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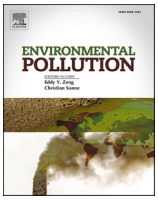

Understanding the ecological effects of the fungicide difenoconazole on soil and *Enchytraeus crypticus* gut microbiome☆

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ABSTRACT

Increasing knowledge of the impacts of pesticides on soil ecological communities is fundamental to a comprehensive understanding of the functional changes in the global agroecosystem industry. In this study, we examined microbial community shifts in the gut of the soil-dwelling organism *Enchytraeus crypticus* and functional shifts in the soil microbiome (bacteria and viruses) after 21 d of exposure to difenoconazole, one of the main fungicides in intensified agriculture. Our results demonstrated reduced body weight and increased oxidative stress levels of *E. crypticus* under difenoconazole treatment. Meanwhile, difenoconazole not only altered the composition and structure of the gut microbial community, but also interfered with the soil-soil fauna microecology stability by impairing the abundance of beneficial bacteria. Using soil metagenomics, we revealed that bacterial genes encoding detoxification and viruses encoding carbon cycle genes exhibited a dependent enrichment in the toxicity of pesticides via metabolism. Taken together, these findings advance the understanding of the ecotoxicological impact of residual difenoconazole on the soil-soil fauna micro-ecology, and the ecological importance of virus-encoded auxiliary metabolic genes under pesticide stress.

1. Introduction

Soil fauna account for nearly a quarter of all known animals on the Earth, and provide many essential ecosystem functions in soil, such as decomposition, the cycling of organic matter, and storage of nitrogen (Ding et al., 2020; Zhang et al., 2020, 2021a; Zhu et al., 2018a). As the first witness of soil contamination, soil fauna is a sensitive indicator of the impacts of pesticides, antibiotics, heavy metals, etc. Contamination induces the expression of oxidative stress biomarkers (Zhang et al., 2021b, 2022a), avoidance behavior (Zhu et al., 2018a), and detoxification genes (Wang et al., 2021a) in soil invertebrates. The gut microbiome of soil fauna was the pivotal factor in maintaining host health (Ma et al., 2019; Zhang et al., 2021b), which was closely related to gene expression (Zhang et al., 2019a), pathogen colonization (Ding et al., 2020), and soil nutrient cycling (Xiang et al., 2019). Numerous studies characterized the gut microbiome in soil invertebrates that were associated with soil-ecological functions (Zhu et al., 2018b), host digestion (Zhang et al., 2021a), metabolism (Zhu et al., 2021), and immunity (Kan et al., 2015). Meanwhile, some studies have demonstrated that the gut microbiome of soil invertebrates may be a more effective indicator of exogenous contamination than that of the host (Anslan et al., 2016; Ding et al., 2020; Zhang et al., 2019b). Therefore, the characterization of the gut microbiome is essential to understand overall gut homeostasis and soil health.

The application of pesticides is an effective strategy to implement sustainable agricultural intensification, which could reduce plant diseases caused by pathogenic bacteria and meet the goal of increased crop yield (Boulanger et al., 2018; Mohring et al., 2020; Xu et al., 2022a). Excessive use of pesticides led to residue in the soil environment, damaging the ecological health of the soil and even threatening human health through the food chain (Ke et al., 2022; Qiu et al., 2022; Xu et al., 2022b). At present, the national production of chemical pesticide active agents reached nearly 2.5 million tons, of which the annual output of fungicides accounted for 11% (http://www.chinapesticide.org.cn/;

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accessed on January 25, 2022). Meanwhile, pesticides could inhibit soil microbial activity, accelerate pathogen invasion, and even disturb soil ecological networks (Ke et al., 2022; Xu et al., 2022b; Xue et al., 2021), which poses a threat to the ecological function. The slight variation in soil microbiome composition and function played a decisive role in the outcome of soil-pathogen interactions under natural field conditions (Wei et al., 2019; Xu et al., 2022b). Soil has been recognized as a hidden reservoir for antibiotic resistance genes (ARGs), pesticides induced microorganisms to develop antibiotic resistance-related gene mutations to obtain antibiotic resistance, and ARGs may be transferred through horizontal gene transfer from the environment to human bacteria (Qiu et al., 2022; Yu et al., 2023; Zhang et al., 2022b).

Difenoconazole, inhibiting demethylase and stopping fungal ergosterol synthesis, is a typical triazole fungicide with high efficiency and a broad spectrum action, was applied to control diseases of vegetables, fruits, and other field crops (Dong et al., 2013; Yun et al., 2012). Due to the high octanol-water partition coefficient and the strong retention of difenoconazole in soil, the environmental concentration of difenoconazole in farmland soil reached levels as high as approximately 5.0 mg difenoconazole kg^{-1} dry soil (Bending et al., 2006; Zhang et al., 2021c) (https://cn.agropages.com/; accessed on June 27, 2017), and its residual half-life in the soil was reported to be about 40–100 d (Chang et al., 2019; Zhang et al., 2021c). Difenoconazole could affect non-target organisms, and inhibit the growth and development of fish and algae (Jiang et al., 2020; Kan et al., 2015), reduce the ability of earthworms to reduce the impacts of oxidative stress (Dong et al., 2013), and alter the composition and structure of soil communities (Zhang et al., 2021c). However, these studies cannot fully reflect the risks of difenoconazole in soil, especially for soil invertebrate animals. *Enchytraeus crypticus* (*E. crypticus*) is a model organism for evaluating soil ecotoxicology (Ding et al., 2020; Zhang et al., 2022a). Due to the symbiotic relationship between intestinal bacteria and *E. crypticus*, the variation of intestinal bacteria responds more accurately to pollutants than physiological indicators of the hosts (Jin et al., 2023). Therefore, obtaining information on the impact of difenoconazole exposure on the soil-soil fauna micro-ecosystem is most important for the comprehensive evaluation of the ecological risks of application of difenoconazole.

In the present study, we selected different doses of difenoconazole as typical treatments to evaluate the effects of fungicide exposure on the soil and the invertebrate gut microecology. Soil and gut bacterial communities were identified via 16S rRNA high-throughput sequencing and soil functional genes were analyzed using metagenomic sequencing. The objectives of this study are as follows (1) to investigate the effects of difenoconazole exposure on survival, body weight, and oxidative stress reduction capacity of *E. crypticus*; (2) to determine the response of the structure and the network interactions of soil and the *E. crypticus* gut bacterial communities to different difenoconazole treatments; (3) to decipher the detoxification mechanism of functional genes encoded by soil bacteria and viruses. We attempt to provide a theoretical basis for the ecological risks to soil and soil fauna caused by residual fungicides in soil.

