Research Article

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# *Metarhizium anisopliae* is a valuable grist for biocontrol in beta-cypermethrin-resistant *Blattella germanica* (L.)

**Xian Cui Zhang,[a,b†] Meng Jiang,[a†] Ya Nan Zang,[a] Hai Zheng Zhao,[a] Cai Xia Liu,[a] Bao Rui Liu,[a] Hua Xue,[a] Coby Schal,[c] Xing Meng Lu,[b] Dong Qin Zhao,[a] Xue Xia Zhang[d*] and Fan Zhang[a*]**

## Abstract

**BACKGROUND: The widespread use of chemical insecticides has resulted in the development of resistance in German cockroaches worldwide, and biopesticides based on entomopathogenic fungi as active ingredients have become a promising alternative strategy. Resistance can change many of the physiological and biochemical characteristics of insect pests, such as cuticle thickness, detoxification enzyme activity, and even intestinal flora composition. Thus, potential interactions between pathogenic fungi and insecticide resistance may lead to unpredictable changes in pest susceptibility to fungi.**

**RESULTS: Beta-cypermethrin-resistant German cockroaches were more susceptible to infection with the fungus *Metarhizium anisopliae* regardless of age and sex. Histopathological results showed that the infection of resistant strains (R) by *M. anisopliae* was visibly faster than that of susceptible strains (S). The gut microbiota of the S strain indicated a stronger ability to inhibit fungi *in vitro*. The abundance of *Parabacteroides*, *Lachnoclostridium*, and *Tyzzerella*_3 decreased significantly in the R strain, and most demonstrated the ability to regulate glucose and lipid metabolism, and antifungal infections. The expression levels of Akirin, BgTPS, and BgPo genes in the R strain were significantly lower than those in the S strain, while BgChi and CYP4G19 gene expression were significantly higher. The mortality of cockroaches infected with *M. anisopliae* decreased to varying degrees after RNA interference, reflecting the role of these genes in antifungal infection.**

**CONCLUSIONS: Results confirmed that insecticide resistance may enhance cockroach susceptibility to fungi by altering intestinal flora and gene expression. Fungal biopesticides have high utilization value in pest control and insecticide resistance management strategies.**

*Supporting information may be found in the online version of this article.*

**Keywords:** German cockroach; beta-cypermethrin; *Metarhizium anisopliae*; susceptibility; microbiota; immunity

## 1 INTRODUCTION

The German cockroach, *Blattella germanica* (L.) (Blattaria: Blattellidae), is an extremely important indoor sanitary pest worldwide that can carry and transmit various parasites, pathogenic microorganism viruses, fungi, and bacteria to human life through biological or mechanical transmission.[1,2] The frequent application of insecticides has led to the ongoing development of high resistance in cockroaches in the past two decades, which has increased the urgent need for an environmentally friendly long-term alternative strategy to control them.[3–5] With the development of resistance, biological control strategies that use naturally occurring entomopathogenic fungi have therefore become a promising strategy, including *Metarhizium anisopliae* and *Beauveria bassiana*.[6,7]

Pathogenic fungi penetrate the cuticle through the formation of germ tubes and cuticle-degrading enzymes, grow in the hemocoel, destroy the important structure of the host, and secrete a series of toxins such as cyclic peptides, eventually leading to the death of insects.[8,9] Compared with susceptible insects, the physiological and biochemical characteristics, such as body weight, detoxification enzymes and cuticle thickness, of insecticide-resistant insects may change, which may affect the sensitivity of resistant insects to parasites

* *Correspondence to: XX Zhang, School of Soil and Water Conservation Beijing Forestry University, Beijing 100083, China, E-mail: xuexiazh@yeah.net; or F Zhang, Shandong Normal University, 88 East Wenhua Road, Jinan 250014, China. E-mail: zhangfan0531@163.com*

† *Xian Cui Zhang and Meng Jiang contributed equally to this study.*

a *Key Laboratory of Animal Resistance Biology of Shandong Province, College of Life Science, Shandong Normal University, Jinan, China*

b *Laboratory of Invertebrate Pathology, College of Animal Sciences, Zhejiang University, Hangzhou, China*

c *Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, USA*

d *School of Soil and Water Conservation, Beijing Forestry University, Beijing, China*

