



# A Neurotoxic Insecticide Promotes Fungal Infection in *Aedes aegypti* Larvae by Altering the Bacterial Community

Y. A. Noskov<sup>1,2</sup> · M. R. Kabilov<sup>3</sup> · O. V. Polenogova<sup>1</sup> · Y. A. Yurchenko<sup>1</sup> · O. E. Belevich<sup>1</sup> · O. N. Yaroslavtseva<sup>1</sup> · T. Y. Alikina<sup>3</sup> · A. M. Byvaltsev<sup>4</sup> · U. N. Rotskaya<sup>1</sup> · V. V. Morozova<sup>3</sup> · V. V. Glupov<sup>1</sup> · V. Y. Kryukov<sup>1</sup>

Received: 1 May 2020 / Accepted: 27 July 2020 / Published online: 24 August 2020  
© Springer Science+Business Media, LLC, part of Springer Nature 2020

## Abstract

Symbiotic bacteria have a significant impact on the formation of defensive mechanisms against fungal pathogens and insecticides. The microbiome of the mosquito *Aedes aegypti* has been well studied; however, there are no data on the influence of insecticides and pathogenic fungi on its structure. The fungus *Metarhizium robertsii* and a neurotoxic insecticide (avermectin complex) interact synergistically, and the colonization of larvae with hyphal bodies is observed after fungal and combined (conidia + avermectins) treatments. The changes in the bacterial communities (16S rRNA) of *Ae. aegypti* larvae under the influence of fungal infection, avermectin toxicosis, and their combination were studied. In addition, we studied the interactions between the fungus and the predominant cultivable bacteria in vitro and in vivo after the coinfection of the larvae. Avermectins increased the total bacterial load and diversity. The fungus decreased the diversity and insignificantly increased the bacterial load. Importantly, avermectins reduced the relative abundance of *Microbacterium* (Actinobacteria), which exhibited a strong antagonistic effect towards the fungus in in vitro and in vivo assays. The avermectin treatment led to an increased abundance of *Chryseobacterium* (Flavobacteria), which exerted a neutral effect on mycosis development. In addition, avermectin treatment led to an elevation of some subdominant bacteria (*Pseudomonas*) that interacted synergistically with the fungus. We suggest that avermectins change the bacterial community to favor the development of fungal infection.

**Keywords** Avermectins · Mosquito · Microbiome · Entomopathogens · *Metarhizium*

## Introduction

Interactions between bacteria and fungi in various habitats are important for the functioning of biological systems [1]. It is well known that in vertebrate animals, these interactions may be modulated by different factors, including infectious

diseases and toxicant stresses [2]. However, these effects have been insufficiently studied in invertebrates. Insect bacterial communities are known to play an important role in different aspects of the host's life, from beneficial to detrimental, and their nature can be shifted depending on biotic and abiotic factors. The most important beneficial functions include the promotion of digestion, host metabolism regulation, the detoxification of plant allelochemicals, the stimulation of growth and development, and the induction of immune response protection from pathogenic microorganisms [3, 4]. In addition to these beneficial properties, the insect gut microbiota may be harmful to their hosts under certain conditions [5]. Alterations in the bacterial community structure may modulate host susceptibility to fungal [6] and bacterial pathogens [7]. It is also known that symbiotic bacteria participate in response to toxicoses caused by chemical insecticides [8, 9], and the microbiome structure of insects may be changed significantly under the influence of insecticides [10]. Importantly, insects are exposed to various toxicants and pathogens during their

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00248-020-01567-w>) contains supplementary material, which is available to authorized users.

✉ Y. A. Noskov  
yunoskov@gmail.com

- <sup>1</sup> Institute of Systematics and Ecology of Animals, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia 630091
- <sup>2</sup> National Research Tomsk State University, Tomsk, Russia 634050
- <sup>3</sup> Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia 630090
- <sup>4</sup> Novosibirsk State University, Novosibirsk, Russia 630090

development, which can lead to complex effects on the host's microbiota that will ultimately affect their survival.

Interactions between symbiotic insect bacteria and pathogens include (i) suppression or competitive exclusion [11, 12], (ii) the stimulation of the host's immune system [13], (iii) the production of antimicrobial compounds [14, 15], and (iiii) synergy between symbiotic and pathogenic microorganisms [6, 16, 17]. The influence of the microbial community on the development of mycoses may be different depending on the insect and fungal species and on environmental factors. The mortality rate of axenic insects infected with entomopathogenic fungi was shown to be significantly higher [18, 19] or significantly lower [6, 16] compared with non-axenic insects. It is likely that antagonistic or synergistic effects between fungi and bacteria depend on the type of fungal infection. With oral administration, a direct interaction between the fungi and gut bacteria occurs, which leads primarily to an antagonistic effect since many gut bacteria inhibit fungal growth and differentiation of infection structures [11, 15, 19, 20]. Topical fungal infections lead to more complicated interactions through the host's immunity as well as through direct interactions between microorganisms and their metabolites. They can cause both types of interactions between fungi and bacteria, antagonistic [18] and synergistic [6, 16, 17]. Despite the possible presence of antagonistic symbionts in insects, the antifungal effect may be absent for a number of reasons, such as (i) a low relative abundance of these bacteria, (ii) interactions with other organisms, (iii) the influence of other environmental factors, and/or (iiii) the production of antibacterial compounds by the fungi [21].

Blood-sucking mosquitoes are an important component of aquatic ecosystems. Many species, and in particular *Aedes aegypti*, play a significant role as a vector of serious human diseases. In the past few decades, entomopathogenic ascomycetes have often been used as an alternative to chemical insecticides for mosquito control. Among them, entomopathogenic fungi from the *Metarhizium* and *Beauveria* genera are actively being developed for use against mosquito larvae and adults [22, 23]. The effectiveness of *Metarhizium* conidia and blastospores in the biocontrol of *Ae. aegypti* larvae and adults has been recently reviewed by Aw and Hue [23].

The influence of mycoses on the microbiota of mosquito was explored in study by Frankel-Bricker and coworkers [24], who showed that the fungal colonization of *Ae. aegypti* larvae by the trichomycete *Zancudomyces culisetae* reduced the microbial community variation across individuals and influenced on adult microbiomes. The effects of topical infections by the ascomycetes *Beauveria* and *Isaria* on the microbiota of mosquito adults has been studied in detail in *Anopheles* and *Aedes* species [6, 25]. Wei and coworkers [6] demonstrated that topical infections of *Anopheles stephensi* with *Beauveria bassiana* lead to the increased

proliferation of *Serratia* in the gut followed by bacterial penetration into the hemocoel, which accelerates the host's death from mycosis. Ramirez and coauthors [25] also demonstrated an increase in the total bacterial load in the gut of *Ae. aegypti* adults after *Beauveria* and *Isaria* infection; however, no changes in the bacterial community structure were registered. The abovementioned authors explained these changes based on the prioritization of the immune response between the cuticle and the gut, as well as by the action of fungal secondary metabolites. It is important to note that in mosquito larvae, unlike adults, fungal infections of *Metarhizium* develop in a completely different way, through the accumulation of fungal conidia in the gut [26] or fungal growth in the siphon with subsequent suffocation [27]. In *Ae. aegypti* larvae, *Metarhizium* conidia are unable to adhere to the body surface, including the siphon, and the primary organ of conidia accumulation is the gut [26, 28]. The reason for the subsequent death of *Ae. aegypti* larvae may be protease-induced stress without the differentiation of infectious structures [26] or the colonization of the hemocoel by the fungus [29, 30]. Because the gut is the major reservoir of bacteria in insects, the fungus interacts directly with the bacteria in the mosquito larvae under this type of pathogenesis. It is possible that these bacteria may exhibit fungistatic properties, or, alternatively, they may act as synergists of the fungi.

Because insecticides can lead to shifts in the bacterial community within insects, it is reasonable to expect a change in their susceptibility to fungi. However, there are no data on the combined effects of entomopathogenic fungi and insecticides on the microbiota of mosquito larvae. Previously, we showed a synergistic effect between the entomopathogenic fungus *Metarhizium robertsii* and the neurotoxic insecticide avermectin in the mortality of *Ae. aegypti* larvae [30]. We suggested that this synergistic effect occurs because of the disturbance of the detoxifying and immune responses at the initial stage of toxicosis and mycosis. We also showed that the fungus was able to colonize the hemocoel in larvae and to form external sporulation. We registered a decrease in the antibacterial activity and an increase in the CFUs of cultivable bacteria from mosquito larvae under the influence of these agents, and thus we hypothesized that the synergistic effect can be mediated by changes in the bacterial community.

In this study, we examined the changes in the bacterial community structure of *Ae. aegypti* larvae under the influence of *M. robertsii* and avermectins. In addition, we identified cultivable bacteria from the predominant taxa and investigated the relationships of these bacteria with the fungus in vitro and in vivo. This study shows

the impact from the bacterial associates of *Ae. aegypti* larvae during fungal pathogenesis.

## Materials and Methods

### Mosquito, Fungus and Insecticide

*Ae. aegypti* larvae from the collection at the Institute of Systematics and Ecology of Animals SB RAS (Novosibirsk, Russia) were reared under controlled laboratory conditions in settled tap water ( $24 \pm 1$  °C,  $30 \pm 10\%$  RH, 12:12 L/D photoperiod). The larvae were fed daily with Tetramin Junior fish food (Tetra, Germany). The adults were fed with 10% sugar solution ad libitum and given blood meal by feeding on a human hand. The adult female mosquitoes laid their eggs on wet filter paper or in a Petri dish containing settled tap water. The hatched larvae were transferred to a glass container containing 1 L of settled tap water and fed daily. Fourth-instar larvae were used in the experiments, as this age is the longest. This precludes the influence of molting on pathogenesis.

