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 significantly reducing the abundance of [redacted] the primary genus in GWM, and induced immune stress in GWM. This phenomenon led to

the proliferation of opportunistic pathogens such as [redacted] 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, 2004). 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

[redacted]

[redacted]

[redacted]

[redacted]

2.3. Individual and mixed exposure experiment

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

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[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

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.

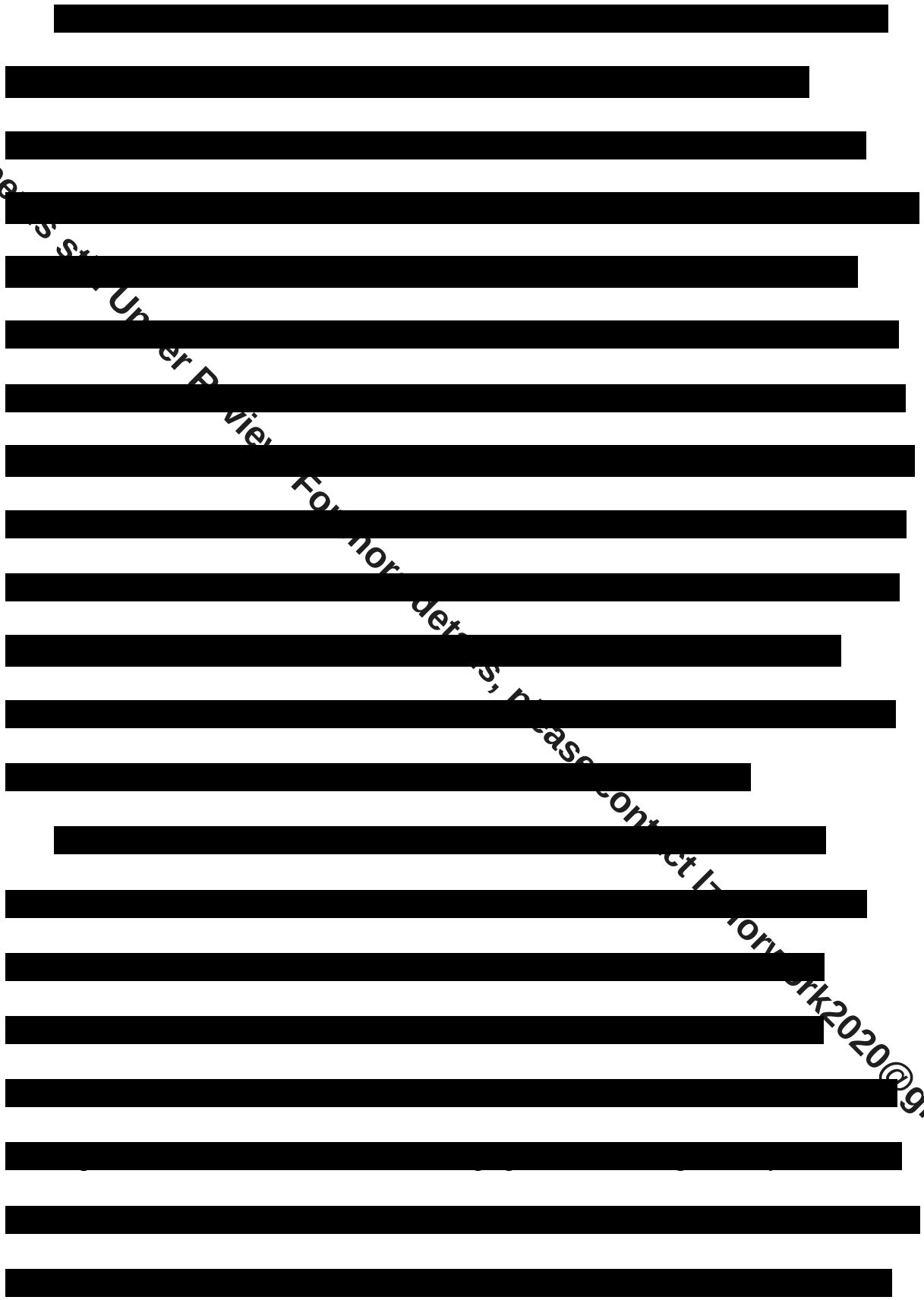
Correlations between gut microbes and differential AMPs gene expression from the GWM guts were performed at the genus level using the R function 'Cor' based on Spearman correlation coefficient analysis.

