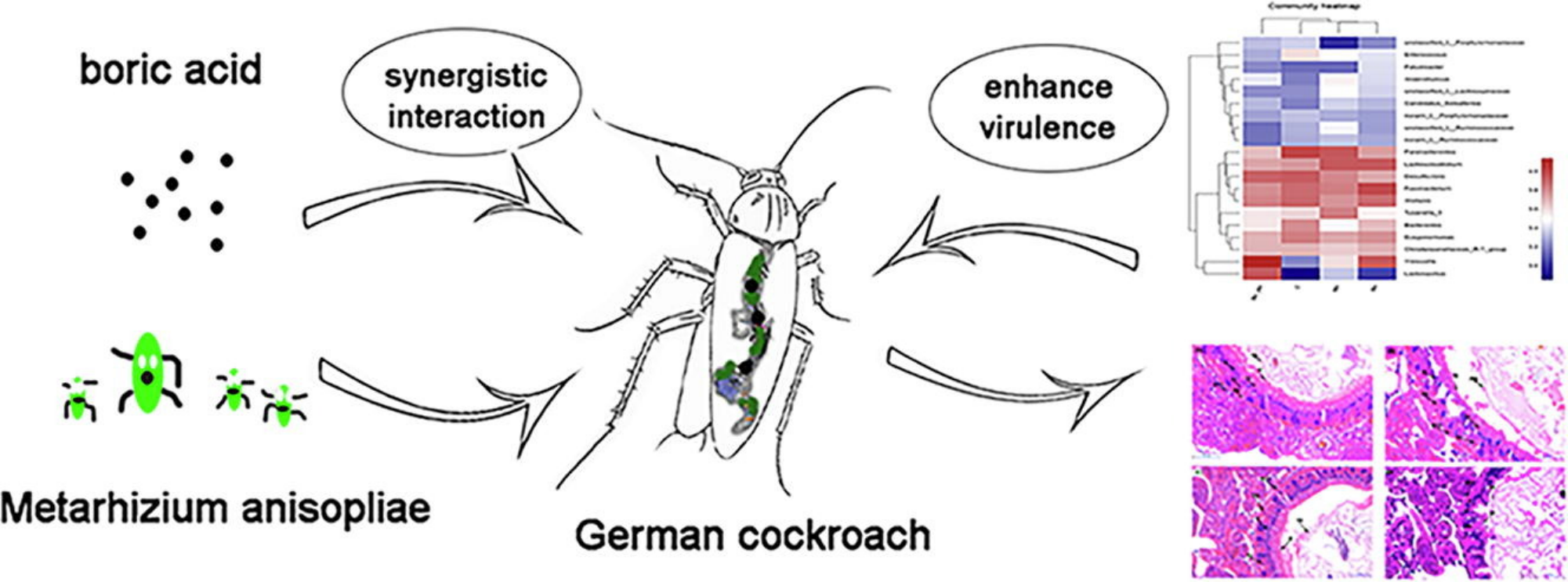

boric acid
synergistic interaction
enhance virulence
Metarhizium anisopliae
German cockroach

Biological Control 152 (2021) 104430

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

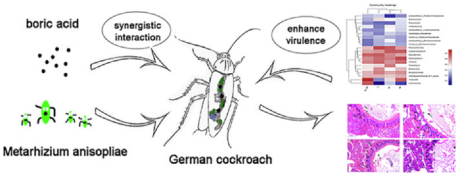
Boric acid enhances *Metarhizium anisopliae* virulence in *Blattella germanica* (L.) by disrupting the gut and altering its microbial community

Rongai Yang[a,1], Meijuan Zhang[a,1], Coby Schal[b], Meng Jiang[a], Tong Cai[a], Fan Zhang[a,*]

[a] Key Laboratory of Animal Resistance Biology of Shandong Province, College of Life Science, Shandong Normal University, 88 East Wenhua Road, Jinan 250014, People's Republic of China

[b] Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, USA

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Blattella germanica
Metarhizium anisopliae
Boric acid
Gut microbiota
Synergism

ABSTRACT

Extensive use of insecticides has caused widespread resistance in German cockroach (*Blattella germanica*) populations on a global scale. Biological control has potential to mitigate insecticide resistance, and *Metarhizium anisopliae*, an entomopathogenic fungus, has shown good efficacy against cockroaches alone and in combination with various insecticides, including boric acid. To investigate the mechanism(s) that underlie synergism between *M. anisopliae* and boric acid, we conducted dose-response assays with combinations of fungus and boric acid fed to cockroaches and histological observations of the midgut and we characterized the gut microbiome of treated cockroaches. The combination treatments were synergistic with co-toxicity factors > 20 at 4 out of 12 treatments and LT_{50} values of 5 days at the highest concentration of boric acid. *M. anisopliae* reached the hemocoel faster when it was ingested with boric acid, likely because boric acid disrupted the epithelial cells of the midgut. The gut microbiome was also altered by these treatments. The abundance of *Parabacteroides* and *Enterococcus*, with known anti-inflammatory and antifungal activity, declined in boric acid and combination treatments, whereas *Weissella*, an opportunistic pathogen, significantly increased in these treatments. We conclude that two major mechanisms underlie this synergism: (1) boric acid facilitates the penetration of *M. anisopliae* by physically and chemically disrupting the midgut, and (2) by altering the gut microbiome, boric acid promotes survival and virulence of *M. anisopliae* in the harsh gut environment.

1. Introduction

The German cockroach, *Blattella germanica*, is a common pest of economic and health importance in human dwellings and other human-built structures in urban and agricultural environments. Its impact on public health is mainly related to its ability to transmit pathogenic microbes and produce potent allergens that cause allergic disease and asthma (Ahmad et al., 2011; Pomés and Schal, 2019; Zhang et al., 2013), and the large amounts of insecticides used in the indoor environment to control cockroach infestations. The use of broad-spectrum insecticides, most recently pyrethroids and neonicotinoids, has led to widespread evolution of resistance (DeVries et al., 2019; Fardisi et al.,

* Corresponding author.
E-mail address: zhangfan0531@163.com (F. Zhang).
[1] Rongai Yang and Meijuan Zhang contributed equally to this study.

https://doi.org/10.1016/j.biocontrol.2020.104430
Received 3 April 2020; Received in revised form 27 August 2020; Accepted 31 August 2020
Available online 03 September 2020

2019; Zhu et al., 2016), and an urgent need to explore alternative strategies of cockroach control, including biological control (Pan and Zhang, 2019; Pereira et al., 2017; Suiter, 1997; Zhang and Zhang, 2018).

Several independent studies have shown that the entomopathogenic fungus *Metarhizium anisopliae* causes high mortality in cockroaches (Gutierrez et al., 2016; Zhang et al., 2018b; Zhang and Zhang, 2018; Zurek et al., 2002) and it has been deployed in commercial biocontrol products (Faria and Wraight, 2007). The main pathway of infection by *M. anisopliae* involves penetration through the cuticle. Although ingestion appears to be a rare route of infection and only reported in a few species of insect (Batta, 2018; Jeffs et al., 1997; Lacey et al., 1988; Zhang et al., 2018a), it is nonetheless important, particularly in the context of delivering conidia in baits. For example, conidia of *M. anisopliae* or *Beauveria bassiana* fed to *Sitophilus granarius* (wheat weevil) caused 50% mortality after 2.79 and 3.05 weeks, respectively (Batta, 2018). For cockroach, ingestion of 1.0×10^8 *M. anisopliae* conidia per ml of water caused 18% mortality 15 d post-inoculation, which was lower than by topical application, but nevertheless significant (Zhang et al., 2018a). Moreover, genome sequences show a series of homologous genes exhibited by *B. bassiana* that are shared with *Bacillus thuringiensis*, a bacterial pathogen that invades the host through ingestion. Finally, *Metarhizium robertsii* has at least six heat-labile bacteria-like enterotoxin genes, suggesting that entomopathogenic fungi might have oral toxicity (Gao et al., 2011; Hu et al., 2014; Li et al., 2020; Xiao et al., 2012). The deployment of entomopathogenic fungi against cockroaches has not been competitive with conventional insecticide formulations because of their slow effects. However, the rapid evolution of insecticide resistance and the advent of highly effective bait formulations raise the question whether entomopathogenic fungi can be effective in baits, especially in combination with other insecticides.

