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ARTICLE

Analyses of the gut bacteriomes of four important *Drosophila* pests

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Abstract

Several *Drosophila* species (Diptera: Drosophilidae) have become serious economic pests of berry and soft-skinned stone fruits around the world. Prominent examples are *Drosophila suzukii* (Matsumura), *D. melanogaster* (Meigen), *D. hydei* (Sturtevant), and *D. immigrans* (Sturtevant). Information on the biology and ecology of *Drosophila* is important for a better understanding of these important fruit pests and, ultimately, for fruit protection. In this study, the gut bacteriomes of these four *Drosophila* species were surveyed and the differences among bacterial communities were characterised. The *16S* rRNA genes of gut microbes were sequenced by Illumina MiSeq technology (Illumina, San Diego, California, United States of America), followed by α-diversity and β-diversity analyses. The results show that bacteria of the family Enterobacteriaceae (*Kluyvera* and *Providencia*; phylum Proteobacteria) dominated all four *Drosophila* species. Specific dominant gut bacterial communities were found in each *Drosophila* species. The dominant families in *D. melanogaster* and *D. suzukii* were Enterobacteriaceae, Comamonadaceae, and Acetobacteraceae. In the intestine of *D. hydei*, Enterobacteriaceae had a proportion of 56.99%, followed by Acetobacteraceae, Spiroplasmataceae, and Bacillales Incertae Sedis XII. In *D. immigrans*, besides Enterobacteriaceae, Alcaligenaceae, Flavobacteriaceae, Xanthomonadaceae, Comamonadaceae, and Sphingobacteriaceae also had high relative abundance. These data expand current knowledge about the putative function related to gut microbes – for example, the metabolism of carbohydrates, amino acids, inorganic ions, lipids, and secondary metabolites. This knowledge provides a basis for further metatranscriptomic and metaproteomic investigations.

Introduction

Flies of the genus *Drosophila* (Diptera: Drosophilidae) have a wide range of hosts and species, many of which exert economically important impacts on cultivated fruits (Parshad and Paika 1964). For example, *D. melanogaster* (Meigen) prefers to lay eggs on rotten fruits (Mitsui *et al.* 2006; Atallah *et al.* 2014), especially on grapes. This may cause grape sour rot and other diseases that can cause serious economic loss. Rombaut *et al.* (2017) suggested that in grapes, sour rot is attributed not only to *D. melanogaster* but also to previous *D. suzukii* (Matsumura) invasion. *Drosophila suzukii* originated in East Asia and spread to America and Europe (Zhai *et al.* 2016). *Drosophila suzukii* has become an economically important invasive pest for berry and stone fruits because flies lay their eggs on fresh fruit, on which larvae feed (Schetelig *et al.* 2018). Moreover, *D. immigrans* (Sturtevant) and *D. hydei* (Sturtevant) are also widely found in Japan and East Asia (Katoh *et al.* 2007) and have been found in cherry orchards in different regions of China (Ren *et al.* 2014; Wang *et al.* 2017). *Drosophila hydei* and

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D. immigrans survive throughout the growing season (from grape and cherry sprouting to the falling of leaves), but their population numbers are lower than those of *D. suzukii* and *D. melanogaster* (Gao *et al.* 2018). To control these *Drosophila* species and to protect fruit, it is necessary to better understand the ecological strategies these important fruit pests employ.

The intestinal tract of insects is a dynamic environment involved in feeding, digestion, and excretion (Savage 1977), and it is inhabited by many microorganisms. In insect guts, bacteria play an important role on the growth, reproduction, digestion, absorption, resistance to pathogenic bacteria, enhancement of immunity, and even resistance to pesticides of hosts (Dillon *et al.* 2005; Warnecke *et al.* 2007; Sharon *et al.* 2010). As an excellent model system to study the effects of gut microbiota on host nutrition and metabolism, many studies assessed *Drosophila*–microbe interactions during the last decade (Douglas 2018). One way in which *D. melanogaster* adapts to the rotten-fruit environment is to feed on microbes, including yeasts and bacteria (*e.g.*, *Acetobacter* and *Gluconobacter*) growing on rotting fruits (Becher *et al.* 2012). The ethanol content is high in rotten fruits infested by *D. melanogaster*. The gut bacterium *Acetobacter pomorum* enables *D. melanogaster* to overcome the detrimental effects of ethanol by regulating the activity of alcohol dehydrogenase (PQ-ADH) in the insulin/insulin-like growth factor signalling pathway (Shin *et al.* 2011). Fresh fruit is rich in sugars and other carbohydrates while also being relatively deficient in proteins and amino acids (Goodhue *et al.* 2011; Milan *et al.* 2012). For this reason, *D. suzukii* must use other strategies, such as bacterial microsymbionts, to adapt to a nutrient-deficient habitat. However, very little information exists on the biological characteristics and adaptive strategies *D. hydei* and *D. immigrans* employ for their survival, especially those associated with their gut bacteria. Moreover, the gut microbiota contributes to the B vitamin requirements of *Drosophila* (Wong *et al.* 2014; Douglas 2017). The role of the gut microbiota for *Drosophila* energy storage has been testified by analyses of the major macromolecular energy stores (*i.e.,* lipid and glycogen) and two free sugars (*i.e.,* glucose and trehalose; Shin *et al.* 2011; Ridley *et al.* 2012; Newell and Douglas 2014). For example, the gut microbiota of wild *D. suzukii* (consisting of, for example, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Comamonadaceae*, and *Enterobacteriaceae*) reared on fresh fruit provides key proteins required for the development of *D. suzukii* (Bing *et al.* 2018). In-depth knowledge on the association between *Drosophila* and their gut core symbionts is therefore important for understanding the adaptive ecology of *Drosophila*.

Over the course of evolution, the microbial diversity in insect guts and the relationship between gut microbes and insects may have been affected by both the diet and habitat of hosts (Yun *et al.* 2014). Many taxonomic differences have been identified between the microbiota of laboratory-reared and wild *Drosophila* (Winans *et al.* 2017). Field-captured flies had a greater diversity of gut microbiota, which varied significantly with sampling location and season. In contrast, laboratory-reared flies possessed strikingly lower bacterial abundance and diversity (Bing *et al.* 2018). To eliminate interference of other factors, in the present study, laboratory populations of *D. suzukii*, *D. melanogaster*, *D. hydei*, and *D. immigrans* were selected. The differences in core bacterial composition were analysed, and the function of intestinal bacteria was predicted. The stable and core bacteria are of the utmost importance not only from the perspective of enhancing the biological understanding of important fruit pests but also from a long-term *Drosophila* management perspective.

