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## Research Article

# Microbiota of Honeybees, *Apis mellifera adansonii* (Hymenoptera: Apidae) from Selected Ecozones, South West Nigeria

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## Abstract

**Background and Objectives:** Honeybees explore their environment in search of nectar and pollens including water whose sources are contaminated by variety of microbes. This has both negative and positive economic implications on health status of honeybees, the consumers of the products including the quality of the products. Hence, the study was designed to investigate the effect of geographical locations on the occurrence and diversity of microbiota on the external integument and the digestive gut of adult worker honeybees, *Apis mellifera adansonii*. **Materials and Methods:** The honeybee samples were sourced from Ondo, Ogun, Oyo and Osun of South West Nigeria. The samples were subjected to microbiological analyses using standard techniques. Two-ways ANOVA was used for statistical test of the data. **Results:** The microbiota of the studied samples includes *Corynebacterium kutscheri*, *Corynebacterium xerosis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Micrococcus luteus*, *Micrococcus varians* and *Staphylococcus aureus* which were gram-positive bacteria, while gram-negative bacteria include *Aeromonas veronii*, *Citrobacter diversus*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Fungi isolates include *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Rhizopus stolonifer*. Most microbiota were not cosmopolitan across selected ecozones of south west, Nigeria except for *Aspergillus fumigatus*. The number of identified species of microbiota either in the digestive guts or on the external integument of worker honeybees from selected ecozones varies from one ecozone to another. There was significant species diversity of microbiota on external integument than in digestive guts of adult workers of *A. mellifera adansonii* from selected ecozones in South West, Nigeria. **Conclusion:** The study showed that both the external integument and the digestive guts of *A. mellifera adansonii* exhibited variety of microbial flora providing research exploration opportunity for probable microbes of economic and geographical importance.

**Key words:** Digestive gut, external integument, honeybees, microbiota, diversity, *Lactobacillus* spp.

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**Competing Interest:** The author has declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Honeybees are of great economic and ecological importance as pollinators of many crops and wild plants<sup>1,2</sup>. Beeswax, honey, pollen, propolis and royal jelly are economic products from honeybees and the beehives. The hives products served as sources of economic empowerment to beekeepers globally in beekeeping regions including South West, Nigeria<sup>3</sup>. Honey is natural substance processed and produced by honeybees from either honeydew, pollens, nectar or from plants' exudation. Honeybees collect nectars and pollens, transform and combine with specific substances from their gut to produce ripe honey<sup>4</sup>. During gathering of pollens and nectars by honeybees within their ecological zones, they interact with the environment by probing for sources of food for survival not mindful of distance involved. Most, if not all, metazoans including honeybees are host to both or either beneficial and/or harmful microbes. This could be on body surfaces or in the digestive guts of such eukaryotic animals such as honeybees. Microbial communities associated with honeybees are acquired from foraging environment, through trophallaxis as social insects and the hive micro environment. Grubbs *et al.*<sup>5</sup> reported that, in addition to the microbial communities present in individual adult workers of honeybees, beehives have resident microbial communities associated with different colony components. These microbial communities reflect in the by-products of honeybees as contaminants. Identified sources of microbial contamination of honey include digestive guts of honeybees, nectars, pollens, dirt, dust, air and flowers during honeybees' interaction with their environment<sup>6,7</sup>. Honeybees, in desperation for food and water, visit human fecal materials (Field observation during severe harmattan seasons). Pollens and nectars of flowers which are part of the environment were contaminated by air borne microbes and fecal matters on plant vegetation. These are automatic sources of contamination of honey when gathered by honeybees during their foraging activities (Field observation). Bacteria associated with honeybees were reported to be widely distributed in the air, soil, water and honeybees' food and external integument<sup>2,8</sup>. The complexity of bacterial communities occurring in the digestive guts of the Japanese honeybees, *Apis cerana japonica* was studied using histological and 16S rRNA gene sequence analyses. It was reported that most of the antagonistic bacteria belonged to *Bacillus* species, suggesting that the bacterial isolates obtained in the study appeared to be potential candidates for the biological control of *Paenibacillus larvae*<sup>9</sup>.