Likewise, the use of boric acid has been limited because of its slow action against cockroaches. Nevertheless, this inorganic insecticide has demonstrated good efficacy against cockroaches (Cochran, 1995; Ebeling, 1995; Gore et al., 2004; Zurek et al., 2003) and low mammalian toxicity (Cox, 2004; Murray, 1998). Its low cost, high solubility in water, safety (particularly as a component of cockroach baits), and no evidence of resistance to boric acid despite more than a century of use, favor the continued use of boric acid in the indoor environment. The mechanism(s) of action of boric acid against cockroaches remains to determined, although several hypotheses have been proposed, including abrasion or destruction of the cellular lining of the foregut leading to starvation (Cochran, 1995; Ebeling, 1995), structural disruption of the midgut, neurotoxicity (poisoning symptom and reduction in acetyl cholinesterase activity , induction of oxidative stress, alteration of protein activity (Büyükgüzel et al., 2013; Hyršl et al., 2007), and mitochondrial dysfunction (Ali et al., 2014). In addition, Gwokyalya and Altuntaş (2019) showed that boric acid can significantly inhibit the hemocyte-mediated immune response of *Galleria mellonella* and poses risks of genotoxicity at high concentrations.

Zurek et al. (2002) demonstrated that the addition of boric acid to *M. anisopliae* synergistically accelerated mycosis in *B. germanica*. Cockroaches treated topically with *M. anisopliae* died in > 28 days (LT_{50} = 10 days), but the addition of boric acid killed 100% of the cockroaches in only 8 days (LT_{50} = 5 days), without compromising the capacity of the fungus to grow from cadavers and induce epizootics. The synergistic interaction was extended to the co-ingestion of boric acid and *M. anisopliae* in baits, which accelerated the LT_{50} by 14 days relative to the fungus alone and by 26 days relative to boric acid alone (Dayer and Karvandian, 2016).

The synergistic interactions between boric acid and *M. anisopliae* against cockroaches appear to be promising for implementation in cockroach pest management (Dayer and Karvandian, 2016; Zurek et al., 2002). Our experimental design aimed to address three previously unaddressed questions related to the mechanisms of this synergy. This design also differentiates our investigation from previous studies. First, since the boric acid-*M. anisopliae* combination will likely be formulated as an ingestible bait, we wanted to mimic this route of delivery, rather than use topical application, with the alimentary canal as the focus of the interaction. For cockroaches, the oral infection of fungus seems to be an uncommon infection route besides cuticular penetration, yet little is known about it (Huang et al., 2013; Mannino et al., 2019; Zhang et al., 2018a). Second, some evidence that high doses of boric acid damage the gut (Ebeling, 1995; Habes et al., 2006) compelled us to assess cellular changes in the midgut in response to lower doses of boric acid. And third, the recognition that the gut microbiome is a significant participant in insecticide toxicology prompted us to compare the microbiomes of cockroaches exposed to various boric acid and *M. anisopliae* combinations. So we investigated the effects of ingesting *M. anisopliae* and boric acid in *B. germanica*. This approach is particularly timely because the oral route of infection with entomopathogenic fungi is poorly understood in insects (Mannino et al., 2019), as are strategies to enhance oral infection.

2. Materials and methods

2.1. Insects

German cockroaches were supplied by Shandong Center for Disease Control and Prevention and maintained on water and rat pellet feed in a growth chamber (60 ± 5% relative humidity; 27 ± 1 °C; 12 : 12 h light : dark cycle) as previously described Yang et al. (2019). All the tests were conducted on adult male German cockroaches.

2.2. Entomopathogenic fungi

The fungal isolate of *M. anisopliae* (isolate EB0732) was obtained from the China General Microbiological Culture Collection Center (CGMCC), cultured in potato dextrose agar (PDA) medium and incubated for 8 days at 28 °C, and the fungal conidia were in logarithmic growth period (Zhang et al., 2018b). Conidia were harvested with a sterile metal loop and suspended in sterile phosphate buffered saline (PBS, 3 mM) containing 0.1% (v/v) Tween 80. The required conidium concentration was determined using a Neubauer hemocytometer (Kanwin Biotechnology Co., Ltd., Shanghai, China).

2.3. Boric acid baits

The inert ingredients of the experimental boric acid baits were selected based on common food choices for rearing *B. germanica* and our preliminary assays (data not shown). To obtain baits containing 0.4, 0.8, 1.2 and 1.6% boric acid in rat pellet feed, boric acid was solubilized in 2 ml PBS, and incorporated into 2 g rat pellet feed powder (v/w) to generate 4, 8, 12, and 16 mg boric acid, respectively, per g of semisolid bait. The baits were left to dry at room temperature for 12 h. At these low doses, boric acid did not deter cockroaches from feeding on the bait. Bioassays were conducted in a no-choice format so only treated bait or untreated food was provided to cockroaches in the bioassay. Cockroaches were provided fresh treated or untreated baits every 5 days, and the old baits were removed.

2.4. Experimental design

For bioassays with boric acid baits alone, cockroaches were divided into four groups of 20 cockroaches, and each group was provided 0.4, 0.8, 1.2 or 1.6% (w/w) of boric acid bait. Control cockroaches were fed a diet treated only with PBS solution. For bioassays with *M. anisopliae* alone, cockroaches were divided into three groups of 20 cockroaches, and each group was then treated with 1×10^7, 1×10^8 or 1×10^9 conidia/ml *M. anisopliae* conidial suspensions. Each cockroach was provided the conidia in 2 μl applied with a microinjector between the paraglossae (mouthparts) (Zhang et al., 2018a). Sterile PBS [0.1% (v/v)

Tween 80] was used as a negative control. For synergy bioassays, three concentrations of *M. anisopliae* (1×10^7, 1×10^8 and 1×10^9 conidia/ml) and four concentrations of boric acid (0.4, 0.8, 1.2 and 1.6% w/w) were combined in binary pairs for 12 treatment combinations, each of which was replicated 3 times (20 adult males per replicate). Each cockroach was first fed 2 µl of *M. anisopliae* conidial suspension, surface sterilized with 0.1% mercuric chloride and rinsed three times with sterile water to remove conidia that adhered to its external surface, and then placed in a group of 20 and continuously provided boric acid bait. Bioassays were monitored daily for 15 days. The data were subjected to probit analysis using SPSS 20.0 for Windows, and the LT_{50} values were estimated with 95% confidence intervals.

The joint action of two insecticides was determined by the Mansour co-toxicity factor method (Mansour et al., 1966). There is no co-operation between the boric acid and *M. anisopliae*. The additive value for the expected mortality of *M. anisopliae* (Ma) and Boric Acid (Ba) is Probability Mortality Ma + Probability Mortality Ba – (Probability Mortality Ma × Probability Mortality Ba) based on probabilities of infection or toxic effects (Berenbaum, 1981). The co-toxicity factor is equal to (actual mortality of the mixture minus theoretical additive mortality of the mixture) / theoretical additive mortality of the mixture) × 100. Co-toxicity factors of a mixture near 20 (–20 to + 20) indicate the probability of additive effects; antagonism is generally associated with factors less than − 20, while factors significantly above + 20 strongly indicate synergism.

2.5. Histology

German cockroaches were fed 2 µl of 1×10^9 conidia/ml of *M. anisopliae* only, 1.2% (w/w) boric acid baits only, or the combination treatment. Sterile PBS [0.1% (v/v) Tween 80] was used as a negative control. The insect's abdomen (3 males from each treatment) was dissected 4 days after treatment, and fixed in Carnoy's fluid for 24 h. Following decalcification, the tissue was dehydrated in an ethanol series (75 to 100%), cleared, embedded in wax, sectioned, mounted on a slide, and stained with hematoxylin-eosin. Cytological observations were made, and photographs were taken with a digital slide scanner. The image observation and acquisition is finally done by CaseViewer 2.4 software (https://www.3dhistech.com/caseviewer).