Materials and methods

Insects and diet

Drosophila suzukii, *D. melanogaster*, *D. hydei*, and *D. immigrans* adults were collected in May 2016 from a cherry orchard in Taian (121° 27′ E, 37° 27′ N), Shandong Province, China. *Drosophila* adults were attracted by semisolid bait provided in a plastic bottle with a small

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hole. The semisolid bait was composed of rice vinegar, red wine, sugar, berry juice, and agar. One male and one female adult of each *Drosophila* species were reared in a tissue-culture bottle (ø 5.5 cm × 9 cm) in the laboratory and received artificial diet (see below) for 5–6 generations. The colony was maintained in a climate-controlled growth chamber at 25 ± 0.5 °C, 70% ± 0.5% relative humidity, and a photoperiod of 16:8 hours (light:dark). These colonies of four *Drosophila* species formed the laboratory populations.

The artificial diet was composed of mashed bananas and apples, corn flour, sucrose, yeast extract, sorbitol, and agar, as described in Zhai *et al.* (2014). Female adults (five days after eclosion) of the laboratory population were used for the isolation and identification of microorganisms. These species are common agricultural pests and are not listed on the "List of Protected Animals in China". No specific permissions were required, as these fields are experimental plots that belong to Shandong Academy of Agricultural Sciences, Jinan, Shandong, China.

Sample collection and dissection

Adults of *D. suzukii*, *D. melanogaster*, *D. hydei*, and *D. immigrans* (five days after hatching) from the same generation were anaesthetised by chilling before dissection. Directly before dissection, to eliminate bacteria on the surface, adults were sterilised with 75% alcohol for 90 seconds and rinsed with distilled water for two minutes. The gut tissues of each individual were then dissected in phosphate-buffered saline (0.2 M, pH 7.2) under a stereomicroscope (SZX2-ILLB; Olympus, Hatagaya, Japan) in sterile conditions. Ten guts were pooled per replicate in each group and were sampled and collected in 2-mL sterile centrifuge tubes, with three biological replicates (*i.e.,* tubes) per group. The samples were frozen at –80 °C.

DNA extraction and *16S* rRNA gene Illumina MiSeq sequencing

The DNA of each sample was extracted with an insect DNA kit (OMEGA Bio-Tek, Norcross, Georgia, United States of America) and purified by the MoBio PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Extracted high-quality DNA from all *Drosophila* species (three biological replicates per species) was high-throughput amplicon sequenced by Sangon Biotech Co., Ltd., Shanghai, China. The DNA was diluted to 1 ng/μL using DNase-free water. Polymerase chain reaction was conducted using bacterial universal primers of the V3 + V4 region: 341*F* (5'-CCTACACGACGCTCTTCCGA-TCTN (barcode) CCTACGGGNGGCWG CAG-3') and 805*R* (5'-GACTGGAGTTCCTTGGCACCCGAGAAT TCCA (barcode) GACTACHVGGGTATCTAA-TCC-3') to amplify the bacterial *16S* rRNA gene. In the primer sequence, the barcode was used to sort the groups in a single run. The sequencing library was generated by NEBNext Ultra DNA Library Pre-Kit (New England BioLabs, Ipswich, Massachusetts, United States of America) following the manufacturer's protocols. The quantity and quality of the library were assessed by the Qubit 2.0 Fluorometer (ThermoFisher Scientific, Waltham, Massachusetts, United States of America) and Agilent Bioanalyzer 2100 system (Agilent, Santa Clara, California, United States of America). The library was sequenced on an Illumina Miseq sequencing platform (Hiseq 2000, Illumina, San Diego, California, United States of America) with 2 × 250 bases, and using the paired-end version. After removal of low-quality reads containing primer/adaptor sequences (identified using cutadapt, version 1.2.1 (https://pypi.org/project/cutadapt/1.2.1/)), paired-end reads were assembled using PEAR, version 0.9.6 (https://cme.h-its.org/exelixis/web/software/pear/). Individual samples then were assigned, based on unique sample-specific barcodes. Quality filtering was performed on the assembled sequences using PRINSEQ, version 0.20.4 (http://prinseq.sourceforge.net/) to obtain high-quality clean sequences. For this, the barcode, primer sequences,

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chimeras, low-quality read sequences, and trimming of read lengths were cut off. Then, these clean data were used for the analyses described below.

Operational taxonomic unit cluster and bacterial taxonomy analysis

Sequences of clean data were clustered into the same operational taxonomic units, applying a 97% identity threshold (3% dissimilarity level) using Mothur software, version 1.30.1. Operational taxonomic unit abundance was normalised using the sequence number corresponding to the sample with the least sequences. A Venn diagram was generated by the VennDiagram package of R software, version 1.6.16. To evaluate the phylogenetic relationships among different operational taxonomic units, multiple sequence alignment was performed between the first 50 operational taxonomic units with the largest overall abundance and the NCBI 16SrRNA database (http://ncbi.nlm.nih.gov/) using MUSCLE software, version 3.8.31 (https://www.drive5.com/muscle/).

The representative sequence for each operational taxonomic unit was screened for species annotation using the NCBI 16SrRNA database at each taxonomic rank (threshold: 0.81). The sequences that satisfied the standard (similarity > 90% and coverage > 90%) were used for the next classification, and sequences that did not satisfy the standard were categorised as "unclassified". Then, the species and relative richness of each microorganism were analysed, and the microbial community structures were depicted as histograms. Furthermore, variation analysis of bacterial abundance based on genera among different *Drosophila* species was estimated using one-way analysis of variance ($\alpha = 0.05$) and Student–Newman–Keuls multiple comparisons using the SPSS 20.0 statistical analysis package.

Alpha diversity analysis

According to operational taxonomic unit abundance, the Shannon and Simpson indexes were used to indicate the community diversity of these four *Drosophila* groups. The Chao1 and Ace indexes (which indicate community richness) and all other indexes were calculated by the software Mothur, version 1.30.1 (http://mothur.org/). Rarefaction analysis of all samples was also performed using Mothur software, version 1.30.1.