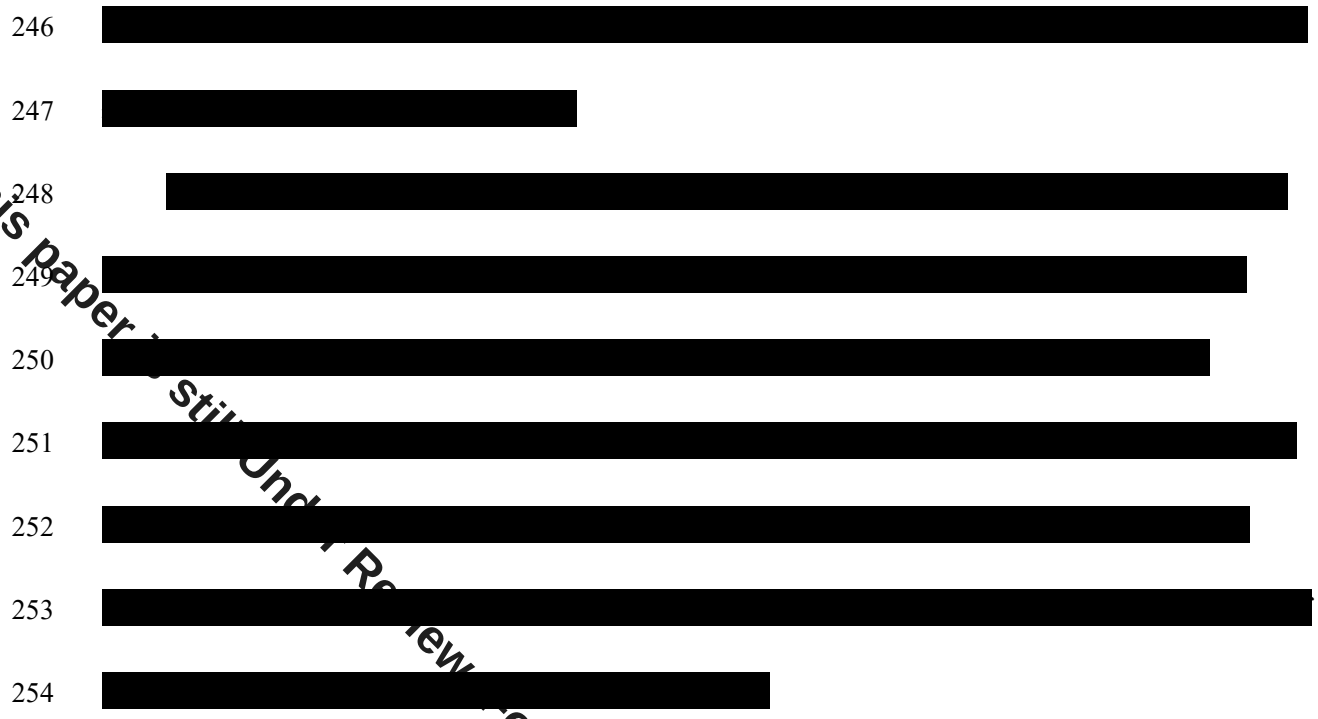
3. Results

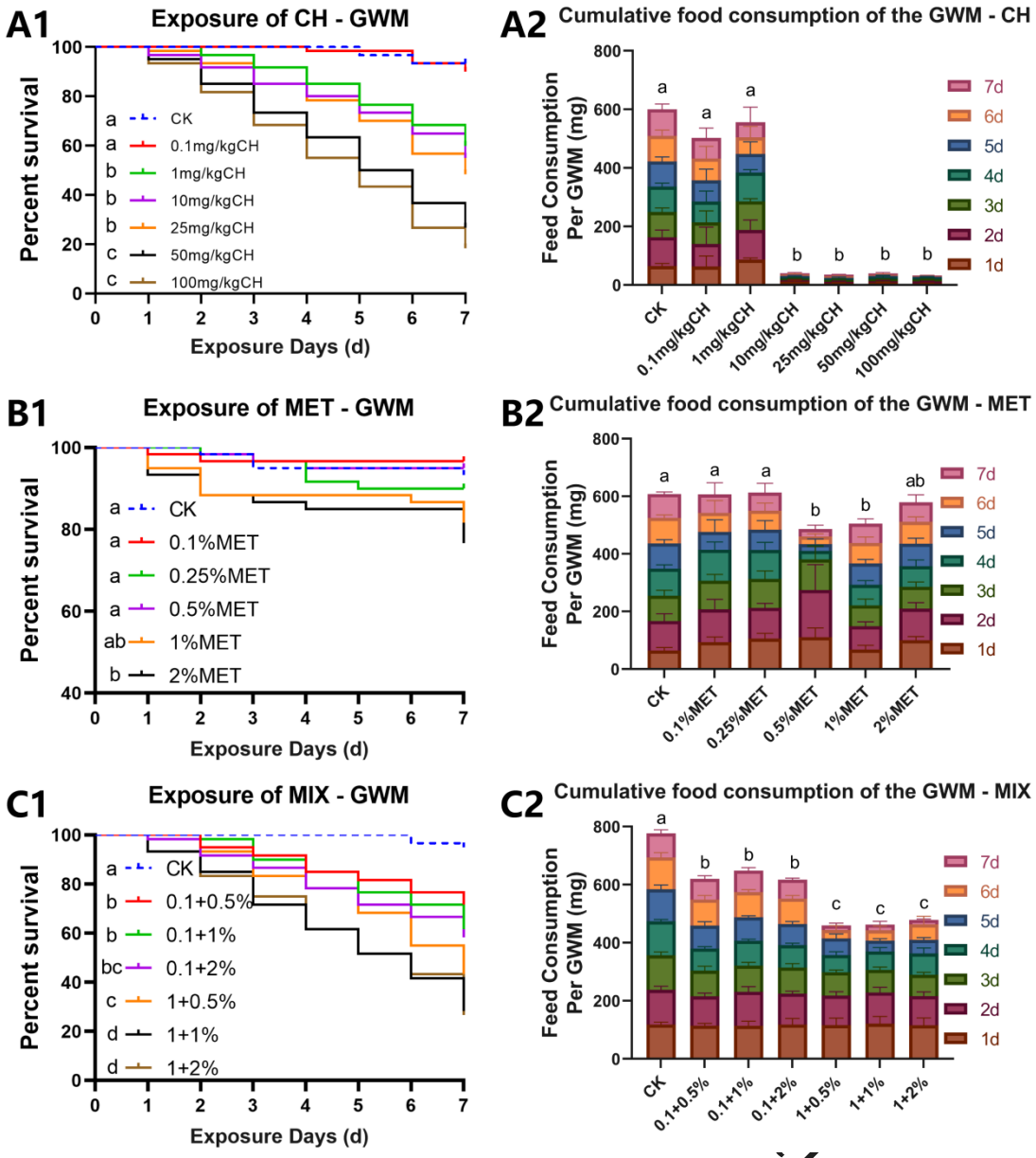
3.1. Result of individual exposure experiment

[redacted]

3.2. Results of mixed experiment







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256 **Fig. 1. The results of the exposure experiment with GWM.** (A) The impact of CH

257 exposure alone for 7 days on the survival rate and feeding behavior of GWM (N = 6, n =

