Original article

Colonization dynamics of the gut flora in western honey bee workers within 7-day post-emergence

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Abstract – Western honey bee (*Apis mellifera*) is widely used in investigations involved in gut flora, and the gut flora of their workers have been demonstrated to be completely colonized within 7 days of emergence. However, colonization rules of the gut flora remain largely unknown. Here, metagenomic 16S rRNA gene data were generated to study colonization rules of the gut flora in workers by detecting dynamics in diversity and the relative abundance of gut bacteria across 14 time points post-emergence from pupal case, including 0 pupation-hours/control, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, and 156 post-emergence-hours (peh). Via comparison analysis of bacterial operational taxonomic units across these time points, diversity values of the gut flora presented the maximum at 0 peh. Among these points, 0–24 peh maintained the relatively higher diversity than the other time points. Furthermore, dynamic changes in several core gut bacteria (e.g., *Lactobacillus*, *Bifidobacterium*, *Frischella*, *Snodgrassella*, and *Gilliamella*) were analyzed in detail, and their biological function was linked to several host biological characteristics such as ecological adaptation, aging, gut health, dietary alterations and diversity, digestion and absorption, and metabolisms during colonization. This study is valuable for the understanding of colonization rules and the contribution of the gut flora to workers.

Apis mellifera workers / Gut flora / Colonization / Metagenomic 16S rRNA / Dynamics

1. INTRODUCTION

The gut flora has been widely considered the second genome of insects, playing key roles in ecology (Ravenscraft et al. 2019), development and evolution (Dong et al. 2020a), and metabolism (Tremaroli and Bäckhed 2012). Functions of the gut flora substantially changed with variation in diversity and the relative abundance, and colonization is the basis of the formation of community structure (Anderson et al. 2016;

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Dong et al. 2020a, b). Many studies have focused on dynamic changes in the gut flora structure during development in insects. For example, the core (shared) gut microbiota significantly changes across different developmental stages in *Apis mellifera* workers, and the diversity of the bacterial community was significantly changed (Powell et al. 2014; Yun et al. 2018; Dong et al. 2020a). Similar investigations have been conducted in the gut flora across all developmental stages of the beetle (Chouaia et al. 2019) and the egg–pupal stages of *Bactrocera minax* (Andongma et al. 2019), and a significant decrease in microbial diversity and increase of the relative abundance of core gut flora were detected with the development of the insect host.

Research on dynamic changes of gut microbial community of bumblebee queens showed a significant difference in diversity and composition of the gut flora between bumblebee queens at different ages and physiological stages (Wang et al. 2019). However, these studies primarily explored dynamic changes of the gut flora among different developmental stages of host, covering a large time interval. Little is known about dynamics (particularly a short time interval) in gut microbial colonization (generally defined as a shift from rare or new species to a stable community (Anderson et al. 2016; Powell et al. 2014)).

The genus *Apis* (Insecta: Hymenoptera: Apidae) contains nine known species, commonly referred to as honey bees. Owing to the fast breeding cycle, relatively simple composition of the gut flora, and easy control of genetics and diet, honey bees have been widely used as an ideal model for investigating the insect gut flora (Vojvodic et al. 2013; Saraithong et al. 2017; Tarpy et al. 2015), particularly western honey bees (*Apis mellifera*) (Dong et al. 2020a, b; Zheng et al. 2018; Anderson et al. 2018). The gut of the western honey bees is primarily composed of nine core bacterial species, accounting for greater than 95% of bacterial sequences (Martinson et al. 2011; Sabree et al. 2012). Five species in the group of core bacteria are generally found in the gut of the western honey bee workers (hereafter collectively referred to as workers), including two gram-negative species, *Snodgrassella alvi* and *Gilliamella apicola* (Kwong and Moran 2012), two major groups of lactic acid bacteria (*Lactobacillus* Firm-4 and Firm-5) (Martinson et al. 2011), and *Bifidobacterium* species (Kwong and Moran 2015). Previous studies have also found another three core bacterial species in the gut of workers, including *Parasaccharibacter apium* (Corby-Harris et al. 2014), *Frischella perrara* (Engel et al. 2013), and *Bartonella apis* (Jeyaprakash et al. 2003). Notably, few microbial taxa are found in newly emerged adult workers (Martinson et al. 2012), and colonization of the gut flora in workers is completed within 4–7 days after emergence (Dong et al. 2020b; Anderson et al. 2016; Powell et al. 2014). Therefore, the advantages of workers with respect to the breeding characteristics, composition of

the gut flora, genetic and dietary factors, and the short colonization cycle of the gut flora present an opportunity to study colonization of the gut flora, particularly for the core gut bacteria.

