



Midgut Bacterial Communities of *Vespa velutina* Lepeletier (Hymenoptera: Vespidae)

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Vespa velutina nigrithorax and *Vespa velutina auraria* are two subspecies of *Vespa velutina* Lepeletier. *V. velutina* preys managed honey bees, other pollinators, and insects. However, the *Vespa* midgut microbiota of three forms, namely queens, workers, and males have not been reported, thus the objective of this study was to analyze the midgut bacterial diversity of the three forms of *V. v. nigrithorax* and *V. v. auraria*. Our results showed that *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Tenericutes*, and *Actinobacteria* were the most abundant phyla, and *Lactobacillus* (17.21%) and *Sphingomonas* (11.39%) were the most abundant genera in the midgut of *V. v. nigrithorax* and *V. v. auraria*. We found that the midgut bacterial compositions of the *V. velutina* males were special, in terms of richness and diversity of bacterial communities, as well as the content of lactic acid bacteria. By comparing the gut bacterial compositions of *Vespa* from different regions (Japan, South Korea, Italy, and China), it was discovered that the gut bacterial compositions were very similar at the phylum and class level, and Gammaproteobacteria, Bacilli, and Alphaproteobacteria were the most abundant classes of bacteria and consistent in the genus *Vespa*. Besides, though *Vespa* from different regions had quite different gut bacterial communities at the genus level, *Lactobacillus* and other lactic acid bacteria were abundant and played important roles in protection and metabolism in *V. velutina* collected from different regions. This is the first report of midgut bacterial diversity of three forms queens, workers, and males of *V. velutina*. Our findings provide insight that *Proteobacteria* and *Firmicutes* (especially *Lactobacillus* and other lactic acid bacteria) are consistent and may play important roles in the genus *Vespa*. The understanding of the microbiome in the midgut of *Vespa* and the discovery of the vital bacteria would provide useful information to design pest biological control agents. Thus, the significance of this study is to provide a basis for the study of the relationship between gut microbiota and physiology and health of *Vespa*, as well as the control of *Vespa*.

Keywords: *Vespa*, three forms, midgut bacterial community, *Proteobacteria*, *Firmicutes*, *Lactobacillus*

INTRODUCTION

Vespa velutina Lepeletier is an Asian hornet that includes three forms, namely queens, workers, and males. The hornet heavily preys *Apis mellifera* (Tan et al., 2007; Monceau et al., 2013), and *Apis cerana* (Tan et al., 2007) in both native and invaded areas. Some reviews have summarized the information on *V. velutina* (Monceau et al., 2014; Kishi and Goka, 2017).

Vespa velutina nigrithorax du Buysson and *Vespa velutina auraria* Smith are subspecies of *V. velutina* (Archer, 1993; Nguyen et al., 2006). The yellow-legged hornet, *V. v. nigrithorax* has established its population in parts of Europe (Villemant et al., 2011) including France (Villemant et al., 2011; Robinet et al., 2017), Spain (Lopez et al., 2011; Goldarazena et al., 2015; Leza et al., 2018), Italy (Bertolino et al., 2016), Germany (Bertolino et al., 2016), the United Kingdom (Budge et al., 2017), and the Netherlands and Switzerland (Robinet et al., 2019). *V. v. nigrithorax* has also established its population in South Korea (Kim et al., 2006; Choi et al., 2012, 2013). The invasion risk of *V. v. nigrithorax* has been assessed by Villemant et al. (2011) and Barbet-Massin et al. (2013). The yellow-legged hornet preys on managed honey bees and other insects (Monceau et al., 2013, 2014; Arca et al., 2014, 2015).