Gram-positive bacteria such as *Bacillus*, *Streptococcus* and *Clostridium* species; gram-negative or gram variable

bacteria including *Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus* and *Pseudomonas* which were isolated and identified in the gut of honeybees were implicated to influence the growth, development, pathogenesis and environmental adaptation of the host insects<sup>10,11</sup>. The environment which includes water, plant vegetation and flowers visited by foraging honeybees were identified as major sources<sup>12,13</sup>. These were responsible for the existence of variety of microbial flora of honeybees (producers of hive products) which probably influenced the quality of honey and health status of both the honeybees and honey consumers. There are published reports on microbiota of honeybees and their impacts on the products and health status of honeybees in other regions excluding Nigeria. Nigeria has dearth of reliable information on microbiota of the honeybees and their products. Hence, there is need to study the microbiota of *A. mellifera adansonii* in South West Nigeria. The study will also provide basic preliminary information as data base on bio-diversity and occurrence of indigenous microbes of *A. mellifera adansonii* for economic uses with respect to fermentation process and bio-control of pests of food crops and health importance. The study compared the occurrence and diversity of microbiota on the external integument (EI) and digestive guts (DG) of adult workers of *A. mellifera adansonii* from different selected ecozones within South West, Nigeria.

## MATERIALS AND METHODS

**Duration of study:** The study was conducted from April, 2013 to July, 2015.

**Sources of adult workers of *A. mellifera adansonii* and aliquots preparation:** Samples of adult workers of *A. mellifera adansonii* were collected from apiaries in selected ecozones such as Ondo, Ogun, Oyo and Osun states respectively. Aliquots of the external integument (EI) of honeybees' samples were prepared using sterilized 200 mL conical flask which contained twenty honeybees with 20 mL of distilled water shake thrice for every 5 min with 10 min pause at a time. This process was repeated for other sample batches. For aliquots of digestive guts (DG), another twenty honeybees of each sample batch were dissected and their digestive guts (DG) were removed into sterilized crucible which contained 20 mL distilled water and crushed with mortar. Homogenized samples of the digestive gut (DG) were prepared by sequential dilution based on decimal dilution system application for microbiological analyses. This was

repeated for other batches of honeybee samples. Adult workers of *A. mellifera adansonii* were targeted for microbial analyses because they are the foragers, processors and nurses in the colony.

### Microbiological analysis of samples of adult workers of

***A. mellifera adansonii***: One milliliter of appropriate four-fold serial dilutions of the sample was inoculated unto nutrient agar, MacConkey agar, Mannitol Salt Agar, Eosin Methylene Blue Agar and Sabouraud Dextrose agar plates in triplicate using the spread plate methods. Inoculated plates were incubated at 35-37°C for 18-24 h for bacteria, while for molds, the inoculated plates of Sabouraud Dextrose agar were incubated at 20-25°C for 5 days. Visible and discrete colonies in incubated plates were counted. After counting, each distinct colony was picked with a sterile wire loop and streaked on Nutrient and Sabouraud Dextrose agar respectively. The plates were incubated at 35-37°C for 18-24 h for bacteria and at 20-25°C for 5 days for yeast and molds respectively. Discrete colonies were purified by repeated sub-culture and then inoculated unto appropriate agar slants and stored in the refrigerator at a temperature of 2-4°C for further tests such as morphological and biochemical characterization. The biochemical tests include catalase test, mannitol sugar fermentation test, starch agar test, anaerobic test, blood agar test, voges-proskauer test, 6.5% NaCl test, citrate utilization tests, coagulase test, sucrose test and deoxyribonuclease (DNase) test. This was repeated for other aliquots. The bacteria were identified according to Bergey's Manual of determinative Bacteriology<sup>14</sup>, while molds were identified using 'Atlas of clinical fungi'<sup>15</sup> and 'Introduction to food-borne fungi', Central Bureau of Fungi culture<sup>16</sup>.

**Determination of CFU/SFU counts on the external integument (EI) and in the Digestive guts (DG) of honeybees**: Plate dilution method was applied for colony forming unit/spore forming unit (CFU/SFU) counts determination of respective groups of microorganisms in 1 g of aliquot sample.

**Statistical analysis**: Data on occurrence and diversity of microbiota of *A. mellifera adansonii* were subjected to two ways analysis of variance at 5% significance level.

