Gut Microbiome of Two Different Honeybee Workers Subspecies in Saudi Arabia

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Honeybees play a vital role in the world’s food supply by acting as essential pollinators in the agricultural fields. Interestingly, more than one third of the world’s essential crops are honeybee’s dependant. The adult honeybee workers harbour a simple specific bacterial spectrum in their guts with vital role in bees’ health. Gut microbial diversity of adult honeybee workers was studied through targeting the V3 and V4 regions of the 16S rRNA gene via Illumina MiSeq. The study identified four phyla of the gut microbiomes in adult workers of the two-honeybee subspecies A.m. jemenitica and A.m. carnica. The most abundant phylum in microbiome of A.m. jemenitica was Firmicutes (48%), while Proteobacteria and Actinobacteriaphyla were less abundant at figures of 31% and 10%, respectively. In microbiome of A.m. carnica, Firmicutes (57%) was also the most dominant phylum, while Proteobacteria and Actinobacteria had lower prevalence at figures of 31% and 10%, respectively. At genus level, adult honeybee workers harboured a number of Lactobacillus spp. in their guts with relative abundance of 80% in A.m. jemenitica workers compared to 52% for A.m. carnica workers. Up to our knowledge, this is the first study of its kind on gut microbiome diversity in honeybee workers of different origins conducted in Saudi Arabia using high-throughput 16S rRNA gene sequencing technology. The results indicated that the variability in monophyletic origin of host of honeybee workers affected gut microbiota composition.

Keywords: A.m. jemenitica, A.m. carnica, 16S rRNA, high-throughput sequencing, diversity.

Honeybees belong to the genus Apis, which is known for its tremendous role in pollination. Unfortunately, honeybee population is recently declining with a potential risk on the agricultural service and subsequently the food supply, not only locally in Saudi but also globally. There is a known mutually beneficial relationship between honeybee gut microbiome and its host. The host provides...
the optimum environment for bacterial growth, while the bacterial community in honeybee guts aids in efficacy of nutrients absorption, optimum growth and development of the host and its ability to defend pathogens, and its adaptation to surrounding environment. Honeybee gut represents a simple model system to study the relationship between gut microbiome with honeybee hosts. The bacterial community in adult honeybee workers is diverse and estimated to reach one billion bacterial cells in each worker’s gut. Such a diversity in bacterial community is dependent on the type of flower that hosts the insect, as well as many other environmental factors. Gut microbiome of honeybee (Apis mellifera) workers is composed of eight to nine core species, e.g., Bartonella apis, Acetobacteraceae, Parasaccharibacter, Snodgrassella alvi, Bifidobacterium asteroides, Lactobacillus sp., Frischella perrara, and Gilliamella apicola.

The two most common bee species that are widely distributed throughout the kingdom of Saudi Arabia are the indigenous Apis mellifera jemenitica, which is a native species, and Apis mellifera carnica, which is imported from Egypt as honey production of domestic bees does not meet the growing demands in Saudi Arabia. Moreover, the production cost is relatively high. Exotic bee colonies have been imported over time, reaching 200,000 bee packages annually. It is well known for local beekeepers that the indigenous bees A.m. jemenitica highly tolerates local stressful conditions when compared with exogenous races A.m. carnica, particularly during summer when the air temperature becomes extremely high. It is also noticed that at high temperatures, indigenous bees continue to forage for pollen and collect nectar, whereas imported bees will stop foraging. Initial reports revealed that the subspecies of exotic honeybees have lower heat tolerance, shorter foraging durations and are more susceptible to Varroa mites when compared with indigenous bees.

In the present study, we compared the gut microbiome composition and diversity of the adult honeybees of Apis mellifera jemenitica and Apis mellifera carnica in Saudi Arabia using high-throughput 16S rRNA gene sequencing technology.

**MATERIAL AND METHODS**

**Sample collection, isolation of guts microbiota and DNA extraction**

Five samples each from honeybee workers of A.m. jemenitica and A.m. carnica were collected in November 2019 from a single hive of Beekeeper Cooperative Association at Al Baha, Saudi Arabia. The collected samples were immediately stored at “80°C. For whole gut dissection of honeybee workers, surface disinfection was done using 1 ml aqueous ethanol (70%, v/v) for 45 sec. Dissected guts were then placed in a pre-frozen mortar and 700 μl S1 lysis buffer (Invitrogen, Thermo Fischer Scientific, USA) were added and guts were transferred to bead tube for extraction process. DNA of gut samples was extracted by the genomic DNA extraction kit (Invitrogen, Thermo Fischer Scientific, USA), and stored at -20°C for further molecular analysis.

