Combination of L-Methionine and Chlorantraniliprole Enhances the Abundance of Opportunistic Pathogenic Bacteria in the Intestine of Greater Wax Moth Leading to Increased Mortality Risk

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ABSTRACT: Honey bees play an essential role in global crop production and agro-economic development due to their pollination properties. However, empirical evidence indicates a worldwide decline in bee colonies. The greater wax moth (GWM), a lepidopteran insect and natural enemy of honey bees, significantly contributes to this decline. Chlorantraniliprole (CH) is commonly used to control GWM in apiaries due to its efficacy and low toxicity to bees. However, long-term use of CH may lead to environmental pollution and GWM resistance. To enhance beekeeping safety and mitigate the risk of GWM resistance from prolonged CH use, we investigated the toxic effects of combining methionine (MET), which has demonstrated insecticidal activity against some lepidopteran pests, with CH on GWM. We conducted both individual and mixed exposure tests of MET and CH on GWM to finally evaluate the toxic effects of the combined treatment (MIX). The results indicated that the combination of MET and CH produced a synergistic lethal effect on GWM. Subsequent microbiome and immune-related gene expression assays, along with correlation analysis, revealed that the MIX treatment induced immune stress in GWM, significantly reducing the abundance of *Enterococcus spp.*, the primary genus in GWM. This phenomenon led to the proliferation of opportunistic pathogens such as *Pseudomonas spp.*, ultimately leading to synergistic lethal effects on GWM mortality. This study provides new insights and data supporting the development of MET as a potential insecticide.

***Keywords*: Insecticide, Microbiome, Mechanism, Synergistic effects.**

1. Introduction

Honeybees are an important part of global agricultural production, and in addition to being an important pollinator of crops, also produce a range of high-value bee products such as honey, beeswax, and bee pollen, which play an indispensable role in agricultural economic development. However, recent decades have witnessed notable declines in both feral and wild honey bee populations, sparking concerns among beekeepers, scientists, and the general public (Potts et al., 2016, 2010; vanEngelsdorp and Meixner, 2010).

Extensive evidence indicates that the decrease in bee populations may be ascribed to various factors, including pathogens, parasites, pests, predators, and chemical pesticides (Chantawannakul et al., 2016; Goulson et al., 2015; Pirk et al., 2016). The Greater Wax moth (GWM, *Galleria mellonella*) is of particular a significant threat to the honeybee populations (Kwadha et al., 2017). GWM is a lepidopteran insect that is a natural enemy of honey bees. Its larvae infest the comb and harm the bees within cells, severely affecting the quality and yield of bee products (Ellis et al., 2013). Moreover, GWM's high adaptability, frequent larval activity, and wide distribution

significantly complicate its prevention and control.

There are currently some methods for the control of GWM, among which pesticides are extensively employed in apicultural practice due to their cost-effectiveness and efficiency (Kwadha et al., 2017). The anthranilic diamide insecticide, chlorantraniliprole (CAS Number 500008-45-7, CH), which was developed by DuPont, exhibits exceptional effectiveness in the elimination of lepidopteran pests and frequently employed in the control of GWM (Bassi et al., 2009; Han et al., 2012; Luo et al., 2020). CH works by excessively activating insect ryanodine receptors (RyRs), causing the sarcoplasmic reticulum to release excessive calcium, leading to feeding cessation and paralysis, ultimately resulting in insect mortality (Lahm et al., 2009). The global market for CH was valued at USD 1800.8 million in 2023 and is projected to grow to USD 2443 million by 2030 (*Chlorantraniliprole Market, Report Size, Worth, Revenue, Growth, Industry Value, Share 2024*, 2024). However, the widespread application of CH has raised concerns regarding its cumulative toxicity to honeybees. Scientific evidence indicate that honeybees exposed to CH can result in mortality, apathy, lethargy, and uncoordinated movement in honeybees, posing significant risks to the colony (EPSA, 2008).

Considering the increasing risks posed by CH to honeybee colonies and the critical necessity for controlling GWM, there exists a demand to explore compounds capable of augmenting the insecticidal potency of CH against pests while ensuring their safety or, at the very least, avoiding an exacerbation of toxicity to honeybees (Liu et al., 2023). Research indicates that MET exhibits insecticidal activity against some Lepidoptera pests, such as *Princeps (Papilio) demoleus* and *Manduca sexta (L.)* by affecting ion flux and neurotransmitter transport (Lewis et al., 2011; Long et al., 2003; Quick and Stevens, 2001). These findings suggest that MET could potentially enhance the insecticidal efficacy of CH against GWM while ensuring the safety of honeybee colonies. Besides, both adult and larval honeybees require the essential amino acid MET for development and growth. Pollen lacking MET can hinder brood rearing, leading bees to avoid collecting pollen or nectar from flowers deficient in MET (Weeks et al., 2018). Consequently, combining MET with CH is not expected to increase the risks posed by CH to honeybees, instead, it has the potential to enhance the effectiveness of CH against GWM.

To the best of our knowledge, limited research has investigated the combined effects of MET and CH on both GWM and honeybees. In our previous study, we initially explored the synergistic effects of CH at commercially recommended doses and the reported maximum safe concentration of MET for honeybees on GWM and honeybees (Liu et al., 2023). However, further exploration is warranted to ascertain the most effective combination concentrations of CH and MET that strike an optimal balance between cost and efficacy. To achieve this objective, we utilized a gradient concentration approach for individual exposure in this study. Subsequently, guided by the outcomes of individual exposure assessments, a mixed exposure approach was adopted to identify the optimal concentration combination that elicits the desired toxic effects on GWM larvae. Furthermore, we elucidated the potential synergistic mechanisms of MIX-treated GWM through microbiome analysis and transcription of target antimicrobial peptides (AMPs)genes. This study is expected to offer new insights into novel pesticides suitable for the control of GWM.

2. Methods and materials

2.1. Chemicals, solvents and devices

L-methionine (99% purity, Macklin Biochemical Technology Co., Ltd., Shanghai, China),

Chlorantraniliprole (200 g/L suspension concentrate, FMC Corporation, Jiangsu, China) and stored at 4°C in the dark. GWM artificial feed (Keyun Biology, Ltd., Henan, China), TransZol Up (ET111-01-V2, TransScript), One-Step gDNA Removal and cDNA Synthesis SuperMix (AT311-02, TransScript), 2 × Tsingke Master qPCR Mix SYBR Green I (TSE201, Tsingke). All the primers were synthesis by Beijing Tsingke Biotech Co., Ltd. MagPure Stool DNA KF kit B (MD5115-02B, MAGEN), Qubit™ dsDNA BR Assay Kit (Q32850, Invitrogen), 2 × Phanta Max Master Mix (P515-03, VAZYME), Magnetic beads (LB00V60, BGI), Qubit® ssDNA Assay Kit (Q10212, Invitrogen), MGISEQ-2000RS High-throughput Sequencing Set (FCS PE300, 940-000039-00, BGI).