The conidia from the MB-1 strain of *Metarhizium robertsii* from the institute's collection were grown on autoclaved millet for 10 days at 26 °C in the dark, followed by drying and sifting, and then stored at 4 °C. The industrial product "Phytoverm" 0.2% (SPC "Pharmbiomed", Russia) was used in the experiments as a chemical insecticide. This product includes a complex of natural avermectins (A1a (9%), A2a (18%), B1a (46%), and B2a (27%)) that are produced by *Streptomyces avermitilis*.

### Treatment Procedures and Colonization Assessment

The treatments were conducted using 200-mL plastic containers containing 100 mL of settled tap water with 15 fourth-instar larvae. The experiment included four treatments, that is, the control, fungus, avermectins, and fungus + avermectins (combined treatment). Both the fungal conidia and avermectins were suspended in distilled water, vortexed, and applied separately or in combination at equal volume (1 mL per container). The final concentrations were 6 µg/L for avermectins (active substance) and  $10^6$  conidia/mL for the fungus. The same amount of distilled water was added to the control treatment. The mortality assay was performed using at least 6 replicates for each of the treatments (one replicate included 15 larvae). The mortality data were recorded daily for 6 days.

The fungal colonization of the mosquitoes was assessed from the 3rd to 6th days posttreatment. Newly dead larvae were squeezed onto a glass slide and the contents were observed for the presence of fungal hyphal bodies using light microscopy (Total— $n = 52$  for the fungus and fungus + avermectins treatments). To visualize the fungal structures,

the mosquito larvae (2–3 days posttreatment) were fixed in a 0.2% solution of glutaraldehyde and 0.1 M Na-cacodylate buffer (pH 7.2). The sample preparation for making ultrathin sections included washing with cacodylate buffer followed by postfixation in 1% (w/v) OsO<sub>4</sub> in the cacodylate buffer. The samples were then dehydrated in an ethanol series, placed in an Epon-Araldite 812 mixture, and sectioned with a Reichert Ultracut S ultramicrotome (Leica, Nussloch, Germany). The ultrathin sections were stained with lead uranyl acetate and lead citrate and viewed on a Hitachi-300 or JEM-100CX electron microscope.

### DNA Extraction and Illumina Sequencing

Forty-eight hours posttreatment, the mosquito larvae were dipped in 0.5% chlorhexidine for 10 s, washed in sterile water, and frozen in liquid nitrogen. Four biological replicates (one replicate = 10 larvae) from each treatment were used for the analysis. The total DNA was extracted using a DNeasy PowerSoil Kit (Qiagen). Bead beating was performed using TissueLyser II (Qiagen) for 10 min at 30 Hz.

The V3-V4 region of the 16S rRNA genes was amplified with the 343F and 806R primer pair combined with Illumina adapter sequences as described previously [31]. A total of 200 ng of PCR product from each sample (a mix of three technical replicates) was pooled together and purified with a MinElute Gel Extraction Kit (Qiagen). The 16S libraries were sequenced with  $2 \times 300$ -bp paired-ends reagents on MiSeq (Illumina) in the SB RAS Genomics Core Facility (Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russia).

The raw sequences were analyzed using the UPARSE pipeline [32] with Usearch v11.0.667. The UPARSE pipeline included the merging of paired reads, read quality filtering, length trimming, merging of identical reads (dereplication), discarding of singleton reads, removal of chimeras, and OTU clustering using the UNOISE algorithm [33]. The alpha diversity metrics were also calculated in Usearch. Rarefaction and extrapolated curves were generated using the "iNEXT" package [34]. The final data set contained 215 OTUs and 446.856 reads ( $27.929 \pm 1.800$  reads per sample, see Appendix A). All the rarefaction curves followed a trend of approaching the saturation plateau, which indicated a reasonable volume of reads (Appendix B, Fig. S1). The raw MiSeq reads were deposited in GenBank under project accession number PRJNA625381.

### Bacterial Load Determination by qPCR

The larvae were collected at 48 h posttreatment. Sample preparation was performed as described in the previous section. The total bacterial 16S rRNA was determined in relation to the mosquito reference genes as described by Ramirez et al.

(2018). Ten larvae were pooled in one sample. The RNA was extracted with QIAzol® Reagent (Qiagen) for DNA, RNA, and protein isolation according to the manufacturer's instructions. The RNA concentration and purity were detected spectrophotometrically. DNase treatment was performed with RQ1 RNase-free DNase (Promega). The RNA was converted to cDNA using 6 mg of DNA-free RNA, 2 µL of 100 nM random nanomers, and 1 µL of RevertedAid™ M-MuLV Reverse Transcriptase (Fermentas, Lithuania).

qPCR was performed in three technical replicates under the following conditions: 95 °C for 3 min; followed by 40 cycles of 94 °C for 15 s and annealing and elongation at 62 °C for 30 s; and then melting curves were generated (70–90 °C). The qPCR was developed with HS-qPCR SYBR Blue (2×) mix (BioLabMix, Russia) by CFX96 Touch (Bio-Rad). Two genes from *Ae. aegypti* were used as reference genes, namely, the 60S ribosomal protein L32 (*Rp49*) and 40S ribosomal protein S17 (*Rps17*). To detect the total bacterial cDNA, universal primers for 16S were used. The primer sequences are shown in Appendix B (Table S1). The primer properties were tested using IDT OligoAnalyzer 3.1 (<http://eu.idtdna.com/calc/analyzer>). Seven replicates per treatment were used for the analysis.

### CFU Counts and Identification of Cultivable Bacteria

The larvae were collected at 48 h posttreatment. The sample preparation was conducted as described in the previous sections, but the insects were not frozen. The larvae were homogenized in 1 mL of cooled, sterile 150 mM sodium chloride (1 sample = 3 larvae). One hundred microliters of the suspensions in  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  dilutions were inoculated in 90-mm Petri dishes containing Blood agar™, *Aeromonas* medium™, Endo agar™ (Himedia Lab., India) and Flavobacteria medium. The Flavobacteria medium was composed of (g/L) 5—tryptose, 5—trehalose, 2—yeast extract, 3—sodium chloride, 0.2—magnesium sulfate, 0.2—Iron (III) chloride, 0.1—bromothymol blue, and 15—agar. Colony counting was performed after 72 h of incubation at 28 °C. Morphologically different colonies were isolated into pure cultures after three passages.

The putative species were determined by sequencing the 16S rRNA gene sequences and comparing them with those available in the NCBI GenBank Database ([www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank)). Bacterial suspensions prepared from the individual colonies were boiled and lysates were used as a template to amplify the 16S rRNA gene. PCR was performed using the primers 16S-8-f-b 5'-AGRGTGATCCCGGCTCA-3' and 16S-1350-r-B 5'-ACGGCGGGTGTGTACAANG, and it resulted in 1342-bp DNA fragments. The sequencing of the resulting PCR fragments was performed using the same primers and a Terminator v.3.1 BigDye™ kit (Applied Biosystems, USA) according to the

manufacturer's protocols. Capillary electrophoresis was performed on ABI 3500 Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequences were verified using Sequencher v.4.0.5. The closest bacterial sequences from the GenBank Database (at a sequence similarity level of at least 98%) were used to determine the putative species. Twenty-four sequences were deposited in the GenBank Database (MT040033–MT040056).

### Antagonistic Effects of the Cultivable Bacteria Towards Fungus In Vitro

The bacteria *Chryseobacterium cucumeris*, *Microbacterium paraoxydans*, *Delftia* sp., and *Pseudomonas putida* were isolated from the laboratory lines of *Ae. aegypti*. The bacteria *Aeromonas hydrophila* (CEMTC 2088) were obtained from a collection of extremophilic microorganisms and type cultures at the Institute of Chemical Biology and Fundamental Medicine SB RAS (Novosibirsk, Russia); together, they were analyzed for antagonistic activity. The plugs of 1-day-old bacterial cultures (Nutrient agar™; Himedia Lab., India) were placed on a freshly plated culture of *M. robertsii* in 90-mm Petri dishes with Sabouraud dextrose agar. The Petri dishes were incubated at 28 °C in the dark. The inhibition zone of mycelial growth (mm) was evaluated after 144 h of incubation. At least 4 replicates were used in the assay.

### Combined Effects of Bacteria and *M. robertsii* on Mosquito Larvae

The bacteria *C. cucumeris*, *M. paraoxydans*, *A. hydrophila*, *Delftia* sp., and *P. putida* were cultivated on Nutrient agar™ (Himedia). One-day-old colonies were collected with an inoculation loop in cooled sterile sodium chloride, vortexed, and washed twice by centrifugation for 5 min at 6,000 g. Last, the bacteria were suspended in sterile water and their cell concentrations were determined using a Neubauer hemocytometer.

Prior to the application of bacteria and fungi, the mosquito larvae were exposed to an antibiotic cocktail (amikacin (OJSC “Sintez,” RF) at 10 mg/L and penicillin (PanReac, Applichem) 10 mg/L) for 24 h. Then, the water containing the antibiotics was exchanged with sterile tap water. The eradication efficacy was controlled by plating larval homogenates on Nutrient agar as described in the section called “CFU Counts and Identification of Cultivable Bacteria.” Suspensions of conidia and bacterial cells were added separately or in combination to containers containing the mosquito larvae. The final concentrations were  $5 \times 10^8$  bacterial cells and  $1 \times 10^6$  conidia per milliliter of water. The control was treated with the equivalent amount of sterile water. Thus, the experiment with each bacteria included four treatments: control, bacteria, fungus, and fungus + bacteria. The mortality data were collected daily for 6 days. At least 30 insects (1

replicate included 10 larvae) from each of the treatments were used for the assays.