**PCR amplification**

PCR was run to amplify bacterial 16S rRNA gene of the variable regions V3-V4. The two universal primers used for PCR are 341F 5'-ACTCCTACGGGAGGCAGCAG-3' (forward primer) and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (reverse primer). The PCR conditions were set as the following: one cycle for initial denaturation at 95°C for 5 min; 25 cycles of denaturation at 95°C for 30 sec followed by annealing at 56°C for 30 sec and primer extension at 72°C for 40 sec; and a one cycle for final extension at 72°C for 10 min. The generated PCR products were checked for quality and selected products were utilized in preparing Illumina DNA libraries. DNA sequencing was run using Illumina Miseq platform (Illumina, San Diego, CA) at Beijing Genome Institute (BGI), China to generate high-quality pair-ends of ~300 bp.

**Statistical analysis**

The high quality paired reads produced in fasta files as raw data were de-multiplexed, quality-filtered and trimmed by trimmomatic package (Version 0.33) through Quantitative Insights Into Microbial Ecology 2 pipeline (QIIME2, v1.80). Obtained reads were merged into single sequence files by the Fast Length Adjustment of SHort
reads (FLASH, Version 1.2.11). In order to assign generated unique sequences into operational taxonomic units (OTUs), reads were tagged and clustered into OTUs with similarity cut off of 97% using the de novo OTU piking procedure. Usearch (Version 7.0.1090) was, then, used to remove Chimeric sequences. Taxonomies were plotted against the gut Microbiome Database (HOMD RefSeq, Version 13.2) through the RDP classifier (Version 2.2) and the Green-genes database (version 2013051816S rDNA database, http://qiime.org/home_static/dataFiles.html) with a cut off of 70%. Alpha diversity indeces were measured in order to assess the intra-species variations within a given sample using Mothur (v1.31.2). Alpha diversity and rarefaction curve boxplots were constructed using software R (v3.1.1). To investigate the inter-species variations within samples, the beta diversity matrices were conducted and visualized using principal coordinate analysis (PCoA) by package ‘ade4’ of software R (v3.1.1). Also, heat maps were generated using the package ‘gplots’ of software R (v3.1.1), and, then, sequence alignments were searched against the Silva core set (Silva_108_core_aligned_seqs) by using PyNAST ‘align_seqs.py’. The obtained OTU phylogenetic tree was, then, plotted by software R (v3.1.1), and visualized through QIIME2 (v1.80).

Annotation of generated OTUs was done in order to detect the relative abundance at different taxonomical levels (phylum, genus and species). Finally, Metastats, PERMANOVA and Benjamini–Hochberg false discovery rate (FDR) correction were also used to correct for multiple hypothesis. The Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was applied using software LEfSe with the online interface Galaxy (version 1.0.0; http://huttenhower.sph.harvard.edu/galaxy/root), to discriminate the two taxonomic races determining highly presented bacterial taxon within each race depending on statistical significance.

RESULTS

Statistics of 16S rRNA Sequence data

The five gut microbiome samples of A.m. carnica were identified as C1 to C5, while the five gut microbiome samples of A.m. jemenitica were identified as J1 to J5. Illumina MiSeq was used in sequencing the partial 16S rRNA gene

![Boxplots of alpha diversity indices illustrates richness and evenness at the group level of gut microbiomes of adult honeybee workers of A.m. carnica (red) and A.m. jemenitica (blue)](image)
Table 1. Statistics of deep sequencing data generated for gut microbiomes of 10 adult honeybee workers from subspecies A.m.carnica (C1-C5) and A.m.jemenitica (J1-J5).