2.6. Preparation of gut homogenates, 16S rRNA gene amplification and pyrosequencing

Gut homogenates from 240 cockroaches, representing 12 samples (3 replicates of 20 cockroaches each; 4 treatment groups) were prepared on the 4th day of exposure. Cockroaches were starved for 24 h before use. Before dissection cockroaches were briefly cleansed with 75% ethanol for surface disinfection and thoroughly washed two times with sterile water to remove the disinfectant. Then, the whole alimentary canal (gut) was dissected with sterile dissection tools.

DNA was extracted using the K2306 Karroten Microbial Genomic DNA extraction kit (Zhang and Yang, 2019). The V6 variable region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR). The primers used were 515F-907R (5′-GTGCCAGCMGCCGCGG-3′ and 5′-CCGTCAATTCMTTTRAGTTT-3′). The PCR was performed in a total volume of 20 µl containing 4 µl 5 × FastPfu Buffer, 2 µl dNTPs (2.5 mM), 10 ng DNA template, 0.8 µl of each primer (0.5 µM), 0.4 µl FastPfu Polymerase and deionized ultrapure water (to 20 µl). The PCR conditions were as follows: 95 °C for 3 min, 27 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. The amplification process was examined by electrophoresis in 2% agarose gel. After purification and quantification of the PCR products, samples were pooled at equal concentrations. Samples were subjected to parallel tagged sequencing by Miseq Sequencing in MAJORBIO based on Solexa Sequencing Technology (Illumina, San Diego, CA, USA).

2.7. Bioinformatic and statistical analysis

Using FLASH software (http://ccb.jhu.edu/software/FLASH), the paired sequences were combined and set to the minimum overlap of 10 bp. The other parameters were at default settings. The original pyrosequencing data were preprocessed for filtration and optimization to obtain trimmed and valid sequences using Trimmomatic and Mothur (http://www.mothur.org). The pyrosequencing chimeras were discarded using UCHIME. The remaining clean reads were used for further analysis. The valid sequences were simplified and aligned using the 'unique.seqs' and the 'align.seqs' command and then compared to the Bacterial SILVA database (http://www.arb-silva.de). The unique sequences were clustered into operational taxonomic units (OTUs). OTUs were calculated at the 97% similarity threshold with chopseq and Mothur (MAJORBIO). Rarefaction analysis showed clear asymptotes using MOTHUR and plot-rarefaction (MAJORBIO). The microbial communities were compared using the UniFrac Server. Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) with clustering analysis were carried out using the R package vegan (Oksanen et al., 2012). The Wilcoxon signed-rank test with Benjamini-Hochberg false discovery rate (FDR) correction was used to examine differential abundance of bacteria and genes between the two strain groups implemented in the R software (R Development Core Team, 2011). One-way ANOVA for more than two groups was performed using SPSS version 20 for Windows. All results are presented as the mean ± standard error (SE). Results with $P < 0.05$ were declared statistically significant between groups.

3. Results

3.1. Mortality bioassays across 12 treatment groups

Bioassays showed that ingested boric acid killed *B. germanica* males in a dose-dependent manner (Table 1), with 0.4% boric acid causing only 6.7% cumulative mortality after 15 days and 1.6% boric acid killing 88.3% of the cockroaches, with an LT_{50} of 7 days. There were significant differences between the two lower concentrations of boric acid (0.4 and 0.8%) and the higher concentrations (1.2 and 1.6%) ($P < 0.01$).

M. anisopliae, also delivered by ingestion, was much slower at killing *B. germanica* males. The lowest concentration of conidia (1×10^7 conidia/ml) killed only 11.7% of the cockroaches over the 15 day observation period, whereas the highest concentration (1×10^9 conidia/ml) killed 26.7% of the treated males (Table 1).

The combination treatments of four boric acid concentrations and three *M. anisopliae* concentrations showed that all treatments resulted in much greater mortality than the summed mortality with boric acid alone and *M. anisopliae* alone, especially at the lowest concentrations of both [1×10^7 conidia/ml *M. anisopliae* and 0.4% boric acid (Table 1)]. Statistical analysis demonstrated synergistic interactions between *M. anisopliae* and boric acid in 4 combination treatments, the remaining combination treatments were determined to have additive effects, and no antagonistic effects were found in any of the groups. All synergistic groups were found in *M. anisopliae* combined with the lower concentrations of boric acid (0.4 and 0.8%).

3.2. Histological examination

The effects of 1.2% boric acid, 1×10^9 conidia/ml *M. anisopliae* and their combination on gut structure of adult cockroaches was investigated after 4 days of exposure in comparison to untreated controls. Transverse sections of the midgut revealed major differences among these treatments (Fig. 1). The midgut of the control samples (untreated cockroaches) was intact (Fig. 1A), showing large cells with central nuclei in the epithelium of the midgut, apical microvilli adjoining the peritrophic membrane, and regeneration cells at the base of the

Table 1
Bioassays assessing the interactive effects of combination treatments of *M. anisopliae* and boric acid on adult male German cockroaches.

Treatment[1]		Actual mortality[2] (%)	Expected mortality (%)	LT_{50} (95% CI)[3] (day)	Slope ± SE	χ^2	Co-toxicity factor	Interaction effect
Ma (cfu·ml^{-1})	Ba (%)							
0	0.4	6.67 ± 1.67^{a}	NA	NA	NA	NA		
0	0.8	63.33 ± 3.33^{b}	NA	9 (7.2–10.1)	0.226 ± 0.034	2.739		
0	1.2	86.67 ± 1.67^{c}	NA	8 (7.1–8.9)	0.398 ± 0.061	1.977		
0	1.6	88.33 ± 4.41^{c}	NA	7 (5.4–8.5)	0.416 ± 0.064	2.473		
1×10^7	0	11.67 ± 3.33^{a}	NA	NA	NA	NA		
1×10^8	0	16.67 ± 4.41^{b}	NA	NA	NA	NA		
1×10^9	0	26.67 ± 3.33^{c}	NA	NA	NA	NA		
1×10^7	0.4	36.67 ± 8.33^{a}	17.6	NA	NA	NA	108.81	Synergistic
	0.8	83.33 ± 1.67^{b}	67.6	8 (6.9–8.9)	0.382 ± 0.070	12.197	23.25	Synergistic
	1.2	93.33 ± 3.33^{bc}	88.2	6 (4.9–7.1)	0.426 ± 0.068	4.179	5.79	Additive
	1.6	100.00 ± 0.00^{c}	89.7	5 (4.3–5.7)	0.546 ± 0.088	5.857	11.49	Additive
1×10^8	0.4	56.67 ± 1.67^{a}	22.2	10 (9.1–11.3)	0.209 ± 0.032	2.194	154.95	Synergistic
	0.8	75.00 ± 2.89^{b}	69.4	7 (5.9–7.8)	0.288 ± 0.045	4.402	8.00	Additive
	1.2	93.33 ± 1.67^{c}	88.9	6 (5.3–6.8)	0.565 ± 0.085	2.240	4.99	Additive
	1.6	95.00 ± 2.89^{c}	90.3	5 (4.3–6.2)	0.621 ± 0.095	3.058	5.23	Additive
1×10^9	0.4	66.67 ± 4.41^{a}	31.6	7 (6.1–8.2)	0.229 ± 0.036	3.516	111.24	Synergistic
	0.8	78.33 ± 3.33^{b}	73.1	7 (6.3–8.7)	0.217 ± 0.039	2.758	7.14	Additive
	1.2	95.00 ± 2.89^{c}	90.2	5 (4.2–6.3)	0.386 ± 0.060	4.624	5.29	Additive
	1.6	98.33 ± 1.67^{c}	91.4	5 (4.7–6.1)	0.465 ± 0.083	1.859	7.53	Additive

[1] Ba = boric acid; Ma = *M. anisopliae*.
[2] Cumulative 15-day mortality. Treatments within each *M. anisopliae* dose that do not share common letters are significantly different (Tukey's HSD; $P < 0.05$).
[3] LT_{50} = days until 50% mortality occurred; CI = 95% confidence interval for LT_{50}; NA = not applicable (mortality did not reach 50%).

epithelium that grouped as crypts. An intact orderly basement lamina and muscular-conjunctive system separated the midgut epithelium from the hemolymph (Fig. 1A). In contrast, ingestion of 1.2% boric acid caused substantial disruption of the midgut epithelium, with loose microvilli in the midgut lumen, vacuoles between epithelial cells, disorganized nuclei, and the integrity of the basal lamina was disrupted (Fig. 1B). Ingestion of *M. anisopliae* alone had no discernible effect on the midgut, and we did not find conidia in any of the midgut sections (Fig. 1C). The *M. anisopliae*-boric acid combination treatments were characterized by a disrupted midgut, as in the boric acid only treatment (Fig. 1B), and conidia were found at the midgut microvilli, apparently having traversed the peritrophic membrane (CO in Fig. 1D).

