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ORIGINAL RESEARCH ARTICLE

Impacts of *Apis cerana* gut microbes on *Nosema ceranae* proliferation in *Apis mellifera*

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ABSTRACT

The gut microbiota is a complex ecosystem including both beneficial and harmful microbes, which is essential for the metabolism, health and immunity of the host. Fecal microbiota transplantation (FMT) is an effective treatment for diseases of altered intestinal microbiota. *Nosema ceranae*, a parasitic fungus in intestinal epithelial cells, destroys the honey bee's gut integrity. This study provided the first report that the number of spores was significantly lower in the native host (Asian honey bee) compared with the novel host (European honey bee). The treatment effect of FMT was denoted by feeding the gut tissue solution from the original host to the new host. The results showed that FMT did not significantly decrease the spore load but enhanced the expression levels of immune genes in the Toll pathway. Our data confirmed that the native host inhibits *N. ceranae* proliferation. Our data suggest microbes of the native host could be an alternative approach to treat the parasite in the novel host.

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KEYWORDS
Fecal microbiota transplantation; *Nosema ceranae*; immune system; *Apis cerana*; *Apis mellifera*

Introduction

Nosema ceranae is an obligatory intracellular parasite of honey bees, and its original host is the Asian honey bee (*Apis cerana*) (Burri et al., 2006; Feng et al., 2014; Sinpoo et al., 2018). *N. ceranae* has successfully infected the European honey bee *A. mellifera*, where high virulence has been reported (BenVau & Nieh, 2017; Paxton et al., 2007). Honey bees showed a decline in foraging activity ahead of time, reduced life, and suppressed immune response after being infected by *N. ceranae* (Goblirsch et al., 2013; Mayack & Naug, 2009; McDonnell et al., 2013; Naug & Gibbs, 2009). *N. ceranae* drives colony loss in Spain (Mañes, 2017).

The gut microbiota is emerging as a key player governing host health, which affects host metabolism, immune system modulation and disease resistance (Harris et al., 2012; Heintz-Buschart & Wilmes, 2018). For instance, *Bombus terrestris* treated with antibiotics or aseptic showed increased susceptibility to the trypanosomatid parasite *Crithidia bombi* (Koch & Schmid-Hempel, 2011). It suggests that the normal microbiota has an inhibitory effect on pathogenic microorganisms. In addition, some specific gut microbes could effectively suppress the pathogens (Rangan & Hang, 2017). *Escherichia coli Nissle1917* competitively reduces *Salmonella* colonization (Deriu et al., 2013). *Enterococcus fecalis* prevents *Staphylococcus aureus* and *Salmonella enterica* infections in worms (*Caenorhabditis elegans*) (Deriu et al., 2013; King et al., 2016). Five bacterial species groups (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus Firm-4*, *Lactobacillus Firm-5* and *Bifidobacterium asteroides*) constitute mainly the gut microbiota of *Apis mellifera*. Four bacterial species groups (*Bifidobacterium*, *Snodgrassella alvi*, *Gilliamella apicola*, and *Lactobacillus*) are considered to be the major gut bacteria of *Apis cerana* (Guo et al., 2015). *Lactobacillus* and *Bifidobacterium* could inhibit the growth of *Paenibacillus larvae* in honey bee larvae (Forsgren et al., 2010). Honey bees with a healthy gut microbiota showed significantly higher survival compared with dysbiosis ones after virus infection (Dosch et al., 2021).

Four immune pathways (Toll, Imd, JNK, and JAK/STAT) in *A. mellifera* respond to bacteria and *Nosema* infection (Antúnez et al., 2009; Evans et al., 2006).

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Z.W. collected field data, carried out statistical analyses, participated in the design of the study and drafted the manuscript; X.W. carried out the molecular lab work, participated in data analysis; Q.H. critically revised the manuscript and proved financial support; L.Z, Z.Z. and W.Y. conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

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Honey bees showed suppressed immune response after being infected by *N. ceranae*. But the *Apis mellifera* gut microbiota can promote the expression of *apidaecin* and *hymenoptaecin* (Kwong et al., 2017), two antimicrobial peptides, and the gut bacteria *Snodgrassella alvi* and *Frischella perrara* can activate the host's immune system (Emery et al., 2017). In addition, *A. cerana* enlisted a higher immune response and fewer offspring spores compared with *A. mellifera* (Sinpoo et al., 2018).

