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Heavy metal exposure reduces larval gut microbiota diversity of the rice striped stem borer, *Chilo suppressalis*

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Abstract

Cadmium (Cd), a widely distributed environmental pollutant in agroecosystems, causes negative effects on crops and herbivores through bottom-up processes. The gut microbial community of an insect can play a critical role in response to metal stress. To understand how microbiota affect the stress responses of organisms to heavy metals in agroecosystems, we initially used 16S rRNA sequencing to characterize the larval gut microbiota of *Chilo suppressalis*, an important agricultural pest, exposed to a diet containing Cd. The species richness, diversity, and composition of the gut microbial community was then analyzed. Results revealed that while the richness (Chao1 and ACE) of gut microbiota in larvae exposed to Cd was not significantly affected, diversity (Shannon and Simpson) was reduced due to changes in species distribution and relative abundance. Overall, the most abundant genus was *Enterococcus*, while the abundance of the genera *Micrococcaceae* and *Faecalibaculum* in the control significantly superior to that in Cd-exposed pests. Phylogenetic investigation of microbial communities by the reconstruction of unobserved states (PICRUSt) showed that the intestinal microorganisms appear to participate in 34 pathways, especially those used in environmental information processing and the metabolism of the organism. This study suggests that the gut microbiota of *C. suppressalis* are significantly impacted by Cd exposure and highlights the importance of the gut microbiome in host stress responses and negative effects of Cd pollution in agroecosystems.

Keywords Heavy metal · *Chilo suppressalis* · Gut microbiota · Stress response · Bottom-up effect

Introduction

Heavy metal pollution is a serious and widespread problem worldwide (Lefcort et al. 2010; Nogawa et al. 2019; Zhao et al. 2022), contaminating agricultural soils and interfering with plant growth, thereby threatening crop production and food safety (Horiguchi et al. 1994; Huybrechts et al. 2019; Liu et al. 2020; Yu et al. 2022). More importantly, heavy metals can be absorbed and accumulated in plants, and subsequently transferred to higher trophic levels (herbivores and natural enemies), triggering multiple indirect bottom-up effects along food chains (Butler and Trumble 2008; Han et al. 2022; Tibbett et al. 2021). The effects triggered by

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heavy metals are key ecological forces driving crop–arthropod community dynamics, ultimately impacting the fitness of organisms in agricultural systems (Dar et al. 2019; Han et al. 2022; Khan et al.2023).

Among heavy metals, Cd ranks 7th in the list of the top 20 most damaging substances owing to its extraordinarily negative health effects (Faroon et al. 2012; Xiao et al. 2013). Notably, Cd may be detrimental to the survival, development, behavior, and population dynamics of herbivores (Di et al. 2020; Lin et al. 2020; Yan et al. 2023). Moreover, accumulation in herbivores leads to this heavy metal cascading through the food chain thereby exposing higher-level organisms to the contaminant. The mild stress of Cd exposure can also induce hermetic effects in insects (Cutler et al. 2022; Wang et al. 2024; Wei et al. 2020). For example, Su et al. (2014) founded that low doses of Cd in artificial diets increase populations of *Spodoptera exigua* (Lepidoptera: Noctuidae), whereas high doses produced the opposite effect. However, very few studies have focused on specific mechanisms driving increased or reduced fitness of arthropods following exposure to Cd.

The gut microbiota makes crucial contributions to functions of host fitness, including development, digestion, behavior, and immune system (Dominguez-Bello et al. 2019; Li et al. 2024; Luo et al. 2021; Morais et al. 2021). In general, some gut microbiotas are necessary for herbivores' growth and reproduction (Bing et al. 2024; Li et al. 2022; Luo et al. 2019), but can also strongly affect resistance to environmental stressors (e.g. pesticide, Sharma et al. 2023) and even natural enemies (Frago and Zytynska 2023; Luo et al. 2022; Man et al. 2023). Herbivores suffer directly from bottom-up forces triggered by heavy metals in the environment (Dar et al. 2019; Tibbett et al. 2021) and although these contaminants may alter the composition of intestinal microbiota (Guo et al. 2023; Li et al. 2021; Wu et al. 2020), the effect of heavy metals on the composition and diversity of the intestinal microbial community has received relatively little attention.

Lepidopteran insects represent some of the most important pest species in agriculture and forestry, with eight species ranked in the top 20 insect pests in publications in Centre for Agriculture and Biosciences International (Royal Botanic Gardens 2017). These pests are geographically widespread and often have a large host range, and traits which could exacerbate bottom-up effects triggered by heavy metals. The rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), is one of the most destructive rice pests worldwide (Lu et al. 2019; Ma et al. 2020; Wang et al. 2021). Previous studies have indicated that exposure to Cd in artificial diets inhibits the fitness of *C. suppressalis* (Liu 2020; Huang et al. 2023) but understanding the mechanisms driving these negative effects have not been fully characterized. To understand how gut microbiota affect the stress responses of this pest to heavy metals in agroecosystems, we first used 16S rRNA sequencing to characterize the larval gut microbiota of *C. suppressalis* exposed to artificial diet containing Cd. Then, we analyzed the species diversity, richness, and composition of the gut microbial community and ultimately, discuss the potential role of gut microbiota in pest management under heavy metal stress in agroecosystems.