## RESULTS

Adult workers of *A. mellifera adansonii* harbored *Corynebacterium kutscheri*, *Corynebacterium xerosis*,

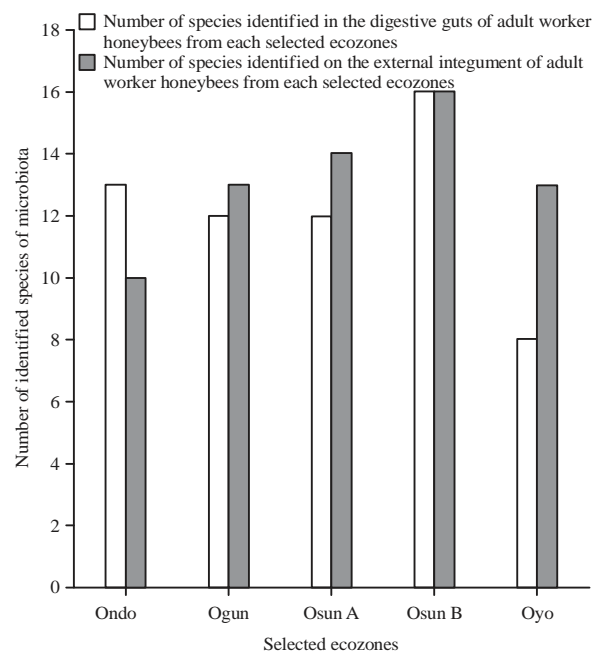


Fig. 1: Number of identified species of microbiota of adult workers of *A. mellifera adansonii* from selected ecozones, South West, Nigeria

*Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Micrococcus luteus*, *Micrococcus varians* and *Staphylococcus aureus* which were gram-positive bacteria, while gram-negative bacteria include *Aeromonas veronii*, *Citrobacter diversus*, *Citrobacter freundii*, *Klebsiella oxytoca*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. The fungi isolates include *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Rhizopus stolonifer* (Table 1). The Gram-positive bacteria such as *C. kutscheri*, *C. xerosis*, *L. bulgaricus*, *L. casei*, *L. fermentum*, *M. luteus* and *M. varians* were more associated with adult workers of *A. mellifera adansonii* in Osun state apart from *C. kutscheri* and *C. xerosis* in Ondo state. Ten species of microbiota exist on the external integument of adult workers of *A. mellifera adansonii* sourced from Ondo state, while Ogun, Osun A (Osogbo), Osun B (Gbongan) and Oyo states respectively exhibited 13, 14, 16 and 13 identified species of microbiota on the external integument of the studied honey bees. The digestive guts of adult workers of *A. mellifera adansonii* from Ondo, Ogun, Osun A (Osogbo), Osun B (Gbongan) and Oyo states harbored 13, 12, 12, 16 and 8 species of microbiota respectively. Adult workers of *A. mellifera adansonii* from Osun state has the highest number of species of microbiota (Fig. 1). There was significant species diversity of microbiota ( $F_{cal}: 6.21$ ;  $F_{tab(2, 7, 0.05): 4.73}$ ) on the external integument compared to that of the

Table 1: Occurrence and diversity of microbiota of adult workers of *A. mellifera adansonii* within South West, Nigeria

Locations	Ondo		Ogun		Osun A (Osogbo)		Osun B (Gbongan)		Oyo	
Categories of microorganisms	El	DG	El	DG	El	DG	El	DG	El	DG
<b>Gram-positive bacteria</b>										
<i>Corynebacterium kutscheri</i>	+	+	-	+	+	+	+	+	-	+
<i>Corynebacterium xerosis</i>	+	+	+	-	+	+	+	-	+	-
<i>Lactobacillus bulgaricus</i>	-	+	-	+	-	-	+	+	-	+
<i>Lactobacillus casei</i>	-	+	-	-	+	+	+	+	+	-
<i>Lactobacillus fermentum</i>	-	+	-	+	+	+	+	+	-	+
<i>Micrococcus luteus</i>	-	-	+	-	+	-	+	+	+	-
<i>Micrococcus varians</i>	+	-	+	-	+	-	+	+	+	-
<i>Staphylococcus aureus</i>	-	+	-	+	+	+	-	+	-	-
<b>Gram-negative bacteria</b>										
<i>Aeromonas veronii</i>	+	-	+	-	+	-	+	+	+	-
<i>Citrobacter diversus</i>	-	+	+	+	-	+	-	+	+	-
<i>Citrobacter freundii</i>	-	+	+	+	-	+	-	+	+	-
<i>Klebsiella oxytoca</i>	+	-	+	-	+	-	+	+	+	-
<i>Klebsiella pneumonia</i>	-	+	-	+	-	+	+	+	-	+
<i>Pseudomonas aeruginosa</i>	+	+	+	+	+	+	+	+	+	-
<b>Fungi</b>										
<i>Aspergillus parasiticus</i>	-	+	+	+	-	+	+	+	+	-
<i>Aspergillus flavus</i>	+	+	+	+	+	+	+	-	+	+
<i>Aspergillus fumigatus</i>	+	+	+	+	+	+	+	+	+	+
<i>Aspergillus niger</i>	+	-	+	+	+	-	+	+	-	+
<i>Rhizopus stolonifer</i>	+	-	+	-	+	-	+	-	+	+