3.3. Gut microbiome

After quality control filtering and pyrosequencing, 634,123 sequences and 1927 OTUs were obtained from the 12 samples. The rarefaction curves showed clear asymptotes, which demonstrated that all samples reached sufficient sampling depth and near-complete sampling of the bacterial community (Fig. 2A). Based on Mothur software with the default settings, all sequences were classified into 20 phyla and 132 genera (Fig. 2B and 2C). The most common phyla in all samples were Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria, and the relative abundances of Firmicutes and Bacteroidetes were significantly different among the four treatment groups (Firmicutes: boric acid-fungus – 50.5%, boric acid – 44.3%, fungus – 47.6%, control – 32.8%; Bacteroidetes: boric acid-fungus – 26.1%, boric acid – 30.6%, fungus –

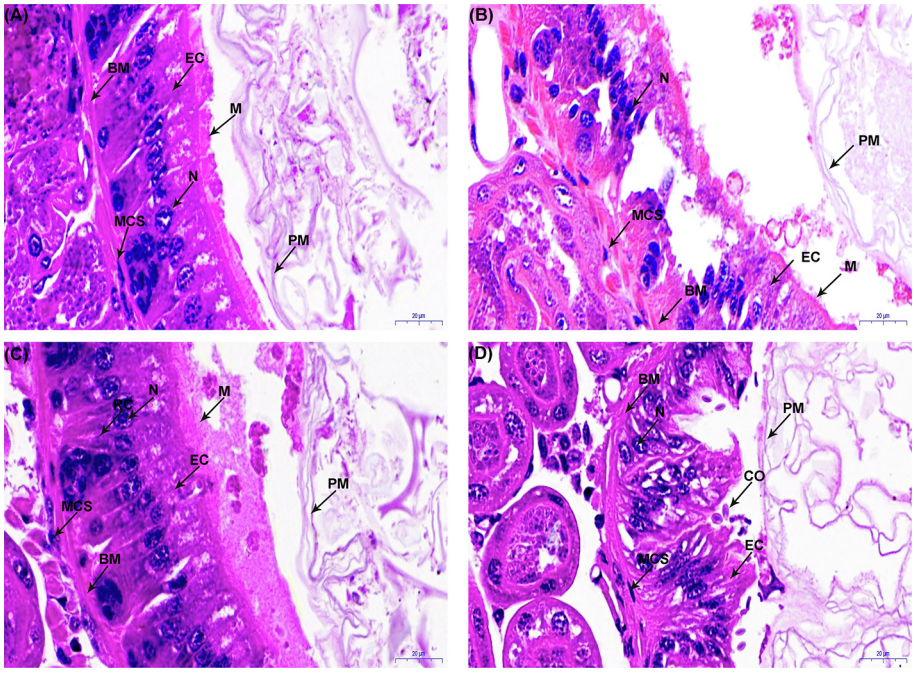


Fig. 1. Histological sections of midguts from *B. germanica* adult males exposed to four treatments by ingestion. (A) Control; (B) boric acid (1.2%) alone; (C) *M. anisopliae* (1×10^9 conidia/ml) alone; (D) *M. anisopliae* (1×10^9 conidia/ml) and boric acid (1.2%). BM, basement membrane; PM, peritrophic membrane; M, microvilli; N, nuclei; RC, regeneration cells; MCS, muscular-conjunctive system; EC, epithelial cells; CO, *M. anisopliae* conidium.

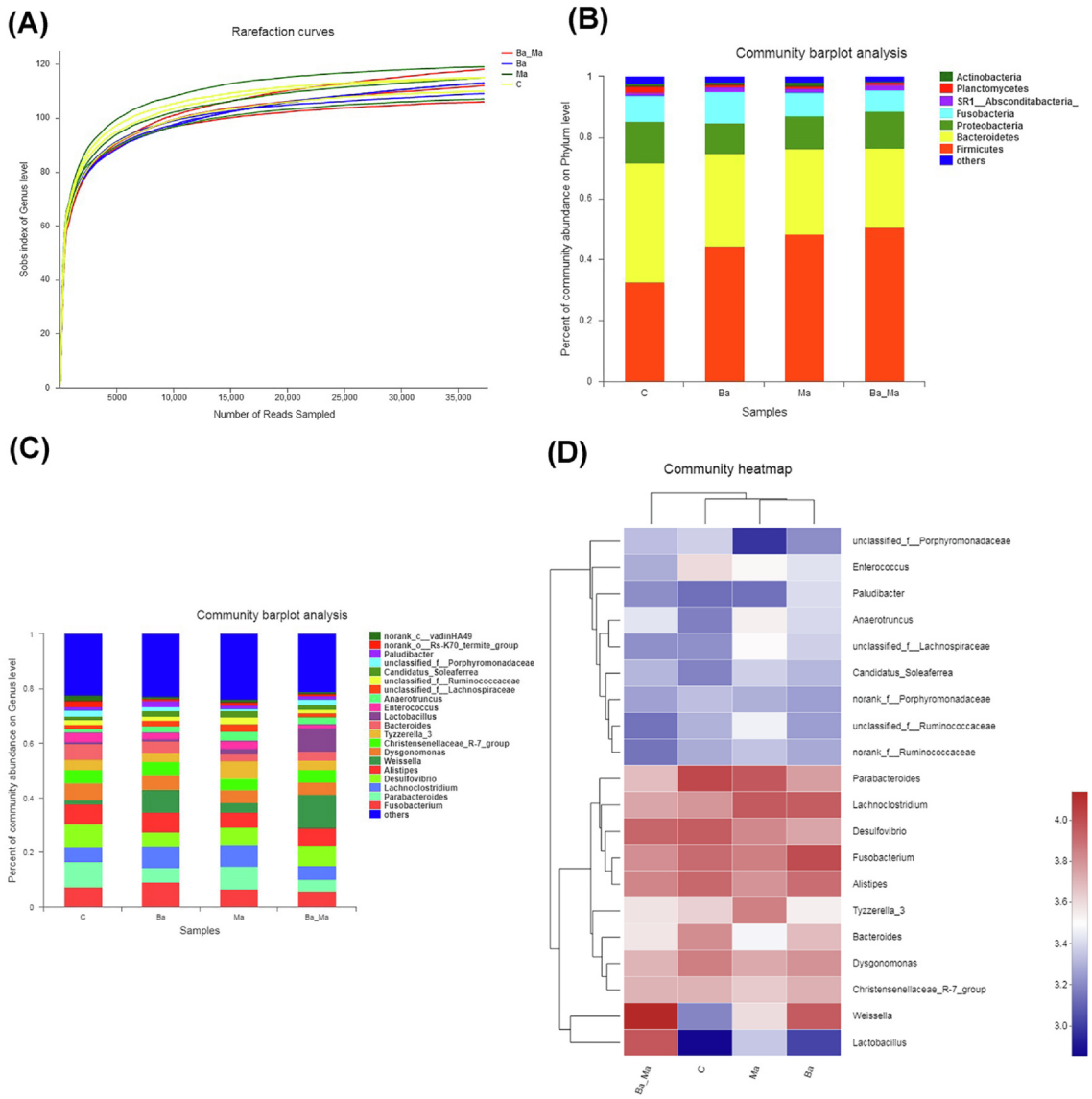


Fig. 2. The gut microbiota varies among the four treatment groups of *B. germanica*, as determined by pyrotag sequencing. Twelve samples are represented by 3 replicates of each of 4 treatment groups. (A) Rarefaction analysis of the different group samples. Sobs represents the observed number of species (OTUs). Rarefaction curves of OTUs clustered at 97% identity that showed clear asymptotes. (B) Bacterial composition of the four communities at the phylum level. (C) Community barplot analysis showing the relative abundance of gut microbiota in each treatment group at the genus level. Taxa with an abundance < 2% are included in "others". (D) Heatmap of the top 20 most abundant genera in bacterial communities detected in the 12 samples. The top and left of the figure are dendrograms for hierarchical cluster analysis grouping genera and sample locations, respectively. C: control sample; Ba: 1.2% (w/w) boric acid sample; Ma: 1×10^9 conidia/ml *M. anisopliae* sample; Ba + Ma: 1×10^9 conidia/ml *M. anisopliae* with 1.2% (w/w) boric acid sample.

