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Effect of transient exposure to carbaryl wettable powder on the gut microbial community of honey bees

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Abstract

Bees are important pollinators in agriculture. The bee population has recently begun to decline possibly due to pesticides. The bee gut microbiota strongly influences the health of bees. The gut microbiota of bees is composed of distinct members belonging to selective taxa. Chemicals like pesticides can alter the gut microbiota. The present study investigated the effect of carbaryl pesticides on gut microbiota of honey bees, which had come in contact with rapeseed plants (*Brassica napus*) sprayed with carbaryl wettable powder during the honey bee brood test under semi-field condition. Molecular techniques (conventional and quantitative polymerase chain reaction (PCR), clone library method, and DNA sequencing) were employed to analyze changes in the microbial communities between the pesticide-exposed and unexposed bees. Phylogenetic analysis of 16S rRNA genes of the clones from both groups, showed differences in their respective compositions of core and non-core bacteria. Both groups contained carbohydrate-degrading bacteria such as *Gilliamella apicola* and *Lactobacillus*. However, the unexposed bees harbored *Alphaproteobacteria*, which were absent in the exposed bees. Microorganisms found in honey bee guts such as *Snodgrassella alvi* and *L. kullabergensis*, however, were observed only in the exposed bees, but not in the unexposed bees. The difference between the two groups was distinctly recognized when copy numbers of 16S rRNA genes were compared by quantitative PCR. Results showed that the average gene copy number for the unexposed bees was higher than that for the exposed bees. This may indicate the toxic effect of pesticides on bees and gut microbiota.

Keywords: Gut microbiota, Honey bee, Pesticide, Toxicity

Introduction

Pollinators that move pollen from anther to stigma in flowers include ants, bats, beetles, birds, butterflies, flies, moths, wasps, and bees. Among these organisms, bees play a major role as pollinators in the agricultural sector [1–3]. One-third of the crops for food consumption rely on bee pollination. A rough estimation of their contribution to the economy worldwide is more than $200 billion [4, 5]. In recent years, there has been a decline in the population of both feral and managed bees [1, 2, 5]. Diverse causes have been proposed for the decline, including pests such as *Varroa* mites, diseases such as the foul brood, and pesticides [6–8]. Use of pesticides for crop protection has affected non-target organisms such as bees. Exposure of bees to pesticides would involve direct acute exposure as well as steady accumulation of the agrochemicals [9]. An example of this agrochemical is carbamates which have been commonly applied as insecticides among orchards. However, timing of the carbamates application should be taken into consideration especially during the stage of blossom [10]. The members of the honey bee hives subjected to high incidence of exposure to pesticides are the worker bees [2, 11]. This could be supported by the study of Johnson et al. [2] stating that carbaryl was detected up to about 1.4 ppm from

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pollen in hives. The worker bees collect nectar and pollen from different flowers and bring it back to the colony hives. They are also tasked with looking after the larvae, specifically in feeding them through the process known as trophallaxis [12]. This feeding behavior strongly influences the development of microbial community in guts of the juvenile honey bees. The uniformity of microbial composition in the guts of *Apis* and non-*Apis* bees is a result of this feeding behavior [13, 14].

The significance of gut microbiota lies in its symbiotic relationship with the host, and one familiar example for this is the beneficial flora in human gut [15, 16]. Likewise, in lower organism, the host insect provides the microbiota with the necessary nutrients. In return, the microorganisms metabolize the substances that the hosts are usually incapable of digesting [17]. Gut microbiota also provides protection from the invasion of opportunistic pathogenic bacteria, which the insects acquire from the environment [12]. These symbiotic microbes, initially established in the gut, prevent the proliferation of unwanted microorganisms [18]. Similar to other organisms, the health of the honey bees is greatly influenced by the gut microbiota. Disruption of balance in gut microbiome or dysbiosis could result in accrued susceptibility of honey bees to diseases and pathogenic microorganisms [12, 13, 19]. The gut microbiota of honey bees is less complex but highly specialized compared to gut microbiota of other organisms [3, 15]. It has been reported that the honey bee gut microbiota is dominated by nine bacterial species clusters, each species performing specific functions relating to carbohydrate breakdown, host defense, and immunity [3, 17, 18].

