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ORIGINAL ARTICLE

Fungi on the cuticle surface increase the resistance of *Aedes albopictus* to deltamethrin

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Abstract *Aedes albopictus* (*Ae. albopictus*) is widely distributed and can transmit many infectious diseases, and insecticide-based interventions play an important role in vector control. However, increased insecticide resistance has become a severe public health problem, and the clarification of its detailed mechanism is a matter of urgence. This study found that target-site resistance and metabolic resistance could not fully explain insecticide resistance in field *Ae. albopictus*, and there were likely other resistance mechanisms involved. The 16S and internal transcribed spacer sequencing revealed significant differences in the species compositions of the cuticle surface symbiotic bacteria and fungi between deltamethrin (DM)-resistant (DR) and DM-susceptible (DS) *Ae. albopictus*. Additionally, the abundances of *Serratia* spp. and *Candida* spp. significantly increased after DM treatment. Furthermore, 2 fungi (*Rhodotorula mucilaginosa* and *Candida melibiosica*) and 3 bacteria (*Serratia marcescens*, *Klebsiella aerogenes*, and *Serratia* sp.) isolated from DR *Ae. albopictus* can use DM as their sole carbon source. After reinoculation onto the cuticle surface of DS *Ae. albopictus*, *R. mucilaginosa* and *C. melibiosica* significantly enhanced the DM resistance of *Ae. albopictus*. Moreover, transcriptome sequencing of the surviving *Ae. albopictus* after DM exposure revealed that the gene expression of cytochrome P450 enzymes and glutathione-S-transferases increased, suggesting that besides the direct degradation, the candidate degrading microbes could also cause insecticide resistance via indirect enhancement of mosquito gene expression. In conclusion, we demonstrated that the cuticle surface symbiotic microbes were involved in the development of insecticide resistance in *Ae. albopictus*, providing novel and supplementary insights into insecticide resistance mechanisms.

Key words *Aedes albopictus*; cuticle surface symbiotic; deltamethrin resistance; fungi

Introduction

Mosquito-borne infectious diseases are globally widespread and cause severe harm to humans (Liu *et al.*, 2021). Among mosquito species that transmit diseases, *Aedes albopictus* is highly invasive and widely distributed, and it transmits a wide range of viruses such as dengue, Zika, and chikungunya in most countries, resulting in an enormous public health burden and

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immense economic losses (Roiz *et al.*, 2024). To solve this problem, the application of insecticides, the reduction of larval sources, and other methods are often used for management (Sarker *et al.*, 2024).

Insecticide treatment is an effective chemical mosquito-control method and has been widely used because of its rapid effects and ease of operation; however, severe insecticide resistance problems urgently need to be solved worldwide (Deng *et al.*, 2021; Asgarian *et al.*, 2023). Currently, there are 4 recognized insecticide resistance mechanisms, namely: (i) metabolic resistance, in which the insecticides are metabolized and degraded by a series of chemical reactions due to the increase in metabolic enzyme activities in the mosquitoes (Schluep & Buckner, 2021); (ii) target resistance, in which the level of insecticide sensitivity in the mosquitoes is decreased by mutations in the insecticide target sites (Auteri *et al.*, 2018); (iii) cuticular resistance, in which the penetration of insecticides is reduced, and the time for insecticides to reach the site of action is prolonged by alterations in the epidermal thickness or composition of the mosquito (Dang *et al.*, 2017); and (iv) behavioral resistance, in which mosquitoes develop behavior to avoid contact with the insecticides (Dhiman *et al.*, 2021). Research is the most in-depth and widely accepted on the first two of these four mechanisms. Although studies on mosquito-related resistance mechanisms are quite thorough, these studies still cannot fully explain the overall mechanism involved (Toé *et al.*, 2022; Wang *et al.*, 2023).

In recent years, increasing attention has been given to the interaction between symbiotic microbes and insects in the field, and the results revealed that the symbiotic microbes not only participate in a variety of the biological activities of insects but can also increase insecticide resistance in insects (Chen *et al.*, 2020; Sato *et al.*, 2021). For example, *Citrobacter* (CF-BD) in *Bactrocera dorsalis* (Hendel) enhances host resistance to trichlorfon by degrading trichlorfon directly into chloral hydrate and dimethyl phosphite (Cheng *et al.*, 2017). An indirect mechanism involves *Lactobacilli* spp. in the gut of *Drosophila melanogaster*, in which the expression of the host *NADPH oxidase 1* gene is induced via the secretion of reactive oxygen species. This in turn mediates the Nrf2 signaling pathway and promotes the upregulation of the detoxification metabolism enzyme-encoding genes *CYP6A18* and *GSTZ2*, thereby resisting paraquat stress (Jones *et al.*, 2015).

Currently, studies on the relationship between symbiotic microbes and insecticide resistance of mosquitoes are limited, and most have focused on the function of the mosquito gut microbes (Strand, 2018). However, the influence of the cuticle surface of mosquitoes, another important location of mosquito microbes, cannot be ignored. Previous studies have focused more heavily on gut bacteria, but the cuticle surface is the primary barrier to mosquito defense, so its role in insecticide resistance may be underestimated (Shi *et al.*, 2023). Some epidermal symbiotic bacteria can help the host resist fungal infection and greatly increase the survival rate of the host (Janke *et al.*, 2022). The cuticle is the first protective barrier against mosquito exposure to insecticides, especially the contact-killing type, and the changes in its commensal microbes may more intuitively reflect their response to insecticides. A previous study revealed that, after exposure to insecticides, the epidermal symbiotic bacteria of mosquitoes significantly change (Dada *et al.*, 2019). Therefore, we hypothesize that the symbiotic microbes on the cuticle surface of *Ae. albopictus* may contribute to enhancing insecticide resistance.

In this study, the symbiotic microbes on the cuticle surface of deltamethrin (DM)-resistant (DR) *Ae. albopictus* was screened and identified, and the candidate DM-degrading organisms were isolated. Functional analysis experiments were then carried out *in vivo* and *in vitro*, which provided a novel research perspective for the discovery of insecticide resistance mechanisms in *Ae. albopictus* and other mosquitoes.

Materials and methods

Mosquito collection and rearing

A laboratory colony of *Ae. albopictus* (Lab) was provided by the National Health Commission Key Laboratory of Parasitic Disease Control and Prevention, Jiangsu, China. It was collected in Wuxi City (WX) in the 1980s and has not been exposed to any insecticides since then. The field populations of *Ae. albopictus* were collected in WX, Changzhou City (CZ), and Suzhou City (SZ) of Jiangsu Province from 2022 to 2023. The human bait and black trap collection methods were used for adult and larval mosquito collection, respectively, in the F0 generation. Taking advantage of the preference of *Ae. albopictus* to oviposit in dark containers, we put some water in these containers in advance to attract them, and then their larvae could be captured after about 1 week. All the test mosquitoes were subsequently morphologically verified and reared in the laboratory to the F1 generation (F1). The adult rearing conditions were as follows: temperature, 25 ± 1 °C; relative humidity, 75% ± 5%; and photoperiod, 10 h : 14 h (light : dark). The larvae were fed pig liver powder and yeast powder (2 : 1). After pupating, the pupae were transferred to mosquito-rearing cages and reared with 5%–10% glucose to the adult stage.

Insecticide resistance bioassays and the adult synergist assay

The laboratory-colonized *Ae. albopictus* and 3 field-collected *Ae. albopictus* populations were tested via the World Health Organization-recommended tube test method (WHO, 2022). The SZ and WX field populations were used to test metabolic enzyme activities, and all of them were subjected to 16S and internal transcribed spacer (ITS) sequencing. For each tube, approximately 30 3- to 5-d-old F1 female mosquitoes without a blood meal were exposed to 0.05% DM-treated film (Chinese Center for Disease Control and Prevention, China) for 1 h. After 24 h of recovery, the mortality rate was recorded. For the control group, *Ae. albopictus* was not exposed to the insecticide-treated film, and each experiment was repeated 3 times (resistant: mortality < 90%; possibly resistant: 90% < mortality< 98%; susceptible: > 98%). All the surviving and dead *Ae. albopictus* were stored at −80 °C.

According to the CDC bottle bioassay (Centers for Disease Control and Prevention [U.S.], 2022), 2 synergists, 400 μg/bottle piperonyl butoxide (PBO, MCE, USA) and 80 μg/bottle diethyl maleate (DEM, MCE, USA), were prepared. S,S,S-tributyl phosphorotrithioate (DEF) was not used because of purchase restrictions. Approximately 125 3- to 5-d-old F1 female mosquitoes without a blood meal were exposed to the synergist for 1 h and then placed in a recovery tube for 1 h. Then, the test mosquitoes were exposed to 0.05% DM film for 1 h, and the mortality rate of each group was recorded after 24 h of recovery. The mosquitoes in the control group were in contact with synergists only, with no contact with DM.

Determination of metabolic enzyme activity

The protein concentration of *Ae. albopictus* was determined with a Pierce BCA Protein Assay Kit (Thermo Scientific, USA). The activities of glutathione-S-transferase (GST) and carboxylesterase (CarE) in DS and DR *Ae. albopictus* were determined via an enzyme-linked immunosorbent assay (ELISA) kit (Jianglaibio, China). Five female mosquitoes per sample were added to 500 μL of buffer and 0.5 mm ceramic beads. All the samples were ground with a frozen grinder. The program was set to 1 000 rad/min for 10 s, 1 500 rad/min for 10 s, and 2 000 rad/min for 10 s, after which the mixture was suspended for 20 s, cycled 5–10 times, and fully ground until large tissue fragments were not visible. After centrifuging at 9391 × *g* (10 000 r/min, Eppendorf Centrifuge 5424 R) for 10 min at 4 °C, the supernatant was selected for protein concentration determination and metabolic enzyme activity determination. The metabolic enzyme activity was calculated from the protein concentration according to the instructions of the ELISA kit.

