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Influence of elevation in structuring the gut bacterial communities of *Apis cerana* Fab

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Abstract
Apis cerana F., a native honey bee of India, is an important crop pollinator and also managed for honey production and other bee products. In present study, 13 population samples were collected from different agro climatic regions of South India, with varied elevation ranging from 1 to 2268 m Mean Sea Level (MSL). The research work was carried out at the Division of
Entomology and Nematology, ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru during 2014-16 to understand the influence of habitat elevation on the gut colonizing bacterial communities of *A. cerana*. By culturing and 16S rDNA sequencing the major bacterial isolates of the gut were identified. Forty six isolates of culturable bacteria belonging to phyla Proteobacteria and Firmicutes were identified. *Bacillus* sp. (Firmicutes) was predominant among higher elevation populations, while Proteobacteria (*Serratia* sp., *Klebsiella* sp. and *Enterobacter* sp.) was dominant bacterial phylotype in plain and coastal populations. From the results it was evident that variability existed in gut microbial communities among populations inhabiting different elevations. They give an insight into the gut associated microbiota niche in relation to elevation gradient, leading to honey bee health and their ecological adaptations, which contributes to conservation strategy.

Keywords: 16S rDNA, *Apis cerana*, gut colonizing bacteria, Proteobacteria, Firmicutes

1. Introduction
Indian honey bee, *Apis cerana* F. is a highly evolved social insect and a major pollinator of several economically important crops, distributed across varied environments [1]. The gut associated bacterial communities are critical for the health of many insect species. Gut microbiome of honey bee influence their physiology and behaviour [2]. The nutrient assimilation, removal of toxins, potentially in their defence against pathogens and immune response of bee are depending on their associated gut microbes [3-5]. There is growing appreciation that the gut microbial community (*i.e.* microbiota) may have played an important role in the evolution of host species [6]. Although the gut microbial composition is quite distinctive between host species there is also considerable variation within host species [7-10].
The adult worker bee of *Apis mellifera* hosts up to 109 bacterial isolates of eight abundant phylotypes making up to 95% of total bacteria, which appear to be specific to social bees [11]. The maintenance of this stable and distinct microbial community depends on the nutrition and social lifestyle, environment of these insects [5, 12-13]. Thirty five bacterial isolates were identified from Japanese honey bee, *A. cerana japonica* by culture based 16S rRNA method [14]. A plethora of diversity was seen including the phyla firmicutes, actinobacteria, and alpha, beta, and gamma proteobacteria [15]. The developmental stages of *A. cerana* also influences increased in colonization of gut microbes (*Bifidobacterium*, *S. alvi*, *G. apicola* and *Lactobacillus*) which can be measured effectively by quantitative PCR [16]. Changes in the composition of intestinal microbiota may be associated with indicators of an immunological challenge and may result in an increased health risk of mountaineers during exposure to very high altitude [17].
In nature *A. cerana* occurs in morphoforms viz., hill and plain strains distinct from each other predominantly in the colouration of the first three abdominal tergites, hill strain being black and that of plain being yellow [18]. The plain population has completely yellow morph, whereas the high elevation population has black or black with yellow patches [19]. Indian sub-continent is rich in bio-diversity due to varied ecological constraints and elevations from mean sea levels [18]. The gut flora diversity may also influence the adaptability of honey bee to the temperate and tropical climatic zones of India.

Previous studies on bacterial populations of honey bee gut symbionts have not examined the variations between altitudes. Geographic isolation and habitat variability affect symbiotic community structure. However, so far there have been no conclusive studies in respect to gut colonizing bacteria of different geographical populations from different altitudes. Hence a study was carried out to explore the gut colonizing bacterial community in *A. cerana* populations from different elevation gradient of South India using 16S rDNA molecular technique.