Beta diversity analysis

Beta (β) diversity analysis was used to evaluate variances in species complexity in samples of different *Drosophila* species. The weighted and unweighted uniFrac distances were calculated using QIIME software, version 1.8.0 (http://qiime.org/) to measure the dissimilarity coefficient between pairwise samples. Nonmetric multidimensional scaling analysis, a nonlinear model designed to improve the representation of the nonlinear biological data structure, was performed to obtain principal coordinates and to visualise complex multidimensional data.

Functional prediction

According to the microbial community structure generated from *16S* rRNA sequencing, functional prediction of the metagenome was conducted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the software PICRUS, version 1.0.0 (http://picrust.github.io/picrust/). The histogram generated by KEGG was used to depict differences in microbial function of the four groups. Variation analysis of the top 10 gene functions contributed by relative operational taxonomic unit abundance was assessed with one-way analysis of variance ($\alpha = 0.05$) and Student–Newman–Keuls multiple comparisons using the SPSS 20.0 statistical analysis package.

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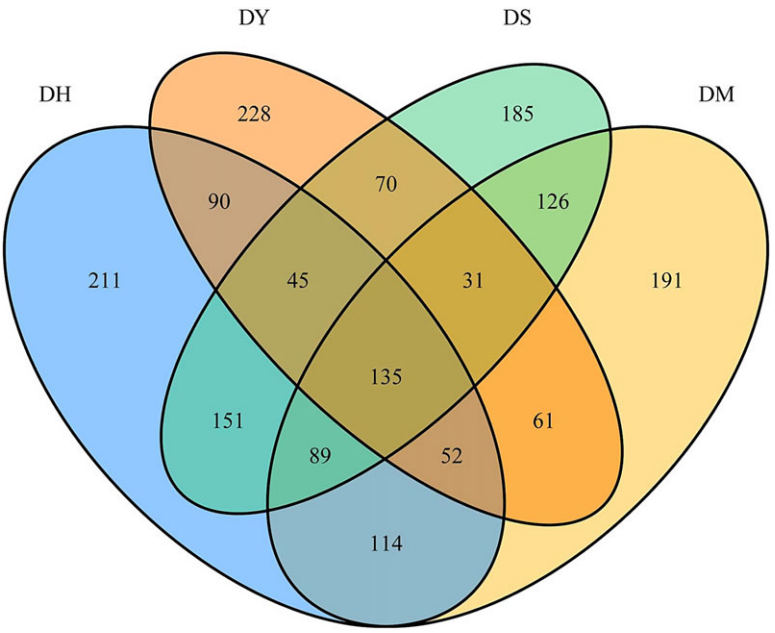

Fig. 1. Venn diagram of operational taxonomic units in four *Drosophila* species. DH, *Drosophila hydei*; DM, *D. melanogaster*; DY, *D. immigrans*; DS, *D. suzukii*.

Results

Sequencing information

Raw data were generated by Illumina MiSeq sequencing of the *16S* rRNA genes of intestinal samples of four *Drosophila* species (*i.e., D. hydei*, *D. immigrans*, *D. suzukii*, and *D. melanogaster*). After quality control, in which samples with a quality score of $Q < 30$ were discarded, filtered clean reads found in each *Drosophila* species (*D. hydei*: 58191 reads; *D. immigrans*: 59780 reads; *D. suzukii*: 58577 reads; *D. melanogaster*: 59776 reads) were then used for downstream bioinformatic processing (Supplemental material, Table S1). The datasets generated in the current study are available under NCBI Bio-project PRJNA719706.

Operational taxonomic unit abundance and bacterial taxonomy analysis

A Venn diagram of operational taxonomic units for all four groups is shown in Fig. 1. A total of 1779 operational taxonomic units was clustered in the samples of four *Drosophila* species. The numbers of operational taxonomic units found in *D. hydei*, *D. immigrans*, *D. suzukii*, and *D. melanogaster* were 887, 712, 832, and 799, respectively. Four *Drosophila* species shared 135 operational taxonomic units, including 69 bacterial genera (11 *Lampropedia*, eight *Acetobacter*, six *Clostridium*, and five *Paenibacillus*), with most of the remaining genera having more than two operational taxonomic units. Moreover, 420 operational taxonomic units were found in both *D. hydei* and *D. suzukii* groups, but only 279 were found both in *D. immigrans* and *D. melanogaster* groups. The phylogenetic tree of the first 50 abundant operational taxonomic units found in all samples is shown in Fig. 2. Among the first 50 operational taxonomic units, 32 belonged to the phylum Proteobacteria, 10 belonged to the phylum Bacteroidetes, and five belonged to the phylum Firmicutes, with one for each of Actinobacteria, Candidatus Saccharibacteria, and Tenericutes. The taxonomic information of operational taxonomic units

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Fig. 2. Circular phylogenetic tree of the first 50 operational taxonomic units representative sequences and database sequences for the genus. Multiple sequence alignment was performed between the first 50 operational taxonomic units with the largest overall abundance and NCBI 16SrRNA database using MUSCLE software. OTU, operational taxonomic unit.

for the four *Drosophila* species and the data for generating Venn plot of the four *Drosophila* species can be found in Supplemental material, Files S1 and S2.

The community structure of phyla in these four *Drosophila* species is shown in Fig. 3A. Proteobacteria, Bacteroidetes, and Firmicutes were the dominant phyla among 16 identified phyla in each species. The sum of the percentages of the Proteobacteria, Bacteroidetes, and Firmicutes phyla was 92.48%, 98.09%, 99.21%, and 98.51% in the intestines of *D. hydei*, *D. melanogaster*, *D. immigrans*, and *D. suzukii*, respectively. Family-level community structure is shown in Fig. 3B. Enterobacteriaceae was the most abundant family in the four species. In the intestine of *D. hydei*, Enterobacteriaceae had a proportion of 56.99%, followed by Acetobacteraceae (7.49%), Spiroplasmataceae (4.78%), and Bacillales Incertae