29.2%, control – 38.8%; $P < 0.05$ for both).

The detected OTUs were distributed among 132 different bacterial genera, and the numbers of genera were similar across the four treatment groups (boric acid-fungus – 125, boric acid – 124, fungus – 127, control – 124) (Fig. 2C). Although the composition of bacterial genera was also similar across the four treatment groups, their relative abundance differed significantly. In pairwise comparisons of the four treatments at the genus level, there were 7 statistically significant differences (Table 2). In the boric acid treatment the relative abundance of *Parabacteroides* (5.24%) declined significantly relative to the control samples (9.41%; $P < 0.01$), as did *Enterococcus* (2.34% vs. 3.56%, respectively; $P < 0.05$). However, the relative abundance of three genera increased significantly in boric acid treatments relative to the control samples: *Weissella* (8.11% vs. 1.40%, respectively; $P < 0.05$), *Paludibacter* (2.29% vs. 1.21%, respectively; $P < 0.05$) and *Anaerotruncus* (2.24% vs. 1.33%, respectively; $P < 0.01$). In the *M. anisopliae* treatment, the relative abundance of *Alistipes* was significantly lower (4.57%) than in the control samples (7.41%; $P < 0.01$). In the boric acid-*M. anisopliae* combination treatment *Parabacteroides* significantly declined (4.32% vs. 9.40%; $P < 0.01$), as did *Enterococcus* (1.74% vs. 3.54%; $P < 0.05$) compared to the control samples. However, *Weissella* occurred at a significantly higher level in the combination treatment samples relative to control samples (12.05% vs. 1.41%, $P < 0.05$) as well as in the boric acid alone samples (Fig. 2C, Table 2). In addition, the abundance of *Weissella* also significantly increased in the combination treatment samples compared with the *M. anisopliae*-alone samples (8.14% vs. 4.30%; $P < 0.05$). However, the relative abundance of *Parabacteroides* was lower in the combination treatment samples.

According to the weighted UniFrac distance and clustering analysis, PCA analysis also confirmed that the bacterial communities in the control samples were significantly different from samples from the other 3 treatment groups (Fig. 3A and 3B). The PCA score plot indicated

Table 2
Relative abundances of bacterial genera that showed significant differences among treatments.[1]

Genus	Ba+Ma			Ba		Ma
	Ba	Ma	C	Ma	C	C
Parabacteroides	0.292	0.022*	0.004**	0.038*	0.011*	0.523
Weissella	0.199	0.044*	0.013*	0.429	0.013*	0.477
Alistipes	0.155	0.075	0.113	0.002**	0.634	0.008**
Fusobacterium	0.023*	0.717	0.527	0.277	0.455	0.759
Enterococcus	0.311	0.150	0.047*	0.347	0.047*	0.111
Paludibacter	0.024*	0.404	0.373	0.052	0.012*	0.804
Anaerotruncus	0.809	0.565	0.133	0.627	0.009**	0.321

*$P < 0.05$, **$P < 0.01$ (one-way ANOVA).
[1] Ba = boric acid; Ma = *M. anisopliae*; Ba + Ma = combination boric acid and *M. anisopliae*; C = control. The sample in the first row is compared with the sample in the second row, the abundance of genus increases (red) and decreases (blue).

that the *M. anisopliae* and control groups were closely related, and on the right of the graph among PC1, the boric acid-*M. anisopliae* samples were closely related to the boric acid-alone and *M. anisopliae*-alone samples. Fig. 3A shows that PC1 represented 49.97% of the total variation and it separated the three groups, but not *M. anisopliae*-alone. PC2, which accounted for 16.58% of the total variation, separated *M. anisopliae*-alone, boric acid-alone and the control group. Overall, the two PCA axes explained 66.55% of the variation between the different communities (Fig. 3A). According to the binary Pearson distance, the NMDS analysis also confirmed that bacterial communities in the control samples were significantly different from those in the other three treatments (Fig. 3B). In addition, the hierarchical heatmap indicated that the bacterial community profiles were most different at the genus level between the boric acid-*M. anisopliae* samples, *M. anisopliae*-alone and control samples (Fig. 2D).

4. Discussion

With the rapid development of insecticide resistance by *B. germanica*, including the aversion to bait impregnated with certain insecticides, it became necessary to find alternatives to control cockroaches among biological agents and chemicals with different modes of action (Cai et al., 2020; Dayer and Karvandian, 2016). In this study, co-ingestion of *M. anisopliae* and boric acid showed additive interactions with boric acid over all treatments and synergism was noted at the lower doses, which was consistent with the results from topical application (Zurek et al., 2002; Dayer and Karvandian, 2016), especially when boric acid was applied at lower doses. In fact, *M. anisopliae* has good compatibility with many chemical insecticides, such as chlorpyrifos, hydramethylnon, propetamphos, fipronil and permethrin (Pari and Kamble, 2000; Schumacher and Poehling, 2012; Chao et al., 2020). However, the physicochemical properties and concentration of insecticides would affect conidial viability, and higher concentrations of pesticides would inhibit the conidial germination or sporulation in general (Schumacher and Poehling, 2012; Udayababu et al., 2012). For example, the conidial viability of *M. anisopliae* in indoxacarb, spinosad, novaluron and cartap hydrochloride treated media was 84.6, 89.2, 80.4 and 77.4 per cent, respectively, and the sporulation was reduced at most by approximately 50% by 40 or 200 ppm of fipronil or amitraz, respectively (Schumacher and Poehling, 2012; Udayababu et al., 2012). We hypothesized that lower concentrations of boric acid may increase the conidial viability of *M. anisopliae* or boric acid intake (good palatability) and lead to a better synergistic effect.

The midgut of insects is considered the major site of digestion and

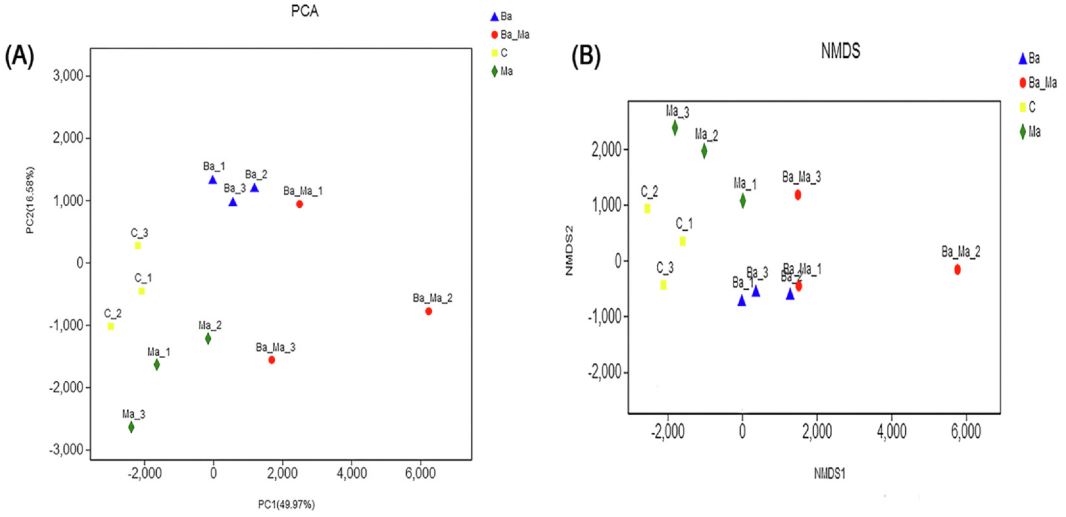


Fig. 3. *Blattella germanica* gut microbiota sample sorting analysis by PCA and NMDS. Twelve samples are represented by 3 replicates of each of 4 treatment groups. (A) Scatter plot of PCA scores shows similarity of the 12 bacterial communities based on Unifrac distance. Principal components (PCs) 1 and 2 explained 49.97% and 16.58% of the variance, respectively. (B) NMDS shows the difference among bacterial communities according to binary Pearson distance. C: control sample; Ba: 1.2% (w/w) boric acid sample; Ma: 1×10^9 (conidia/ml) *M. anisopliae* sample; Ba + Ma: 1×10^9 (conidia/ml) of *M. anisopliae* with 1.2% (w/w) boric acid sample.