2. Materials and Methods

2.1 Insect collection

The forager bees were collected by net sweep method from 13 populations at different elevation, ranging from 1 to 2268 metre altitude (Cochin, Kanyakumari, Vijayarai, Thuraiyur, Peechi, Puthalapattu, Sangareddy, Ananthagiri, Gandhipuram, Hessaraghatta, Valparai, Kodaikanal and Ooty) during course of research 2014-16 and the samples were preserved at -20 °C for further study (Table 1). The research work was carried out at the Ddivision of Entomology and Nematology, ICAR-Indian Institute of Horticultural Research, Bengaluru, to understand the influence of elevation on gut colonizing bacterial communities of *A. cerana*. Populations were categorised into four groups based on altitudes (<100, 100-500, 500-1000 and >1000 metre Mean Sea Level) of their place of collection.

2.2 Gut isolation

The samples from -20 °C were removed, thawed and surface sterilized with 70% ethanol. The stored samples were mounted on wax plate and using the fine dissection scissors the entire gut region was detached carefully and transferred in to phosphate buffer (10mM) with pH 7.4.

2.3 Culturing and genomic DNA isolation of gut microbes

The entire gut region (10mm) (Fig. 1) was transferred to 1.5 ml microfuge tube containing 200µl of sterile water and crushed thoroughly using micro pestle. The crude extract was enriched by incubating at 37 °C with 10ml of nutrient broth overnight and 100 µl of each dilution (serial dilution of 10^3, 10^4 and 10^5) were spread plated on Nutrient Agar (NA) and Luria-Bertani (LB) agar medium in triplicate and incubated under 37 °C [17]. After 2 days of incubation the colonies were isolated morphologically and sub-cultured on NA plate. After 48 hours of incubation, the individual pure colony was picked and inoculated in screw cap culture tubes containing 10 ml Nutrient Broth (NB) and Luria-Bertani (LB) broth, incubated at 37 °C for 48 hours. After incubation period the cultures were centrifuged individually and the pellet was crushed in lysis buffer using micro pestle. The genomic DNA was isolated from the lysed cells by using MN NucleoSpin Tissue kit (Macherey-Negel Pvt. Ltd.) as per manufacturer guidelines.

2.4 16S rDNA PCR amplification

The conservative 16S rDNA region was amplified for the identification of bacterial isolates using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R primers (5'-ACGGTTACCTTGTTAGGACTT-3') [18]. The reaction mixture (25 µl) containing 1 µl of sample DNA, 1 µl of forward and reverse primers (10mM), 3 µl of 10X buffer with 15mM of $MgCl_2$, 1 µl of dNTPs (10mM), 18.5 µl of DEPC (Diethylpyrocarbonate) treated nuclease free water and 0.5 µl of 1U of Taq polymerase was used for the amplification by using the standard protocol (Initial denaturation at 95 °C for 4 min followed by denaturation step at 94 °C for 45 sec, annealing 51.5 °C for 45 sec and extention at 72 °C for 1 min for 36 cycles and final extention at 72 °C for 10 mins) in Thermal cycler (ProS Eppendorf India Limited, Chennai).

The amplified product was visualized in 1.5% agarose gel electrophoresis (50V for initial 10 mins and 100V for 2 hours 20 mins) and visualized under UV illuminator GelDoc system (Syngene G:Box, Bengaluru). The amplified product was purified using MN-NucleoSpin Gel and PCR cleanup kit (Macherey-Negel Pvt. Ltd.) and sequenced by Sanger method (Amnion Pvt. Ltd., Bengaluru).

2.5 Sequence analysis

A BLAST search algorithm was used to study the homology of the obtained 16S rDNA sequences (1339 bps) with the already available sequences at National Centre for Biotechnology Information (NCBI). Based on sequence comparison with GenBank, isolates were identified at genus and species level. The isolates exhibits >99% sequence homology with deposited sequence at NCBI.

2.6 Statistical analysis

Total of 14 isolates with 16 rDNA sequence length of 1339 bps were analysed in the present study and retrieved sequences were aligned in BioEdit v7.0.5.3 program using Clustal W. The aligned sequences were deposited in the GenBank with accession number KY411699 – KY411712. Phylogeny tree was constructed using MEGA v5.01 to obtain specific genetic distance by using best fit model Kimura 2 + Gamma (G) correction to construct the Neighbour-joining tree with the outgroup *Alcaligenes faecalis* with boostrap value for nodal support [19].