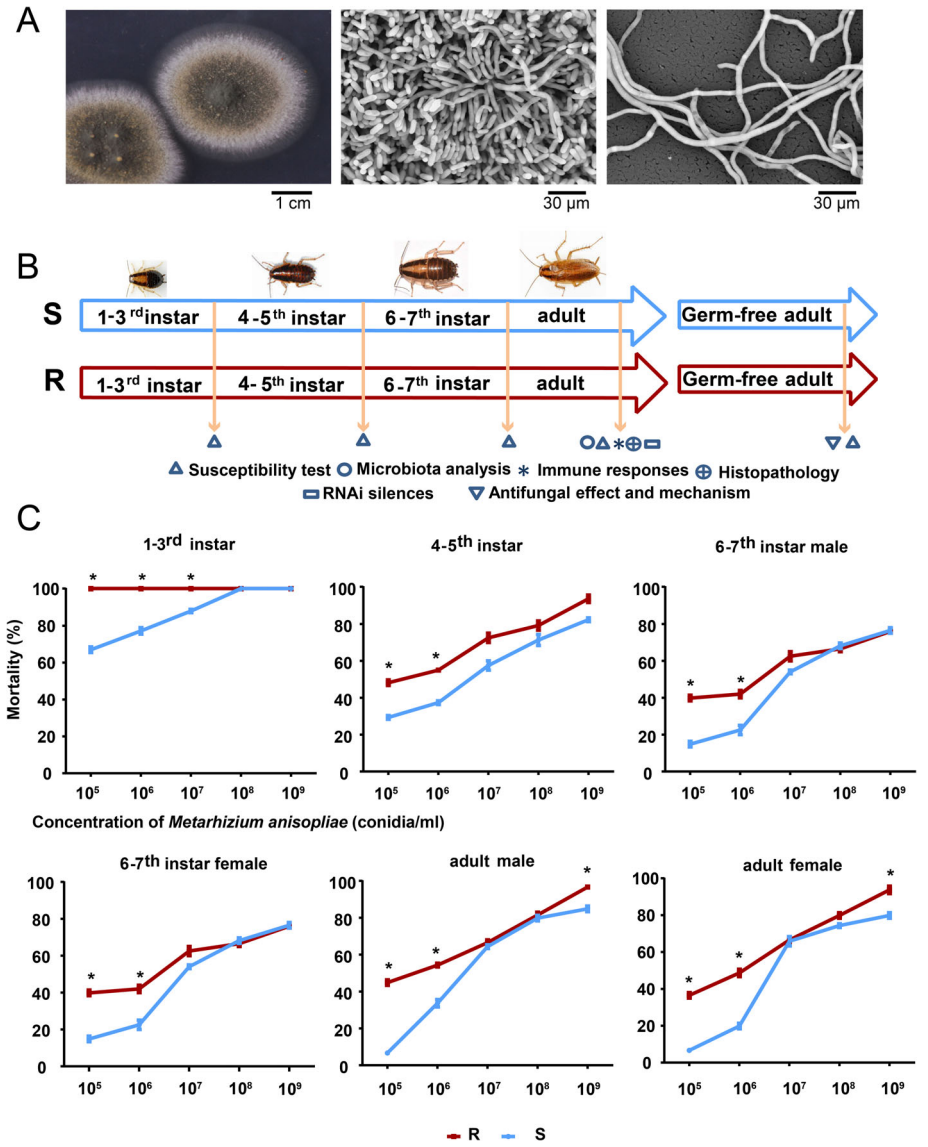


**Figure 1.** Susceptibility of R and S strain cockroaches to *Metarhizium anisopliae* applied through topical applications. (A) Characterization of *M. anisopliae*. (B) Experimental design showing the morphological characteristics of *M. anisopliae* and *Blattella germanica* development (top), treatments (middle), and experiments (bottom) during the study. Blue, conventional strain (S); red, *β*-CYP-resistant strain (R). (C) The mortality of R and S strains infected by *M. anisopliae* at 15 days postinoculation. Error bars represent the mean and SE, mean with 95% confidence intervals, $n = 20$ individuals per treatment group. Variation analysis was performed by student's t test . *Indicates that the data are significantly different ($P < 0.05$) from that of the *β*-CYP-resistant cockroach. Blue, Conventional strain treated with *M. anisopliae* (ST); red, *β*-CYP-resistant strain treated with *M. anisopliae* (RT).

and pathogens.[10–13] For example, the high level of esterase expression in organophosphate-resistant *Culex* mosquitoes results in changes in the redox potential of cells, which limits the growth of parasites such as filarias.[14] Monooxygenase levels in pyrethroid-resistant *Anopheles* species increase oxidative stress, which impair the survival of *Plasmodium*.[15] Insecticide-resistant *Anopheles* mosquitoes and bed bugs, *Cimex lectularius*, remain equally susceptible to infection with the fungus *B. bassiana*, but the mechanism is not clear.[16,17] Because enzymatic detoxification of insecticides is also an important mechanism of resistance, enhanced detoxification may interact with fungal metabolites such as cyclic peptide toxins and reduce the influence of toxic factors.[18] In addition, pyrethroid resistance can increase cuticular hydrocarbons (CHCs) and the uniform lipid layer arrangement in cockroaches, resulting in reduced cuticle permeability, which may also have a potential impact on the cuticle penetration of pathogenic fungi.[19] Thus, when resistance encounters fungal infection, it may have unpredictable consequences for the pest.

There are many symbiotic microbiota in the insect gut that play important roles in host nutrition, food digestion, and synthesizing vitamins, and also contribute positively by protecting the host against pathogens, detoxifying defense chemical insecticides, and stimulating the host immune response.[20–23] Gut bacteria can actively interact with resistance *via* direct degradation of insecticides or passive changes to adapt to insecticide resistance. For example, nymphs of *Riptortus pedestris* can obtain fenitrothion-degrading *Burkholderia* from environmental soil and establish a specific and beneficial symbiosis with it, thus acquiring resistance to fenitrothion.[24] In the oriental fruit fly,

  

insecticide resistance has been attributed to detoxifying enzymes produced by Proteobacteria located in the midgut.[25] For cockroaches, the gut microbial community of susceptible *B. germanica* was significantly different from that of indoxacarb-resistant and beta-cypermethrin-resistant (*β*-CYP-resistant) strains in which treatment with antibiotics increased susceptibility to orally administered insecticides.[26,27] Gut bacteria such as *Bacillus subtilis* and *Pseudomonas* can also secrete fengymycin, bacilysin,[28] bacillomycin,[29] mycosubtilin,[30] and pyrrolidone carboxyl peptidases (pcps) to fight fungal infection in cockroaches.[31–33] However, the effect of insecticide-induced changes in intestinal flora on the susceptibility of *B. germanica* to fungi is still unknown.

Considering that resistance can change many physiological and biochemical characteristics of pests, understanding whether such changes will affect the biocontrol effect of pathogenic fungi is important. In this study, we describe the differential susceptibility of *β*-CYP-resistant cockroach (R) and susceptible cockroach (S) nymphs and adults to *M. anisopliae*. The interaction between *M. anisopliae* and gut microbiota and the response of host immunity in resistant cockroaches were also investigated.

## 2 MATERIALS AND METHODS

### 2.1 Insects

Two German cockroach strains were provided by the Key Laboratory of Animal Resistance Biology of Shandong Province, Shandong Normal University, China. The susceptible strain German cockroach (S) was an inbred line for more than 40 years and was not exposed to any insecticides. The *β*-CYP-resistant strains (R) were selected from strains S, and more than 3000 individuals were used in the experiment in order to minimize the effects of genetic drift. The R strain German cockroach model was selected *via* topical application of *β*-CYP with a 50–70% mortality rate every generation and the resistance levels were increased 22.8-fold for $F_{16}$ generations.[32]

All strains were fed rat pellet feed and water (26 ± 1 °C, 65 ± 5% relative humidity). Tested insects were one- to three-instar nymphs, four- to five-instar nymphs, six- to seven-instar male and female nymphs, adult males, and nongravid female S and R strain German cockroaches.[33]

### 2.2 Entomopathogenic fungi

*M. anisopliae* isolate EB0732 was obtained from mycotized *Eupolyphaga sinensis* cadavers and stored in the China General Microbiological Culture Collection Center (CGMCC) NO.: 13170 Fig. 1(A).[33] The detailed experimental design is shown in Fig. 1(B).

### 2.3 Susceptibility of cockroach to *M. anisopliae*

R and S strain cockroaches (one- to three-instar nymphs, four- to five-instar nymphs, six- to seven-instar male and female nymphs, adult males, and adult females) were each divided into five groups of 20 cockroaches and 3 μL of *M. anisopliae* conidial suspension ($1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$ or $1 \times 10^9$ conidia/mL) was applied to each cockroach with a microinjector through its dorsal cuticle surface (ST, susceptible strain treated with *M. anisopliae*; RT, *β*-CYP-resistant strain treated with *M. anisopliae*). Sterile water supplemented with 0.05% Tween 80 was used as a negative control. After treatment, the insects were fed normally. Mortality was monitored daily for 15 days, and only dead insects that were covered with mycelium were considered a result of a fungal infection. The histopathological study and antifungal assay were performed on adult male cockroaches due to their relatively stable physiological state and were less affected by the regulation of hormones compared to nymphs and adult females.[34,35]

### 2.4 Histopathological study

The abdomens of adult male *B. germanica* were separated from the thorax under a stereomicroscope and preserved in 2.5% glutaraldehyde fixative solution in 300 μL of sterile phosphate-buffered saline (PBS). The samples were rinsed in sterile PBS (pH = 7.4), dehydrated in a series of graded ethanol solutions (75%, 85%, 95%, 100%), vitrified with dimethyl benzene, and embedded in Leica paraffin at 62 °C. Periodic acid-Schiff polysaccharide and glycoconjugates (PAS) and toluidine blue-borax separate staining methods were used for histochemical analyses.[36]

### 2.5 Anti-entomopathogenic fungal assay *in vitro*

Dissected gut tissue from S strain and R strain cockroaches was homogenized in PBS buffer. The supernatants of gut homogenates were passed through a 0.22-μm pore size filter (Millipore Corp., Billerica, MA, USA). The gut filtrates were then collected, and the agar well diffusion method was used to assess anti-*M. anisopliae* activity. A total of 20 μL of each sample was pipetted into each well. The plates were then incubated at 28 °C for 3 days, and the diameter of the inhibition zones was measured. PBS buffer was used as a negative control.