258 6*10). (B) The impact of MET exposure alone for 7 days on the survival rate and feeding

259 behavior of GWM (N = 6, n = 6*10). (C) The impact of combined exposure to CH and MET

260 for 7 days on the survival rate and feeding behavior of GWM (N = 6, n = 6*10). CK: Blank

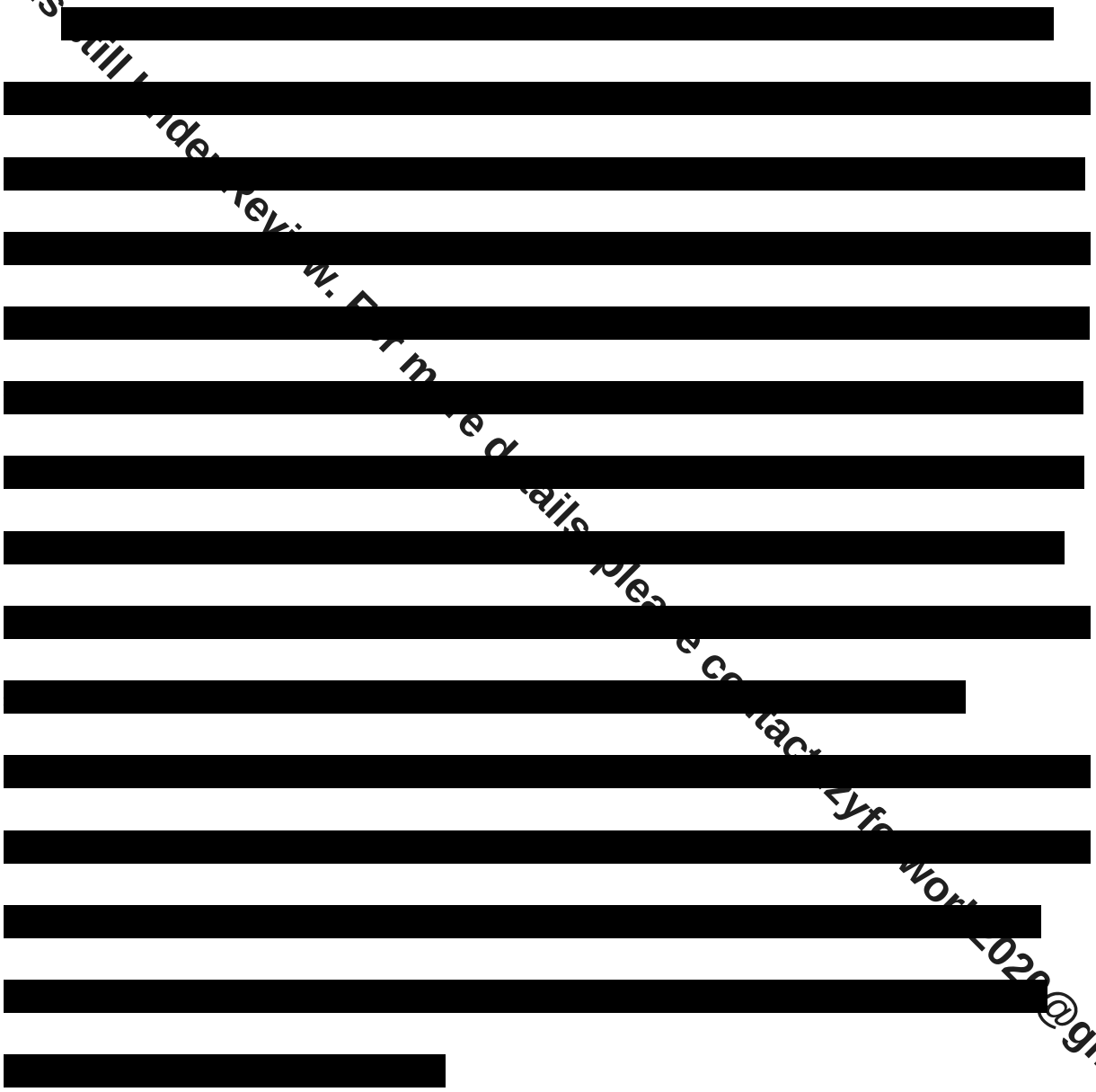
261 control. All data were recorded daily. Statistical analysis was performed using the Kruskal-