| Sample ID | Reads length (bp) | Raw Data (Mbp) | N Base (%) | Low quality (%) | Clean Data (Mbp) | Data utilization (%) | Raw reads | Clean reads | Read utilization (%) |
|-----------|-------------------|----------------|------------|----------------|-----------------|----------------------|-----------|-------------|---------------------|
| C1        | 300:300           | 157.62         | 0.035      | 3.863          | 143.01          | 90.73                | 262698^2 | 243471^2   | 92.68               |
| C2        | 299:300           | 160.13         | 0.028      | 4.042          | 144.97          | 90.53                | 267327^2 | 247247^2   | 92.49               |
| C3        | 298:300           | 145.38         | 0.024      | 4.128          | 131.32          | 90.33                | 243118^2 | 224437^2   | 92.32               |
| C4        | 297:300           | 154.8          | 0.022      | 4.032          | 140.04          | 90.47                | 259289^2 | 239737^2   | 92.46               |
| C5        | 296:300           | 90.52          | 0.025      | 4.629          | 80.83           | 89.29                | 151884^2 | 138813^2   | 91.39               |
| J1        | 297:293           | 149.45         | 0.021      | 3.73           | 136.59          | 91.4                 | 253305^2 | 235671^2   | 93.04               |
| J2        | 296:293           | 162.1          | 0.026      | 3.963          | 147.33          | 90.89                | 275209^2 | 254716^2   | 92.55               |
| J3        | 294:293           | 145.85         | 0.02      | 3.664          | 133.58          | 91.59                | 248468^2 | 231607^2   | 93.21               |
| J4        | 293:293           | 135.37         | 0.021      | 3.797          | 123.52          | 91.25                | 231011^2 | 214843^2   | 93                 |
| J5        | 300:293           | 158.64         | 0.022      | 3.702          | 145.15          | 91.5                 | 267523^2 | 248977^2   | 93.07               |

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of PC1 and negative portion of PC2, whereas A.M.J group was mainly localized in the positive portion of PC2 (Figure 2). The principal coordinate analysis (PCoA) plots were created using a Bray-Curtis distance matrix and the samples were plotted to represent the microbial community compositional differences between samples. The plots are dimensionally scattered in accordance to their gut microbiome compositional relationships. The results of the present study indicate that the differences in gut microbiomes between these two groups are possibly due to the different origins of worker honeybees of the two subspecies.

The stacked number of OTUs and the number of observed species for different samples as rarefaction measures are shown in Figure S2. When the refraction curves inclines (Figure S2a) or stops climbing (Figure S2b), the produced data would be enough for further analysis. However, as long as the curve is still climbing, the complexity of the data in samples become higher; since more species being detected throughout sequencing analysis. The two rarefaction curve measures refer to the maximum number of sequences attained for all samples that allows to study taxonomic relative abundance and to assess eligibility of such data to represent all species of any microbial community. The findings from both rarefaction measures show that 54,000 is the maximum number of sequence reads that can be used further in studying taxonomic abundance (Figure S2).

**Structure of gut microbiomes across the two honeybee workers**

Two taxonomic ranks (phylum and species) were used in the comparison of gut microbiomes between adult honeybee workers A.M.C and A.M.J at the phylogenetic level (Figure 3). The results indicate that phylum *Firmicutes* harbours 24 genera, while *Proteobacteria, Actinobacteria, Bacteroidetes and Therman* harbour 23, 8, 6 and 2 genera.
Differential abundance of microbes due to different origin of worker

The observed microbial taxa along with their redundancies across different samples identified after OTU annotation are described in Table S2. The taxa refer to phylum, class, order, family, genus, and species. Eight phyla of the gut bacteria were identified according to relative abundance. They are Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria, TM7, Tenericutes and Thermi (Figure 4). Aligning with the number of genera of each phylum shown in Figure 3, the most abundant phylum were Firmicutes (57%), Proteobacteria (31%) and Actinobacteria (10%) in A.M.C group (Figure 4). Meanwhile, Firmicutes (48%), Proteobacteria (44%) and Actinobacteria (6%) were the most abundant in A.M.J group (Figure 4). The comparison at phylum level revealed a significant increase in Cyanobacteria in the A.M.C group (P-value = 0.031746), while a significant increase of Proteobacteria in the A.M.J group (P-value = 0.037724)(Table S3). Interestingly, Table S3 also indicates the existence of the three phyla TM7, Tenericutes and Thermi only in A.M.C group. The previous results align with those of the heat map at phylum level as Firmicutes, Proteobacteria and
Actinobacteria were shown to be the most abundant phyla across samples and groups (Figure S3).

In terms of species relative abundance in the gut microbiomes of two groups A.M.C and A.M.J Bacteroides_fragilis, Bacteroides_ovoatus, Commissalibacter_intestini, Blautia_producta, Melissococcus_plutonius, Ruminococcus_gnavus, Saccharibacter_floricola and Snodgrassella_alvi were shown to be the most abundant (Figure 5). The figure also indicates that a large proportion of the OTUs were not assigned to a certain species (93.80% for A.M.C and 86.20% for A.M.J). We have no explanation for these results except that a large number of species in workers of honeybee was not identified or classified before. The results in Table S4 indicates a significant increase of Melissococcus_plutonius in the gut microbiome of A.M.C (P-value = 0.034454), while Snodgrassella_alvi in the A.M.J group (P-value = 0.008948). Results for the latter species Snodgrassella_alvi align with that presented in Figure 5c. The Ruminococcus_gnavus and Saccharibacter_floricola were not existed in the A.M.J group. The heat map at species level indicates that Snodgrassella_alvi harbours the highest relative abundance across all samples (Figures S4).