Recent studies have given significant attention to the identification of the factors affecting the lability of the composition of the symbiotic gut microbes. One such factor is exposure to chemicals such as pesticides and antibiotics [13, 19]. This exposure includes not only the direct application of the chemicals onto the hives but also the pesticides-applied plants, that the honey bees come in contact with during nectar collection [2, 17]. Exposure to these chemicals can possibly alter the gut microbial composition of honey bees, consequently affecting immune function, metabolism, and susceptibility of these bees to pathogens as well [12].

Since the composition of the gut microbiome of honey bees is rather simple compared to the other organisms, shifts in the microbial communities can be detected more easily. In the present study, the effect of carbaryl, a broad-spectrum insecticide, on the size and composition of bacterial community in the guts of honey bees was investigated.

Materials and methods

Exposure of honey bees to pesticide under semi-field conditions

The semi-field test was performed to assess the effect of carbaryl wettable powder (WP) 50%, Detailed experimental conditions were maintained as described previously [4]. The experiment consisted of three treatment groups (negative control without pesticide exposure, positive control treated with diflubenzuron WP, and the experiment treated with carbaryl WP), with each group in three replicate tunnels. The honey bee colonies were placed in tunnels covering an area of 70 m^2 and containing *Brassica napus*. Negative controls were sprayed with tap water (400 L/ha), while the experiments were sprayed with carbaryl (250 g a.i./ha in 400 L tap water/ha) during active flight of bees. Bees were collected from the negative control and the carbaryl-treated groups, after 2 h of exposure.

Genomic DNA extraction, PCR and clone library construction

The collected bees were placed in 50-mL conical centrifuge tubes and kept in −80 °C prior to dissection. Each honey bee was surface sterilized by washing with 70% ethanol and rinsed with sterile distilled water. A total of 10 honey bees from each group were dissected individually in 70% ethanol to isolate the gut. All the isolated guts were pooled in a 1.5-mL microcentrifuge tube. Total genomic DNA from the pooled isolated guts was extracted according to the manufacturer's instructions indicated in PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA).

The 16S rRNA gene was amplified using the bacterial universal primers 27F and 1492R in T100™ Thermal Cycler (Bio-Rad Laboratories, Inc., Forster City, CA, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 5 min. The final extension was set at 72 °C for 5 min.

Clone libraries were constructed for 16S rRNA genes from the two groups of genomic DNAs. PCR products of 16S rRNA gene were ligated into pLUG-Prime® TA cloning vector (iNtRON Biotechnology, Seongnam, South Korea). The ligated PCR products in the plasmid were then transformed into chemically competent *Escherichia coli* DH5-α cells (Enzynomics, Daejeon, South Korea). The transformed cells were plated onto Lysogeny (LB) agar, supplemented with 100 μg/mL ampicillin (iNtRON Biotechnology, Seongnam, South Korea). Clones containing the insert were selected by blue and white screening.

DNA sequencing and phylogenetic analysis

In order to confirm the insert DNA, randomly selected representative white colonies were subjected to colony PCR using the primers M13F and M13R, and the fragment size of PCR products was checked using agarose gel electrophoresis. After confirmation of the desired band, the PCR products from all the selected white colonies were purified using AccuPrep® PCR Purification Kit (Bioneer Corporation, Daejeon, South Korea). Following quantification of DNA concentration using Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, US), the purified DNA samples were sent for sequencing at Genotech (Daejeon, South Korea).

The obtained contigs for the DNA sequences were first assembled using BioEdit version 7.0.5.3, followed by manual editing. The edited assembled DNA sequences were then searched for similar DNA sequences using National Center for Biotechnology Information (NCBI) BLAST. The sequences of both the clones and the obtained similar sequences were aligned using MUSCLE in MEGA7. The phylogenetic trees were then constructed using the neighbor-joining clustering method and Kimura 2-parameter distance model with 1000 bootstrap replications.