262 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



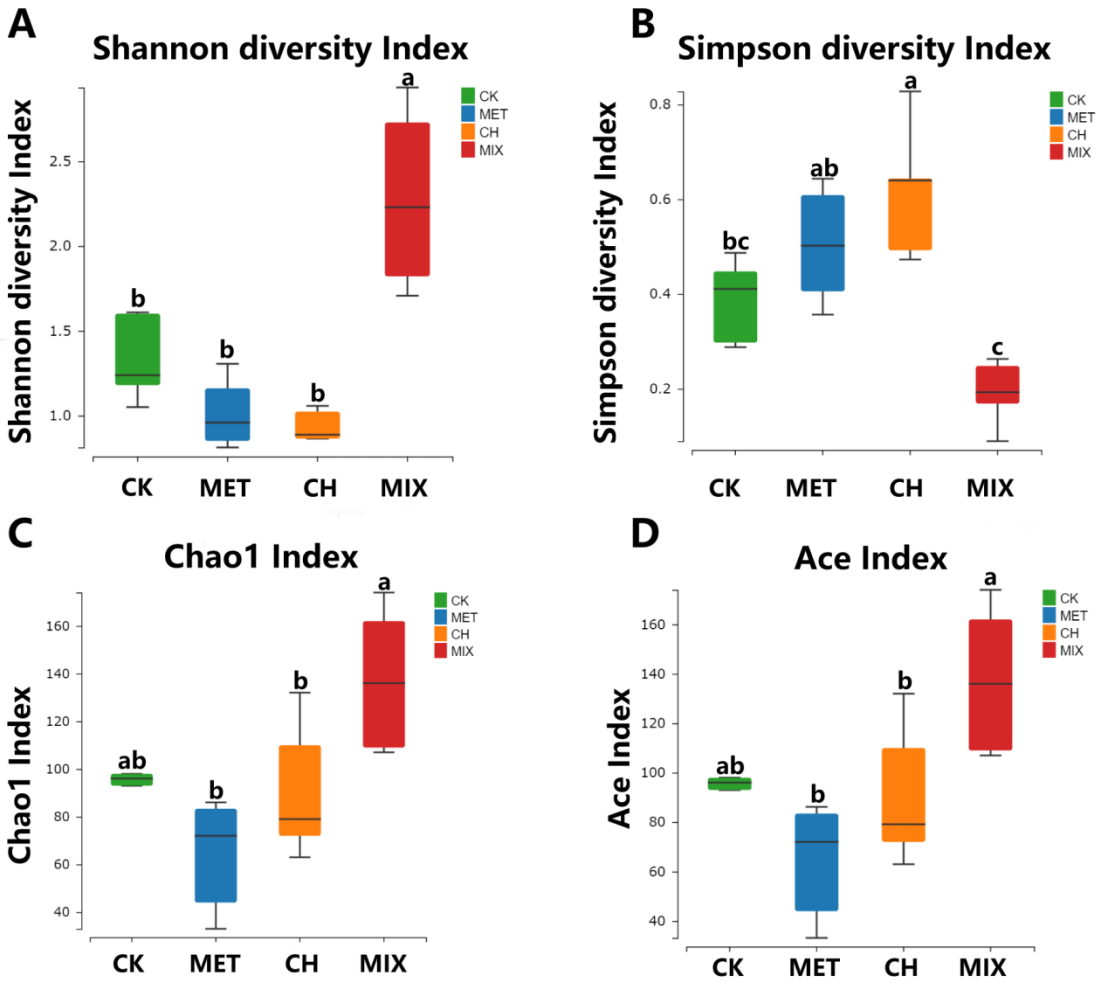
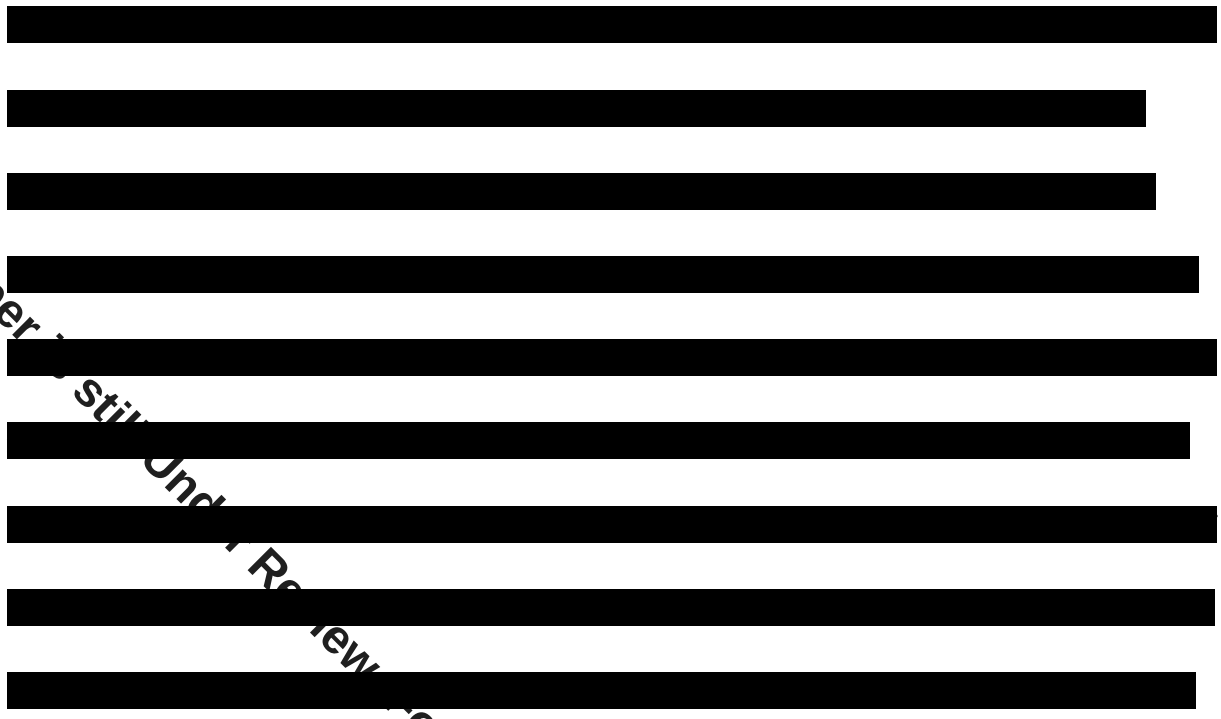


Fig. 2. Boxplot of alpha-diversity indices of the gut microbial community in GWM exposed by CK, MET, CH and MIX. GWM were orally administrated with CK, MET, CH and MIX for 7 d, and digest from the gut was collected for 16S rRNA sequencing. (A) The Shannon and (B) Simpson indices show the ASV diversity 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, 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