3. Results

Forty six isolates of culturable bacteria were identified from collected populations of *A. cerana* using 16S rDNA primers, which belonged to two phyla viz., Proteobacteria and Firmicutes. All the sequences obtained in the present study were BLAST searched at NCBI and aligned in BioEdit v7.0.5.3 program using Clustal W. The aligned sequences were deposited in the GenBank and obtained the accession numbers KY411699 – KY411712 (Table 2). Phylogeny tree was constructed using MEGA v5.01 to obtain specific genetic distance by using best fit model Kimura 2 + Gamma (G) correction to construct the Neighbour-joining tree with *Alcaligenes faecalis* (KF500593). From the phylogeny it was observed that all the *Bacillus* sp. were clustered together with separate clade which belongs to phyla Firmicutes, whereas *Serratia* sp., *Klebsiella* sp., *Pantoea* sp. and other *Enterobacter* sp. clustered into separate clade, which belongs to phyla γ-Proteobacteria (Fig. 2). *Acidovorax* sp. formed separate clade which belongs to β-Proteobacteria and an out-group *Alcaligenes faecalis* used to root the tree. Most of the high elevation populations had *Bacillus* sp. which belongs to Firmicutes while the plain region honey bee gut was dominated by *Enterobacter* sp. belonging to γ-Proteobacteria (Table 3). The higher elevation (>1000 meter MSL) population (Ooty, Kodaikanal and Valparai) carried predominantly *Bacillus* sp. viz., *Bacillus subtilis*, *B. aryabhattai*, *B. magaterium*, *B. cereus*, *B. odysseyi*, *B. pumilus* and *B. velezensis*. These isolates were gram positive bacterium belongs to phyla Firmicutes. Whereas moderate elevation populations (<1000 meter MSL) recorded gram negative isolates (*Serratia marcescens*, *Klebsiella* sp.,

Serratia nematodiphila and *Enterobacter* sp.). In moderate elevation (500 – 1000 meter MSL) population (Hessaraghatta, Ananthagiri and Sangareddy) carried predominantly gram negative bacterial isolates belongs to Phyla γ-Proteobacteria viz., *Klebsiella oxytoca, Serratia marcescens, Klebsiella variicola* and other unidentified *Enterobacter* sp. also the Hessaraghatta population hosts gram negative bacterium, *B. cereus* and *B. magaterium*.

In plain region (100 – 500 meter MSL) population (Gandhipuram and Puthalapattu) carried the unknown *Klebsiella* sp., *Serratia marcescens,* and other unknown *Enterobacter* sp. along with the Gandhipuram population also hosts *B. cereus* and other unknown *Bacillus* sp. The coastal region (<100 meter MSL) population (Peechi, Thuraiyur, Vijayarai, Kanyakumari and Cochin) hosts predominantly γ-Proteobacteria such as *Serratia nematodiphila, Serratia marcescens,* unknown *Klebsiella* sp. and other unknown *Enterobacter* sp. also the Kanyakumari population hosts unknown *Bacillus* sp. while, Peechi population carries *B. cereus*. *Pantoea* sp., a biocontrol agent isolated from Thuraiyur population. The pathogen *Paenibacillus thiaminolyticus* (firmicutes) from Vijayarai and *Acidovorax* sp. belongs to β-Proteobacteria from Ooty were isolated.

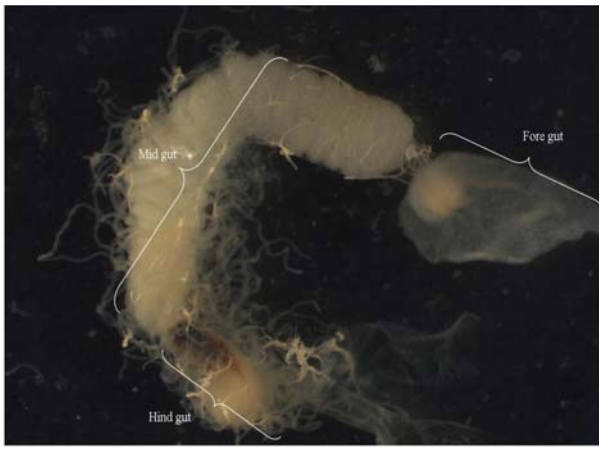

Fig 1: Gut specimen isolated from *A. cerana* (Forager)