2. Materials and methods

2.1. Soil, the test animals, and chemicals used

Soil samples were collected from agricultural land located in Hangzhou China (120°16′E, 30°29′N). Fresh soil samples were taken from the top 5–20 cm and stones and weeds were carefully expunged. All soil samples were air-dried in a ventilated place and sieved to 2 mm for further analysis. Adult *E. crypticus* was used as the model organism, which was acquired from Aarhus University, Denmark. The model organism has been cultivated in the laboratory for over 3 years (Ma et al., 2019). The *E. crypticus* were cultured in an incubator under controlled conditions at 20 ± 1 °C, with a photoperiod of 16:8 h (light: dark, 800 lx). Synchronized juveniles produced by adults on the same day were selected and cultured in the medium for 30 d to obtain test organisms for the experiment (Zhang et al., 2019b; Zhu et al., 2018a). The culture medium of *E. crypticus* has been previously described (Zhang et al., 2022a, 2019b).

Difenoconazole (CAS:119,446-68-3; white powder; >98% purity), was obtained from Aladdin® (Shanghai, China). Considering that residual pesticide doses in the field are often both above or below the environmentally relevant concentration (5 mg/kg), we selected 0.2, 1, and 2-fold (1, 5, 10 mg/kg) as recommended doses for exposure (Chang et al., 2019; Zhang et al., 2021c). Difenoconazole was dissolved in methanolic/sterile water (1:20) to prepare a stock solution, the same volume of methanolic was also added to the control group with an exposure ratio (methanolic/soil) of less than 1 in 1000. Then, the groups were set according to the intended treatment concentration of difenoconazole (DZ) as follows: control, DZ1 (1 mg difenoconazole/kg dry soil), DZ5 (5 mg difenoconazole/kg dry soil), and DZ10 (10 mg difenoconazole/kg dry soil).

2.2. Experimental design

The prepared difenoconazole solution was mixed with sterile water, added and homogenized to the soil at a dose of 1, 5, and 10 mg difenoconazole/kg dry soil, respectively. Soil without difenoconazole exposure served as the control group. Each microcosm consisted of a glass beaker containing 65 g of soil-difenoconazole mixture. Four replicates of each treatment group were conducted in this study. The treated soil was chemically equilibrated in an incubator for 3 d before the experiment started.

To guarantee sufficient individuals for analysis, 20 similar body sizes of adult *E. crypticus* were placed in each beaker according to the OECD guidelines (2004). The treatment groups were incubated in the artificial climate chamber for 21 d and the soil water content was adjusted three times a week. No nutrients were added to the samples during the exposure period to simulate the conditions in the field. At the end of exposure, the surviving adults were selected from the sample and washed three times with sterile phosphate buffer saline (pH = 7.3–7.5), transferred to a sterile centrifuge tube, followed by the calculation of their survival rate. Simultaneously, the tissues of five randomly selected *E. crypticus* were homogenized and used to measure the reactive oxygen species (ROS) of *E. crypticus*. The specific operation was based on the instructions of the enzyme-linked immunosorbent assay (ELISA) kit and slightly modified according to the actual situation.

2.3. Analysis of soil properties and enzymatic activity

The ratio of dry soil to water for determining soil pH was 1:2.5 (pH = 7.76), and the water content was 55–65% of the maximum water-holding capacity of the soil. Soil organic matter (SOM), nitrate nitrogen (NO_3^--N), and available phosphorus (A-P) are the main factors in determining to gain a more detailed understanding of difenoconazole effects in soil physicochemical properties. We used fresh soil (0.5 g) to add the extract at 1:10 to determine the physical and chemical properties of the soil after 3 weeks.

In this study, soil-neutral phosphatase (S-NP) and urease (S-UE) were used to evaluate the effects of difenoconazole exposure on soil microbial communities. We took 2 g of soil from each group after 1, 7, and 21 d, respectively, for air-drying and sieving to measure soil enzyme activity. The SOM, NO_3^--N, A-P, S-NP, and S-UE kits were purchased from Comin Biotechnology (Suzhou, China).

2.4. DNA extraction from E. crypticus gut and soil

Seven randomly selected adult *E. crypticus* from each sample were killed and fixed with ethanol, washed three times, and placed in a centrifugation tube. The bodies were homogenized with microelectronic tissue to obtain gut tissue. Finally, proteinase K and protein lysis buffer

were added to these centrifugation tubes and incubated at 55 °C for 5–6 h with repeated shaking. DNA extraction for *E. crypticus* gut and soil was performed using the FastPure® Cell & Tissue DNA Isolation Mini Kit (Vazyme, China) and FastDNA® Spin Kit for Soil (MP Biomedicals Inc., Santa Ana, CA, USA) following the instructions with minor modifications to volume, respectively. After extraction, the concentration and purity of DNA were measured via fluorescent quantitative analysis (Nanodrop; Thermo Fisher Scientific, Inc.). Finally, the DNA samples were kept at −40 °C until analysis.

2.5. Amplification, high-throughput sequencing, and bioinformatic analysis

We selected the barcoded primer 515F-806R (515 F: GTGCCAGCMGCCGCGGTAA and 806 R: GGACTACHVGGGTWTCTAAT) to target the V4 region to amplify the bacterial 16S rRNA gene. The amplification process was based on previous studies (Zhang et al., 2019b; Zhu et al., 2018a). Finally, the high-quality products were quantified and purified for sequencing on the Illumina Hiseq2500 platform (Novogene, China).

The raw reads of sequencing were confirmed by Quantitative Insights Into Microbial Ecology (version 1.9.1) (Caporaso et al., 2010). In order to guarantee the authenticity of the downstream analysis, clean data were obtained by removing primer sequences, ambiguous nucleotides, and low-quality reads. In QIIME, high-quality sequences based on 97% sequence similarity were identified as operational taxonomic units (OTUs) by the UCLUST algorithm (Edgar, 2010). Singleton OTUs were discarded from the OTU table. Representative sequences for PyNAST alignment were assigned via the Greengenes16S rRNA gene database (version 13.8), and their taxonomic annotation was processed with RDP classifier 2.2 (Nilsson et al., 2019). We excluded 5% of the total archaeal sequences and unassigned them from our downstream analysis.

2.6. Metagenomic analysis

To further decipher the function of the soil microbiome, eight soil samples including the control and DZ5 (close to environmental residual concentrations), were selected for complete metagenomic analysis using Illumina HiSeq platforms at Tianke Technology (Hangzhou, China). The resulting clean reads were assembled using MEGAHIT (Li et al., 2015), and the length of scaftigs over 500 bp was selected to be the final assembling result. The assembled scaftigs were predicted using MetaGeneMark. Finally, the unigenes with *e*-value $\geq 1e^{-5}$ were selected to obtain functional information by aligning with the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000) and CAZymes (CAZ) (Tatusov et al., 2000) using DIAMOND. Functional genes linked to carbon (C), nitrogen (N), and sulfur (S) metabolism were identified according to the metabolic pathways mapped against KEGG orthologs (KO) (Zheng et al., 2022). The target gene sequence was compared with the Comprehensive Antibiotic Resistance Database (CARD) using BLAST software to identify the ARGs (*e*-value $<10^{-5}$, identity ≥70%, coverage ≥80%).