DNA sequences from the clones were deposited at NCBI GenBank with accession numbers MH842171 to MH842195 and MH879846 to MH879858.

Quantitative real-time PCR

For quantification of 16S rRNA gene, quantitative real-time PCR (qPCR) was performed with a standard curve for absolute quantification in CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Forster City, CA, USA). The 16S rRNA gene region was first amplified with some modification from genomic DNA of *E. coli* DH5-α using PCR with primers 27F and 519R [20]. The 16S rRNA gene was cloned into the plasmid as described above and subsequently transformed into competent cells. The plasmid containing 16S rRNA gene (491 bp using 27F and 519R) was utilized to construct the standard curve for absolute quantification of 16S rRNA gene copy numbers. Five series of 10-fold dilution of concentration of the plasmid with the inserted 16S rRNA gene, with three replicates, were analyzed. The master mix for the qPCR was prepared as follows: a total of 25 µL consisting of 10 µL AccuPower® 2X GreenStar qPCR Master Mix (Bioneer Corporation, Daejeon, South Korea), 1 µL each of 27F and 519R (10 µM), 8 µL sterile distilled water, and 5 µL plasmid template. The conditions for the qPCR were set as follows: initial denaturation at 95 °C for 5 min, 24 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s, and extension at 72 °C for 1 min. The melting curve analysis was set between 65 and 95 °C with an increment of 0.5 °C for 5 s. Gene copy number was calculated using the cycle of quantification (Cq) based on the formula indicated by Ritalahti et al. [21]. For the bee samples, qPCR was performed using the same conditions.

Statistical analysis

The single-factor analysis of variance (ANOVA) was performed for the statistical analysis of the gene copy number in Microsoft Excel.

Results

Bacterial community composition

Thirty clones from each clone library of the exposed and unexposed groups were used for the DNA sequence analysis. The primers 27F and 1492R were used to amplify the entire hypervariable regions V1–V9 of the 16S rRNA gene. Each of the 30 sequences from the unexposed and

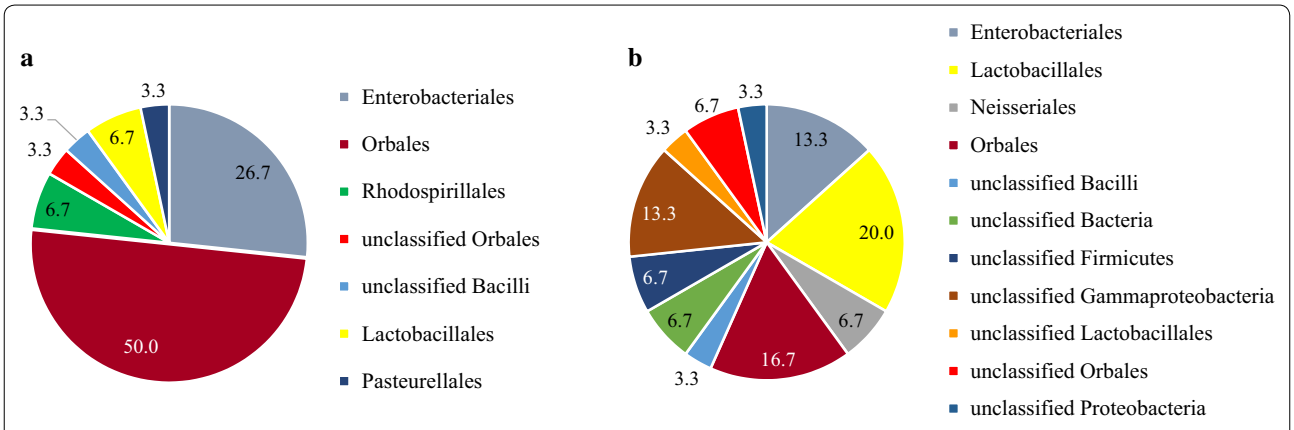

Fig. 1 Compositions of 30 BLAST-matched sequences of 30 DNA sequences from the unexposed bees (**a**), and 30 BLAST-matched sequences of 30 DNA sequences from the exposed bees (**b**) at the order level

