ORIGINAL ARTICLE

# Differences in the intestinal microbiota between insecticide-resistant and -sensitive *Aedes albopictus* based on full-length 16S rRNA sequencing

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## Abstract

The intestinal symbiotic bacteria of *Aedes albopictus* play a potential role in host resistance to insecticides. In this study, we sequenced the full-length of 16S rRNA and analyzed the differences in the intestinal microbiota between deltamethrin-resistant and -sensitive *Ae. albopictus*. Symbiotic bacteria were cultured and analyzed using six types of culture media in aerobic and anaerobic environments. We found significant differences in the diversity and abundance of the intestinal microbiota of the two strains of *Ae. albopictus*. The symbiotic bacteria cultured in vitro were found to be mainly facultative anaerobes. The cultured bacteria such as *Serratia oryzae* and *Acinetobacter junii* may function to promote the development of insecticide resistance. This work indicates that intestinal bacteria may contribute to the enhancement of insecticide resistance of *Ae. albopictus* It also highlights the analytical advantage of full-length 16S rRNA sequencing to study the intestinal microbiota of mosquitoes.

**KEYWORDS**
16S rRNA, full-length sequencing, intestinal microbiota, resistance

## 1 | INTRODUCTION

*Aedes albopictus* is an important medical arthropod and the main vector of viruses such as dengue, Zika, and chikungunya. Dengue fever remains prevalent and outbreaks often occur in subtropical and temperate countries with around 390 million people being infected worldwide annually (Bhatt et al., 2013). To date, effective and safe vaccines have not been developed for the prevention and control of mosquito-borne viruses and the control of such viruses relies mainly on the suppression of mosquito vectors and chemical methods. Pyrethroid insecticides have been widely used for mosquito control owing to their insecticide efficacy and non-toxicity in mammals. However, the overuse and abuse of these insecticides

have resulted in widespread resistance, thereby defeating the very purpose of vector prevention and mosquito control.

Previous studies have indicated that symbiotic bacteria in insects, particularly in the gut, endow the host with improved adaptability to the environment (Tsuchida et al., 2014), enhanced immunity against external pathogenic microorganisms, and increased detoxification rates (Oliver et al., 2003; Vorburger & Rouchet, 2016). Symbiotic bacteria in the host gut induce the production of detoxification enzymes to develop common metabolism and mineralization processes that act to improve host resistance (Scates et al., 2019). Studies on agricultural pests showed that the presence of Burkholderia (Kikuchi et al., 2012) and *Stenotrophomonas maltophilia* (Shen et al., 2010; Zhao et al., 2009)

in Riptortus pedestris (Hemiptera) and Citrobacter in the intestine of _Plutella xylostella_ (Cheng et al., 2017) increased insecticide tolerance in the host.

A particular species of symbiotic bacteria in the gut of _Ae. albopictus_ has reduced the titer of Dengue virus and alter the ability of mosquitoes to transmit vector viruses (Apte-Deshpande et al., 2014; Ramirez et al., 2012, 2014). Studies have also confirmed the differences in the bacterial diversity between pyrethroid-sensitive and resistant strains of wild _Anopheles gambiae_, _An. albimanus_, and _An. arabiensis_ (Minard et al., 2013). Fenitrothion-resistant Burkholderia acquired from the environment may help their hosts degrade insecticides (Kikuchi et al., 2011). Alternatively, Wolbachia, as intestinal microbiota of _Ae. aegypti_ and _Culex pipiens_ positively correlated with insecticide resistance by regulating esterase genes (Hoffmann & Turelli, 2013; Minard et al., 2013). Based on these studies, we hypothesize that the diversity and abundance of _Ae. albopictus_ gut bacteria may be related to the insecticide resistance and that the gut bacteria of deltamethrin-sensitive and -resistant _Ae. albopictus_ are different.

To test this hypothesis, we isolated and analyzed symbiotic bacteria from the intestinal tracts of deltamethrin-resistant and -sensitive larvae. The isolated bacteria from both larval strains were treated with deltamethrin and analyzed to explore the complex interactions between bacteria and insecticide resistance. We showed that not only the intestinal bacteria of the two strains had differences in diversity and abundance, but specific strains were also prevalent in the intestinal tracts.

In comparison with the full length of 16S rRNA, short-read amplicon sequencing in the variable region (V3–V4) of 16S rRNA can affect sequencing accuracy and impact the identification of bacterial species (Klemetsen et al., 2019). In this study, the full-length of the 16S rRNA was sequenced with a single-molecule sequencer (PacBio Sequel II; Burke & Darling, 2016). The PacBio circular consensus sequencing (CCS), a long-read sequencing model that can be applied for 16S rRNA genes, was used to significantly improve the resolution of the bacteria. This is the first study to demonstrate the use of next-generation sequencing techniques to analyze the full length of 16S rRNA genes in _Ae. albopictus_ intestinal microbiota and insecticide resistance.

Besides molecular analysis, gut bacteria have been extensively cultured and identified using a combination of various culture media and environmental conditions. Only the bacteria that can be cultured in vitro can be used for in vitro functional assays. This is therefore particularly important for the isolation and identification of bacteria with insecticide-degrading capabilities.

## 2 | METHODS

### 2.1 | Mosquito rearing and bioassays

The insecticide-sensitive strain of _Ae. albopictus_ (denoted as the S strain) was donated by the Disease Control and Prevention Center (CDC) in Shandong. The strain has been reared for more than 20 years in the mosquito breeding room of the Shandong Institute of Parasitic Diseases during which _Ae. albopictus_ was not exposed to insecticides. Insecticide resistance was screened in each generation using deltamethrin at a lethal concentration of 50% (LC50), and 52 generations were screened to obtain the resistant strain (denoted as R strain). Figure 1 shows a summary schematic of the selection process used in this study. The breeding conditions of the mosquitoes were as follows: temperature = 26°C ± 2, relative humidity = 75% ± 5, a photoperiod of 12:12 hr (light:dark). The larvae were fed with a mixture of pork liver powder and yeast powder in a 1:3 ratio. Adult mosquitoes were transferred to the mosquito cages, fed with a 10% glucose solution, and defibrinated sheep blood through a Hemotek unit.