A. mellifera and *A. cerana* are two sister honey bee species (Lourenço et al., 2005). Although the two bee species are in close contact, they maintained their unique microbiome characteristics (Casteels et al., 1989). The gut microbial composition of honey bees based on 16S rRNA gene sequencing showed that *A. mellifera* has relatively fewer microbiota in the midgut compared with *A. cerana*. *A. mellifera* has a lower level of Lactobacillus in the midgut (Ahn et al., 2012). In addition, the contribution of non-culturable and other bacteria could not be neglected in host health (Kwong & Moran, 2016)

Fecal microbiota transplantation (FMT) is a method for the treatment of diseases caused by intestinal microbial defects, that transfer stool from a "healthy" donor to a recipient (Vindigni & Surawicz, 2017). This provides conditions and values for exchange host colonization induced by internal bacteria in the laboratory (Antúnez et al., 2009). In *A. cerana*, the workers inoculated with intestinal bacteria were more resistant to *N. ceranae* than germ-free gut microbiota deficient workers (Wu et al., 2020). The life of older African turquoise killifish (*Nothobranchius furzeri*) can be increased by transplanting the gut microbes of younger fish to older fish (Smith et al., 2017).

We hypothesize that the gut microbes in native hosts might be involved in inhibiting parasite proliferation. To verify the hypothesis, we first proved a smaller number of spores can be found in native hosts compared with novel hosts in natural conditions. Then the gut microbe of the native host was transferred to the novel host, followed by the parasite inoculation, to quantify the parasite offspring spores and host immune responses.

Materials and methods

Quantification of *N. ceranae* spores in natural condition

The two honey bee species (*A. mellifera* and *A. cerana*) were collected from the experimental apiaries in the Honey bee Research Institute, Jiangxi Agricultural University (28.77° N, 115.83° E). For each colony, 30 foragers with pollen loads were captured at the hive entrance (Kaneko et al., 2010). In total, 1140 foragers were collected from 19 *A. mellifera* and 19 *A. cerana* colonies. *N. ceranae* spores were isolated from honey bee midgut tissues. Spores were purified using Percoll (Supplementary Material) (Cornman et al., 2009). The parasite species was determined using *N. ceranae* specific PCR method (Fries et al., 2013). The spore load was counted using 400× optical microscope (Nikon, Tokyo, Japan) and 25×16 type hemacytometer (Qiujing, Shanghai, China). The method of counting the spore number is described in Supplementary Materials.

Gut tissue solution preparation

Sixteen *A. cerana* foragers with pollen loads were captured at the hive entrance (Kaneko et al., 2010). The midguts were dissected, pooled and homogenized in 1 ml PBS. The solution was centrifuged at 1000 g for 10 min. The supernatant was removed to collect the pellet (Wu et al., 2020), which was again resuspended in 1 ml PBS. The suspension was diluted in 24 ml 50% sterile sucrose solution.

N. ceranae spore and gut tissue inoculation

Sealed brood combs from an *A. mellifera* colony were kept in the incubator with 34 ± 1 °C and 50% humidity until the emergence of young workers. The newly emerged (< 24 h) workers were randomly distributed into rearing cups, with 40 bees per cup. Three experimental groups and an additional blank group were constructed. The blank group of workers received no treatment. The honey bees inoculated with gut tissue solution was defined as Group I. The honey bees inoculated with gut tissue solution with 10^5 *N. ceranae* spores were defined as Group II. The honey bees inoculated with 10^5 *N. ceranae* spores without gut tissue were defined as Group III. The blank group of workers was Group IV that received no treatment (Supplementary Table S1). The purified spores were diluted to a concentration of 5.0×10^4 spores/μl in sucrose solution. The workers of Group II and Group III were inoculated with 2 μl spores sucrose solution alone (Fries et al., 2013). During 14 days experimental period, sucrose solution was the only food *ad libitum*. The number of dead bees was recorded and removed daily. Ten bees per cup were collected to count the spore load at 14 days post infection (dpi). The remaining bees were used for RNA extraction to quantify the expression level of immune genes.