exposed bees was compared with the most similar DNA sequence retrieved from BLAST. Both groups showed bacteria belonging to *Enterobacteriales*, *Lactobacillales*, and *Orbales* as the common taxonomic groups. Moreover, the latter two taxa composed the majority of the identity of bacterial species in the unexposed and exposed bees. However, DNA sequences from the unexposed bees were more diverse at bacterial species than those observed in the exposed bees (Fig. 1). The taxonomic groups detected in the unexposed bees which consisted of *Bacillales*, *Pasteurellales*, and *Rhodospirillales* were absent in the exposed bees. Conversely, species from the order *Neisseriales* were detected only in the exposed bees, but not in the unexposed bees.

Phylogenetic analysis of the clone sequences

Differences in the composition of microbial communities between the bee groups were examined based on phylogenetic affiliations. The phylogenetic tree for the unexposed group was split into two as there were nine clones that diverged from the main tree. However, the second tree for the unexposed bees constructed for these nine clones still diverged from the second set of reference bacteria. Hence, this tree was not used for describing the microbial community in the unexposed bees. On the other hand, there was only one phylogenetic tree constructed for the exposed bees.

In the phylogenetic tree of the unexposed bees (Fig. 2), there were six clusters observed. These clusters were grouped into three classes. The first group was class *Alphaproteobacteria* with sequences similar to a genus *Commensalibacter*. The second cluster was class *Bacilli* including sequences similar to genus *Lactobacillus*. The remaining four clusters were grouped under class *Gammaproteobacteria* composed mainly of sequences similar to *Gilliamella apicola*, *Frischella perrara*, and *Klebsiella oxytoca*. There was only one phylogenetic tree generated for the exposed bee group (Fig. 3). In comparison to the phylogenetic tree of the unexposed bees, more clusters were observed in the exposed bees, but the nine clusters could further be grouped into three taxonomic classes; namely classes *Bacilli*, *Gammaproteobacteria*, and *Betaproteobacteria*. Interestingly, class *Alphaproteobacteria* was not observed in the exposed bees, and *Betaproteobacteria* was only recognized in the exposed bees. Although order *Enterobacteriales* was observed in both the unexposed and the exposed bees, more genera were distinguished in the exposed bees as exemplified by *Cronobacter*, *Edwardsiella*, *Erwinia*, *Pantoea*, *Providencia*, *Serratia*, and *Snodgrasella*. In addition, the copy number of the 16S rRNA gene per honey bee gut was analyzed in both groups to suggest relative bacterial abundance between the two groups. The average gene copy number of 16S rRNA gene was higher in the unexposed bees ($2.83 \times 10^6 \pm 1.20 \times 10^6$ per gut) as compared with that in the exposed bees ($5.25 \times 10^5 \pm 9.81 \times 10^3$ per gut) (Fig. 4). The single-factor ANOVA showed that the difference in the average gene copy of 16S rRNA gene was statistically significant with a p value of 0.028 at 95% confidence interval.

Discussion

Previous studies have shown detrimental effects of carbaryl to honey bees. In this study, the potential effect of exposure to carbaryl pesticide on the health condition of the honey bee was looked into. The compositions of microbial communities in both the unexposed and exposed groups of bees were examined by comparing phylogenetic trees from randomly selected clones. According to Engel and Moran [22], bacterial community of honey bee gut was suggested to be composed of only nine species. These species were mostly members of phyla *Firmicutes* and *Bacteroidetes*; classes *Gammaproteobacteria*, *Betaproteobacteria*, and *Alphaproteobacteria*; and genus *Bifidobacterium* [12, 23, 24]. In this study, these bacterial groups, except for *Bifidobacterium* and *Bacteroidetes*, were recognized in both the bee groups. The two bacterial groups in exception have been reported to be often less abundant in bee microbiota [3].