Tissue grinder (FK-A, Jingtan instrument manufacturing Co., Ltd., Shanghai, China), Automatic sample rapid grinder (JXFSTPRP-48, Jingxin Technology, Shanghai, China), Eppendorf ThermoMixer (Comfort 5355, Eppendorf), Centrifuge (5417R, Eppendorf), KingFisher Flex (KingFisher Flex, Thermo Fisher), Eppendorf Reference (Eppendorf), Qubit™ 3 Fluorescence Quantifier (Q33216, Thermo Fisher), Genetic sequencer (MGISEQ-2000, MGI).

2.2. GWM larvae and experiments feeds preparation

The GWM was purchased from Keyun Biology Company (Henan, China) and cultivated in a thermostatic incubator under specific conditions for succession (30 ± 1°C, darkness) (MATSUMOTO and YANO, 1995; Sehnal F., 1996). The forth instar larvae were selected for the exposure experiments.

The artificial feed was initially weighed with MET and/or CH and added to the grinder. After grinding, the feed was shaped into particles suitable for GWM consumption. The individual exposure experiment was set up with 0.1 mg/kg, 1 mg/kg, 10 mg/kg, 25 mg/kg, 50 mg/kg, and 100 mg/kg of CH feed treatment concentration and 0.1%, 0.25%, 0.5%, 1%, and 2% of MET feed treatment concentration, and a blank control was set up, respectively. The feed concentrations of the CH and MET used in the mixed exposure test were derived from the results of the individual exposure test, i.e., two levels of 0.1 mg/kg and 1 mg/kg for CH and three levels of 0.5%, 1% and 2% for MET were used in the mixed exposure test to determine the optimal compounding concentration, with a blank control included.

2.3. Individual and mixed exposure experiment

The fourth GWM larvae were utilized for both individual exposure and mixed exposure experiment. Each treatment comprised six biological replicates, with 10 GWM larvae assigned to each replicate. The larvae were incubated in a thermostatic incubator at 30°C throughout the exposure test period, which lasted for 7 days. The difference between the feed used in individual and mixed exposure experiments was described in section 2.2. Larval mortality and feeding were recorded every 24 h.

After the experimental period, larvae, except those used for microbiome analysis, were frozen in liquid nitrogen, briefly thawed on ice, and rinsed with anhydrous ethanol. After gentle drying, the larvae were then transferred to a new sterile petri dish, dissected along the midline from head to tail, and the complete intestines were exposed. The intestines from the same petri dish were delicately removed with sterile forceps and placed in centrifuge tubes. These samples were promptly frozen in liquid nitrogen and stored at -80°C for further analysis.

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2.4. Gut microbiota analysis

Digesta samples were collected from the hindguts of treatment-surviving GWM for microbiome analysis. The analysis procedure was supported by the BGI Co. Ltd (Shenzhen China)which is responsible for PCR amplification and library preparation of the 16S rRNA gene. Prime pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3')and 806R (5'-GGACTACHVGGGTWTCTAAT-3') embedded with barcodes were chosen to target the V3-V4 regions. After the gene amplification, quantitation, qualification, and purification, high quality sample sequence information was obtained. reads splicing from each sample was performed using FLASH (Fast Length Adjustment of Short reads, v1.2.11) and denoised by the DADA2 (Divisive Amplicon Denoising Algorithm) method in Qiime2 to obtain ASVs (Amplicon Sequence Variants), and then the ASVs were compared with the Silva v138 SSU rRNA database for species annotation analysis by RDP classifer v2.2, with the confidence value threshold set to 0.6, to obtain taxonomic information of the samples and to count the community composition of the samples at the genus level. Based on the annotated ASVs, we likewise performed alpha diversity analysis (Chao1, Shannon, Simpson, and ACE) for each treatment group, as well as analyzed the Beta diversity of different treatment groups using the PLS-DA method and utilized R for the comparison of keystone species differences, and the analysis of species differences among groups. The LEfSe analysis (Linear discriminant analysis Effect Size) was conducted based on the cloud platform of BGI.

2.5. Transcription of target genes in GWM guts

The total RNA from the GWM midgut was extracted by the Trizol Up (ET111-01-V2, TransScript). Subsequently, 1 ng of these RNA from each sample were used to synthesize cDNA (AT311, TransGen Biotech). Lastly, the RT-qPCR was performed based on QuantStudio 5 Real-Time System (Applied Biosystems), using 2 × Master qPCR Mix SYBR Green I (TSE201, Tsingke)and other necessary reagents according to the protocols. Two-step reaction program for RT-qPCR setup was shown in Table S1.

To ensure experimental reproducibility, each treatment underwent evaluation in five biological replicates, each incorporating three technical replicates, and results were analyzed using the ddCT method. The sequences of primers employed in the RT-qPCR experiment are *Gloverin*, *Gallerimycin, 6-tox*, *Galiomicin*, and *Cecropin-D*. The translation elongation factor 1-alpha (*EF1α*)was employed as a housekeeping gene for normalization of target gene expression levels (Krams et al., 2017). All the primers of these genes are presented in Table S2.

2.6. Statistical analysis

The data distribution normality assumptions for the survival rate, cumulative food consumption, and the transcription level of target genes of GWM were analysed using the Kolmogorov-Smirnov test. Intergroup statistical differences for the aforementioned experimental parameters were determined by the Kruskal-Wallis test or one-way analysis of variance (ANOVA), followed by Turkey's post-hoc test, using SPSS 26.0 software. Survival analysis was conducted using the Log-Rank test implemented within the R package 'survminer' (V.4.2.2), and the p-values were adjusted using the Benjamini-Hochberg method to account for a 5 % false-discovery rate (FDR).

The microbiome analysis of the GWM was detailed in Section 2.4.

Correlations between gut microbes and differential AMPs gene expression from the GWM

guts were performed at the genus level using the R function 'Cor' based on Spearman correlation coefficient analysis.