The expression levels of immune genes

The midgut of individual bees was dissected. Two midgut tissues were pooled for total RNA extraction using TRIzol (Invitrogen, USA). RNA reverse transcription and Real-time quantitative PCR were carried out using Reverse Transcription Kit and TB Green Premix Ex TaqTM II (Takara). The 20 μl reaction system was

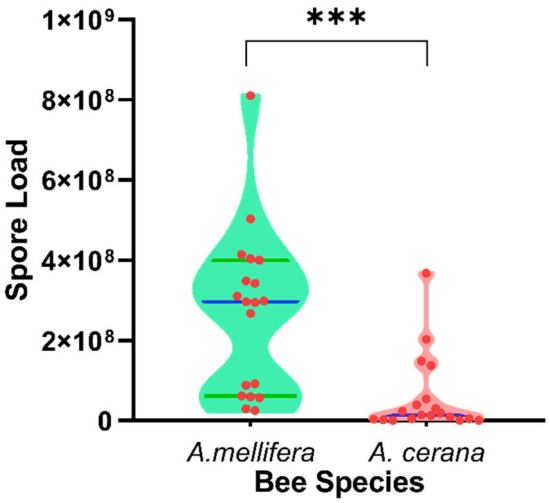

Figure 1. The number of *N. ceranae* spores found in the two honey bee species. The number of spores was significantly higher in the honey bee *A. mellifera* compared with *A. cerana* (Mann Whitney *U*-test: $U = 41$; $P < 0.001$). ***represents the significant level at $P < 0.001$.

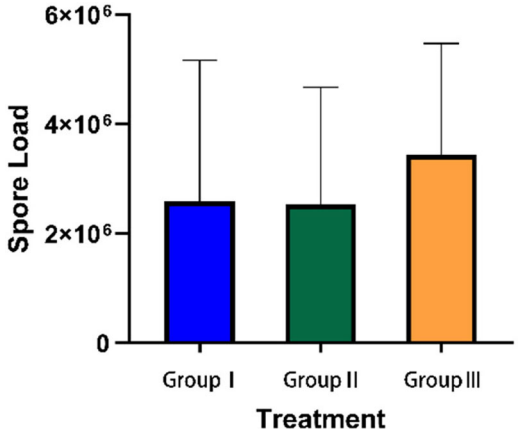

Figure 2. Effect of gut tissue solution on spore load of *A. mellifera*. On average, Group III showed the highest number of spores ($3.431 \times 10^6 \pm 6.452 \times 10^5$). Even though statistically not significant compared with Group I and Group II. Bars indicate mean values ± standard deviation. Group I: Gut tissue solution, Group II: Gut tissue solution+ 10^5 *N. ceranae*, Group III: 10^5 *N. ceranae*.

composed of 10 µl TB Green Fast qPCR Mix, ROX Reference Dye (0.4 µl), upstream primer (0.8 µl), downstream primer (0.8 µl), cDNA (2 µl) and ddH_2O (6 µl). The PCR protocol was as follows: Initial denaturation at 94 °C for 30 sec; denaturation at 94 °C for 5 sec and 40 cycles for elongation at 60 °C for 60 sec. A total of 22 immune genes (Evans, 2006) and 2 reference genes (Liu et al., 2020; Van Hiel et al., 2009) were quantified (Supplementary Table S2).

Data analysis and statistics

All data were tested for normal distribution with the Kolmogorov–Smirnov test. Data were analyzed for normal distribution. Parametric tests were used for normally distributed data, otherwise, non-parametric statistics were applied. The spore load variance between the-two honey bee species in natural conditions was analysed with the non-parametric Mann-Whitney *U*-test. The spore load variance among the three treatment groups was analysed with one-way ANOVA test. The amplification efficiency for each qPCR reaction was calculated based on the strength of the fluorescence for each cycle using qPCR package, R (Pabinger et al., 2014; Spiess & Spiess, 2018). The average amplification efficiency of all reactions in a gene was used to calculate the relative gene expression. The relative expression of immune genes among the three groups was analysed with the Kruskal-Wallis test. All statistical analysis was performed using SPSS version 26.

Results

Spore loads in natural condition

An average of $5{,}651 \times 10^7$ spores per bee were counted in *A. cerana*, whereas $2{,}686 \times 10^8$ spores were found in *A. mellifera* foragers. After normalizing the body weight, a significantly higher number of spores was found in the honey bee *A. mellifera* compared with *A. cerana* (Mann Whitney *U*-test: $U = 41$; $P < 0.001$, Figure 1).