## Statistical Analysis

The differences in the survival of the mosquito larvae were analyzed by the log-rank test followed by a Holm-Sidak adjustment. The synergistic and antagonistic effects between the fungus and insecticide as well as between the fungus and bacteria were determined by comparing the observed and expected mortality using the  $\chi^2$  criterion, as described by Robertson and Preisler [35]. A comparison of portions of larvae in which hyphal bodies were detected and not detected was performed using Fisher's exact test. Data on the bacterial OTU abundance, total 16S rRNA, CFU count, and antagonistic activity of the bacteria in vitro were checked for normal distribution using a Shapiro-Wilk *W* test. The MiSeq data were analyzed by the two methods: (i) comparisons of relative abundances and (ii) comparisons of actual read counts per taxon after rarefying. The effects were considered as significant ( $P < 0.05$ ) if they were registered in the both cases only. The data for predominant taxa had normal distribution and were analyzed by the two-way ANOVA followed by Tukey's post hoc test. The data for subdominant taxa had mainly abnormal distribution and were analyzed using a non-parametric analog of the two-way ANOVA—Scheirer-Ray-Hare test [36], followed by Dunn's post hoc test. In the results, the levels of significance are presented for the relative abundances comparisons. Data on total 16S rRNA and CFU count were also analyzed by the Scheirer-Ray-Hare test. Differences in the antagonistic activity of the bacteria towards *M. robertsii* in vitro were determined by the one-way ANOVA followed by Tukey's test. The software PAST 3 [37], SigmaStat 3.1 (Systat Software Inc., USA), STATISTICA 8 (StatSoft Inc., USA), and AtteStat v.12.5 [38] were used for the analyses.

## Results

### Mortality and Colonization Assay

The mortality of the untreated larvae was 3.3% on the 6th day posttreatment (Fig. 1a). Fungal infection led 50.8% mortality of mosquito larvae, and treatment with avermectins led to 46.7% mortality by the 6th day posttreatment. The mosquito mortality increased significantly after the combined treatment (85% at the 6th day posttreatment), and a synergistic effect was registered from the 3rd to the 6th days ( $\chi^2 > 6.5$ ,  $df = 1$ ,  $P < 0.001$ ). The log-rank test also showed significant differences between the combined treatment and treatments with avermectins or fungus alone ( $\chi^2 > 9.9$ ,  $df = 1$ ,  $P < 0.001$ ). The median-lethal survival time ( $LT_{50}$ ) decreased 1.5-fold (from 6 to  $4 \pm 0.2$  days) after the combined treatment,

compared with the single treatments. Thus, the effects in mosquito mortality were consistent with those in our previous work [30].

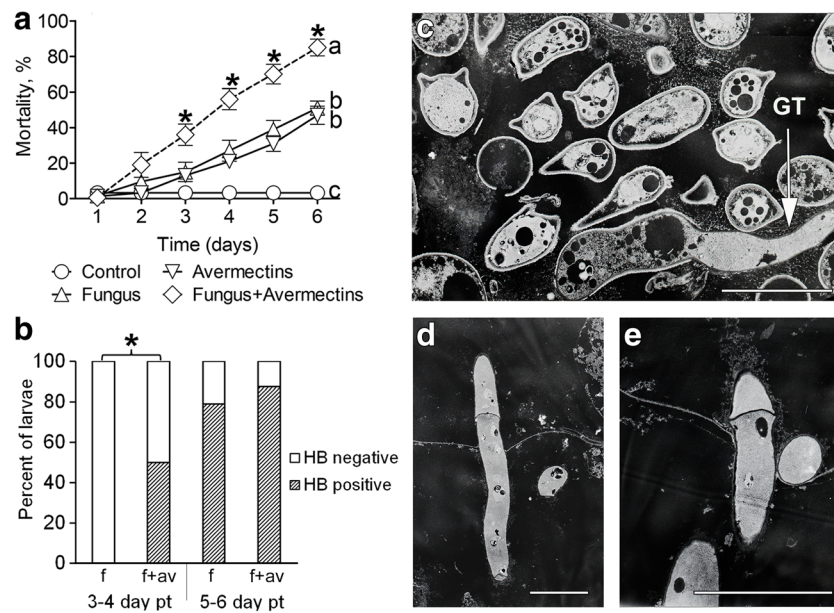
Fungal germ tubes and hyphal bodies were detected in both the gut lumen and the hemocoel (Figs. 1c–e). The number of hyphal body-positive larvae was significantly higher after the combined treatment compared with the treatment with fungus alone by the 3rd and 4th days posttreatment (Fisher's exact two-sided test,  $df = 1$ ,  $P = 0.014$ , Fig. 1b). At the 5th–6th days posttreatment, this trend was also observed, but the differences between treatments were insignificant ( $P = 0.23$ ). No hyphal bodies were detected in the fungus-free treatments.

### Changes in the Bacterial Communities After Fungal and Avermectin Treatments

The classification based on 16S rRNA sequencing revealed 215 operational taxonomic units (OTUs) in mosquito larvae that were grouped into 73 genera, 48 families, and 11 classes (Appendix A). Three classes were the most abundant, namely Flavobacteria (phylum Bacteroidetes), Actinobacteria (phylum Actinobacteria), and Gammaproteobacteria (phylum Proteobacteria). The most abundant genus (51.6%) was *Chryseobacterium* (Flavobacteriaceae), which was represented by five OTUs. In addition, two other genera, *Microbacterium* (Microbacteriaceae) as represented by only one OTU (abundance 13.9%) and *Aeromonas* (four OTUs, abundance 13.4%), were identified among the predominant ones.

The number of OTUs decreased significantly under the influence of fungal infection (two-way ANOVA, effect of fungus:  $F_{1,12} = 7.0$ ,  $P = 0.02$ , Table 1), and by contrast, the influence of avermectins led to an increase in the number of OTUs ( $F_{1,12} = 28.8$ ,  $P = 0.0002$ , Table 1). Under fungal infection, we observed a trend towards an increase in the Shannon index ( $F_{1,12} = 2.9$ ,  $P = 0.11$ ), but treatment with avermectins led to a decrease in the index ( $F_{1,12} = 6.4$ ,  $P = 0.03$ ). Fungal infection decreased the Chao1 index ( $F_{1,12} = 5.5$ ,  $P = 0.04$ ), but treatments with avermectins led to an increase in this index ( $F_{1,12} = 13.0$ ,  $P = 0.004$ ). No significant interactions were observed between the two factors on the diversity indexes.

Treatments with *M. robertsii* and avermectins led to significant shifts in the structure of the mosquito microbiota (Fig. 2). Regarding the predominant groups, fungal infection led to a decrease in the relative abundance of the most abundant group (Flavobacteria, *Chryseobacterium*), while toxicosis caused by avermectins led to an increase in the abundance of this group of bacteria (effect of the fungus:  $F_{1,12} = 7.8$ ,  $P = 0.015$ ; effect of the avermectins:  $F_{1,12} = 46.5$ ,  $P < 0.0001$ , Fig. 2a, b); however, the factor interaction between the fungal and avermectin treatments was not significant ( $F_{1,12} = 3.4$ ,  $P = 0.09$ ). After the fungal treatment, we observed a sharp increase in the abundance of Gammaproteobacteria, which was represented



**Fig. 1** Mortality dynamics of *Aedes aegypti* larvae and a colonization assay. **a**—Dynamic of *Aedes aegypti* larval mortality after separate and/or combined treatments with the fungus *Metarhizium robertsii* ( $1 \times 10^6$  conidia/mL) and avermectins (6  $\mu\text{g/L}$ ). Different letters indicate significant differences between treatments (log-rank test,  $\chi^2 > 9.9$ ,  $df = 1$ ,  $P < 0.001$ ). The asterisks indicate a synergistic effect on the larval mortality between the fungus and avermectins ( $\chi^2 > 6.5$ ,  $df = 1$ ,  $P < 0.01$ ).

**b**—Proportion of freshly dead larvae in which hyphal bodies were detected (HB-positive) and not detected (HB-negative) after treatment with the fungus alone (f) and in combination with avermectins (f + av). The asterisk (\*) indicates a significant difference between treatments ( $P = 0.014$ , Fisher's exact two-sided test). **c**—Electron microscopy of the gut lumen showing the conidia and germ tubes (GT). **d**, **e**—Electron microscopy of a hemocoel showing the presence of hyphal bodies. Scale bar, 5  $\mu\text{m}$

primarily by *Aeromonas* (Tukey's HSD,  $P < 0.0005$  compared with the other treatments), but we did not observe this elevation after the combined treatment with fungus and avermectins (factor interaction  $F_{1,12} = 88.4$ ,  $P < 0.0001$ ), i.e., avermectins limited the *Aeromonas* increase caused by fungal infection. Importantly, the avermectin treatment led to a significant 3.3–3.6-fold decrease in the relative abundance of Actinobacteria, which were primarily represented by *Microbacterium* ( $F_{1,12} = 12.4$ ,  $P = 0.004$ ).