Recently, Bonilla-Rosso and Engel [24] further categorized them into core and non-core members of the gut microbiota. The core members included *Gilliamella apicola*, *Lactobacillus* Firm-5 and Firm-4, *Snodgrassella alvi*, and *Bifidobacterium*. These core microbial members facilitate the acquisition of nutrients via carbohydrate metabolism [17]. The results of this study showed slight differences with respect to the previously described core gut microbial taxa. In this study, *G. apicola* and *Lactobacillus* were observed in both the groups. This was in accordance with the results of previous studies, since the two bacterial taxa have been known to be associated with the metabolism of sugars, derived from nectar, honey, or pollen present in honey bee diets [12, 17, 24]. Additionally, *G. apicola* is also known to contain pectinase, which catabolizes pectin, a component of pollen. The honey bee, itself cannot degrade the pectin, and therefore *G. apicola* might assist the honey bee in obtaining nutrients from the pollen. *Snodgrasella alvi* is another bacterium categorized as core gut member of honey bees. However, this bacterium was absent in the unexposed group. It was recognized only in the exposed group [18, 25]. This result was somewhat contradictory to the previous studies, since *S. alvi* was also abundant in honey bee guts, while working together with *G. apicola* for acquisition of nutrients and for providing defense against pathogens by biofilm formation on the epithelium of the gut [25, 26]. A