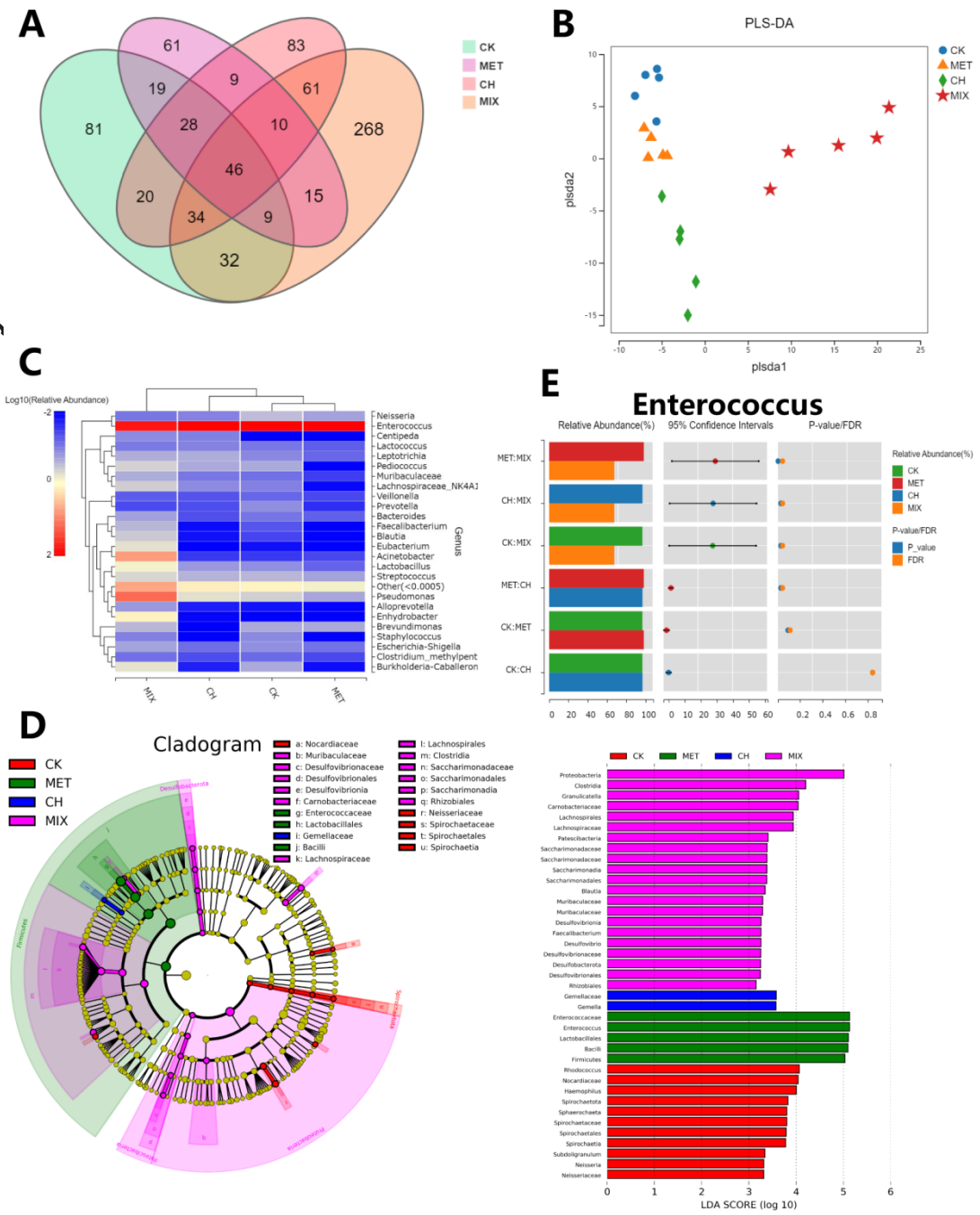