2.7. Statistical analysis

The data of *E. crypticus* physiological indicators, soil properties, and enzymatic activity were presented via mean ± standard deviation. The differences between groups were determined by two-tailed Welch's *t*-test and one-way analysis of variance (ANOVA) with Tukey's post-hoc test. We attributed $P < 0.05$ to a significant difference between treatments and control. The alpha diversity of the gut and soil microbial communities was assessed at the genus level using the vegan package in R 4.3.1. Both the Adonis test and the Anosim test were applied to evaluate the effects of difenoconazole on the bacterial community structure of gut and soil. The differences between the groups were compared based on the Bray-Curtis dissimilarity. Volcano plots of genera were completed by the ggplot2 package in R3.4.1, based on the difference analysis, the genera with gradient response were selected for further analysis. Redundancy analysis (RDA) was used to reveal the relationship between soil physicochemical properties (SOM, NO_3^--N, A-P, S-NP, S-UE) and shared dominant families. The line chart, linear fitting, and boxplots were visualized using GraphPad Prism version 8.0.2. The heatmaps showing the number of ARGs and Sankey diagrams were generated on the Lianchuan Biological Cloud Online Platform (https://www.omicstudio.cn/tool/24).

For network analysis, we chose the psych package in R to calculate Pearson correlation coefficients between bacterial communities at the genus levels ($P < 0.05$, $|r| > 0.6$), and used Gephi v0.9 to obtain the degree and betweenness centrality of each node, based on the genera with relative abundance >0.5% in at least one sample. Co-occurrence network diagrams were generated by Cytoscape V3.9.1 to characterize the effects of significantly different genera ($n = 50$, the relative abundance >0.5%, $P < 0.05$) in bacterial communities. Structural equation models (SEM) were developed to estimate the total effect between different concentrations of difenoconazole, gut bacteria (PCoA1 of Bray-Curtis distance), soil bacteria (PCoA1 of Bray-Curtis distance), F/B (the ratio of the relative abundances of Firmicutes and Bacteroidetes in gut bacteria communities), ROS, and the body weight of *E. crypticus*. SEM was constructed using the piecewise SEM package in R 4.3.1, in which $P > 0.05$ indicates a good fit of the model. The piecewise SEM model fitting and total effect algorithms were previously described (Li et al., 2022; Matthews et al., 2019).

3. Results

3.1. Effects of difenoconazole exposure on physicochemical properties of E. crypticus and soil

The content of A-P after 21 d under difenoconazole treatment was significantly increased compared to the control (Table S1, $P < 0.05$, ANOVA with Tukey's post-hoc test). Difenoconazole did not remarkably change the contents of SOM, NO_3^--N in soil samples (Table S1, $P > 0.05$, ANOVA with Tukey's post-hoc test). The activity of S-UE was significantly inhibited in the 5 and 10 mg/kg difenoconazole treatments after 1 d, and the 10 mg/kg difenoconazole treatment rapidly stimulated the activity of S-NP (Table S2, $P < 0.05$, ANOVA with Tukey's post-hoc test). Difenoconazole did not significantly alter the activities of S-NP and S-UE in soil samples over 7 d ($P > 0.05$, ANOVA with Tukey's post-hoc test).

Difenoconazole exposure did not cause severe mortality in *E. crypticus*, while the dried weight of *E. crypticus* in DZ10-treated groups decreased significantly after 21 d of exposure, compared to the control (Fig. 1a, $P < 0.05$, ANOVA with Tukey's post-hoc test). There was no significant effect on the survival rate of *E. crypticus* (Fig. 1a, $P > 0.05$, ANOVA with Tukey's post-hoc test). Notably, the concentration of ROS displayed a significant upward trend in the DZ5 and DZ10-treated groups (Fig. 1a, $P < 0.05$, ANOVA with Tukey's post-hoc test).

3.2. Effects of difenoconazole exposure on the E. crypticus gut and soil microbial community

After assembling and filtering, we obtained 940,099 and 1,382,232 bacterial sequences from the soil and gut microbiomes, respectively. Proteobacteria (32.8%) and Acidobacteria (28.34%) were the most abundant bacterial phyla in the gut and soil microbiomes, respectively (Fig. 1b). Furthermore, the relative abundance of Firmicutes was negatively correlated with the difenoconazole dose (Fig. S1a, $P < 0.05$, $R^2 = 0.3434$). Acidobacteriota were negatively correlated with the different treatment doses of difenoconazole in the gut microbiome (Fig. S1a, $P < 0.05$, $R^2 = 0.3776$, Spearman correlation analysis). Difenoconazole significantly increased the relative abundance of Acidobacteria and Chloroflexi, and the relative abundance of Bacteroidota and Myxococcota decreased in the DZ10-treated soil sample (Fig. S1b, *P*