### 2.6 Viability of gut-passaged conidia and *M. anisopliae* dry matter content

A 100-μl conidial suspension of *M. anisopliae* (strain EB0732, $1 \times 10^9$ conidia/mL) was added to 200 mg of rat feed powder, which was fed freely to the adult male cockroach and was eaten within 12 h. The fungal load in feces was assayed at 24 h, and the 20-mg samples were spread on PDA agar plates with ampicillin (100 mg/mL), which were incubated for 3 days at 26 °C. *M. anisopliae* dry matter content was determined to form PDA plates by drying to constant weight using a microwave oven as previously described.[37]

### 2.7 Survival assay of conventional and germ-free cockroaches to *M. anisopliae* applied through topical applications

To determine whether gut microbiota benefit R and S host survival, adult male cockroaches were challenged with *M. anisopliae* (strain EB0732, $1 \times 10^9$ conidia/mL), as described in section 2.3. Treatment cohorts of R and S *B. germanica* in this study were designated into two groups: (i) conventionally reared larvae containing their inherent gut microbiota and (ii) germ-free larvae completely devoid of gut microbiota.[38]

### 2.8 DNA extraction, Illumina sequencing, and community analysis

The two strains of adult male cockroaches were each divided into three groups of 100 cockroaches and 3 μL of *M. anisopliae* conidial suspension ($1 \times 10^9$ conidia/mL) was applied topically on the dorsal cuticle surface. Then, gut tissue from the R and S strains was prepared after infection with *M. anisopliae* for 5 days and cleansed with 70% ethanol and sterile water. DNA was extracted using the Takara Mini BEST Universal Genomic DNA Extraction Kit Ver. 5.0 (Takara Bio Inc., Dalian, China).

The primer set 27F/1492R (Table S2) for the entire length of bacterial 16S rRNA was used for PCR analysis to check the bacterial DNA quality. To construct 16S rRNA clone libraries, the V3-V4 region of the 16S rRNA gene was amplified with universal primer

sets 338F and 806R (Table S2) containing a 12-base universal tailed tag and Illumina adaptor. PCR amplification was performed in triplicate using the isolated 16S rRNA gene primers. PCR conditions were as follows: 95 °C for 3 min, followed by 28 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. A final elongation step of 10 min at 72 °C was included. A broad range of microorganisms was identified, and identical volumes and concentrations were determined using Illumina MiSeq sequencing (Majorbio, Shanghai, China). Specifically, uninfected cockroaches were processed by the same DNA extraction and PCR amplification kits under the same conditions as the controls to avoid contamination.

Pair-end raw sequencing data were processed with QIIME version 1.8.0. Demultiplexing, adaptor trimming, and read merging were performed by the default QIIME pipeline. Chimeric sequences of merged reads were detected and removed by USEARCH (version 11.0.667) in QIIME (Edgar 2010). The clean sequences were subsequently clustered into operational taxonomic units (OTUs) at 97% similarity by USEARCH. Representative sequences from each OTU cluster were aligned and annotated by the Ribosomal Database Project (RDP) classifier against the SILVA database, with a minimum confidence of 0.8. Mitochondrial and chloroplast reads were then removed, and an OTU table was resampled to obtain an equal number of read numbers per sample. To investigate bacterial community richness and species diversity, an OTU table was created and input to MOTHUR (version 1.42.0) to calculate observed OTUs (Sobs) and Shannon.[39] Principal coordinate analysis (PCA) was based on the OTU abundance of sequenced samples, and Bray–Curtis dissimilarities were calculated by the 'vegan' package in R. Variation analysis in PCA was calculated by PERMANOVA with 999 permutations.[40] Gut microbial composition at the phylum level and genus level was performed using the UniFrac Server. Hierarchical cluster analysis used the Bray–Curtis distance and complete-linkage method, and the significantly enriched pathways of microbial functional metabolic pathways were identified from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Raw sequencing data were added to the NCBI Short Read Archive (SRA) BioProject PRJNA757708.

### 2.9 RT-qPCR validation of immune genes

To measure the immune gene expression levels of S and R strain cockroaches at 0, 12, 36, 48, 72, 84 and 108 h post *M. anisopliae* infection, adult males were divided into five groups: Akirin, BgTPS, BgPo, BgChi, and CYP4G19. RNA was isolated from German cockroaches using the MiniBEST UniveR Sal RNA Extraction Kit 9767 (Takara Bio Inc.). After quantification by a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA), 1000 ng of RNA was collected from each sample to synthesize cDNA using the PrimeScript RT reagent Kit (Perfect Real Time) RR037A (Takara Bio Inc., Dalian, China) according to the manufacturer's instructions. Real-time PCR was performed using a Roche LC480 real-time PCR system (Roche, Switzerland) with a 20-μL reaction system that included 10 μL of 2X LightCycler 480 SYBR Green I Master, 2 μL cDNA, 0.5 μL forward and reverse primer, and 7 μL sterile water. Each run was performed in triplicate. The PCR reaction comprised 10 min of denaturation at 95 °C, followed by 40 cycles of 10 s at 95 °C, 60 °C for 10 s, and 72 °C for 10 s. To generate a melting curve, the temperature was increased from 60 to 85 °C with a ramp rate of 0.2 °C/s. $\beta$-actin was used as a housekeeping gene in this study, and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression.[41] All primers used for relative gene expression analysis are listed in Table S3.

### 2.10 RNAi silences antifungal immune genes

For RNAi, 300–500 bp fragments of the target genes were amplified *via* PCR from the cDNA. Then, primers attached to the T7 promoter sequence were used for PCR amplification of the double-stranded RNA (dsRNA) templates. The dsRNA was synthesized using the MEGA script T7 Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A 42 bp noncoding sequence from GFP was used as a control dsRNA. dsRNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and was injected into the abdomen of the cockroach.