The bioassay of insecticide resistance of the larva was determined following the World Health Organization (WHO) guidelines according to the following formula:

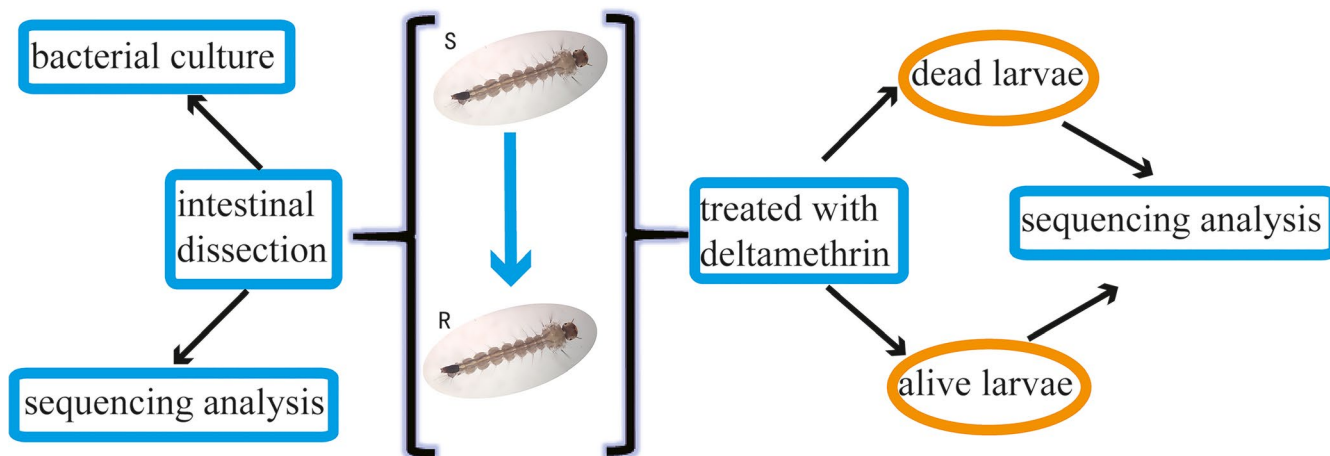$$RR = \text{resistant strain LC50/sensitive strain LC50}.$$



**FIGURE 1** Test flow chart. Blue arrows mean screening and breeding with LC50 concentration of deltamethrin

After exposure to deltamethrin, a stable symbiotic relationship with larvae was maintained by gradually increasing the abundance of symbiotic bacteria that can degrade deltamethrin insecticides. For bacterial isolation, the third instar larvae were treated with deltamethrin at LC50 dose for 24 h. After being lightly touched with a glass rod, the motionless larvae were treated as dead. Upon deltamethrin treatment, dead and alive individuals were collected for subsequent analysis of the bacteria.

## 2.2 | Intestinal anatomy and bacterial culture

The larvae of two strains at the third instar were selected and rinsed with sterile distilled water. The larvae were then soaked in 75% ethanol for 3 min and finally rinsed with sterile distilled water for 2 min. The mosquitoes were dissected in sterile conditions and the mid-gut removed and placed into 1.5 ml reaction tubes. For each strain at least five biological replicates and 20 mid-guts of each sample were collected for subsequent microbiota analysis.

After the samples for bacterial culturing had been processed, the samples were aliquoted into 1.5 ml reaction tubes containing 100 μl of distilled water. The mid-guts were ground and shaken to fully release the bacteria from the intestine. The samples were then diluted to concentrations of $10^{-2}$, $10^{-3}$, $10^{-4}$, and $10^{-5}$, and 10 μl of each concentration was plated onto the prepared six types of culture media. For each dilution, four replicates were separately cultured for 48 h in aerobic (air, 37°C) and anaerobic environments (5% $CO_2$, 37°C). All of the above experiments were performed under sterile conditions.

After incubation, 1000 μl of sterile normal saline was added to each culture medium. Colonies were picked using an inoculation needle and dissolved in normal saline by gently shaking. The solutions were pipetted into 1.5 ml reaction tubes and centrifuged at 5300 rcf for 5 min to pellet the bacteria.

## 2.3 | DNA extraction and sequencing of 16S rRNA

An ultrasonic disruptor (SinapTec®, France) was used to disrupt each intestinal tissue sample. The total DNA from the tissues was extracted according to the instructions of the PowerSoil® DNA Isolation kit. The primer sequences of the full-length 16S rRNA were as follows: 27F (AGRGTTTGATYNTGGCTCAG) and 1492R (TASGGHTACCTTGTTASGACTT). The PCR reaction conditions were 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, with 30 cycles in a reaction volume of 10 μl.

The PCR products were purified, quantified, and homogenized to form a sequencing library (SMRT Bell). After the library was quantified, the next-generation high-throughput sequencing method was followed using the single-molecule sequencer (PacBio Sequel II) (Kingan et al., 2019). The off-machine data were in the bam file format and the CCS (Circular Consensus Sequencing) file was exported by smrtlink analysis software (SMRT Link, version 8.0). The

data from different samples were identified according to the barcode sequence and converted into fastq datasets. The lima (v1.7.0) software was used to identify the CCS sequence of the different samples through the barcode sequence and to remove the chimera (UCHIME, version 8.1; Edgar et al., 2011), allowing the generation of a high-quality CCS sequence.

## 2.4 | Species notes and taxonomic analysis

UCLUST (Edgar, 2010) in the QIIME software (version 1.8.0; Caporaso et al., 2010) was used to cluster tags at a similarity level of 97% and to obtain OTU (Operational taxonomic units). The taxonomic annotation of OTU was performed based on the Silva (http://www.arb-silva.de) database.

## 2.5 | Analysis of microbial diversity and KEGG functional gene prediction

The abundance of symbiotic bacteria and bacterial diversity in the samples from different strains was determined by alpha diversity analysis. The Shannon and the Simpson indices are commonly used to evaluate species diversity. In the case of the same species abundance, the greater the uniformity of species in the community, the greater the diversity and higher the Shannon index. Conversely, a low Simpson index indicates a higher level of species diversity in the sample. The Chao1 index was used to measure species abundance and was defined as the number of species in the sample.

Mothur (Hiltemann et al., 2019; version v.1.30; http://www.mothur.org/) software was used to evaluate sample alpha diversity. To compare the diversity between the samples, the number of sequences contained in the samples was standardized during analysis. The alpha diversity of each sample was counted at the similarity level of 97%.

Metastats software was used to perform a $t$ test on the species abundance between the groups and the calculated $p$-value ($p \leq 0.05$) was further corrected to obtain the $q$-value. Species that caused differences in the composition of the two groups were screened out based on the $p$- or $q$-values. The significance analysis between the groups was performed at the levels of phylum, class, order, family, genus, and species.

The functional gene composition was assessed from the samples using the PICRUSt (Wilkinson et al., 2018) software as well as to analyze the potential differences. The generated OTU-table was standardized. Then, through the greengene ID corresponding to each OTU, KEGG (Kyoto Encyclopaedia of Genes and Genomes) information corresponding to the OTU was obtained. These data were used to calculate the abundance of KEGG. An independent sample $t$ test was used to analyze the significant differences between the groups with a $p$-value threshold of 0.05.

# 3 | RESULTS

## 3.1 | Sample characteristics and sequencing reads of 16S rRNA

The LC50 of *Ae. albopictus* larva of resistant and sensitive strains was 219.2 and 2 μg/L, respectively. Before treatment with deltamethrin, 205 *Ae. albopictus* larvae from the two strains were analyzed by 16S rRNA full-length sequencing. A total of 63,299 sequences were produced (an average of 308.78 sequences per mosquito) that decreased to a total of 62,835 sequences after filtering and purification. After treatment with deltamethrin, the dead and surviving individuals were collected from both strains of larvae. A total of 156,453 sequences were collected and 151,133 sequences were obtained after filtering and purification. From the analysis of the six sample groups, and removing outliers, the Shannon diversity index dilution curves of 26 samples were obtained (see Figure 2). The curves had become flatter, indicating that the sequencing was sufficient and could be used for subsequent analysis. The dissection, sample, and sequence numbers before and after filtering are shown in Table 1.

