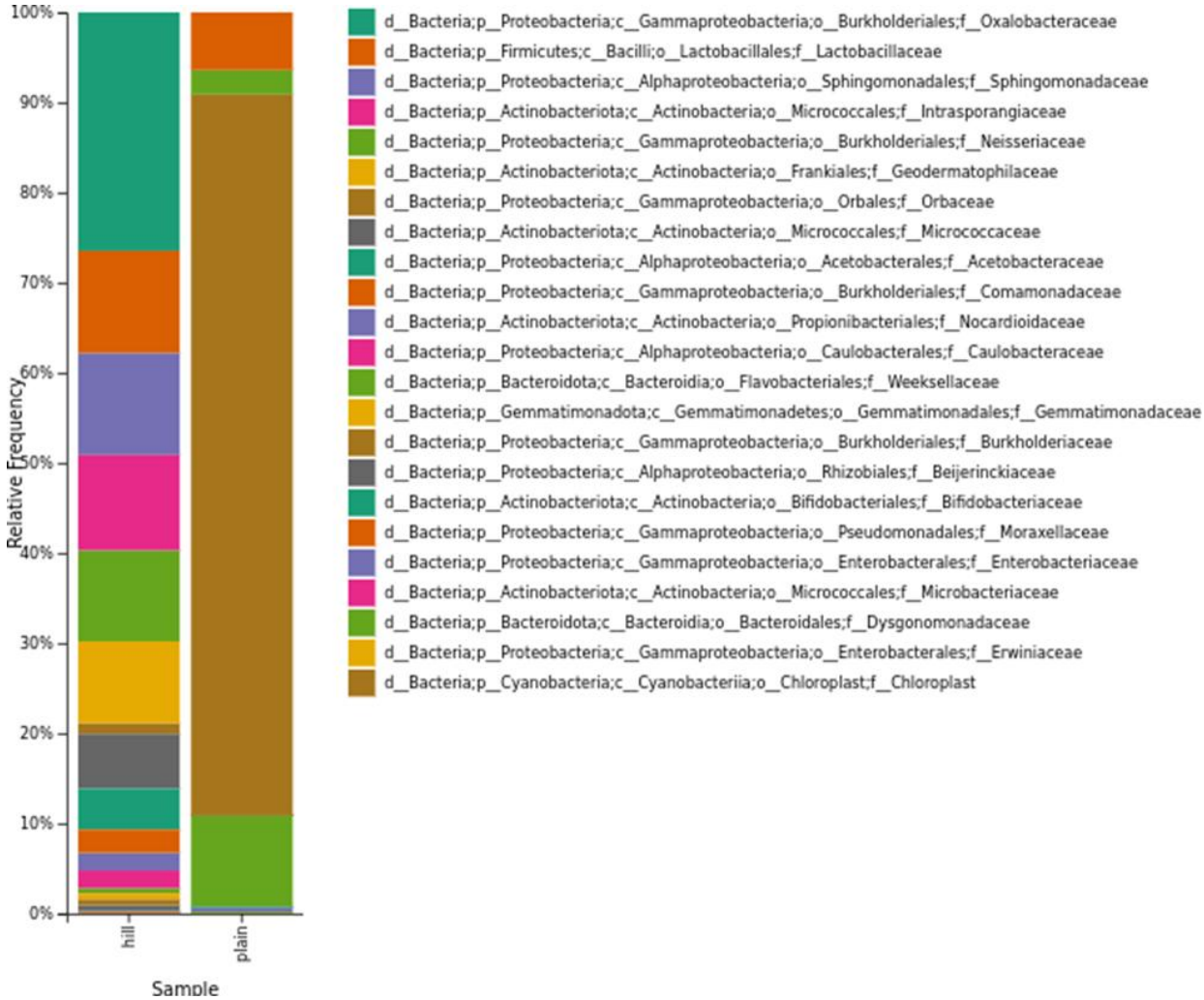


Fig. 2. The bacterial composition in the gut of *Apis cerena* at the genus level with highest abundance of *Oxalobacteraceae* and *Orbaceae* in the hill and plain race honey bees respectively