**Table 1** Diversity characteristics of bacterial communities in *Aedes aegypti* larvae at 48 h posttreatment with avermectins, *Metarhizium robertsii*, and their combination

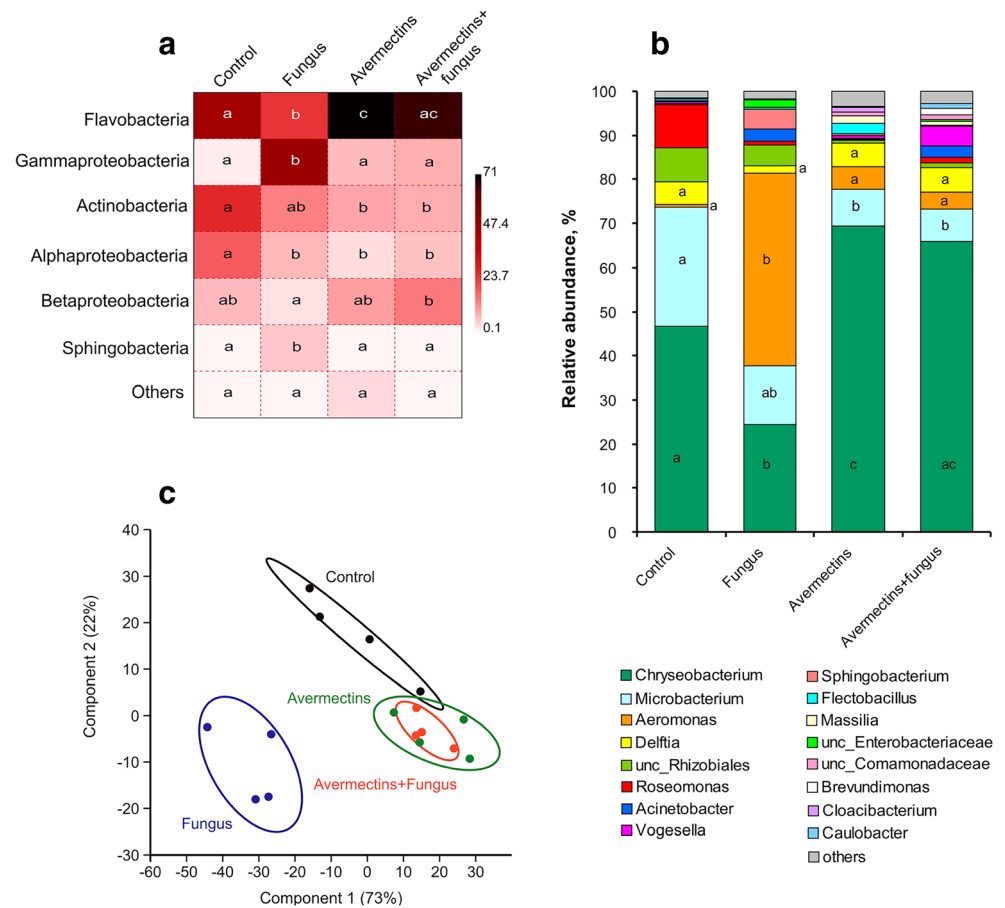
Treatments	Diversity indexes		
	OTUs	Shannon*	Chao1*
Control	78 $\pm$ 5.8 <sup>ab</sup>	1.6 $\pm$ 0.09 <sup>ab</sup>	101 $\pm$ 7.7 <sup>ab</sup>
Fungus	70 $\pm$ 1.9 <sup>a</sup>	2.1 $\pm$ 0.05 <sup>a</sup>	78 $\pm$ 5.1 <sup>a</sup>
Avermectins	106 $\pm$ 5.6 <sup>c</sup>	1.5 $\pm$ 0.25 <sup>b</sup>	121 $\pm$ 3.5 <sup>b</sup>
Avermectins + fungus	90 $\pm$ 3.1 <sup>bc</sup>	1.6 $\pm$ 0.11 <sup>ab</sup>	110 $\pm$ 10.8 <sup>b</sup>

\*The Shannon and Chao1 indexes were calculated for the OTU level. The Chao1 index was calculated in adjusted version [39]. The standard errors ( $\pm$  SE) were calculated for four replicates. Different letters indicate significant differences among treatments (Tukey's test,  $P < 0.05$ )

Regarding the less abundant groups, the fungal treatment led to a significant decrease in the relative abundance of Alphaproteobacteria and increased Sphingobacteria ( $F_{1,12} > 4.9$ ,  $P < 0.05$ , Fig. 2a) as well as the elevation of certain Gammaproteobacteria such as *Acinetobacter* and unc. *Enterobacteriaceae* ( $H_{1,15} > 11.3$ ,  $P < 0.001$ , Appendix B, Fig. S2). Under the influence of avermectins, a decrease in the abundance of Alphaproteobacteria followed by an increase in the abundance of Betaproteobacteria was observed ( $F_{1,12} > 10.5$ ,  $P < 0.007$ , Fig. 2a). In particular, a decrease in the proportion of subdominant *Roseomonas* and unc. Rhizobiales (Alphaproteobacteria) was accompanied by a significant increase in the abundance of a number of low-abundance bacteria, namely *Vogesella*, *Massilia*, unc. *Comamonadaceae*, unc. *Burkholderiales*, and *Pseudoduganella* (Betaproteobacteria) ( $H_{1,15} > 4.4$ ,  $P < 0.035$ , Appendix B, Fig. S2). In addition, avermectin treatment led to a significant increase in the proportion of *Pseudomonas* (Gammaproteobacteria) as well as *Brevundimonas* and *Asticcacaulis* (Alphaproteobacteria) ( $H_{1,15} > 3.8$ ,  $P < 0.05$ , Appendix B, Fig. S2).

A principal component analysis (PCA) showed a clear distance between the bacterial communities of the control group, the group treated with the fungus, and the groups treated with avermectins (Fig. 2c). Larval bacterial communities treated with avermectins and the combination (avermectins + fungus) were significantly overlapped, which confirms the higher

**Fig. 2** Changes in the bacterial communities of *Aedes aegypti* larvae whole-body homogenates at 48 h posttreatment with *Metarhizium robertsii* conidia, avermectins, and their combination. **a**—Relative abundance of the bacterial classes. **b**—Relative abundance of the genera. Different letters indicate significant differences between treatments for specific groups of bacteria, as calculated for 4 biological replicates (Tukey's post hoc test,  $P < 0.05$ ). **c**—Principal component analysis for the OTU level



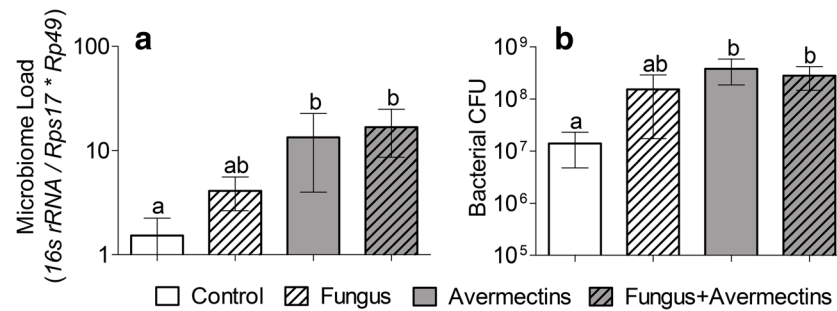
similarity of the bacterial communities between these two groups. The first component explained 73% of the variation, and OTU-1 (*Chryseobacterium*) made the largest contribution. The second component explained 22% of the variation, with the largest contributions from OTU-2 (*Microbacterium*) and OTU-6 (*Aeromonas*).

### Total Bacterial Load and Identification of Cultivable Bacteria

The total bacterial load in mosquito larvae from different treatments was analyzed by measuring the bacterial 16S rRNA (qPCR) and counting the bacterial CFUs on different media. Both analyses revealed a significant increase in the bacterial load under toxicosis caused by avermectins (Fig. 3). Specifically, the total bacterial 16S rRNA increased significantly under the influence of avermectins and was insignificant under the influence of the fungus ( $H_{1,27} = 5.7$ ,  $P = 0.01$  and  $H_{1,27} = 1.8$ ,  $P = 0.18$ , respectively). The plating of larvae homogenates on nonselective blood agar also showed a significant increase in the CFU count under avermectin treatment ( $H_{1,19} = 8.7$ ,  $P = 0.003$ ) and insignificant elevation under fungal infection ( $H_{1,19} = 0.3$ ,  $P = 0.59$ ). No significant factor interactions between

the fungus and avermectins on the bacterial load was revealed. Similar effects were obtained for the CFU counts on Endo agar, Flavobacteria media, and *Aeromonas* media (Appendix B, Fig. S3).

To establish the effects of the bacteria on the development of mycosis, we isolated and identified the bacteria from the predominant groups present in the microbiome, primarily *Chryseobacterium*, *Microbacterium*, *Aeromonas*, and *Delftia*. From the *Chryseobacterium* genus, we identified four isolates of *C. cucumeris* (Appendix B, Table S2) that were obtained from blood agar and Flavobacteria media. Among the *Microbacterium*, two isolates of *M. paraoxydans* were obtained from blood agar. From the *Delftia* genus, we isolated six cultures from Endo agar and *Aeromonas* media. These cultures demonstrate 100% shared identity for the 16S rRNA sequences between two species, *Delftia tsuruhatensis* and *D. lacustris*. In addition, *Pseudomonas alcaligenes*, *P. putida*, *P. protegens*, *P. aeruginosa*, *P. mosselii*, *Stenotrophomonas maltophilia*, and *Acinetobacter* sp. were isolated from different media (Appendix B, Table S2). We could not isolate any *Aeromonas* cultures using the media mentioned above. Primarily, *Pseudomonas* colonies were obtained from the *Aeromonas* media. Therefore, we used a museum strain of *Aeromonas hydrophila* (see section called



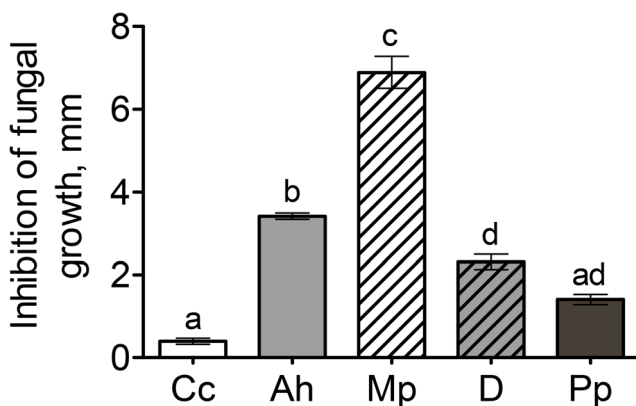
**Fig. 3** Total bacterial load for whole-body homogenates of *Aedes aegypti* larvae at 48 h posttreatment with *Metarhizium robertsii* conidia, avermectins, and their combination. a—16S rRNA normalized

expression of two mosquito reference genes, *Rsp17* and *Rp49*. b—CFU count on blood agar. Different letters above each column indicate significant differences between treatments (Dunn's test,  $P < 0.05$ )

“Antagonistic Effects of the Cultivable Bacteria Towards Fungus In Vitro”) in the subsequent experiments.