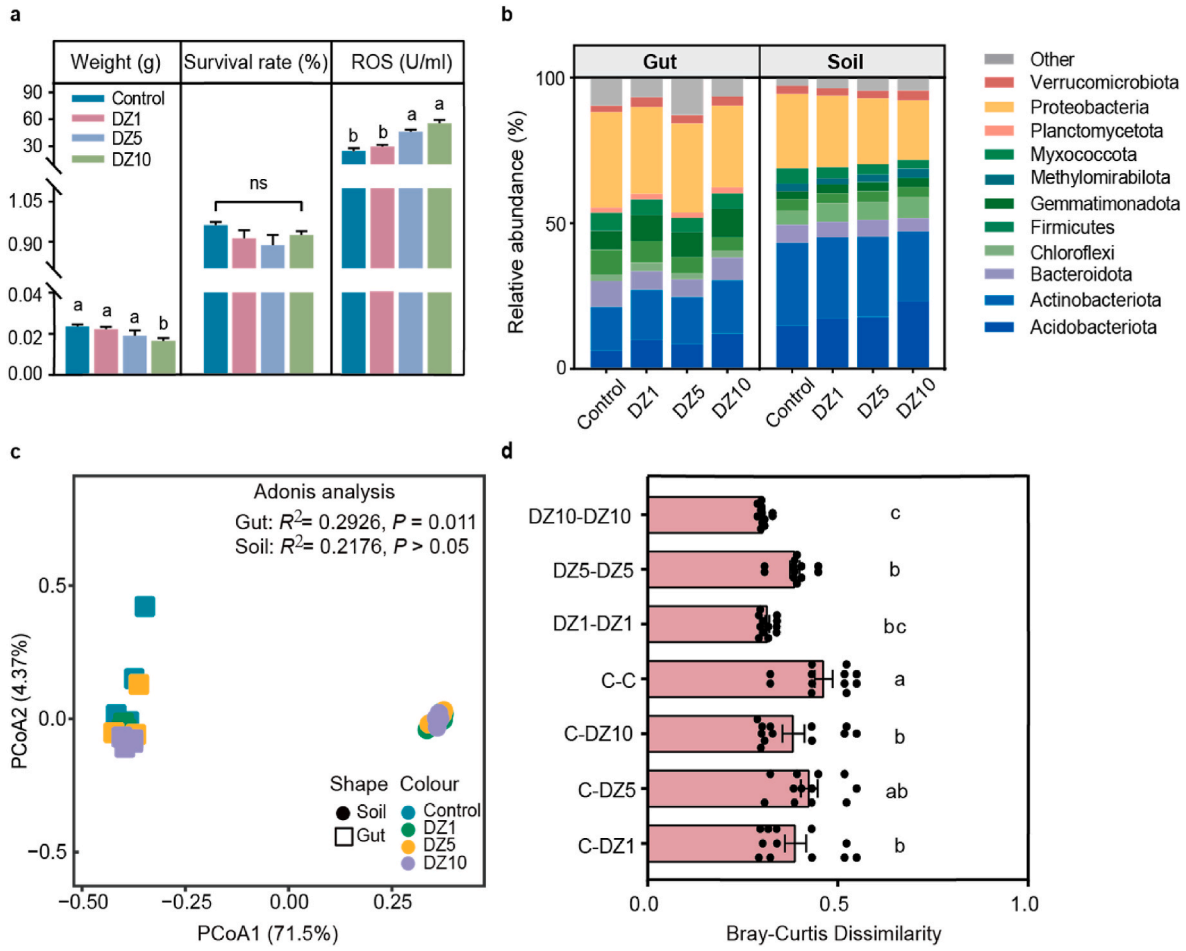

Fig. 1. Effects of difenoconazole exposure on body weight, survival rate, and reactive oxygen species (ROS) concentrations of *E. crypticus* in treated groups (a). Relative abundance of phylum (top 10) in gut and soil bacterial communities (b). PCoA based on genus level revealed significant separation of soil and gut bacterial communities (c). Intragroup and intergroup differences of gut bacterial communities based on Bray-Curtis dissimilarity (d). The letters represent differences between various treatment groups by one-way ANOVA with Tukey's post-hoc test, $P < 0.05$.

< 0.05, ANOVA with Tukey's post-hoc test).

The Shannon index showed that the diversity of soil and gut bacteria was not significantly affected by difenoconazole exposure compared with the control (Table S3, $P > 0.05$, ANOVA with Tukey's post-hoc test). The principal coordinates analysis (PCoA) using the Bray–Curtis distance illustrated that gut bacterial communities were distinct from the surrounding soil and difenoconazole significantly affected the gut bacterial communities of *E. crypticus* (Fig. 1c, $P < 0.05$, Adonis analysis). Meanwhile, Bray-Curtis dissimilarity using the gut bacterial files showed significant separation between the DZ5-and DZ10-treated groups, and control (Fig. 1d, $P < 0.05$, two-tailed Welch's *t*-test). Moreover, the gut microbiome was significantly more sensitive than the soil microbiome in responding to difenoconazole treatments (Fig. 1c and d). Variations of the bacterial community were more pronounced after high-dose difenoconazole exposure than in low-dose bacterial communities (Fig. 1 and Fig. S1).

As shown in Fig. 2a, the effects of difenoconazole on the abundance of gut bacteria families showed an increase in *Gemmatimondaceae*, *Nitrosomonadaceae*, and *Chitinophagaceae* compared with the corresponding soil samples ($P < 0.05$, two-tailed Welch's *t*-test). The relative abundance of the shared families *Dongiaceae*, and *Nitrosomonadaceae* in the gut microbiome were significantly shifted compared to treated soil samples, but there was no difference in the gut and soil control groups. As for *E. crypticus* gut samples, the relative abundance of the dominant families *Xanthobacteraceae*, *Beijerinckiaceae,* and *Polyangiaceae* significantly decreased in difenoconazole treatments, compared with the control (Fig. S2, $P < 0.05$, two-tailed Welch's *t*-test). Moreover, RDA results further indicated that, in addition to dominant families of gut bacteria, S-NP and A-P in soil were potential indicators of gut microbiome changes under pesticide contamination (Fig. S3, $P < 0.05$, Permutation = 999).

To characterize the impact of difenoconazole on the *E. crypticus* gut and soil bacterial communities, we pooled significantly altered genera under difenoconazole treatment [$P < 0.05$, $|\log2(\text{fold change})| > 1$]. A total of 20, 14, 38 genera in the gut and 8, 13, 14 in soil bacterial communities, respectively, were significantly changed under difenoconazole exposure compared to the control (Fig. S4, $P < 0.05$). The genera with dose-dependent effects were used for further analysis. Furthermore, difenoconazole decreased the relative abundance of *Polyangium*, *Bradyrhizobium,* and *Kaistia* in gut samples, and the abundance of the *Nocardioides* decreased significantly in the DZ5 and DZ10 soil sample groups (Fig. 2b, $P < 0.05$, ANOVA with Tukey's post-hoc test).

3.3. The effect of difenoconazole on microbial interactions

To discriminate the effects of difenoconazole on the gut and soil bacterial communities, we applied bacterial co-occurrence network analysis to reveal the interactions between soil-soil fauna communities and significantly different genera in difenoconazole treatments (Fig. 3a). In the co-occurrence network of gut and soils bacterial communities, the proportion of the positive and negative correlations of significantly different genera in the total bacterial degrees were 13.7 and 10.8%,