DNA extraction and knockdown resistance genotyping

The genomic DNA from the surviving (DR) and dead (DS) samples of *Ae. albopictus* from WX and SZ field populations was extracted via a DNeasy Blood and Tissue Kit (Qiagen, Germany) and stored at −80 °C. Referring to Kasai *et al.* (2011) and other methods, 3 pairs of primers were synthesized to amplify the partial fragments of transmembrane domains II, III, and IV of the VGSC gene. AegSCF20 (gacaatgtggatcgcttccc) and aegSCR21 (gcaatctggcttgttaacttg) were used to amplify domain II, aegSCF7 (gagaactcgccgatgaaactt) and aegSCR7 (gacgacgaaatcgaacaggt) were used to amplify domain III, and albSCF6 (tcgaagtacttcgtgtcg) and albSCR8 (aacagcaggatcatgctg) were used to amplify domain IV. The polymerase chain reaction (PCR) conditions were 95 °C for 5 min followed by 30 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 1 min. The PCR products were sequenced by ExSyn-bio Technology Co., Ltd. (Shanghai, China).

AegSCF20 (gacaatgtggatcgcttccc) (forward primer for domain II), aegSCR8 (tagctttcagcgcttcttc) (reverse primer for domain III) and albSCF6 (tcgaaggaagtacttcgtgtcg) (forward primer for domain IV) were used for sequencing. BioEdit (v 7.2.5) software was used to compare and analyze the peak patterns of the sample sequences and the reference sequences of the *Ae. albopictus* VGSC gene (GenBank accession numbers: KC152045.1, KC152046.1, and KC152047.1). Mutations in the known knockdown resistance (*kdr)* mutation sites (S989, I1011, L1014, V1016, I1532, F1534, D1763) were observed. The allele types and genotypes were subsequently determined, and the frequencies of *kdr* in each region were counted and calculated.

Differences in the symbiotic microbes between DS and DR Ae. albopictus

The DNA of the symbiotic microbes on the cuticle surface of *Ae. albopictus* with different resistance phenotypes after treatment with 0.03% DM (L), 0.05% DM (H), and no DM (control) were collected and analyzed via 16S sequencing and ITS sequencing. 100 μL of phosphate-buffered saline (PBS) was added to a pool of 20 female *Ae. albopictus* samples, which were subsequently vortexed and mixed, and this process was repeated 3

times. Finally, 300 μL of the epidermal microbes mixture was obtained, and 100 μL of the mixture was left for subsequent culture and screening. Next, DNA was extracted from the symbiotic microbes via a DNeasy Blood and Tissue Kit (Qiagen, Germany). The DNA concentration and integrity were measured via a NanoDrop 2000 (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. The extracted DNA was stored at −80 °C.

The extracted DNA was used as a template for PCR amplification of bacterial 16S ribosomal RNA (rRNA) genes with barcoded primers and Takara Ex Taq (Takara, Japan). For bacterial diversity analysis, the V3–V4 variable regions of 16S rRNA genes were amplified with the universal primers 343F (5′-TACGGRAGCCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′) (Nossa *et al.*, 2010). The extracted DNA was used as a template for the PCR amplification of the fungal ITS genes using barcoded primers and Takara Ex Taq (Takara). For fungal diversity analysis, the ITS1 variable regions of the ITS genes were amplified with the universal primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Mukherjee *et al.*, 2014).

The amplicon quality was visualized via agarose gel electrophoresis. The PCR products were purified with AMPure XP beads (Agencourt) and amplified via another round of PCR. After being purified with AMPure XP beads again, the final amplicon was quantified via the Qubit double-stranded DNA (dsDNA) Assay Kit (Thermo Fisher Scientific, USA). The concentrations were then adjusted for sequencing. Sequencing was performed on an Illumina NovaSeq 6000 with 250 bp paired-end reads (Illumina Inc., USA; OE Biotech Company, China).

Library sequencing and data processing were conducted by OE Biotech Co., Ltd. (Shanghai, China). The raw sequencing data were generated in FASTQ format. Paired-end reads were then preprocessed via Cutadapt software to detect and cut off the adapter. After trimming, the paired-end reads were filtered into low-quality sequences, denoised, merged, and detected, and the chimeric reads were cut off via DADA2 (Callahan *et al.*, 2016) with the default parameters of QIIME2 (Bolyen *et al.*, 2019). Finally, the software outputs the representative reads and the amplicon sequence variant (ASV) abundance table. The representative read of each ASV was selected via the QIIME2 package. All representative reads were annotated and subjected to BLAST searches against the Silva database (v 138) using a q2-feature classifier with the default parameters.

QIIME2 software was used for alpha and beta diversity analysis. The microbial diversity in the samples was estimated via alpha diversity, which includes the Chao1 index (Chao & Bunge, 2002) and Shannon index (Hill *et al.*, 2003). The unweighted UniFrac distance matrix generated via the R package was used for unweighted UniFrac principal coordinate analysis (PCoA) to estimate the beta diversity. The R package was subsequently used to analyze the significance of differences between different groups via analysis of variance (ANOVA). The linear discriminant analysis effect size (LEfSe) method was used to compare the spectrum of taxonomic abundance.

Screening, isolation, and identification of DM-degrading symbiotic microbes on the cuticle surface of DR Ae. albopictus

The surviving *Ae. albopictus* of the WX field population were collected after insecticide resistance bioassays, and each sample consisted of the cuticle microbes' mixture of 20 female mosquitoes. In accordance with the methods (Guo *et al.*, 2009), this mixture was inoculated into Luria-Bertani (LB) liquid medium (Solarbio, China), 100 mL medium containing tryptone (1 g), yeast extract (0.5 g), NaCl (1 g), for enrichment culture for 2–3 d at 30 °C and 200 r/min, after which the culture was inoculated into carbon source test fluid (methylsulfonyl methane [MSM]) medium (REBIO, China) containing 10 μg/mL DM at a volume ratio of 5%. After one week of culture at 30 °C and 200 r/min, the culture was transferred again at a volume ratio of 5%, and the process was repeated 3 times. Finally, the final culture was inoculated on LB plating medium and Sabouraud's Glucose Agar plating medium (SDA, REBIO, China) for careful identification of different colony characteristics.

These microbes were isolated and purified until all the colonies were single in shape and consistent in appearance. Gram staining was performed on all the purified microbes (Bartholomew & Mittwer, 1952), and the morphological characteristics of the candidate degrading microbes were observed to distinguish between bacteria and fungi, with *Escherichia coli* as the Gram-negative control. The Gram staining kit was purchased from Solarbio (China).

The strains were subsequently identified via 16S sequencing (Janda & Abbott, 2007) and ITS sequencing (Nilsson *et al.*, 2019). The full-length 16S rRNA gene was amplified using primers 27F (5′-AGAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). ITS1 (5′-GTAGGT GAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) were used to amplify the ITS. The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s, and

72 °C for 1 min. The PCR products were sequenced by ExSyn-bio Technology Co., Ltd. (Shanghai, China), and the sequencing results were submitted to GenBank for homology analysis.

To evaluate whether the candidate degrading microbes could use DM as the sole carbon source for growth, we investigated their effect on mosquitoes *in vitro*. The candidate degrading microbes were cultured in MSM supplemented with DM (10 μg/mL). The optical density at 600 nm (OD_{600}) of the microbes were measured by a microplate reader every day, and a growth curve was drawn for each strain. The experimental steps were performed according to the microbiology experiment tutorial (Xu *et al.*, 2019). Nutrient medium was used as a positive control (LB medium for bacteria and SDA medium for fungi) to ensure that all the candidate microbes were alive when inoculated. MSM without DM was used as a negative control. Next, the DM-degrading microbes were enriched and cultured, seeded with 25% glycerol and stored at −80 °C.

Bioassays of metabolic enzyme activity in DM-degrading microbes

The microbes were inoculated into nutrient medium (LB medium for bacteria and SDA medium for fungi), LB medium or SDA medium supplemented with DM (10 μg/mL), or MSM supplemented with DM (10 μg/mL). All degrading microbes were cultured for 12 h at 30 °C and 200 r/min to ensure that they were in the logarithmic growth phase. The precipitation in a 1–3 mL culture of each strain was then collected, centrifuged at 9 391 × *g* (10 000 r/min, Eppendorf Centrifuge 5424 R) for 1 min, and washed with PBS; this process was repeated 3 times to ensure that no residual medium remained in the precipitate. Next, 500 μL of buffer was added to the microbial precipitate, which was then ultrasonically crushed in an ice bath according to the ELISA kit instructions. The program was set to 200 W ultrasonication for 3 s at intervals of 10 s, which was repeated 30 times. The samples were then centrifuged at 13 523 × *g* (12 000 r/min, Eppendorf Centrifuge 5424 R) at 4 °C for 10 min, after which the supernatants were placed on ice for testing. The other steps were the same as those used for the bioassays of metabolic enzyme activity in mosquitoes.