## 3.2 | Differences in the diversity and abundance of intestinal microbiota

Statistically significant differences were observed in the diversity and abundance of the intestinal microbiota of *Ae. albopictus* larvae from resistant and sensitive strains. The differences were mainly reflected in the following aspects: 124 OTUs were present, of which 50 were shared, and 53 and 21 were obtained from resistant and sensitive strains, respectively (Figure 3a). Significant differences were observed in the Shannon ($t$ = 8.26, $df$ = 6, $p$ < 0.001) and the Simpson indices ($t$ = 6.475, $df$ = 6, $p$ = 0.001) and the Chao1 index ($t$ = 3.811, $df$ = 6, $p$ = 0.009) of the two strains. We observed that the bacterial diversity of the resistant strain was significantly greater than that of the sensitive strain (Figure 4).

At the level of order classification (Figure 5a), 10 bacterial phyla such as Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were mainly annotated. The relative abundance of Proteobacteria in the resistant strain (71.57 ± 11.21%) was significantly higher than the sensitive strain ($t$ = 6.865, $df$ = 6, $p$ < 0.001). This was followed by Firmicutes that had relative abundances in the resistant and sensitive strains of 11.03 ± 10.30% and 0.58 ± 0.19%, respectively (no statistically significant difference: $t$ = 2.028, $df$ = 6, $p$ = 0.089).

At the genus level (Figure 5b), Serratia had the highest proportion in the resistant strain (32.24 ± 7.94%) and its prevalence was significantly higher than in the sensitive strain ($t$ = 3.805, $df$ = 6, $p$ = 0.009). This was followed by Acinetobacter, which ranked second in the resistant strain with an abundance of 16.58 ± 5.16% ($t$ = 6.20, $df$ = 6, $p$ = 0.001). Aeromonas had an abundance of 11.19 ± 2.29% that ranked third in the resistant strain and was significantly higher than in the sensitive strain ($t$ = 5.480, $df$ = 6, $p$ = 0.002).

At the species classification level (Figure 5c), *S. oryzae* had the highest abundance in the resistant strain (32.24 ± 7.94%) and was significantly higher than in the sensitive strain ($t$ = 3.805, $df$ = 6, $p$ = 0.009). This was followed by *Aeromonas hydrophila* that had an abundance of 11.19 ± 2.29% in the resistant strain and was significantly higher than the sensitive strain ($t$ = 5.48, $df$ = 6, $p$ = 0.002). Among the resistant strain, *A. junii* had an abundance value of 11.39 ± 6.46%, which was still significantly higher than the sensitive strain (0.17 ± 0.08%; $t$ = 3.474, $df$ = 6, $p$ = 0.013).

## 3.3 | Changes in intestinal microbiota before and after deltamethrin treatment

A total of 116 OTUs were obtained from the sensitive strain of which 45 were shared. 26 and 45 OTUs were unique to the



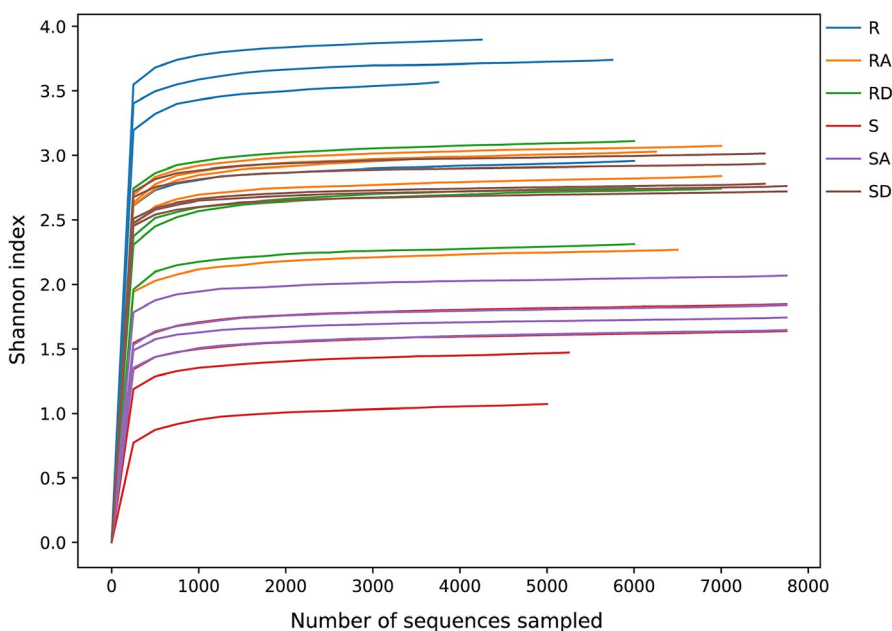**FIGURE 2** Rarefaction curves from each group. The letters S, SA, SD, R, RA, and RD represent the sensitive strain, surviving groups of sensitive strain after deltamethrin treatment, dead groups of sensitive strain after deltamethrin treatment, resistance strain, surviving groups of resistance strain after deltamethrin treatment, dead groups of resistance strain after deltamethrin treatment, respectively

**TABLE 1** Sample information sheet

| Strain | LC$_{50}$ / RR | Untreated sample | | | | | Treated with deltamethrin | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | | | Live strains | | | | | Dead strains | | | | |
| | | Sample ID | D | T | Barcode-CCS | Optimization-CCS | Sample ID | D | T | Barcode-CCS | Optimization-CCS | Sample ID | D | T | Barcode-CCS | Optimization-CCS |
| S | 2 μg/L / 1 | S1 | 23 | 20 | 8004 | 8003 | SA1 | 22 | 20 | 7998 | 7973 | SD1 | 21 | 20 | 8038 | 7970 |
| | | S2 | 25 | 21 | 5614 | 5613 | SA2 | 23 | 20 | 6671 | 6667 | SD2 | 22 | 21 | 7937 | 7800 |
| | | S3 | 20 | 20 | 7955 | 7946 | SA3 | 22 | 21 | 7995 | 7995 | SD3 | 20 | 20 | 7871 | 7853 |
| | | S4 | 21 | 21 | 5297 | 5293 | SA4 | 22 | 20 | 8034 | 7988 | SD4 | 20 | 20 | 7997 | 7990 |
| | | S5 | 23 | 22 | 7975 | 7974 | SA5 | 25 | 21 | 8029 | 8018 | SD5 | 22 | 20 | 7942 | 7743 |
| R | 219.2 μg/L / 109.6 | R1 | 23 | 20 | 4199 | 4189 | RA1 | 23 | 20 | 7948 | 7714 | RD1 | 20 | 20 | 8002 | 6846 |
| | | R2 | 22 | 21 | 6721 | 6678 | RA2 | 24 | 21 | 6113 | 5861 | RD2 | 21 | 21 | 7994 | 7129 |
| | | R3 | 22 | 20 | 5158 | 5053 | RA3 | 20 | 20 | 7992 | 7861 | RD3 | 23 | 22 | 7943 | 7770 |
| | | R4 | 20 | 20 | 5396 | 5361 | RA4 | 23 | 20 | 8014 | 8002 | RD4 | 23 | 20 | 7975 | 7132 |
| | | R5 | 20 | 20 | 6980 | 6725 | RA5 | 22 | 21 | 8042 | 7786 | RD5 | 21 | 20 | 7918 | 7035 |