Materials and methods

Insect rearing

Chilo suppressalis females, initially collected in 2022 from rice fields (Nanchang County, Nanchang, Jiangxi Province, China), were maintained under laboratory conditions as described in detail in Huang et al. (2023). Larvae were reared on artificial diets and maintained at 28 ± 1 °C and 70 ± 5% relative humidity with a 16:8 h (L:D) photoperiod (Han et al. 2012). Newly hatched neonates were used for the next experiment.

Cadmium treatment

Based on data from the China Food Safety National Standard for Maximum Levels of Contaminants in Foods (SAMR and NHCC 2022), Cd concentrations in stalks of rice in paddies in China (Liu 2020), and a previously published study (Huang et al. 2023), we used the environmentally-relevant concentration of 1.0 mg/kg of $CdCl_2$ in artificial diets. These diets consisted of cadmium chloride ($CdCl_2$, Aladdin Bio-Chem Technology, China) or a distilled water control and were prepared following the protocols published in Huang et al. (2023). Fifty larvae were used in each treatment with three replications, representing 150 per treatment ($N = 3$). Ten 4th instar larvae (2nd day in 4th instar, 8 d after treatment) per replications were randomly selected for gut tissue collection.

Gut tissue collection

Samples were collected following protocols described by Chen et al. (2022). The larvae were surface-sterilized in 75% ethanol for 120 s and rinsed three times with sterile water for 30 s. The entire gut tissues were dissected in sterile petri dishes under a stereoscope (Stemi DV4, Zeiss, Germany) with sterile forceps, and the dissected midgut tissues were rinsed with sterile phosphate buffered saline (PBS, 0.01 M, Solarbio, Beijing). These samples were transferred into a sterile 2-mL Eppendorf tube containing 500 μL PBS and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent DNA extraction. These 10 larvae were then pooled as a biological sample, with 3 such pools (representing 3 biological replicates) being collected (N = 3).

DNA extraction and sequencing

DNA was extracted using a TGuide S96 Magnetic Soil/Stool DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's protocol and DNA quality was assessed using a fluorometer (synergy HTX, Gene Company Limited, Hong Kong). The V3 + V4 regions of 16S rRNA genes were amplified with the specific primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Kozich et al. 2013). The polymerase chain reaction (PCR) conditions followed those published by Wang et al. (2022): 98 °C for 30 s, followed by 10 cycles of 98 °C for 10 s, 65 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. Then PCR products were purified using VAHTSTM DNA clean beads (Vazyme, Nanjing, China). The PCR products were determined on 1.8% agarose gels, quantified by ImageJ (Schneider et al. 2012), and further purified using the E.Z.N.A. Cycle Pure Kit (Omega, Norcross, GA) according to the manufacturer's instructions. 150 ng per sample in each treatment was mixed to form a sequencing library. Illumina NovaSeq 6000 was used to sequence by Biomarker Technologies (Beijing, China).

Bioinformatic analysis

After sequencing, raw data were first analyzed using Trimmomatic (var. 0.33) (Bolger et al. 2014). Subsequently, primer sequence recognition and adapters removal were performed for high-quality reads using Cutadapt (var. 1.9.1) (Martin 2011). Paired-end reads were merged using USEARCH (var. 10.0) (Edgar 2013) based on 97% sequence similarity, and filtered at 0.005% of the total number of sequences. The chimera sequences were identified and removed using UCHIME (var. 8.1) (Edgar et al. 2011). After sequence assembling, amplicon sequence variants (ASVs) data were filtered to remove chimera, then divided with DADA2 (Callahan et al. 2016) in QIIME2 2020.6 (Bolyen et al. 2019) at an 80% confidence threshold. Paired reads were trimmed and filtered with a maximum of 2 expected errors per read. The RDP (ribosomal database project) classifier (http://rdp.cme.msu.edu/) was used to analyze the phylogenetic affiliation of each 16S rRNA gene sequence (ASVs) against the Silva (SSU132) 16S rRNA database using a confidence threshold of 70%. According to results of the taxonomic analysis, the species composition of different samples at each taxonomic level (including phylum, class, order, family, genus and species levels) was obtained and the relative abundance map of each species composition was drawn using R software (var. 3.3.1). Species with an abundance less than 1% were merged.

The reconstruction of unobserved states (PICRUSt) was used to generate a functional profile based on 16S rRNA data. All sequences were first reclassified to the Greengenes gene database (DeSantis et al. 2006) and an ASVs biom table was created using Mothur. Following this, normalized ASVs tables were generated using PICRUSt (Langille et al. 2013), and these were used to create a functional metagenome prediction file for each sample. The functional metagenome predictions files were analyzed using the "categorize by function" command (level 3) using the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways.