3. Results

3.1. Result of individual exposure experiment

According to Fig. 1A1, n the CH single exposure experiment, no significant difference in survival rate was observed between the 0.1 mg/kg CH treatment group and the blank control group ($p = 0.52$). The seven-day survival rate was 90.00% ± 5.00% for the CH treatment group and 93.33% ± 11.55% for the control group. However, when the concentration increased to 1 mg/kg, the survival rate decreased significantly to 60.00% ± 5.00%, compared to the control group ($p < 0.05$). The survival rates under exposure to 10 mg/kg and 25 mg/kg CH were 55.00% ± 5.00% and 48.33% ± 5.77%, respectively, with no significant difference from the 1 mg/kg group ($p = 0.52$, $p = 0.22$). Exposure to 50 mg/kg and 100 mg/kg CH significantly reduced the survival rates to 26.67% ± 2.89% and 18.33% ± 7.64%, respectively, compared to both the blank control and the 1 mg/kg CH treatment groups ($p < 0.05$).

Differences in feeding behavior among the CH treatment groups were also evident. The blank control group exhibited the highest average daily intake, at 85.73 ± 18.41 mg/GWM/Day, with no significant variation in daily intake. The 1 mg/kg CH treatment group had the next highest intake, at 75.09 ± 38.13 mg/GWM/Day. As shown in Fig. 1A2. when the CH concentration was less than 1 mg/kg, there was no significant impact on the overall feeding behavior of GWM. However, when the CH concentration exceeded 1 mg/kg, the feeding rate of GWM significantly decreased, averaging 5.25 ± 2.95 mg/GWM/Day.

According to Fig. 1B1, in the MET single exposure experiment, there was no significant difference in GWM survival rates for the 0.1%, 0.25%, 0.5%, and 1% MET treatment groups compared to the blank control group ($p = 0.57$, $p = 0.59$, $p = 0.70$, $p = 0.11$). After seven days of exposure, the survival rates were 93.33% ± 5.77% for the control group, meanwhile, 96.67% ± 5.77%, 90.00% ± 13.23%, 95.00% ± 5.00%, and 78.33% ± 2.89% for the 0.1%, 0.25%, 0.5%, and 1% MET treatment groups, respectively. The 2% MET treatment group exhibited a survival rate of 76.67% ± 12.58%, which was significantly different from the control group ($p < 0.05$)."

As indicated in Fig. 1B2, the average daily intake for the 0.1%, 0.25%, and 2% MET treatment groups showed no significant difference compared to the control group ($p = 0.773$, $p = 0.786$, $p = 0.535$), with intake rates of 86.61 ± 37.60, 87.57 ± 30.73, and 82.73 ± 23.48 mg/GWM/Day, respectively. The control group exhibited an average daily intake of 86.81 ± 17.90 mg/GWM/Day. However, significant differences were observed in the average daily intake for the 0.5% and 1% MET treatment groups compared to the control group ($p < 0.05$), with the 1% MET treatment group showing a consistent average daily intake of 72.1487 ± 16.69 mg/GWM/Day throughout the entire exposure period.

Overall, in the CH single exposure experiment, a concentration of 1 mg/kg CH significantly reduced the survival rate of GWM to 60%, while only inhibiting the feeding behavior of GWM in the later stages of exposure. Although concentrations higher than 1 mg/kg CH could increase mortality rates, the feeding behavior of larvae was strongly suppressed, which is unfavorable for the effective intake of MET in mixed experiments. In the MET single exposure experiment, both the 1% and 2% MET treatments reduced GWM survival rates to below 80% within seven days, with no significant difference between them. Additionally, it was found that methionine

concentrations above 3% were difficult to completely dissolve in water at room temperature. Considering the lethal effects of both compounds on GWM, ensuring the total intake of active substances, and practical applicability, two CH levels (0.1 mg/kg and 1 mg/kg) and three MET levels (0.5%, 1%, and 2%) were selected for the mixed exposure experiments to determine the optimal compound concentration.

3.2. Results of mixed experiment

From Fig. 1C1, during the mixed exposure experiment, the survival rate of the blank control group was 95.00% ± 5.00%, and the treatment groups of 0.1 mg/kg CH compounded with 0.5%, 1%, and 2% MET all significantly reduced the survival rate of GWMs compared to the blank control ($p < 0.05$), which were 71.67% ± 10.41%, 61.67% ± 2.89% and 58.33% ± 15.28%, respectively. In contrast, in the 1 mg/kg CH level, the difference between the 1 mg/kg CH + 0.5% MET treatment group and the 0.1 mg/kg + 2% MET treatment group was not significant, with the former having a survival rate of 43.33% ± 11.55%. In contrast, the GWM survival rate of the 1 mg/kg CH compounded with 1% or 2% MET was significantly lower in the former than in the compounded treatment at the 0.1 mg/kg CH level, with the GWM survival rates of the 1 mg/kg CH + 1% MET and 1 mg/kg CH + 2% MET treatment groups being 28.33% ± 12.58% and 26.67% ± 7.64%, respectively. However, the difference in survival rate between the 1 mg/kg CH + 1% MET and 1 mg/kg CH + 2% MET treatment groups was not significant ($p = 0.96$).

Fig. 1C2 shows that in the mixed exposure experiment, the average daily food consumption for the blank control group was 111.89 ± 15.90 mg/GWM/Day. All mixed treatment groups significantly reduced GWM food consumption. The average food consumption for all mixed treatments at the 0.1 mg/kg CH level was 90.95 ± 19.49 mg/GWM/Day, significantly different from the control group ($p < 0.05$). The average food consumption for all mixed treatments at the 1 mg/kg CH level was significantly lower than those at the 0.1 mg/kg CH level ($p < 0.05$), with an average of 69.04 ± 35.85 mg/GWM/Day. Additionally, food consumption in the 1 mg/kg CH treatment groups showed a decreasing trend over time, with an average of 120.79 ± 21.81 mg/GWM on the first day and only 16.13 ± 7.54 mg/GWM on the seventh day.

Through the mixed exposure experiment, the 1 mg/kg CH + 1% MET treatment group significantly reduced GWM survival and food consumption over seven experiment days compared to other combined treatments. Although the 1 mg/kg CH + 2% MET group exhibited higher toxicity, the difference between them was not significant. Considering both cost and efficacy, the 1 mg/kg CH + 1% MET combination was identified as the optimal concentration for controlling GWM. microbiome research will future investigate the impact of this combination on GWM gut microbiota structure.