### Antagonistic Effect of Cultivable Bacteria Towards *Metarhizium robertsii* In Vitro

An analysis of interactions between the cultivable bacteria and *M. robertsii* using an agar plug assay showed that all of the bacteria exhibit antagonistic effects of varying strength (Fig. 4, see also Appendix B, Fig. S4). The weakest antagonism was registered for *C. cucumeris*, which only inhibited fungal growth to  $0.4 \pm 0.07$  mm. The strongest antagonism was exhibited by *M. paraoxydans* ( $6.9 \pm 0.4$  mm), and it was significantly different from that of the other bacteria (Tukey's test,  $P < 0.001$ ). The bacterium *A. hydrophila* also showed a relatively strong antagonistic effect towards *M. robertsii* ( $3.4 \pm 0.07$  mm). Cultures of *Delftia* sp. and *P. putida* inhibited fungal growth to  $2.3 \pm 0.18$  mm and  $1.4 \pm 0.12$  mm, respectively.



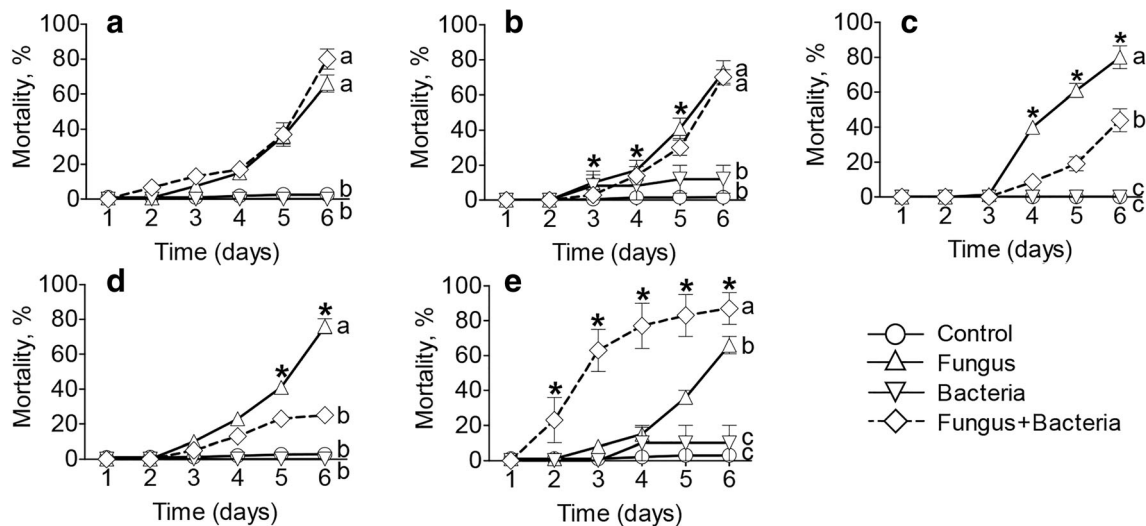
**Fig. 4** Antagonistic effect of predominant bacteria from *Aedes aegypti* larvae towards *Metarhizium robertsii* as estimated by agar plug method. Cc, *Chryseobacterium cucumeris*; Ah, *Aeromonas hydrophila*; Mp, *Microbacterium paraoxydans*; D, *Delftia* sp.; and Pp, *Pseudomonas putida*. Different letters indicate significant differences (Tukey's test,  $P < 0.05$ )

### Combinative Effects of Bacteria and *M. robertsii* on the Mortality of Mosquito Larvae

Treating with the antibiotic cocktail significantly inhibited the bacterial associates of the *Ae. aegypti* larvae. No bacterial colonies were registered after plating the larvae treated with the antibiotics on LB media. Treatment with antibiotic cocktail had no effect on the larval survival throughout the experiment (Fig. 5S). Larvae treated with antibiotics were more susceptible to *M. robertsii* compared with native larvae. A significant synergistic effect between the antibiotics and fungus was observed from the 5th to 7th days posttreatment ( $\chi^2 > 33.6$ ,  $df = 1$ ,  $P < 0.05$ ; Fig. 5S).

In the following experiments, we studied the combined effects between cultivable bacteria and *M. robertsii* on larvae that were pretreated with antibiotics. None of the tested bacteria caused larval mortality compared with the antibiotic-treated control (Fig. 5). However, treatments with different bacteria along with the *M. robertsii* conidia led to synergistic, antagonistic, or neutral effects on the mortality of *Ae. aegypti* larvae. Specifically, treating the larvae with *C. cucumeris* did not have any effect on the larval mortality compared with the fungal treatment alone (log-rank test,  $\chi^2 < 0.9$ ,  $df = 1$ ,  $P > 0.34$ ) (Fig. 5a). Treating with *A. hydrophila* led to a slight decrease in susceptibility to fungal infection. A weak but significant antagonistic effect from the 3rd to 5th days posttreatment was registered ( $\chi^2 > 6.8$ ,  $P < 0.01$ ) (Fig. 5b). Importantly, the mortality of fungus-treated larvae was significantly reduced after treating with *M. paraoxydans* (Fig. 5c). A significant antagonistic effect was recorded from the 4th to 6th days posttreatment ( $\chi^2 > 6.5$ ,  $P < 0.01$ ). The reintroduction of *Delftia* sp. also led to reduced mortality due to the fungus (Fig. 5d). A significant antagonistic effect was registered from the 5th to 6th days posttreatment ( $\chi^2 > 6.9$ ,  $P < 0.01$ ). The mortality rate of the conidia-treated larvae was significantly higher after the reintroduction of *P. putida* (Fig. 5e). A significant synergistic effect was observed from the 2nd to 6th days posttreatment ( $\chi^2 > 4.5$ ,  $P < 0.04$ ).





**Fig. 5** Mortality dynamics of germ-free *Aedes aegypti* larvae treated with conidia from *Metarhizium robertsii* ( $1 \times 10^6$  conidia/mL), cultivable bacteria ( $5 \times 10^8$  cells/mL), and their combination. The control treatment included mosquito larvae pre-treated with antibiotic cocktail (amikacin + penicilin) for 24 h. a—*Chryseobacterium cucumeris*; b—*Aeromonas hydrophila*; c—*Microbacterium paraoxydans*; d—*Delftia* sp.; and e—

*Pseudomonas putida*. Asterisks indicate significant antagonistic or synergistic effects between the bacteria and fungus on the mortality of the mosquito larvae ( $\chi^2 > 3.85$ ,  $df = 1$ ,  $P < 0.05$ ). Different letters next to each line indicate significant differences between treatments as estimated by log-rank test ( $P < 0.05$ )

## Discussion

This study shows significant changes in the structure of the bacterial community of *Ae. aegypti* larvae under the influence of avermectins and *M. robertsii* infection. We suggest that the synergy between these agents is linked to the shifts in the bacterial composition. In particular, avermectins decreased the relative abundance of strong fungal antagonists (Actinobacteria, *Microbacterium*) and increased the abundance of bacteria with neutral effects on the fungus (Flavobacteria, *Chryseobacterium*), which may create favorable conditions for the differentiation of the fungal infection structures and promote a more active penetration of the fungus through the gut wall into the hemocoel. It should be noted that there are contrasting data on the germination of fungal conidia in the gut of *Ae. aegypti* larvae and the colonization of the hemocoel by hyphal bodies in experiments by various researchers. In particular, Butt and coauthors [26] observed conidia accumulation in the larval gut without the differentiation of fungal structures, while Riba and coworkers [29] registered typical mycosis with hyphal growth. In our previous study [30] on the same laboratory line of *Ae. aegypti* (as in the present study), we observed a colonization of the hemocoel by hyphal bodies of *M. robertsii* after treating with the fungal conidia. The hyphal bodies were recorded for both living and recently dead larvae [30]. The proportion of hyphal body-positive larvae in the late stages of pathogenesis was high (more than 83%), which indicates the stability of fungal colonization for this line of mosquitoes. It is possible that contrasting data on the colonization of mosquito larvae may be

associated with significant differences in the microbiome structures of different mosquito lines.

Significant variation in the composition of bacterial communities in natural and laboratory populations of *Ae. aegypti* larvae has been shown [40, 41]. The bacterial communities of *Ae. aegypti* larvae are mostly represented by three primary phyla: Bacteroidetes, Actinobacteria, and Proteobacteria. However, the composition and abundance of different classes, families, and genera varied significantly [40, 42]. The high abundance of *Chryseobacterium* and *Microbacterium* was shown for some laboratory lines of *Ae. aegypti* [42], which is consistent with our research. In the present study, we registered the OTUs of some groups, such as Burkholderiales, *Comamonas*, and *Rhizobium*, that may be in a mutualistic relationship with insects and may constitute a significant part in the reproductive organs, salivary glands, or guts [43–45]. However, there is no evidence that different mosquito populations have a particular set of bacteria that is typical for all individuals, regardless of the habitat [46].