Diversity analyses

The diversity analyses followed those published by Wang et al. (2022). The alpha diversity was evaluated using QIIME2 (Bolyen et al. 2019), including ACE, Chao1 for species richness, and Shannon and Simpson Indices for species diversity. The beta diversity analysis, based on the weighted UniFrac distance, was analyzed by nonmetric multidimensional scaling (NMDS). Metagenomic biomarker discovery by way of class comparison, tests of biological consistency and effect size estimation were conducted by Line Discriminant Analysis Effect Size (LEfSe) (Segata et al. 2011).

Statistical analysis

A Student's t test was used to analyze alpha diversity indices between the control and each Cd treatment ($P < 0.05$). The Wilcoxon rank test was used to analyze abundance at the phylum and genus levels between the control and each Cd treatment ($P < 0.05$). SPSS statistical software (var. 25.0, IBM, USA) was used for statistical analyses.

Results

Sequencing and ASVs distribution

A total of 473,619 pairs of raw reads were obtained from 6 samples. A total of 73,281–80,271 clean reads were produced overall. High-quality reads were clustered into ASVs with a 97% identity, with 417–1176 ASVs produced for each sample (Supplementary Table S1).

Sample rarefaction curves were created and demonstrated that the number of ASVs gradually flattened and entered the plateau stage, indicating the sequencing depth used captured the majority of midgut bacterial diversity in