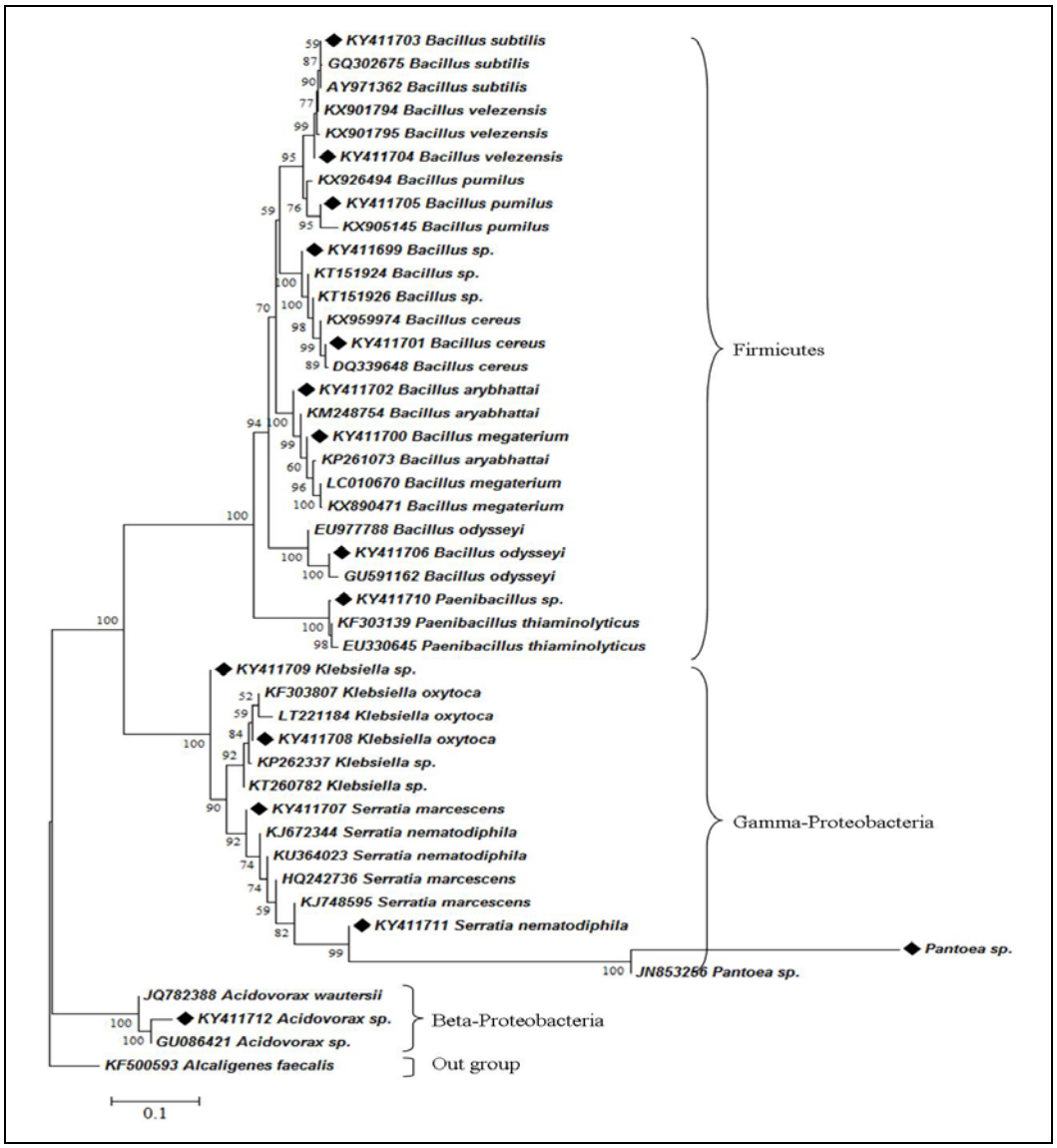

Fig 2: Phylogenetic tree based on the sequence of the 16S rDNA gene (Neighbour-joining analysis with Kimura 2-parameter model; Gamma Distributed (G); aligned 1339 bp) using MEGA 5.01. Beta- and gamma-Proteobacteria and Firmicutes bacterial phyla are clustered separately on the tree which is rooted with *Alcaligenes faecalis* (outgroup). The bootstrap support value expressed at nodes. The scale bar represents 0.1 changes per base.