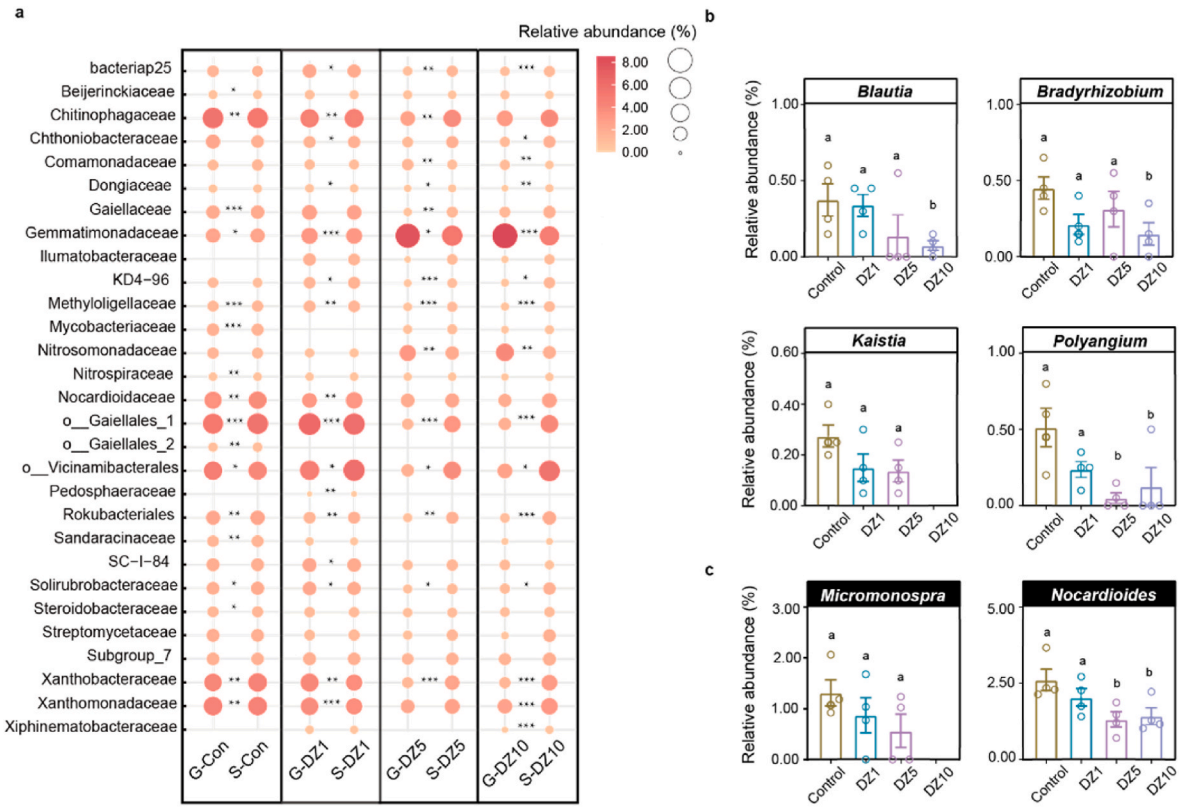

Fig. 2. Shared bacterial families (the relative abundance >1%) between *E. crypticus* gut and soil groups, G:gut; S:soil. (a). Decreased relative abundance of beneficial bacteria in gut (white background) and soil (black background)) after exposure, respectively (b, c). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, represents the significant difference between gut and soil treatments (two-tailed Welch's *t*-test, $P < 0.05$). The letters represent differences between various treatment groups by one-way ANOVA with Tukey's post-hoc test, $P < 0.05$.

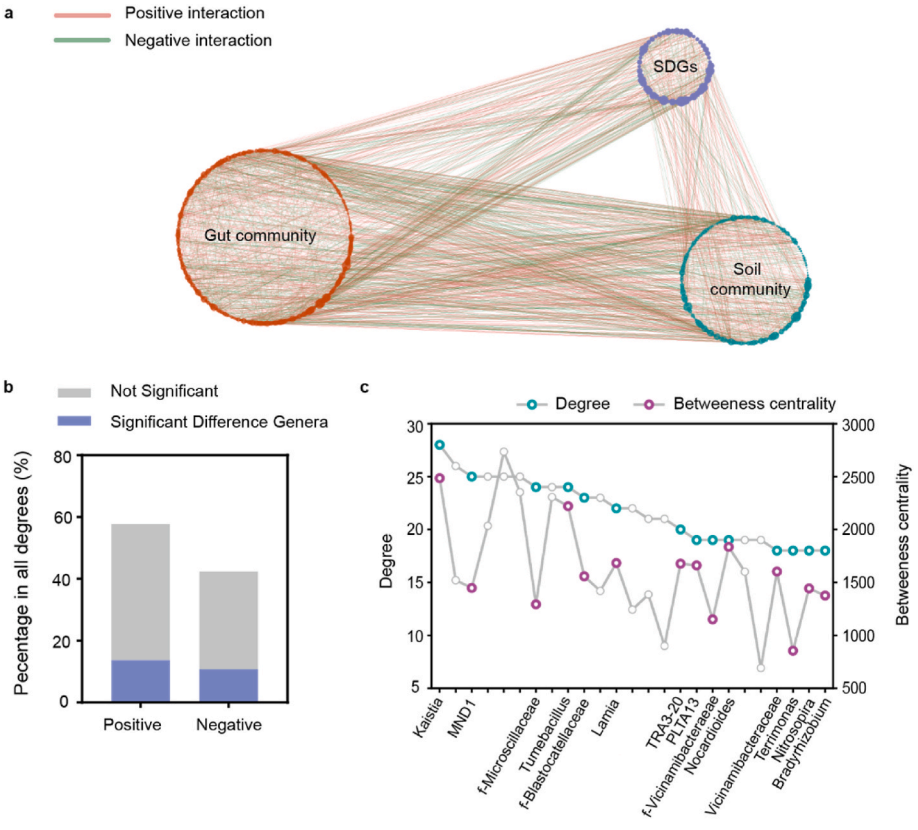

Fig. 3. Co-occurrence network analysis revealing the correlation between significantly different genera and soil-gut bacterial communities, the size of the node was determined according to the degree of each genus, SDGs: significantly different genera (a). The proportion of significantly different genera with positive and negative correlations in the whole community (b). According to soil-soil fauna gut network analysis to investigate the degree and betweenness centrality of significantly different genera (c).

respectively (Fig. 3b). Notably, significantly different genera accounted for a large proportion in the top 25 (14/25) (Fig. 3c).

As mentioned above, exposure to difenoconazole developed oxidative stress capacity, resulting in the alteration of the gut community structure. SEM was performed to calculate the total effect of gut bacterial communities, soil bacterial communities, F/B, and ROS on body weight (Fig. 4). A total of 73% variance in weight was explained in the model. SEM also revealed that the difenoconazole treatment affected the gut community structure of *E. crypticus*. Moreover, different doses of difenoconazole affected the body weight due to the induction of ROS and F/B of *E. crypticus*.

3.4. Effect of difenoconazole on soil microbial functioning

We used a metagenomic sequencing approach to explore the possible functional shift of soil-associated microbiomes after exposure to difenoconazole. The percentage of soil bacteria, archaea, and viruses were 81.1%, 6.2%, and 1.1% in the control and 81.8%, 5.5%, and 0.8% in the DZ5 treatment, respectively (Fig. S5).