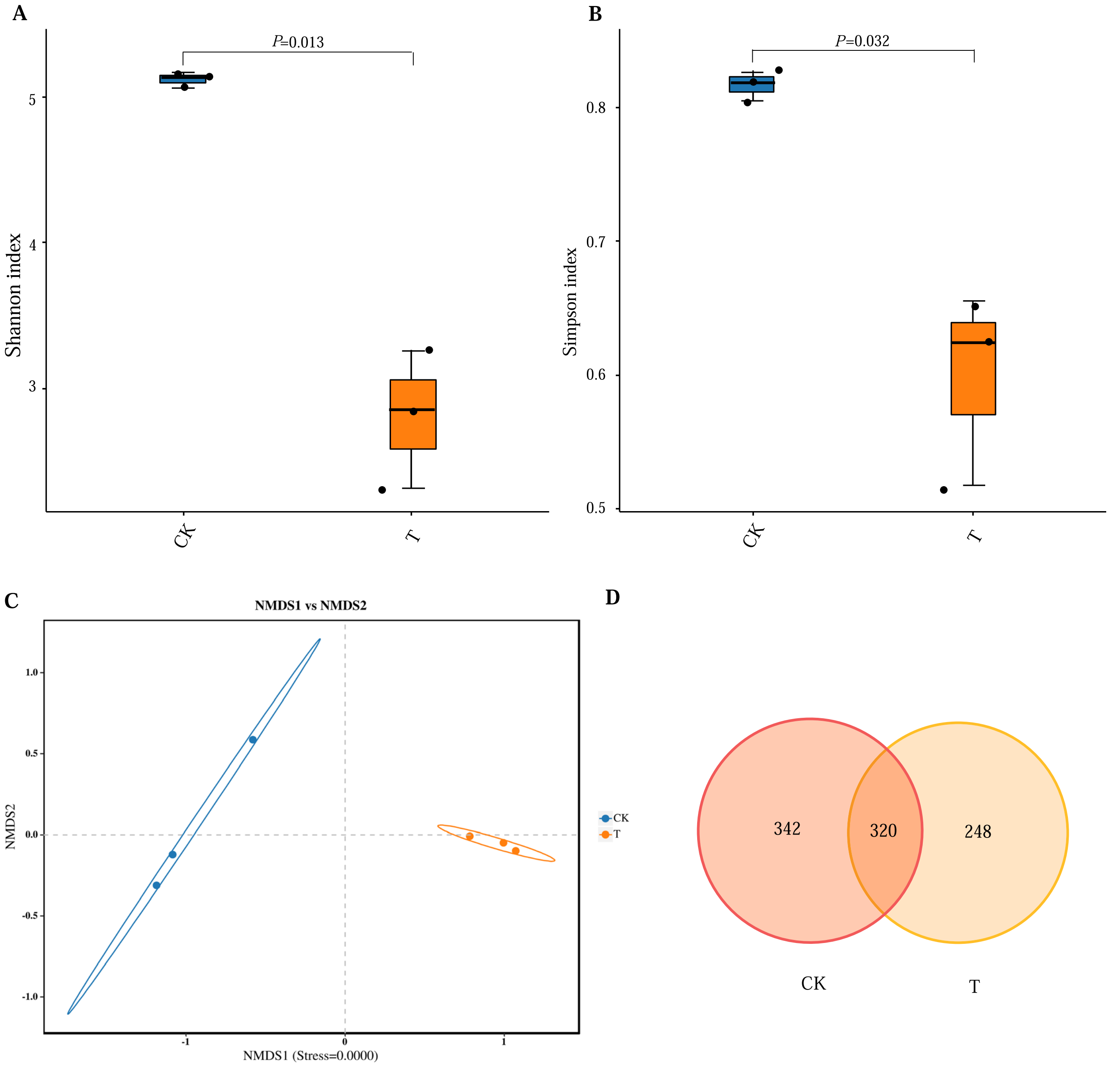

Fig. 1 Comparison of the diversity and composition of overall bacterial communities in cadmium-exposed and control larvae of *Chilo suppressalis*. **A** Shannon's diversity index; **B** Simpson's diversity index (Student's *t* test was used to compare the difference between control and treatment); **C** Nonmetric multidimensional scaling (NMDS) of bacterial communities; **D** Venn diagram comparing the numbers of genera of gut microbiota shared and unique in the control (CK) and cadmium exposed treatment (T)

the samples (Supplementary Fig. S1A). The Shannon index rarefaction curve also indicated the sequencing depth was sufficient to cover most of the microbial information in our study (Supplementary Fig. S1B).

Gut microbial diversity

The alpha diversity indexes were calculated based on ASVs table and were used to analyze species diversity and richness between Cd exposed and unexposed larvae. Although the richness (Chao1 and ACE) was not significantly affected (Supplementary Table S2, Chao1, $t = 2.515$, $df = 4$, $P = 0.066$, ACE, $t = 2.516$, $df = 4$, $P = 0.066$), diversity (Shannon and Simpson) was reduced due to changes in species distribution and relative abundance (Fig. 1A, B, Supplementary Table S2, Shannon, $t = 8.383$, $df = 4$, $P < 0.001$, Simpson, $t = 5.174$, $df = 4$, $P = 0.007$). Therefore, Cd exposure altered the composition and evenness of species in the intestinal microbiome but did not eliminate entire species. Additionally, the midgut microbiota in the control group differed significantly and was significant separately clustered from Cd-exposed larvae (Fig. 1C,

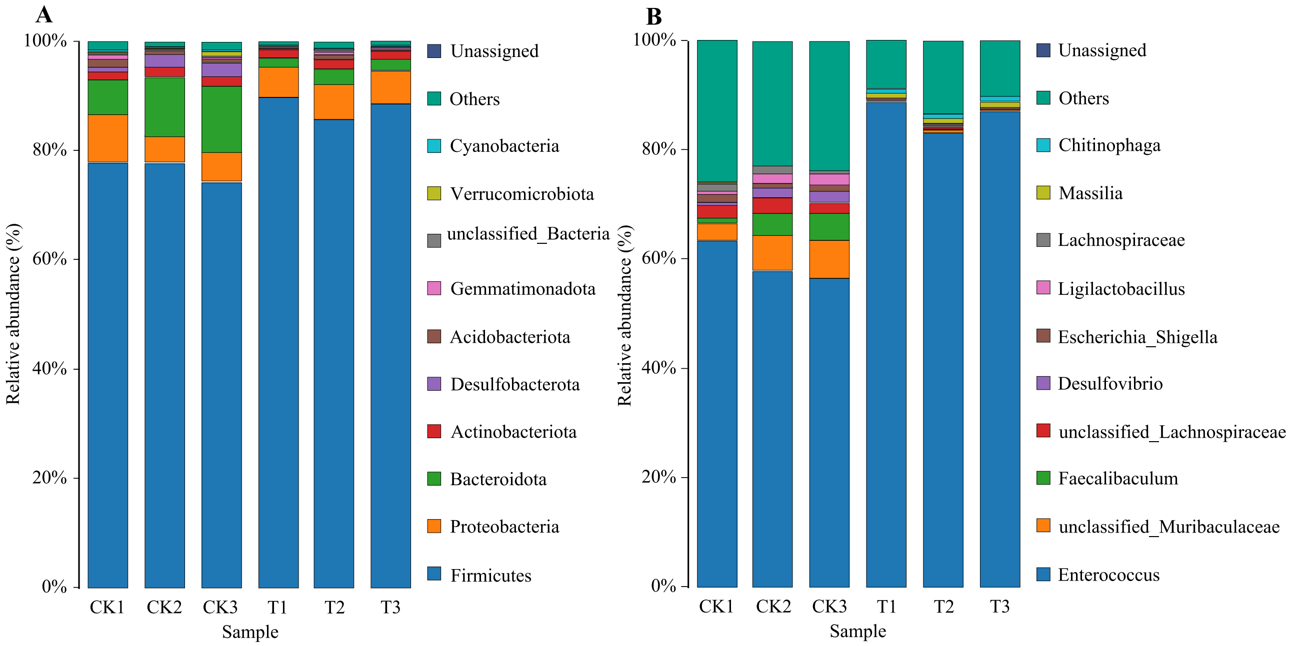

Fig. 2 Relative abundance of the ten most frequently observed phyla (**A**) and the ten most abundant genera (**B**) of gut microbiota of *Chilo suppressalis* exposed to cadmium (T1-3) or in the control group with no exposure (CK1-3)

AMOVA, $P < 0.001$). Venn diagrams illustrated that there were 248 and 342 unique genera for the control and Cd-exposed treatments, respectively, with 320 genera being shared (Fig. 1D).

