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Dynamic change of gut microbiota in the male bee of *Bombus terrestris* (Hymenoptera: Apidae)

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Abstract

Background

The gut microbiota play a key role in the development and health of bumble bees. Male bees are important for the reproductive activity of a colony, yet there are few studies on their gut microbiota.

Results

By using qPCR, we found there are significant changes in total bacteria and six important bacteria genera from different developmental stages compared to workers bees. The results indicate that *Gilliamella*, *Snodgrassella*, and *Lactobacillus* are the dominant gut bacteria in male bees, which is consistent with the previous studies in worker bees, however, there are more total bacteria in male bees. Another gut bacteria genus, *Bacillus* may be a probiotic bacteria for reproduction in male bees, although the possible function of these bacteria require further study.

Conclusions

This research can provide insight into the relationship between the bacterial community and the physiological health and reproductive capacity of male bumble bees.

Key words: bumble bee, gut microbiota, change, male bees

Background

Bumble bees are important pollinators and play a key role in maintaining ecological balance and plants diversity [1]. The members of the colony are divided into three different castes, each with specialized duties: a queen, workers, and males. After the queen has founded a colony and her first clutch of workers emerges to help her in providing for the hive, the queen's primary job is to lay eggs. The workers gather food, care for the young, and clean and defend the nest. The males' purpose is

to leave to mate with virgin queens from other nests, ensuring future genetic diversity.

Many studies have suggested that there are close relationships between a host and their gut microbiota. Some gut bacteria can generate a probiotic effect for the host assisting the host to digest food, while some cause the host to produce anti-microbiotal peptides and defend against pathogens [2, 3]. Similarly, there is a relatively simple yet specialized gut microbiota in the gut microorganisms of bumble bees [4, 5]. Studies have shown that *Gilliamella, Snodgrassella,* and *Lactobacillus* are the dominant gut bacteria in bumble bee workers, and these bacteria have an important impact on the bumble bee's development and physiology [2]. In addition, there is a significant difference between the gut bacteria in unmated and mated bumble bee queens. *Gilliamella, Snodgrassella,* and *Lactobacillus* are the dominant genera in unmated queens; however, *Bacillus*, *Pseudomonas*, and *Lactococcus* are the main gut bacteria in mated queens [6]. Additionally, *Gilliamella, Snodgrassella,* and *Lactobacillus* have been found to assist the bumble bee degrading the pollen wall, absorbing nutrition and protect the host against parasites [4, 7, 8]. Further research is required on the possible function of *Bacillus*, *Pseudomonas*, and *Lactococcus* in the bumble bee.

In contrast to workers bees, few studies have examined microbial communities that are associated with bumble bee males, even though their health and proper function are important to the productivity of their colonies.

A better understanding of the gut microbiota composition in different physiological states of bumble bee males would shed light on the complex interplay between the microbiota and male health. Using a targeted qPCR approach, we assessed the abundance in the identified predominant bacteria (including *Gilliamella, Snodgrassella, Lactobacillus*, *Bacillus*, *Pseudomonas*, and *Lactococcus*) at different physiological stages of unmated and mated male bees, which may help further understanding of the relationship between male bees and gut bacteria. Our study is the first to explore dynamic changes of the gut microbiota across different life stages of bumble bee males, from adult eclosion to mating. It shows dynamic diversity and variation of gut bacterial communities and improves our understanding of possible relationships between the gut microbial communities and different developmental and physiological states of bumble bee males.

Results

Copy number variations of differentially abundant bacterial genera in the different developmental stages of unmated males.