Table 1: Details of sample collection

Place of collection	District	State	Altitude (m)	Latitude and Longitude	Habitat of sample collection
Ooty	Nilgiris	Tamilnadu	2268	11.41°N, 76.70°E	Weeds
Kodaikanal	Dindigul		2133	10.23°N, 77.48°E	Ornamental tree
Valparai	Coimbatore		1193	10.32°N, 76.95°E	Tea shop
Hessaraghatta	Bangalore	Karnataka	890	13.07° N, 77.03°E	Bee hive
Ananthagiri	Vikarabad	Telangana	638	17.31°N, 77.86°E	Bee hive
Sangareddy	Sangareddy		496	17.58°N, 78.06°E	Ornamental tree
Gandhipuram	Coimbatore	Tamilnadu	411	11.09°N, 76.97°E	Ornamental tree
Puthalapattu	Chittoor	Andhra Pradesh	330	13.38°N, 79.08°E	Mango tree
Peechi park	Thrissur	Kerala	100	10.53°N, 76.36°E	Ornamental tree
Thuraiyur	Tiruchirappalli	Tamilnadu	85	11.16°N, 78.62°E	Ornamental shrub
Vijayarai	West Godavari	Andhra Pradesh	16	16.53°N, 80.63°E	Bee hive
Kanyakumari	Kanyakumari	Tamilnadu	2	8.08°N, 77.55°E	Ornamental tree
Cochin	Ernakulam	Kerala	1	9.97°N, 76.28°E	Juice shop

Table 2: Accession numbers for bacterial isolates at NCBI

		NCBI		
Sl. No.	**Bacterial isolates**	**Accession number**	**Query coverage (%)**	**Identity/ Similarity (%)**
1	*Bacillus* sp.	KY411699	100	100
2	*Bacillus megaterium*	KY411700	100	100
3	*Bacillus cereus*	KY411701	100	100
4	*Bacillus aryabhattai*	KY411702	100	100
5	*Bacillus subtilis*	KY411703	100	100
6	*Bacillus velezensis*	KY411704	100	100
7	*Bacillus pumilus*	KY411705	100	99
8	*Bacillus odysseyi*	KY411706	100	100
9	*Serratia marcescens*	KY411707	100	99
10	*Klebsiella oxytoca*	KY411708	100	99
11	*Klebsiella* sp.	KY411709	100	100
12	*Paenibacillus* sp.	KY411710	99	99
13	*Serratia nematodiphila*	KY411711	100	100
14	*Acidovorax* sp.	KY411712	100	99

Table 3: Identified gut bacteria from populations of *Apis cerana* at different elevation

Sl. No.	Elevation (metre)	Place of sample collection	Identified culturable bacteria	Phylum/Class
1	>1000 MSL	Ooty	*Bacillus cereus*	Firmicutes
			Bacillus sp.	
			Acidovorax sp.	Proteobacteria
			Bacillus subtilis	Firmicutes
		Kodaikanal	*Bacillus cereus*	
			Bacillus subtilis	
			Bacillus pumilus	
			Bacillus aryabhattai	
			Bacillus odysseyi	
		Valparai	*Bacillus velezensis*	
			Bacillus aryabhattai	
			Bacillus subtilis	
			Bacillus magaterium	
			Bacillus cereus	
2	500 – 1000 MSL	Hessaraghatta	*Bacillus magaterium*	
			Bacillus cereus	
			Klebsiella sp.	γ-Proteobacteria
			Enterobacter sp.	
		Ananthagiri	*Klebsiella oxytoca*	
			Serratia marcescens	
			Enterobacter sp.	
		Sangareddy	*Serratia marcescens*	
			Klebsiella variicola	
			Klebsiella sp.	
			Enterobacter sp.	
3	100 – 500 MSL	Gandhipuram	*Bacillus cereus*	Firmicutes
			Bacillus sp.	
			Enterobacter sp.	
		Puthalapattu	*Klebsiella* sp.	γ-Proteobacteria
			Enterobacter sp.	
			Serratia marcescens	
4	<100 MSL	Peechi	*Bacillus cereus*	Firmicutes