Gut microbial community composition

The ASVs of the midgut microbiota from Cd-exposed and unexposed *C. suppressalis* were classified into 32 phyla and 860 genera. The three most abundant phyla were *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, accounting for more than 92% of bacterial sequences obtained (Fig. 2A). The dominant bacterial phylum was *Firmicutes* in both the control and treatment groups. Remarkably, the *Bacteroidetes* phylum was the second most abundant microbiota in the control group, accounting for 9.7% of the total bacterial sequences, and was significantly higher than the Cd exposed larvae (2.2%, $P < 0.05$, Fig. 2A). The most abundant genera were *Enterococcus*, while the genus *Micrococcaceae* and *Faecalibaculum* had a significantly higher abundance in the control group ($P < 0.05$, Fig. 2B). In addition, hierarchical cluster analysis based on weighted UniFrac distance showed that there were two main branches of the microbial communities from *C. suppressalis* larvae. Samples from the Cd exposed larvae clustered into one branch and samples from non-exposed larvae clustered into the other (Fig. 3).

The LEfSe analysis showed that significant differences at the genus, family, order, class, and phylum levels was led by differences at the species level directly (Fig. 4A). The microbial abundance of one single order, family, and genus in the Cd-exposed group was significantly higher in this group than that in the control group. However, the microbial abundance of 4 orders, 5 families, and 3 genera from the control group was significantly higher than those of the Cd-exposed group (Fig. 4A), and the abundances of the class *Enterococcus* was significantly enriched in the Cd-exposed group (Fig. 4B, LDAscore = 5.211 > 4.0). The abundances of the order *Bacteroidales* was significantly higher in the control group compared to the Cd-exposed group (LDA score = 4.618 > 4.0).

Prediction and differences in gut microbial metabolism pathways

According to PICRUSt, intestinal microorganisms mainly participated in 34 pathways responsible for cellular processes, environmental information processing, genetic information processing, metabolism, and organic systems, especially those used in environmental information processing and the metabolism of the organism (Fig. 5).

Discussion

Our research revealed that exposure to Cd significantly lowered the gut microbiota diversity, but did not significantly influence their richness; therefore, Cd exposure altered the