The total bacterial copies and the single bacterial genera of *Gilliamella, Snodgrassella Lactobacillus*, *Bacillus*, *Pesudomonas*, and *Lactococcus* were detected in unmated males over a period of 1–25 days. The results show that the mean absolute number (± s.d.) of the overall bacterial rRNA genes of each male age and six predominant genera were quite low at the first day post-eclosion. The copies of total bacteria gradually increased and peaked at the ninth day ($1.01\times10^{10}\pm 3.53\times 10^{9}$), after then they began to decline and persisted at a relatively low level during the remaining stages (Fig.1). The change in abundance of *Gilliamella* and *Snodgrassella* is similar, both began to increase gradually and peaked at the seventh day ($3.38\times10^{9}\pm5.64\times10^{8}$, $5.31\times10^{9}\pm7.02\times10^{8}$), which is a significant difference to other stages ($p<0.05$). Levels then gradually diminished until the fifteenth day ($5.35\times10^{8}\pm1.92\times10^{8}$, $9.91\times10^{8}\pm2.66\times10^{7}$), after then they maintained a relatively high level. The change in quantities of *Lactobacillus* and *Lactococcus* were similar with the highest concentration at the third day ($1.13\times10^{9}\pm1.52\times10^{8}$, $1.87\times10^{5}\pm5.65\times10^{4}$), which is significantly different to other stages ($p<0.05$), the copy numbers remained at a relatively low level at other stages (Fig. 1). However, the highest abundance of *Bacillus* was at the nineteenth day ($3.09\times10^{8}\pm7.65\times10^{7}$), which is significantly different to other stages ($p<0.05$),. The *Pseudomonas* bacteria was present in greatest amounts at the seventeenth day ($2.31\times10^{6}\pm2.39\times10^{5}$) (Fig. 1).

Copy number variation of differentially abundant bacterial genera in the different developmental stages of mated males.

In this study, the male bee first mated with the queen at the twelfth day after eclosion [14]. Male bees were detected at the thirteenth day when they emerged from the pupa, and act as the first day of the mated male. The results showed that there was no significant different for the total bacterial copies among the different male stages, but

they maintained a relatively low level after the first day of mating. However, the *Gilliamella* and *Snodgrassella* genera had the highest copy numbers at the first day of mating ($3.06\times10^{9}\pm1.02\times10^{9}$, $8.54\times10^{9}\pm2.94\times10^{9}$), which was a significant difference to other stages ($p<0.05$) (Fig. 2). However, the genera of *Lactobacillus* ($2.19\times10^{8}\pm4.73\times10^{7}$), *Lactococcus* ($1.23\times10^{4}\pm4.83\times10^{3}$) and *Pseudomonas* ($6.70\times10^{7}\pm9.75\times10^{5}$) had the highest copy numbers at the sixth day for mated males, which was a significant difference to other stages($p<0.05$), ; they were relatively low in abundance at other stages (Fig. 2). *Bacillus* had the highest copy numbers at the eleventh ($9.15\times10^{7}\pm5.39\times10^{6}$) and sixteenth days ($8.64\times10^{7}\pm1.01\times10^{7}$), and there was significant differences to other stages ($p<0.05$) (Fig. 2).

Discussion

A close relationship between gut microorganisms and their host was detected; this has important impacts on the development and physiology of the host [15-19]. However, the host can also influence the abundance and composition of gut microorganisms [4, 12, 20]. In this study, the dynamic change of the total bacteria and the each bacterial genus *Gilliamella*, *Snodgrassella*, *Lactobacillus*, *Bacillus Lactococcus*, and *Pseudomonas* was identified at different developmental stages of unmated and mated male bumble bees. The results showed there were different dynamic changes in gut bacteria at different developmental stages, it might be explained that different genus of gut bacteria have different functions in their host at different stages of development. Many studies have found that gut symbionts potentially affect reproductive behaviors in insects. For example, in *Drosophila melanogaster,* commensal bacteria play a role in mating preferences [21, 22], and the alteration of female microbiota counteracts a default male outbreeding strategy by inhibiting female sexual signaling [23]. This study estimated the average absolute number of total bacterial rRNA genes at each stage with $6.98\times10^{7}\pm 1.27\times10^{7}$ —$1.01\times10^{10}\pm 3.54\times10^{9}$ copies per gut, which are much higher than the worker's gut identified in previous ($1.2\times10^{8}\pm1.1\times10^{8}$ per gut) [24] research. The differences are intriguing; however, further work is required to clarify if the microbiota influences male bumble bee mating behavior and chemical

communication required for copulation – such as male sex pheromone production.

Many studies have demonstrated that the bacterial genus of *Gilliamella*, *Snodgrassella*, and *Lactobacillus* play a key role in the health of worker bees [15-18]. In this study, the bacterial genus of *Bacillus*, *Lactococcus*, and *Pseudomonas* shows an obvious increase in the mated bumble bee queens [6]. Likewise, the genus of *Gilliamella* and *Snodgrassella* are also the major gut bacteria in male bees, which is consistent with the previous study on worker bees [7, 11, 25]. The content of *Lactobacillus* and *Bacillus* is relatively low when compared with *Gilliamella* and *Snodgrassella*; the metabolic pathway shows that *Lactobacillus* can transform various carbohydrates into lactic acid [8, 26], and the function is closely associated with the larvae-feeding process, which is done by the workers [27]. Therefore, levels are higher in the guts of workers than in male bees [4]. In addition, there are fewer copies of *Pseudomonas* and *Lactococcus*, indicating that perhaps they are not the main gut bacteria in male bees.