absorption of nutrients, but is also involved in osmoregulation and immunity. It is also the most permeable and vulnerable part of the alimentary tract, unprotected by cuticle, where epithelial cells are protected from ingested xenobiotics and pathogens by a thin chitin/protein matrix – the peritrophic membrane. Our histological results revealed that ingested boric acid caused major cytological perturbations, and even destroyed the midgut epithelium, which were consistent with previous observations on the German cockroach exposed to higher concentration of boric acid (Habes et al., 2006). It also showed that a fair number of conidia appeared at the midgut microvilli on the 4th day in the *M. anisopliae*-boric acid combination treatments, though we didn't find any germinated conidia in gut and hemocoel of cockroach due to short sampling time. Researchers have different views on the survival status of *M. anisopliae* in the gut of host insects, because of the complexity of the gut structure and properties (e.g., pH, oxygen, nutrient deficiency and digestive enzymes, etc.), as well as insects have different immune adaptations to *M. anisopliae* (Mannino et al., 2019; Zhang et al., 2018a). Some studies showed that there was no conidial germination in gut, such as for *Aedes aegypti* and *Reticulitermes flavipes*, in which ingested *M. anisopliae* occludes the gut and kills the host without ever germinating or penetrating to the hemocoel to undergo normal vegetative development in the body cavity of *A. aegypti* (Chouvenc et al., 2009; Tariq, 2013). However, Lacey et al. (1988) observed that the conidia in the midgut of moribund *Culex quinquefasciatus* larvae were at the germination stage, but there was no obvious tissue invasion. Our histological evidence supports the idea that the *M. anisopliae*-boric acid combination significantly accelerated the penetration rate and germination of *M. anisopliae*, mainly because boric acid (1) damaged the gut structure, including the peritrophic membrane, microvilli, epithelial cells and basal lamina; (2) interfered with osmoregulation by altering the pH and osmolarity of the gut and hemocoel environment; (3) interfered with host immunity by decreasing the activity of detoxifying enzyme at the late infection stage of *M. anisopliae*, then the conidia eventually invade the hemolymph of the insect, together with the various toxic effects of boric acid, accelerate the death of the host insect.

We also hypothesized that boric acid and/or *M. anisopliae* might disrupt the gut microbial community. We analyzed the gut microbiota of four different treatment groups of cockroaches following Solexa high-throughput sequencing. Boric acid ingestion lowered the relative abundance of *Parabacteroides* and *Enterococcus* compared with the control samples. The abundance of *Parabacteroides* and *Enterococcus* was also lower in the combined boric acid-*M. anisopliae* treatment, suggesting that boric acid was responsible for these changes. Interestingly, both bacterial taxa are known to have anti-inflammatory effects and protect the host from fungal invasion (e.g., Wu et al., 2019). *Enterococcus* sp. has antifungal activity against several fungi including *Candida albicans*, *Debaryomyces hansenii*, *Fusarium culmorum* and *Penicillium roqueforti*. It also produces three bacteriocins: EntV, durancin A5-11a and durancin A5-11b, which have similar antimicrobial properties (Belguesmia et al., 2013; Graham et al., 2017; Huang et al., 2013; Xie and Zhou, 2018). *Parabacteroides* were involved in the degradation of complex organic matter, provide amino acids for the nutrition of the cockroach and produce a variety of antimicrobial agents such as bacteriocins, adhesion organic and acids inhibitors (Allaker and Douglas, 2009; Berlanga et al., 2016; Yuki et al., 2015). All of these functions of the bacteria provide supplementary pathways for nutrient metabolism and immune defense of *B. germanica*. Given the concomitant suppression of *Parabacteroides* and *Enterococcus* in the presence of boric acid, it is reasonable to speculate that boric acid was responsible for physical and chemical changes in the gut that facilitated *M. anisopliae* penetration of the midgut.

On the other hand, the abundance of *Weissella* significantly increased in both the boric acid and the boric acid-*M. anisopliae* treatments. *Weissella* is an opportunistic pathogen that infects its host after the mucosal barrier of the gut had been disrupted (Kamboj et al., 2015; Yang et al., 2017; Zhang et al., 2014, 2020), so its increased abundance in the cockroach gut may be related to changes in gut chemistry, especially pH, effected by boric acid; *Weissella* thus would be expected to enhance the synergism of the boric acid-*M. anisopliae* combination. Similar enhancement effects have been described in other systems. *Serratia marcescens*, a commensal bacterium in the mosquito gut, enhances arbovirus acquisition by secreting a protein, *Sm*Enhancin, which digests membrane-bound mucins on the insect gut epithelium, which facilitates viral dissemination (Wu et al., 2019a). Finally, the gut microbiota of *B. germanica* was little affected by *M. anisopliae* alone; only the abundance of *Alistipes* decreased compared with the control samples. The genus *Alistipes* resembles the *Bacteroides fragilis* group, and appears to be involved in carbohydrate metabolism. Of particular interest is that *Alistipes* species can hydrolyse chitin, which is a fungal cell wall component (Li et al., 2013; Liu et al., 2013). It is possible that toxins secreted by *Metarhizium* (destruxins) (Chen et al., 2014; Wang et al., 2012) alter gut conditions to disfavor *Alistipes* and thus benefit *Metarhizium*.

The mechanisms that underlie the synergism between *M. anisopliae* and boric acid have not been thoroughly investigated. One hypothesis was that topically applied conidia might stimulate cockroaches to groom and thus ingest more topically applied boric acid. However, the synergism was eliminated by replacing *M. anisopliae* with a dust (flour) or heat-killed *M. anisopliae* that would also stimulate grooming (Zurek et al., 2002). Because it appeared that low concentrations of boric acid enhance the activity of *M. anisopliae*, and not vice versa, other potential interactions might include (1) boric acid facilitates the penetration of *M. anisopliae* by physically and chemically disrupting the midgut, and (2) by altering the gut microbiome, boric acid promotes survival and virulence of *M. anisopliae* in the harsh gut environment. A recent study revealed that the cytotoxic effect of boric acid can significantly suppresses the hemocyte mediated immune responses of *G. mellonella*, such as melanization, nodule and capsule formation, thereby lowers the spreading ability of host hemocytes (Gwokyalya and Altuntaş, 2019). boric acid able to assist *M. anisopliae* quickly escape hemocyte encapsulation in this context, a previously unsuspected immune evasion strategy that remains to be investigated. Interestingly, synergism was also observed with co-injections of low doses of *M. anisopliae* and boric acid directly into the hemolymph, suggesting that the gut may not be the site of the synergism (Zurek et al., 2002). We hypothesized that while multiple mechanisms may be involved, a major site of the synergism may be boric acid disrupting the structure of the midgut and altering the gut microbiota of *B. germanica*, thus enhancing penetration and virulence of *M. anisopliae*. Overall, our results demonstrate that the combined applications of boric acid and fungal conidia in baits provide safer and effective methods of cockroach control, and the mechanism(s) of action of boric acid suggests that it might facilitate the penetration of other natural and synthetic insecticides and synergize their activity. Moreover, disruption of the integrity of the midgut by boric acid should facilitate pathogen penetration, predisposing cockroaches to diseases and septicemia.