Here, we sequenced mass partial 16S rRNA gene sequences of the gut flora in workers across 14 time points (12-h intervals) during the whole colonization process within 7 days after emergence. We evaluated diversity, taxonomic annotations, and the relative abundance of 16S rRNA gene sequences in each group at different time points. Furthermore, we explored the colonization rules of gut bacteria from the perspectives of dynamic changes in diversity and the relative abundance in workers.

2. MATERIALS AND METHODS

2.1. Collection of Apis mellifera workers

Apis mellifera workers were collected in Chenggong district, Kunming city, Yunnan province, China, as previously described (Dong et al. 2020a, b). Next, three frames were removed from a colony and place into an observation hive, as described in Rangel et al. (2009). Briefly, three groups of healthy western honey bees were selected from three different hives containing nearly emerged adult worker individuals. From each hive, three frames were removed and placed in a smaller observation hive. The total size of each observation hive was approximately 5,000 healthy adult individuals, and the frames used were composed of roughly half brood as well as half honey and pollen to mimic the natural state of a colony. Next, the hives were placed in the dark at 34 °C (to mimic the hive environment). To avoid cross-contamination from other bees and nesting materials in hives, late-stage pupae (thinned honey cup, and emergence will occur after 0.5 ~ 1 day in our experience) were removed from cup cages. Then, five individuals were randomly picked, placed in 10-mL plastic centrifuge tubes using sterile tweezers (as 0 pupation-hours (peh)/control), as previously described (Powell et al. 2014). The samples collected at this time point did not come into contact with

the nurse bees and nesting materials. After that, the frame was returned to the dark incubation environments. Subsequently, 300 newly emerging worker individuals (natural emergence, 100 individuals per group) were collected, and their backs were marked by red enamel paint. These marked individuals were placed back in the original hives. Five worker individuals from each of three hives were randomly collected at 12, 24, 48, 60, 72, 84, 96, 108, 120, 132, 144, and 156 post-emergence-hours (peh, covering day 7 after emergence). Sample collection in each of three hives/groups was independently performed as three biological replicates. All samples were stored at -80 °C for DNA extraction.

2.2. DNA extraction and PCR amplification

Before removing the whole gut from worker individuals, the sample surface was individually sterilized using 70% and 90% ethanol solution for 1 min, respectively, followed by multiple washes using double-distilled water in a laminar flow hood under a stereomicroscope. The abdomen was dissected using sterilized scissors and tweezers, and the whole gut was removed, transferred into a 1.5-mL microcentrifuge tube filled with 100 µL double-distilled water and ceramic beads (0.1 mm) (Cenotec, Gyeongsangnam-Do, Korea) for the subsequent DNA extraction.

Gut tissues were homogenized in liquid nitrogen. Total genomic DNA was extracted from the gut using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. Total DNA quality was evaluated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Broadly conserved barcode primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWCTAA-3′) were used for the PCR amplification of variable region V4 ($\sim$ 390 bp) of the 16S rRNA gene (Walters et al. 2016). PCRs were carried out in 20-µL reactions in triplicate, and each reaction tube contained 4 µL of 5×FastPfu Buffer, 2 µL of 2.5 mM dNTPs,

0.8 µL of each primer (5 µM), 0.4 µL of Pfu DNA polymerase (Fermentas, Waltham, MA, USA), and 10 ng of template DNA. Reaction conditions were as follows: an initial denaturation step at 95 °C for 3 min; 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; and a final extension of 72 °C for 5 min. The PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and then quantified using QuantiFluor-ST Kits (Promega, Madison, WI, USA) according to the manufacturers' protocols. The concentrations of PCR products were checked using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Darmstadt, Germany) and quality control was performed using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). To maximize the diversity of bacteria from the population sample rather than from an individual sample (Andongma et al. 2019; Awawing et al. 2015), equal amounts of DNA samples from five individuals from each group were pooled and used for PCR amplification. For each sampling time points, DNA of five individuals in each biological replicate was mixed as one downstream sequencing sample. Thus, three samples were sequenced as three biological replicates for each sampling time point.

2.3. 16S rRNA gene sequencing

The PCR products were assessed by electrophoresis on a 2% agarose gel. The PCR products were recovered using a GeneJET Gel Extraction Kit (Thermo Scientific). High-throughput sequencing libraries were constructed using an Ion Plus Fragment Library Kit (Thermo Scientific). In brief, the procedure included (1) ligation of Y-adaptors, (2) removal of self-ligation fragments using magnetic beads, (3) enrichment of the template library by PCR amplification, and (4) denaturation by sodium hydroxide to generate single-stranded DNA fragments. All of the libraries were sequenced on the Ion S5 XL platform (Thermo Scientific) at Novogene Biotechnology Co., Ltd. (Beijing, China).