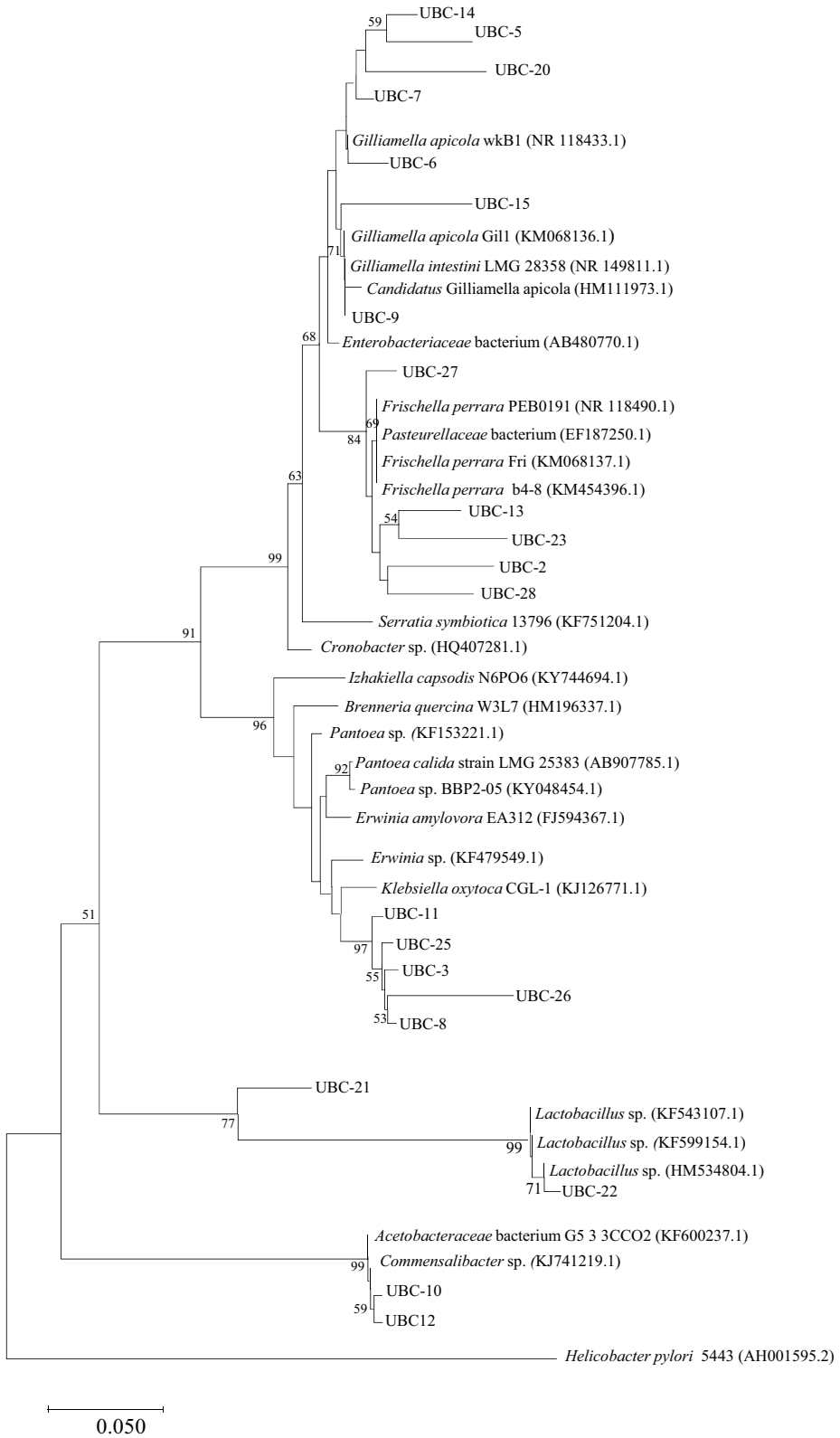

Fig. 2 Phylogenetic tree of the gut microbial clones of the unexposed bees. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances were computed using the Kimura-2 parameter. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