			Klebsiella sp.	γ-Proteobacteria
			Enterobacter sp.	
			Serratia marcescens	
		Thuraiyur	*Serratia marcescens*	
			Enterobacter sp.	
			Pantoea sp.	
		Vijayarai	*Klebsiella* sp.	
			Enterobacter sp.	
			Serratia marcescens	
			Serratia nematodiphila	
			Paenibacillus thiaminolyticus	Proteobacteria
		Kanyakumari	*Serratia marcescens*	γ-Proteobacteria
			Enterobacter sp.	
			Bacillus sp.	Firmicutes
		Cochin	*Serratia marcescens*	γ-Proteobacteria
			Enterobacter sp.	

4. Discussion

Based on the 16S rDNA sequence obtained from each isolates which were BLAST searched at NCBI for sequence identity (>95%) and query coverage (>95%) (Table 2). Phylogenetic tree constructed using best fit model Kimura 2 + Gamma (G) correction to construct the Neighbour-joining tree. The gram positive bacterial isolates (*Bacillus subtilis*, *B. aryabhattai*, *B. magaterium*, *B. cereus*, *B. amyloliquefaciens*, *B. odysseyi*, *B. pumilus*, *B. subtilis*) dominated among higher elevation populations (>1000 meter MSL), namely Ooty, Kodaikanal and Valparai. Whereas plain populations (<1000 meter MSL) recorded gram negative isolates (*Serratia marcescens*, *Klebsiella* sp., *Serratia nematodiphila* and *Enterobacter* sp.). Similarly, previous study reported that change in altitude contribute in shaping gut microbiota [20]. Even though Hessarahgatta and Gandhipuram were moderately elevated regions (500 – 1000 metre MSL), *Bacillus* sp. was found predominantly. Though the elevation varies 1 to 2268 meters, foot mark of *Bacillus* sp. was observed in both coastal Kanyakumari and plain Gandhipuram, Hessaraghatta population. Because *bacillus*, common bacteria found in gut and plays essential role in digestion. Evident to this is reported by several researchers observed that both *Bacilli* and γ-Proteobacteria were most active classes in bee environment [10]. Literature revealed that, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus, B. megaterium*, *Brevibacillus laterosporus* as normal gut micro biome associated with honeybees [21]. Previous studies on *B. subtilis* have shown that surfactin, a lipopeptide produced by the bacterium, inhibited honey bee pathogens, including *P. larvae*, *Ascosphaera apis* and *Nosema ceranae* [22]. And also the commercial formulations based on *Bacillus* sp. preparations are used for the biocontrol of fungal and bacterial plant pathogens [23]. *Bacillus* isolates, showed promising antagonistic effect on *Paenibacillus*, a causative agent of American foulbrood disease [12]. The presence of *Paenibacillus* also recorded in Vijayarai, a costal population.

Serratia marcescens and other unidentified *Enterobacter* sp. was found predominantly in coastal region to moderate elevation (0-650 meter MSL) compared to higher elevation. From the present study, it clearly indicates that the abundance of the *Bacillus* sp. is directly proportional to altitude. Further, *Serratia marcescens*, *Klebsiella* sp. and other *Enterobacter* sp. were the foremost microbial isolate identified mostly from plain regions from Cochin, Thuraiyur, Kanyakumari, Vijayarai, Ananthagiri, Sangareddy, Peechi and Chittoor populations. Also *Serratia nematodiphila,* a microbial pathogen was isolated in Vijayarai population. Adult honey bees were the major carriers and dispersers of pathogen, *S. marcescens* [24, 25]. *Klebsellia oxytoca* (gram negative) which is present in the reproductive organs of insects was found in the gut of Ananthagiri, Sangareddy populations of honey bee [26, 27]. A similar bacterial phylotype *Klebsiella variicola*, was recorded from Sangareddy population, the bacteria plays an important role in digestion or cellulotytic activity in gut [28]. Presence of *Klebsiella* signifies its role in coping up with food source of high cellulose and high ambient temperatures which raises upto 42 °C.