All dsRNA synthesis primers used in this study are listed in Table S4. In the experiments on innate immunity, for each gene newly emerged adult males were injected with a dose of 3 μg of dsRNA per insect within 10 days. Adult male cockroaches of R and S strains were divided into three groups of 30 individuals. A $1 \times 10^9$ conidia/mL suspension was made by mixing the conidia with 0.01% (v/v) Tween 80 and distilled water. Then, 3 μL of conidial suspension was applied topically on the dorsal cuticle surface. Mortality was monitored daily for 15 days.

### 2.11 Bioinformatic and statistical analysis

All statistics were performed using GraphPad Prism version 9.00 for Windows. The independent sample T-test was performed to compare absolute quantification of susceptibility tests, antifungal effect activity, gut microbiota, *M. anisopliae* dry matter content, immune gene expression, and RNAi silences across R and S, or RT and ST groups. One-way ANOVA for more than two groups was performed using SPSS version 20 for Windows. Results are shown as the mean ± standard error (SE), and results with $P < 0.05$ were declared statistically significant between groups. All experiments were repeated at least three times.

## 3 RESULTS

### 3.1 Susceptibility of R and S strain cockroaches to *M. anisopliae*

R strain German cockroaches were more susceptible to infection with the fungus *M. anisopliae* regardless of age and sex ($P < 0.05$). The mortality of the R strain cockroach was higher than that of the S strain cockroach after treatment with different concentrations of *M. anisopliae*, particularly at lower concentrations ($1.0 \times 10^5$ conidia/mL) (Fig. 1(C)). For example, the cumulative mortality rate of R strain cockroaches (one- to three-instar nymphs) reached 100% at $1.0 \times 10^5$ conidia/mL 15 days postinoculation (dpi) and that of the S strain was 66.67 ± 0.79% ($t = 2.100$, df = 38, $P < 0.0001$). However, at $1.0 \times 10^9$ conidia/mL, the mortality rates of the R and S strains (adult male) were 96.67 ± 0.00% and 84.33 ± 2.38% ($t = 2.196$, df = 38, $P = 0.0082$), respectively (Fig. 1(C)). In addition, younger nymphs were observably less *M. anisopliae* resistant than the last instar nymphs and adults at the same doses ($P < 0.05$). The *M. anisopliae* had the strongest toxicity to one- to three-instar nymph at a concentration of $1 \times 10^9$ conidia/mL; mortality increased in the following days and reached 100% and 100% after 15 days (RT *vs* ST). However, the six- to seven-instar female *B. germanica* was not susceptible to infection, which caused 75% and 75% mortality (RT *vs* ST) (one- to three-instar nymphs *vs*. six- to seven-instar females, $t = 5.511$, df = 38, $P = 0.0006$).

### 3.2 Histopathological changes of German cockroaches

Histological sections showed general and detailed views of the abdomen of newly emerged adult cockroaches (Fig. 2). Results



  

showed that the infection process of *M. anisopliae* against the R strain cockroach (Fig. 2(D)–(F)) was visibly faster than that against the S strain cockroach (Fig. 2(A)–(C)). For the S strain cockroach, the condia of *M. anisopliae* had not penetrated the exoskeleton completely on the first day but had penetrated the exoskeleton of strain R and entered the hemolymph (Fig. 2(A),

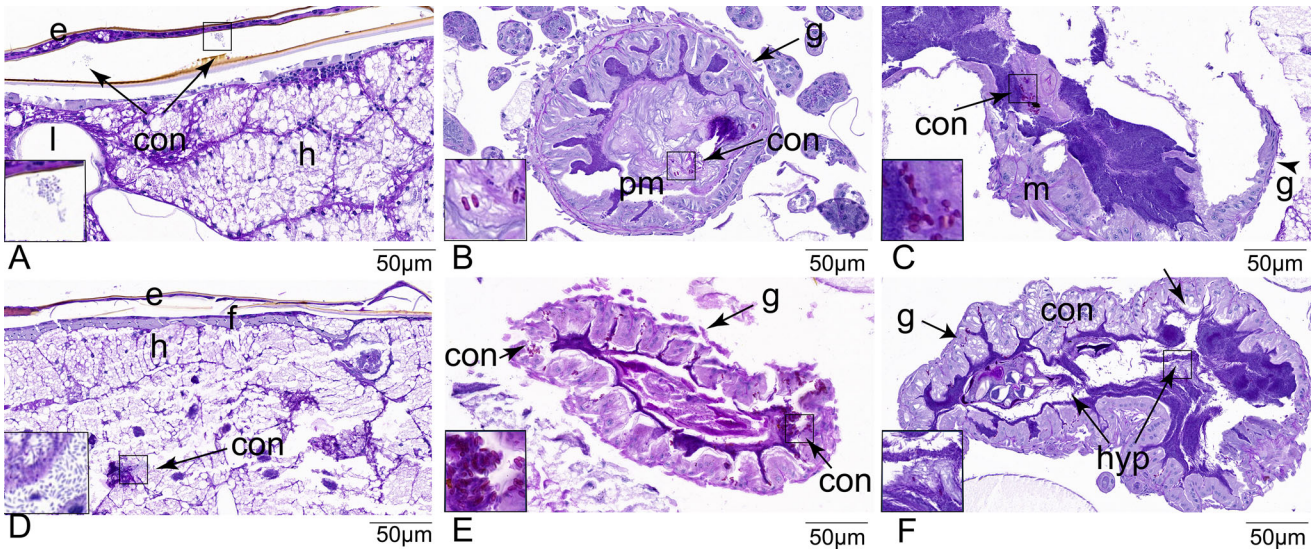


**Figure 2.** Pathological changes in males of the abdominal R and S strains through percutaneous host infection with *M. anisopliae* after 1 days (A, D), 3 days (B, E), and 5 days (C, F). Sections were stained with the PAS test (A, B, C, S males; D, E, F, R males). con, conidia; hyp, hypha; e, exoskeleton; f, fat body; g, gut; h, hemocoel; l, lipid droplet; m, muscle. Boxes show details. Scale bar = 50 μm.