2.4. Bioinformatics and amplicon analyses

Raw fastq files were quality-filtered by Trimmomatic (version 0.36) to remove adapters and primers (Bolger et al. 2014). Next, the overall analysis pipeline was implemented in the mothur software package (v.1.44.3) (Schloss et al. 2006) and are illustrated on the mothur website (http://www.mothur.org/wiki/MiSeq_SOP). Contigs between read pairs were assembled using the *make.contigs* command, and any contigs with an ambiguous base N (i.e., below quality score 6) or less than 200 bp were discarded using the *screen.seqs* command. If two sequences have the same identical sequence, they were considered duplicates and will be merged using the *unique.seqs* command. Sequences then were aligned to a reference set (the SILVA database v138.1: https://www.arb-silva.de/) (Quast et al. 2013) using the *align.seqs* command, and those sequences that could not be aligned to the correct region were removed using the *screen.seqs*. Subsequently, sequences from each sample were further denoised (pre-cluster) using the *pre.cluster* command. The chimera sequences were removed using UCHIME (Edgar et al. 2011) after the VSEARCH alignment that is called within mothur using the *chimera.vsearch* command against the SILVA database. Then, a naive Bayesian classifier was employed to classify each sequence against 16S rRNA gene set stored at the Ribosomal Database Project (release 11) (this set includes rRNA gene sequences from mitochondria and chloroplasts) using the *classify.seqs* command. A pseudobootstrap confidence score of 80% was used. The chloroplast sequences, mitochondrial sequences, *Archaea* sequences, and *Eukaryota* sequences were removed using the *remove.lineage* command. Finally, sequences were split into groups corresponding to their taxonomy at the level of order (splitmethod = classify, taxlevel = 4) and then assigned to operational taxonomic units (OTUs) at a 97% similarity (cutoff = 0.03) using the *cluster.split* command. After taxonomic assignment of each OTU, the proportions of each bacterial lineage were calculated in each sample.

Finally, to measure the gut microbiota diversity (including alpha and beta diversity) and quantify the taxonomic composition of the samples, all sequences were randomly resampled to 57,002 sequences (the samples obtained the minimum of quality-filtered reads) per sample for sequencing data normalization. The subsequent analyses for diversity and the relative abundance were conducted based on the normalized data. For calculation of the relative abundance (that is percentage abundance), it means ratio of reads of each OTU to total number of reads in the normalized data of each sample/time point. The taxonomic comparisons between groups were conducted with the relative abundance of OUTs of each bacterial group in each sample group (Eun et al. 2016).

Alpha diversity indices used for evaluation of the diversity (Shannon and Simpson indices) and richness (Chao 1 and ACE indices) were calculated with the unique sequences of each sample. Subsequently, unique sequences were selected using the mothur *unique.seqs* command and distance matrix was generated using the *dist.seqs* command and clustered using the mothur phylip. Rarefaction metrics were calculated using the mothur *rarefaction.single* command. The coverage and alpha diversity indices were calculated using the mothur *summary.single* command. For analysis of a non-metric multidimensional scale (NMDS), the OTUs in each sample were rarefied to a common number of sequences using the mothur *dist.shared* command. Next, NMDS analysis was implemented using the mothur *nmds* command.

A linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al. 2011) was used to identify genera that were consistently different between sample types. LEfSe results were visualized using a taxonomy bar chart, as generated using the LEfSe website, http://huttenhower.sph.harvard.edu/galaxy/, with the default LDA score of 4.

2.5. Statistical analyses

Statistical significance in the relative abundances of OTUs in different samples was analyzed by a nonparametric Kruskal–Wallis test, with analyses implemented using MedCalc (version 19.6) statistical software (https://www.medcalc.org/index.php). The Student's *t* test implemented in MedCalc was used to compare the alpha diversity metrics and the relative abundance between the two groups. Pearson correlation test was assessed using the *cor.test* function with "method = pearson" parameter in the R platform. The results were statistically significant when *p* values were less than 0.05.

3. RESULTS

3.1. Summary of sequencing data

Forty-two samples were obtained from workers from 0 to 156 peh. High-throughput sequencing was performed to generate 3,405,257 raw reads. Subsequently, 3,227,420 clean reads were obtained after data filtering, with an average read length of 253 bp. In total, 18,930 OTUs were detected from all samples, with an average of 450 OTUs per sample (Supplementary Table 1). The Good's coverage index of all samples was above 0.99, indicating that adequate data were obtained for further analyses.

3.2. Alpha and beta diversity

The diversity and richness of the gut bacterial communities in each sample were presented in Supplementary Table 1. In particular, the diversity and abundance of taxa in the gut flora of workers at 0–24 peh were significantly higher than those at the other time points (0–24 peh vs. other pehs, Student's *t* test, $p < 0.001$), with maximum values at 0 peh: ACE, $p = 0.0055$; Chao, $p = 0.0076$; Shannon, $p = 0.0331$; Simpson, $p = 0.0427$ (Fig. 1). These results indicated that diversity and the relative abundance of gut microbes significantly changed over colonization in workers. The

observed species were also significantly different (observed species, $p = 0.0014$) among different time points.