Recently, many studies focused on the hornet such as microsatellite variation (Choi et al., 2013), the complete mitochondrial genome (Kim et al., 2017), rapid molecular methods for identification (Stainton et al., 2018), flight capacities (Sauvard et al., 2018), control (Turchi and Derijard, 2018), as well as the gut microbiota. Gut bacteria play an essential role in nutrition, digestion, detoxification, memory variation, and the efficiency of protection against pathogens (Cardoza et al., 2006; Engel et al., 2012), for example, bacteria in honey bee gut (Engel and Moran, 2013; Kwong et al., 2014; Jia et al., 2016, 2017; Li et al., 2021). Suenami et al. (2019) characterized and compared the gut microbiome in two hornet species, *Vespa mandarinia* and *Vespa simillima*. The two species have simple gut microbiota, composed of seven or eight “core” operational taxonomic units (OTUs). Seo et al. (2018) briefly compared the intestine bacterial microbiota of Asian hornets (*V. v. nigrithorax*) and honey bees and found out that the relative ratio of bacterial populations of Asian hornets was different from that of honey bees. However, the intestine bacterial diversity in three forms of hornet has not been investigated. Although the gut microbial composition in different castes of the *V. v. nigrithorax* was reported recently (Cini et al., 2020), the gut microbial composition of the males, one of the three forms, was not investigated. Besides, the gut microbial community of *V. v. auraria* has also not been reported. Furthermore, the midgut is the largest digestive organ of bees, and the microbes in it are very important for their health and fitness, while the above studies collected the whole intestine as samples and did not focus on the microbial composition in the midgut of *V. velutina*. Therefore, the studies of *Vespa* midgut bacteria are still limited.

In this study, we analyzed the midgut bacterial communities of queens, workers, and males of *V. v. nigrithorax* and *V. v. auraria*

through sequencing technology. We found that the midgut bacterial compositions of the *V. velutina* males were special, in terms of richness and diversity of bacterial communities, as well as the content of lactic acid bacteria. When considering the queens, differences in forms did not seem to have an obvious effect on the midgut bacterial communities of *V. velutina*. Moreover, the midgut bacterial communities of *V. v. nigrithorax* and *V. v. auraria* were similar. Besides, the gut bacterial compositions of *Vespa* from different species or countries were quite different at the genus level. It was remarkable that *Lactobacillus* and other lactic acid bacteria were abundant and might play important roles in studies related to *V. velutina*. Here we provide a characterization of the midgut bacterial composition of *V. velutina* and found the specificity of the midgut microbiome of *V. velutina* males. The understanding of the microbiome in the midgut of *Vespa* and the discovery of the vital bacteria would provide useful information to design a more efficient method of control through the development of a set of natural enemies as biological control agents.

MATERIALS AND METHODS

Vespa Samples

The experimental samples were collected in November 2018, *V. v. auraria* from Shijingshan (N40°13'35.10", E116°04'15.63") in Beijing, and *V. v. nigrithorax* from Nanchang (N28°25'9.78", E114°41'29.94") in Jiangxi Province, China.

Dissection and DNA Extraction

The queens, workers, and males of *V. v. auraria* and *V. v. nigrithorax* were dissected. Five males, five queens, and five workers were taken from each of the five colonies of *V. v. nigrithorax* and five colonies of *V. v. auraria*. All of the queen samples in this study were really gynes. A total of 150 individuals, which included 75 samples of each subspecies, were placed in the refrigerator for 60 s at -20°C , and the midguts were dissected on ice using sterile forceps. Five midguts were pooled in a 2 ml sterile centrifuge tube and treated with liquid nitrogen before being stored in the freezer at -80°C for subsequent DNA extraction. The DNA of midgut bacteria was extracted referring to the previous methods (Dai et al., 2018). Total genomic DNA was quantified by BioSpectrometerR kinetic (Eppendorf, Germany), and their integrity was verified using agarose electrophoresis. The bacterial 16S rRNA gene V3–V4 region was targeted with the primer pair 341f/806r (341F: CCTAYGGGRBGCASCAG, 806R: GGACTACNNGGTATCTAAT) for the microbial community diversity analysis, and the purified products were sequenced on the Illumina HiSeq 2500 platform at Novogene Bioinformatics Technology Co., Ltd., Beijing, China (Dai et al., 2018). The obtained sequences were normalized to make the samples comparable at the same sequencing depth for the following analysis. To identify specific taxa in the midgut microbiota, we performed a linear discriminant analysis effect size (LEFSe) analysis that revealed changes in the abundance of bacterial taxa accounted for the observed differences in the midgut microbiota.