Effect of gut tissue solution on N. ceranae proliferation in A. mellifera

At 14 dpi, 10 bees from each group were collected to count the spores. The number of spores in Group III ($3.431 \times 10^6 \pm 6.452 \times 10^5$) was higher than Group I ($2.594 \times 10^6 \pm 8.134 \times 10^5$) and Group II ($2.538 \times 10^6 \pm 6.755 \times 10^5$). Overall, the variance of spores was not statistically significant among the three Groups (ANOVA, $F(2,27) = 0.848$, $P > 0.05$) (Figure 2).

Effect of gut tissue solution and N. ceranae inoculation on immune gene expression

Among the 22 immune genes in the three experimental groups (Group I, II, III), three genes were significantly upregulated in bees provided with gut tissue solution (Figure 3). The relative expression level of *AmPPo* was significantly higher in Group II than Group I. The relative expression level of *dorsal-1* was again significantly higher in Group II than Group III. For the gene *Apidaecin*, the relative expression level in group III was significantly lower than Group I and group II.

Compared with the control group, the relative expression level of *dorsal-1* was significantly up-regulated in Group I ($P < 0.05$) and Group II ($P < 0.05$), and up-regulated in Group III ($P > 0.05$). The relative expression level of *Apidaecin* was again significantly up-regulated in Group I ($P < 0.05$) and Group II

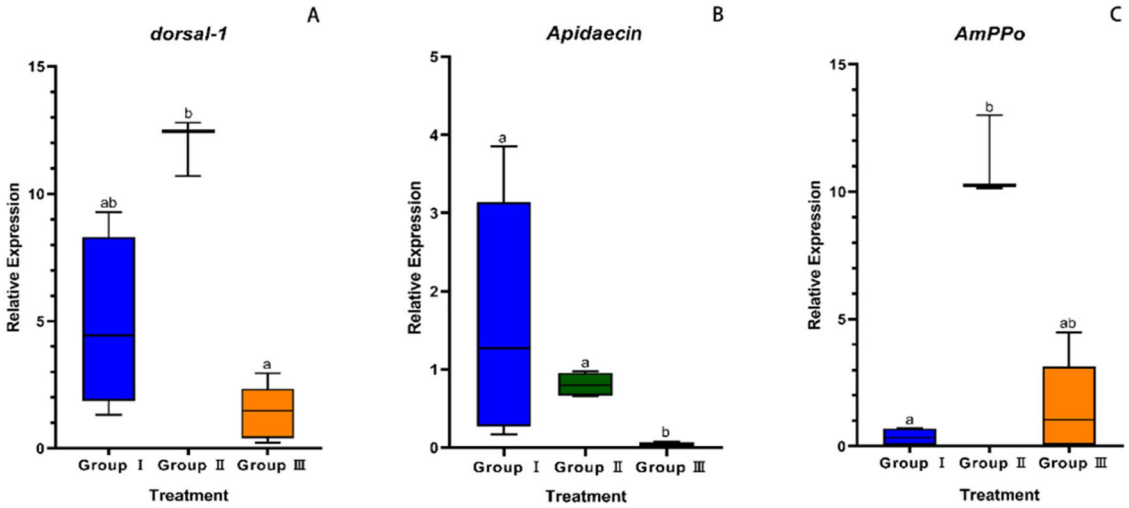

Figure 3. The relative gene expression level of *AmPPo*, *dorsal-1* and *Apidaecin* among the three groups. Overall, the feeding of gut tissue solution enhanced the expression level of the three genes. The relative expression was analyzed using the non-parametric Kruskal-Wallis test. Data are represented as lower quartile, median and upper quartile (boxes), and minimum and maximum ranges (whiskers). Group I: Gut tissue solution, Group II: Gut tissue solution+ 10^5 *N. ceranae*, Goup III: 10^5 *N. ceranae*. The different letters indicated significant differences, $P < 0.05$.

($P < 0.05$), and up-regulated in Group III ($P > 0.05$). The relative expression level of *AmPPo* was significantly down-regulated in Group IV than Group II ($P < 0.05$) and Group III ($P < 0.05$), down-regulated in Group I ($P > 0.05$).