Based on Bray-Curtis distances, a reliable (stress = 0.079) NMDS analysis was performed (Fig. 2). In particular, samples obtained at 0–24 peh were separated from other groups, and samples from the same biological replicates were clustered. These results indicated reliable biological replicates and structural heterogeneity of the gut flora among different time points.

3.3. Relative abundance of the gut flora

In total, 131 OTUs were shared across all time points and these were identified as core OTUs. Most of these OTUs could be annotated to the genus level (Supplementary Table 2), including *Lactobacillus*, *Snodgrassella*, *Bombella*, *Enterobacteriaceae*, *Commensalibacter*, *Bartonella*, *Fructobacillus*, *Bifidobacterium*, *Gilliamella*, and *Frischella*.

According to annotation results for OTU taxonomic assignments, the predominant phyla and the top 10 genera with respect to the relative abundance in all samples were selected for downstream analyses. The relative abundance of the phylum Firmicutes increased over time after emergence (Table I). The relative abundance of Firmicutes reached a maximum at 24 peh (66%). The relative abundance of the phylum Proteobacteria showed a similar trend to that of Firmicutes and showed a maximum value of 77% at 156 peh. Conversely, the relative abundances of the remaining predominant phyla significantly decreased over time after emergence, including Bacteroidetes and Actinobacteria. These two phyla presented the highest relative abundances at 0 peh. As determined by Kruskal–Wallis tests, significant changes in the relative abundance of gut bacteria across time points were observed for the following phyla: Proteobacteria ($p = 0.0369$), Bacteroidetes ($p = 0.0046$), and Acidobacteria ($p = 0.0113$).

At the genus level, the gut flora of workers at 0 peh was primarily composed of Lactobacillus (16%) and non-core microbiota (Table II).
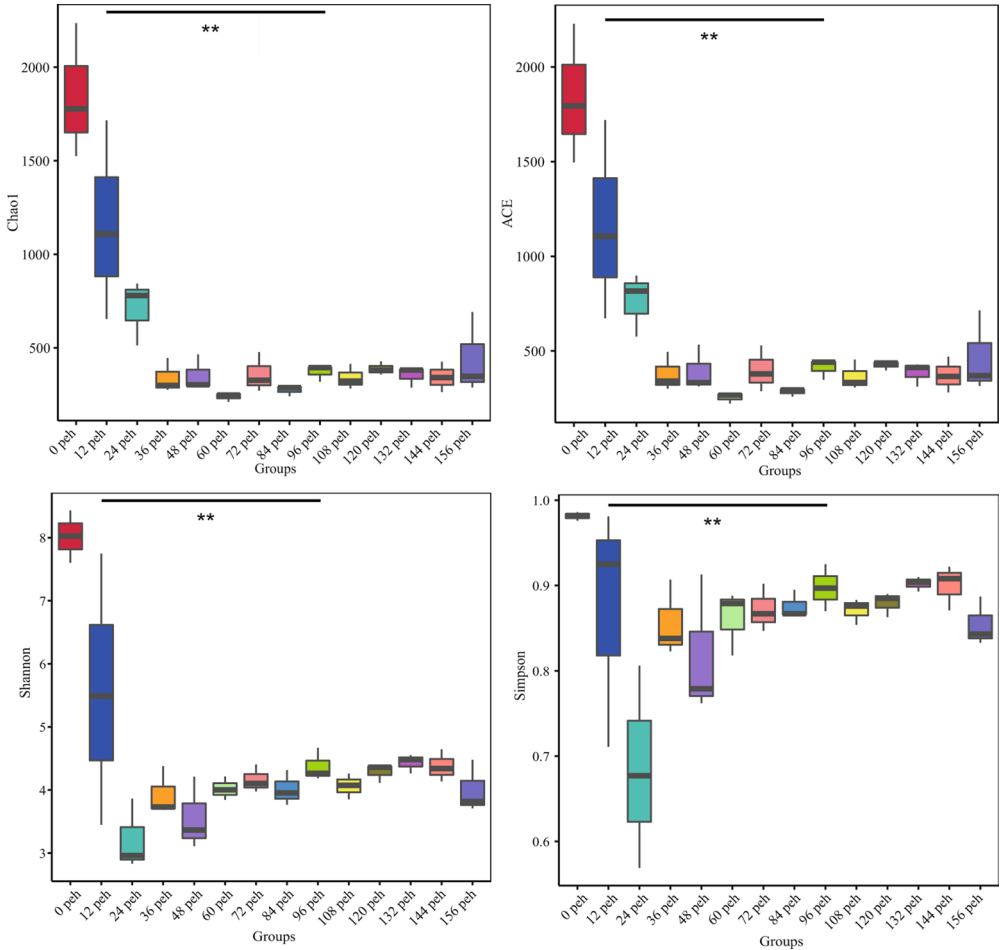
Figure 1. Diversity (Shannon and Simpson) and richness indices (Chao1 and ACE) of the gut flora of workers collected at different time points during colonization. The abscissa shows group names and the ordinate shows the exponential average values for each group. Significant levels of 0–24 peh vs. other pehs, Student's *t* test: **$p \leq 0.01$, *$p \leq 0.05$. Higher values for the Shannon, Chao1, and ACE indices indicate higher diversity, while higher values for the Simpson index indicate lower diversit