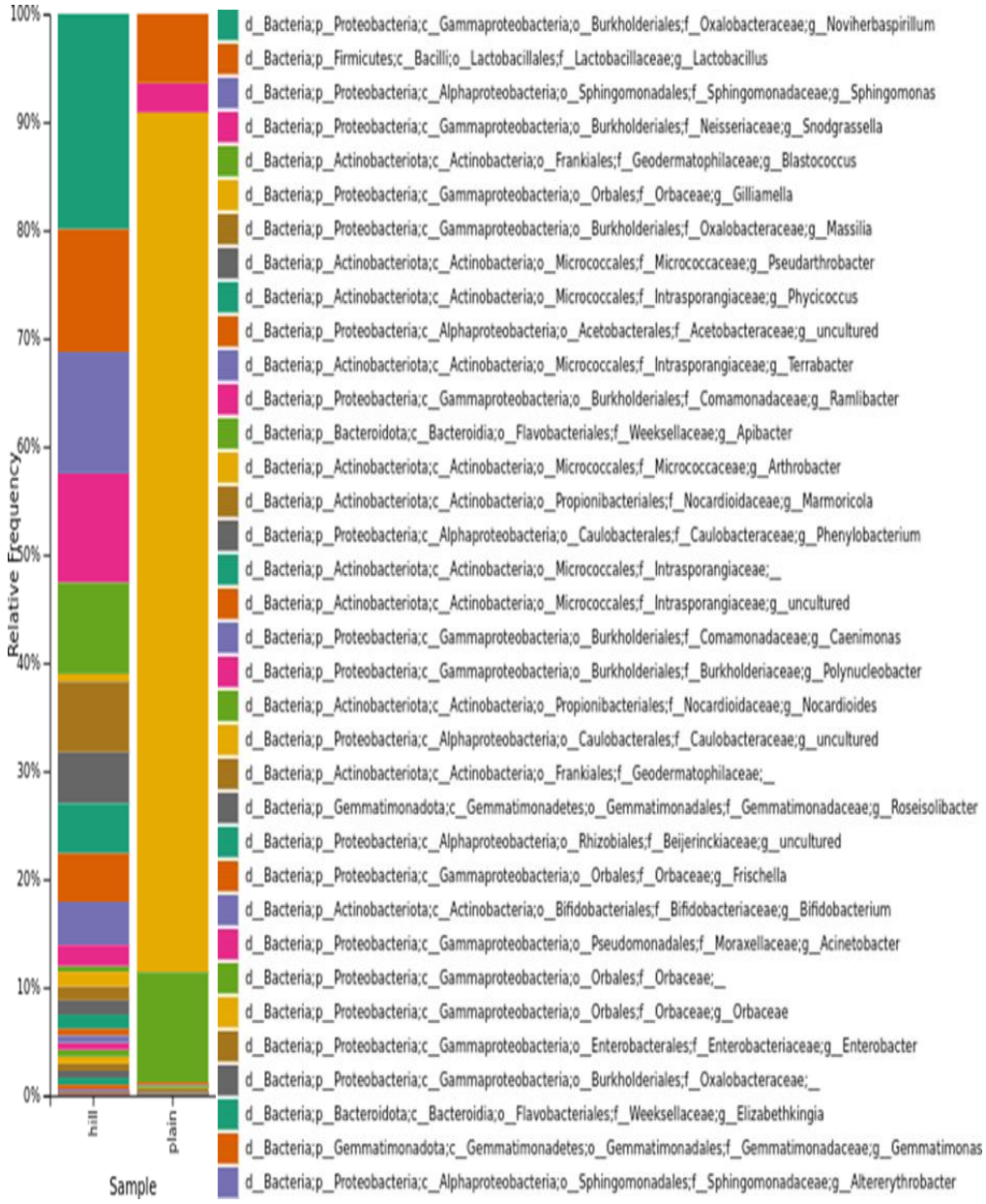
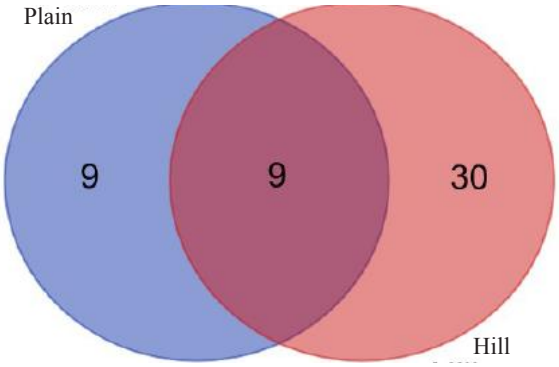


Fig. 3. The composition of the gut bacteriome. The microbiome was dominated by *Noviherbaspirillum* in the hill race and by *Gilliamella* in the plain race honeybees.