In conclusion, the virulence of *M. anisopliae* to German cockroaches can be synergistically accelerated with boric acid; both insecticidal agents have favorable human and mammalian safety records. The main advantage of the *M. anisopliae*-boric acid combination is that it can accelerate the mechanism of action of the fungus without compromising fungus viability in cadavers, which is crucial for inducing epizootics in cockroach populations. These should compel the development of more effective alternative fungus-based baits against the German cockroach by using boric acid as a synergistic agent to change the gut microecology of the cockroach. However, more research is necessary to optimize this formulation, determine its efficacy under field conditions, and also generalize it to other pest species.

Author statement

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

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81572027), the Key Programs for Science and Technology Development of Shandong Province (2017GSF221012) and the National Critical Project for Science and Technology on Infectious Diseases of P. R. China (2017ZX100303404).

References

Ahmad, A., Ghosh, A., Schal, C., Zurek, L., 2011. Insects in confined swine operations carry a large antibiotic resistant and potentially virulent enterococcal community. BMC Microbiol. 11, 23.

Ali, S.E., Thoen, E., Evensen, O., Wiik-Nielsen, J., Gamil, A.A.A., Skaar, I., 2014. Mitochondrial dysfunction is involved in the toxic activity of boric acid against *Saprolegnia*. PLoS One. 9, e110343.

Allaker, R.P., Douglas, C.W.I., 2009. Novel anti-microbial therapies for dental plaque-related diseases. Int. J. Antimicrob. Agents. 33, 8–13.

Batta, Y.A., 2018. Efficacy of two species of entomopathogenic fungi against the stored-grain pest, *Sitophilus granarius* L. (Curculionidae: Coleoptera), via oral ingestion. Egypt. J. Biol. Pest. Control. 28, 44.

Belguesmia, Y., Choiset, Y., Rabesona, H., Baudy-Floc'h, M., Le Blay, G., Haertle, T., Chobert, J.M., 2013. Antifungal properties of durancins isolated from *Enterococcus durans* A5–11 and of its synthetic fragments. Lett. Appl. Microbiol. 56, 237–244.

Berenbaum, M.C., 1981. Criteria for analyzing interaction between biologically active agents. Adv. Cancer Res. 35, 269–335.

Berlanga, M., Llorens, C., Comas, J., Guerrero, R., 2016. Gut bacterial community of the Xylophagous cockroaches *Cryptocercus punctulatus* and *Parasphaeria boleiriana*. PLoS One. 11.

Büyükgüzel, E., Büyükgüzel, K., Snela, M., Erdem, M., Adamski, Z., 2013. Effect of boric acid on antioxidant enzyme activity, lipid peroxidation, and ultrastructure of midgut and fat body of *Galleria mellonella*. Cell Biol. Toxicol. 29, 117–129.

Cai, T., Huang, Y.H., Zhang, F., 2020. Ovarian morphological features and proteome reveal fecundity fitness disadvantages in β-cypermethrin-resistant strains of *Blattella germanica* (L.) (Blattodea: Blattellidae). Pestic. Biochem. Physiol. (in press).

Chao, Y., Wang, M.Y., Dai, W., Dong, F.Y., Wang, X.J., Zhang, F., 2020. Synergism between hydramethylnon and *Metarhizium anisopliae* and their influence on the gut microbiome of *Blattella germanica* (L.). Insects 11, 538.

Chen, X.R., Hu, Q.B., Yu, X.Q., Ren, S.X., 2014. Effects of destruxins on free calcium and hydrogen ions in insect hemocytes. Insect Sci. 21, 31–38.

Chouvenc, T., Su, N.Y., Robert, A., 2009. Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. J. Invertebr. Pathol. 101.

Cochran, D.G., 1995. Toxic effects of boric acid on the German cockroach. Experientia. 51, 561–563.

Cox, C., 2004. Boric acid and borates. J. Pesticide Reform. 24, 10–15.

Dayer, M.S., Karvandian, K., 2016. Toxicity of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) and boric acid against nosocomial cockroaches, *Blattella germanica*. Arthropods 5, 114–124.

DeVries, Z.C., Santangelo, R.G., Crissman, J., Suazo, A., Kakumanu, M.L., Schal, C., 2019. Pervasive resistance to pyrethroids in German cockroaches (Blattodea: Ectobiidae) related to lack of efficacy of total release foggers. J. Econ. Entomol. 112, 2295–2301.

Ebeling, W., 1995. Inorganic insecticides and dusts. In: Rust, M.K., Reierson, D.A., Owens, J.M. (Eds.), Understanding and Controlling the German Cockroach. Oxford University Press, New York, New York, pp. 193–230.

Fardisi, M., Gondhalekar, A.D., Ashbrook, A.R., Scharf, M.E., 2019. Rapid evolutionary responses to insecticide resistance management interventions by the German cockroach (*Blattella germanica* L.). Sci. Rep. 9.

Faria, M., Wraight, S.P., 2007. Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control. 43, 237–256.

Gao, Q., Jin, K., Ying, S.-H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.-Q., Zhou, G., 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genetics. 7, e1001264.

Gore, J.C., Zurek, L., Santangelo, R.G., Stringham, S.M., Watson, D.W., Schal, C., 2004. Water solutions of boric acid and sugar for management of German cockroach populations in livestock production systems. J. Econ. Entomol. 97, 715–720.

Graham, C.E., Cruz, M.R., Garsin, D.A., Lorenz, M.C., 2017. *Enterococcus faecalis* bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*. Proc. Natl. Acad. Sci. USA 114, 4507–4512.

Gutierrez, A., Machado, J., Hubner-Campos, R., Pennisi, M., Rodrigues, J., López Lástra, C., García, J., Fernandes, É., Luz, C., 2016. New insights into the infection of the American cockroach *Periplaneta americana* nymphs with *Metarhizium anisopliae* s.l. (Ascomycota: Hypocreales). J. Appl. Microbiol. 121, 1373–1383.

Gwokyalya, R., Altuntaş, H., 2019. Boric acid induced immunotoxicity and genotoxicity in model insect *Galleria mellonella* L. (Lepidoptera: Pyralidae). Arch. Insect. Biochem. Physiol. 101, e21588.

Habes, D., Morakchi, S., Aribi, N., Farine, J.P., Soltani, N., 2006. Boric acid toxicity to the German cockroach, *Blattella germanica*: alterations in midgut structure, and acetylcholinesterase and glutatbione S-transferase activity. Pestic. Biochem. Physiol. 84, 17–24.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., Leger, R.J.S., Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl Acad. Sci. 111, 16796–16801.

Huang, Y.H., Wang, X.J., Zhang, F., Huo, X.B., Fu, R.S., Liu, J.J., Sun, W.B., Kang, D.M., Jing, X., 2013. The identification of a bacterial strain BGI-1 isolated from the intestinal flora of *Blattella germanica*, and its anti-entomopathogenic fungi activity. J. Econ. Entomol. 106, 43–49.

Hyršl, P., Büyükgüzel, E., Büyükgüzel, K., 2007. The effects of boric acid-induced oxidative stress on antioxidant enzymes and survivorship in *Galleria mellonella*. Arch. Insect. Biochem. Physiol. 66.

Jeffs, L.B., Feng, M.-G., Falkowsky, J.E., Khachatourians, G.G., 1997. Infection of the migratory grasshopper (Orthoptera: Acrididae) by ingestion of the entomopathogenic fungus *Beauveria bassiana*. J. Econ. Entomol. 90, 383–390.

Kamboj, K., Vasquez, A., Balada-Llasat, J.-M., 2015. Identification and significance of *Weissella* species infections. Front. Microbiol. 6 1204–1204.

Lacey, C.M., Lacey, L.A., Roberts, D.R., 1988. Route of invasion and histopathology of *Metarhizium anisopliae* in *Culex quinquefasciatus*. J. Invertebr. Pathol. 52, 108–118.

Li, Y.R., Coby, S., Pan, X.Y., Huang, Y.H., Zhang, F., 2020. The dynamic balance of intestinal bacteria and fungi in the German cockroach and the response of metabolic enzymes after treatment with antibiotic. J. Econ. Entomol (in press).