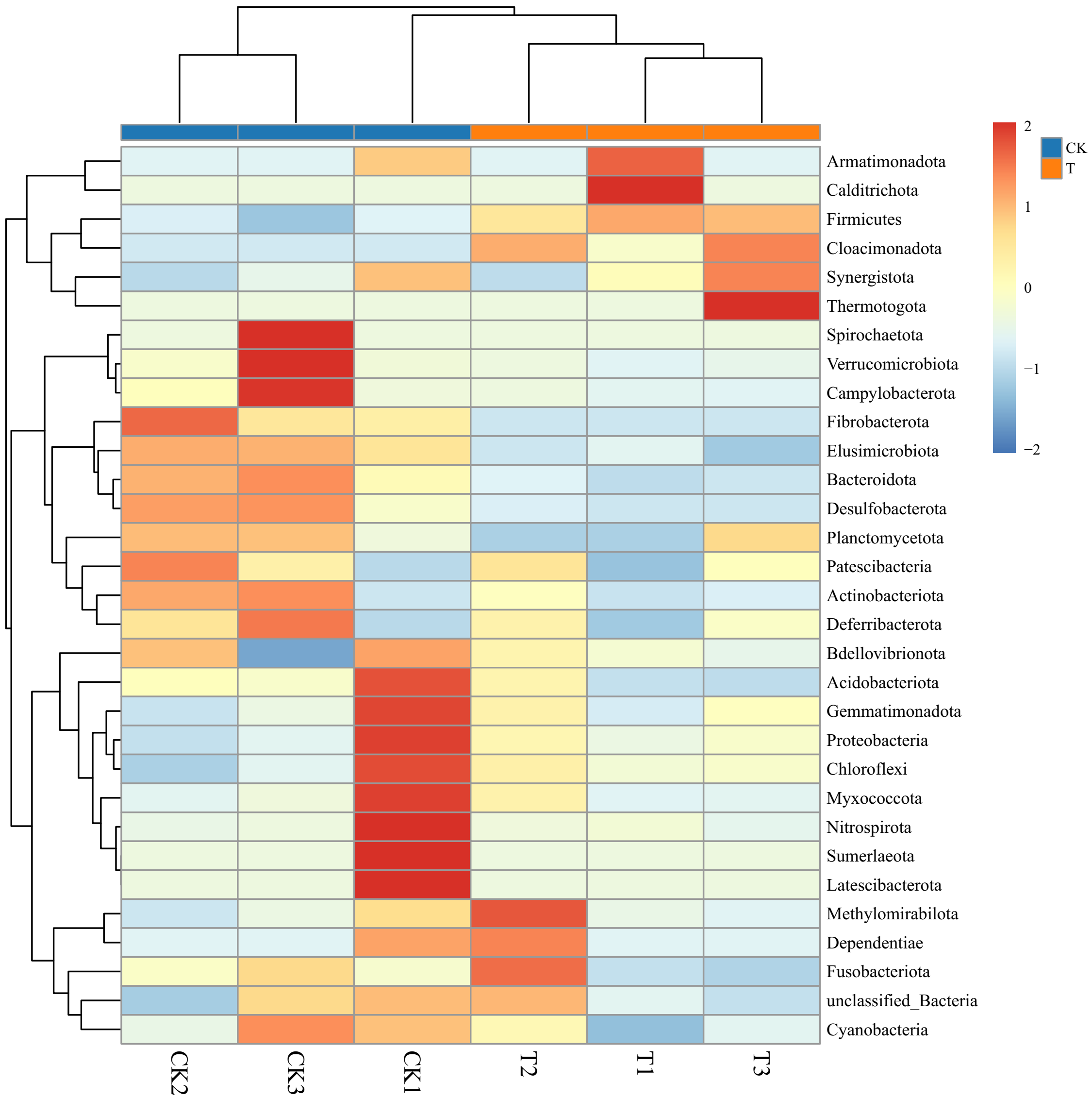

Fig. 3 Heat map of microbial species abundance at the genus level from cadmium-exposed (T1-3) and control (CK1-3) larvae of *Chilo suppressalis*. The color code indicates relative abundance ranging from low (blue) to high (red)

composition and evenness of species in the intestinal microbiome but did not eliminate entire species. These intestinal microorganisms mainly participated in 34 pathways, especially in the environmental information processing and metabolism pathways. This study therefore suggests that gut microbiota affects the response of *C. suppressalis* to Cd exposure and highlights the importance of the gut microbiome in host stress responses in agroecosystems.

An important result of this study was the significant differences observed in the microbial community structure and diversity of *C. suppressalis* following Cd exposure. This indicates that the heavy metal exposure has a significant influence on the microbiome in *C. suppressalis*. Our results were largely consistent with a report by Wu et al. (2020), in which exposure to Cd substantially decreased gut microbiota diversity of the black soldier fly larvae, *Hermetia illucens* (Diptera: Stratiomyidae). Similar results have been reported for the pygmy grasshopper, *Eucriotettix oculatus* (Orthoptera: Tetrigidae), in which gut microbial community diversity in habitats polluted by heavy metals was reduced (Li et al. 2021). In addition, different indexes were used to describe the richness (number of species) and evenness (relative abundance of species). Our results found that while richness (Chao1 and ACE) was not significantly affected, diversity (Shannon and Simpson) was reduced due to changes in species distribution and relative abundance. Therefore, Cd exposure altered the composition and evenness of species in the intestinal microbiome but did not eliminate entire species. However, species richness was not affected in the Wu et al. (2020) study that the black soldier