It has been previously shown that biological (*Bacillus thuringiensis*) and chemical (methoprene) insecticides led to specific shifts in the bacterial communities of mosquito larvae [10]. Avermectins affect glutamate and other ligand-gated and voltage-dependent chloride channels in invertebrates [47], and they also cause the inhibition of cellular immunity [48] and the destruction of intestinal epithelial cells [49]. In addition, this insecticide leads to a decrease in food consumption and thereby causes starvation, which was shown in studies on terrestrial insects [50]. Slowing the passage of food through the gut leads to the creation of more isolated and less aerated conditions for the

development of bacteria as well as to changes in the pH and other physicochemical parameters of the microenvironment. Moreover, avermectins cause dysregulation in the humoral immunity and detoxification systems [30, 48, 51], which may also lead to a change in the total bacterial load and the microbiome structure in the gut and other organs.

After the avermectin treatment, we observed an increase in the total bacterial load of the larvae, which was accompanied by an increase in the number of OTUs, the Chao index, and a decrease in the Shannon index. We observed shifts in the community structure towards an increase in the abundance of Flavobacteria and Betaproteobacteria. An increase in the total bacterial load may correlate with a decrease in the Shannon index, since the proliferation of certain groups of bacteria leads to the crowding out of less abundant groups. This trend was shown, for example, in the wax moth *Galleria mellonella* under the influence of another neurotoxin (*Habrobracon hebetor* venom) [17]. Importantly, *Aedes* larvae are able to digest bacteria, in particular Flavobacteria [52]. Therefore, an increase in the total bacterial load and especially *Chryseobacterium* is probably associated with a decrease in digestive activity caused by the destruction of the gut epithelium and the disturbance in the enzymatic system under the influence of avermectins. The elevation of the Betaproteobacteria abundance was due to subdominant bacteria that primarily belonged to Burkholderiales (*Massilia*, *Comamonadaceae*, unc. *Burkholderiales*). This effect remains unclear. It was shown by Kikuchi and coworkers [53], and Muturi and coworkers [54], that Burkholderiales abundance and diversity might increase in different environments (water, soil) after an insecticide treatment. These authors suggest that Burkholderiales can use insecticides as a carbon source. However, the ability of these bacteria to utilize avermectins has not been studied.

Fungal infection led to a significant decrease in the diversity indexes of the bacterial communities and a trend towards the elevation of total bacterial 16S rRNA was registered. Previously, an enhancement in the bacterial load and slight decrease of the diversity indexes were observed during the topical infection of mosquito adults with *Beauveria* and *Isaria* fungi, which was associated with the modulation of immune responses [6, 25]. Interestingly, the proportion of *Aeromonas* was strongly increased by fungal infection. We believe that *Aeromonas* develops in water or in the gut lumen due to the excess of organics, associated with the introduction of fungal conidia. It is well known that *Aeromonas* is more abundant in eutrophic water bodies [55–58]. In addition, *Aeromonas* exhibits high chitinolytic activity and is able to use chitin as a carbon source [59]. Brzezinska and Donderski [57] showed that *Aeromonas* was predominant among other chitinolytic bacteria in freshwater bodies and especially in eutrophic lakes. Therefore, the elevation of

*Aeromonas* may be caused by an increase in the fungal chitin content after *M. robertsii* treatment. It is noteworthy that the larvae treated with the combination of the fungus and avermectins did not show a high abundance of *Aeromonas*. This result correlates with the fact that under the influence of avermectins, *Ae. aegypti* larvae accumulate fewer fungal conidia compared with larvae treated with fungus alone [30]. Interestingly, mycosis development was often accompanied by an increase in the abundance of chitinolytic bacteria in insects, such as *Serratia marcescens* [6], *Erwinia* [16], and *Aeromonas* (present study).

The changes in the bacterial community of *Ae. aegypti* larvae under the influence of *M. robertsii* may also be associated with the microbiome of the fungus. Bacterial communities of entomopathogenic fungi are poorly studied. However, Chen et al. [60] showed that the bacteriomes of the entomophthoralean fungus *Pandora* are represented primarily by Enterobacteriaceae and *Acinetobacter*. According to our preliminary data, the conidia of *Metarhizium* are characterized by a relatively rich bacteriome (> 70 OTUs) and by a high abundance of Enterobacteriaceae and *Acinetobacter* (Appendix B, Fig. S6). This result correlates with the fact that fungal treatment increased the abundance of unc. Enterobacteriaceae and *Acinetobacter* in *Ae. aegypti* larvae (Appendix B, Fig. S1).

Bioassays on the predominant bacteria showed different strengths of antagonism to *M. robertsii* in in vitro tests and different effects (from antagonism to synergism) under the combined larval treatment when using the bacteria and *M. robertsii*. Germ-free larvae were more susceptible to fungus than native larvae (Appendix B, Fig. S5). This finding is highly consistent with other studies [19], which show the contribution of *Blattella germanica* gut bacteria to fungal resistance after the oral administration of *Metarhizium anisopliae*. Most importantly, following avermectin treatment, the abundantly proliferating *Chryseobacterium* was relatively inert to the fungus, i.e., it did not have an antagonistic effect on larval mortality and showed the weakest antagonism in vitro (Fig. 4, Fig. S4). However, *Microbacterium* (Actinobacteria), which decreased under avermectin toxicosis, was the strongest antagonist of the fungus in both in vitro and in vivo assays. We suggest that the decrease in Actinobacteria abundance after avermectin treatment could have caused a decrease in fungistatic activity in mosquito larvae and contributed to a more active differentiation of the infectious fungal structures. Actinobacteria are known to secrete broad-spectrum metabolites that exhibit antifungal activity [61, 62]. In particular, *Microbacterium* produces isoflavones (Genestin and Daidzin) with activity against the phytopathogenic ascomycete *Phyllosticta citricarpa* [63]. The ability of *Microbacterium* to reduce the growth of *Aspergillus* [64, 65], *Penicillium* [66], and *Candida albicans* [67] was shown, and here we demonstrate antagonistic activity against

*Metarhizium* for the first time. Regarding other bacteria, *Aeromonas* showed antagonism towards *Metarhizium* in an in vitro test and weak antagonism in a bioassay on *Ae. aegypti* larvae. The increase in *Aeromonas* abundance was impeded by avermectins after the combined treatment, which may also have had an impact on the promotion of fungal infection. The bacterium *Delftia* exhibited antagonism during the coinfection assay; however, the abundance of these bacteria in the microbiome structure did not change significantly under the influence of the fungus and avermectins. Therefore, the contribution of *Delftia* to the synergism between these agents is not obvious. Among the subdominant bacteria, we observed an increase in the abundance of *Pseudomonas* after avermectin treatment. These bacteria showed strong synergism with the fungus on mosquito mortality, despite their antagonistic interactions in vitro. Previously, a synergistic effect between *Beauveria bassiana* and *Pseudomonas* sp. was shown on locusts despite their antagonistic effect in vitro [68]. This finding confirms the more complicated relationships between fungi and bacteria in insects compared with direct interactions under cocultivation on artificial media.

In conclusion, this is the first study to show changes in the bacterial communities of mosquitoes in response to the combined action of insecticides and entomopathogenic fungi. The obtained results indicate the significant effects of both *Metarhizium* and avermectins on the microbiota in *Ae. aegypti* larvae, and these changes are expressed both as changes in the total bacterial load and in the diversity and structure of the bacterial community. The influence of avermectins led to a shift in the bacterial community that was more favorable for the development of fungal infection. In particular, it reduces the abundance of fungal antagonists (Actinobacteria) and increases the abundance of bacteria with a neutral effect on *Metarhizium* (Flavobacteria). The latter was correlated with more active differentiation of fungal infection structures, and, accordingly, the faster death of the larvae after combined treatment with insecticide and the fungus. Future studies may focus on functional changes in the microbial community under the influence of insecticides and entomopathogenic fungi. It should be noted that some bacteria (*Aeromonas*) are potential pathogens of vertebrates, and they demonstrated a sharp increase after *M. robertsii* treatment. Therefore, subsequent studies may also focus on changes in the abundance of these bacteria in water bodies after fungal treatments against mosquito larvae.

**Acknowledgments** We are grateful to Dr. Vladimir A. Shilo (Karasuk biological station of ISEA SB RAS) for assisting in the experiments organization and Elena Balzovskaya for technical assistance. We also acknowledge Dr. Andrey A. Miller for preparation of ultrathin sections of mosquito larvae, Dr. Aleksandr A. Alekseev for determining the exact ratio of avermectins' isomers in an industrial product Phytoverm using HPLC, and Daria Noskova for help in performing the experiments.

**Authors' Contributions** Experimental design (VYK, VVG, YAN), performing the experiments (MRK, OVP, YAY, OEB, ONY, TYA, AMB, UNR, VVM), data analysis (VYK, YAN, MRK, UNR, VVM, AMB), writing the manuscript (YAN, VYK, MRK, OVP), and obtain funding (YAN, VVG, VVM).

**Funding Information** This work was supported by the Russian Science Foundation (project No. 18-74-00090). The maintenance of the mosquito line and microorganism collections at Institute of Systematics and Ecology of Animals SB RAS were supported by Federal Fundamental Scientific Research Programs (No. AAAA-A16-116121410124-8 and No. AAAA-A16-116121410123-1). A collection of extremophilic microorganisms and type cultures at Institute of Chemical Biology and Fundamental Medicine SB RAS was supported by the project AAAA-A17-117020210027-9.