Statistics

The normalized sequences were classified into OTUs at 97% similarity using UCLUST (Version 1.2.22). The taxonomy of the OTUs was assigned by blasting against SILVA database 132 with default parameters. Alpha diversity observed species (richness) and the Shannon index (diversity indices) were performed with Mothur (Version v.1.30). Beta diversity including unweighted unifrac distances between samples was performed with QIIME (Version 1.8.0). The UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) cluster analysis was performed using the Unweighted Unifrac Distance matrix.

RESULTS

Sequencing Results and Quality Control

Paired-end sequencing of 16S rRNA V3–V4 gene was produced from 30 samples. After trimming the barcodes, primers and filtering chimeras, short and low-quality reads, 75,715 effective sequences were obtained, and the average length of effective sequence reads was 416 bp. In all samples of three forms of *V. v. nigrithorax* and *V. v. auraria*, 93.9% of the sequences were assigned to the phylum level, 90.6% to the class level, 84.4% to the order level, 76.6% to the family level, 51.0% to the genus level, and 16.4% to the species level.

Alpha Diversity

Beeswarm plots of observed OTUs and the Shannon index for individual sample groups are shown in **Figure 1**. There were significant differences between *V. v. auraria* males and workers ($p = 0.006$), between males of *V. v. auraria* and *V. v. nigrithorax* ($p = 0.007$) in the observed species (**Figure 1A**). There were significant differences between *V. v. auraria* males and workers ($p = 0.0141$), and between *V. v. nigrithorax* males and workers ($p = 0.0155$) in the Shannon index (**Figure 1B**). It was suggested that in *V. v. auraria*, males showed significantly higher richness rank and a higher level of diversity compared to the workers. On the contrary, in *V. v. nigrithorax*, workers showed a significantly higher level of diversity compared to the males. Moreover, when comparing the two subspecies, the richness and diversity of bacterial communities in the males of *V. v. auraria* were significantly higher than that in the males of *V. v. nigrithorax*.

Intestinal Bacterial Communities

Based on the average relative abundance, *Proteobacteria* (51.13%), *Firmicutes* (36.35%), *Bacteroidetes* (5.12%), *Tenericutes* (3.15%), and *Actinobacteria* (2.22%) were the most abundant phyla in the midgut of three types of *V. v. nigrithorax* and *V. v. auraria* (**Figure 2**). γ -Proteobacteria (32.51%), Bacilli (28.05%), and α -Proteobacteria (18.46%) were the highest abundant classes. Enterobacteriaceae (23.83%), Streptococcaceae (17.92%), and Sphingomonadaceae (11.46%) were the most abundant family. Major genera (abundant >1%) were *Lactobacillus*, *Sphingomonas*, *Spiroplasma*, *Weissella*, *Serratia*, *Megamonas*, *Fructobacillus*, *Moraxella*, and *Bombella*. *Lactobacillus* (17.21%) and *Sphingomonas* (11.39%) were the most abundant genera.