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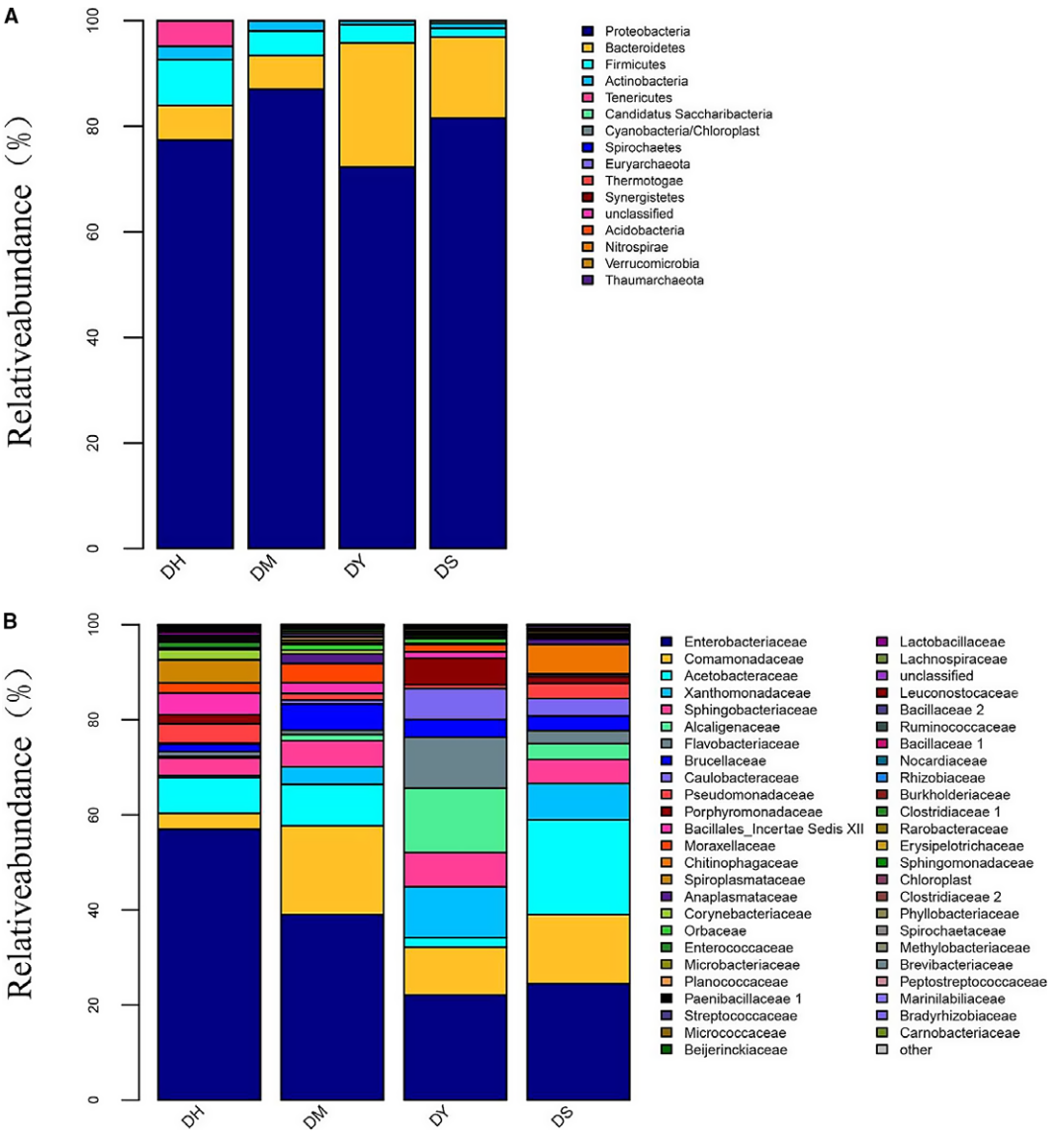

Fig. 3. A, Bar diagram of the community structure at the phylum level in four *Drosophila* species. **B,** Bar diagram of the community structure at the family level in four *Drosophila* species.

Sedis XII (4.58%). Besides Enterobacteriaceae (22.11%), other dominant families in *D. immigrans* (*e.g.*, Alcaligenaceae, Flavobacteriaceae, Xanthomonadaceae, Comamonadaceae, and Sphingobacteriaceae) had similar relative abundances. The dominant families in both *D. melanogaster* and *D. suzukii* were Enterobacteriaceae, Comamonadaceae, and Acetobacteraceae, with total relative abundances of 66.40% and 58.96%, respectively.

A total of 211 bacterial genera were found in the gut microbiota of the four *Drosophila* species, and the dominant species and amounts differed between these four species. The dominant intestinal bacteria that comprised a total percentage above 50% included four bacterial genera in *D. melanogaster*, including *Kluyvera*, *Providencia*, *Lampropedia*, and *Acetobacter*. The dominant bacterial genera in the intestines of *D. immigrans* (50.02%) were *Providencia*,

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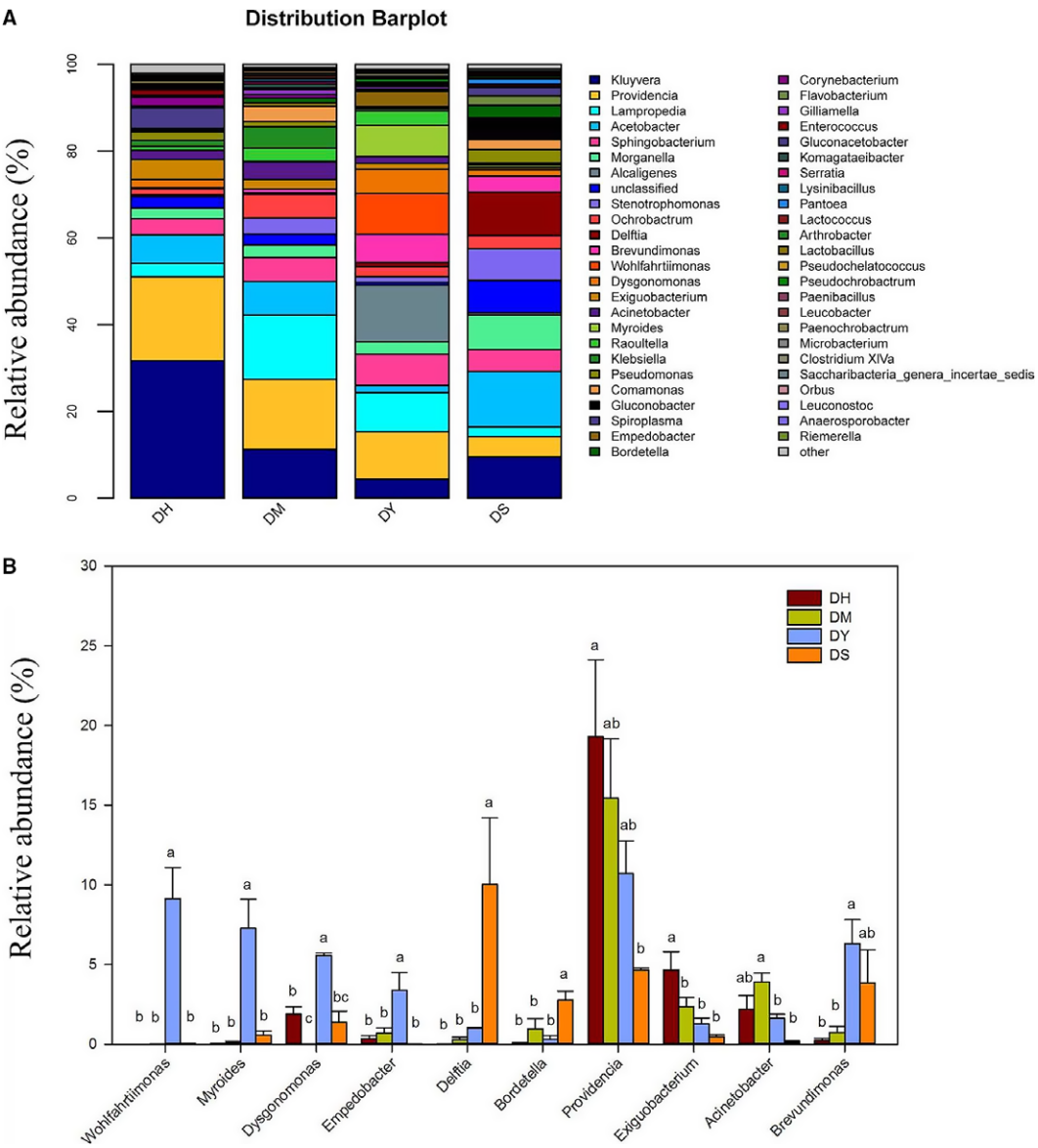