Discussion

Our investigation verifies the hypothesis that the parasite load differs between *A. cerana* and *A. mellifera* under natural conditions. Artificial inoculation of *N. ceranae* in two honey bee species also indicated the native host defends *N. ceranae* infection more effectively by a stronger immune response compared with the novel host (Sinpoo et al., 2018). A balance of intestinal microbial flora plays a vital role in host health, it depends on the composition of intestinal microflora and the bidirectional interactions between the host immune system and the microbiota (Cotter, 2011). In our data, the spores load of Group II was 26% lower than Group III, even though statistically not significant. This result may be related to the activation of the *A. mellifera* immune response by the gut tissue solution.

The Toll pathway of honey bees and *Drosophila* play critical roles in sensing infection by fungi, and trigger a series of follow-up reactions (Beutler, 2004; Evans et al., 2006; Li et al., 2018). The interactions between gut microbiota and the toll-like receptors (TLRs) help maintain the homeostasis of the host immune system (Yiu et al., 2017). For the three immune genes (*AmPPo*, *dorsal-1* and *Apidaecin*), the expression levels were higher in the treatment groups than Group IV. Our results clearly demonstrate that feeding gut tissue and *N. ceranae* infection upregulate the expression levels of immune genes. But the three immune genes expression levels of Group III were lower than Group II. This phenomenon may be related to the immunosuppression of *N. ceranae* on the host (Antúnez et al., 2009; Chaimanee et al., 2012), but it cannot be ruled out that there are not enough gut microbes in the midgut of Group III to promote the expression of host immune genes. The change in upstream *dorsal-1* gene expression initiates responses to the downstream genes *AmPPo* and *Apidaecin* in the Toll immune pathway. Insect melanogenesis is an important defense against pathogenic microorganism (Eleftherianos & Revenis, 2011). Phenoloxidase (PO) is the most important enzyme in melanin biosynthesis (Sussman, 1949). *AmPPo* is involved in the melanisation immune response by regulating the activity of PO (Lourenço et al., 2005). *Apidaecin* is in the class of proline rich antimicrobial peptides, and plays an important role in resisting microbial infections (Casteels et al., 1989).

A. mellifera and *A. cerana* are two sister honey bee species (Liu et al., 2014). Although the two bee species are in close contact, they maintained their unique microbiome characteristics (Ellegaard et al., 2020). Gut microbial composition of honey bees based on 16S rRNA gene sequencing showed that *A. mellifera* and *A. cerana* have 97% similarity (Evans et al., 2006). There may be more gut microbiota colonization derived from the gut tissue solution. The colonizing microbiota required validation by genome sequencing.

A. cerana is the original host of *N. ceranae*, and has evolved tolerance toward microspores compared with *A. mellifera* in both natural and experimental conditions (Fries et al., 1996; Sinpoo et al., 2018). Among the three immune genes we considered, the gene

expression of Group III was always maintained at a low level, but the gut tissue solution changed this situation. The gene expression of *dorsal-1* and *Apidaecin* in Group I and Group II is higher than Group III, but gut tissue solution did not elevate expression levels of *AmPPo*. The initial inoculation dose of spores affected the melanin biosynthesis. This result may provide good proof for further interpreting the competitive role of *A. cerana* gut bacteria and spores.

Nosemosis is an infectious disease caused by *Nosema apis* and *N. ceranae*. But the effects of *N. ceranae* on bee colony health have been reported to be more severe compared to infections by *N. apis* (Chaimanee et al., 2010; Chen et al., 2008; Fries et al., 2006). Our results for the first time the parasite load difference between *A. cerana* and *A. mellifera* under natural conditions. However, more survey results are required to draw definitive conclusions. Transplantation of gut microbes could be an alternative method to treat gut parasites.

Data accessibility

All the data generated and used in our study are included in the electronic supplementary material: table S1 includes the spore inoculation quantity and food type, table S2 includes the primer sequence of 22 immune genes and two reference genes, table S3 includes the spore loads in natural condition, table S4 includes the spore loads by treatment, table S5 includes the average expression level of 22 immune-related genes in each group and table S6 includes the expression level of 22 immune-related genes in each honey bee.

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Disclosure statement

We have no competing interests.

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