Linear discriminant analysis effect size (LEfSe) and its LDA scores (≥ 3) were used to identify possible biomarkers in gut microbiota that refer to the origin of the host (Figure 6). The results in cladogram indicate that the possible marker in gut microbiome of A.M.C is Enterococcaceae family, while Neisseriaceae, Neisseriales and Betaproteobacteria taxa of A.M.J (Figure 6a). Biomarkers in A.M.C based on LDA score include Enterococcaceae, Saccharibacter_sp., Saccharibacter_floricola, Firmicutes (Melissococcus_sp. and Melissococcus_plutonius) and Cyanobacteria.
Fig. 5. Relative abundance of gut microbiomes of adult honeybee workers of *A.m.carnica* and *A.m.jemenitica* among (a and b, respectively) and across species (c) as measured by Metastats. **High significant difference between microbiomes of *A.m.carnica* and *A.m.jemenitica*.**
while Betaproteobacteria (Neisseriales, Neisseriaceae, Snodgrassella sp. and Snodgrassella_alvi) in A.M.J (Figure 6b).

**DISCUSSION**

The gut microbiome structure of honeybee workers is dependent upon monophyletic origin of...
the host9, social interactions and the type of diet consumed, whether workers are bee bread, pollen or nectar. In the present study, high-throughput sequencing was carried out for samples taken from the two honeybee subspecies A.m. carnica and A.m. jemenitica and statistical analysis proved that the diversity of the bacterial community composition of A.m. carnica and A.m. jemenitica was statistically significant.

Four major bacterial phyla (Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes) were recognized in the guts of honeybee workers of the two subspecies A.m. carnica and A.m. jemenitica. The dominant phylum in gut microbiomes of the two subspecies was Firmicutes with values of 57.2 and 48.5%, respectively. This conclusion was also drawn in several previous reports9,21,22,23. Genus Lactobacillus, gram-positive bacteria belonging to the family Lactobacillaceae (Firmicutes), was found to have a high relative abundance in adult workers of both A.m. carnica and A.m. jemenitica with values of 52% and 80%, respectively. It is a core gut bacterium that is dominant in the rectum of honeybee workers. Within this context, Ahnet al.24 concluded that Lactobacillacea dominates in both of A. cerana and A. mellifera species. This genus produces several compounds in honeybee gut with known antimicrobial activities such as organic acids, hydrogen peroxide, bacteriocin, reutericycin and reuterin that mostly inhibit decaying and protects against pathogenic bacteria, as well as some fungi25,26. Therefore, Honeybees likely use lactobacilli as probiotic27. In the present study, the dominance of Lactobacilli in both A.m. carnica and A.m. jemenitica adult workers is supported by the presence of low pH (3.9) of honey and nectar28. This is concluded because of the ability of lactobacilli to ferment sugar in the gut of honeybee workers and, hence, to generate acidic environment29, which inhibits the growth of many other bacteria. The low abundance in Lactobacillacea was reported to be associated with the presence of pathogenic bacteria30.

Genus Bifidobacterium, gram-positive bacteria belonging to the Actinobacteria phylum, was also identified in gut of both A.m. carnica and A.m. jemenitica adult workers. Again, it is dominant in rectum, and a core gut bacteria of honeybee workers. Bifidobacterium strains carry large surface proteins, which have a role in adhesion or degradation of plant materials7,31,32. Additionally, Bifidobacterium carries gene clusters that are responsible for the production and utilization of trehalose, which is a disaccharide molecule used by insects as an energy reservoir, in comparison to glycogen, which is the energy storage form in mammals33.

Family Neisseriaceae and its descendent Snodgrassella_alvi(S. alvi), gram-negative bacteria belonging to Betaproteobacteria phylum, significantly increased in A.m. jemenitica. These bacteria participate in oxidation of carbohydrates. However, the pathway for the uptake and glycolytic breakdown of carbohydrates does not exist in S. alvi, thus, this bacterium is located consistently within the periphery of the insect’s gut lumen. This area has high oxygen concentrations and this environment is preferable for S. alvi due to its dependence on aerobic respiration34,35. Insects depend on the aerobic oxidation of carboxylates rather than breaking down carbohydrates resulting in various products such as citrate, malate, acetate and lactic acid that serve as energy sources12,27. The steady co-exits of S. alvi with other fermentative bacterial taxa in the same gastrointestinal environment can result from utilizing separate sets of resources.