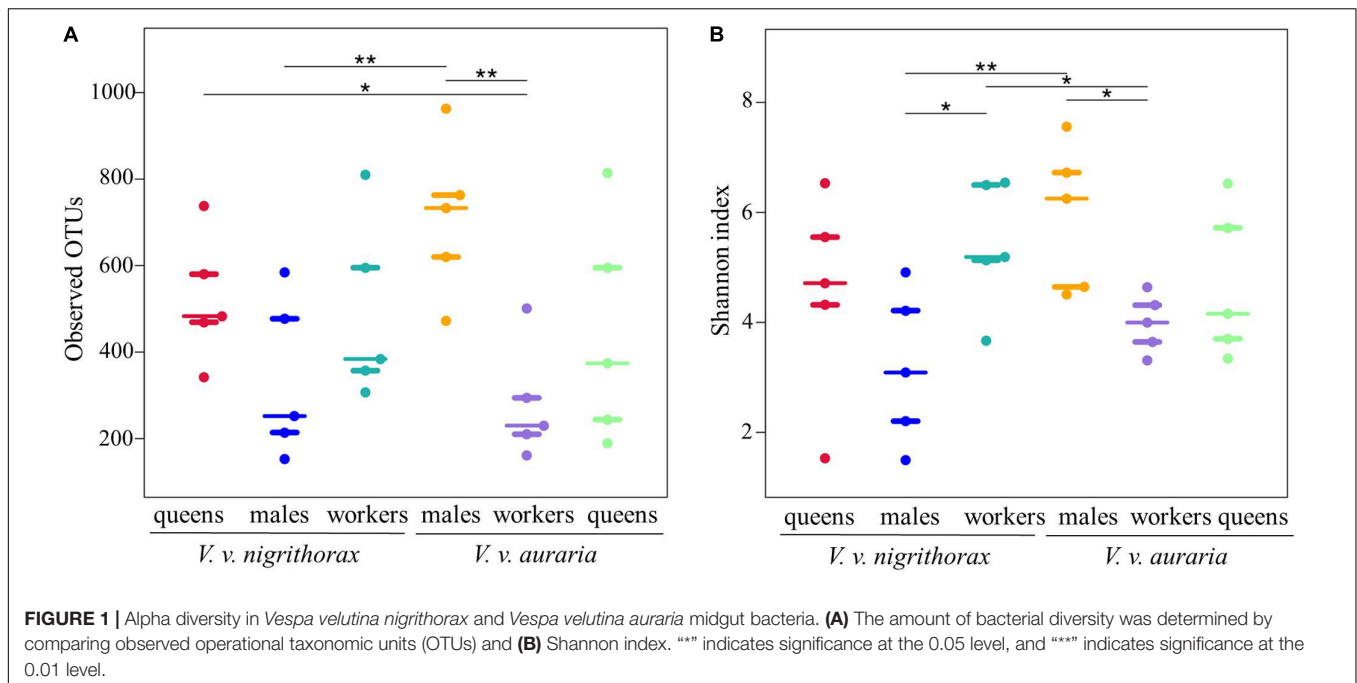
Linear Discriminant Analysis Effect Size

To detect the classified bacterial taxa with significant abundance differences among the different forms of *V. velutina*, a biomarker analysis based on the linear discriminant analysis (LDA) effect size (LEfSe) method was used. As shown in **Figure 3**, 48 bacterial clades presented statistically significant differences with an LDA threshold of 4.0. The largest number of bacterial taxa were presented in the males of *V. v. auraria* (AD), followed by the workers of *V. v. nigrithorax* (NW). A total of 13 bacterial taxa were significantly enriched in the males of *V. v. auraria*, including Clostridiales, Bacteroidales, *Actinobacteria*, Lachnospiraceae, and Bacillales. Most of the above bacteria are involved in nutrient metabolism, especially for protein degradation, sugar fermentation, and defending insects against harmful microorganisms by producing antimicrobial compounds (Visser et al., 2012; Wang et al., 2020). In addition, 10 bacterial taxa were significantly enriched in the workers of *V. v. nigrithorax*. It was interesting to note that seven bacterial taxa were significantly enriched in the males of *V. v. nigrithorax* (ND), and most of them belonged to lactic acid bacteria, including *Lactococcus*, Streptococcaceae, Lactobacillales, *Lactococcus lactis*, and *Weissella cibaria*, which are considered beneficial bacteria and are associated with fermentation.