The relative abundances of the other four core bacteria were less than 0.005% at 0 peh. At 12 peh, the relative abundance of *Lactobacillus* significantly increased to 51% (0 vs. 12 peh, Student's *t* test, $p < 0.05$); and the relative abundance of the genera *Snodgrassella*, *Bombella*, *Fructobacillus*, and *Frischella* significantly increased to 7%, 4%, 7%, and 1%, respectively (0 vs. 12 peh, Student's t test, $p < 0.05$). Interestingly, the increase (15–70%) in the relative

abundance of five core gut bacterial species (*Snodgrassella alvi* and *Gilliamella apicola*, two species of *Lactobacillus*, and one species of *Bifidobacterium*) was significantly related to a decrease in the relative abundance of non-core flora (from 83 to 28%) (Pearson correlation coefficient$=0.891$, $p < 0.01$). In addition, the relative abundance of *Commensalibacter*, *Bartonella*, *Gilliamella,* and *Bifidobacterium* increased at 24 peh, 36 peh, 36 peh, and 36 peh, respectively,
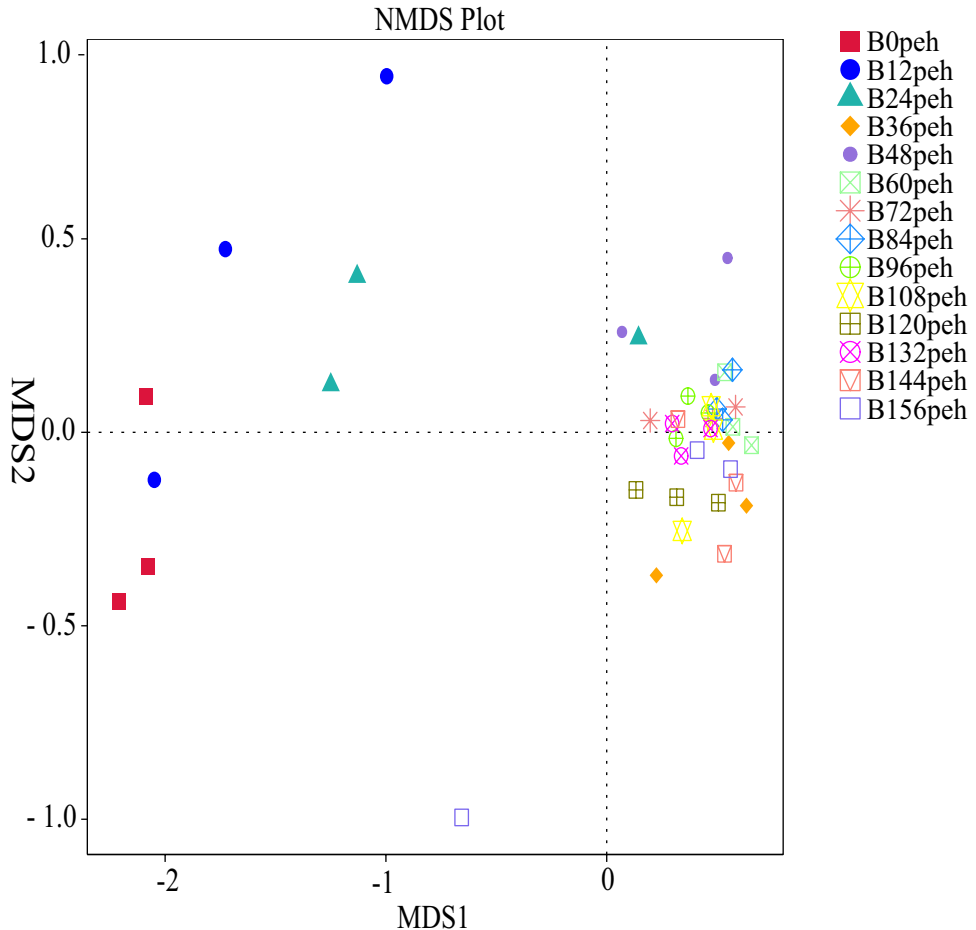
Figure 2. Multidimensional scaling analysis of all samples collected at different time points during the colonization of workers. Each point in the figure represents a sample, and the distance between the points represents the degree of divergence. Samples belonging to the same biological replicate are represented by the same color

in comparison with levels at earlier time points (Table II). According to Kruskal–Wallis tests, significance changes in relative abundance across time points were obtained for the following genera: *Lactobacillus* ($p = 0.0241$), *Commensalibacter* ($p = 0.0058$), *Snodgrassella* ($p = 0.0112$), *Bartonella* ($p = 0.0008$), *Bombella* ($p = 0.1571$), *Fructobacillus* ($p = 0.5073$), *Frischella* ($p = 0.2166$), *Gilliamella* ($p = 0.0127$), *Sphingomonas* ($p = 0.0015$), *Bifidobacterium* ($p = 0.0113$), and others.