_Note:_ D: dissection number; T: test number; RR: resistance ratio. The number means the serial number of the biological replicate of the sample; S means refers to the sensitive strain, R refers to the resistant strain, SA and RA refer to the surviving strains of the sensitive and resistant strains treated with deltamethrin, respectively. SD and RD indicate the dead strains of the sensitive and resistant strains treated with deltamethrin, respectively.

untreated and treated group, respectively (Figure 3b). In the resistant strain, 186 OTUs were obtained, of which 74 were shared. Twenty-nine and 83 OTUs were unique to the untreated and treated group, respectively (Figure 3c). To screen out the strains potentially involved in insecticide resistance, strains with significantly increased abundance after deltamethrin treatment were compared. At the species level, the relative abundance of _A. junii_ (5.28 ± 4.12%) in the treatment group of the sensitive strain was significantly higher than the control group (0.17 ± 0.09%; $t = 2.476$, $df = 6$, $p = 0.048$). For the resistant strain, the relative abundance of _A. junii_ ($t = 6.044$, $df = 6$, $p = 0.009$) and _S. oryzae_ ($t = 10.392$, $df = 6$, $p = 0.002$) showed a statistically significant increase after deltamethrin treatment.

## 3.4 | Differences in microbiota between dead and surviving individuals after deltamethrin treatment

Deltamethrin treatment seemed to create differences in the microbiota of the dead and surviving individuals in the sensitive strain. A total of 124 OTUs were obtained, of which 68 were shared while 34 and 22 OTUs were unique to the dead and surviving individuals, respectively (Figure 3d). For the resistant strains, 177 OTUs were obtained, of which 113 were shared by both, and 20 and 44 were unique to the dead or surviving individuals, respectively (Figure 3e). Compared to the surviving individuals, the relative abundance values of _S. oryzae_, _A. junii_, and _C. indologenes_ in the dead individuals were slightly higher than in the surviving individuals; however, the difference was not statistically significant.

## 3.5 | Functional gene prediction by KEGG analysis

Besides the identification of strains that may promote insecticide degradation, KEGG functional gene prediction was also carried out. The relative abundance of xenobiotics biodegradation and metabolism functional genes was compared before and after treatment with deltamethrin. Significant differences in the resistant ($t = 4.558$, $df = 8$, $p = 0.02$) and sensitive strains ($t = 8.433$, $df = 6$, $p < 0.001$) were observed. These results are in accordance with the analysis of alpha diversity before and after treatment.

## 3.6 | Cultivable bacteria and screening of strains

Six media (Appendix Table A1) were used to culture the bacteria, which led to the isolation of 28 types of bacteria in vitro. Most of these strains were facultative anaerobes and mainly Gram-negative bacteria. The culture results are summarized in Appendix Table A1. After screening the resistant and sensitive strains between the groups, as well as before and after deltamethrin treatment, three strains were identified. These strains may help _Ae. albopictus_ larvae to resist deltamethrin and were identified as _S. oryzae_, _A. junii_, and

(a)



53    50    21

R         S

(b)



45    45    26

Treated S    Untreated S

(c)



83    74    29

Treated R    Untreated R

(d)



34    68    22

Dead S      Alive S
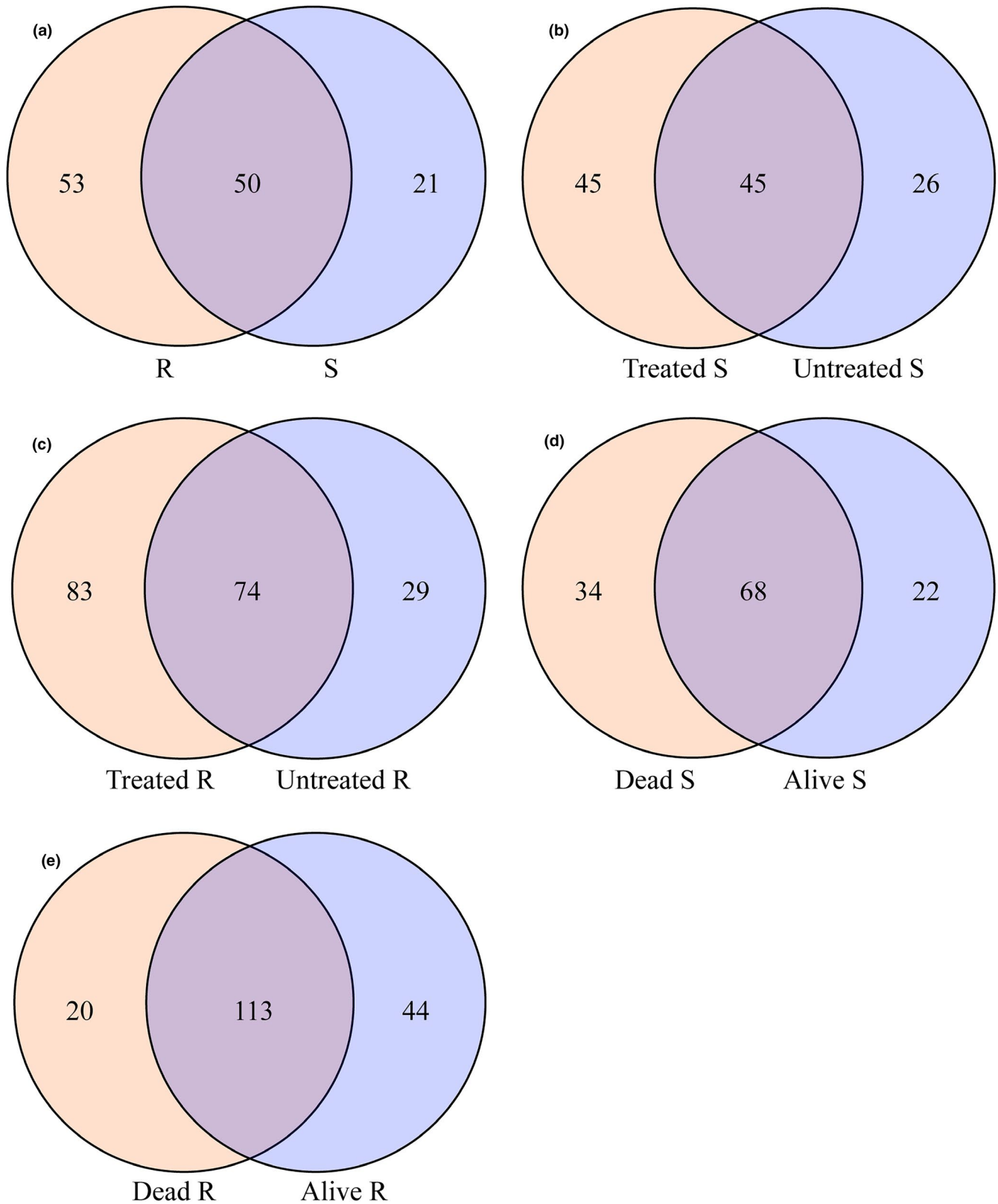
(e)