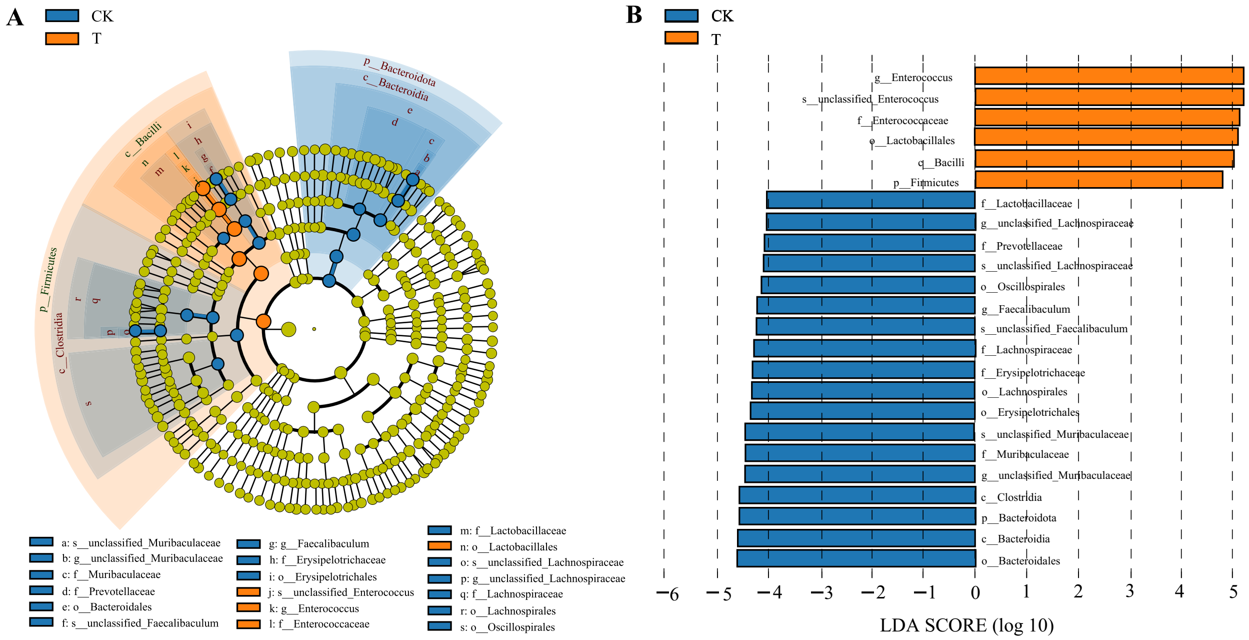

Fig. 4 Evolutionary branching diagram of linear discriminant analysis effect size (LEfSe) analyses to detect significant differences between microbial relative abundance between cadmium-exposed (T) and non-exposed (CK) larvae of *Chilo suppressalis*. Cladogram representation of the differential abundances at different taxonomic levels (**A**); LEfSe analysis shows differentially abundant genera as biomarkers determined using the LEfSe analysis with an LDA score >4.0 (**B**). From the center to outside (**A**), the circle represents the taxonomic level from phylum to species and the diameter of the small circle is proportional to relative abundance

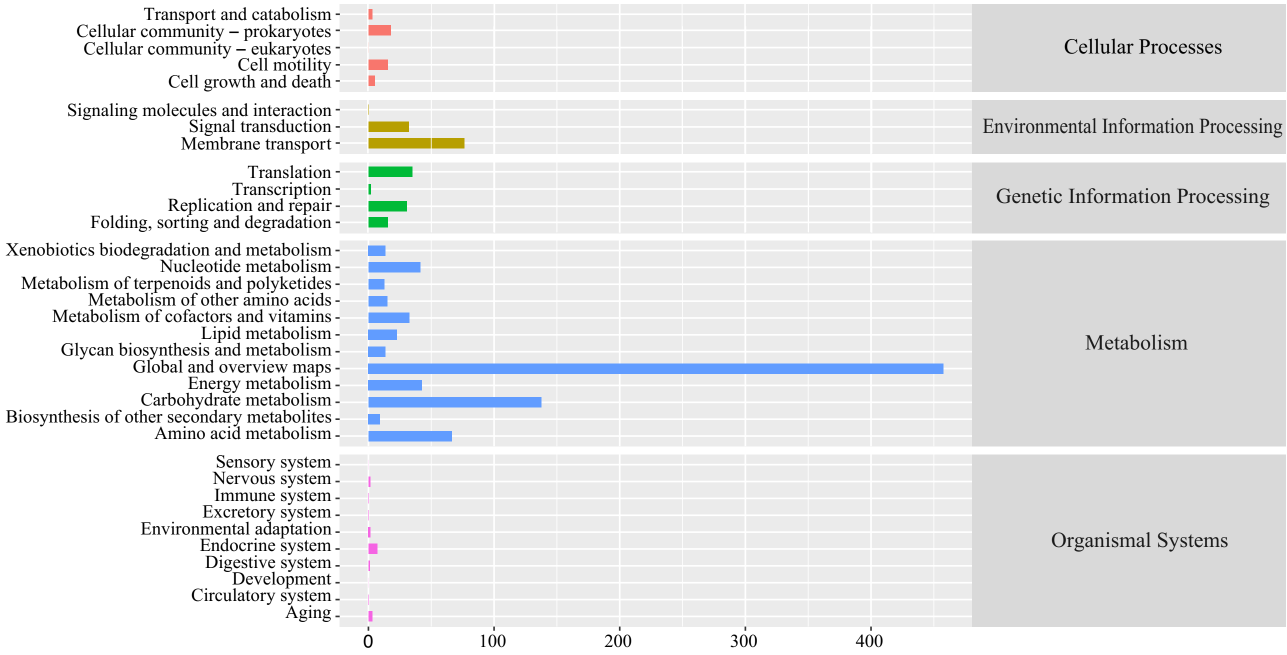

Fig. 5 Predicted KEGG (Kyoto Encyclopedia of Genes and Genomes) secondary functional pathway abundance map. The abscissa represents the abundance of functional pathways (unit: 1 million KO), the ordinate represents the functional pathways of the second classification level of KEGG, the rightmost box represents the first level to which the pathways belong

fly larvae was exposed to Cd and Cu. These results suggest that gut microbiota of arthropods can interact to environmental stressors across different agroecosystems, but varies with insect species.

Previous studies have shown that the gut bacterial communities of *C. suppressalis* consist mainly of *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* (Chen et al. 2023a; Zhang et al. 2022; Zhong et al. 2021).