Fig. 4. A, Bar diagram of the community structure based on the genus level for four *Drosophila* species. **B,** One-way analysis of variance of community richness based on the genus level in four *Drosophila* species. Different letters in the same genus indicate significant differences (Student–Newman–Keuls multiple comparisons, $P < 0.05$).

Sphingobacterium, *Alcaligenes*, *Wohlfahrtiimonas*, and *Myroides*. Six bacterial genera (*Kluyvera*, *Providencia*, *Acetobacter*, *Stenotrophomonas*, *Delftia*, and *Gluconobacter*) comprised a total percentage of 54.58% and were the dominant bacteria in the intestines of *D. suzukii*. The dominant bacterial genera in the intestines of *D. hydei* were *Kluyvera* and *Providencia*, comprising a total of 50.21% (Fig. 4A).

According to the results of the one-way analysis of variance, a significant difference existed in the relative abundance of specific genera among the four *Drosophila* species (Fig. 4B). The percentages of *Wohlfahrtiimonas*, *Myroides*, *Dysgonomonas*, and *Empedobacter* in the

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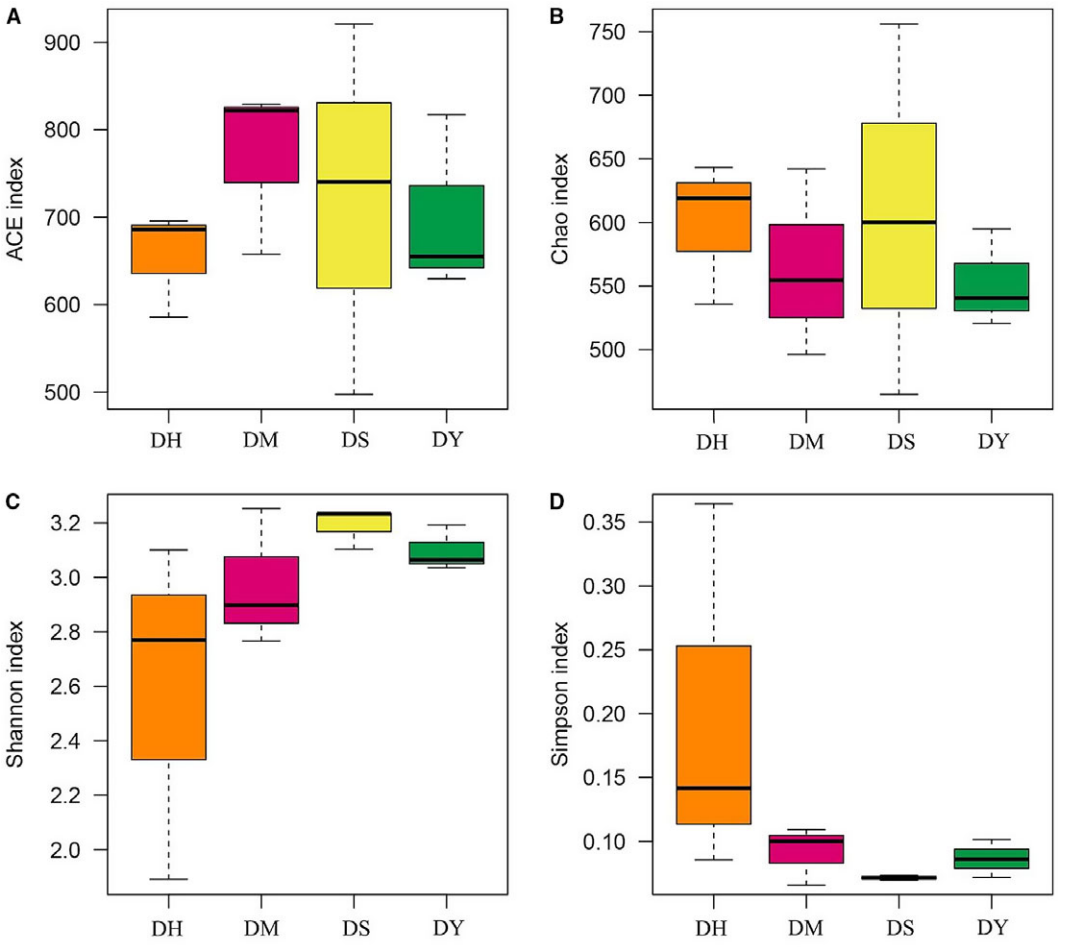

Fig. 5. Boxplot illustrating the α-diversity indexes of four *Drosophila* species: **A,** ACE index; **B,** Chao1 index; **C,** Shannon index; **D,** Simpson index.