20    113    44

Dead R       Alive R

**FIGURE 3** OTUs Venn diagram between samples. The letters R and S represent the deltamethrin-resistant and -sensitive strains, respectively. 3a represents the Venn diagram between the resistant and sensitive strains; 3b and 3c represent the Venn diagram of the sensitive and resistant strains before and after treatment, respectively; 3d represents the Venn diagram of the dead and surviving groups of the sensitive strain after treatment. 3e represents the Venn diagram of the dead and surviving strains of the resistant strain after treatment
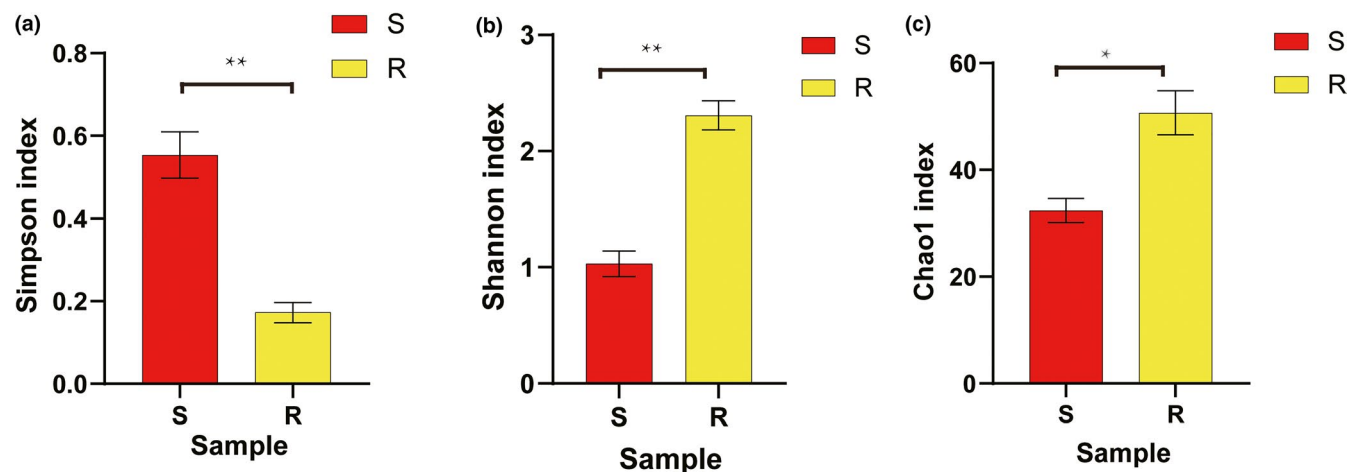
**FIGURE 4** Difference in diversity index between sensitive and resistant strains. **$p < 0.001$, *$p < 0.05$. 4a, 4b, and 4c represent the Simpson, Shannon, and Chao 1 index, respectively. Letters S and R represent sensitive and resistant strains, respectively
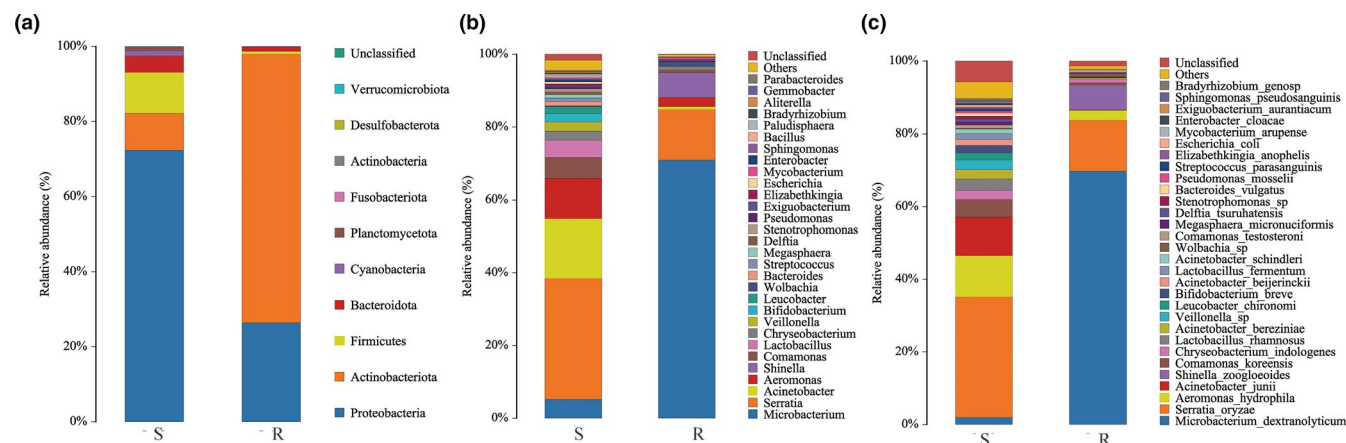


**FIGURE 5** Histogram of species abundance at different taxonomic levels. 5a, 5b, and 5c show the histograms of species abundance at the levels of order, genus, and species. Letters S and R represent sensitive and resistant strains, respectively

*C. indologenes*. The ability to culture and isolate these bacteria served as an important factor for further in vitro verification experiments.

## 4 | DISCUSSION

*Aedes albopictus* is a critical vector for various mosquito-borne viruses and is also an important model species for basic mosquito research (Barrett & Higgs, 2007; Dutra et al., 2016). There is a need for research focusing on the mechanisms of prevention and control of insecticide resistance in mosquitoes. This is essential for developing improved prevention and control measures of mosquito-borne diseases. Previous studies on the role of intestinal microbiota in mosquito insecticide resistance have not achieved great results compared with agricultural pests. This area has garnered renewed interest with recent reports suggesting that the internal and epidermal microbes of *Ae. albopictus* and *An. albimanus* were significantly altered after exposure to different insecticides (Achee et al., 2008; Dada et al., 2019). Besides, the presence of symbiotic bacteria in the

mid-gut of *An. arabiensis* and *An. stephensi* contributes to insecticide resistance (Barnard et al., 2019; Soltani et al., 2017).

For mosquitoes captured in the wild, the composition and abundance of intestinal microbiota is a dynamic process, impacted by sampling time, habitat environments, genetic background, physiological state, and developmental stage (Ben Ami et al., 2010; Estes et al., 2012; Morrow et al., 2015). To avoid interference of these non-experimental factors, the *Ae. albopictus* larvae used in this study were bred in the laboratory and obtained at the third instar stage for both the sensitive and resistant strains. For the complete metamorphic mosquito, the absorption and metabolism of nutrients by the larva intestine can directly affect the morphology of adults and their ability to adapt to different environments (Christiana et al., 2010; Sandra et al., 2015). Earlier studies have shown that intestinal microbiota plays an important role in the metabolism of external toxic substances. Therefore, selecting larvae as a research model can be used to screen out different strains based on insecticide resistance that can also be used to study the metabolism of biologically active substances (Ceja-Navarro et al., 2015).