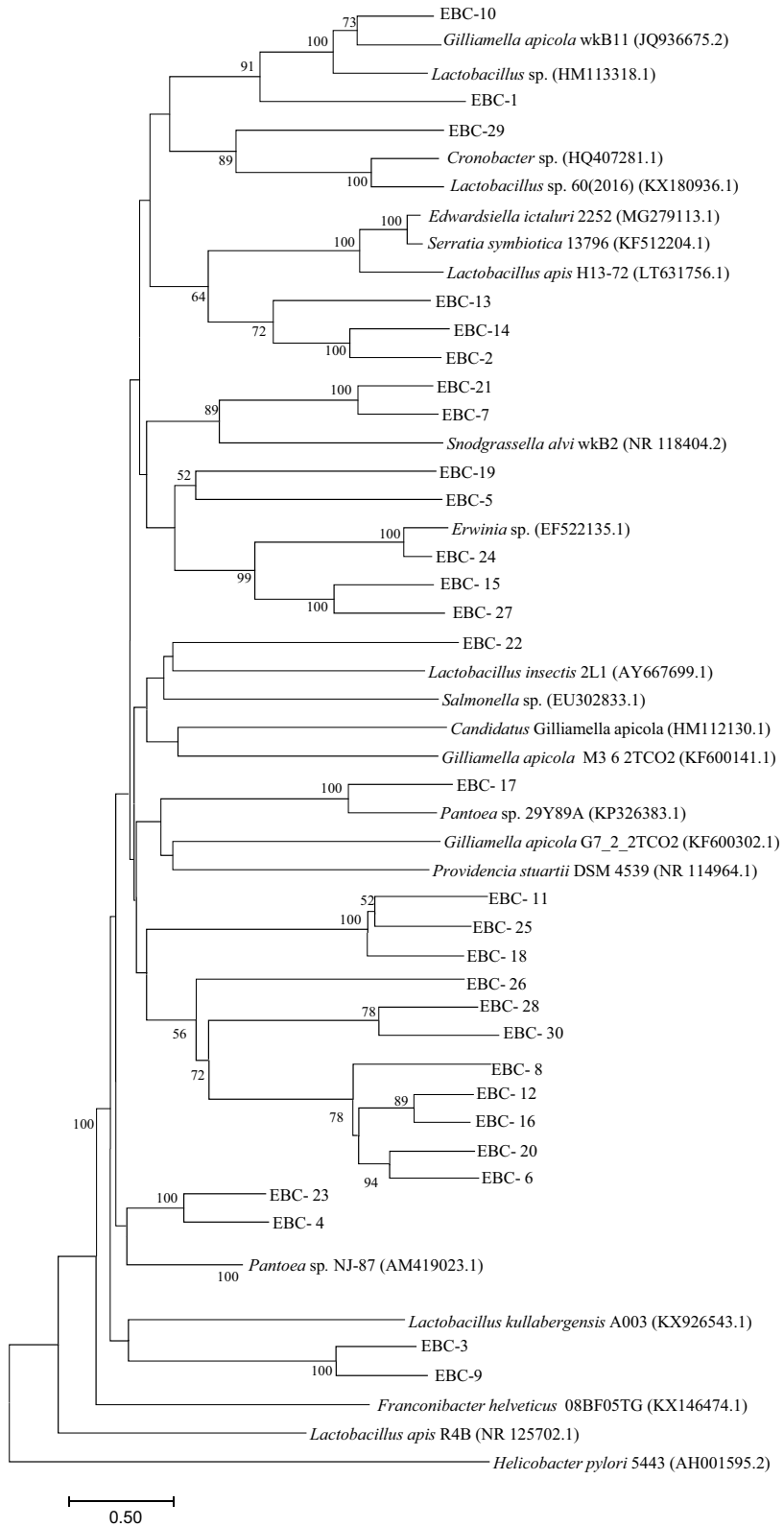

Fig. 3 Phylogenetic tree of the gut microbial clones of the exposed bees. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances were computed using the Kimura-2 parameter. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

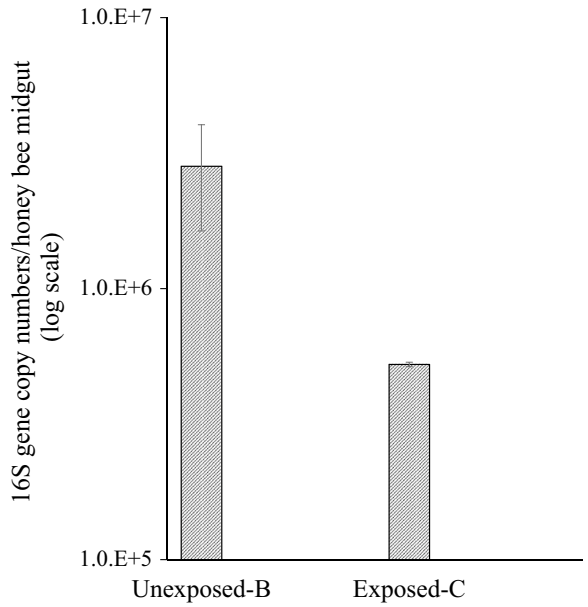

Fig. 4 Average copy numbers of 16S rRNA gene per gut of the unexposed and exposed honey bees to the pesticide of carbaryl. Single-factor ANOVA at 95% confidence interval

prudent explanation to the absence of *S. alvi* in the unexposed bees might be that proportion of this core phylotypes occurred differently among individual bees, even if was is of the same age and from the same colony [3].

As enumerated by Bonilla-Rosso and Engel [24], the non-core bacterial groups included *Frischella perrara*, *Bartonella apis*, *Lactobacillus kunkeei*, *Bombella apis*, and *Commensalibacter* sp. Among these five groups of bacteria, only two were observed in this study. These were *Commensalibacter* and *Frischella perrara*, observed only in the unexposed bees. The *Commensalibacter* is an acetic acid bacterium (AAB), known as a commensal bacterium residing in the guts of insects whose diet is mainly derived from sugar-rich compounds like honey [27]. In relation to the result obtained in this study, the absence of this AAB in the exposed bees can possibly be suggestive of the health status of the exposed bees as explained by Crotti et al. [27]. On the other hand, *F. perrara* has been suggested to be responsible in scab formation in the ileum of the honey bee gut [25]. The scab formation is suggestive of immune regulation of the honey bees against the proliferation of *F. perrara* in the gut, although no study has yet reported any negative effect of *F. perrara* on honey bees.