Degrading microbes were inoculated back into the cuticle surface of DS-treated Ae. albopictus

A plasmid containing enhanced green fluorescent protein (EGFP) was used to label each microbial strain, and then the colonization of the symbiotic microbes was observed via fluorescence microscopy. Plasmid pET28a-EGFP (FENGHUISHENGWU, China) simultaneously expressing EGFP and a kanamycin resistance label (50 μg/mL) was transformed via electroporation (Trevors, 1990; Sakural & Komatsubara, 1996; Bando *et al.*, 2013).

Overnight cultures of *Serratia marcescens*, *Klebsiella aerogenes*, and *Serratia* sp. were centrifuged to precipitate the microbes, which were washed 3 times with precooled glycerol to remove all residual medium. A 100 μL glycerol–microbe mixture and 2 μL plasmid ($\leq$ 50 ng/μL) were added to a precooled 0.2 cm Gene Pulser/MicroPulser Cuvettes (Bio-Rad, USA), and the parameters of the Gene Pulser Xcell Total System (Bio-Rad, USA) were set to 12.5 kV/cm, 25 μF, and 200 Ω. The electroporation mixture was immediately inoculated into LB liquid medium and recovered at 30 °C for 30 min, after which the culture was inoculated onto LB plating medium supplemented with 100 μg/mL kanamycin and cultured overnight at 30 °C.

The bacteria that were successfully transformed with the plasmid on the resistance plating medium were preserved with 25% glycerol and stored at −80 °C. The EGFP-labeled bacterial strains were cultured to an OD_{600} of 0.4–0.6. The bacterial precipitate in 5 mL of culture was collected, and the bacterial mixture was resuspended in 500 μL of sterile PBS buffer after washing. The bacterial mixture was reinoculated onto the cuticle surface of Lab *Ae. albopictus* by spraying, and the mosquitoes were kept in an exposure tube recommended by the WHO for insecticide resistance tests. The upper and lower ends of the tube were sealed with nylon gauze, and 5% glucose cotton covered the upper end of the tube. The microbe solution was sprayed from the 1st d to the 5th d. A 3 mL spray pot was used, and the volume of solution sprayed was 500 μL each time. One spray was done in the morning and 1 spray in the evening, for a total of 10 times.

Each group of mosquitoes was subsequently exposed to 0.03% DM film, and the mortality rate was recorded to evaluate the changes in resistance of the mosquitoes sprayed with the degrading microbes. The control group was sprayed with PBS. The fluorescently labeled degrading bacteria were observed via fluorescence microscopy, and the colonial morphology of the degrading fungi were observed on SDA plating medium.

Evaluation of the degradation effect of degrading microbes

High-performance liquid chromatography – mass spectrometry (HPLC–MS) was used to evaluate the effects of

DM degradation by microbes. The degrading microbes in the logarithmic growth phase were inoculated into MSM liquid medium supplemented with DM (20–50 μg/mL) and cultured at 30 °C and 200 r/min for 24 h. The degraded cultures of the microbes were collected at 0 h and 24 h and recorded. The control group was blank medium without inoculation. These samples were stored at −80 °C.

For sample pretreatment, the samples were naturally thawed in the dark, and 0.1 mL of the degradation culture was transferred to a 10 mL volumetric flask with acetonitrile to scale and then mixed up and down to ensure homogenization. After ultrasonic extraction for 30 min, the samples were centrifuged at 9 391 × *g* (10 000 r/min, Eppendorf Centrifuge 5424 R) for 10 min at 4 °C. The supernatant was passed through a 0.22 μm organic filter membrane and tested via Anhui Sci-rule Analysis and Studying Technology Co., Ltd (Anhui, China).

To evaluate whether the degrading microbes could further degrade the metabolites of DM, 3-phenoxybenzoic acid (3-PBA, MCE, USA) and 3-phenoxybenzaldehyde (MCE, USA), the above experiments involving carbon source utilization were used for verification.

Transcriptome sequencing analysis (RNA-seq) of microbe-treated Ae. albopictus

The surviving *Ae. albopictus* exposed to 0.03% DM film after being sprayed with PBS and degrading microbes were collected. The RNA extraction, library construction, and transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). Total RNA was extracted via TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated via a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed via an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Libraries were subsequently constructed via the VAHTS Universal V5 RNA-seq Library Prep Kit according to the manufacturer's instructions. Next, the library was sequenced via the Illumina NovaSeq 6000 sequencing platform, and Fastp software (Chen *et al.*, 2018) was used to process the raw reads to obtain clean reads for subsequent data analysis. The *Ae. albopictus* reference genome (GCA_001444175.2) was aligned using HISAT2 software (Kim *et al.*, 2015), and the gene expression level (fragments per kilobase of transcript per million mapped reads) was calculated (Roberts *et al.*, 2011). The read count of each gene was obtained via HTSeq-count (Anders *et al.*, 2015). Differential expression was analyzed by DESeq2 software (Love *et al.*, 2014), and genes with a Q-value < 0.05 and a fold change > 2 were defined as differentially expressed genes (DEGs).

On the basis of the hypergeometric distribution algorithm, GO (The Gene Ontology Consortium, 2019), Reactome, and WikiPathways enrichment analyses were subsequently performed on the DEGs to screen for significantly enriched functional items, and the $\log_{10}$ *P*-values corresponding to each item were sorted from large to small. PCA was performed in R (v 3.2.0), and hierarchical clustering analysis was performed on the DEGs. Radar maps were drawn to show the upregulation and downregulation of DEGs, and histograms, chords, or enrichment analysis circles were drawn for significantly enriched functional entries. Gene set enrichment analysis was performed via GSEA software (Mootha *et al.*, 2003; Subramanian *et al.*, 2005).

Data analysis

One-way ANOVA was used to analyze the differences in mortality and metabolic enzyme activity between groups. The comparison of *kdr* mutation sites among different regions was performed via the χ^2 test. A *t*-test was used for other statistical analyses. All analyses were performed via GraphPad Prism or SPSS 25, and a *P*-value < 0.05 was considered statistically significant.

Results

The 3 field populations of Ae. albopictus were resistant to DM

After contact with 0.05% DM film for 1 h and recovery for 24 h, the average mortalities of F1 individuals from the 3 field populations of CZ, SZ, and WX were 47.95% (211/440), 33.77% (78/231), and 49.11% (138/281), respectively, indicating that the 3 populations were resistant to DM (Fig. S1).

Metabolic enzymes are partially related to DM resistance in Ae. albopictus field populations

One-way ANOVA revealed no significant difference in GST and CarE activities between the susceptible Lab samples and resistant samples of the WX and SZ field populations without DM treatment ($P > 0.05$, Fig. 1A, B). However, after exposure to DM, the GST and CarE activities of the 2 resistant strains were significantly greater than those of the Lab samples (Fig. 1A, B). When the WX population was exposed to a low concentration of DM film (0.03%), the activities of GST and