For our study, 16S rRNA full-length sequencing based on the third-generation sequencing technique was carried out. Compared to the V3–V4 region of the 16S rRNA gene amplicon, full-length sequencing has a higher resolution and annotation rate with less noise and inaccurate sequences (Burke & Darling, 2016), suggesting that it can be used to detected rarer species (Deutscher et al., 2018). Results showed that only short region sequencing of the V3–V4 region greatly reduced the reliability and accuracy of species annotation. This also affected the annotation of the genus and species that were 78% and 8%, respectively, leading to incorrect estimations of bacterial diversity (Pernice et al., 2014; Youssef et al., 2009). However, full-length 16S rRNA gene sequencing using a long-read, single-molecule sequencer improved the annotations of the genus and species, which were more than 95% and 60%, respectively. The accuracy of the annotation was also significantly improved.

Molecular studies have shown that the diversity and relative abundance of intestinal microbiota in resistant strains are significantly higher than the sensitive strains. These differences are mainly reflected at the genus and species level and have been confirmed in many agricultural pests such as *Aphis gossypii* (Zhang et al., 2019). The relative abundance of Proteobacteria and Firmicutes had an absolute advantage in resistant strains. Acinetobacter and Serratia in Proteobacteria showed significant differences between the two strains. Bacteria from these two genera possess degrading effects on pyrethroid insecticide in vitro, particularly deltamethrin and cypermethrin (Cycoń & Piotrowska-Seget, 2016; Cycoń et al., 2013; Zhang et al., 2010).

Wolbachia is the most widely studied symbiont in arthropods and has been reported to colonize nearly 65% of all arthropods (Hilgenboecker et al., 2008). Although such bacteria were detected in all samples, it has no specific contribution to the metabolism of exogenous toxic substances and functions mainly in reproductive regulation (e.g., cytoplasmic incompatibility, male-killing, feminization; Hunter et al., 2003; Kageyama et al., 2002; Werren et al., 2008). However, it has been reported that the higher the Wolbachia density, the more genes relative to resistance are expressed in *Culex pipiens* pallens (Duron et al., 2006).

The cultivation and identification of bacteria is the most intuitive and classic method for studying microorganisms. Although the diversity and abundance of bacteria are affected by culture media and the culture environment (Deutscher et al., 2018), it can objectively reflect the composition of different cultivable bacteria. It has been reported that for most mosquito intestinal microbes, lysogeny broth (LB) culture medium cannot meet culture growth requirements (Short et al., 2017). To make the cultured bacteria more diverse, six culture media with different nutrients and nutritional gradients, along with two culture environments were used. Our results showed that most of the *Ae. albopictus* intestinal microbiota could not be cultured in vitro.

When larvae encounter harmful substances such as insecticides, they actively mobilize various functions to fight against these substances to prevent death. Intestinal bacteria are required to make adaptive changes to their composition and abundance to help the host improve tolerance and maintain stable parasitic relationships (Cator et al., 2020). This process forms the basis of insecticide resistance and after exposure to insecticides, the bacteria that undergo compensatory changes might affect resistance. This process may be understood as a selective pressure (Chandrasegaran & Juliano, 2019). When hosts face selective pressure, the abundance of symbiotic bacteria changes rapidly to adapt to environmental changes, and microbial populations usually accelerate the evolution of the host (Gressel, 2018). During the mosquito screening, some biological characteristics are amplified that may be due to the survival of larvae with specific microbiota. For instance, *Asaia* accelerates the development of *An. gambiae* larvae by affecting the expression of host genes related to the epidermis formation (Mitraka et al., 2013). In this context, the two strains of *Ae. albopictus* were treated with deltamethrin insecticide at the LC50 concentration for 24 h. Our results showed that the diversity of the symbiotic bacteria in the intestine changed after exposure to deltamethrin. These data suggest that insecticide promoted the growth of specific bacteria, particularly bacterial communities with the ability to degrade insecticides (Muturi et al., 2017). Our studies found that *S. oryzae* and *A. junii* had the most significant changes in relative abundance after exposure to insecticides. Consistent with our results, Rauglas et al. (2016), found that the abundance of Acidovorax also increased significantly after exposure to insecticides.

The gene variations among the intestinal microbiota occurred at a higher frequency compared with the host genes, which might stabilize the intestinal environment and modulate the ability of the host to adapt to dynamic external environments (Rosenberg & Zilber-Rosenberg, 2011; Zilber-Rosenberg & Rosenberg, 2008). This genetic feature may be exploited for engineering the intestinal microbiota to develop novel strategies for the prevention and control of insecticide resistance. However, a limitation of our study was the lack of functional validation of symbiotic bacteria both in vitro and in vivo.

## 5 | CONCLUSIONS

The increased diversity of intestinal microbiota in response to the insecticide pressure may contribute to insecticide resistance in *Ae. albopictus*. The relative abundance of Serratia and Acinetobacter in the resistant strain was higher than that in the sensitive strain, suggesting that the bacteria from these two genera had a positive effect on host resistance to deltamethrin. We also present evidence that the full-length 16S rRNA sequencing technology can be used as an ideal method to study the intestinal microbiota of mosquitoes.

## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

**Haiyang Wang:** Data curation (lead); Writing-original draft (lead); Writing-review & editing (lead). **Chongxing Zhang:** Data curation (supporting); Formal analysis (supporting). **Peng Cheng:** Resources (supporting); Software (supporting). **Yang Wang:** Resources (supporting); Software (supporting). **Hongmei Liu:** Supervision (supporting); Validation (supporting); Visualization (supporting). **Haifang Wang:** Methodology (supporting). **Huaiwei Wang:** Supervision (supporting). **Maoqing Gong:** Funding acquisition (lead); Investigation (lead); Methodology (lead); Supervision (lead); Validation (lead); Visualization (lead).

## ETHICS STATEMENT

None required.

## DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. Raw sequence reads have been deposited into the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA687827: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA687827

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## REFERENCES

Achee, N. L., Grieco, J. P., Vatandoost, H., Seixas, G., Pinto, J., Ching-NG, L., Martins, A. J., Juntarajumnong, W., Corbel, V., Gouagna, C., David, J. P., Logan, J. G., Orsborne, J., Marois, E., Devine, G. J., Vontas, J., & Kittayapong, P. (2019). Alternative strategies for mosquito-borne arbovirus control. _PLOS Neglected Tropical Diseases_, _13_(1), e0006822. https://doi.org/10.1371/journal.pntd.0006822

Apte-Deshpande, A. D., Paingankar, M. S., Gokhale, M. D., & Deobagkar, D. N. (2014). Serratia odorifera mediated enhancement in susceptibility of _Aedes aegypti_ for chikungunya virus. _Indian Journal of Medical Research_, _139_, 762–768.

Barnard, K., Jeanrenaud, A. C. S. N., Brooke, B. D., & Oliver, S. V. (2019). The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector _Anopheles arabiensis_ (Diptera: Culicidae). _Scientific Reports_, _9_, 9117.

Barrett, A. D. T., & Higgs, S. (2007). Yellow fever: A disease that has yet to be conquered. _Annual Review of Entomology_, _52_, 209–229.