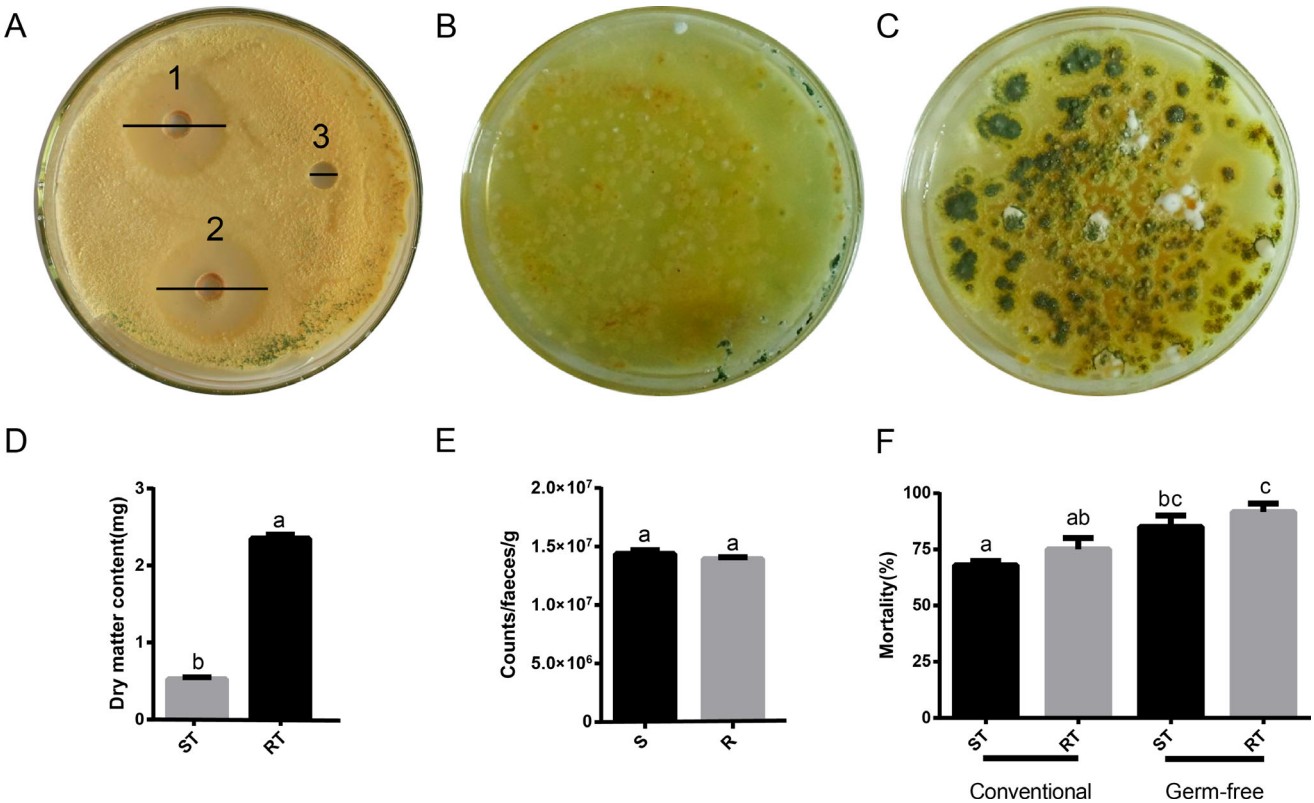


**Figure 3.** Gut microbiota antifungal effect activity *in vivo*. (A) Antifungal activity of 20-μl gut homogenates from R strain (1) and S strain (2) cockroaches against *M. anisopliae* on PDA plates 3 days postincubation. An equal amount of PBS buffer was used as a control (3). The growth (B, C) and dry weight (D) of *M. anisopliae* from ST (B) and RT (C) strain faces on PDA agar plates with ampicillin (100 mg/mL), which were incubated for 3 days at 26 °C. S, conidia from S strain feces; R, conidia from R strain feces. Variation analysis was performed by student's *t*-test. (E) Viability of gut-passaged conidia. S, feces from S strains; R, feces from R strains (Student's *t*-test). (F) Mortality of conventional (S, R) and germ-free cockroaches (S, R) infected with *M. anisopliae* through topical applications at 15 days postinoculation. The same lowercase letters are not significantly different according to Tukey's HSD test.



  

(D)). On the third day, only sporadic condia were found in the intestinal tract of the S strain, but a large number of condia had invaded the intestinal tract of the R strain (Fig. 2(B),(E)). On the fifth day of infection, there were only condia in the intestinal tract of the S strain, but a large number of mycelia had propagated in the R strain (Fig. 2(C),(F)).

### 3.3 Gut microbiota antifungal effect activity of S and R strains *in vivo*

Antifungal experiments showed that both S and R strain cockroaches of gut homogenates exhibited inhibition activity against *M. anisopliae*. The gut samples of the S strain showed stronger antifungal activity than those of the R strain (2.70 ± 0.15 cm *vs* 2.41 ± 0.12 cm) ($t = 56.69$, df = 8, $P < 0.0001$) (Fig. 3(A)). After oral feeding for 24 h, there was no difference in the number of condia collected from the feces of the two strains (Fig. 3(E)). However, condia collected from the feces of the R strain grew more vigorously on PDA medium (Fig. 3(B),(C)), and the dry matter mass of *M. anisopliae* cultured for three days was 2.35 ± 0.12 mg and 0.525 ± 0.03 mg, respectively ($t = 10.22$, df = 8, $P < 0.0001$) (Fig. 3(D)). In mortality assay tests, as shown in Fig. 3(F), the survival rate of conventional cockroaches was markedly higher than that of germ-free cockroaches after 15 days of treatment with *M. anisopliae* (70 ± 1.08 *vs* 75 ± 2.88% for the S strain, $P = 0.08$; 75 ± 2.88% *vs* 92.5 ± 2.21%, $P = 0.14$ for the R strain).

### 3.4 . Composition of the microbiota of R, S strains

Entire gut homogenates of R and S strain cockroach adults were extracted to compare their associated microbial communities after infection with *M. anisopliae* for 72 h. Initial rarefaction curves of OTU diversity estimates revealed a sufficient and saturated sampling depth. Good's coverage (sample coverage), which estimates what percentages of the total species are represented in the 12 samples, averaged 99%, suggesting that the majority of samples sequenced were reliable in this study (Fig. 4(A)). We assessed similar community pattern diversity using richness estimators and diversity indices (ACE, species richness, Shannon) for the 12 groups of cockroaches (Table S1) (Fig. 4(B),(C)). For global comparison of the gut microbiota derived from all 12 samples, we performed PCA. The first two principal components (PC1 and PC2) accounted for 95.02% of the total variance (Fig. 4(D)). PC1 accounted for 89.06% of all variance in the data and separated the S strain samples from the R strain samples. The results from the PCA suggest that there is a different gut microbiota between the S strains and R strains ($P = 0.001$) (Fig. 4(D)).