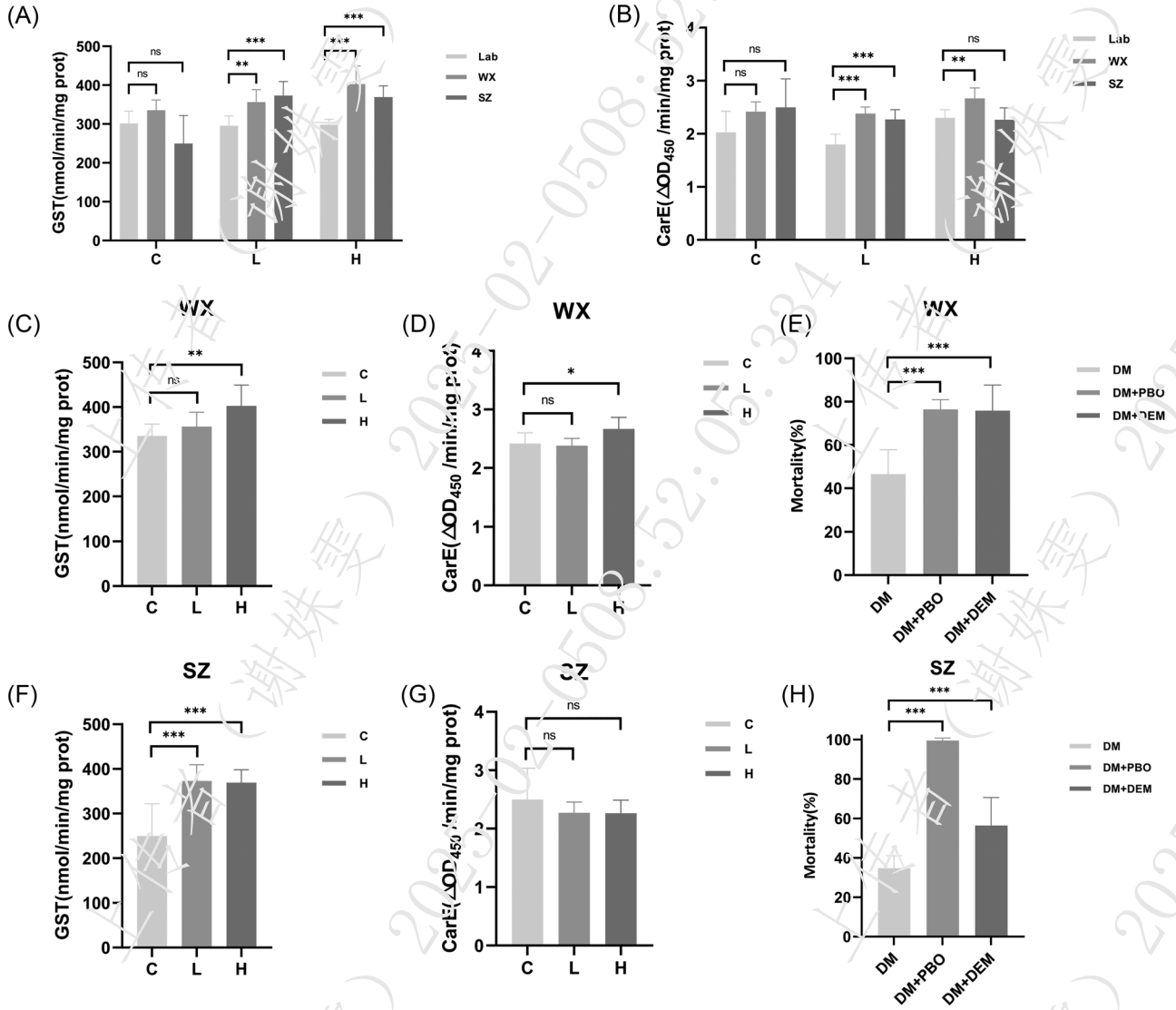

Fig. 1 GST activity (A) and CarE activity (B) of Lab *Aedes albopictus* and field *Ae. albopictus* of WX (Wuxi City) and SZ (Suzhou City) after treatment with different concentrations of DM. The GST activity (C) and CarE activity (D) of the surviving *Ae. albopictus* of WX after treatment with different concentrations of DM; the GST activity (F) and CarE activity (G) of surviving mosquitoes after treatment with different concentrations of DM of SZ; mortality of *Ae. albopictus* of WX (E) and SZ (H) after exposure to DM combined with synergists PBO and DEM, respectively. Group C (Control): blank, Group L (low concentration of deltamethrin film): 0.03% DM, Group H (high concentration of deltamethrin film): 0.05% DM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. CarE, carboxylesterase; DM, deltamethrin; DEM, diethyl maleate; GST, glutathione-S-transferase; ns, not significant; PBO, piperonyl butoxide.

CarE did not change. However, when the WX population was exposed to a high concentration of DM film (0.05%), the activities of both GST and CarE increased (Fig. 1C, D). When exposed to low or high concentrations of DM, the GST activity of the SZ population significantly increased, whereas that of CarE did not significantly change (Fig. 1F, G). After the 2 resistant strains were treated with the synergists PBO and DEM, the average mortality rates of the WX field population were 76.25% (199/261) and 74.92% (248/331), respectively, and the average mortality rates of the SZ field population were 99.45% (182/183) and 56.54% (134/237), respectively. Comparatively, without synergist treatment, the mortality rates of the 2 resistant strains increased to different degrees after exposure to DM and PBO or DEM (Fig. 1E, H).

Target resistance is not involved in the DM resistance of Ae. albopictus

A total of 124 female F1 mosquitoes from the WX and SZ populations were detected, and 372 DNA sequences were obtained that were 99.0% consistent with the partial sequences of VGSC domains II, III, and IV of *Ae. albopictus*. The sequencing results revealed there were no mutations at the S989, I1011, L1014, V1016, or D1763 sites in any of the samples, whereas there were different degrees of mutations at the I1532 and F1534 sites (*i.e.*, I1532T, F1534S, and F1534C) (Table 1). The test revealed there was no significant difference between the mosquitoes with different resistance phenotypes in the 2 field populations and the mutations at the I1532 and F1534 sites ($P > 0.05$).

Species composition of the symbiotic microbes on the cuticle surface significantly differed between the sensitive and resistant phenotypes of Ae. albopictus

The 16S sequencing results revealed that there was not a significant difference in the number of symbiotic bacteria on the cuticle surface of *Ae. albopictus* between the sensitive phenotypes (Lab) and the resistant phenotypes (F0 generation) of CZ and WX field populations without DM exposure. Only the SZ samples presented greater species diversity and abundance (Fig. 2A, B). Moreover, the ITS sequencing results revealed significant differences in the species diversity of the symbiotic fungi between the DS mosquitoes (Lab) and the DR mosquitoes (F0 generation) of CZ and SZ field populations. The species diversity of the CZ samples was lower than that of the Lab samples, but their abundance was greater than that of the Lab samples. The species diversity and abundance in the SZ samples were also lower than those in the Lab samples. However, those of the WX samples were not significantly different from those of the Lab samples (Fig. 2D, E). PCoA revealed that the repeated samples of *Ae. albopictus* with different resistance phenotypes were clearly clustered (Fig. 2C, F). The species composition of the symbiotic microbes of each group was subsequently analyzed, revealing significant differences in the symbiotic bacteria and the symbiotic fungi at the genus level (Fig. 2G, H).

Analysis of the community structure distribution revealed that after exposure to DM, the abundance of *Candida* spp. in the 3 field strains (Fig. S2A–2C) significantly increased, whereas the abundance of *Serratia* spp. only increased in the CZ (Fig. S3A) and SZ samples (Fig. S3B).

Symbiotic microbes degrade DM on the cuticle surface of DR Ae. albopictus

Through enrichment and screening by carbon source utilization experiments, 7 DM-tolerant symbiotic microbe strains (10 μg/mL) were isolated and purified. Gram staining revealed that 2 strains had obvious spore structures and were identified as fungi (Fig. S4B and 4C), and the other 5 strains were Gram-negative bacilli identified as bacteria (Fig. S4D–4H). Next, the amplification products of the 16S rRNA gene and ITS gene of all the strains were recovered and sequenced to obtain 14 sequences, which were submitted to GenBank for homology analysis. The microbes were identified as *Rhodotorula mucilaginosa*, *Candida melibiosica*, *S. marcescens*, *K. aerogenes*, *Serratia* sp., *Chryseobacterium* sp., and *Pantoea dispersa*. Moreover, growth curve experiments revealed that *R. mucilaginosa*, *C. melibiosica*, *S. marcescens*, *K. aerogenes*, and *Serratia* sp. could use DM as the sole carbon source (Fig. 3A, B, E–G). Furthermore, *R. mucilaginosa* and *C. melibiosica* grew most vigorously in MSM (Fig. 3A, B).

Metabolic enzymes assist microbes in degrading DM in vitro

The GST and CarE activities of *R. mucilaginosa* (Fig. 4A, B), *C. melibiosica* (Fig. 4D, E), *S. marcescens* (Fig. 4G, H), and *K. aerogenes* (Fig. 4J, K) in MSM containing DM were significantly greater than that in MSM lacking DM. However, *Serratia* sp. only exhibited slightly increased CarE activity (Fig. 4N) in LB medium supplemented with DM compared with that in LB medium without DM (Fig. 4). Further, PBO and DEM had no obvious inhibitory effects on the metabolic enzymes of *R. mucilaginosa* (Fig. 4C), *C. melibiosica* (Fig. 4F), or *K. aerogenes* (Fig. 4L). It inhibited *S. marcescens* (Fig. 4I) for 24 h but promoted *Serratia* sp. infection (Fig. 4O).

R. mucilaginosa and C. melibiosica can increase the insecticide resistance of Lab Ae. albopictus

Fluorescence microscopy experiments revealed that the 3 bacteria without EGFP labeling under both white light and fluorescence (Fig. 5C) and the 3 EGFP-labeled bacterial strains successfully colonized the cuticle surface of *Ae. albopictus* (Fig. 5A). Cuticle fungal culture after spraying revealed that *R. mucilaginosa* and *C. melibiosica* (Fig. 5B) could also colonize the cuticle surface of mosquitoes. After contact with 0.03%

Table 1 *kdr* mutant allele frequency at codons 1 532 and 1 534 from *Aedes albopictus* of WX and SZ field populations from Jiangsu Province, China.

			Kdr (1532)				Genotype		
Insecticide	Phenotype	*N*	Wild ATC(I)	Mutant ACC(T)	Mutant frequency (%)	*P*- value	I/I	I/T	T/T
Deltamethrin	WX-S	32	60	4	6.25	1.000	28	4	0
	WX-R	30	57	3	5.00		27	3	0
	SZ-S	32	64	0	0.00	0.484	32	0	0
	SZ-R	30	58	2	3.33		29	0	1
			Kdr (1534)				Genotype		
Insecticide	Phenotype	*N*	Wild TTC(F)	Mutant TCC(S)	Mutant frequency (%)	*P*- value	F/F	F/S	S/S
Deltamethrin	WX-S	32	48	16	25.00	0.611	17	14	1
	WX-R	30	40	20	33.33		14	12	4
	SZ-S	32	55	9	14.06	0.319	24	7	1
	SZ-R	30	49	11	18.33		19	11	0
			Kdr (1 534)				Genotype		
Insecticide	Phenotype	*N*	Wild TTC(F)	Mutant TGC(C)	Mutant frequency (%)	*P*- value	F/F	F/C	C/C
Deltamethrin	WX-S	32	61	3	4.69	1.000	29	3	0
	WX-R	30	57	3	5.00		27	3	0
	SZ-S	32	62	2	3.13	0.492	30	2	0
	SZ-R	30	60	0	0.00		30	0	0

kdr, knockdown resistance; WX-S, deltamethrin-susceptible (DS) samples of Wuxi (WX) field population; WX-R, deltamethrin-resistant (DR) samples of WX field population; SZ-S, DS samples of Suzhou (SZ) field population; SZ-R, DR samples of SZ field population.

