boric acid

synergistic interaction

enhance virulence

Metarhizium anisopliae

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

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**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.,...
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 × 10⁷, 1 × 10⁸ or 1 × 10⁹ 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 × 10⁷, 1 × 10⁸ and 1 × 10⁹ 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 LT₅₀ values were estimated with 95% confidence intervals.

The joint action of two insecticides was determined by the Mansour co-operation 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 × 10⁹ conidia/ml of M. anisopliae only, 1.2% (w/w) boric acid 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 Karrot 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 Trimomatic 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 chesopeq 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 LT₅₀ 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 × 10⁷ conidia/ml) killed only 11.7% of the cockroaches over the 15 day observation period, whereas the highest concentration (1 × 10⁹ 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 × 10⁷ 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 × 10⁹ 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
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 –

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### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Actual mortality (%)</th>
<th>Expected mortality (%)</th>
<th>LT$_{50}$ (95% CI) (day)</th>
<th>Slope ± SE</th>
<th>Co-toxicity factor</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Ma 0.4 Ba</td>
<td>6.67 ± 1.67 $^a$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0 Ma 0.8 Ba</td>
<td>33.33 ± 3.33 $^b$</td>
<td>NA</td>
<td>NA</td>
<td>2.26 ± 0.034</td>
<td>2.739</td>
<td>Synergistic</td>
</tr>
<tr>
<td>0 Ma 1.2 Ba</td>
<td>86.67 ± 1.67 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>0.398 ± 0.061</td>
<td>1.977</td>
<td>Additive</td>
</tr>
<tr>
<td>0 Ma 1.6 Ba</td>
<td>88.33 ± 4.41 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>0.416 ± 0.064</td>
<td>2.473</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^7$ Ma 0 Ba</td>
<td>11.67 ± 3.33 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 × 10$^7$ Ma 0.4 Ba</td>
<td>16.67 ± 4.41 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 × 10$^7$ Ma 0.8 Ba</td>
<td>26.67 ± 3.33 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 × 10$^7$ Ma 1.2 Ba</td>
<td>36.67 ± 3.33 $^c$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1 × 10$^8$ Ma 0.4 Ba</td>
<td>67.66 ± 1.67 $^a$</td>
<td>8 (6.9–9.9)</td>
<td>0.382 ± 0.070</td>
<td>12.197</td>
<td>23.25</td>
<td>Synergistic</td>
</tr>
<tr>
<td>1 × 10$^8$ Ma 0.8 Ba</td>
<td>33.33 ± 3.33 $^b$</td>
<td>6 (4.9–7.1)</td>
<td>0.426 ± 0.068</td>
<td>4.179</td>
<td>5.79</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^8$ Ma 1.2 Ba</td>
<td>100.0 ± 0.00 $^c$</td>
<td>4 (3.5–7.7)</td>
<td>0.546 ± 0.088</td>
<td>5.857</td>
<td>11.49</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 0.4 Ba</td>
<td>56.67 ± 1.67 $^c$</td>
<td>10 (9.1–11.3)</td>
<td>0.209 ± 0.032</td>
<td>2.194</td>
<td>154.95</td>
<td>Synergistic</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 0.8 Ba</td>
<td>75.00 ± 2.89 $^d$</td>
<td>9 (5.5–7.8)</td>
<td>0.288 ± 0.045</td>
<td>4.402</td>
<td>8.00</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 1.2 Ba</td>
<td>93.33 ± 3.33 $^b$</td>
<td>6 (5.3–6.8)</td>
<td>0.565 ± 0.085</td>
<td>2.240</td>
<td>4.99</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 1.6 Ba</td>
<td>90.00 ± 0.00 $^c$</td>
<td>5 (4.3–6.2)</td>
<td>0.621 ± 0.095</td>
<td>3.058</td>
<td>5.23</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 0.4 Ba</td>
<td>66.67 ± 4.41 $^c$</td>
<td>6 (5.1–8.2)</td>
<td>0.229 ± 0.036</td>
<td>3.516</td>
<td>111.24</td>
<td>Synergistic</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 0.8 Ba</td>
<td>78.33 ± 3.33 $^b$</td>
<td>7 (6.3–8.7)</td>
<td>0.217 ± 0.039</td>
<td>2.758</td>
<td>7.14</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 1.2 Ba</td>
<td>85.00 ± 2.89 $^c$</td>
<td>5 (4.2–6.3)</td>
<td>0.386 ± 0.060</td>
<td>4.624</td>
<td>5.29</td>
<td>Additive</td>
</tr>
<tr>
<td>1 × 10$^9$ Ma 1.6 Ba</td>
<td>98.33 ± 4.41 $^c$</td>
<td>5 (4.7–6.1)</td>
<td>0.465 ± 0.083</td>
<td>1.859</td>
<td>7.53</td>
<td>Additive</td>
</tr>
</tbody>
</table>

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%).
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
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

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

*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).
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 osmolality 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 microbital 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, duracin A5-11a and duracin 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 mucus barrier of the gut had been disrupted (Ramboj 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, SmEnhancin, 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 (destruexins) (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 (Gwokalya 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

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