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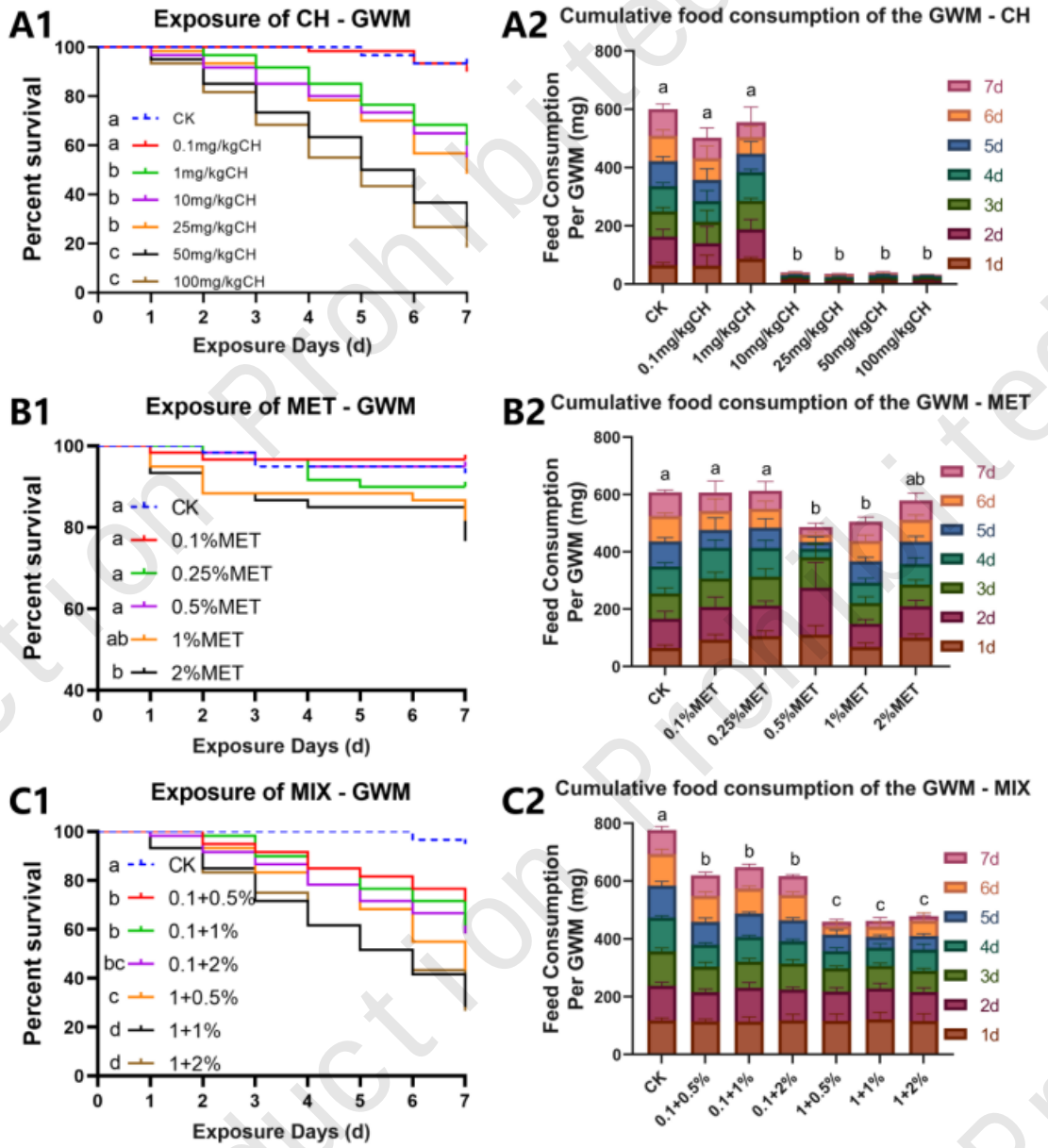

Fig. 1. The results of the exposure experiment with GWM. (A) The impact of CH exposure alone for 7 days on the survival rate and feeding behavior of GWM (N = 6, n = 6*10). (B) The impact of MET exposure alone for 7 days on the survival rate and feeding behavior of GWM (N = 6, n = 6*10). (C) The impact of combined exposure to CH and MET for 7 days on the survival rate and feeding behavior of GWM (N = 6, n = 6*10). CK: Blank control. All data were recorded daily. Statistical analysis was performed using the Kruskal-Wallis test, survival analysis using the Log-Rank test, with Benjamini-Hochberg correction for p-values. Different letters indicate significant differences between two groups, while the same letters indicate no significant differences ($\alpha = 0.05$).

3.3. Microbiome profiles of the GWM hindgut

3.3.1. Effects of CH and MET exposure on gut microbial diversity and abundance in the GWM

The effects of various treatments on the gut microbial diversity and abundance of the GWM were evaluated using alpha diversity indices, including Shannon, Simpson, Chao1, and Ace. The experimental groups consisted of the blank control (CK), 1% MET exposure group (MET), 1

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mg/kg CH exposure group (CH), and the mixed exposure group of 1 mg/kg CH and 1% MET (MIX). The Shannon and Simpson indices measure microbial community diversity, while the Chao1 and Ace indices reflect microbial community richness. As illustrated in Fig. 2A, the Shannon index of the MIX treatment group was significantly higher compared to the CH treatment group (2.28 ± 0.54 vs. 1.34 ± 0.26, $p < 0.05$). Conversely, the Simpson index (Fig. 2B) was significantly lower in the MIX group than in the CH group (0.19 ± 0.07 vs. 0.62 ± 0.14, $p < 0.05$), aligning closely with CK (0.19 ± 0.07 vs. 0.39 ± 0.09, $p < 0.05$). Additionally, as dipicted in Figure 2-2C and D, the MIX treatment led to a substantial increase in Chao1 and Ace indices relative to the CH treatment group (137 ± 30.32 vs. 91.20 ± 28.86, $p < 0.05$) and (137 ± 30.32 vs. 91.23 ± 28.84, $p < 0.05$), respectively. Consequently, GWM exhibited a notable elevation in gut microbial diversity and abundance when exposed to a mixture of CH compared to CH alone.