-, Absence, +: Presence, El: External integument of honeybees, DG: Digestive gut of honeybees, ++: Occurrence in digestive gut (DG) within selected ecozones, +++: Occurrence on external integument (El) within selected ecozones

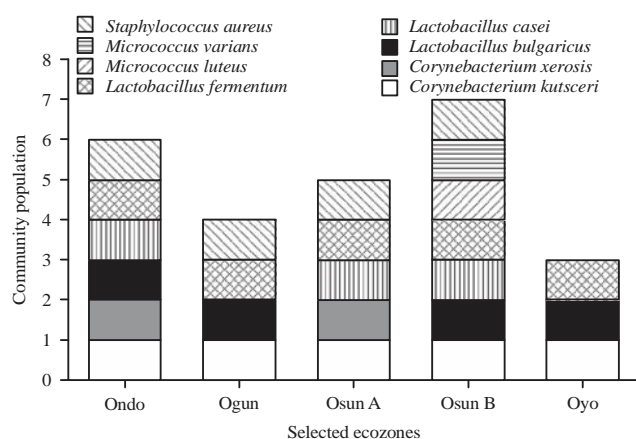


Fig. 2: Gram-positive bacterial community profile in the digestive guts of adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

digestive guts of the adult workers of *A. mellifera adansonii* from selected ecozones of South West, Nigeria. Digestive guts of adult workers of *A. mellifera adansonii* in Ondo and Osun (B) states accommodate more species of Gram-positive bacteria (Fig. 2), while more Gram-negative bacteria was observed in Osun B (Fig. 3). Comparatively, Osun (B) has both gram-positive and negative bacteria species in abundance. Fungi (molds) community profile in the digestive gut of adult worker honeybees in each ecozones was

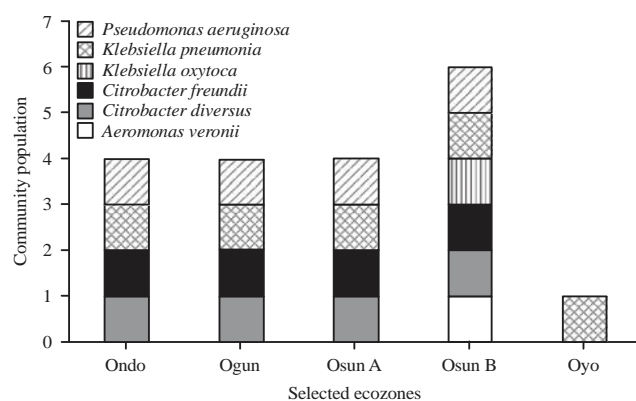


Fig. 3: Gram-negative bacterial community profile in the digestive guts of Adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

exhibited in Fig. 4. Also, the external integument of adult workers of *A. mellifera adansonii* from Osun state harbored more species of Gram-positive bacteria than those in other ecozones (Ondo, Ogun and Oyo) (Fig. 5). *Pseudomonas aeruginosa*, *K. oxytoca* and *A. veronii* exist generally on the external integument of adult workers of *A. mellifera adansonii* across all selected ecozones (Fig. 6). Most samples of adult workers of *A. mellifera adansonii* from all the selected ecozones almost have similar species of fungi on the external integument (Fig. 7).