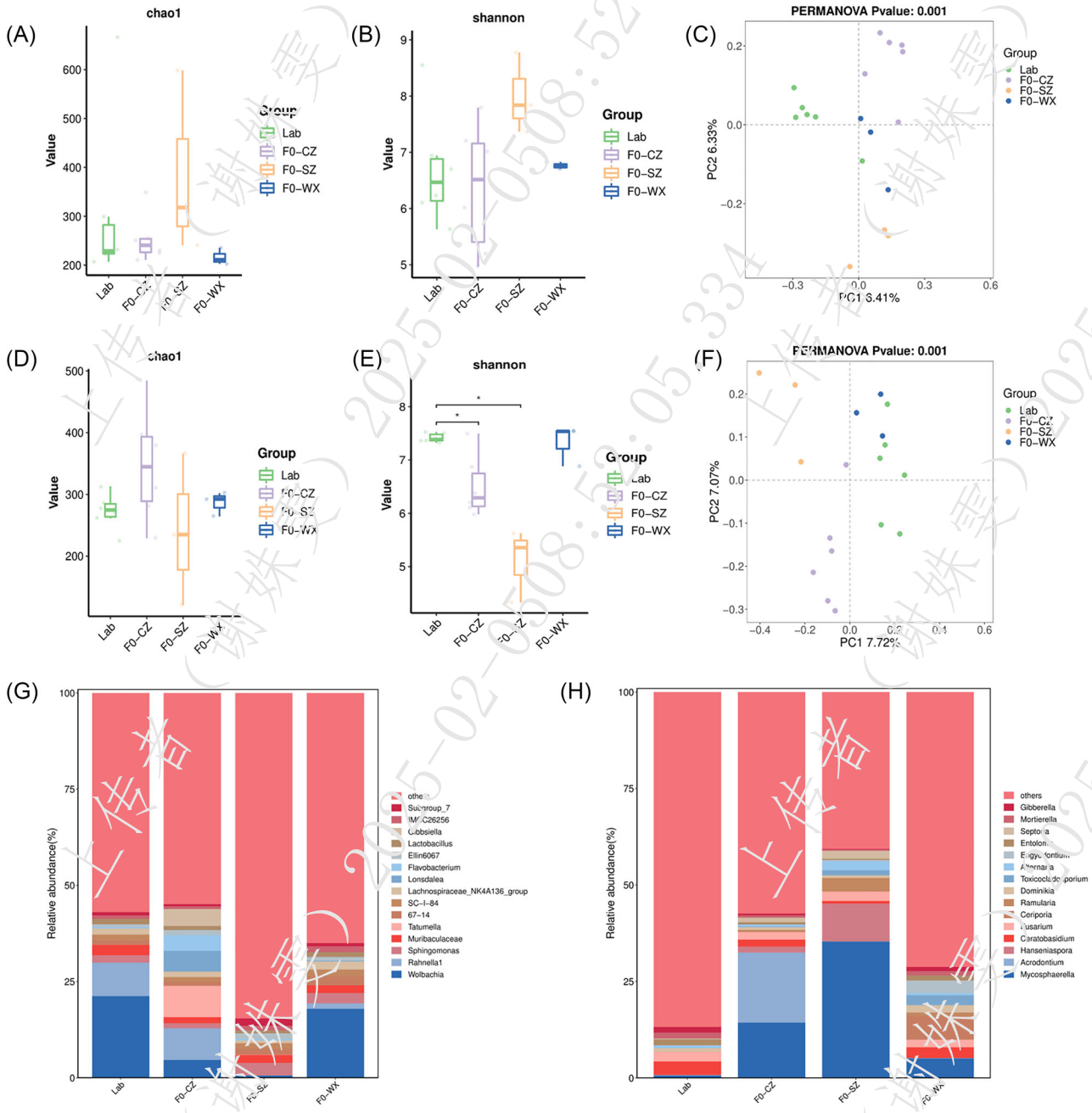

Fig. 2 Boxplot diagram of the cuticle surface symbiotic microbe α diversity of *Aedes albopictus* with different resistance phenotypes: bacteria (A, B) and fungi (D, E). Principal component analysis at the genus level of the cuticle surface symbiotic microbes of *Ae. albopictus* with different resistance phenotypes: bacteria (C) and fungi (F). Column diagram of the community structure at the genus level of the cuticle surface symbiotic microbes of *Ae. albopictus* with different resistance phenotypes: bacteria (G) and fungi (H). Lab: sensitive laboratory colony; F0-WX: F0 generation of the Wuxi field population, F0-SZ: F0 generation of the Suzhou field population, F0-CZ: F0 generation of the Changzhou field population. *$P < 0.05$.

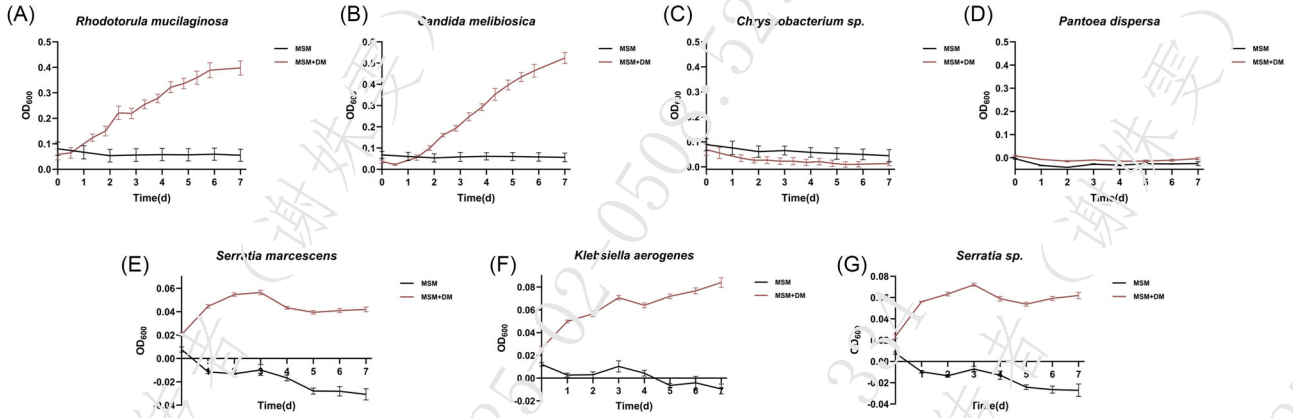

Fig. 3 Growth curve assay of the primarily screened microbes. (A) *Rhodotorula mucilaginosa*, (B) *Candida melibiosica*, (C) *Chryseobacterium* sp., (D) *Pantoea dispersa*, (E) *Serratia marcescens*, (F) *Klebsiella aerogenes*, (G) *Serratia* sp. DM, deltamethrin; MSM, carbon source test fluid medium.

DM and after 24 h of recovery, the average mortalities were 96.47% (355/368), 84.83% (123/145), 82.31% (107/130), 93.79% (151/161), 90.27% (102/113), and 95.93% (118/123) in the control, *R. mucilaginosa*, *C. melibiosica*, *S. marcescens*, *K. aerogenes*, and *Serratia* sp. groups, respectively (Fig. 6A). A *t*-test analysis revealed that the mortalities of *Ae. albopictus* sprayed with *R. mucilaginosa* and *C. melibiosica* were significantly lower than those in the control group.

Different effects of DM and its metabolites among candidate degrading microbes

We observed the utilization of the DM metabolites 3-phenoxybenzaldehyde and 3-PBA by degrading microbes and found that some microbes could use these 2 metabolites as the sole carbon source; in particular, *Candida* sp. and *Serratia* sp. highly utilized 3-phenoxybenzaldehyde (Fig. 6E). Both *C. melibiosica* and *R. mucilaginosa* had the greatest ability to degrade 3-PBA (Fig. 6D). Subsequently, HPLC–MS analysis revealed that the average 24 h degradation rates of DM by *R. mucilaginosa* (Fig. 6B) and *C. melibiosica* (Fig. 6C) were 20.17% and 70.45%, respectively.

Degrading microbes increase the gene expression of specific metabolic enzymes in Ae. albopictus

The comparison rate of all treatment reads was 76.18%–76.89%, and the numbers of DEGs observed in the 2 groups (T1 *vs*. C and T2 *vs*. C) were 1 613 and 834, respectively.

Differential expression analysis

The T1 versus C comparison revealed 48 DEGs related to metabolic enzymes, most of which were CYPs and GSTs; 40 were upregulated and 8 were downregulated. In the T2 versus C comparison, 37 DEGs related to metabolic enzymes, which were similar to those in the T1 versus C comparison, were identified, of which 31 genes were upregulated and 6 genes were downregulated (Fig. 7A, B). In the T1 versus C and the T2 versus C comparisons, 21 genes were upregulated and 5 genes were downregulated (Fig. 7C, D).

GO enrichment analysis

In the T1 versus C and the T2 versus C comparisons, positive regulation of cholesterol efflux (GO: 0010875) was the most significantly enriched biological process. In the cellular component category, the extracellular space (GO: 0005615) and the external side of the plasma membrane (GO: 0009897) were the most significantly enriched terms. In the molecular function category, iron ion binding (GO: 0005506); oxidoreductase activity, acting on paired donors, incorporation or reduction of molecular oxygen (GO: 0016705); monooxygenase activity (GO: 0004497); and haem binding (GO: 0020037) were the most significantly enriched terms (Fig. 7E, F).