Li, H., Zhang, F.M., Guo, H.Y., Zhu, Y.Y., Yuan, J.D., Yang, G.W., An, L.G., 2013. Molecular characterization of hepcidin gene in common carp (Cyprinus carpio L.) and its expression pattern responding to bacterial challenge. Fish. Shellfish Immun. 35, 1030–1038.

Liu, N., Zhang, L., Zhou, H.K., Zhang, M.L., Yan, X., Wang, Q., Long, Y.H., Xie, L., Wang, S.Y., Huang, Y.P., Zhou, Z.H., 2013. Metagenomic insights into metabolic capacities of the gut microbiota in a fungus-cultivating termite (*Odontotermes yunnanensis*). PLoS One. 8, e69184..

Mannino, M.C., Huarte-Bonnet, C., Davyt-Colo, B., Pedrini, N., 2019. Is the insect cuticle the only entry gate for fungal infection? insights into alternative modes of action of entomopathogenic fungi. J. Fungi. 5, 33.

Mansour, N.A., Eldefrawi, M.E., Toppozada, A., Zeid, M., 1966. Toxicological studies on the Egyptian cotton leaf worm, *Prodenia litura*. VI. Potentiation and antagonism of organophosphorus and carbamate insecticides. J. Econ. Entomol. 59, 307–311.

Murray, F.J., 1998. A comparative review of the pharmacokinetics of boric acid in rodents and humans. Biol. Trace Elem. Res. 66, 331–341.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H., Wagner, H., 2012. Vegan: Community Ecology Package. R package version 2.0-5. http://CRAN.R-project.org/package = vegan.

Pan, X.Y., Zhang, F., 2019. Advances in Biological Control of the German cockroach, *Blattella germanica* (L.). Biol. Control. 142, 104104.

Pari, P., Kamble, S.T., 2000. *In vivo* study on combined toxicity of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) strain ESC-1 with sublethal doses of chlorpyrifos, Propetamphos, and Cyfluthrin Against German Cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 60–70.

Pereira, R.M., Oi, D.H., Baggio, M.V., Koehler, P.G., 2017. Microbial control of structural insect pests. In: Lacey, L.A. (Ed.), Microbial Control of Insect and Mite Pests: From Theory to Practice. Elsevier, New York, pp. 431–442.

Pomés, A., Schal, C., 2019. Cockroach and other inhalant insect allergens. In: Lockey, R.F., Ledford, D.K.Eds.) (Eds.), Allergens and Allergen Immunotherapy. Taylor & Francis Group, New York.

Development Core Team, R., 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Schumacher, V., Poehling, H.M., 2012. In vitro effect of pesticides on the germination, vegetative growth, and conidial production of two strains of *Metarhizium anisopliae*. Fungal. Biol. 116.

Suiter, D.R., 1997. Biological suppression of synanthropic cockroaches. J. Agri. Entomol. 14, 259–270.

Tariq, M.B., 2013. *Metarhizium anisopliae* pathogenesis of mosquito larvae: a verdict of accidental death. PloS One 8.

Udayababu, P., Goud, C.R., Pedada, D., 2012. Effect of Selected Pesticides on the Growth Parameters of *Metarhizium anisopliae* (Metch.) Sorokin. J. Biol. Control 26, 380–385.

Wang, B., Kang, Q., Lu, Y., Bai, L., Wang, C., 2012. Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. Proc. Natl. Acad. Sci. U.S.A. 109, 1287–1292.

Wu, T.-R., Lin, C.-S., Chang, C.-J., Lin, T.-L., Martel, J., Ko, Y.-F., Ojcius, D.M., Lu, C.-C., Young, J.D., Lai, H.-C., 2019. Gut commensal *Parabacteroides goldsteinii* plays a predominant role in the anti-obesity effects of polysaccharides isolated from *Hirsutella sinensis*. Gut. 68, 248–262.

Wu, P., Sun, P., Nie, K.X., Zhu, Y.B., Shi, M.Y., Xiao, C.G., Liu, H., Liu, Q.Y., Zhao, T.Y., Chen, X.G., Zhou, H.N., Wang, P.H., Cheng, G., 2019a. A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses. Cell. Host. Microbe. 25, 101–102.

Xiao, G., Ying, S.-H., Zheng, P., Wang, Z.-L., Zhang, S., Xie, X.-Q., Shang, Y., Leger, R.J.S., Zhao, G.-P., Wang, C., 2012. Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci. Rep. 2, 483.

Xie, W., Zhou, J., 2018. Aberrant regulation of autophagy in mammalian diseases. Biol. Letters 14.

Yang, C.L., Zhu, H.Y., Zhang, F., 2019. Comparative proteomics analysis between the short-term stress and long-term adaptation of the *Blattella germanica* (Blattodea: Blattellidae) in response to beta-cypermethrin. J. Econ. Entomol. 112, 1396–1402.

Yang, H.T., Zou, S.S., Zhai, L.J., Wang, Y., Zhang, F.M., An, L.G., Yang, G.W., 2017. Pathogen invasion changes the intestinal microbiota composition and induces innate immune responses in the zebrafish intestine. Fish. Shellfish. Immun. 71, 35–42.
Yuki, M., Kuwahara, H., Shintani, M., Izawa, K., Sato, T., Starns, D., Hongoh, Y., Ohkuma, M., 2015. Dominant ectosymbiotic bacteria of cellulolytic protists in the termite gut also have the potential to digest lignocellulose. Environ. Microbiol. 17.
Zhang, F., Huang, Y.H., Liu, S.Z., Zhang, L., Li, B.T., Zhao, X.X., Fu, Y., Liu, J.J., Zhang, X.X., 2013. *Pseudomonas reactans*, a bacterial strain isolated from the intestinal flora of *Blattella germanica* with anti-*Beauveria bassiana* activity. Environ. Entomol. 42, 453–459.
Zhang, F., Sun, X.X., Zhang, X.C., Zhang, S., Lu, J., Xia, Y.M., Huang, Y.H., Wang, X.J., 2018a. The interactions between gut microbiota and entomopathogenic fungi: a potential approach for biological control of *Blattella germanica* (L.). Pest. Manag. Sci. 74, 438–447.
Zhang, F., Wang, X.J., Huang, Y.H., Zhao, Z.G., Zhang, S.S., Gong, X.S., Xie, L., Kang, D.M., Jing, X., 2014. Differential expression of hemolymph proteins between susceptible and insecticide-resistant *Blattella germanica* (Blattodea: Blattellidae). Environ. Entomology. 43, 1117–1123.
Zhang, F., Yang, R., 2019. Life history and functional capacity of the microbiome are altered in beta-cypermethrin-resistant cockroaches. Inter. J. Parasitol. 49, 715–723.
Zhang, X.C., Li, X.X., Gong, Y.W., Li, Y.R., Zhang, K.L., Huang, Y.H., Zhang, F., 2018b. Isolation, identification, and virulence of a new *Metarhizium anisopliae* strain on the german cockroach. J. Econ. Entomol. 111, 2611–2616.
Zhang, X.C., Zhang, F., 2018. The potential control strategies based on the interaction between indoor cockroaches and their symbionts in China. Adv. In. Insect. Phys. 55, 55–122.
Zhang, F., Yang, C.L, Zhang, X.C, Zhu, H.Y, Huang, Y.H., 2020. Isolation of an anti-entomopathogenic fungal protein secreted from *Pseudomonas aeruginosa* BGf-2: an intestinal bacteriam of *Blattella germanica* (L.). J. Invertebr. Pathol. 173, 107371.
Zhu, F., Lavine, L., O'Neal, S., Lavine, M., Foss, C., Walsh, D., 2016. Insecticide resistance and management strategies in urban ecosystems. Insects. 7, 1–26.
Zurek, L., Gore, J.C., Stringham, S.M., Watson, D.W., Waldvogel, M.G., Schal, C., 2003. Boric acid dust as a component of an integrated cockroach management program in confined swine production. J. Econ. Entomol. 96, 1362–1366.
Zurek, L., Watson, D.W., Schal, C., 2002. Synergism between *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) and boric acid against the German cockroach (Dictyoptera : Blattellidae). Biol. Control. 23, 296–302.