DISCUSSION

Vespa velutina is considered a pest of *A. cerana* in parts of China (Tan et al., 2007), and an invasive species in Europe (Monceau et al., 2014). *V. velutina* may have adverse impacts on domestic *Vespa* species or other insect populations (Wilson et al., 2009; Beggs et al., 2011; Choi et al., 2012). This is the first report of midgut bacterial diversity of three forms queens, workers, and males of *V. velutina*.

The whole gut (midgut and hindgut) microbial composition of *V. velutina* has been reported by Cini et al. (2020). They found that microbial communities showed differences in composition across reproductive phenotypes (workers and future queens) (Cini et al., 2020). While in this study, the midgut bacterial composition of queens showed no significant differences in richness rank and level of diversity compared to that of the workers and males, so it seems that the differences in forms have no obvious effect on the midgut bacterial communities of *V. velutina*. Cini et al. (2020) also reported that the worker stage harbored a significantly higher number of bacterial OTUs compared to all other sample types (larva, newly emerged females, gyne, nest, and meconium), while there were no differences displayed by Shannon and Evenness indices among all sample types. However, in the present study, the richness and diversity of midgut bacterial communities of males were significantly higher than that of workers in *V. v. auraria*. In addition, the lactic acid bacteria were significantly enriched in the males of *V. v. nigrithorax*. Therefore, in *V. velutina*, the midgut bacterial communities of males were special and their specific roles in metabolism and health are also worthy of further study. Besides, based on the results of principal component analysis (PCA), principal coordinate analysis (PCoA) of Unweighted