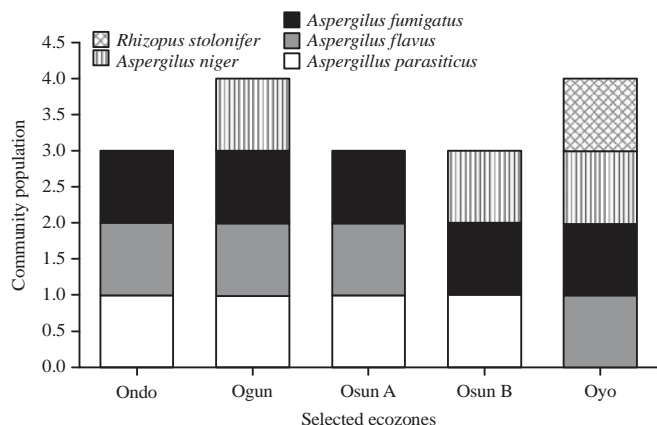


Fig. 4: Fungi (molds) community profile in the digestive gut of adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

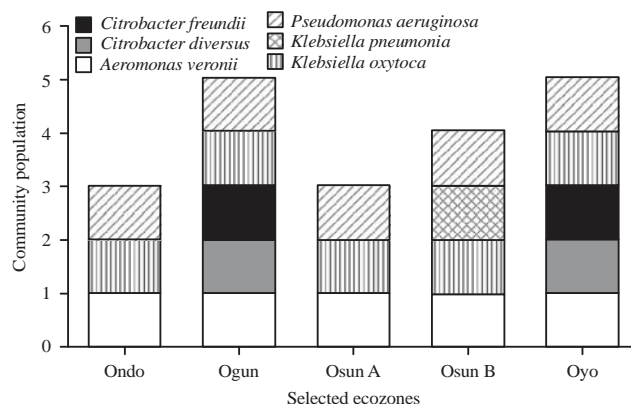


Fig. 6: Gram-negative bacterial community profile on the external integument of adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

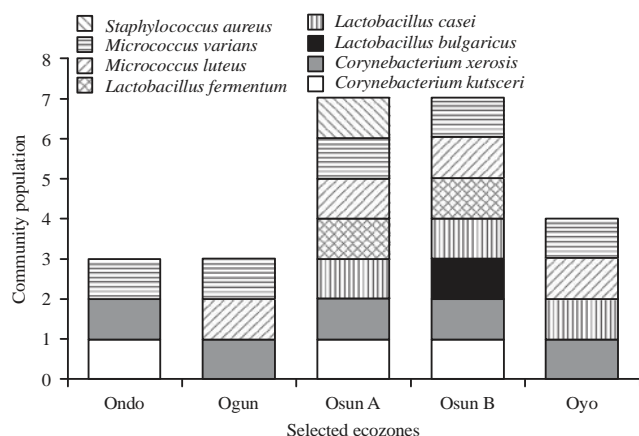


Fig. 5: Gram-positive bacterial community profile on the external integument of adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

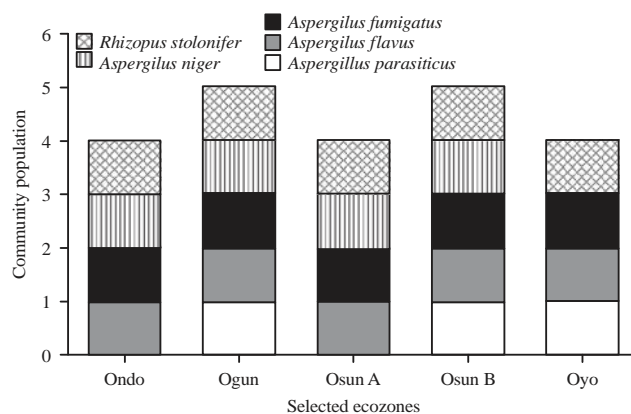


Fig. 7: Fungi (molds) community profile on the external integument of adult workers of *A. mellifera adansonii* in each ecozones, South West, Nigeria

The mean colony forming unit of bacteria on the external integument ( $4.12 \times 10^3$  CFU mL<sup>-1</sup>) of honeybees was relatively high compared to that of the digestive guts ( $3.64 \times 10^3$  CFU mL<sup>-1</sup>). This indicates that honeybees gathered variety of microbes from their environment during foraging activities for nectars and pollens. Also, the corbicular apparatus which was part of the external integument of honeybees for pollen collection have regular contact with their environment during foraging activities. Hence, the corbicular apparatus (pollen baskets) probably gather more microbes before the microbes selectively get into the digestive gut through trophallaxis or exposure to hive components (combs, honeybee bread and fecal materials).