intestines of *D. immigrans* were 3.39%, 7.26%, 5.57%, and 9.12%, respectively, which differed significantly from the percentages of the other three species (*Wohlfahrtiimonas*: $F = 21.46$, $P < 0.01$; *Myroides*: $F = 14.60$, $P < 0.01$; *Dysgonomonas*: $F = 30.28$, $P < 0.01$; *Empedobacter*: $F = 7.12$, $P = 0.01$). In addition, the relative abundances of the genera *Delftia* (10.01%) and *Bordetella* (2.76%) were much higher in *D. suzukii* than in the other three flies (*Delftia*: $F = 5.25$, $P = 0.03$; *Bordetella*: $F = 7.48$, $P = 0.01$). However, the relative abundances of *Providencia* and *Exiguobacterium* in *D. suzukii* were significantly lower than those among *D. hydei* intestinal microbes (*Providencia*: $F = 3.83$, $P = 0.06$; *Exiguobacterium*: $F = 7.41$, $P = 0.01$). No significant difference was found among other bacterial genera.

Alpha-diversity and beta-diversity analyses

The α-diversity indexes illustrating the microbial community richness (ACE and Chao1) and diversity (Shannon and Simpson indexes) for each of the four *Drosophila* species are shown in Fig. 5. Although *D. melanogaster* had a higher ACE index (769.54 ± 56.06), no significant difference was found compared to the other three *Drosophila* species (ACE: $F = 0.39$, $df = 2$,

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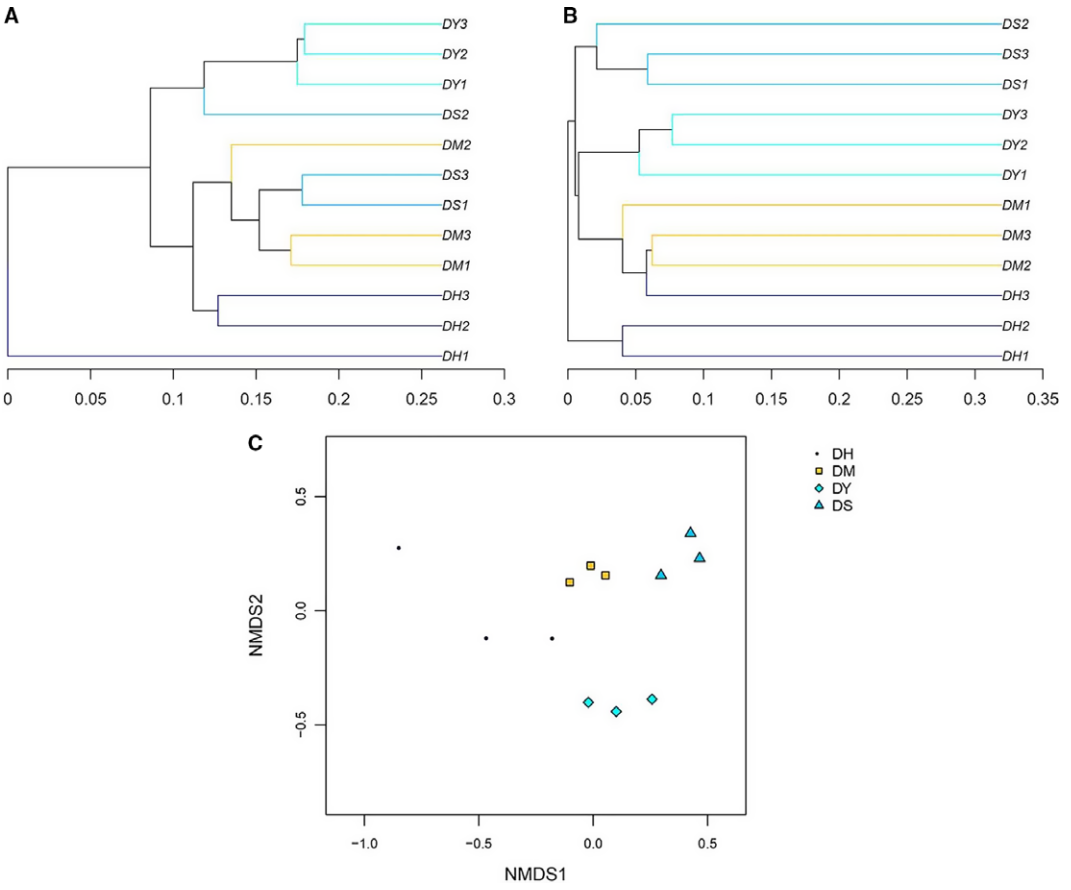

Fig. 6. A, Weighted tree plot of all samples found in four *Drosophila* species. **B,** Unweighted tree plot of all samples in four *Drosophila* species. Weighted and unweighted uniFrac distances were calculated using QIIME software. **C,** Nonmetric multidimensional scaling (NMDS) analysis reflecting the extent of variation in the gut bacterial communities among four *Drosophila* species.

$P = 0.77$). The Chao1 index did not differ significantly among the four *Drosophila* species (Chao1: $F = 0.27$, $df = 2$, $P = 0.84$). However, bacterial diversity also did not differ significantly among the four *Drosophila* species (Shannon: $F = 1.81$, $df = 2$, $P = 0.22$; Simpson: $F = 1.77$, $df = 2$, $P = 0.23$). Richness rarefaction curves of all samples tended to be flat, indicating that the amount of sequencing data was reasonable and that operational taxonomic unit coverage was sufficient (Supplemental material, Fig. S1).

Beta (β) diversity evaluating the differences in microbial communities among different *Drosophila* species was represented by a Bray tree plot based on weighted uniFrac (Fig. 6A) and unweighted uniFrac (Fig. 6B) distances between samples. Similarly, according to nonmetric multidimensional scaling analysis, the clustering result of gut bacteria in the four *Drosophila* species suggests that the gut microbial community was affected by the host species (Fig. 6C).