Similarly, the top three abundant phyla of microbiota in our study were *Firmicutes*, *Proteobacteria* and *Bacteroidetes*, accounting for more than 92% of the bacterial sequences. However, there is difference in the dominant phyla of microbiota of *C. suppressalis* reared on different plants. The predominant phyla were *Proteobacteria* and *Firmicutes* when reared on water-oat fruit, while *Proteobacteria* dominated when the insects were reared on rice seedlings (Zhong et al. 2021). The three most dominant bacterial phyla of *C. suppressalis* reared on artificial diets were *Firmicutes*, *Proteobacteria* and *Bacteroidetes* (Chen et al. 2023a), and *Proteobacteria*, *Firmicutes* and *Actinobacteria* were most common in overwintering larvae (Zhang et al. 2022). This suggests that the compositional change could be, in part, an evolutionary mechanism to allow adaptation to a wide range of host plants.

Several functions of the dominant gut microbiota that could affect host plant acceptability are evident. Firstly, our study found that the *Firmicutes* and *Bacteroidetes* phyla were the most abundant microbiota. *Bacteroidetes* and *Firmicutes* have been shown to be necessary components in mammal lipid metabolism, suggesting that a change in their abundance could affect the body weight of animals (Wu et al. 2020; Zhu et al. 2018). Accordingly, the difference in the abundance of *Bacteroidetes* and *Firmicutes* could explain the developmental differences in *C. suppressalis* reported after exposure to Cd (Huang et al. 2023), where Cd exposure induced a significantly higher emergence rate and female pupa ratio compared with those in the control. Secondly, the most abundant genus recorded in our study was *Enterococcus*, which showed significantly increased abundance following exposure to Cd. Similar phenomena have been observed in *Bacillus thuringiensis* (Bt)-susceptible strains of *C. suppressalis* (Chen et al. 2023a). The *Enterococcus* has been proven to be associated with insecticide and pathogen resistance (Zhang et al. 2013; Shao et al. 2014). However, the abundance of *Muribaculaceae*, a family involved in resistance to environmental stressors (Jiang et al. 2023; Zhou et al. 2023), in the gut microbiota of *C. suppressalis* decreased sharply under Cd exposure. That is, the presence of these two genera in *C. suppressalis* may account for the resistance of this pest under environmental stress (i.e. Bt toxins, heavy metals), suggesting complex and closely-linked interactions between environmental stressors, insect hosts and midgut microbes.

Although the two groups had differential enriched bacterial microbial communities, the functional potentials of the gut microbiota from two groups seem to converge and can be categorized into two main groups as environmental information processing and metabolism. Gene function analyses in rice striped stem borer microbiota after Cd exposure indicated that intestinal microbiota primarily participate in 34 pathways responsible for five functions. Similar results have been found in the pygmy grasshopper, *E. oculatus*, exposed to heavy metal pollution (Li et al. 2021) and the wolf spider, *Pardosa pseudoannulata* (Araneae: Lycosidae), under Cd exposure (Wang et al. 2023). A high diversity of gut microbiota in field-collected insecticide-resistant populations of the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae), has been observed when compared to laboratory-reared populations, and enriched bacteria in resistant individuals was related to detoxification (Malathi et al. 2018). Similar result has also been found in the bean bug, *Riptortus pedestris* (Hemiptera: Alydidae) (Kikuchi et al. 2012), the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Gomes et al. 2020) and *C. suppressalis* (Chen et al. 2023a). Specific bacterial communities of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) played crucial roles in the adaptation to Cd detoxification and immune regulation, following exposure to Cd in mulberry leaves (Chen et al. 2023b). These previous findings, coupled with the results presented from our study, indicate that gut microbes likely have crucially important roles when herbivores are exposed to environmental stresses such as heavy metals.

Conclusions

Our results documented that larval gut microbiota of an important agricultural pest, *C. suppressalis*, were significantly affected when exposed to an artificial diet containing Cd. Importantly, gut microbiota diversity was significantly reduced in Cd-exposed larvae as compared to that in the control treatment without exposure, but not in overall richness. The most abundant genus of microbiota was *Enterococcus*, and *Micrococcaceae* may account for the observed Cd resistance and degradation. Intestinal microorganisms mainly participated in 34 pathways, particularly the environmental information processing and metabolism pathways. This knowledge could be valuable for understanding the connection between Cd exposure, changes in intestinal microbiota, herbivore stress responses in agroecosystems, and developing strategies to manage pest populations while mitigating the negative effects of Cd pollution. Such data suggest heavy metal exposure can have profound implications in biological control and species interactions in the field, possibly driven through changes in the microbiome of pest species. Additional studies to explore the ecological performance (i.e., duration, survival, fecundity and resistance to stressors) of *C. suppressalis* with changes in gut microbe communities stressed by Cd exposure are needed, and could be used to determine the responsible gut bacteria species for Cd resistance and/or degradation using culture-dependent assays.

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Compliance with ethical standards

Competing interests The authors declare no competing interests.

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