**Data Availability** The MiSeq data were deposited in GenBank under the study accession number PRJNA625381. The sequences of the 16S rDNA genes were deposited in the GenBank database under accession numbers MT040033–MT040053. Experimental data are presented in Appendix A.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethics Approval** Not applicable

**Consent to Participate** Not applicable

**Consent for Publication** Not applicable

**Code Availability** Not applicable

## References

1. Deveau A, Bonito G, Uehling J, Paoletti M, Becker M, Bindschedler S, Hacquard S, Hervé V, Labbé J, Lastovetsky OA, Mieszkin S, Millet LJ, Vajna B, Junier P, Bonfante P, Krom BP, Olsson S, van Elsas JD, Wick LY (2018) Bacterial - fungal interactions: ecology, mechanisms and challenges. *FEMS Microbiol Rev* 42:335–352. <https://doi.org/10.1093/femsre/fuy008>
2. Karl JP, Hatch AM, Arcidiacono SM, Pearce SC, Pantoja-Feliciano IG, Doherty LA, Soares JW (2018) Effects of psychological, environmental and physical stressors on the gut microbiota. *Front Microbiol* 9:2013. <https://doi.org/10.3389/fmicb.2018.02013>
3. Engel P, Moran NA (2013) The gut microbiota of insects—diversity in structure and function. *FEMS Microbiol Rev* 37:699–735. <https://doi.org/10.1111/1574-6976.12025>
4. Strand MR (2018) Composition and functional roles of the gut microbiota in mosquitoes. *Curr Opin Insect Sci* 28:59–65. <https://doi.org/10.1016/j.cois.2018.05.008>
5. Mason KL, Stepien TA, Blum JE, Holt JF, Labbe NH, Rush JS, Raffa KF, Handelsman J (2011) From commensal to pathogen: translocation of *Enterococcus faecalis* from the midgut to the hemocoel of *Manduca sexta*. *MBio* 2:e00065–e00011. <https://doi.org/10.1128/mBio.00065-11>
6. Wei G, Lai Y, Wang G, Chen H, Li F, Wang S (2017) Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. *Proc Natl Acad Sci* 114:5994–5999. <https://doi.org/10.1073/pnas.1703546114>

7. Broderick NA, Robinson CJ, McMahon MD, Holt J, Handelsman J, Raffa KF (2009) Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. *BMC Biol* 7:11. <https://doi.org/10.1186/1741-7007-7-11>
8. Cheng D, Guo Z, Riegler M, Xi Z, Liang G, Xu Y (2017) Gut symbiont enhances insecticide resistance in a significant pest, the oriental fruit fly *Bactrocera dorsalis* (Hendel). *Microbiome* 5:13. <https://doi.org/10.1186/s40168-017-0236-z>
9. Fernández MDM, Meeus I, Billiet A, Van Nieuwerburgh F, Deforce D, Vandamme P, Viñuela E, Smagghe G (2019) Influence of microbiota in the susceptibility of parasitic wasps to abamectin insecticide: deep sequencing, esterase and toxicity tests. *Pest Manag Sci* 75:79–86. <https://doi.org/10.1002/ps.5195>
10. Receveur JP, Pechal JL, Benbow ME, Donato G, Rainey T, Wallace JR (2018) Changes in larval mosquito microbiota reveal non-target effects of insecticide treatments in hurricane-created habitats. *Microb Ecol* 76:719–728. <https://doi.org/10.1007/s00248-018-1175-3>
11. Sivakumar G, Rangeshwaran R, Yandigeri MS, Mohan M, Venkatesan T, Ballal CR, Verghese A (2017) Characterization and role of gut bacterium *Bacillus pumilus* on nutrition and defense of leafhopper (*Amrasca biguttula biguttula*) of cotton. *Indian J Agric Sci* 87:534–539
12. Łukasik P, van Asch M, Guo H, Ferrari J, Charles J, Godfray H (2013) Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecol Lett* 16:214–218. <https://doi.org/10.1111/ele.12031>
13. Kwong WK, Mancenido AL, Moran NA (2017) Immune system stimulation by the native gut microbiota of honey bees. *R Soc Open Sci* 4:170003. <https://doi.org/10.1098/rsos.170003>
14. Arango RA, Carlson CM, Currie CR, McDonald BR, Book AJ, Green F, Lebow NK, Raffa KF (2016) Antimicrobial activity of actinobacteria isolated from the guts of subterranean termites. *Environ Entomol* 45:1415–1423. <https://doi.org/10.1093/ee/nvw126>
15. Moraes APR, Videira SS, Bittencourt VREP, Bittencourt AJ (2014) Antifungal activity of *Stenotrophomonas maltophilia* in *Stomoxys calcitrans* larvae. *Rev Bras Parasitol Vet* 23:194–199. <https://doi.org/10.1590/S1984-29612014037>
16. Xu L, Deng J, Zhou F, Cheng C, Zhang L, Zhang J, Lu M (2019) Gut microbiota in an invasive bark beetle infected by a pathogenic fungus accelerates beetle mortality. *J Pest Sci* 92:343–351. <https://doi.org/10.1007/s10340-018-0999-4>
17. Polenogova OV, Kabilov MR, Tyurin MV, Rotskaya UN, Krivopalov AV, Morozova VV, Mozhaitseva K, Alikina T, Kryukov VY, Glupov VV (2019) Parasitoid envenomation alters the *Galleria mellonella* midgut microbiota and immunity, thereby promoting fungal infection. *Sci Rep* 9:1–12. <https://doi.org/10.1038/s41598-019-40301-6>
18. Zhou F, Wu X, Xu L, Guo S, Chen G, Zhang X (2018) Repressed *Beauveria bassiana* infections in *Delia antiqua* due to associated microbiota. *Pest Manag Sci* 75:170–179. <https://doi.org/10.1002/ps.5084>
19. Zhang F, Sun XX, Zhang XC, Zhang S, Lu J, Xia YM, Huang YH, Wang XJ (2018) The interactions between gut microbiota and entomopathogenic fungi: a potential approach for biological control of *Blattella germanica* (L.). *Pest Manag Sci* 74:438–447. <https://doi.org/10.1002/ps.4726>
20. Blackburn MB, Gundersen-Rindal DE, Weber DC, Martin PA, Farrar Jr RR (2008) Enteric bacteria of field-collected Colorado potato beetle larvae inhibit growth of the entomopathogens *Photobacterium temperata* and *Beauveria bassiana*. *Biol Control* 46: 434–441. <https://doi.org/10.1016/j.biocontrol.2008.05.005>
21. Boucias DG, Zhou Y, Huang S, Keyhani NO (2018) Microbiota in insect fungal pathology. *Appl Microbiol Biotechnol* 102:5873–5888. <https://doi.org/10.1007/s00253-018-9089-z>
22. Scholte EJ, Knols BG, Samson RA, Takken W (2004) Entomopathogenic fungi for mosquito control: a review. *J Insect Sci* 4:19. <https://doi.org/10.1093/jis/4.1.19>
23. Aw KMS, Hue SM (2017) Mode of infection of *Metarhizium* spp. fungus and their potential as biological control agents. *J Fungi* 3:30. <https://doi.org/10.3390/jof3020030>
24. Frankel-Bricker J, Buerki S, Feris KP, White MM (2020) Influences of a prolific gut fungus (*Zancudomyces culisetiae*) on larval and adult mosquito (*Aedes aegypti*)-associated microbiota. *Appl Environ Microbiol* 86:e02334–e02319. <https://doi.org/10.1128/AEM.02334-19>
25. Ramirez JL, Dunlap CA, Muturi EJ, Barletta AB, Rooney AP (2018) Entomopathogenic fungal infection leads to temporospatial modulation of the mosquito immune system. *PLoS Negl Trop Dis* 12:e0006433. <https://doi.org/10.1371/journal.pntd.0006433>
26. Butt TM, Greenfield BP, Greig C, Maffei TG, Taylor JW, Piasecka J, Dudley E, Abdulla A, Dubovskiy IM, Garrido-Jurado I, Quesada-Moraga E, Penny MW, Eastwood DC (2013) *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. *PLoS One* 8:e81686. <https://doi.org/10.1371/journal.pone.0081686>
27. Lacey CM, Lacey LA, Roberts DR (1988) Route of invasion and histopathology of *Metarhizium anisopliae* in *Culex quinquefasciatus*. *J Invertebr Pathol* 52:108–118. [https://doi.org/10.1016/0022-2011\(88\)90109-7](https://doi.org/10.1016/0022-2011(88)90109-7)
28. Greenfield BP, Lord AM, Dudley E, Butt TM (2014) Conidia of the insect pathogenic fungus, *Metarhizium anisopliae*, fail to adhere to mosquito larval cuticle. *R Soc Open Sci* 1:140193. <https://doi.org/10.1098/rsos.140193>
29. Riba G, Keita A, Soares Jr GG, Ferron P (1986) Comparative studies of *Metarhizium anisopliae* and *Toxopneustes cylindrosporum* as pathogens of mosquito larvae. *J Am Mosq Control Assoc* 2:469–473
30. Noskov YA, Polenogova OV, Yaroslavtseva ON, Belevich OE, Yurchenko YA, Chertkova EA, Kryukova NA, Vyu K, Glupov VV (2019) Combined effect of the entomopathogenic fungus *Metarhizium robertsii* and avermectins on the survival and immune response of *Aedes aegypti* larvae. *PeerJ* 7:e7931. <https://doi.org/10.7717/peerj.7931>
31. Brouchkov A, Kabilov M, Filippova S, Baturina O, Rogov V, Galchenko V, Mulyukin A, Fursova O, Pogorelko G (2017) Bacterial community in ancient permafrost alluvium at the Mammoth Mountain (eastern Siberia). *Gene* 636:48–53. <https://doi.org/10.1016/j.gene.2017.09.021>
32. Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods* 10:996–998. <https://doi.org/10.1038/nmeth.2604>
33. Edgar RC (2016) UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv* 081257. <https://doi.org/10.1101/081257>
34. Hsieh TC, Ma KH, Chao A (2016) iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods Ecol Evol* 7:1451–1456. <https://doi.org/10.1111/2041-210X.12613>
35. Robertson JL, Preisler HK (1992) Pesticide bioassays with arthropods. CRC, Boca Raton. <https://doi.org/10.1201/9781315373775>
36. Scheirer CJ, Ray WS, Hare N (1976) The analysis of ranked data derived from completely randomized factorial designs. *Biometrics* 32:429–434. <https://doi.org/10.2307/2529511>
37. Hammer O, Harper DA, Ryan PD (2001) Palaeontological statistics software package for education and data analysis. *Palaeontol Electron* 4:1–9