The indication of the presence of *Lactobacillus* in the present study is an indication of the usage of these as probiotics in the gut of the honey bees (Lombogia et al., 2020). The abundance of such probiotics can be used as an important aspect to bee health (Lombogia et al., 2020). For the Actinobacteria phylum, the most occurring genera were constituted by *Blastococcus* for the hill race. Apart from *Gilliamella*, the plain race bees also reported *Lactobacillus, Snodgressela* and *Blastococcus* to some extent. The number of OTUs (Operational Taxonomical Units) determined in our study at the genus level for the bacterial composition has been displayed in Fig. 4. A total of 48 OTUs were determined, nine unified OTUs belonging to the plain race bees and 30 OTUs were from the hill race honey bees. Common OTUs for both the races were only nine in number which were part of both the races. More distinctive level of variation was observed in the hill race honey bees as compared to the plain race. The alpha diversity in terms of the OTUs was determined using the Shannon vector algorithms. Generally, the Shannon index ranges from 1.0 to 3.5 (Lombogia et al., 2020) and for our study the Shannon entropy for Hill samples was 2.8 and 1.2 for Plain race honey bees



Hill race	OTUs	Plain race	OTUs
Noviherbaspirillum	5119	*Enterobacter*	53
Sphingomonas	2933	*Salmonella*	2
Phycicoccus	1206	*Neokomagataea*	1
Massilia	1641	*Klebsiella*	11
Polynucleobacter	177	*Escherichia-Shigella*	1
Caenimonas	159	*Dysgonomonas*	25
Arthrobacter	359	*Variovorax*	1
Terrabacter	1059	*Pantoea*	1

Fig. 4. Unified OTUs reported in the hill and plain race honey bees at the genus level from Coimbatore, Tamil Nadu

(Fig. 5). These results showed good diversity in the Hill race bees but a low microbiome diversity in the plain race honey bees. As the value of OTUs increases, the distribution of the individuals in the taxa becomes more even (Lombogia et al., 2020).

The core members of the bee gut microbiome observed have been highlighted herein and these are conserved across the different geographies. Proteobacteria was the most dominated phylum reported in the present work, along with Firmicutes, Actinobacteria and Bacteroidetes. The community structure in the gut indicates the possible insight of how these bacteria affect the host health. This study concludes that the Hill race has a diverse nectar and pollen source from a wide array of bee pasturage which is absolutely lacking in the plain race of honey bee within the city campus of Coimbatore. So, the hill and plain race are not distinct but the food source makes the difference. Present results highlight the importance of future studies on honey bees in India to harness the potential of this microbiome for improving bee health. More studies will contribute to a better understanding of the role of single members to the health of the host. A better understanding of the indigenous bee can also help with combating the global issues such as the decline in the bee population and the enhancement of pollination services. Honey bees can be used as good tractable model systems that can offer numerous parallels to the human gut microbiota.

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AUTHOR CONTRIBUTION STATEMENT