Taxonomic analysis showed that the most widespread phylum was Firmicutes, which, together with Planctomycetes, Fusobacteria, Bacteroidetes, and Proteobacteria, was detected in all samples. Firmicutes was the most abundant division in the R and S strains (R 39.37% *vs* S 32.53%, RT 47.42% *vs* ST 34.91%), and the second most abundant bacterial phylum in the R and S strains

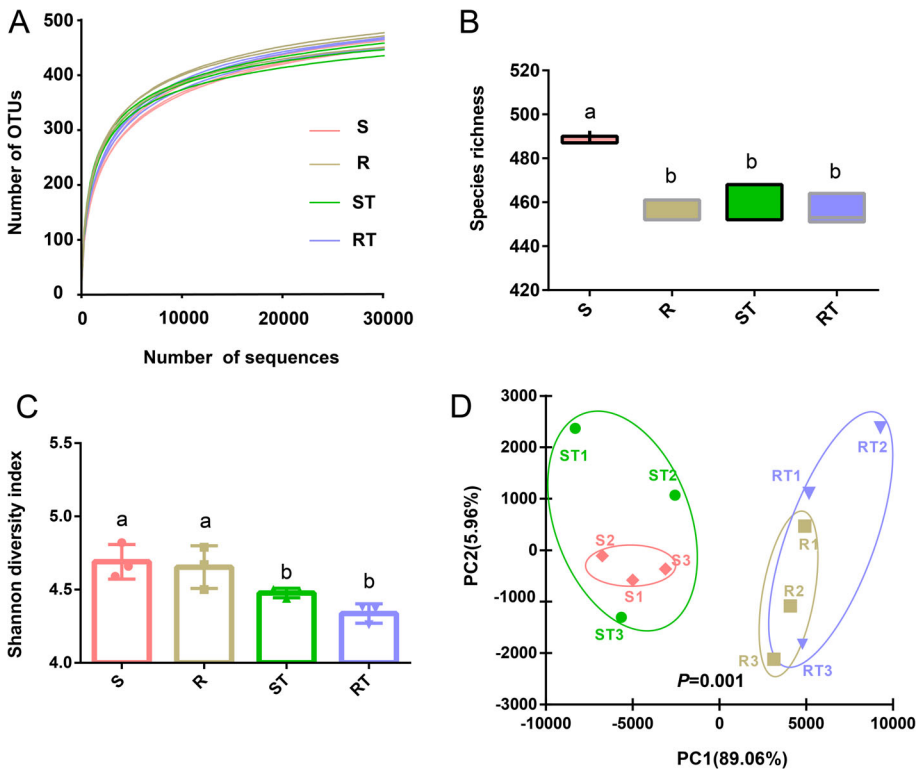


**Figure 4.** Richness and diversity indices are relative to each gut sample. (A) Rarefaction analysis of the different samples. Rarefaction curves of OTUs clustered at the 97% phylotype similarity level. (B) Spatial variation in bacterial species richness and diversity. (C) The Shannon diversity index is relative to each gut sample. (D) *B. germanica* gut microbiota sample sorting analysis by PCA. Twelve samples are represented by three replicates of each of four treatment groups. The scatter plot of PCA scores shows the similarity of the 12 bacterial communities based on UniFrac distance. Principal components (PCs) 1 and 2 explained 89.06% and 5.96% of the variance, respectively. S, susceptible strain; R, *β*-CYP-resistant strain; ST, susceptible strain treated with *M. anisopliae*; RT, *β*-CYP-resistant strain treated with *M. anisopliae*. Different letters indicate significant differences and the same letters indicate no significant difference (one-way ANOVA, LSD *post hoc* test, $P < 0.05$).

  

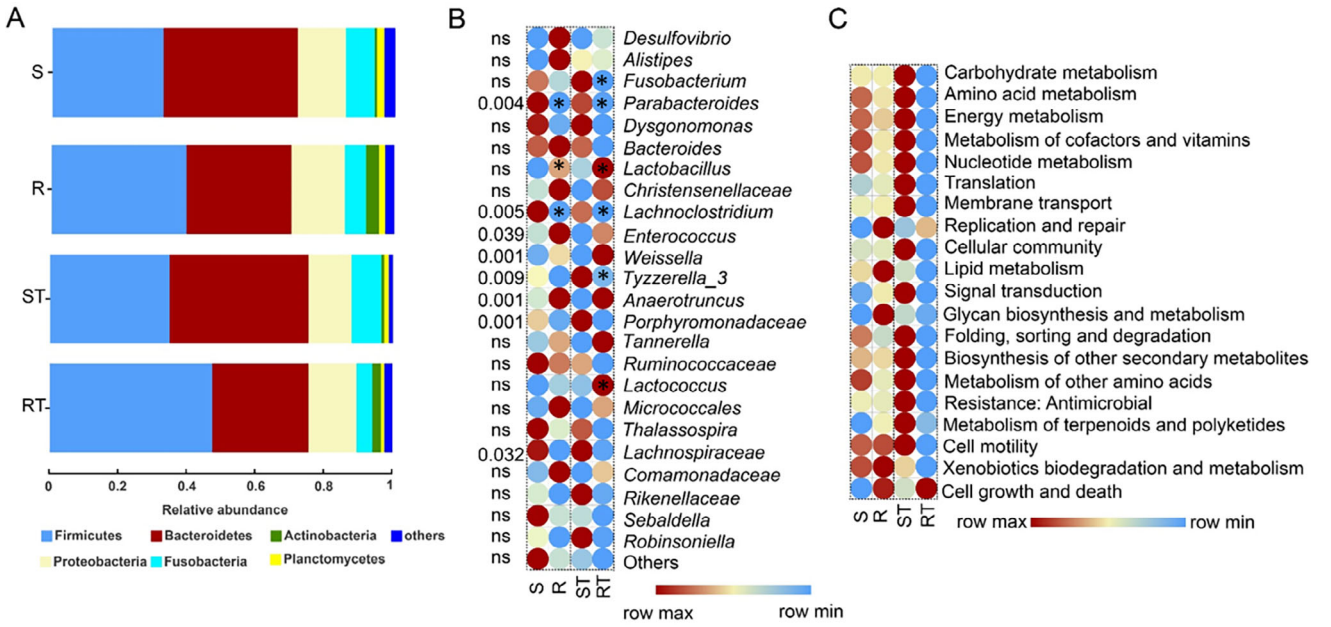


**Figure 5.** Gut microbial composition and functional analysis of *B. germanica*. (A) Bacterial composition of the different communities at the phylum level (PERMANOVA test with 999 permutations, $P \leq 0.05$). (B) Relative abundances of genera that showed significant differences among samples from the gut. One-way ANOVA was used to evaluate the significance of differences between the indicated groups. Hierarchical cluster analysis used the Bray–Curtis distance and complete-linkage method. Each bar or column represents each group (three individuals for every group). (C) The top 20 KEGG enriched pathway comparisons between S, R, ST, and RT at level 3 for the intersection of target gut microflora.