38. Gaidyshev IP (2004) Solution of scientific and engineering tasks by means of Excel, VBA and C/C++ St. BKhV-Petersburg, Petersburg
39. Chiu CH, Chao A (2016) Estimating and comparing microbial diversity in the presence of sequencing errors. *PeerJ* 4:e1634. <https://doi.org/10.7717/peerj.1634>
40. Coon KL, Brown MR, Strand MR (2016) Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. *Mol Ecol* 25:5806–5826. <https://doi.org/10.1111/mec.13877>
41. Dada N, Jumas-Bilak E, Manguin S, Seidu R, Stenström TA, Overgaard HJ (2014) Comparative assessment of the bacterial communities associated with *Aedes aegypti* larvae and water from domestic water storage containers. *Parasit Vectors* 7:391. <https://doi.org/10.1186/1756-3305-7-391>
42. Coon KL, Vogel KJ, Brown MR, Strand MR (2014) Mosquitoes rely on their gut microbiota for development. *Mol Ecol* 23:2727–2739. <https://doi.org/10.1111/mec.12771>
43. Gimonneau G, Tchioffo MT, Abate L, Boissière A, Awono-Ambéné PH, Nsango SE, Christen R, Morlais I (2014) Composition of *Anopheles coluzzii* and *Anopheles gambiae* microbiota from larval to adult stages. *Infect Genet Evol* 28:715–724. <https://doi.org/10.1016/j.meegid.2014.09.029>
44. Tchioffo MT, Boissière A, Abate L, Nsango SE, Bayibéki AN, Awono-Ambéné PH, Christen R, Gimonneau G, Morlais I (2016) Dynamics of bacterial community composition in the malaria mosquito's epithelia. *Front Microbiol* 6:1500. <https://doi.org/10.3389/fmicb.2015.01500>
45. Kaltenpoth M, Flórez LV (2020) Versatile and dynamic symbioses between insects and Burkholderia Bacteria. *Annu Rev Entomol* 65:145–170. <https://doi.org/10.1146/annurev-ento-011019-025025>
46. Strand MR (2017) The gut microbiota of mosquitoes: diversity and function. In *arthropod vector: controller of disease transmission 1*: 185–199. Academic Press. <https://doi.org/10.1016/B978-0-12-805350-8.00011-8>
47. Clark JM, Scott JG, Campos F, Bloomquist JR (1995) Resistance to avermectins: extent, mechanisms, and management implications. *Annu Rev Entomol* 40:1–30. <https://doi.org/10.1146/annurev.en.40.010195.000245>
48. Tomilova OG, Kryukov VY, Duisembekov BA, Yaroslavtseva ON, Tyurin MV, Kryukova NA, Skorokhod V, Dubovskiy IM, Glupov VV (2016) Immune-physiological aspects of synergy between avermectins and the entomopathogenic fungus *Metarhizium robertsii* in Colorado potato beetle larvae. *J Invertebr Pathol* 140:8–15. <https://doi.org/10.1016/j.jip.2016.08.008>
49. Alves SN, Serrão JE, Melo AL (2010) Alterations in the fat body and midgut of *Culex quinquefasciatus* larvae following exposure to different insecticides. *Micron* 41:592–597. <https://doi.org/10.1016/j.micron.2010.04.004>
50. Akhanaev YB, Tomilova OG, Yaroslavtseva ON, Duisembekov BA, Kryukov VY, Glupov VV (2017) Combined action of the entomopathogenic fungus *Metarhizium robertsii* and avermectins on the larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera, Chrysomelidae). *Entomol Rev* 97:158–165. <https://doi.org/10.1134/S0013873817020026>
51. Tang G, Xiong Y, Liu Y, Song Z, Yang Y, Shen G, Wang J, Jiang H (2019) The transcription factor MafB regulates the susceptibility of *Bactrocera dorsalis* to avermectin via GSTz2. *Front Physiol* 10:1068. <https://doi.org/10.3389/fphys.2019.01068>
52. Chen S, Kaufman MG, Korir ML, Walker ED (2014) Ingestibility, digestibility, and engineered biological control potential of *Flavobacterium hibernum*, isolated from larval mosquito habitats. *Appl Environ Microbiol* 80:1150–1158. <https://doi.org/10.1128/AEM.03319-13>
53. Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T (2012) Symbiont-mediated insecticide resistance. *Proc Natl Acad Sci* 109:8618–8622. <https://doi.org/10.1073/pnas.1200231109>
54. Muturi EJ, Donthu RK, Fields CJ, Moise IK, Kim CH (2017) Effect of pesticides on microbial communities in container aquatic habitats. *Sci Rep* 7:44565. <https://doi.org/10.1038/srep44565>
55. Ramteke PW, Pathak SP, Gautam AR, Bhattacharjee JW (1993) Association of *Aeromonas caviae* with sewage pollution. *J Environ Sci Health A* 28:859–870. <https://doi.org/10.1080/10934529309375916>
56. Popovic NT, Kazacic SP, Strunjak-Perovic I, Barisic J, Klobucar RS, Kepec S, Coz-Rakovac R (2015) Detection and diversity of aeromonads from treated wastewater and fish inhabiting effluent and downstream waters. *Ecotoxicol Environ Saf* 120:235–242. <https://doi.org/10.1016/j.ecoenv.2015.06.011>
57. Brzezinska MS, Donderski W (2006) Chitinolytic bacteria in two lakes of different trophic status. *Pol J Ecol* 54:295–301
58. VandeWalle JL, Goetz GW, Huse SM, Morrison HG, Sogin ML, Hoffmann RG, Yan K, McLellan SL (2012) *Acinetobacter*, *Aeromonas* and *Trichococcus* populations dominate the microbial community within urban sewer infrastructure. *Environ Microbiol* 14:2538–2552. <https://doi.org/10.1111/j.1462-2920.2012.02757.x>
59. Someya N, Ikeda S, Morohoshi T, Tsujimoto MN, Yoshida T, Sawada H, Ikeda T, Tsuchiya K (2009) Diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan. *Microbes Environ* 26:1011040239. <https://doi.org/10.1264/jsm2.ME10149>
60. Chen C, Chen X, Xie T, Hatting JL, Yu X, Ye S, Wang Z, Shentu X (2016) Diverse bacterial symbionts of insect-pathogenic fungi and possible impact on the maintenance of virulence during infection. *Symbiosis* 69:47–58. <https://doi.org/10.1007/s13199-015-0371-x>
61. Kerr JR (1999) Bacterial inhibition of fungal growth and pathogenicity. *Microb Ecol Health Dis* 11:129–142. <https://doi.org/10.1080/089106099435709>
62. Zhao P, Xue Y, Gao W, Li J, Zu X, Fu D, Feng S, Bai X, Zuo Y, Li P (2018) Actinobacteria-derived peptide antibiotics since 2000. *Peptides* 103:48–59. <https://doi.org/10.1016/j.peptides.2018.03.011>
63. Savi DC, Shaaban KA, Gos FM, Thorson JS, Glienke C, Rohr J (2019) Secondary metabolites produced by *Microbacterium* sp. LGMB471 with antifungal activity against the phytopathogen *Phyllosticta citricarpa*. *Folia Microbiol* 64:453–460. <https://doi.org/10.1007/s12223-018-00668-x>
64. Arfaoui M, Vallance J, Bruez E, Rezugui A, Melki I, Chebil S, Sadfi-Zouaoui N, Rey P (2019) Isolation, identification and in vitro characterization of grapevine rhizobacteria to control ochratoxigenic *Aspergillus* spp. on grapes. *Biol Control* 129:201–211. <https://doi.org/10.1016/j.biocontrol.2018.10.019>
65. Manna M, Oh JY, Kim KD (2017) Microbe-mediated control of *Aspergillus flavus* in stored rice grains with a focus on aflatoxin inhibition and biodegradation. *Ann Appl Biol* 171:376–392. <https://doi.org/10.1111/aab.12381>
66. Manna M, Oh JY, Kim KD (2017) Biocontrol activity of volatile-producing *Bacillus megaterium* and *Pseudomonas protegens* against *Aspergillus flavus* and aflatoxin production on stored rice grains. *Mycobiology* 45:213–219. <https://doi.org/10.5941/MYCO.2017.45.3.213>
67. Graça AP, Viana F, Bondoso J, Correia MI, Gomes L, Humanes M, Reis A, Xavier JR, Gaspar H, Lage OM (2015) The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge *Erylus deficiens* (Astrophorida, Geodiidae). *Front Microbiol* 6:389. <https://doi.org/10.3389/fmicb.2015.00389>
68. Lednev GR, Kryukov VY, Khodyrev VP, Levchenko MA, Duisembekov BA, Sagitov AO, Glupov VV (2008) Dynamics of mortality of the migratory locust under synchronous infection with entomopathogenic fungi (*Beauveria bassiana*, *Metarhizium anisopliae*) and bacteria *Pseudomonas* sp. *Contemp Probl Ecol* 1:210–213. <https://doi.org/10.1134/S199542508020069>