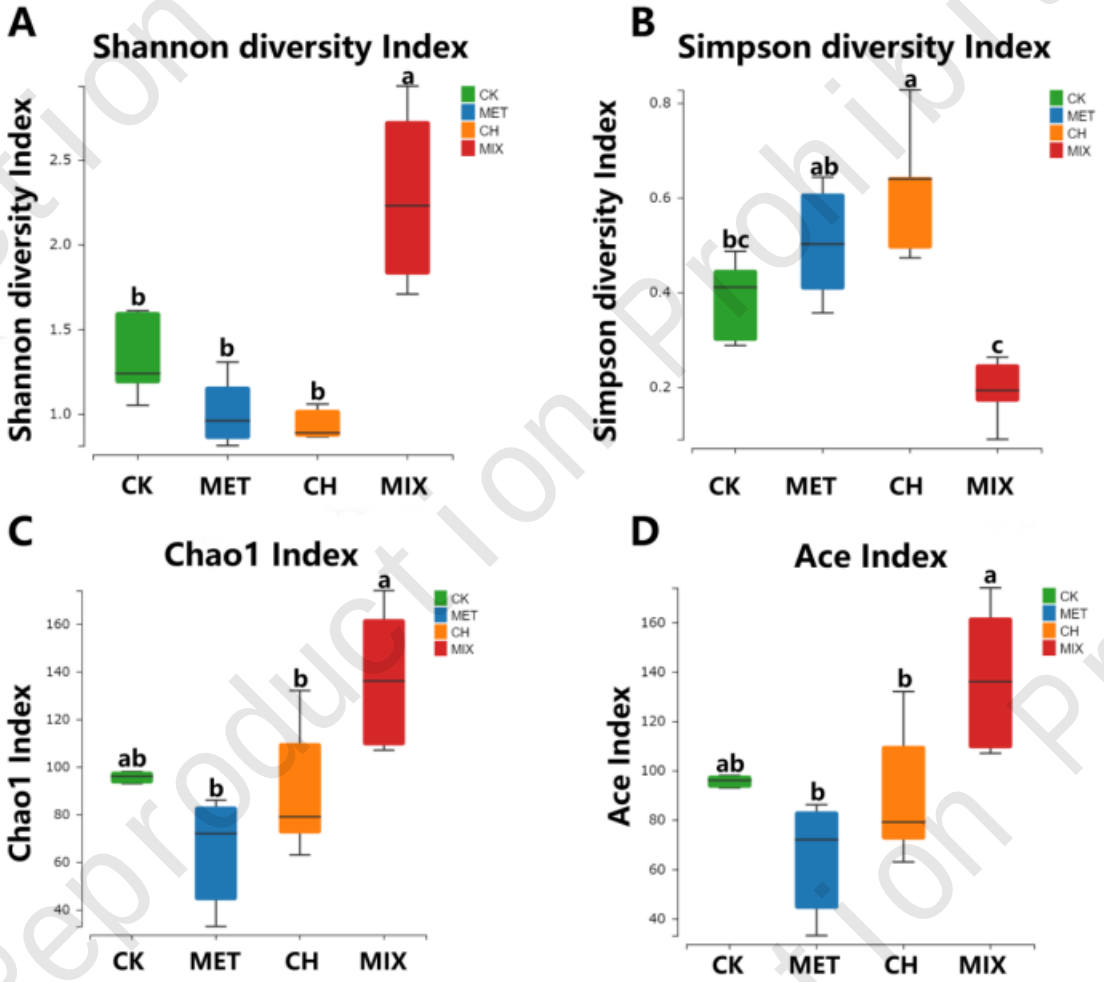

Fig. 2. Boxplot of alpha-diversity indices of the gut microbial community in GWM exposed by CK, MET, CH and MIX. GWM were orally administrated with CK, MET, CH and MIX for 7 d, and digest from the gut was collected for 16S rRNA sequencing. (A) The Shannon and (B) Simpson indices show the ASV diversity and (C) Ace and (D) Chao1 indices show the ASV abundance in all samples (n = 5 in each group). Box plots depict the medians (central horizontal lines), interquartile ranges (boxes), and 95 % confidence intervals (whiskers). A one-way ANOVA followed by a Turkey post-hoc test was used, different letter marks indicate significant differences between each two groups,

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while the same letter does not ($\alpha = 0.05$).

3.3.2. Alterations in the gut microbial composition of the GWM by exposure to CH and MET

In Fig. 3A, following denoising by the DADA2 method, a total of 776 ASVs were identified across all samples, with 46 ASVs common to the CK, MET, CH, and MIX treatment groups. Additionally, the Venn diagram illustrates that the CK treatment group exhibited 81 unique ASVs, while the MIX group demonstrated the highest count of unique ASVs among all groups, reaching 268. Partial Least Squares Discriminant Analysis (PLS-DA) enables discrimination of the relative similarity of microbial compositions among different treatment groups. As shown in Fig. 3B, the results of PLS-DA illustrate distinct gut microbial compositions among various treatment groups, indicating the presence of unique microbial community structures within each group, implying differential impacts of treatments on the gut microbiota of GWM. The analysis of microbial community relative abundances can elucidate the overarching effects of different treatments on GWM gut microbiota, thus, the relative abundances of microbial communities in the four treatment groups were examined. As illustrated in Fig. 3C, at the genus level, *Enterococcus spp.* emerged as the dominant genus in GWM, constituting 96.47% in CK, 97.99% in MET, and 96.57% in CH, while its prevalence was notably lower in MIX, accounting for only 67.43%. Concurrently, differences were observed among the groups in other key genera such as *Pseudomonas spp.* and *Acinetobacter spp.* In the CH group, the proportions of these genera were *Pseudomonas spp.* (0.61%) and *Acinetobacter spp.* (0.04%). Notably, unlike *Enterococcus spp.*, the relative abundances of these genera in the MIX group were elevated, with *Pseudomonas spp.* at 14.93% and *Acinetobacter spp.* at 5.69%. To further identify biomarker genera, Linear Discriminant Analysis Effect Size (LEfSe) was employed to examine the primary changes in gut microbiota and discern the differentially abundant genera across different treatment groups. As illustrated in Fig. 3D, *Enterococcus spp.* exhibited significant differences among the groups, presenting the highest differential contribution value among all genera (LDA: 5.13, $p < 0.01$), and was notably enriched in the MET group. Moreover, the Kruskal-Wallis test revealed that compared to CH, MIX treatment significantly reduced the abundance of *Enterococcus spp.* ($p < 0.05$). *Enterococcus spp.* is recognized as the core genus of GWM gut microbiota and is believed to be associated with the expression of GWM immune-related genes and defense against opportunistic pathogens. Consequently, the decreased abundance of *Enterococcus spp.* in the GWM gut may pose a threat to its survival.