Besides them *Pantoea* sp., *Paenibacillus thiaminolyticus* and *Acidovorax* sp. (pathogen) were recorded. Interestingly *Acidovorax* sp. which cause bacterial fruit blotch disease in cucurbit crops was recorded from Ooty population, which has been reported for the first time from the Asian sub-continent [29]. Further studies are required to know the role in gut and incidence of pathogen in Ooty. In Thuraiyur population, *Pantoea* sp. was identified but there is no clue about its role in gut. *Pantoea agglomerans*, which is a possible biocontrol agent against Fire Blight (*Erwinia amylovora*) [30]. Diet is considered to be one of most important environmental factors that influence the assembly of gut microbiota [4, 31-33]. Therefore presence of this bacterium throws light on the host species present in that ecosystem and the microbiota associated with it.

Also an interesting observation was made during the course of this study, wherein we found that the forager worker bees had a wider gut associated bacterial phylotype library in comparison to the hive and nurse bees, because the worker bee are involved in foraging of several habitats. This is mainly because the forager bees are majorly acquitted with food sourcing of pollen pat and nectar, which are the major store houses for microbial populations; hence it is definite to find a major section of such microbial phylotypes in the class of forager bees. Therefore, they are directly associated with wide host range and its microbiota at different altitudes [34]. Though the source of food habitat is similar in the collection sites, the prevalence of gut bacterial community differed. One of the factors altering the gut microgiota may be varying temperatures at different elevations. For example, dominants of *Serratia* sp., *Klebsiella* sp. and other *Enterobacter* sp. at coastal to higher elevation (<1000m MSL) in compared to prevalence of *Bacillus* sp. above 1000m MSL. This study gives an insight of diversity of microbiota is not only depends on the biotic factor (food source), also influenced by abiotic factor such as decrease in temperature increased in altitude. Our result supports the previous study of role of gut microbiome in bee health by elevation gradient as one of the factor [2].

Different geographic origins of honey bees may result in

diverse compositions of gut microbiota, due to distinctive life environments, genetic background and dietary habits. In this study the influence of abiotic factor (temperature) in compared to biotic factor (food source) reveals structuring of gut bacterial community in relation to elevation gradient. Solely diet may not be the reason behind the clear differences in the presence bacterial species in hill and plain region. *Bacillus* sp. was only present in the guts of the hill region populations and *Enterobacter* sp. in plain region. In contrary to this, Bangalore and Coimbatore populations contain *Bacillus* sp. which may be influenced by other abiotic factors mainly temperature at moderately higher altitude and the food sources prevailing in that region.

In the present study we identified a number of culture dominant bacterial phylotypes isolated from the gut of *A. cerana* at different altitudes of geographically located populations, it revealed that the diversity and richness of the gut associated bacteria in honey bees not only depends on sourcing of food habitat but also ecological adaptations and biotic and abiotic stress reported by centre for pollinator research, PennState, USA. Hence in the future course of time, similar studies to identify bacterial phylotype from not just culture based methods as performed in this study but also employing non culture-based techniques such as FISH (Fluorescence *in situ* hybridization), Metagenomics, and 454 pyrosequencing. This will help to explore and understand the influence of microbiota niche with respect to honey bee health and better understanding the functions of gut colonizing bacteria which contributes to conservation strategy.

5. Conclusions

The current study provides insights of the gut bacterial communities influenced by elevation gradient considering a remarkable factor which leads to honey bee health and conservation aspects. We also found significant differences of the gut microbiome in relation to altitude variation. Firmicutes (*Bacillus* sp.) contributes predominance in higher altitude compare to plain and costal (γ-Proteobacteria) *A. cerana* population, which gives insight to future studies on diversity of the gut microflora for bee health and eco-adaptability with respect to conservation strategy. Gut microbial diversity of *A. cerana* at different geographical populations varying altitudes and their relationship, which needs further studies.

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