Functional prediction

Based on the KEGG databases, the heatmap cluster illustrates the functional diversity of the bacterial communities in the four *Drosophila* species (Fig. 7A). The contribution of each

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operational taxonomic unit is associated with the function of *16S* rRNA. Relative abundance was found for the top 10 gene functions in the four species: "amino acid metabolism", "carbohydrate metabolism", "cellular processes and signalling", "energy metabolism", "lipid metabolism", "membrane transport", "metabolism", "metabolism of other amino acids", "metabolism of terpenoids and polyketides", and "replication and repair". According to the results of the one-way analysis of variance of the top 10 functions, significant differences were found in the relative abundances of gene functions among the four *Drosophila* species (Fig. 7B). The relative abundances of gene functions "lipid metabolism" and "amino acid metabolism" in *D. suzukii* and *D. immigrans* were higher than those in *D. hydei* ($F = 5.23$, $P = 0.03$; $F = 7.51$, $P = 0.01$, respectively). However, the *16S* rRNA gene of intestinal bacterial in *D. hydei* had the highest relative abundance of all four KEGG functions in "membrane transport", "carbohydrate metabolism", and "metabolism" and "cellular processes and signalling". No significant difference was found in the relative abundance of *16S* rRNA gene functions among *D. melanogaster*, *D. immigrans*, and *D. suzukii*.

Discussion

The gut microbiome of insects is increasingly recognised as playing an important role in shaping the ecological adaptability of insects. *Drosophila* has become a valuable model for microbiome research, and relevant research can increase the understanding of how the microbiome influences host traits. Research on the composition of the gut microbiota of *Drosophila* has principally focused on bacteria. Chandler *et al.* (2011) reported that four bacterial families contributed 90% of all sequences of the gut microbiota in 14 wild *Drosophila* species. These include Enterobacteriaceae, Acetobacteraceae, Lactobacillaceae, and Enterococcaceae. All wild populations are dominated by at least one of the four major clades. The gut bacterial communities of field-caught *D. suzukii* were dominated by two families of the phylum Proteobacteria: Acetobacteraceae and Enterobacteriaceae (Martinez-Sañudo *et al.* 2017). The diversity of intestinal microbes in insects may be affected by feeding conditions. Moreover, much taxonomic research has focused on the gut microbiota of laboratory-reared *Drosophila* (Broderick and Lemaitre 2012; Douglas 2018). For example, a core member of microbiota that belongs to the genus *Gluconobacter* is common in wild *D. melanogaster* and *D. simulans* but is absent from laboratory-reared flies (Staubach *et al.* 2013). The family Staphylococcaceae of the phylum Firmicutes mainly prevails in laboratory-reared populations (Martinez-Sañudo *et al.* 2017).

In the present study, the diversities of intestinal bacterial communities were analysed in four laboratory-reared *Drosophila* species fed by an artificial diet that includes corn, fruits, and yeasts. Comamonadaceae, of the phylum Proteobacteria, represented a high proportion of microbes in the gut of *Drosophila* (*e.g., Delftia* in *D. suzukii* and *Lampropedia* in *D. melanogaster*). The abundance of Enterobacteriaceae in laboratory-reared *D. hydei* was only 56.99% in the present study, which differs from a previous report where a single Enterobacteriaceae operational taxonomic unit represented at least 85% of bacterial microbiomes in wild *D. hydei* fed with fruits (Chandler *et al.* 2011). Acetobacteraceae and *Providencia* in the family Enterobacteriaceae were dominant in the intestine of wild *D. immigrans* (Chandler *et al.* 2011), whereas in the present study, the family Acetobacteraceae was rare in laboratory-reared *D. immigrans*. Another difference of *D. immigrans* was that the bacterial genera *Alcaligenes*, *Wohlfahrtiimonas*, *Myroides*, and *Sphingobacterium* were also abundant in laboratory-reared *D. immigrans* in the present study but were rare in wild flies. However, Enterobacteriaceae (*Kluyvera* and *Providencia*) and Acetobacteraceae (*Acetobacter* and *Gluconobacter*), of the phylum Proteobacteria, were still the core families in *D. suzukii* and *D. melanogaster*, which was consistent with previous studies (Broderick and Lemaitre 2012;

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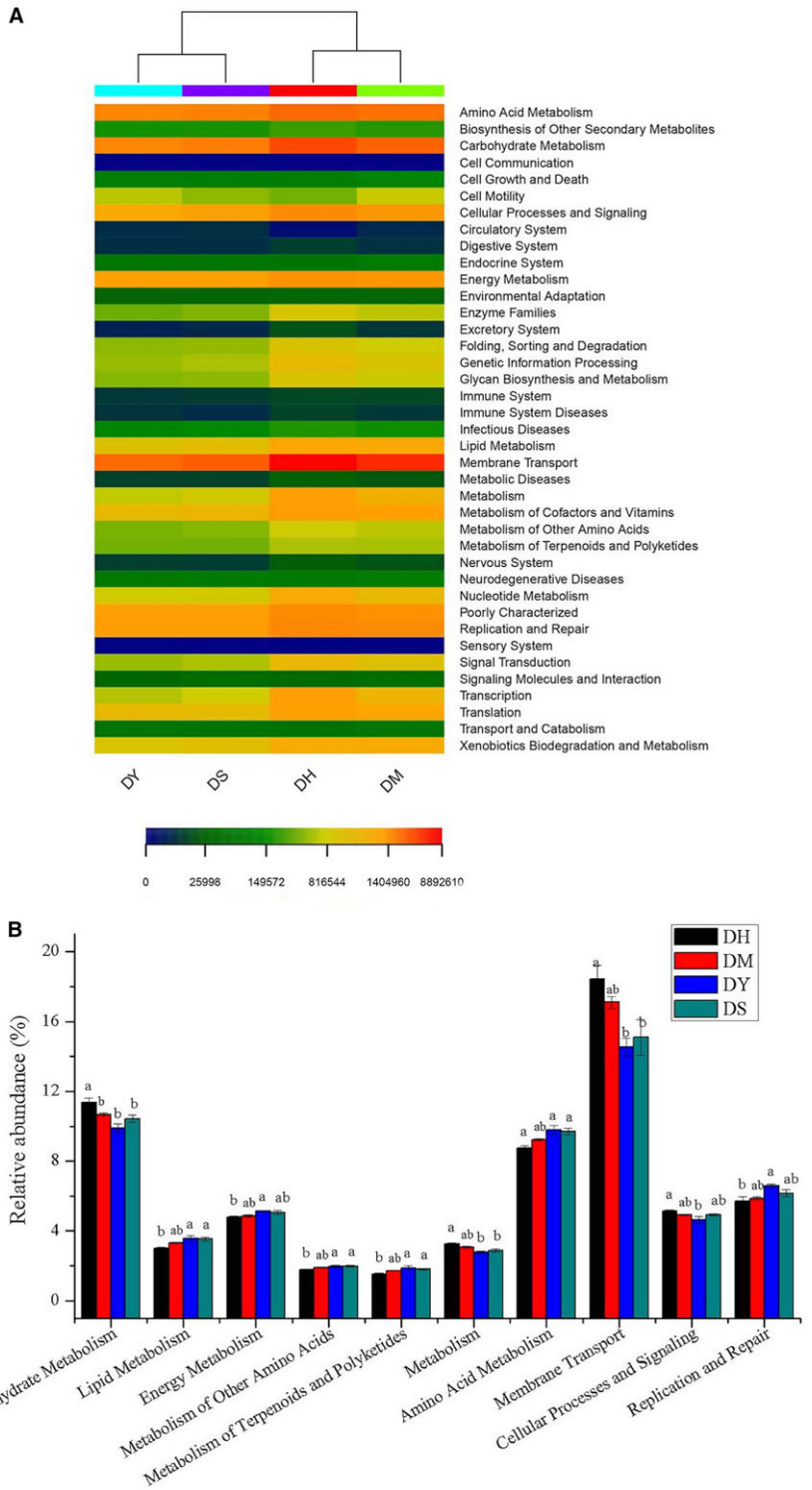