## DISCUSSION

Generally, all the isolated and identified microbiota of adult workers of *A. mellifera adansonii* within selected ecozones exhibited occurrence both in digestive guts and on external integument of honeybees. However, most microbiota are not cosmopolitan across selected ecozones of South West, Nigeria except for *Aspergillus fumigatus*. The number of identified species of microbiota either in the digestive guts (DG) or on the external integument (EI) of adult workers of *A. mellifera adansonii* from selected ecozones varies from one ecozone to another. The findings from this study showed that microbiota component community of adult workers of

*A. mellifera adansonii* in the selected ecozones was composed of more Gram-positive bacteria than Gram-negative bacteria. From the study, dominant digestive guts' microbiota was *C. kutscheri*, *L. bulgaricus*, *L. fermentum*, *S. aureus*, *C. diversus*, *C. freundii*, *K. pneumonia*, *P. aeruginosa*, *A. parasiticus*, *A. flavus* and *A. fumigatus*. The dominant microbiota of the external integument includes *C. xerosis*, *M. luteus*, *M. varians*, *A. veronii*, *K. oxytoca* and *P. aeruginosa*, *A. flavus*, *A. fumigatus*, *A. niger* and *R. stolonifer*. *Klebsiella pneumonia* was more abundant and prominent in the digestive guts of adult honeybees across all selected ecozones. The study showed that Lactobacilli exhibited high dominance and diversity in the digestive guts of adult workers of *A. mellifera adansonii* across selected ecozones. Tysset *et al.*<sup>10</sup> and Gilliam and Prest<sup>17</sup> reported that the intestines of adults and larval stages of honeybees contained gram-positive bacteria such as *Bacillus* spp., *Streptococcus* spp. and *Clostridium* spp. which were different from the gram-positive bacteria such as *C. kutscheri*, *C. xerosis*, *L. bulgaricus*, *L. casei*, *L. fermentum*, *M. luteus*, *M. varians* and *S. aureus* identified in the study. While *C. diversus*, *K. pneumonia*, *P. aeruginosa* were common Gram-negative bacteria in both studies. Kacaniova *et al.*<sup>6</sup>, who studied the microflora of honeybees' gastro-intestinal tracts reported that Anaerobic and aerobic microorganisms, coliforms, enterococci, *Bacillus* spp., *Pseudomonas* spp. and yeasts were found in all bees but lactobacilli, staphylococci and molds were absent. This probably differs slightly from what was observed in the study. Also, Powell *et al.*<sup>18</sup> identified Gram-negative bacteria such as *Snodgrassella alvi*, *Gilliamella apicola* and *Frischella perrara* from the hind gut using deep sequencing of 16S rRNA gene amplicons which differ from Gram-negative bacteria identified in this study. This showed that geographical distribution dictates the kind of microflora of foraging *A. mellifera adansonii*.

Detection of novel probiotic bacterium *Lactobacillus* spp. in the digestive guts of adult workers of *Apis cerana indica* (Indian honeybees) collected from different parts of Karnataka, India, using Partial 16Rdna sequences, revealed the presence of novel bacterial flora composed of lactic acid bacteria (LAB). This was reported to originate in the honey stomach of the Indian honeybees<sup>19</sup>. *Lactobacillus* was one of the two genera reported to be predominantly present in significant numbers in the midgut of *A. mellifera indica*<sup>19</sup>. Although different species of the Genus: *Lactobacillus* was identified in this study. *Lactobacillus bulgaricus*, *L. casei* and *L. fermentum* were Gram-positive bacteria identified from the digestive guts of adult workers of *A. mellifera adansonii* which differs from that of the digestive guts of adult workers of

*A. mellifera indica*. These comparable differences in species were due to their ecological and geographical distributions which reflected on their microbiota diversity in relation to zoogeographical distribution of associated honeybees.

## CONCLUSION

Geographical locations dictate the abundance, occurrence and diversity of microbial communities and their interaction with honeybees. The study provides basic information for future genomic research aimed at understanding the evolutionary diversity and functional roles of microbiota community of honeybees and their economic benefits.

## SIGNIFICANCE STATEMENT

The external integument and digestive guts of honeybees, *Apis mellifera adansonii* harbour significant population of microflora dependent on geographical distribution. The presence of novel lactic acid bacteria which include *Lactobacillus bulgaricus*, *L. casei* and *L. fermentum* can be explored using genomics for pharmacological and fermentation purposes. Honeybees, *A. mellifera adansonii* are future potential sources of micro flora exploration through metagenomics. This will enhance discovery and development of useful products of pharmaceutical importance and microbes for bio-remediation, bio-control of pests of agricultural crops and pests of health importance.

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