LEfSe analysis showed that bacterial groups were significantly (LDA score > 4) enriched at different time points at the genus and species levels, primarily at 0 peh and 12 peh (Fig. 3). *Sphingomonas*, *Enterococcus*, and *Stenotrophomonas* were significantly enriched at 0 peh, and *Lactobacillus kunkeei* and *Fructobacillus* were significantly enriched at 12 peh. The genera *Commensalibacter*, *Gilliamella*, *Bombella*, *Snodgrassella*, and *Bartonella* were significantly enriched at 24 peh, 36 peh, 120 peh, 144 peh, and 156 peh, respectively.

Table I Relative abundances of predominant gut bacterial phyla (relative abundance values of > 5% in at least one sample) across different time points

Phyla	Relative abundance (%)*														p values
	0 peh	12 peh	24 peh	36 peh	48 peh	60 peh	72 peh	84 peh	96 peh	108 peh	120 peh	132 peh	144 peh	156 peh	
Firmicutes	32	64	66	47	62	61	56	59	41	37	26	39	40	21	0.4478
Proteobacteria	31	30	32	51	36	36	43	41	55	62	74	61	59	77	0.0369
Bacteroidetes	27	8	1	0	0	0	0	0	0	0	0	0	0	0	0.0046
Actinobacteria	5	2	0	1	0	5	1	1	3	1	2	2	1	1	0.0113

*Ratio of reads of each OTU to total number of reads in the normalized data of each time point. The same as in the following tables

4. DISCUSSION

Changes of gut microbes have a huge impact on bee biology (Raymann and Moran 2018). For example, the gut flora of honey bees converts dietary compounds to produce short-chain fatty acids that enhance honey bee sucrose responsiveness and stimulate the immune system (Kešnerová et al. 2017; Engel et al. 2012; Zheng et al. 2017; Kwong et al. 2017; Emery et al. 2017). In addition, pesticide exposure or antibiotic treatment can disrupt the composition of the gut flora, increasing the risk of infection by pathogens and thus increasing the mortality of the host (Raymann ey al. 2017; Maes et al. 2016; Motta et al. 2018; Blot et al. 2019). In this study, high-throughput sequencing technology was used to obtain partial 16S rRNA gene sequences from the gut flora of workers during the complete colonization period. An NMDS analysis supported the reliability of the sampling and sequencing data. The NMDS results also indicated a relatively similar structure of the worker gut flora at 36–156 peh. However, this structure was significantly different from that before 24 peh, revealing that 24 peh was a key time point for structural changes in the microbial community.

Our results further revealed that the richness and diversity of the gut flora substantially decreased during the 7-day period after emergence, similar to the trend observed in the gut flora across all developmental stages of the beetle *Popillia japonica* (Chouaia et al. 2019) and the egg–pupal stages of *B. minax* (Andongma et al. 2019). In particular, in this study, the highest alpha diversity of the gut flora in workers was detected at 0 peh, followed by a decline and stable levels after 36 peh. This pattern may be explained by shifting of social work roles of workers, with changes of dietary structure and working conditions. Workers within 2–3 days of age (48–72 peh) depend on other more developed workers for feeding, primarily consume honey, and they are responsible for heat preservation for larvae, hatching, and hive cleaning (Kryger et al. 2000; Yi 2017). Within 3–7 days of age, workers produce the first excretion and depart

Table II Relative abundances of the most abundant 10 gut bacteria genera across different time points