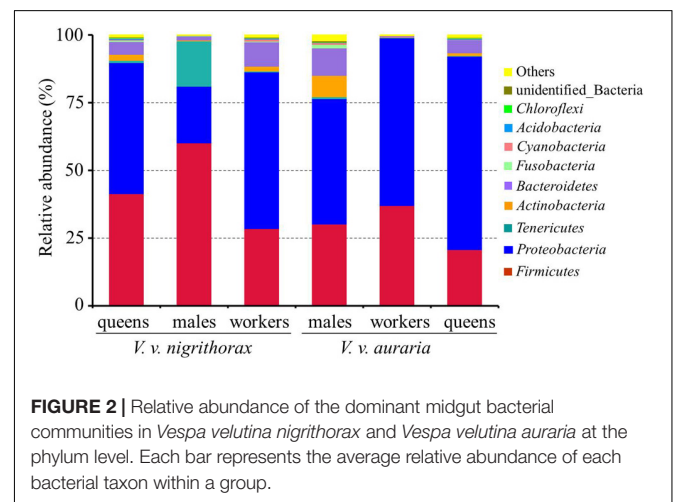


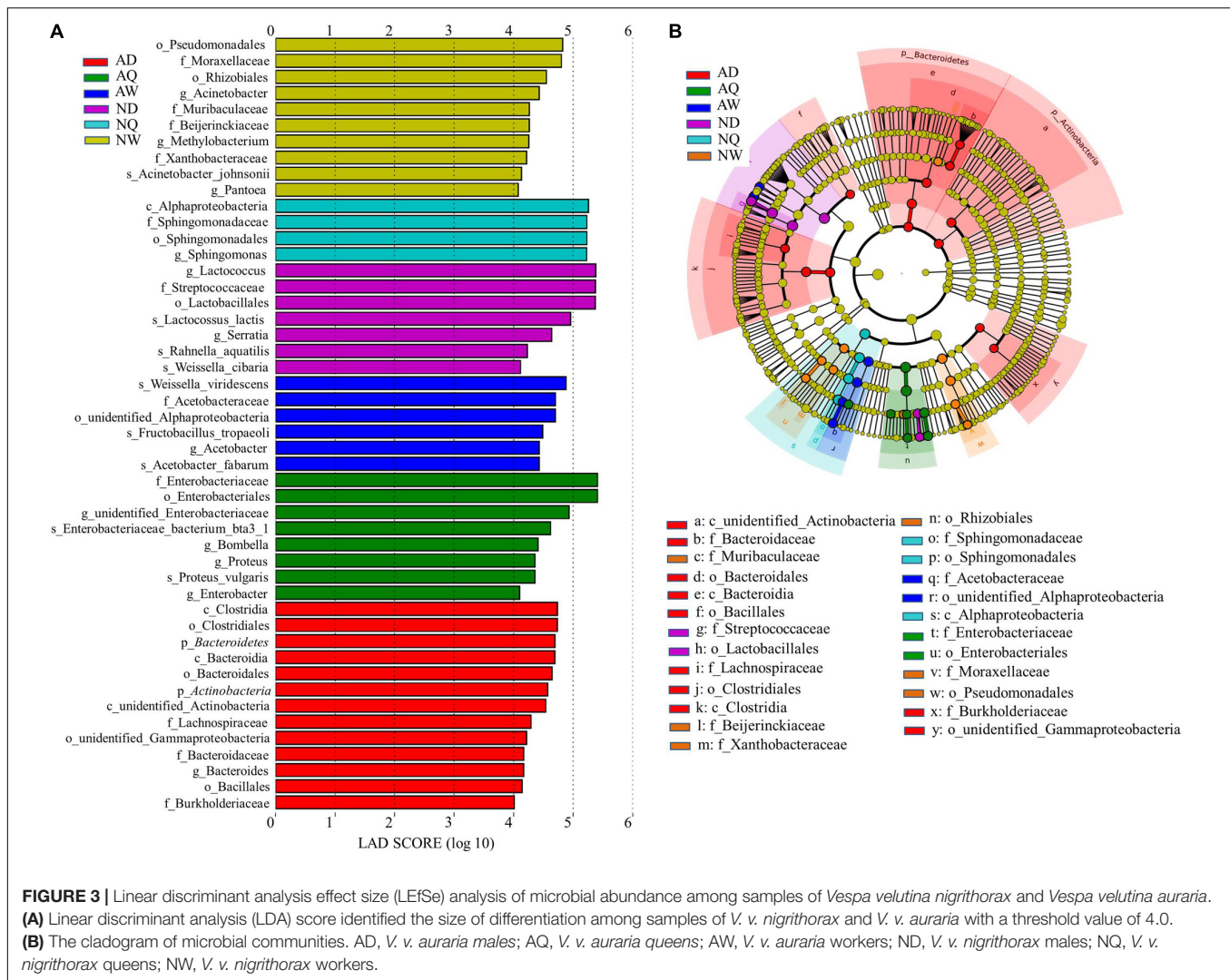
Unifrac Distance (**Supplementary Figure 1**), and cluster analysis (**Supplementary Figure 2**), it was found that the differences inside groups (queens, males, and workers) were greater than that between groups (*V. v. nigrithorax* and *V. v. auraria*), which indicated that the midgut bacterial communities of *V. v. nigrithorax* and *V. v. auraria* were similar.

Proteobacteria, *Firmicutes*, *Bacteroidetes*, *Tenericutes*, and *Actinobacteria* were the most abundant phyla in the midgut of *V. v. nigrithorax* and *V. v. auraria*. When comparing the gut bacterial compositions of the genus *Vespa* from different studies (**Supplementary Table 1**), it was found that the gut bacterial compositions were very similar at the phylum and class level, even if these samples were collected from different species (or subspecies) and regions (from different countries). In *V. mandarinia*, *V. simillima*, *V. v. nigrithorax*, and *V. v. auraria*, the most abundant and shared phyla of bacteria were *Proteobacteria* and *Firmicutes*, and the most abundant and shared classes of bacteria were Gammaproteobacteria, Bacilli, and Alphaproteobacteria. It was suggested that the above bacteria were consistent in the genus *Vespa*. However, when comparing the gut bacterial compositions at the low taxonomic levels, there were differences among different species. It was interesting to discover that the differences in gut bacterial compositions at the genus level were obvious. In our study, the gut bacteria compositions at the genus level were quite different from that in *V. mandarinia*, *V. simillima* (Suenami et al., 2019), and *V. v. nigrithorax* (Seo et al., 2018), but similar to that in *V. v. nigrithorax* (Cini et al., 2020). In fact, *Bifidobacterium* and *Lactobacillus* were the main abundant genera in *V. v. nigrithorax* (Cini et al., 2020), but were rare in the guts of *V. mandarinia*, *V. simillima* (Suenami et al., 2019) and *V. v. nigrithorax* (Seo et al., 2018). In this study, the main abundant genera included *Lactobacillus* and several other lactic acid bacteria