Ben Ami, E., Yuval, B., & Jurkevitch, E. (2010). Manipulation of the microbiota of mass-reared Mediterranean fruit flies _Ceratitis capitata_ (Diptera: Tephritidae) improves sterile male sexual performance. _ISME Journal_, _4_, 28–37.

Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R., Simmons, C. P., Scott, T. W., Farrar, J. J., & Hay, S. I. (2013). The global distribution and burden of dengue. _Nature_, _496_, 504–507.

Burke, C. M., & Darling, A. E. (2016). A method for high precision sequencing of near full-length 16S rRNA genes on an Illumina MiSeq. _PeerJ_, _4_, e2492.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. _Nature Methods_, _7_, 335–336.

Cator, L. J., Johnson, L. R., Mordecai, E. A., Moustaid, F. E., Smallwood, T. R. C., LaDeau, S. L., Johansson, M. A., Hudson, P. J., Boots, M., Thomas, M. B., Power, A. G., & Pawar, S. (2020). The role of vector trait variation in vector-borne disease dynamics. _Frontiers in Ecology and Evolution_, _8_, 189.

Ceja-Navarro, J. A., Vega, F. E., Karaoz, U., Hao, Z., Jenkins, S., Lim, H. C., Kosina, P., Infante, F., Northen, T. R., & Brodie, E. L. (2015). Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. _Nature Communications_, _6_, 1–9.

Chandrasegaran, K., & Juliano, S. A. (2019). How do trait-mediated non-lethal effects of predation affect population-level performance of mosquitoes? _Frontiers in Ecology and Evolution_, _7_, 25.

Cheng, D., Guo, Z., Riegler, M., Xi, Z., Liang, G., & Xu, Y. (2017). Gut symbiont enhances insecticide resistance in a significant pest, the oriental fruit fly _Bactrocera dorsalis_ (Hendel). _Microbiome_, _5_, 13.

Christiana, A., Monika, D., & Rohlfs, M. (2010). Influence of dietary yeasts on _Drosophila melanogaster_ life-history traits. _Entomologia Experimentalis et Applicata_, _136_(1), 1–11.

Cycoń, M., & Piotrowska-Seget, Z. (2016). Pyrethroid-degrading microorganisms and their potential for the bioremediation of contaminated soils: A review. _Frontiers in Microbiology_, _7_, 1463.

Cycoń, M., Żmijowska, A., & Piotrowska-Seget, Z. (2013). Enhancement of deltamethrin degradation by soil bioaugmentation with two different strains of _Serratia marcescens_. _International Journal of Environmental Science & Technology_, _11_, 1305–1316.

Dada, N., Lol, J. C., Benedict, A. C., López, F., Sheth, M., Dzuris, N., Padilla, N., & Lenhart, A. (2019). Pyrethroid exposure alters internal and cuticle surface bacterial communities in _Anopheles albimanus_. _ISME Journal_, _13_, 2447–2464.

Deutscher, A. T., Burke, C. M., Darling, A. E., Riegler, M., Reynolds, O. L., & Chapman, T. A. (2018). Near full-length 16S rRNA gene next-generation sequencing revealed Asaia as a common midgut bacterium of wild and domesticated Queensland fruit fly larvae. _Microbiome_, _6_, 85.

Duron, O., Labbé, P., Berticat, C., Rousset, F., Guillot, S., Raymond, M., & Weill, M. (2006). High Wolbachia density correlates with cost of infection for insecticide resistant _Culex pipiens_ mosquitoes. _Evolution_, _60_, 303–314.

Dutra, H. L., Rocha, M. N., Dias, F. B., Mansur, S. B., Caragata, E. P., & Moreira, L. A. (2016). Wolbachia blocks currently circulating Zika virus isolates in Brazilian _Aedes aegypti_ mosquitoes. _Cell Host & Microbe_, _19_, 771–774.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. _Bioinformatics_, _26_, 2460–2461.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. _Bioinformatics_, _27_, 2194–2200.

Estes, A. M., Hearn, D. J., Burrack, H. J., Rempoulakis, P., & Pierson, E. A. (2012). Prevalence of Candidatus Erwinia dacicola in wild and laboratory olive fruit fly populations and across developmental stages. _Environmental Entomology_, _41_, 265–274.

Gressel, J. (2018). Microbiome facilitated pest resistance: potential problems and uses. _Pest Management Science_, _74_, 511–515.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with Wolbachia?–A statistical analysis of current data. _FEMS Microbiology Letters_, _281_, 215–220.

Hiltemann, S. D., Boers, S. A., van der Spek, P. J., Jansen, R., Hays, J. P., & Stubbs, A. P. (2019). Galaxy mothur Toolset (GmT): A user-friendly

application for 16S rRNA gene sequencing analysis using mothur. *Gigascience*, 8, giy166.

Hoffmann, A. A., & Turelli, M. (2013). Facilitating Wolbachia introductions into mosquito populations through insecticide-resistance selection. *Proceedings of the Royal Society. Biological Sciences*, 280, 20130371.

Hunter, M. S., Perlman, S. J., & Kelly, S. E. (2003). A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proceedings of the Royal Society. Biological Sciences*, 270, 2185–2190.

Kageyama, D., Nishimura, G., Hoshizaki, S., & Ishikawa, Y. (2002). Feminizing Wolbachia in an insect, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Heredity (Edinburgh)*, 88, 444–449.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., & Fukatsu, T. (2012). Symbiont-mediated insecticide resistance. *Proceeding of the National Academy of Science of the United States of America*, 109, 8618–8622.

Kikuchi, Y., Hosokawa, T., & Fukatsu, T. (2011). An ancient but promiscuous host-symbiont association between Burkholderia gut symbionts and their heteropteran hosts. *ISME Journal*, 5, 446–460.

Kingan, S. B., Heaton, H., Cudini, J., Lambert, C. C., Baybayan, P., Galvin, B. D., Durbin, R., Korlach, J., & Lawniczak, M. K. N. (2019). A high-quality de novo genome assembly from a single mosquito using PacBio sequencing. *Genes (Basel)*, 10(1), 62–72. https://doi.org/10.3390/genes10010062

Klemetsen, T., Willassen, N. P., & Karlsen, C. R. (2019). Full-length 16S rRNA gene classification of Atlantic salmon bacteria and effects of using different 16S variable regions on community structure analysis. *Microbiologyopen*, 8, e898.

Minard, G., Mavingui, P., & Moro, C. V. (2013). Diversity and function of bacterial microbiota in the mosquito holobiont. *Parasit Vectors*, 6, 146.

Mitraka, E., Stathopoulos, S., Siden-Kiamos, I., Christophides, G. K., & Louis, C. (2013). Asaia accelerates larval development of *Anopheles gambiae*. *Pathogens and Global Health*, 107, 305–311.

Morrow, J. L., Frommer, M., Shearman, D. C., & Riegler, M. (2015). The microbiome of field-caught and laboratory-adapted Australian tephritid fruit fly species with different host plant use and specialisation. *Microbial Ecology*, 70, 498–508.

Muturi, E. J., Donthu, R. K., Fields, C. J., Moise, I. K., & Kim, C. H. (2017). Effect of pesticides on microbial communities in container aquatic habitats. *Scientific Reports*, 7, 44565.