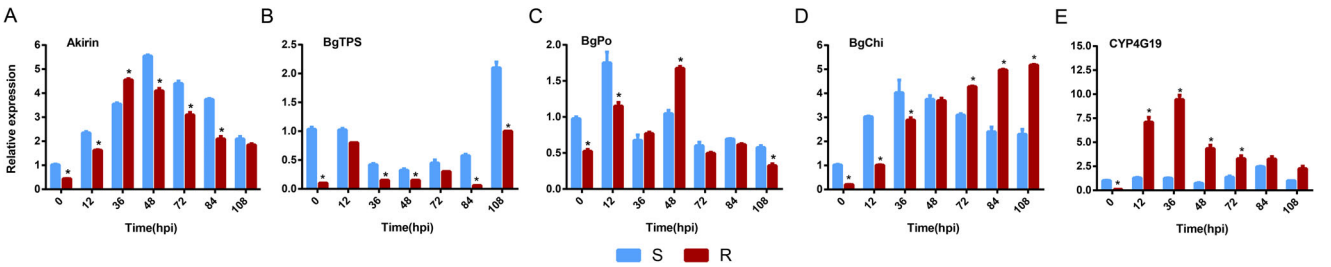


**Figure 6.** Time course of the expression level of different genes (BgPo, Akirin, CYP4G19, BgTPS, BgChi) after *M. anisopliae* treatment in male *B. germanica*. The vertical axis represents the relative gene expression ratios and the horizontal axis represents the times (0, 12, 36, 48, 72, 84 and 108 h) after treatment. The values represent the mean and standard deviation of three replications. Vertical bars are mean ± SE ($n = 3$). *$P < 0.05$ (Student's *t*-test). (A) BgPo, (B) Akirin, (C) CYP4G19, (D) BgTPS, (E) BgChi.

was Proteobacteria (R 30.57% *vs* S 39.25%, RT 28.09% *vs* ST 40.42%) (Fig. 5(A)).

At the genus level, the relative abundances of Parabacteroides (10.61% *vs* 3.39%, S *vs* R, $P = 0.001$), *Lachnoclostridium* (6.79% *vs* 2.85%, $P = 0.004$), and *Tyzzerella_3* (4.40% *vs* 0.76%, $P < 0.001$) were significantly lower in the R strain than in the S strain, while *Lactobacillus* (0.77% *vs* 7.82%, $P = 0.001$) and *Weissella* (1.59% *vs* 5.40%, $P = 0.005$) were both significantly higher in the R strain than in the S strain. After 15 days of treatment with *M. anisopliae*, there were significantly lower levels of *Fusobacterium*, *Parabacteroides*, *Lachnoclostridium*, *Tyzzerella*_3, and *Porphyromonadaceae* in the R strain than in the ST group and significantly higher levels of *Lactobacillus*, *Weissella*, and *Lactococcus* in the RT group ($P < 0.05$).

Across all samples, enriched KEGG pathways included cellular community, translation and membrane transport. KEGG analyses showed that nearly all KEGG pathways of the RT group were significantly lower than those of the other three groups ($P < 0.01$) (Fig. 5(C)).

### 3.5 *M. Anisopliae* exposure-mediated immune gene expression changes

Using RT–qPCR, we validated the expression level of antifungal immune genes (Akirin, BgTPS, BgPo, BgChi, CYP4G19) and showed that the Akirin, BgTPS, and BgPo genes of the S strains were higher than those of the R strains after treatment with *M. anisopliae* at different times post infection ($P < 0.05$) (Fig. 6(A)–(C)). The CYP4G19 and BgChi genes of the R strains were higher than those of the S strains at all stages of infection (Fig. 6(A)–(C)). For the BgChi gene, the expression level in S strains was higher than that in R strains during the early stages of infection (12–48 h) and lower in later stages of infection (72–108 h) (Fig. 6(D)).

### 3.6 RNAi silences anti-fungal immune genes

RNA interference tests showed that the expression levels of these immune genes (AKIRIN, BgPo, CYP4G19, BgChi, BgTPS) in cockroaches significantly decreased after RNAi (Fig. 7(A)). Both R and S strains increased the susceptibility to *M. anisopliae* after RNAi,



  

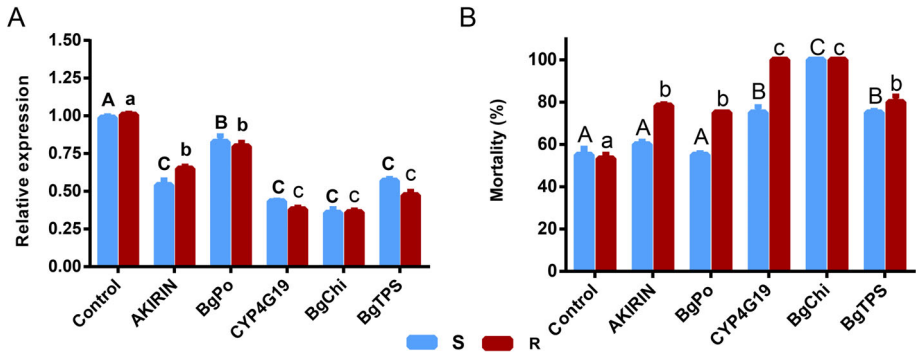


**Figure 7.** Changes in immune-related expression and mortality in male *B. germanica* after RNAi treatment. (A) Changes in immune gene expression levels (BgPo, Akirin, BgChi, BgTPS, CYP4G19) between the RNAi treatment group and the control group after 48 h. (B) Changes in the mortality of RS strains infected with EB0732 compared with the control after RNAi. Values represent the mean of three independent experiments and the error bars represent the SE. The same lowercase or uppercase letter are not significantly different according to Tukey's HSD test ($\alpha = 0.05$, $P > 0.05$) compared with the control.

but the mortality of R strain was significantly higher than that of the S strain ($P < 0.05$). The BgChi and CYP4G19 genes had the best interference effect, and the cumulative mortality rate of R and S strain cockroaches reached 100% ± 0.00% *vs* 100% ±0.00% and 100 ± 0.00% *vs* 73.33 ± 2.89% after 10 days, respectively. In contrast, the interference effect of the BgPo gene was relatively poor, and after infection with *Metarhizium* the mortality of the R and S strains was only 75 ± 0.00% and 55.00 ± 5.00% ($t$ = 3.497, df = 38, $P$ = 0.0081), respectively (Fig. 7(B)).

## 4 DISCUSSION

In this study, we report for the first time that resistance to beta-cypermethrin insecticides does not confer cross-resistance to infection by *M. anisopliae* of *β*-CYP-resistant German cockroaches regardless of age and sex. Similar results have been observed for *Cimex lectularius*[17] and *Bemisia tabaci*.[42] A study confirmed that both pyrethroid-resistant and insecticide-susceptible bed bugs were equally susceptible to infection by the fungal biopesticide Aprehend.[17] This study also corroborates that R strain German cockroaches were more susceptible to infection with the fungus *M. anisopliae*. Similarly, Howard *et al*. found that the insecticide-resistant *Anopheles gambiae* VKPER strain was significantly more susceptible to *B. bassiana* infection than the insecticide-susceptible SKK strain.[43] This study also demonstrated that mosquitoes treated with chemical insecticides exhibited decreased mosquito vectorial capacity by reducing the formation of sporozoites of *Plasmodium*.[16] In addition, younger nymphs were markedly less *M. anisopliae* resistant than the last instar nymphs and adults. This result suggests that the immune defense mechanism of younger nymphs is more imperfect than that of last instar nymphs and adults.[44] Many factors, such as nutrition status, physiological resistance, and nutrition status, may influence the virulence of *M. anisopliae* to German cockroaches, which may contribute to the detoxification of German cockroaches.[45,46]