S. Mohankumar, N Saranya conceived and

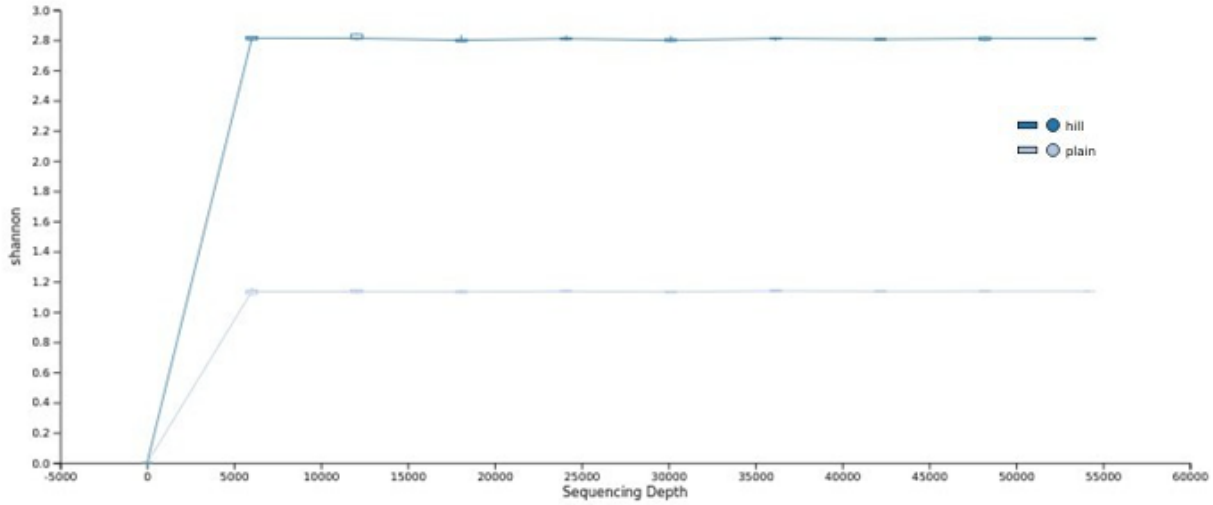

Fig. 5. Shannon vector Algorithm for hill and plain race at 2.8 and 1.1 index respectively displaying good bacterial diversity for hill race honey bees as compared to plain race bees

designed the research. Akanksha Thakur conducted the experiments and wrote the manuscript. S. Nakkeeran, M R Srinivasan and S. Subramaniam contributed to the samples and analysed the results. All authors read and approved the manuscript.

CONFLICT OF INTEREST

Authors have declared no competing interests.

REFERENCES

Anjum S I, Aldakheel F, Shah A H, Khan S, Ullah A, Hussain R, Mohammed O B. 2021. Honey bee gut an unexpected niche of human pathogen. Journal of King Saud University-Science 33(1): 101247.

Anjum S I, Shah A H, Aurungzeb M, Kori J, Azim M K, Ansari M J, Bin L. 2018. Characterization of gut bacterial flora of Apismellifera from north-west Pakistan. Saudi Journal of Biological Sciences 25(2): 388-392.

Coleman J, Juhn J, James A A. 2007. Dissection of midgut and salivary glands from *Ae. aegypti* mosquitoes. Journal of Visualized Experiments 5: 228.

Disayathanoowat T, Yoshiyama M, Kimura K, Chantawannakul P. 2012. Isolation and characterization of bacteria from the midgut of the Asian honey bee (*Apisceranaindica*). Journal of Apicultural Research 51(14): 312-319.

Dong Z X, Li H L, Chen H F, Wang F, Deng X U, Lin L B, Zhang Q L, Li J L, Guo J. 2020. Colonization of the gut microbiota of honey bee *(Apismellifera*) workers at different developmental stages. Microbiological Research 231: 126370.

Duong B T T, Lien N T K, Thu H T, Hoa N T, Lanh N T, Yun B. Yoo M, Cho Y S. 2020. Investigation of the gut microbiome of Apiscerana honey bees from Vietnam. Biotechnology Letters 42(11): 2309-2317.

Ellegaard K M, Engel P. 2019. Genomic diversity landscape of the honey bee gut microbiota. Nature Communications 10(1): 1-13.

Engel P, Bartlett K D, Moran N A. 2015. The bacterium *Frischella perrara* causes scab formation in the gut of its honeybee host. mBio6 (3): e00193-15.

Fakrudin B, Omkar Babu K, Abid Yerimani, Mohankumar S, Lazar KV, Chapalkar S, Shashidhar Viraktamath, Vastrad A S. 2013. Genetic diversity and phylogeography of *Apis cerana* Fabricius. Morphometry and phylogeography of honey bees and stingless bees in India. Department of Agricultural Entomology, University of Agricultural Sciences, Dharwad, Karnataka. pp. 124-157.