### Table 2. Alpha diversity comparison results among groups of gut microbiomes of honeybee workers from subspecies A.m.carnica(A.M.C) and A.m.jemenitica (A.M.J)

| Alpha diversity measure | Mean (A.M.C) | SD (A.M.C) | Mean (A.M.J) | SD (A.M.J) | p-value |
|------------------------|--------------|------------|--------------|------------|---------|
| Sobs                   | 74.6         | 44.59036   | 48.2         | 3.11448    | 0.03175 |
| Chao                   | 79.1         | 42.37209   | 52.01667     | 3.76128    | 0.05556 |
| Ace                    | 83.69118     | 40.78638   | 53.23999     | 3.78296    | 0.01587 |
| Shannon                | 2.4658       | 0.22593    | 2.4769       | 0.21404    | 0.84127 |
| Simpson                | 0.1377       | 0.0347     | 0.12313      | 0.0389     | 0.30952 |
leading to metabolic variations suggesting a syntrophic interaction. For example, S. alvi can utilize some of the substrates such as lactic acid, acetate and formate, which are produced from carbohydrate fermentation\(^36,37\). Furthermore, S. alvi and G. apicola\(^38\) are enriched with genes encoding biofilm formation. The two species inhabit the host’s ileum, indicating that the biofilm can provide a protective layer against pathogens. The bacteria of the family Acetobacteraceae and its descendent genus Commensalibacter (also referred to as Alpha 2.1), gram-negative bacteria belonging to phylum Proteobacteria, were identified as a core member of the gut microbiota in honeybees and bumble bees\(^9,31\). It was observed mainly in the midgut and hindgut of honeybee workers. In our study, Commensalibacter presents in A.m. carnica and A.m. jemenitica. However, Saccharibacter florica (Alpha-2.2) presents only in A.m. carnica. Furthermore, Saccharibacter florica is isolated from pollen, suggesting that this phylotype is associated with flowers\(^39\). The role of these phylotypes (Alpha 2.1 and Alpha-2.2) is associated with their abilities to adapt with fast growing metabolic processes, with two distinctive mechanisms. Alpha2.1 bacteria harvest energy through a wide range of substrates linked and utilized through a flexible oxidative and biosynthetic metabolism. Whereas, Alpha2.2 bacteria, that lack alternative oxidative pathways, determine metabolic processes through oxidative fermentation after harvesting glucose for rapid energy\(^40\).

The bacteria of the family Enterococccaeae and its descendent species Melissococcus plutonius, gram-positive bacteria of phylum Firmicutes, present in low abundance (3%) in gut microbiome of A.m. carnica honeybee workers. This conclusion was also noted in previous reports\(^41\). M. plutonius is known to cause the European foulbrood (EFB) in early stage of honeybee larvae, with assistance from secondary invaders (Enterococcus faecalis, Paenibacillus alvei and Bacillus pumilus). M. plutonius was shown to have 30 different sequence types clustered under three clonal complexes (CC 3, CC12, and CC13)\(^42,44\), where CC13 is the least virulent complex\(^43,45\). Honeybee workers transmit M. plutonius between colonies via robbing and drifting\(^46,47\). Erban et al.\(^45\) compared control samples from the EFB zone with samples from EFB zone without clinical symptoms, and bees from colonies from EFB zone with clinical symptoms. The study identified a 100-fold higher prevalence of M. plutonius in colonies with EFB symptoms, while it only presents in 3 of 16 control colonies that are distant from the EFB zone. This suggests that M. plutonius has lower abundance in healthy honeybee colonies, which is consistent with the results of the present study.

**CONCLUSION**

The present findings indicative that differences in gut microbiome structures of honeybee workers of the two subspecies A.m. carnica and A.m. jemenitica are due to varied monophyletic origin of the host. These findings support previous results suggesting that honeybee workers have a mutual coevolving relationship with specific group of bacteria. This group of bacteria co-exists and is maintained throughout the descending generations of the host. Inclusion of more subspecies inhabited in Saudi Arabia along with ones of this study can further support our findings.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Supplementary Information**

Supplementary information accompanies this article at http://dx.doi.org/10.13005/bbra/2870

Additional file: Additional Table S1, S2, S3 and S4 Additional Figure S1 to S4
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