except *Bifidobacterium*, thus consolidating their presence as one of the leading bacterial communities in the gut of *V. velutina*. The above results indicated that *Vespa* from different regions (Japan, South Korea, Italy, and China) have quite different gut bacterial communities at the genus level, thus we speculated that geographic location might affect the compositions of *Vespa* gut bacteria, but not at the high taxonomic levels, such as the level of phylum and class.

The gut microbiota is important for the health status of the host (Engel et al., 2012; Engel and Moran, 2013; Kwong et al., 2014). *Gilliamella*, *Lactobacillus*, and *Bifidobacterium*, which are the core genera in the gut of honey bees (Kwong and Moran, 2016; Ma et al., 2019), are also the main genera of bacteria in *Vespa*. It has been suggested that *V. simillima*





gut is exposed to honey bee-derived microbial populations and likely selects for specific microbial groups (Suenami et al., 2019). Though Suenami hypothesized that the hornets do not require *Lactobacillus* and *Bifidobacterium* because they may already have alternative fermenters, *Lactobacillus* and *Bifidobacterium* are the two abundant genera of bacteria in *V. v. nigrithorax* and *V. v. auraria* (Cini et al., 2020 and this study). Besides, the results of this study showed that lactic acid bacteria were significantly enriched in the midgut of *V. v. nigrithorax* males. Recent studies show that *Lactobacillus* Firm-5 species enhances bees' memory and honeybee gut *Lactobacillus* modulates host learning and memory behaviors via regulating tryptophan metabolism (Li et al., 2021; Zhang et al., 2022). Besides, *Bifidobacterium* spp. significantly contributes to the honey bee's metabolism and health (Chen et al., 2021). Therefore, we presume that *Lactobacillus* and *Bifidobacterium* still play important roles in the protection and metabolism of *V. velutina*. It is necessary to carry out more research to verify the roles of *Lactobacillus* and *Bifidobacterium* in the life of *V. velutina* in the following study.

This discovery provides insight into the intestinal microbiota of *V. velutina*. Besides, further studies on the relationship between *Vespa* and gut microbial communities (especially Proteobacteria and Firmicutes) are necessary to elucidate how gut microbiota influence *Vespa* physiology and biology. On this basis, it will be feasible and meaningful to design pest biological control agents targeting the key microbes in the gut of *Vespa* to control them. One shortcoming of this study is that *V. v. nigrithorax* and *V. v. auraria* samples were collected from two sites in northern and southern China which were different in geographical environment and climatic conditions. However, it is rare to find two *V. velutina* subspecies in the same wild site.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The datasets generated in this study can be found in the sequence read archive (SRA) archive in GenBank under the BioProject: PRJNA833662.

AUTHOR CONTRIBUTIONS

P-LD and FL conceived this research and designed the experiments. P-LD, LZ, FL, S-LM, and YY participated in the design and interpretation of the data. P-LD, FL, P-HW, S-LM, YY, X-LW, and W-GY performed the experiments and analysis. P-LD, LZ, and FL wrote the manuscript. P-LD, LZ, FL, X-LW, and Q-YD participated in the revisions of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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