Oliver, K. M., Russell, J. A., Moran, N. A., & Hunter, M. S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceeding of the National Academy of Sciences of the United States of America*, 100, 1803–1807.

Pernice, M., Simpson, S. J., & Ponton, F. (2014). Towards an integrated understanding of gut microbiota using insects as model systems. *Journal of Insect Physiology*, 69, 12–18.

Ramirez, J. L., Short, S. M., Bahia, A. C., Saraiva, R. G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G., & Dimopoulos, G. (2014). Chromobacterium Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and in vitro anti-pathogen activities. *PLoS Pathogens*, 10, e1004398.

Ramirez, J. L., Souza-Neto, J., Torres Cosme, R., Rovira, J., Ortiz, A., Pascale, J. M., & Dimopoulos, G. (2012). Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases*, 6, e1561.

Rauglas, E., Martin, S., Bailey, K., Magnuson, M., Phillips, R., & Harper, W. F. Jr (2016). The effect of malathion on the activity, performance, and microbial ecology of activated sludge. *Journal of Environmental Management*, 183, 220–228.

Rosenberg, E., & Zilber-Rosenberg, I. (2011). Symbiosis and development: the hologenome concept. *Birth Defects Research. Part C, Embryo Today: Reviews*, 93, 56–66.

Sandra, L. J., Mehlferber, E., & Moore, P. J. (2015). Life-history trade-offs under different larval diets in *Drosophila suzukii* (Diptera: Drosophilidae). *Physiological Entomology*, 40(1), 2–9. https://doi.org/10.1111/phen.12082

Scates, S. S., O'Neal, S. T., & Anderson, T. D. (2019). Bacteria-mediated modification of insecticide toxicity in the yellow fever mosquito, *Aedes aegypti*. *Pesticide Biochemistry and Physiology*, 161, 77–85.

Shen, Y. J., Lu, P., Mei, H., Yu, H. J., Hong, Q., & Li, S. P. (2010). Isolation of a methyl parathion-degrading strain *Stenotrophomonas* sp. SMSP-1 and cloning of the ophc2 gene. *Biodegradation*, 21, 785–792.

Short, S. M., Mongodin, E. F., MacLeod, H. J., Talyuli, O. A. C., & Dimopoulos, G. (2017). Amino acid metabolic signaling influences *Aedes aegypti* midgut microbiome variability. *PLoS Neglected Tropical Diseases*, 11, e0005677.

Soltani, A., Vatandoost, H., Oshaghi, M. A., Enayati, A. A., & Chavshin, A. R. (2017). The role of midgut symbiotic bacteria in resistance of *Anopheles stephensi* (Diptera: Culicidae) to organophosphate insecticides. *Pathogens and Global Health*, 111, 289–296.

Tsuchida, T., Koga, R., Fujiwara, A., & Fukatsu, T. (2014). Phenotypic effect of "Candidatus Rickettsiella viridis," a facultative symbiont of the pea aphid (*Acyrthosiphon pisum*), and its interaction with a coexisting symbiont. *Applied and Environment Microbiology*, 80, 525–533.

Vorburger, C., & Rouchet, R. (2016). Are aphid parasitoids locally adapted to the prevalence of defensive symbionts in their hosts? *BMC Evolutionary Biology*, 16, 271.

Werren, J. H., Baldo, L., & Clark, M. E. (2008). Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6, 741–751.

Wilkinson, T. J., Huws, S. A., Edwards, J. E., Kingston-Smith, A. H., Siu-Ting, K., Hughes, M., Rubino, F., Friedersdorff, M., & Creevey, C. J. (2018). CowPI: A rumen microbiome focussed version of the PICRUSt functional inference software. *Frontiers in Microbiology*, 9, 1095.

Youssef, N., Sheik, C. S., Krumholz, L. R., Najar, F. Z., Roe, B. A., & Elshahed, M. S. (2009). Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Applied and Environment Microbiology*, 75, 5227–5236.

Zhang, C., Jia, L., Wang, S., Qu, J., Li, K., Xu, L., Shi, Y., & Yan, Y. (2010). Biodegradation of beta-cypermethrin by two *Serratia* spp. with different cell surface hydrophobicity. *Bioresource Technology*, 101, 3423–3429.

Zhang, S., Luo, J., Wang, L., Zhang, L., Zhu, X., Jiang, W., & Cui, J. (2019). Bacterial communities in natural versus pesticide-treated *Aphis gossypii* populations in North China. *Microbiologyopen*, 8, e00652.

Zhao, Y. J., Dai, Y. J., Yu, C. G., Luo, J., Xu, W. P., Ni, J. P., & Yuan, S. (2009). Hydroxylation of thiacloprid by bacterium *Stenotrophomonas maltophilia* CGMCC1.1788. *Biodegradation*, 20, 761–768.

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews*, 32, 723–735.

## APPENDIX

TABLE A1   Cultivable bacteria and suitable media

| The species of culturable bacteria | Media | | | | | |
|---|---|---|---|---|---|---|
| | AA | BHI | GAM | LB | NA | TSA |
| Serratia oryzae | ● | ● | ● | ● | ● | ● |
| Acinetobacter bereziniae | ● | ● | ● | ● | ● | ● |
| Comamonas koreensis | ● | ● | ● | ● | ● | ● |
| Acinetobacter junii | ● | ● | ● | ● | ● | ● |
| Chryseobacterium indologenes | ● | ● | ● | ● | ● | ● |
| Delftia tsuruhatensis | ● | ● | ● | ● | ● | ● |
| Stenotrophomonas sp | ● | ● | ● | ● | ● | ● |
| Elizabethkingia anophelis | ● | ● | ● | ● | ● | |
| Aeromonas hydrophila | ● | ● | ● | ● | ● | ● |
| Acinetobacter schindleri | ● | | | | ● | |
| Comamonas testosteroni | ● | ● | | ● | ● | |
| Pseudomonas mosselii | ● | ● | ● | ● | | ● |
| Shewanella xiamenensis | ● | | | ● | | |
| Microbacterium dextranolyticum | | ● | | ● | | ● |
| Acinetobacter beijerinckii | | ● | ● | ● | ● | ● |
| Leucobacter chironomi | | ● | ● | | ● | |
| Hydrogenophaga palleronii | | ● | | | | |
| Microbacterium esteraromaticum | | ● | | | | |
| Methylorubrum aminovorans | | ● | | | | |
| Acinetobacter schindleri | | | ● | | | |
| Exiguobacterium aurantiacum | | | ● | | | |
| Bacillus anthracis | | | ● | | | |
| Sphingobacterium detergens | | | | ● | | |
| Pseudoxanthomonas sp | | | | | ● | ● |
| Lysinibacillus sphaericus | | | | | | ● |
| Comamonas aquatica | | | | | ● | ● |
| Pseudomonas guguanensis | | | | | ● | |
| Cutibacterium acnes | | | | | ● | |

Note: The black dots mean this strain can be well cultured in this medium.

Abbreviations: AA, anaerobic agar; BHI, brain heart infusion agar; GAM, Gifu anaerobic medium agar; LB, lysogeny broth; NA, nutrient agar; TSA, tryptose soya agar.