Discussion

Ae. albopictus, as one of the most invasive mosquito species, transmits a variety of mosquito-borne diseases worldwide. At present, most insecticide-resistant

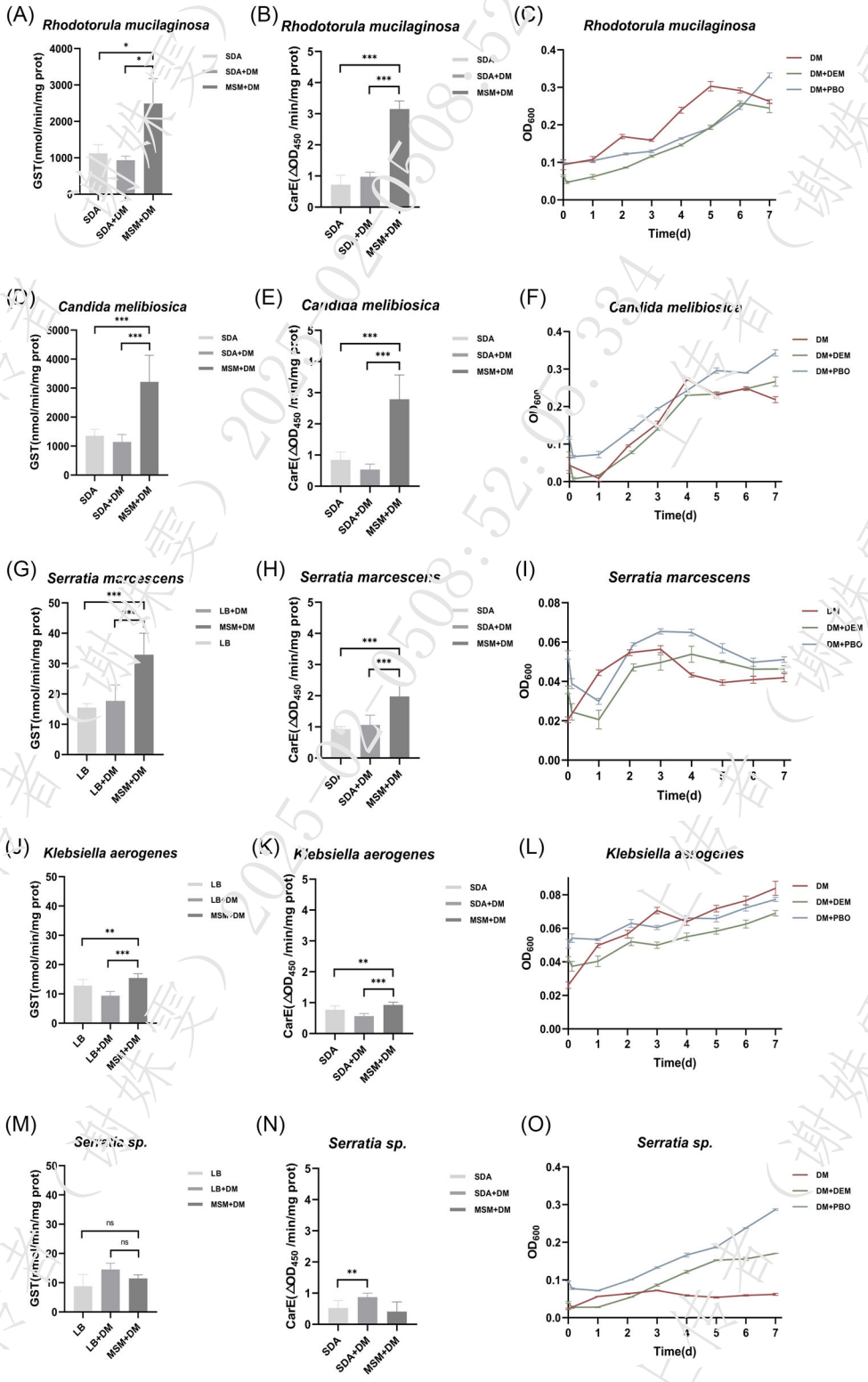

Fig. 4 GST activity (A) *Rhodotorula mucilaginosa*, (D) *Candida melibiosica*, (G) *Serratia marcescens*, (J) *Klebsiella aerogenes*, (M) *Serratia* sp. and CarE activity (B) *R. mucilaginosa*, (E) *C. melibiosica*, (H) *S. marcescens*, (K) *K. aerogenes*, (N) *Serratia* sp. of the degrading microbes after culturing in different media with or without DM. Growth curve assay of the degrading microbes (C)

R. mucilaginosa, (F) *C. melibiosica*, (I) *S. marcescens*, (L) *K. aerogenes*, (O) *Serratia* sp. after exposure to DM combined with the synergists PBO and DEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. CarE, carboxylesterase; DEM, diethyl maleate; DM, deltamethrin; GST, glutathione-S-transferase; ns, not significant; PBO, piperonyl butoxide.

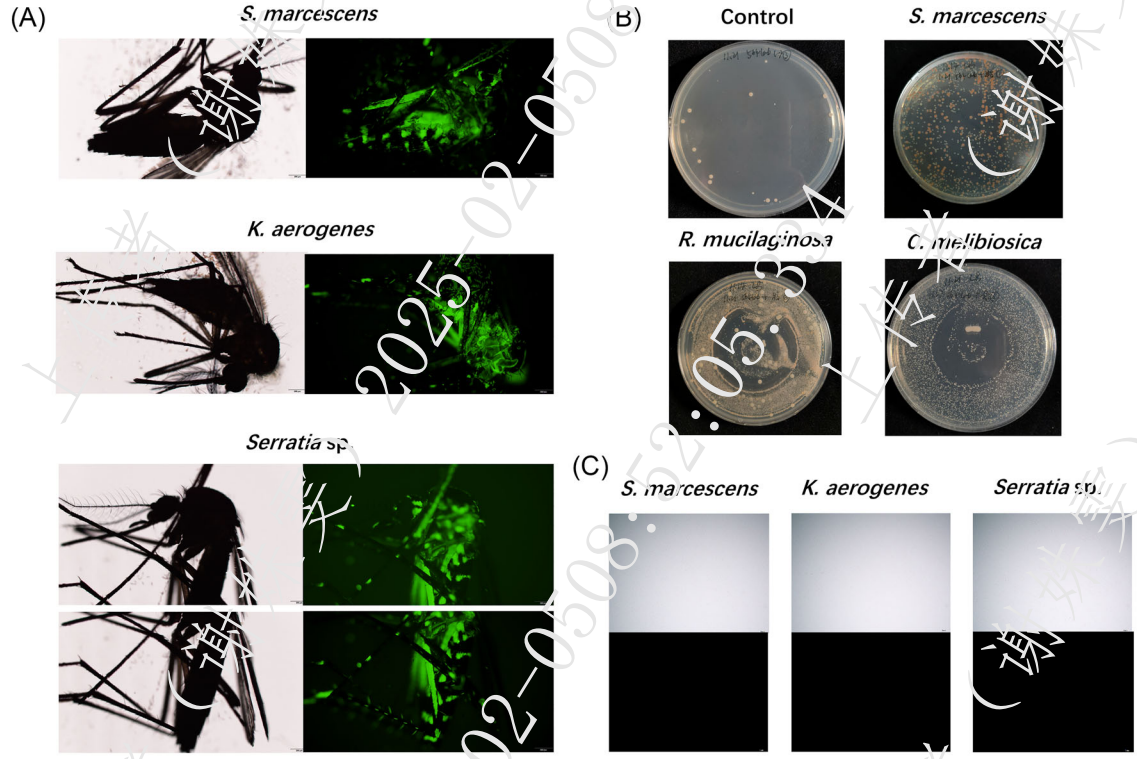

Fig. 5 (A) Fluorescence of DS *Aedes albopictus* after spraying with *Serratia marcescens*, *Klebsiella aerogenes*, and *Serratia* sp. (B) Culture of the cuticle surface symbiotic microbes of DS *Ae. albopictus* after spraying PBS (Control), *S. marcescens*, *Rhodotorula mucilaginosa*, and *Candida melibiosica*. (C) Three bacteria without EGFP under both white light (top) and fluorescence (bottom). DS, deltamethrin-susceptible; EGFP, enhanced green fluorescent protein.

mosquito strains have enhanced detoxification of metabolic enzymes (Nauen *et al.*, 2022) and target-site gene mutations (Auteri *et al.*, 2018), but these processes still cannot fully explain the resistance mechanism. In recent years, the interaction between symbiotic microbes and their host has been widely studied. Some symbiotic bacteria can participate in a variety of host biological activities, such as growth and development (Michalkova *et al.*, 2014; Whittle *et al.*, 2021), nutritional metabolism (Brune & Dietrich, 2015; Zheng *et al.*, 2016), and immune defense (Moreira *et al.*, 2009; Bai *et al.*, 2019a), and they can also increase host resistance to insecticides (Chen *et al.*, 2020; Sato *et al.*, 2021).

In this study, through insecticide resistance bioassays, we found that *Ae. albopictus* from CZ, SZ, and WX field populations had high resistance to DM. Next, we evaluated the metabolic resistance and target-site resistance of *Ae. albopictus* from WX and SZ field populations, but we did not find a significant correlation between *kdr* mutation and insecticide resistance, indicating that target-site resistance may not play a major role in the resistance mechanism. Additionally, the synergistic bioassay results revealed that the metabolic resistance of *Ae. albopictus* mediated by CYPs in *Ae. albopictus* may play a crucial role in the SZ field population; however, the mortality rate of *Ae. albopictus* of the WX field population did not increase in response to synergist treatment, indicating that, in addition to the above resistance mechanisms, other resistance mechanisms may play important roles in this field population. Therefore, *Ae. albopictus* of the WX field population was selected for subsequent resistance-related cuticle surface symbiotic microbe screening.