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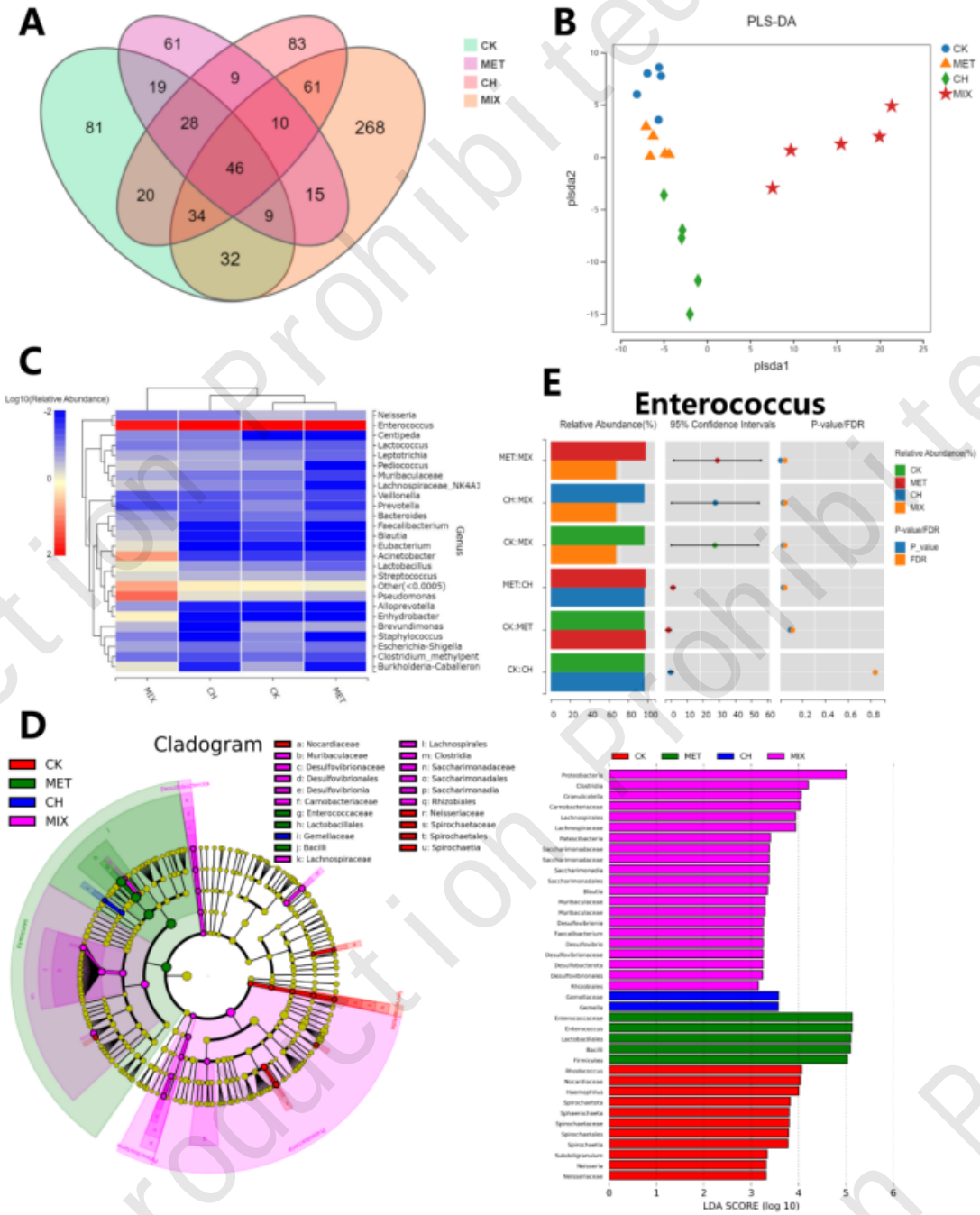

Fig. 3. MET, CH and MIX altered the gut microbiota composition of GWM. (A) The Venn diagram shows the distribution number of each observed ASVs. (B) The differences in microbial beta diversity of GWM gut under the CK, MET, CH and MIX exposure for 7 days based on partial least squares discriminant analysis (PLS-DA). (C) Heatmaps of the relative abundances at the genus level. The 25 most abundant genera of bacteria are shown, and genera not identified were categorized as "Other". (D) Linear discriminant analysis and effect size (LEfSe) analyses were used to compare the different relative abundance of bacterial taxa. CK (red), MET (green), CH (blue), and MIX (pink) were compared (LDA score threshold: 3.0). (E) Differences in the abundance of *Enterococcus* among different treatment exposures by Kruskal-Wallis test.

3.4. The expression of AMPs genes in GWM

The expression of AMPs genes of GWM has variated after exposure to the MET, CH, and

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MIX treatment. The transcription levels of the *Gloverin, Gallerimycin, 6-tox, Galiomicin,* and *Cecropin-D* genes were analyzed using ANOVA followed by the post-hoc Tukey test. As shown in Fig. 4A, MIX significantly reduced *Gloverin* expression levels compared to CK (0.43 ± 0.01 folds, $p < 0.01$), however, it did not significantly differ from MET and CH. In line with the *Gloverin* trend, MIX not only significantly reduced the expression level of *Gallerimycin* compared to CK (0.58 ± 0.05, $p = 0.02$), but also MET (0.56 ± 0.05, $p = 0.01$) from Fig. 4B. In contrast, expression levels of *6-tox* and *Galiomicin* were significantly lower after MIX treated compared to other groups, and specially, compared to CH, MIX reduced the *6-tox* and *Galiomicin* expression levels by 0.55 ± 0.08 folds ($p < 0.01$) and 0.53 ± 0.04 folds ($p < 0.01$), respectively, as illustrated in Fig. 4C and D. Regarding *Cecropin-D*, MIX was significantly reduced its expression levels compared to CK (0.48 ± 0.19, $p < 0.01$) and MET (0.59 ± 0.19, $p < 0.01$), but no significant changes relative to CH were observed.

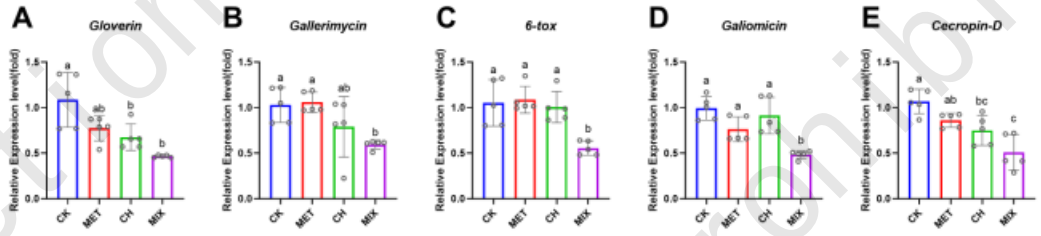

Fig. 4. Effects of MET, CH, and MIX on AMPs gene expression in midguts of GWM after chronic exposure. (A) *Gloverin*, (B*) Gallerimycin*, (C) *6-tox*, (D) *Galiomicin*, (E) *Cecropin-D*. The data presented are derived from five biological replicates for each treatment. Statistical analyses were conducted using ANOVA followed by the post-hoc Tukey test, where different letter notations indicate significant differences between groups, while the same letter does not ($\alpha = 0.05$). Error bars represent the standard deviation of fold changes.