After emerging from the pupa to the point of sexual maturity, male bees rely on bee bread for nutrition. Gut bacteria abundance is initially low; this gradually increases with the host's development. This result is consistent with the study on worker bees [9, 27]. The core gut bacteria are colonized in the male through the host's contact with its nestmates, hive materials, and consumption of bee bread. During early adulthood, male bees are focused on feeding in order to obtain enough energy. The genomics study of *Gilliamella apicola* found that it can assist the host in degrading pollen and obtaining adequate nutrition, and *Snodgrassella* and *Lactobacillus* can protect the host against pathogens [7, 28], Therefore, they present in high abundance in the guts of male bees in order to improve the development and health of their host. *Bacillus* has relatively high copies from the ninth day after eclosion, and the individual also reaches sexual maturity at this Time [14]. Zhang revealed that *Bacillus* can increase the activity and quality of sperm in male mice, which suggests that gut bacteria may be associated with the host's reproductive success. Additionally, *Bacillus* can also enhance the host's immunity and protect against pathogens [29, 30, 31].

Most bumble bee species only mate with one queen in their lifetime (including

Bombus terrestris) [32], and they do not play a role in the colony after mating. This study reveals that the abundance of gut bacteria is maintained at relatively low levels (except for *Bacillus*) after mating; this can be influenced by the physiological status and the roles of the host.

This study suggests that *Gilliamella*, *Snodgrassella*, and *Lactobacillus* are also the core gut bacteria in male bumble bees, as is the case for worker bees. Yet, *Bacillus* is also abundant in the bee gut, possibly because it is another probiotic bacterium for the host. Meanwhile, the possible function of these gut bacteria in the growth and development of the host is an area that requires more study.

Conclusions

Bumblebee males undergo a number of biological changes as they transition through adult emergence, mating, foraging. Therefore, they represent an important system to understand the link between physiological, behavioral, and environmental changes and host-associated microbiota. It is plausible that the bumblebee male gut bacteria play a role in shaping the ability of the male to survive environmental extremes and mating, due to long established coevolutionary relationships between the host and microbiome members.

Our results show that there is a significant difference in diversity and composition of the gut microbial communities in males of Bombus terrestris across different physiological states. This study will give us insight into the relationship between the bacterial community and the physiological states in bumble bee males, and provide the theoretical foundation for the further study of the microbiotal function in the health and mating success of bumble bee males.

Methods

Sample collection

Males of *Bombus terrestris* (Linnaeus) (Hymenoptera: Apidae) were collected from the Institute of Apicultural Research, CAAS, China. The colonies were reared in the

dark at a temperature of 27 ± 1℃ and relative humidity of 50-60%. Sugar water (1:1 v/v) and apricot pollen were provided ad libitum to subsequently produced 100 colonies until males and gynes (new queens) emerged. The different physiological status of male samples, including unmated and mated, were collected with different periods respectively. The samples were divided into two physiological stages, including unmated and mated. In the unmated samples, we collect the 1d, 3d, 5d, 7d, 9d, 11d, 13d, 15d, 17d, 19d, 21d, 23d, and 25d after emergence (n=5, per time point). And the mated samples including 1d, 6d, 11d, 16d, 21d, and 26d after mated with queen (n=5, per time point). All collected samples were treated with liquid nitrogen and then stored at -80°C until used.

Extraction of the gut DNA

The whole gut (including crop, midgut, ileum, and rectum) of bumble bee males were collected at the sterile environment. For each bee, the body surface was disinfected with 70% and 90% ethanol solution for 1min respectively, then using the double-distilled water to wash it for some times. The abdomen was dissected with the disinfectant scissors and tweezers, the whole digestive tract was removed and transferred into a 1.5mL microcentrifuge tube with 100 μL double-distilled water and ceramic beads (0.1mm) for the DNA extraction. After the gut sample was homogenized in the Tissue Lyser (QIAGEN Hilden, Germany), the genomic DNA was isolated with the Wizard® Genomic DNA Purification Kit (Promega, A1120), following the manufacturer's instructions. t30 μL nuclease-free water was used to dissolve the DNA. The concentration and quality of extracted DNA were determined by the Nanodrop 2000 (Thermofisher) and 1% agarose gel electrophoresis. The DNA was stored at -20°C until used.