In this study, no difference in the species diversity of symbiotic bacteria or symbiotic fungi on the cuticle surface was observed between DS and DR F0 *Ae. albopictus* without DM treatment, but a significant difference in

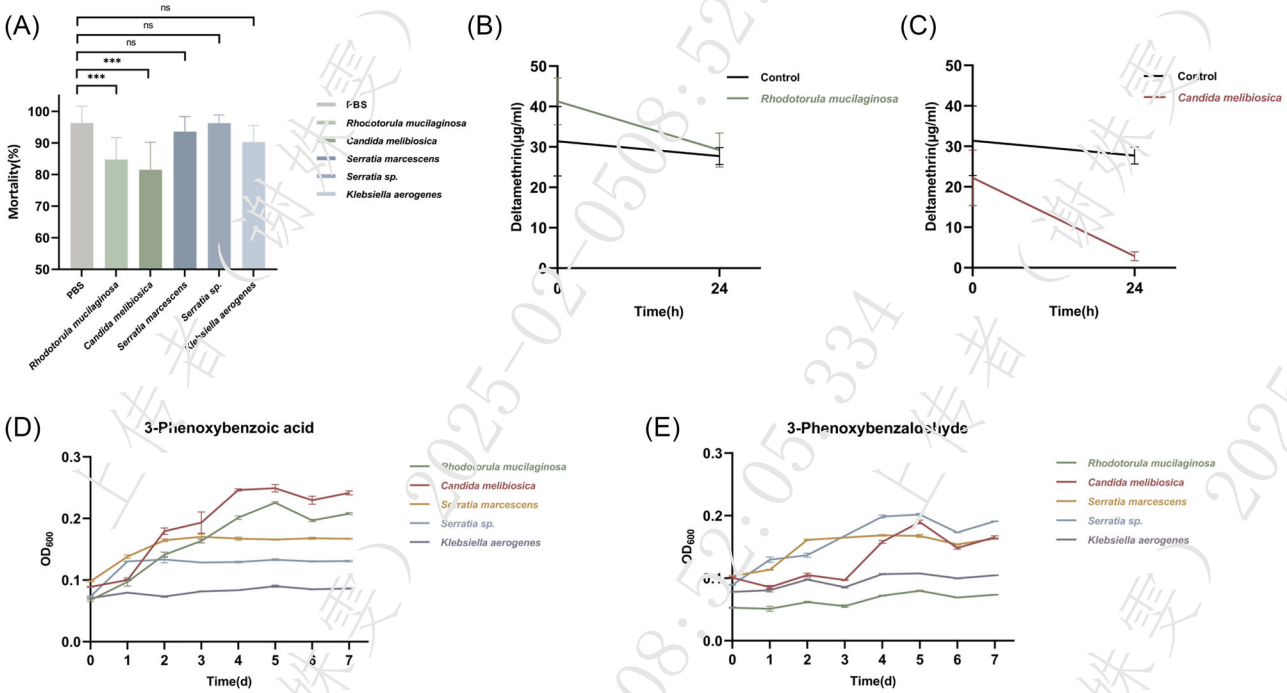

Fig. 6 Average mortalities of DS *Aedes albopictus* after spraying degrading microbes on the cuticle surface (A) utilization of DM (B, C) and metabolites (D) 3-PBA; (E) 3-Phenoxybenzaldehyde by the degrading microbes. ***$P < 0.001$. 3-PBA, 3-phenoxybenzoic acid; DM, deltamethrin; ns, not significant; DS, deltamethrin-susceptible.

species composition was found; this was consistent with the findings of a previous study on *Anopheles gambiae* (Omoke *et al.*, 2021). However, habitat, food, and other factors also affect the colonization of microorganisms in mosquitoes, which may explain why the diversity of mosquito microbes with different phenotypes are similar but the species composition is different. Dada *et al.* (2019) characterized and compared the symbiotic bacteria of *Anopheles albimanus* with different resistance phenotypes. They reported that, compared with the sensitive phenotype, the symbiotic bacteria of fenitrothion-resistant *A. albimanus* presented lower species diversity, but the degrading bacteria and enzymes were significantly enriched, suggesting that the enrichment of symbiotic bacteria may be related to insecticide selection pressure.

To study whether insecticide exposure affects the cuticle surface microbes, we used different concentrations of DM to treat the F1 generation of *Ae. albopictus* field populations. We found that the abundance and composition of the symbiotic microbes significantly differed at the genus level and that the abundance of *Serratia* spp. and *Candida* spp. increased. Moreover, we also detected *Serratia* spp. and *Candida* spp. on the cuticle surface of *Ae. albopictus* in the F0 generation without DM treatment, but they were not dominant microbes on the cuticle surface and their relative abundance was not high.

Serratia spp. contain a gene encoding a hydrolase and can utilize methyl parathion (Mootha *et al.*, 2003; Subramanian *et al.*, 2005). This gene has also been found in *Burkholderia* spp. and is associated with the degradation of organophosphorus insecticides (Hayatsu *et al.*, 2000), indicating that different degrading bacteria may have similar degradation methods for the same type of insecticide. *Candida* spp. can degrade bifenthrin via the hydrolysis of ester bonds and the cleavage of biphenyl and other pyrethroids (Chen *et al.*, 2012). Therefore, we speculate that *Serratia* spp. and *Candida* spp. may be related to DM resistance in *Ae. albopictus*, and the resistance-related degrading microbes may be present at a low level when the mosquitoes are not exposed to DM; once exposed to DM, they may rapidly proliferate to jointly resist the damage to the mosquitoes caused by DM.

Many insecticide-degrading bacteria were originally isolated from contaminated soil and wastewater (Laveglia & Dahm, 1977; Chen *et al.*, 2014; Yang *et al.*, 2018). Therefore, the same method was used in this study; that is, the candidate degrading microbes that can use DM as a single carbon source were screened via carbon

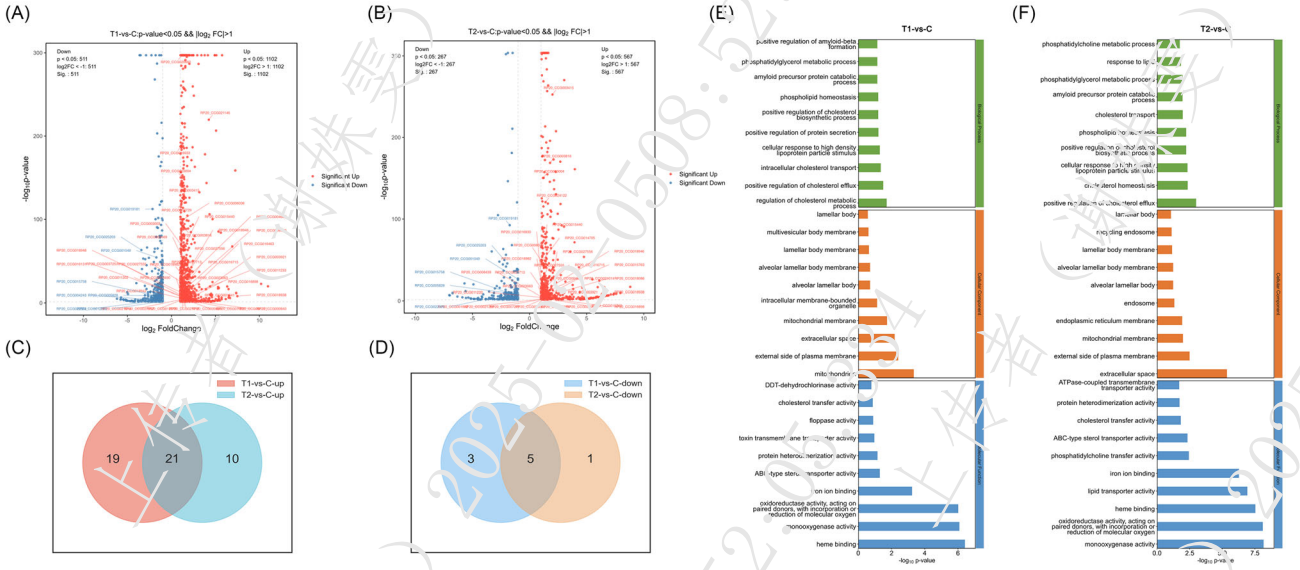

Fig. 7 DEGs related to metabolic enzymes in a volcano map (A) *Rhodotorula mucilaginosa*, (B) *Candida melibiosica* and GO enrichment histogram for comparisons (E) *R. mucilaginosa*, (F) *C. melibiosica* of *Aedes albopictus* after spraying the degrading fungi on the cuticle surface. The differentially upregulated (C) and downregulated (D) metabolic enzymes are displayed in the Venn diagram of *Ae. albopictus* after spraying degrading fungi on the cuticle surface. (C) Control, phosphate-buffered saline, T1: *R. mucilaginosa*, T2: *C. melibiosica*. DEGs, differentially expressed genes; GO, Gene Ontology.

source utilization experiments. Ultimately, 7 strains of DR candidate degrading microbes were obtained, among which *Candida* spp., *Rhodotorula* spp., *Serratia* spp., and *Klebsiella* spp. have been reported to be involved in the degradation of various insecticides. For example, *Candida pseudolambica* can rapidly degrade high concentrations of diazinon (Ebadi *et al.*, 2022), and *Candida* sp. VITJzN04 degrades the organochlorine pesticide lindane (now banned) through a variety of enzymes (Salam & Das, 2014). *R. mucilaginosa* IM-2 can hydrolyze the neonicotinoid insecticides acetamiprid (AAP) and thiacloprid (THI) into the intermediate products IM1-3 and amide derivatives, respectively (Dai *et al.*, 2010). *Rhodotorula glutinis* and *R. rubra* effectively degrade chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) via phosphotriesterases (PTEs), and the degradation rate increases with increasing chlorpyrifos concentration and amount of microbial inoculation (Bempelou *et al.*, 2018). *Serratia liquefaciens* and *S. marcescens* can degrade the insecticide diazinon, and the biodegradation effect is greater in the presence of glucose (Cycoń *et al.*, 2009). *Serratia* spp. SPL-2 can effectively degrade the organophosphorus insecticide methidathion, and its fermentation broth can remove pesticide residues from agricultural products (Li *et al.*, 2013). *Klebsiella oxytoca* KE-8 can effectively degrade endosulfan and its main metabolite endosulfan sulfate (Kwon *et al.*, 2005). *Klebsiella pneumoniae* CP19 can effectively degrade chlorpyrifos (Elshikh *et al.*, 2022). *Klebsiella pneumoniae* BPBA052 can degrade a variety of pyrethroids, the primary metabolites of pyrethroids 3-PBA and the secondary metabolites of pyrethroids phenol and catechol (Tang *et al.*, 2019).