3.5. Correlation between microbiome and AMPs expression of treated GWM

Among all microbial communities in the GWM gut, *Neisseria* and *Enterococcus* exhibited strong positive correlations with AMPs genes. Specifically, *Neisseria* was significantly more positively correlated with *Gallerimycin* ($r = 2.76$, $p < 0.01$) and *Cercropin-D* ($r = 2.52$, $p < 0.01$), whereas *Enterococcus*, the absolute dominant genus in terms of abundance in the GWM, was significantly more positively correlated with *6-tox* ($r = 2.68$, $p < 0.01$), and was also significantly positively correlated with *Gloverin* ($r = 2.24$, $p < 0.05$) and Cecropin-D ($r = 1.99$, $p < 0.05$). In contrast, *Blautia, Muribaculaceae, Alloprevotella, Escherichia.Shigella, Lactobacillus, Pediococcus, Leptotrichia, Pseudomonas* and *Burkholderia.Caballeronia.Paraburkholderia*, on the other hand, were negatively correlated with most of the AMPs genes. Notably, *Blautia* showed the strongest negative correlation with *Galiomicin* ($r = -1.83$, $p < 0.01$), *Lactobacillus*, as the sixth most abundant genus on average in the GWM gut, was significantly negatively correlated with both *6-tox* ($r = -1.54$, $p < 0.01$) and *Cecropin-D* ($r = -1.27$, $p < 0.05$), and *Pseudomonas*, the genus with the second highest mean abundance in the GWM gut, was also significantly negatively correlated with *Gallerimycin* ($r = -1.22$, $p < 0.05$).

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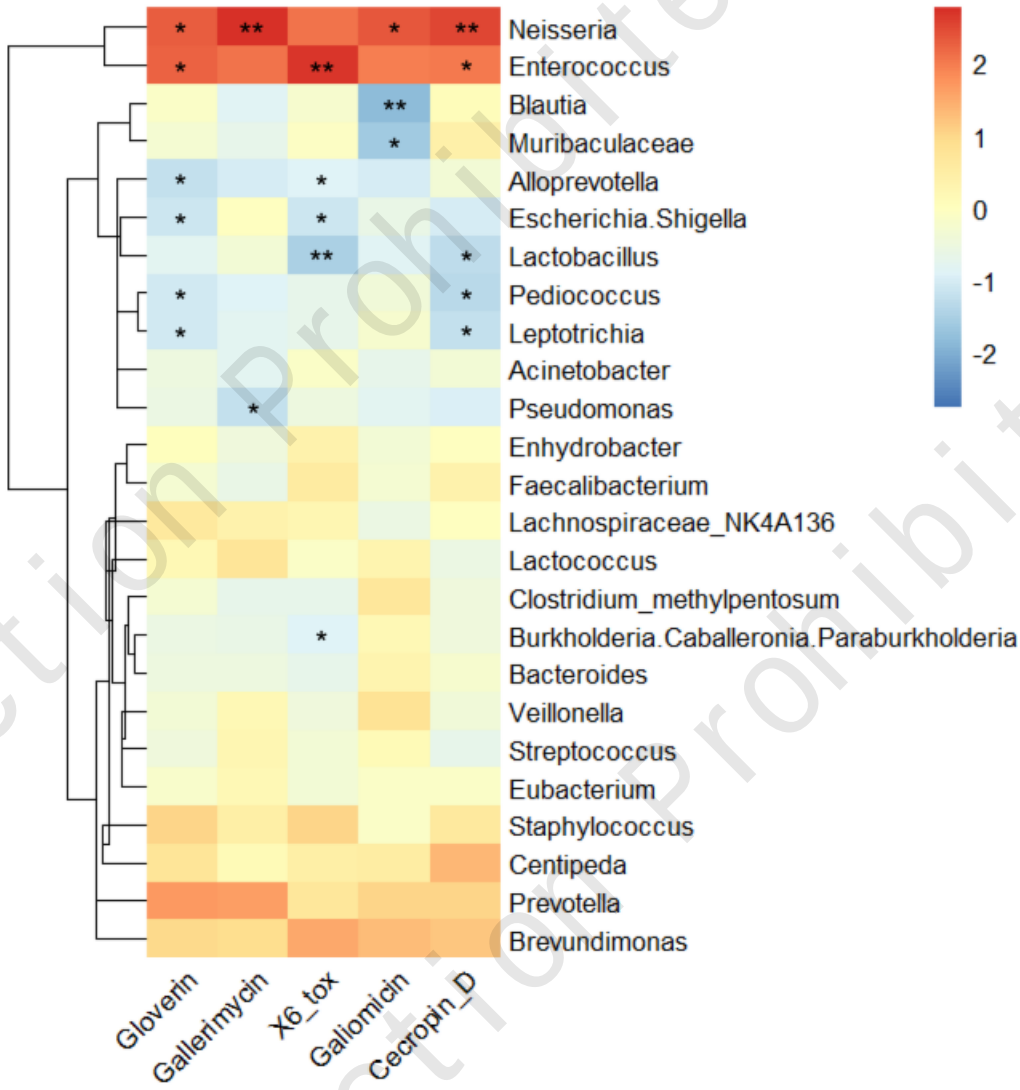

Fig. 5. **Heatmap of Spearman correlation coefficients summarizing the correlations between the relative abundance of gut microbes at the genus level and changes in AMPs genes expression level from the guts of GWM.** Using "median" as the clustering method, all five biological replicates were tested. The color intensity reflects the extent of correlation between alterations in gut microbiota composition and variations in AMP gene expression levels. "*" represents a p-value < 0.05, while "**" represents a p-value < 0.01.

4. Discussion

As a natural adversary of honeybees, the GWM stands as a notable contributor to the global decline in bee populations. Given the crucial role of bees in crop pollination and honey production, the presence of GWM imposes adverse effects on agricultural economic development. Furthermore, with economic development, there arises an escalating demand for both quantity and quality of food. Hence, the effective management of GWM in apiculture assumes critical significance in fostering economic growth and bolstering human well-being. Presently, conventional approaches to GWM control encompass the utilization of chemical insecticides such as CH or biological alternatives like *Bacillus thuringiensis* (Han et al., 2023). Nonetheless, the prolonged use of CH has sparked concerns regarding its sublethal impacts on non-target insects

and the emergence of pest resistance. Biological insecticides, although more environmentally benign and target-specific compared to chemical counterparts, suffer from limitations in terms of variety, efficacy, and application precision, thus contributing to the development of resistance among certain lepidopteran pests. Research has demonstrated the insecticidal properties of MET, a vital amino acid, against lepidopteran pests like the citrus swallowtail (*Princeps Papilio demoleus*) and the tobacco hornworm (*Manduca sexta L.*) (Lewis et al., 2011; Long et al., 2003; Quick and Stevens, 2001). Furthermore, combining pesticides has proven effective in delaying resistance, boosting efficacy, curbing pesticide usage, and reducing control expenses, thereby presenting an opportunity to enhance the efficacy of GWM control by combining MET with CH (Abbas et al., 2015; Ahmad et al., 2009; Khan et al., 2013). This study, conducted through individual exposure and mixed exposure experiments of CH and MET on GWM, determined the optimal combination concentration for comprehensive GWM control to be 1 mg/kg CH and 1% MET, significantly augmenting control efficacy against GWM larvae.