The Primer design and PCR amplification

The major bacterial genus' 16S rRNA gene sequences were acquired from the GenBank database. The conserved regions of each genus was aligned and analyzed by using the software DNAMAN, then using Primer Premier (version 5.0) to identify and design the unique primer pairs. The universal 16S rRNA primer was used to detected the total bacterial copies for every sample which was from the previous studies [9, 10].

The primer sequences of *Bacillus*, *Pseudomonas*, and *Lactococcus* were BacF (GATGCGTAGCCGACCTGAGA) and BacR (GGCGTTGCTCCGTCAGACTT), PseF (CCGTAACTGGTCTGAGAGGATG) and PseR (GCATGGCTGGATCAGGCTTT), LactF (GCGATGATACATAGCCGACCTG) and LactR (AGTTAGCCGTCCCTTTCTGGTT) respectively, primers of 16s, *Gilliamella*, *Snodgrassella*, and *Lactobacillus* were from the previous study [11, 12]. In order to ensure the specificity of these primer pairs, the PCR amplification was performed in a 20 μL mixture system, which consisting of SYBR^{R} Premix Ex Taq II (Tli RNaseH Plus) (2×) (10μL), the forward primer (10μM) (0.8μL), the reverse primer (10μM) (0.8μL), DNA sample (1μL) and the double-distilled water (7.4 μL). And the PCR reaction process was pre-denaturation at 95°C for 30s, then 40 cycles of 95°C, 5s for denaturation and 60°C, 30s for annealing. The specificity of the amplified fragments was checked by melt curve , and the product sizes were determined by 1% agarose gel electrophoresis.

Absolute quantification PCR (qPCR)

Single-band PCR products were purified using the EasyPure PCR Purification Kit and inserted into the T vector by the use of the pEASY-T1 Simple Cloning Kit. The recombinant plasmid DNA was transformed into competent cells . After the mixtures were smeared uniformly on Luria broth (LB) agar plates, they were cultured at 37 °C overnight, then the positive bacterial clones were selected and continued to culture with liquid LB. The recombinant plasmid DNA was isolated by the AxyPrep Plasmid DNA Mini Kit (Axygen, APMNP 50)according to the manufacturer's instruction .The plasmid concentration was measured by spectrophotometry (Nanodrop 2000, Thermofisher) and the quality was visualized via 1% agarose gel electrophoresis. The recombinant plasmid DNA was stored at -80°C for future use.

According to the formula described by Dhanasekaran et al., at the basis of the concentration of recombinant plasmids, the original copy numbers of the recombinant plasmid DNA were calculated and then 10-fold serially diluted to obtain different concentration for constructing the standard curve.

Absolute quantitative PCR was performed with samples and the serially diluted plasmid DAN simultaneously, the PCR reaction mixture and thermocycler conditions were the same as described above. The samples DNA were diluted 10-fold before use and each of them were run in triplicate. The bacterial actual copy numbers in samples were calculated with the Ct value which related to the relevant standard curve [13], and the standard curve was built by forming a liner regression between the copy number of diluted standards (x axis) and the corresponding Ct values (y axis). The amplification efficiency of the plasmid
template was calculated from the slope of the standard curve according to the following formulas: $E = 10^{(-1/slope)} - 1$ (Thermo Fisher Scientific qPCR Efficiency Calculator).

Statistical Analysis

The SPSS software (version 17) was used to analyze the copy numbers of bacterial genera among different samples. The significant differences of bacterial copy numbers at different time points were performed by One-way ANOVAs and Least Significant Difference tests (LSD).

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Abbreviations

Not applicable

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Contributions

Conceived and designed the experiments: JL KL LW. Performed the experiments: LW KL JG YG. Analyzed the data: LW KL DZ YG JL JG ZG. Contributed reagents/materials/analysis tools: LW KL DZ YG JG JL ZG. Wrote the paper: LW JL ZG YC. but all authors contributed to and approved the final version.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional information

Not applicable.