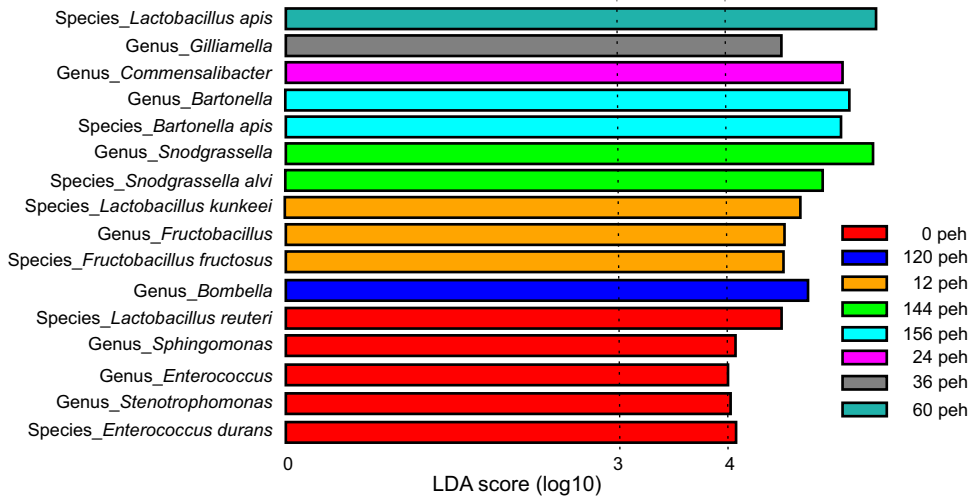| Genera	Relative abundance (%)														p values
	0 peh	12 peh	24 peh	36 peh	48 peh	60 peh	72 peh	84 peh	96 peh	108 peh	120 peh	132 peh	144 peh	156 peh	
Lactobacillus	16	51	65	47	61	59	59	59	41	37	23	35	39	17	0.0241
Commensalibacter	0	0	24	1	1	5	11	6	14	7	5	3	8	4	0.0058
Snodgrassella	0	7	0	25	30	19	20	12	14	19	30	23	35	35	0.0112
Bartonella	0	0	0	4	0	3	0	0	1	2	15	0	3	28	0.0008
Bombella	0	4	6	0	2	1	4	8	10	10	12	11	3	1	0.1572
Fructobacillus	0	7	0	0	3	1	1	2	2	1	4	4	1	0	0.5073
Frischella	0	1	0	1	1	1	0	1	1	1	1	1	3	5	0.2166
Gilliamella	0	0	0	7	0	0	0	0	1	0	1	1	4	4	0.0127
Bifidobacterium	0	0	0	1	0	5	1	1	3	1	2	2	1	1	0.0113
Sphingomonas	0	0	0	1	0	1	2	0	0	2	1	1	1	2	0.0015
Others	84	30	5	13	2	5	2	11	13	20	8	19	2	3	0.0266

Figure 3. Histogram of LDA values. LDA scores for gut bacteria exceeding the threshold value of 4 indicate a significant difference between groups. The *y*-axis shows gut bacteria with a significant difference in relative abundance in comparison with other gut flora at each time point. Histogram length (LDA Score) represents the effect size of gut bacteria

the hive; these more mature workers begin to make bee bread (a mixture of honey and bee pollen) to feed themselves and larvae (Yi 2017). In addition, the relative abundance of core bacteria increased and the relative abundance of non-core bacteria decreased, which may be explained by the gradual occupation of the ecological niche of non-core flora by core flora in worker guts that are a relatively closed and resource-limited space. Previous studies have shown that workers initially lack gut bacteria (Powell et al. 2014; Martinson et al. 2012; Guo et al. 2015). In this study, indeed, most core bacteria were not detected at 0 peh workers, further verifying previous findings.

As core bacterial genera in the worker guts, *Lactobacillus* and *Bifidobacterium* can help honey bees absorb nutrients (Alberoni et al. 2018; Wu et al. 2012). In particular, Lactobacillus species can convert carbohydrates, particularly sugars, to energy in guts, thereby promoting host development (Rodriguez and Nadra 1995), and can promote the absorption of monosaccharides, calcium, magnesium, and proteins, as well as the production of vitamin B in nectar (Engel and Moran 2013; Butler et al. 2013). In the current study, the relative abundance of *Lactobacillus* was substantially higher at 12

peh than at 0 peh, indicating that normal gut functions related to digestion, absorption, and immune defense in workers begin to develop. *Bifidobacterium* stimulates hormone production of the host so that the development of honey bee is regulated (Kešnerová et al. 2017). *Bifidobacterium* also contains many enzymes that promote carbohydrate degradation, such as pectin-degraded enzymes, glycosides, and polysaccharide hydrolases (Slováková et al. 2002). In this study, *Bifidobacterium* was first detected at 36 peh, thereafter, gradually increased in the worker guts, revealing that digestion and absorption ability of host guts was further improved. This result is similar with previous studies of Dong et al. (2020a). Interestingly, the relative abundance of *Bifidobacteria* between 9 and 30 dep decreased with the age of bees. During this period, workers leave from the hive to search for pollen and nectar (Calderone 1998; Robinson 1992). These results suggested that *Bifidobacterium* strains may be a bacterial indicator used for assessing the age of workers (Anderson et al. 2018). The relative abundance of *Lactobacillus* is negatively correlated with host age (Anderson et al. 2018; Anderson and Ricigliano 2017; Guo et al. 2015; Dong et al. 2020a). The