Significant alterations in gut microbes may be a critical factor in insects mortality. The intestinal microbiota of insects plays a crucial role in their immune system and normal life activities. For instance, in the model organism *Drosophila melanogaster*, individuals with a normal microbiota structure, including key genera like *Acetobacter* and *Lactobacillus*, are less susceptible to infections compared to those lacking these bacteria (Blum et al., 2013; Ridley et al., 2012). The gut microbiota continuously colonizes the host's intestines and provides ongoing benefits (Huang and Douglas, 2015; Kau et al., 2011; Masson et al., 2016). It competes with opportunistic microbes for nutrients and attachment sites on the intestinal epithelial surface. When external factors perturb the host, pathogens may replace the mutualistic microbes originally colonizing the gut, leading to inflammation, metabolic imbalance, or even death (Garrett et al., 2010). Additionally, the balance between microbial growth and nutritional processing efficiency in the gut is generally maintained by immune mechanisms, such as reactive oxygen species and antimicrobial peptide production (De Block and Stoks, 2008; Johnston and Rolff, 2015; Makarova et al., 2016; R. M. Jones et al., 2012). This regulatory mechanism is directly linked to the diet of the host. Studies have shown that exposure of GWM larvae to antibiotic-containing feed disrupts their gut microbiota and reduces the transcriptional activation of immune-related genes, resulting in decreased resistance to adverse environmental factors (Vilcinskas, 2013). In this study, the impact of feed treated with different drugs on the intestinal microbial structure of GWM larvae was significant. The analysis of alpha diversity also presents that MIX treatment significantly increased the diversity of larval gut microbiota. Compared to the control group and the group treated with CH, the MIX group significantly reduced the abundance of *Enterococcus spp.* in larval intestines while significantly increasing the abundance of other pathogenic bacteria such as *Pseudomonas spp.* As the dominant genus in the intestines of GWM, the abundance of *Enterococcus spp.* is positively correlated with the expression of some immune-related genes, such as *Gloverin*, which encode antimicrobial peptides (Johnston and Rolff, 2015). When the abundance of *Enterococcus spp.* is high, larvae upregulate the expression of immune-related genes, maintaining the balance of gut microbiota and alleviating the disturbance caused by adverse environmental factors to GWM larvae. Furthermore, *Enterococcus spp.* can secrete enterocins, which exhibit antimicrobial activity against certain Gram-positive bacteria in decay or pathogenic bacteria (De Vuyst and Vandamme, 1994). As an opportunistic pathogen, *Pseudomonas spp.* usually exists in low numbers in insect bodies without causing disease. However, under external

environmental stress or when the insect's immune system is weak, *Pseudomonas spp*. typically causes multi-tissue infections and immune suppression in insects. Studies have shown that GWM larvae are highly sensitive to *Pseudomonas spp.*, with the median lethal dose (LD50) for GWM larvae being only a few dozen colonies (Chadwick, 1967; Jarrell and Kropinski, 1982; Qin et al., 2022; Stephens, 1962). In the present experiment, the abundance of *Pseudomonas spp.* was significantly increased in the intestines of MIX-treated GWM, becoming the second most abundant bacterium on average in the GWM intestines, which may be an important reason for the increased mortality in GWMs

As natural molecules with a wide range of bactericidal activities, Antimicrobial peptides (AMPs) are important regulators of microbiota diversity and abundance in the GWM gut(Zhang and Gallo, 2016). Among all the AMP genes tested in this study, *Gloverin*, an inducible antimicrobial peptide, exhibited strong activity against Gram-positive bacteria (Zitzmann et al., 2017). Similarly, *Gallerimycin* demonstrated a marked fungicidal effect on filamentous fungi (Schuhmann et al., 2003). In contrast, *6-tox,* an atypical defensin-derived immune-related peptide, was specifically expressed in the midgut and acted against invading bacteria(Lee et al., 2010). *Galiomicin* showed potent antifungal activity, although it had limited antibacterial efficacy (Dekkerová-Chupáčová et al., 2018). *Cecropin-D* displayed vigorous antibacterial activity against Gram-negative bacteria and fungi, but its efficacy against Gram-positive bacteria was limited (Oñate-Garzón et al., 2017). According to the results of the microbiome profile, the abundance of *Enterococcus spp.* in the MIX-treated GWM intestines followed the same trend as that of all immune genes, and its abundance was significantly down-regulated compared to the CK-treated group. In contrast, some opportunistic pathogenic bacteria, such as *Pseudomonas spp.* showed an opposite trend to the expression of AMPs. The abundance of some opportunistic pathogens was significantly up-regulated after MIX treatment. This phenomenon, combined with the results of toxicity tests, supports our previous conjecture that the complexation of MET and CH can affect the expression of immune-related genes in GWM, which in turn leads to a decrease in the abundance of *Enterococcus spp.* and an increase in the abundance of opportunistic pathogens, and causing harm to the GWM larvae.

5. Conclusion

This study demonstrates that the combination of MET and CH significantly reduces the survival rate of GWM larvae while down-regulating the expression of AMPs genes, which in turn inhibits the colonization of *Enterococcus spp.*, a symbiotic bacterium in the GWM larval gut, while increasing the abundance of opportunistic pathogens such as *Pseudomonas spp.* These findings offer new insights for pesticide combination innovation and the development of MET as a novel insecticide of GWM.

CRediT author statement

Zhaoyong Liu: Investigation, Methodology, Data curation, Validation, Visualization, Software, Formal analysis, Writing – original draft. **Zhao Dan:** Resources, Validation. **Yue Wei:** Funding acquisition, Conceptualization, Supervision, Writing – review & Editing, Resources, Validation. All authors reviewed the manuscript.

Funding declaration

Supported by Hebei Natural Science Foundation (C2020204129) and Research Program of

Basic Scientific Research Operating Expenses of Provincial Universities in Hebei Province (KY2023016).

Acknowledgement

We thank the State Key Laboratory of North China Crop Improvement and Regulation of Hebei Agricultural University for providing the experimental platform for this study.

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