Gilliam M.1997. Identification and roles of non-pathogenic microflora associated with honey bees. FEMS Microbiology Letters 155 (1): 1–10.

Jeyaprakash A, Hoy M A, Allsopp M H. 2003. Bacterial diversity in worker adults of *Apis mellifera capensis* and *Apis mellifera scutellata* (Insecta: Hymenoptera) assessed using 16S rRNA sequences. Journal of Invertebrate Pathology 84 (2): 96-103.

Johnson J S, Spakowicz D J, Hong B Y, Peterson L M, Demkovicz P, Chen L, Leopold S R, Hanson B M, Agresta H O, Gerstein M, Sodergren E, Weinstock G M. 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature Communications 10(1): 1-11.

Klein A M, Vaissière B E, Cane J H, Dewenter S I, Cunningham S A, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274(1608): 303-313.

Lombogia C A, Tulung M, Posangi J, Tallie T E. 2020. Bacterial composition, Community structure and diversity in *Apis nigrotiana* gut. International Journal of Microbiology 2020 (6906921): 1-8.

Mathialagan M, Edward Y S J T, David P M M, Senthilkumar M, Srinivasan M R, Mohankumar S. 2018. Isolation, characterization and identification of Probiotic Lactic Bacteria (LAB) from Honeybees. International Journal of Current Microbiology and Applied Sciences 7(4): 894-906.

Miroslava K. 2019. Antagonistic effect of gut microbiota of honeybee (*Apis mellifera*) against causative agent for American foulbrood *Paenibacillus* larvae. Journal of Microbiology, Biotechnology and Food Sciences 9 (special issue): 478-481.

Moran N A. 2015. Genomics of the honey bee microbiome. Current Opinion in Insect Science 1: 22-28.

Pattemore D E, Goodwin R M, McBrydie H M, Hoyte S M, Vanneste J L. 2014. Evidence of the role of honey bees (*Apis mellifera*) as vectors of the bacterial plant pathogen *Pseudomonas syringae*. Australasian Plant Pathology 43(5): 571-575.

Shashidhar V, Abrol D P, Vastrad A S, Bhaktibhavana R, Muthuraman M, Devanesan S, Solomon Raju A J. 2013. Morphometry of Indian honey bee, *Apis cerana* Fabricius. Morphometry and phylogeography of honey bees and stingless bees in India. Department of Agricultural Entomology, University of Agricultural Sciences, Dharwad, Karnataka. pp. 26-46.

Shell WA, Rehan SM. 2022. Comparative metagenomics reveals expanded insights into intra- and interspecific variation among wild bee microbiomes. Communication Biology 5: 603.

Subotic S, Boddicker AM, Nguyen VM, Rivers J, Briles CE, Mosier AC. 2019. Honey bee microbiome associated with different hive and sample types over a honey production season. PLoS ONE 14(11): e0223834.

Tola Y H, Waweru J W, Hurst G D D, Slippers B, Paredes J C. 2020. Characterization of the Kenyan honey bee (*Apis mellifera*) gut microbiota: A first look at tropical and sub-Saharan African bee associated microbiomes. Microorganisms 8(11):1721.

Vojvodic S, Rehan S M, Anderson K E. 2013. Microbial gut diversity of Africanized and European honey bee larval instars. PLoS One 8(8): e72106.

Wu J, Lang H, Mu X, Zhang Z, Su Q, Hu Z, Zhang H. 2021. Honey bee genetics shape the strain-level structure of gut microbiota in social transmission. Microbiome 9: 225.

Wu M, Sugimura Y, Iwata K, Takaya N, Takamatsu D, Kobayashi M, Taylor D, Kimura K, Yoshiyama M. 2014. Inhibitory effect of gut bacteria from the Japanese honey bee, *Apis cerana japonica*, against *Melissococcus plutonius*, the causal agent of European foulbrood disease. Journal of Insect Science 14(1): 129.

Zheng H, Steele M I, Leonard S P, Motta E V, Moran N A. 2018. Honey bees as models for gut microbiota research. Lab Animal 47(11): 317-325.

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