To reveal how difenoconazole affects the soil bacteria and virus functional properties, we compared the functional annotations in the control and the DZ5-treated soil. Based on the KEGG database, we identified differentially enriched functional genes annotated to soil bacteria and virus files (Fig. 5a and b). Up-regulated bacterial function genes were mainly annotated in metabolism and signaling transduction, while down-regulated bacterial function genes were involved in xenobiotics biodegradation. Up-regulated viral function genes were mainly annotated in the nervous system and carbohydrate metabolism, while down-regulated viral function genes were connected with folding, sorting, and degradation. Besides, the functional profiles of microbiome associated with KO and CAZ under pesticide stress were significantly enriched or abrogated for analysis (top 10, $P < 0.05$, two-tailed Welch's *t*-test, Table S4). As for bacterial function genes, the *pho*P alkaline phosphatase gene (k07538) was depleted and modules involved in UDP-Glc α-glucosyltransferase (GT4) were enriched in the DZ5 treatment (Fig. 5c, $P < 0.05$, two-tailed Welch's *t*-test).

We analyzed functional genes involved in soil microbial community-related C, N, and S metabolism (Table S5). Compared with the control, the relative abundances of C, N, and S metabolic genes annotated with bacteria did not significantly shift in the DZ5-treated group. The functional genes related to the carbon cycle with virus-encoded genes were significantly enriched in the contaminated soil community (Fig. 5d, $P <$ 0.05, two-tailed Welch's *t*-test). Furthermore, a total of 20 ARGs were detected from soil microcosms, and these identified ARGs were divided into eight classifications and four mechanisms. The numbers of ARGs showed an uprising trend under difenoconazole pressure, mainly assigned to peptide antibiotics and glycopeptide antibiotics (Fig. S6).

4. Discussion

The utility of difenoconazole in controlling crop diseases and water safety is widely accepted (Wang et al., 2022; Zhang et al., 2021c), while the environmental risks of difenoconazole exposure to soil-soil fauna microecology remain less well explored. We relied on the soil planetary system to deeply understand the influence of residual difenoconazole exposure on soil-*E. crypticus* gut microecology health and explore the changes in functional genes of soil microbial annotation via metagenomic approaches.

4.1. Difenoconazole influenced the physiological activity of E. crypticus

Soil enzyme activity is a powerful indicator of mass and microbial metabolic activity, which reflects not only changes in soil physical and chemical properties, biomass, and biodiversity, but also nutrient cycling in ecosystems such as C, N, and P (Xue et al., 2021). The activity of S-NP rapidly increased at 10 mg/kg of difenoconazole for one day, which may be due to the accelerated conversion of organophosphorus by soil phosphatases when difenoconazole exposure caused a temporary loss of phosphorus (Zhang et al., 2014). Difenoconazole did not significantly change the soil available nutrient pools in soil samples after 7 d of soil exposure at a dose ranging from 1 to 10 mg/kg. This indicates that difenoconazole had no effect on the major nutrient supply in the soil. Nevertheless, we observed that exposure to 5 and 10 mg/kg of difenoconazole resulted in elevated oxidative stress of *E. crypticus*. As one kind of triazole fungicide, difenoconazole has been reported to induce DNA damage and apoptosis of non-target organisms. The impacts were associated with ROS production (Jiang et al., 2020; Park et al., 2022; Teng et al., 2018). Besides, residual difenoconazole depressed the dry weight of soil fauna. Variations in oxidative stress capacity and weight could be evaluated as physiological indicators of ecotoxicology, then, to decipher the molecular mechanisms of residual difenoconazole on soil microecology by omics biotechnology.

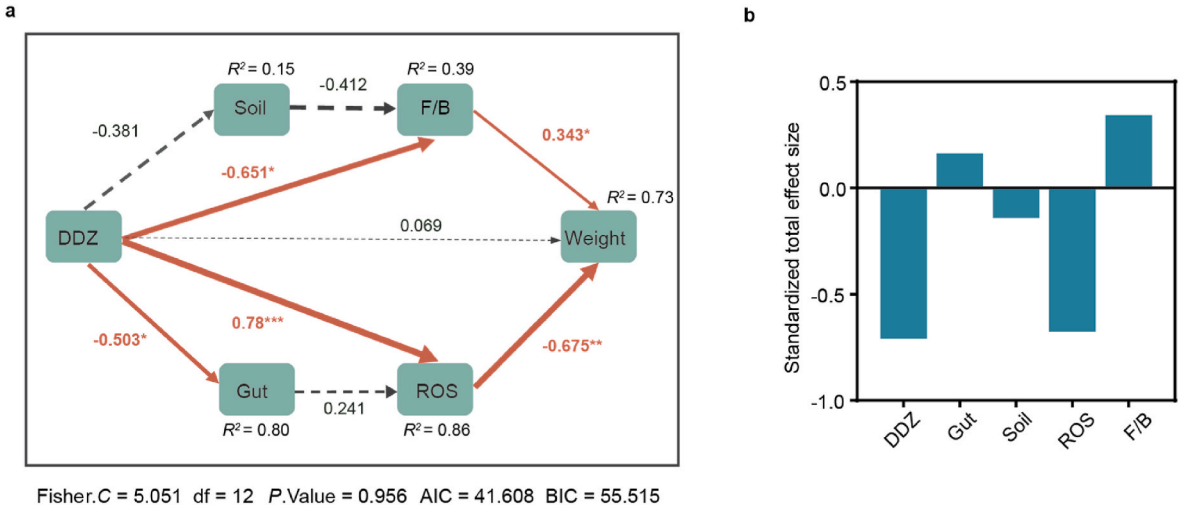

Fig. 4. SEM was applied to evaluate the relationship between difenoconazole exposure dose, soil bacteria (PCoA1 of Bray-Curtis distance), gut bacteria (PCoA1 of Bray-Curtis distance), ROS, F/B (the ratio about the relative abundances of Firmicutes and Bacteroidetes in gut bacteria) and weight of *E. crypticus*. The number on the line represents the path coefficient, which is proportional to the width of the arrow, the R^2 represents the proportion of variance explained. Solid and dashed lines reflect significant and insignificant correlations, respectively (a). Plot showing the total normalized effect of the above indicators on body weight (b).