Results showed that the gut microbiota of the S strain exhibited a stronger ability to inhibit *M. anisopliae* than that of the R strains *in vitro*, which was confirmed by bioassay tests. The abundance of *Parabacteroides*, *Lachnoclostridium*, and *Tyzzerella*_3 decreased significantly in the R strain via high-throughput sequencing, and most showed the ability to regulate glucose and lipid metabolism and antifungal infections. *Parabacteroides* can protect the host from pathogens propagating by accommodating metabolism, repairing intestinal barrier integrity, and stimulating the host immune system, which are essential probiotics for insect growth and development.[47–49] An anti-inflammatory lipopolysaccharide (LPS) molecule purified from *Parabacteroides* can inhibit lung tissue inflammation by reducing intestinal inflammation and mitochondrial functions, enhancing ribosomal activities, and systematically restoring aberrant host amino acid metabolism.[50] *Lachnoclostridium* exists in the guts of numerous species, from invertebrates to vertebrates, such as honey bees and humans, and can reduce gut mucosal inflammation levels and rebalance the intestinal microbiota.[51–53] In contrast, we found that the relative abundances of *Lactobacillus*, *Lactococcus* and *Weissella* were higher in the R strain than in the S strain. *Lactobacillus*, and *Lactococcus* protect intestinal barrier function, promote gut microbiome resilience, and alleviate oxidative stress.[54,55] *Weissella* participates in antibacterial potential and anti-inflammatory efficiency. Several strains of *Weissella* are universal opportunistic pathogens, and the pathogenesis is a multifactorial process that depends on the pathogens, pesticides, and immune status of the host.[56,57] Among the pre-infection and post-infection strains, *Parabacteroides*, *Lachnoclostridium*, *Enterococcus*, *Weissella*, *Tyzzerella*_3, *Anaerotruncus*, and *Porphyromonadaceae* showed significant differences. The commensal bacterium *Enterococcus faecium* triggers enhanced pathogen resistance and intestinal tract epithelial barrier function *via* secreted peptidoglycan hydrolase (SagA).[58] This result indicates that the gut microbiota of German cockroaches is disordered after *M. anisopliae* infection.[59] In addition, the mortality of the conventional cockroach of the R and S strains was lower than that of the germ-free cockroach. The gut microbiota enhances resistance to widely used pathogenic microorganisms.[60]

Histopathological changes showed that the exoskeleton penetration ability of *M. anisopliae* to resistant strains (R) was visibly faster than that of susceptible strains (S). Insecticide treatment results in thickening of the insect cuticle. This mechanism has been reported in pyrethroid-resistant *Cimex lectularius* bugs,[61] *Anopheles gambiae*,[62] and dichloro-diphenyltrichloroethane (DDT)-resistant Drosophila strains.[63] However, entomopathogenic fungi infect insect hosts primarily through intersegmental folds, mouthparts, and spiracles, where conidia preferentially germinate and penetrate.[64] Nonpolar crude compounds from the insect cuticle surface, which contain long-chain fatty acids, wax amino acids, and peptides, are

  

strong activators of conidial germination.[65] Entomopathogenic fungi can also fully degrade epicuticular hydrocarbons and use them as the only carbon source and for incorporation into similar cellular components.[66] This difference may account for the favorable toxicity profiles of *M. anisopliae* and the relatively high mortality of the R strain associated with insecticide treatment.

The dense physical barriers of cuticle proteins cross-linked to exoskeletal chitin and smooth hydrophobic surfaces were destroyed, and reactive oxygen species decreased after long-term beta-cypermethrin treatment.[67,68] Results also showed that the conidia of the R strain grew and reproduced at a faster rate than those of the S strain *in vivo*, such as hemolymph and gut. This phenomenon could occur because the host genes significantly affected by insecticide treatment covered a wide range of functional categories, including metabolism, cellular transport, macromolecular degradation, signal transduction, and immune defense.[69] For example, DDT resistance in these lines is linked to higher levels of expression of the generic detoxifying enzymes glutathione S-transferases (GST) and esterases.[57] Also, an instant immune response of hosts is the melanization reaction observed on the surface of cuticles and hemocoels.[70,71] It is generally assumed that melanization is a defense reaction mechanism to sequester *M. anisopliae* and that the production of toxic substances may be another cause of different infection rates in the two strains. Marmaras found that the formation of melanin seems to be due to quinone (tyrosine reactive intermediate) that is produced by the action of phenoloxidase (*BgPo*) and quinone compounds are one of the primary components of the chitinous (*BgChi*) exoskeleton of insects.[72] Results also show that the expression levels of Akirin, BgTPS, and BgPo genes in the R strain were significantly lower than those in the S strain, while BgChi and CYP4G19 gene expression were significantly higher. These data have allowed for the reconstruction of significant aspects of the host's immune pathways and the nature of the response to *M. anisopliae*. For example, the Akirin gene plays a primary role in immune defense in the epidermis of insects following natural fungal infection, linking the transcription factor NF$\kappa$B in the IMD pathway.[73,74]

In addition, CYP4G19 is well known for its multiple functions, and its roles in $\beta$-CYP resistance are chiefly related to metabolic resistance by enhancing detoxification.[19,75] Further research has also shown that CYP4G genes play an important role in contributing to fungal infection resistance and cuticular penetration in *Locusta migratoria*.[76] Research has shown that overexpressed CYP4G19 in the resistant strain facilitates the production of CHC in pyrethroid-resistant cockroaches and modulates cuticular permeability in pyrethroid-resistant cockroaches.[63] However, other cuticular components and the physical structure of cuticle layers may also contribute to penetration resistance in *B. germanica*. Therefore, enhancement of the cuticular lipid barrier did not mitigate the contributions of other mechanisms, such as immunity and resource constraints.[19,77] It is likely that these mechanisms may cooperate multiplicatively to increase epidermal adhesion of *M. anisopliae*. We report CYP4G19 gene dsRNA-specific nuclease activity after infection with *M. anisopliae* and speculate that rapid interference with the immune pathway of injected dsRNA may increase the sensitivity to *Metarhizium* in this insect. This result further suggested that the German cockroach already initiated the immune response at the early infection stage and that CYP4G19 genes play an important role in the early response against pathogenic fungi. The high efficiency of RNAi in the German cockroach unlocks its potential as a genetic model system for investigating cockroach biology. Taken together, these findings provide convincing evidence that fungal insecticides are effective alternatives for resistance management. The combination of fungal biopesticides and chemical insecticides can be used to compensate for the relatively slow action and low lethality of fungal insecticides. Also, fungal pathogens have been shown to be effective against insecticide-resistant *B. germanica* populations with no known resistance or cross-resistance, making them excellent candidates for use in German cockroach management programs.

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## CONFLICT OF INTEREST DECLARATION

All authors compiled, wrote, and approved this version of the article, and no part of this paper has been published or submitted elsewhere. No conflict of interest exists in the submission of this manuscript.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI at https://www.ncbi.nlm.nih.gov/, reference number BioProject PRJNA757708.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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