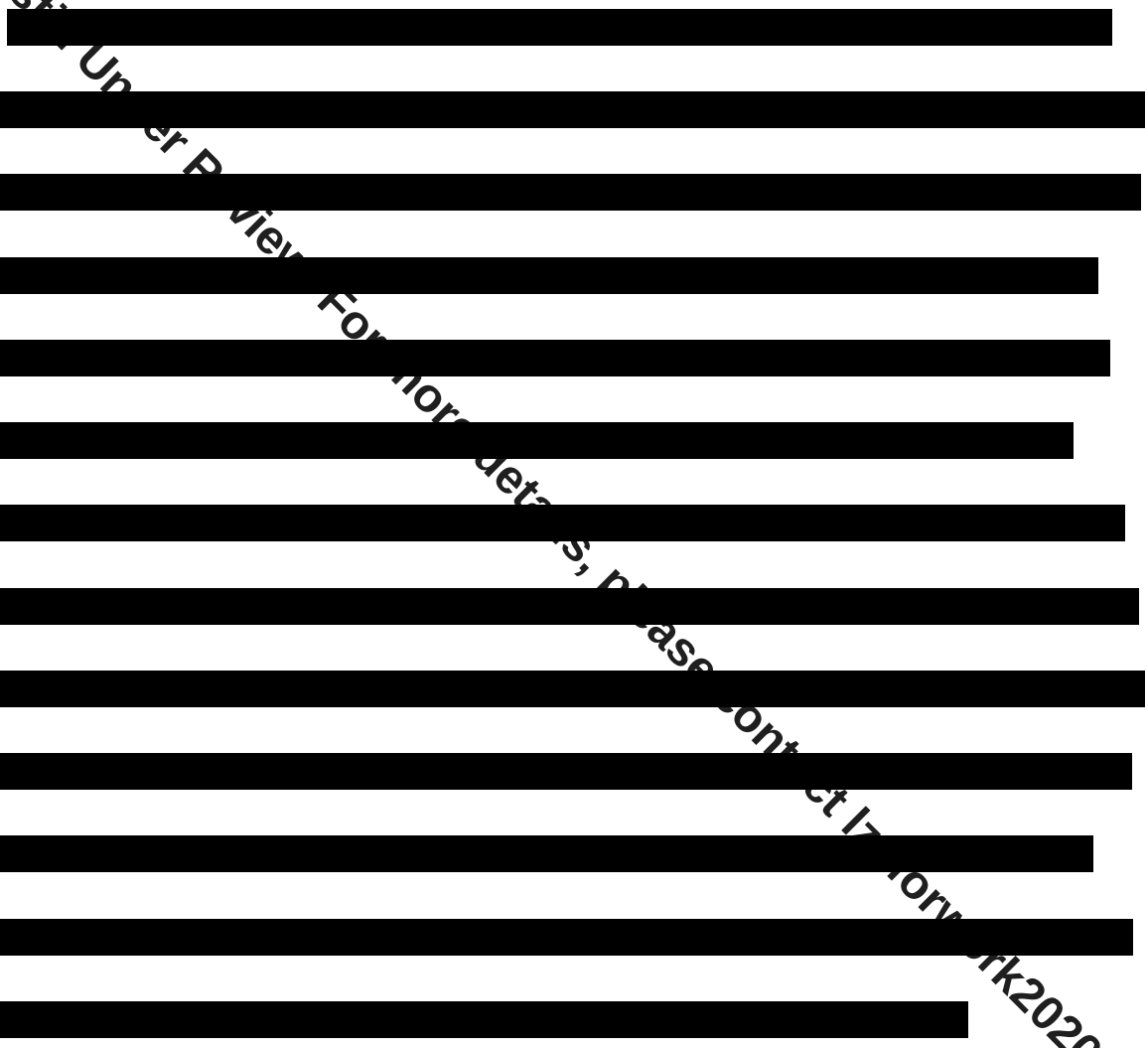