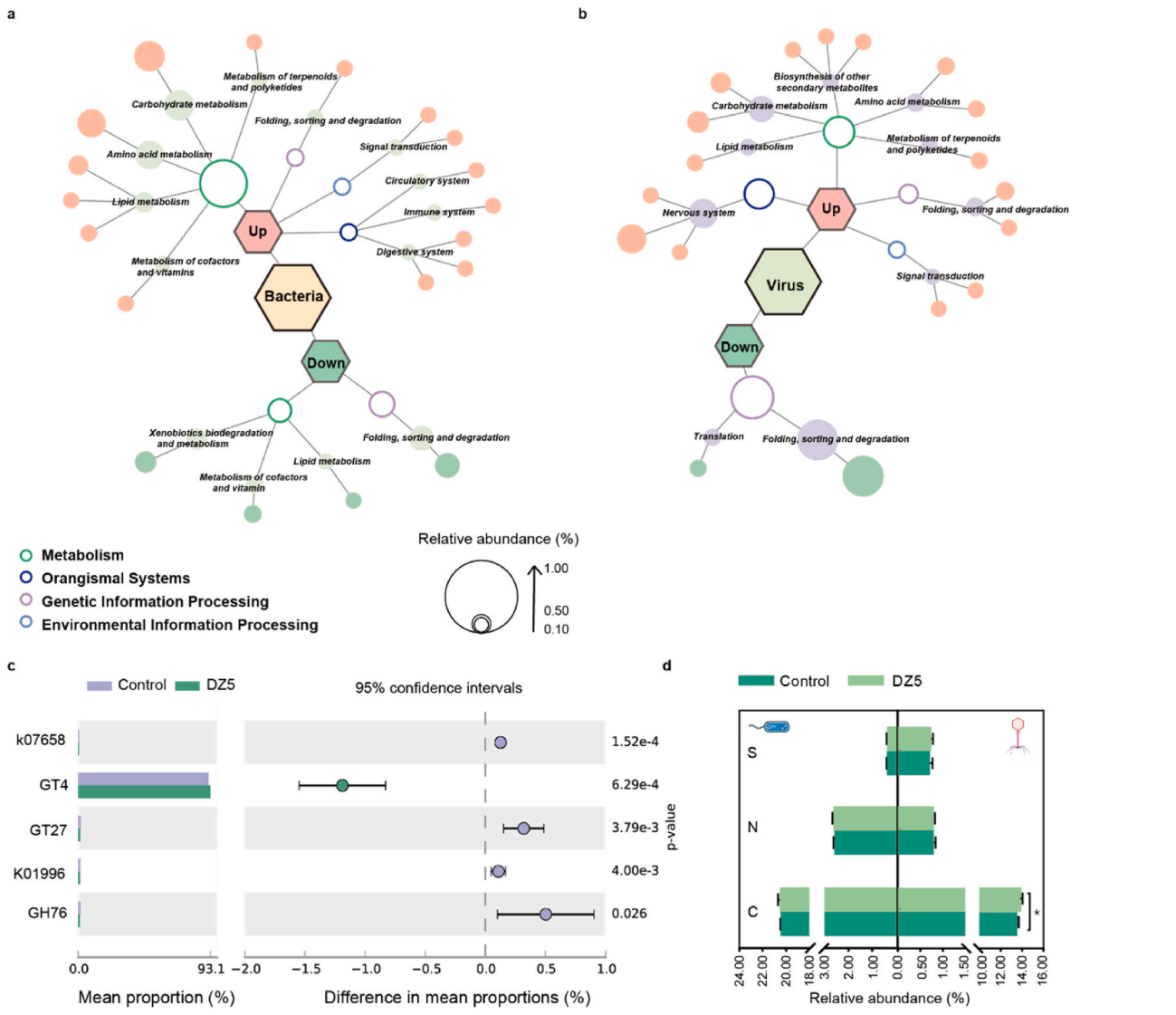

Fig. 5. Soil metagenomes revealed effects of difenoconazole exposure on soil function. Based on level 3 in the KEGG database enrichment of significantly altered soil bacterial community and viral community functional potential in DZ5-treated group compared with control, the size of the bubble is controlled as a reference (a, b). Differential abundance analysis of microbiome functional genes in the DZ5-treated group compared with control (c). The enrichment of bacterial and viral genes encoding CNS metabolic functions (d).

4.2. Difenoconazole affected gut microbial community structure and soil-E. crypticus ecological network

In contrast to the edaphic community, the composition and structure of the gut bacterial community were disturbed by 5 and 10 mg/kg difenoconazole, resulting in a gradient effect of more beneficial bacteria. Meanwhile, SEM-based analysis revealed that the structure of gut bacterial communities was notably impacted by exposure concentration than in soil. These findings demonstrated that the gut microbial communities of *E. crypticus* were more susceptible to pesticide stress compared with the microbial communities in soil. We anticipate that the functional redundancy and complexity of soil communities were critical for maintaining microbial communities under environmental perturbation (Ding et al., 2020; Zhang et al., 2021b), but importantly, host conditions and edaphic effects were the dominant force in regulating the gut microbiome of soil animals (Pass et al., 2015; Zhu et al., 2021), and the invasion of difenoconazole might impact on the gut bacteria through the direct or indirect contact and accumulation of soil fauna. There are previous examples which that support the view that the gut microbial communities of soil fauna are more sensitive than soil microbial communities, in this case following exposure to azoxystrobin, oxytetracycline, and microplastics (Zhang et al., 2019b; Zhu et al., 2018a, 2018b). Dysbiosis in the gut microbiome could interfere with the immune system and metabolic function of the host, thereby potentially undermining the contribution of invertebrates to soil ecological health (Wilschut et al., 2021; Xiang et al., 2019). Together, the symbiosis between host and gut bacteria emphasized that the gut communities of soil invertebrates provide strong evidence for an accurate response to the toxicity of exogenous pollutants.

Taxon-based analysis revealed that difenoconazole treatment altered the relative abundance of dominant gut bacterial taxa. Firmicutes and Bacteroidetes play a dominant role in the lipid metabolism of humans and mammals (De Filippo et al., 2010; Kan et al., 2015; Yin et al., 2015), of which the abundance ratio decreased significantly after treatment with difenoconazole (Fig. S7). This suggests that difenoconazole might affect the growth of soil fauna by interfering with their gut microbiome. Meanwhile, nitrate reduction and nitrogen fixation are remarkable traits of the members of the *Xanthobacteraceae* (Cheng et al., 2022) and *Beijerinckiaceae* (Souza et al., 2021). This finding indicated that gut communities of soil invertebrates that have beneficial roles in terrestrial

ecosystem processes were disrupted with increasing doses of difenoconazole. Besides, we identified the significantly different genera in the microbiome, which presented a gradient response to the difenoconazole dose, such as *Bradyrhizobium*, *Kaistia*, *Polyangium*, and *Blautia*, which decreased gradually in contaminated treatments. Interestingly, *Bradyrhizobium*, *Kaistia*, and *Polyangium* are mainly involved in nitrogen fixation (Favero et al., 2022), biodegrading aromatic compounds (Liao et al., 2021), and promoting plant growth (Wang et al., 2021b). More importantly, the *Blautia* genus contributes to anti-inflammatory responses to maintain intestinal health (Wang et al., 2019; Zhou et al., 2021a). In contrast to the gut microbial community (Fig. 2c), *Nocardioides*, and *Micromonospora* with reduced abundance in treated soils are closely related to metabolizing pollutants (Wozniak-Karczewska et al., 2020) and promoting plant growth (Ortuzar et al., 2020). Together, exposure to difenoconazole damaged the balance of bacterial communities, resulting in the destruction of potentially beneficial bacteria present in soil ecosystems. This implies that difenoconazole exposure interfered with the ecological service functions of soil fauna.