Figure Legend

Fig. 1 Dynamic changes of gut bacteria（*Gilliamella, Snodgrassella, Lactobacillus, Bacillus, Pesudomonas* and *Lactococcus*）in the different time points of unmated male bees. UM: Unmated.

418 **Fig. 2** Dynamic changes of six species of bacteria（*Gilliamella, Snodgrassella, Lactobacillus,*
419 *Bacillus, Pesudomonas* and *Lactococcus*）in different time points of mated male bees. M: Mated.
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Figures

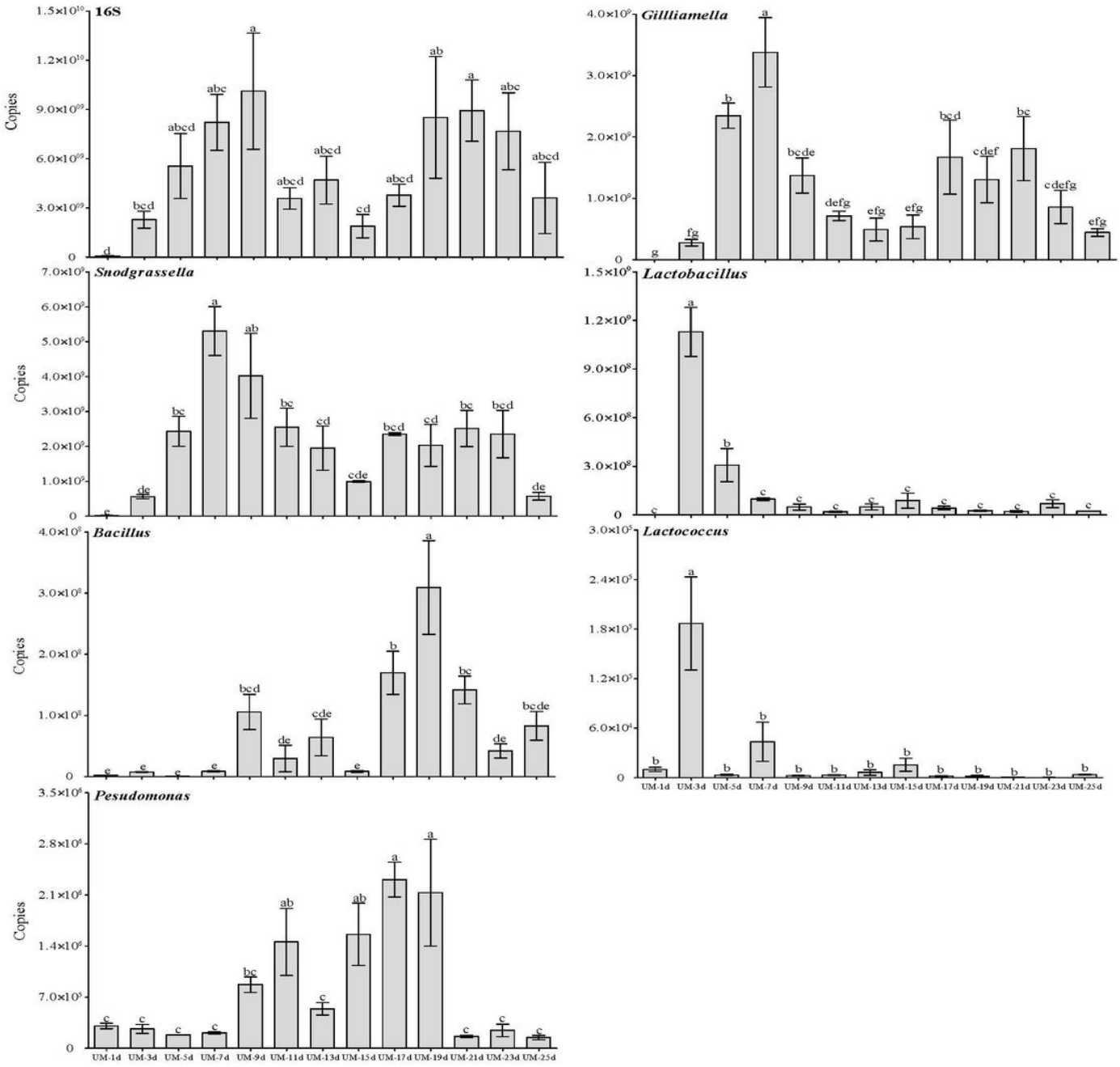

Figure 1

Dynamic changes of gut bacteria(Gilliamella, Snodgrassella, Lactobacillus, Bacillus, Pesudomonas and Lactococcus)in the different time points of unmated male bees. UM: Unmated.