The identification of the degrading microbes, such as *Candida* spp. and *Serratia* spp., in this study is in line with the results of previous microbial sequencing analysis, suggesting that these 2 symbiotic microbes likely participate in the insecticide resistance mechanism of *Ae. albopictus*. However, in complex microbial networks, single degrading microbes do not exist in isolation, and the microbial community shows unique associations and interactions that can degrade pollutants more effectively and systematically (Espinosa-Ortiz *et al.*, 2022). Therefore, it cannot be ruled out that other microbes also play crucial roles in resistance mechanisms. In addition, considering that there is a single set of culture conditions that is universally used to screen degrading microbes, some specific microbes may not be cultured successfully, For instance, some bacteria or fungi may replace the dominant microbes of mosquitoes and inhibit the growth of others during the enrichment process (Sprunt, 1968). This view can also explain why the degrading microbes screened in this study do not fully correspond to our first 16S and ITS sequencing results.

To further study the interaction between the symbiotic microbes and insects, we inoculated the target bacteria and fungi into DS *Ae. albopictus*. Intestinal bacterial colonization can be achieved by feeding glucose water with the target bacteria (Wu *et al.*, 2019). The colonization of symbiotic bacteria on the cuticle surface involves direct spraying after mosquito anesthesia (Carolino *et al.*, 2014). Fungal colonization is mostly carried out by spore infection (Farenhorst & Knols, 2010; Lovett *et al.*, 2019); that is, a black cloth with a fungal spore mixture is hung in places with mosquito infestations so that they can be infected with the target fungus. In this study, we used direct spraying of the symbiotic microbes on the cuticle surface and fluorescent labeling to verify whether the symbiotic bacteria successfully colonized *Ae. albopictus* (Wang *et al.*, 2017; Accoti *et al.*, 2023). Given that it is difficult to transform wild-type fungi with plasmids (Lorang *et al.*, 2001), the method of collecting surface symbiotic bacterial cultures is used to save samples and because it is more intuitive than quantitative PCR. Furthermore, the results of verification experiments demonstrated that the bacteria can indeed successfully colonize the surface of *Ae. albopictus* by spraying. The results of this study showed that spraying *R. mucilaginosa* and *C. melibiosica* significantly increased the resistance level of DS *Ae. albopictus*; this was consistent with the results of previous insecticide degradation experiments and further indicated that the cuticle surface fungi of *Ae. albopictus* may play an important role in resistance mechanisms.

To date, one previous study focused on the bacterial degradation of insecticides, but only a few fungi were used for biodegradation (Bhatt *et al.*, 2019). In some waters or soils polluted by pesticides for a long period of time, a variety of fungi capable of degrading pesticides have been found, and the degradation ability of fungi is greater than that of bacteria (Gangola *et al.*, 2019). We found that on minimal salt medium containing DM, the growth of fungi was faster and more vigorous than that of bacteria, and the growth curve also suggested that fungi can degrade DM better than bacteria. Therefore, we chose 2 fungi for the next degradation rate evaluation. HPLC–MS revealed that the 24 h DM degradation rate by *C. melibiosica* was greater than that of *R. mucilaginosa*. Moreover, the results of MSM culture showed that *C. melibiosica* grew faster than *R. mucilaginosa*.

It has been reported that some degrading bacteria can degrade insecticides themselves and even their metabolites (Bempelou *et al.*, 2018; Tang *et al.*, 2019). Pyrethroids are commonly degraded into 3-PBA and 3-phenoxybenzaldehyde, and fungi mostly use CarE to metabolize DM into 3-PBA (Bhatt *et al.*, 2019). Therefore, we performed 3-PBA detection experiments on the same sample but did not detect this substance. We speculate that this substance may be transient; that is, although it is generated, it is also metabolized by the degrading bacteria, as reported by Chen *et al.* (2011). Therefore, we used the growth curve method to directly evaluate whether the degrading microbes can use these 2 metabolites as the sole carbon source for growth. The results showed that *C. melibiosica* and *R. mucilaginosa* can use 3-PBA, indicating that the degrading fungi can use not only DM but also DM metabolites. Moreover, we found that among the 7 degrading microbes, *C. melibiosica* had the greatest degradation effect.

The degradation of pyrethroid insecticides is related mostly to changes in metabolic enzymes. Esterases can directly metabolize ester insecticides into acids and alcohols by hydrolyzing ester bonds (Tallur *et al.*, 2008; Bai *et al.*, 2019b). GSTs play a role in metabolic detoxification by catalyzing the binding of nucleophilic glutathione to exogenous toxicants. Cytochrome P450 enzymes metabolize and detoxify exogenous toxicants through a series of reactions, such as redox hydrolysis. In this study, we evaluated the metabolic enzyme activities of each degrading bacterial and fungal strain, and found that the GST and CarE activities of *R. mucilaginosa*, *C. melibiosica*, *S. marcescens*, and *K. aerogenes* were significantly greater. Therefore, it is speculated that GST and CarE play important roles in the degradation of DM by the degrading microbes.

Finally, to further study the molecular mechanism by which degrading fungi enhance DM resistance in *Ae. albopictus*, RNA-seq was used to analyze DEGs between Lab mosquitoes sprayed with fungi and those sprayed with PBS, and genes related to DM resistance were screened. The results revealed that the DEGs shared by the 2 groups were significantly enriched in pathways such as "oxidative phosphorylation," "biosynthesis of insect hormones," "ligand–receptor interaction of nerve tissue," and "protein digestion and absorption." Insect hormones are a class of trace active substances secreted by insect glands and are involved in the regulation of various insect biological activities. For example, tanning hormones can participate in body surface hardening and darkening, making the insect cuticle harder. Additionally, enrichment of the "biosynthesis of insect hormones" pathway may indicate that the physiological metabolism of *Ae. albopictus* changes when exposed to DM, which may be used to resist the penetration of DM.

Compared with those in the PBS group, metabolic enzyme-encoding genes, including *CYP6a14*, *GSTD1*, *Esterase B1*, and other genes, were upregulated in *R. mucilaginosa* and *C. melibiosica*. The uniquely upregulated genes in *R. mucilaginosa* included *CYP9E2* and

GSTT1, and those in *C. melibiosica* included *CYP304a1* and *GST1*. According to the literature, the CYP450s related to insecticide resistance are mostly distributed in the CYP4, CYP6, and CYP9 families (Avicor *et al.*, 2014; Ishak *et al.*, 2017; Zou *et al.*, 2019). *Esterase B1* is associated with permethrin resistance in *Culex quinquefasciatus* (Ramkumar *et al.*, 2023). *GSTD1* is associated with DDT resistance in *An. gambiae* (Matzkin, 2008). *GSTT3* is upregulated in permethrin-resistant *Ae. albopictus* (Ishak *et al.*, 2016). These findings indicate that the screened *Ae. albopictus*-degrading microbes may also increase mosquito resistance to insecticides by regulating resistance-related metabolic enzyme-encoding genes such as CYPs and GSTs.

In summary, this study reveals that the degrading microbes on the cuticle surface of *Ae. albopictus* can increase insecticide resistance and may exert synergistic resistance by directly degrading DM and indirectly inducing the expression of metabolic enzyme-encoding genes. To a certain extent, our findings improve the interaction mechanism between the symbiotic microbes and host insecticide resistance. However, there are still some limitations in this study. Although we found that the cuticle microorganisms could enhance the resistance of mosquitoes, we only tested the function of the cuticle and ignored other underlying parts of mosquitoes, such as the gut or the ovary, so we could not say these degrading microorganisms act solely on the surface. The interaction between the cuticle and the gut and other organs' microorganisms leading to insecticide resistance should be studied further.

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Disclosure

All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest, including specific financial interest and relationships and affiliations relevant to the subject of the manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Insecticide resistance bioassays result of 3 field populations of *Aedes albopictus*.

Fig. S2 Circos and heatmap plots of the community structure at the genus level of *Aedes albopictus* epidermal symbiotic bacteria after treatment by different concentrations of deltamethrin (DM).

Fig. S3 Circos and heatmap plots of the community structure at the genus level of *Aedes albopictus* epidermal symbiotic fungi after treatment by different concentrations of deltamethrin (DM).

Fig. S4 Gram staining of the primary screening microbes.