The interactions within microbial communities are the key driver of regulation of the community structure and dynamics, and the presence of negative interactions is an important contributor to reducing the likelihood of community disturbance and increasing stability (Coyte et al., 2015; Ratzke et al., 2020). Degree and the betweenness centrality have been recognized as two important mechanisms in explaining the network nodes, which determine the capability of a node to the information or circulation in the network (Zhang et al., 2021a; Brugman et al., 2022). The constructed co-occurrence network based on the significantly different genera and soil-soil fauna bacterial communities revealed that significantly different genera occupied a dominant position in the bacterial network, which played a part in the competing niches of the network (up to 1/3 in all negative degrees) (Zhang et al., 2021b, 2022a & c). This expanded knowledge on the adverse effects of difenoconazole exposure on bacterial communities in terms of the soil-soil fauna microecological balance by impairing the abundance of potential functional bacteria. Additionally, previous studies reported that the F/B ratio might serve as a parameter to evaluate the weight of soil fauna (Zhu et al., 2018a). SEM analysis explained that difenoconazole doses were positively correlated to body weight by affecting the F/B ratio of the *E. crypticus* gut community. Furthermore, these insights suggested that the applied dose of difenoconazole does not serve as a major factor that directly affects the loss of weight, but interfered with the gut bacterial community or induced oxidative stress of the host to cause variation in the weight of *E. crypticus*. Taken together, these results supported that ROS may be a potential predictor of shifts in the weight of *E. crypticus* to respond to pollutants (Zhang et al., 2020; Zhu et al., 2018a).

4.3. Soil microbiome induced detoxification and metabolic genes to metabolize pesticides

We further deciphered the ecological risk of difenoconazole in edaphic ecosystems based on microbiome function and enrichment of ARGs according to soil metagenomic analysis. We found that difenoconazole significantly affected the metabolism of the soil microbiome. This was partly due to soil bacteria accelerating the metabolism of pesticides through the increased release of amino acids or long-chain organic acids (Gao et al., 2021; Ratzke et al., 2020). Signal transduction was induced under biotic and abiotic stresses to trigger biological detoxification processes (Bickerton et al., 2016; Staubach et al., 2013; Zhang et al., 2022d). In the current study, the alkaline phosphatase gene *pho*P, involved in the recycling of organic phosphorus, was decreased in DZ5-treated soil. This suggests that residual difenoconazole damaged the organophosphorus gene abundance of microorganisms within 21 d due to the destruction of microbial functional activity and turnover, which may have a potential effect on soil phosphorus absorption (Gao et al., 2021; Pathak et al., 2010). Glucuronic acid played a detoxification role in combining exogenous and endogenous products and encoding a family of detoxifying enzymes (Dwivedi et al., 2018; Tephly and Burchell, 1990). UDP-Glc α-glucosyltransferase was enriched in the DZ5-soil group, protecting microbes from the toxicity of pesticides to the soil. As discussed above, our findings demonstrated that residual fungicides could induce detoxification mechanisms in the soil microbiome, among which these metabolisms and signal transduction might be effective strategies for alleviating abiotic stress.

In this study, we reported that the effects of difenoconazole could enhance the abundance of ARGs in soil. Similarly, pesticides were shown to enhance the transfer of mobile gene elements and ARGs through increased cell membrane permeability (Ke et al., 2022; Qiu et al., 2022; Zhou et al., 2021b). Both pesticides and non-antibiotic drugs enhanced ARGs were affiliated with producing ROS and promoted bacterial competence (Wang et al., 2022; Zhang et al., 2022c). These results demonstrated that the use of difenoconazole for plant disease control accelerated the emergence and transmission of ARGs in the edaphic ecosystems.

Notably, viral genes related to carbon metabolism were significantly enriched in contaminated soil, but no effect of the abundance of these genes was observed in bacteria encoding carbon metabolism. The reason may be that viruses enhanced C-cycling and energy conversion by ecosystem-related auxiliary metabolic genes (Nelson et al., 2022; Trubl et al., 2018), or soil-active bacteria were actively targeted by phages, and the release of unstable cellular components after cell lysis due to the influence of pesticides might affect soil C-cycling (Kuzyakov and Mason-Jones, 2018; Nelson et al., 2022). These results verified that residual difenoconazole in the soil environment might pose a potential impact on ecosystem functioning, and viral communities alleviated the toxic mechanism of pesticides via capturing energy (Zheng et al., 2022).

5. Conclusion

In conclusion, this study showed that difenoconazole exposure to soil produced dose-dependent effects on weight and on the oxidative stress mechanism of *E. crypt*icus. Difenoconazole exposure also altered the gut bacterial community composition and structure. The current study improved our understanding of residual difenoconazole threatening the abundance of beneficial bacteria participating in microbial functioning, and interfering with the stability of soil-soil fauna ecological communities. Meanwhile, SEM showed that ROS significantly correlated with the weight of *E. crypticus*. This indicates that the oxidative stress capacity could act as a vector for shifts in the weight of soil fauna. Furthermore, the metagenomic analysis showed significant enrichment of bacterial-encoding detoxification and virus-encoding carbon cycle genes in the contaminated soil. The analysis further revealed the role of viral communities on bacterial ecology in soil microbial communities. These findings shed a novel light on the understanding of the processes underlying soil-soil fauna ecological health due to exposure to difenoconazole for crop disease control.

Credit author statement

Guoyan Qin: Performed the experiments, Visualization, and Original draft; **Qi Zhang**: Conceptualization, Original draft, Designed the research; **Ziyao Zhang:** Data curation, Investigation; **Yiling Chen:** Conceptualization, Supervision; **Jichao Zhu:** Methodology, Investigation; **Yaohui Yang:** Visualization, Investigation; **Willie Peijnenburg:** Manuscript reviewing and editing; **Haifeng Qian:** Funding acquisition, Supervision, Manuscript reviewing and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121518.

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