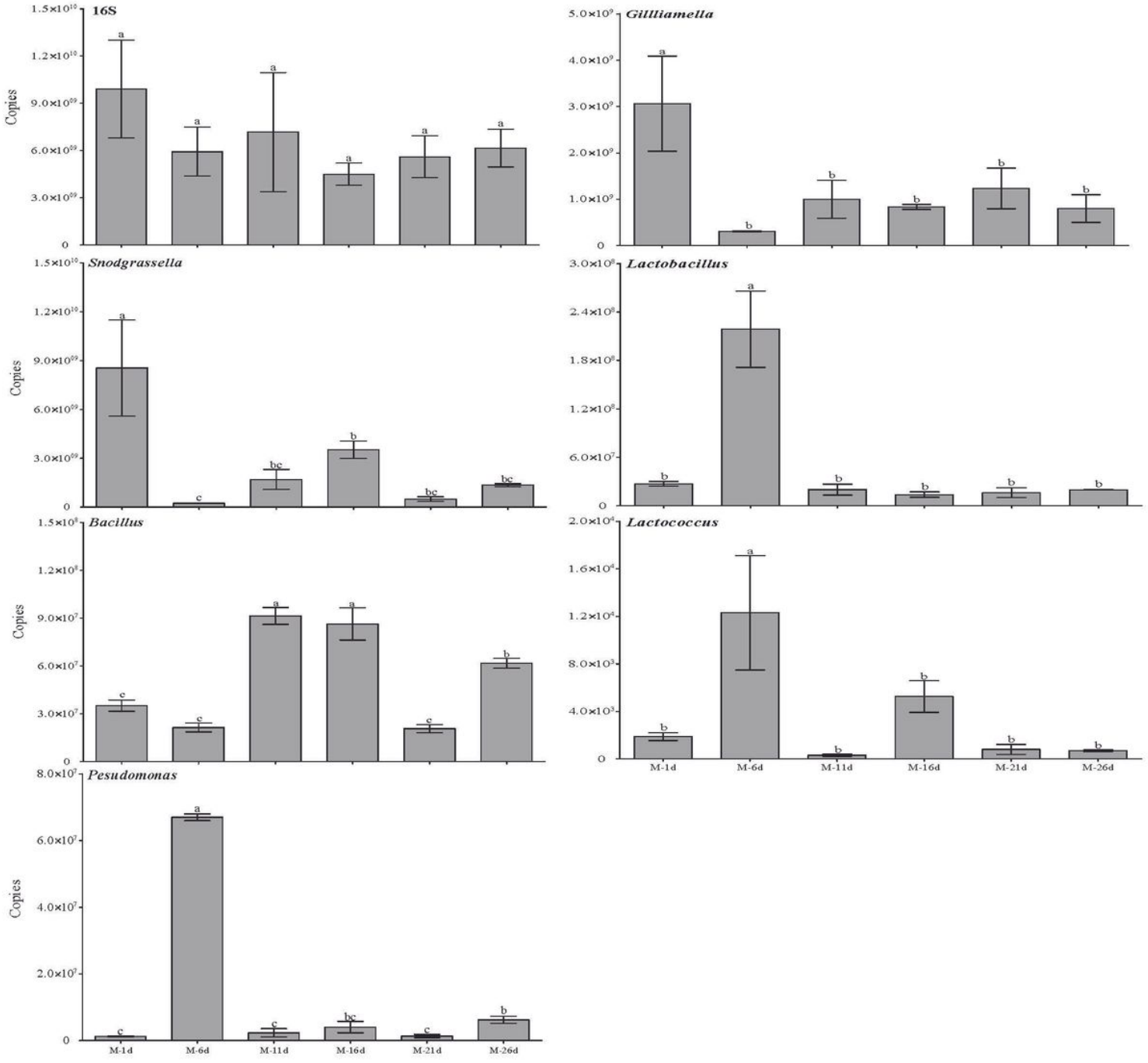

Figure 2

Dynamic changes of six species of bacteria (Gilliamella, Snodgrassella, Lactobacillus, Bacillus, Pesudomonas and Lactococcus)in different time points of mated male bees. M: Mated.