current analyses showed that the relative abundance of *Lactobacillus* initially increased (0–60 peh) and then decreased during colonization of the gut flora. These findings suggested that the relative abundance of *Lactobacillus* may be also a gut bacterial marker for worker aging. Previous reports have shown that the relative abundance of *Snodgrassella* is positively correlated with survival rates of honey bees (Maes et al. 2016). *Snodgrassella* is also abundant in the gut of honey bee. Together with *Gillianella*, it obtains nutrients for bees and can form a biofilm on the gut epithelium to resist pathogens (Engel et al. 2015; Kwong et al. 2014). In this study, the relative abundance of *Snodgrassella* significantly increased at 12 peh and reached a peak at 144 peh, indicating that this genus is a potential indicator of gut microbiome development, with the greatest gut activity at 144 peh. Additionally, the relative abundance of *Frischella* increased at 12 peh and remained stable thereafter. *Frischella* ensures that the immune system is early activated, and *Frischella*-induced immune activation hinders the invasion of foreign microbes in the hindgut (Emery et al. 2017). Moreover, an increase in the abundance of *Frischella* promotes dietary alterations (Maes et al. 2016). Thus, probiotics-mediated gut function involved in the immune response and dietary diversity probably begins to develop in workers at 12 peh or earlier. In this study, the relative abundance of *Gilliamella* increased at 36 peh and colonization was completed at 96 peh (showing a steady state thereafter). After 132 peh, the abundance of *Gilliamella* has increased by comparing with that before 132 peh, but it basically remained in a stable state, as similarly detected in (Martinson et al. 2012). Moreover, the relative abundance of *Gilliamella* was lower at 24 peh than the other time points (Guo et al. 2015), further confirming the accuracy of results obtained in our study. In addition, *Gilliamella* is a core species in the honey bee guts (Kwong and Moran 2012). It participates in the decomposition of pectin (a primary component of the cell walls of pollen associated with digestion and absorption) (Engel and Moran 2013). Therefore, the *Gilliamella*-mediated decomposition of pollen cell walls

was intensively formed at 36 peh, and ability to feed on pollen may be fully realized at 96 peh in workers, resulting in the effective utilization of pollen and nectar.

Interestingly, previous studies have demonstrated that the non-core bacterial *Commensalibacter* has a key role in sugar degradation (Ribière et al. 2019). The relative abundance of *Commensalibacter* was significantly higher at 24 peh than at 0 peh in this study, indicating sugar metabolism in the worker guts substantially increased at 24 peh via colonization of *Commensalibacter*.

Several studies in bees demonstrated substantial changes in gut bacteria and likely indicate that eukaryotic parasites will also alter the gut bacteriome of honey bees (Schwarz et al. 2016; Hubert et al. 2017). Previous studies assessed the effects of globally widespread parasites and pathogens (i.e., the ectoparasitic mite *Varroa destructor*, the fungal pathogens *Nosema apis* and *Nosema ceranae*, and the trypanosome *Lotmaria passim*) on *A. mellifera* bacteriome (Hubert et al. 2017). The results showed that high *Varroa* infestation (varroosis) is more important than other three disease factors in influencing bacteriome and the composition of the bacterial community in adult bees. In particular, varroosis decreased the relative abundance of the bacteria *B. apis* and *Lactobacillus apis*, while increased the relative abundance of *Lactobacillus helsingborgensis*, *Lactobacillus mellis*, *Commensalibacter intestini*, and *S. alvi*. Moreover, previous experiments supported a link between altered core gut microbiota and increased parasite and pathogen prevalence, as observed from honey bee colony collapse disorder (Schwarz et al. 2016). Therefore, changes in the relative abundance of core gut bacteria over colonization contributed to the shape of parasite susceptibility in workers.

5. CONCLUSION

This study revealed dynamic changes in structure and functions of the gut flora using a comparative study of metagenomic 16S rRNA data.

The diversity of the gut flora decreased over time after worker emergence, and remained stable after 36 peh. The relative abundance of core bacterial taxa increased during colonization, while that of non-core bacteria decreased. In particular, the core gut bacteria were analyzed in detail, and their biological function was linked to metabolism, immunity, feeding, digestion, and behavior of host. Notably, many taxa had unknown functions, particularly non-core bacteria. Further investigations using metabolomics and functional genomics are required to confirm their functions.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

Conceived and designed the experiments: Q-LZ, JG, and L-BL. Sampling: S-BC and Z-XD. Analyzed the data: S-BC, Z-XD, GW, and Q-LZ. Contributed reagents/materials/analysis tools: Q-LZ, JG, and GW. Wrote the paper: S-BC, GW, and Q-LZ. All authors have read, commented on, and approved the manuscript.

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DATA AVAILABILITY

All clean sequence data of 42 libraries reported in this study have been deposited in the Sequence Read Archive (SRA) database of NCBI under accession number SRR9698607-SRR9698648.

CODE AVAILABILITY

All code used in this study is included in the software (non-custom code).

DECLARATIONS

Ethics approval Not applicable.

Consent for participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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