Fig. 3. MET, CH and MIX altered the gut microbiota composition of GWM. (A) The Venn diagram shows the distribution number of each observed ASVs. (B) The differences in microbial beta diversity of GWM gut under the CK, MET, CH and MIX exposure for 7 days based on partial least squares discriminant analysis (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



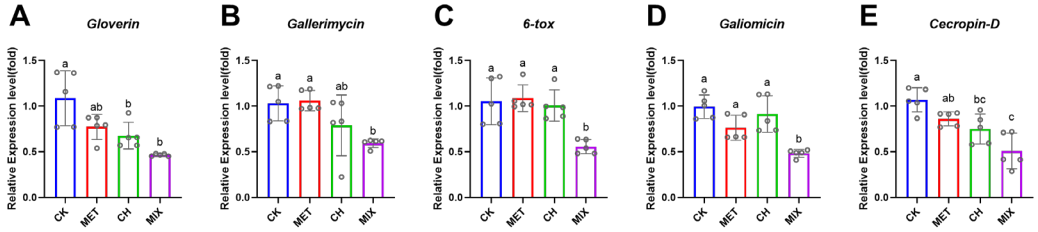


Fig. 4. Effects of MET, CH, and MIX on AMPs gene expression in midguts of GWM after chronic exposure. (A) *Gloverin*, (B*) Gallerimycin*, (C) *6-tox*, (D) *Galiomicin*, (E) *Cecropin-D*. The data presented are derived from five biological replicates for each treatment. Statistical analyses were conducted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