Fig. 7. A, Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis of bacteria in four *Drosophila* species. DH, *Drosophila hydei*; DM, *D. melanogaster*; DY, *D. immigrans*; DS, *D. suzukii*. **B,** Analysis of variance of KEGG functional analysis of the top 10 bacteria in four *Drosophila* species. Different letters in the same genus indicate significant differences (Student–Newman–Keuls multiple comparisons, $P < 0.05$).

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Chandler *et al.* 2014; Martinez-Sañudo *et al.* 2017; Adair *et al.* 2018). Winans *et al.* (2017) also reported that, for Enterobacteriaceae and Acetobacteraceae, laboratory-reared and wild *Drosophila* cannot be easily differentiated by *16S* taxonomy. Therefore, taxonomic differences between the microbiota of laboratory-reared and wild *Drosophila* existed because of differences in diet, ecological environment, and species, whereas core bacteria (*i.e.,* Enterobacteriaceae) remained stable and conserved (Kumar *et al.* 2019).

In the present study, significant differences were found in the relative abundance of bacterial genera among the four *Drosophila* species, which may be related to different ecological adaptations. For example, the relative abundance of the genus *Delftia* in *D. suzukii* was significantly higher than that in the other three species. Gut-associated bacteria are generally acquired from the environment after birth (Broderick and Lemaitre 2012). The genus *Delftia* has been isolated from different environments such as fresh and marine water, soil, infected plants, clinical samples, and activated sludge. This genus has been characterised by the ability of its members to transform (or degrade) multiple organic pollutants (Ubalde *et al.* 2012). Members of the genus *Delftia* have been described as plant growth–promoting bacteria because they promote plant growth either by providing nutrients (*e.g., via* nitrogen fixation, production of phytohormones, siderophores, and organic acids) or by helping with the resistance to infection by pathogens (Morel *et al.* 2011). *Delftia*, with its high nitrogen-fixing activity, is regarded as an endophytic bacterial species that inhabits healthy plant tissue and cannot be found in rotting fruits (Di Fiore and Del Gallo 1995). Based on these results, it can be assumed that *D. suzukii* may obtain the bacteria of the genus *Delftia* from fresh fruit, and bacteria are then retained in the intestines of flies. However, the other three *Drosophila* species, which mainly feed on rotten fruit or other food sources, would not obtain the same amount of genus *Delftia*. *Delftia* was also abundant in wild *D. suzukii*, because it provides key proteins required for the development of *D. suzukii* reared on fresh fruit (Bing *et al.* 2018). Therefore, *D. suzukii* has the unique ecological adaptability to consume fresh fruits, which may contribute to different gut microbes compared to those found in other *Drosophila* species.

Moreover, the intestine of *D. immigrans* had significantly higher percentages of *Empedobacter*, *Myroides*, *Dysgonomonas*, and *Wohlfahrtiimonas* compared to the microbes found in the other three *Drosophila* species. Thomas *et al.* (2011) had reported that the genera *Myroides* and *Empedobacter* contain pathogenic species. *Wohlfahrtiimonas* species were isolated from the bloodstream of a patient with septicemia and wound myiasis (Bonwitt *et al.* 2018). Many findings have provided evidence that *W. chitiniclastica* is transmitted by larvae of house flies, *Musca domestica* (Diptera: Muscidae), and black soldier flies, *Hermetia illucens* (Diptera: Stratiomyidae), during myiasis (Gupta *et al.* 2012; Lee *et al.* 2014). According to the results of the present study, the bacteria in these four genera comprise 25.34% of the intestinal bacteria of *D. immigrans*. However, whether these gut bacteria contribute to the spread of disease needs to be further evaluated.

The gut microbiota plays a very important role in many aspects of host physiology, especially in digestive, nutritional, metabolism-related, and immune functions (Fraune and Bosch 2010; Douglas 2018). Warnecke *et al.* (2007) reported that the gut microbes of *Nasutitermes ephratae* (Blattodea: Termitidae) can help the host degrade lignocellulose to compensate for the shortage of lignocellulose-degradation genes in termites. The herbivorous turtle ants of the genus *Cephalotes* (Hymenoptera: Formicidae) possess a conserved gut microbiome that enriches the nutrient composition by recycling nitrogen-rich metabolic waste to increase the production of amino acids (Duplais *et al.* 2021). The results of the present study identified the metabolism of carbohydrates, amino acids, inorganic ions, lipids, and secondary metabolites as important functions of gut bacteria in *Drosophila*. These results show that gut microbes were probably related to the metabolism of fruit flies. However, the species and distribution of these microbes differed between the four *Drosophila* species. The differences during metabolism of carbohydrates, amino acids, inorganic ions, lipids, and secondary

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metabolites may be related to differences of the living habits and ecological niches among the four *Drosophila* species. However, the specific bacteria related to the adaptation to a specific ecological niche need to be identified in further studies.

In conclusion, taxonomic differences in microbiota were found among four laboratory-reared *Drosophila* species. The predicted function of bacteria can serve as targets for future downstream functional studies of core gut bacterial communities, which would expand the available knowledge about the fruit flies' adaptations for survival in hostile environments. Breaking the relationship between gut bacteria and *Drosophila* species is one of the strategies to control them.

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