Lastly, there were other bacteria which could be classified neither as core nor non-core bacteria. These bacteria included *Cronobacter*, *Erwinia*, *Edwardsiella*, *Klebsiella*, *Providencia*, *Serratia*, and *Pantoea*. The bacteria that are not commonly found in the gut microbiota of honey bees could have been acquired from the environment and could be considered as opportunistic pathogens [3, 25, 28]. These uncategorized bacteria were observed in more abundance in the exposed group as compared to the unexposed group. *Klebsiella* was only observed in the unexposed group, while *Cronobacter*, *Edwardsiella*, *Providencia*, *Serratia*, *Erwinia*, and *Pantoea* were observed in the exposed group. Encountering higher frequency of sequences similar to uncategorized bacteria could probably be indicative of disruption of balance of gut microbiome or disease as mentioned in previous studies in relation to dysbiosis in the presence of a potential cause like chemicals [3, 13, 29]. Although it is difficult to conclude decisively that the differences in the composition of the gut microbial communities from the two groups can be attributed directly to the pesticide exposure, Raymann et al. [13] have suggested that one difference between a healthy colony and a colony suffering from colony collapse disorder (CCD) can be a decrease in *Alphaproteobacteria* in gut bacterial communities [19]. This health condition and AAB proliferation is also attested by Crotti et al. [27]. This is in congruence with what was observed in the exposed bees, where there was absence of *Alphaproteobacteria*. Additional basis to infer variation between the two groups was comparison of the total copy number of bacterial 16S rRNA gene. The result showed that average gene copy number per bee gut was roughly an order higher in the unexposed bees with $2.83 \times 10^6 \pm 1.20 \times 10^6$ copy/bee than in the exposed bees with $5.25 \times 10^5 \pm 9.81 \times 10^3$ copy/bee, which was statistically significant at 95% confidence interval. Difference in the 16S rRNA gene copy numbers seems to demonstrate effect of chemical exposure of the bees on the size of the gut bacterial communities. This result is well supported by the previous study indicating that the total gut bacterial abundance decreased among the bees exposed to tetracycline [13].

The results of this study showed the potential effect of carbaryl to the health of individual honey bees as indicated by the changes in composition of the microbial gut. Cumulative consequence of exposure to pesticides affecting the colony development of bumble bees [30], which is a close relative of honey bees, was clearly related to this. On the other hand, this study laid out a new aspect to look into the effect of pesticide in relation to the health of the individual worker bees which could, in cumulative effect, also affect the colony as worker bees carry out different tasks important to the maintenance of the colony [17, 19]. However, the cumulative effect should be studied further to see if it is transitory or has little or no effect to the whole colony.

In the present study, we investigated the effect of transient exposure of honey bees to a pesticide, carbaryl.

Based on the results obtained, there were differences in the members of the gut microbial communities between the unexposed and the exposed bees. These differences were observed in the composition of the core and non-core bacterial communities, as well as those bacteria not categorized as core or non-core gut members such as putative opportunistic pathogens. Moreover, the differences in the gut microbial communities became more apparent, when 16S rRNA gene copy numbers were compared between those groups. It would be valuable to determine in the future whether or not the effect of exposure to pesticides on the composition of the bacterial community is transitory, and therefore whether the bacterial community can be recovered.

Authors' contributions

KN carried out the molecular experiments and drafted the manuscript. SL participated in the molecular experiments and sequencing analysis. KC planned the study, performed the bee-pesticide experiments in the field chambers, and revised the manuscript. J-HL planned the study and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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