3.5. Correlation between microbiome and AMPs expression of treated GWM

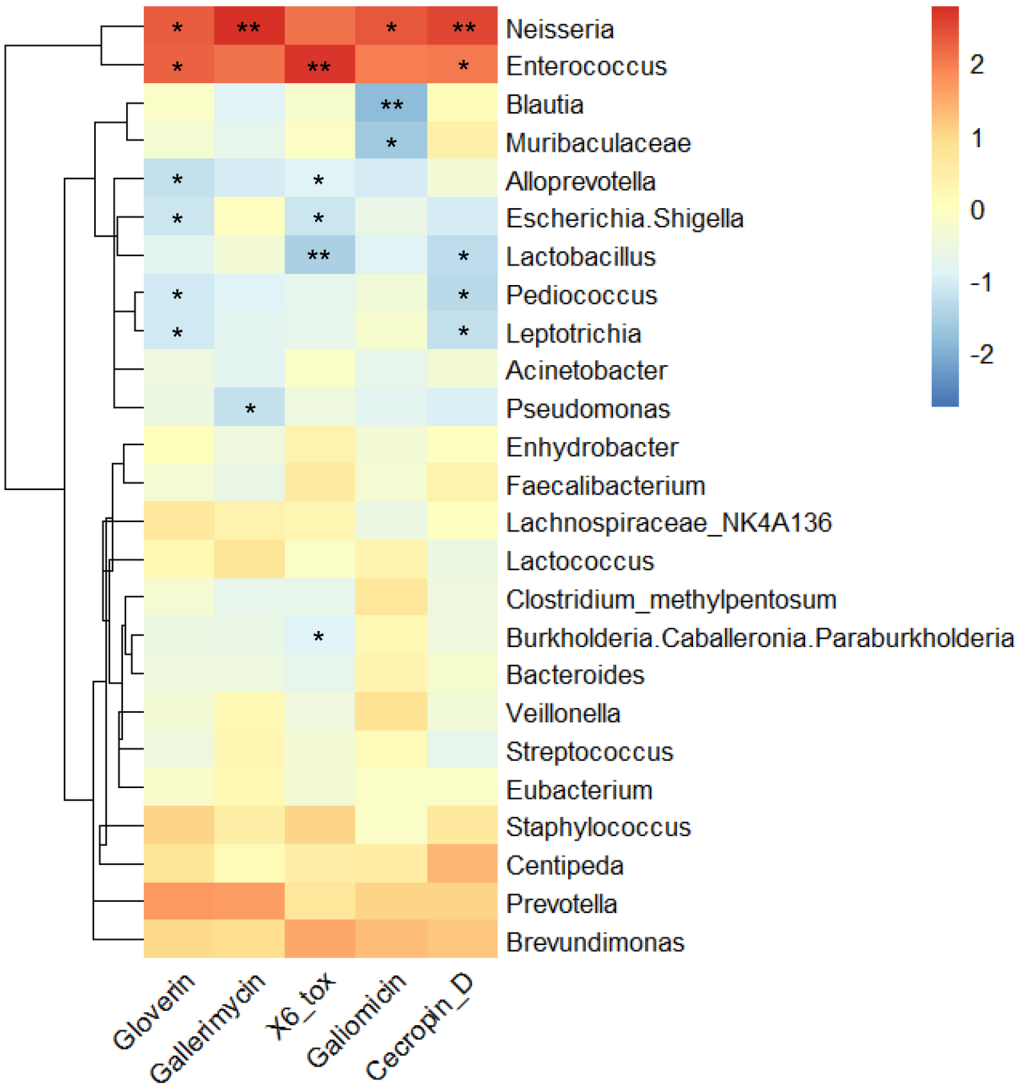


Fig. 5. Heatmap of Spearman correlation coefficients summarizing the correlations between the relative abundance of gut microbes at the genus level and changes in AMPs genes expression level from the guts of GWM. Using "median" as the clustering method, all five biological replicates were tested. The 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 [redacted]

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 [redacted]. in

larval intestines while significantly increasing the abundance of other pathogenic bacteria such as [redacted]. As the dominant genus in the intestines of GWM, the abundance of [redacted]. 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 [redacted] 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, [redacted] can secrete [redacted] which exhibit antimicrobial activity against certain Gram-positive bacteria in decay or pathogenic bacteria (De Vuyst and Vandamme, 1994). As an opportunistic pathogen, [redacted]. 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, [redacted] typically causes multi-tissue infections and immune suppression in insects. Studies have shown that GWM larvae are highly sensitive to [redacted], 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 [redacted] 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, [redacted] an inducible antimicrobial peptide, exhibited strong activity against Gram-positive bacteria

(Zitzmann et al., 2017). Similarly, [redacted]emonstrated a marked fungicidal effect on filamentous fungi (Schuhmann et al., 2003). In contrast[redacted], an atypical defensin-derived immune-related peptide, was specifically expressed in the midgut and acted against invading bacteria(Lee et al., 2010). [redacted] showed potent antifungal activity, although it had limited antibacterial efficacy (Dekkerová-Chupáčová et al., 2018). [redacted] 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 [redacted]. 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[redacted]. 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 [redacted] 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 1% MET and 1 mg/kg CH yields the most optimal integrated effect, significantly reduces the survival rate of GWM larvae while down-regulating the expression of AMPs genes, which in turn inhibits the colonization of [redacted] a symbiotic bacterium in the GWM larval gut, while increasing the

abundance of opportunistic pathogens such [redacted]. 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.

Acknowledgement

This study was supported by Hebei Natural Science Foundation (C2020204129) and Research Program of Basic Scientific Research Operating Expenses of Provincial Universities in Hebei Province (KY2023016).

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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