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Increased Transmission of Antibiotic Resistance Occurs in a Soil Food Chain under Pesticide Stress

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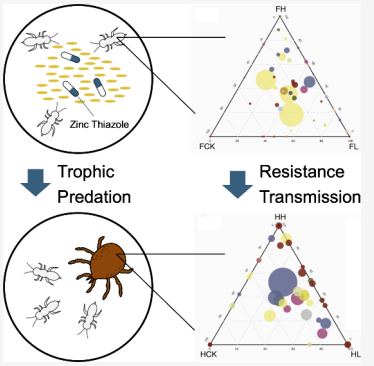
ABSTRACT: The rising spread of antibiotic resistance is a global concern, but the pathways of dissemination within soil ecosystems remain poorly understood. Here, we quantified the occurrence of antibiotic resistance genes (ARGs) in gut microbiomes of soil collembolans (*Folsomia candida*) under pesticide stress (zinc thiazole, ZT) and analyzed the trophic transfer of ARGs to the microbiomes of predatory mites (*Hypoaspis aculeifer*), natural predators of collembolans. High throughput quantitative PCR was used to quantify ARGs, whereas gut microbiomes of collembolans and mites were characterized using 16S rRNA gene amplicon sequencing, and potential pathogens were identified. Our results revealed that ZT exposure significantly elevated the abundance of ARGs (e.g., *AAC(6′)-Ir*) in soil collembolan microbiomes. With the increase of ARGs in prey collembolan microbiomes, an increase of ARGs in predatory mite microbiomes was observed through trophic transfer. Mobile genetic elements (MGEs) significantly contribute to the transmission of ARGs within this food chain. Additionally, co-occurrence analysis indicated a strong association between gut resistomes and pathogens, such as *Brevundimonas diminuta*, in the collembolans and predatory mites. Overall, our study provides evidence for the dissemination of ARGs through the collembolan-predatory mite food chain following pesticide exposure, which is important for understanding the broader dynamics of antibiotic resistance spreading in soil ecosystems.



KEYWORDS: *antibiotic resistance genes, food chain, gut microbiomes, pathogens, predatory mites, collembolans*

1. INTRODUCTION

Antibiotic resistance has emerged as a global health threat.[1,2] Although resistance in microorganisms is a natural phenomenon, increased anthropogenic use of antibiotics since the 1940s has massively increased resistance in human, animal, and plant systems.[3,4] Diverse antibiotic resistance genes (ARGs) that code for different resistance proteins and mechanisms are now ubiquitous in nature, being transmitted and spread across One Health sectors, including soil environments.[5−7] Soils harbor the most diverse naturally evolved ARGs on Earth; however, the transmission and spread of antibiotic resistance within soil ecosystems, particularly among soil fauna, are still poorly understood. This understanding is critical for developing holistic solutions to this global health challenge.[8]

Soil fauna plays a crucial role in essential ecological processes within soil ecosystems,[9,10] and their gut microbiomes have been reported as major reservoirs of ARGs.[11] Anthropogenic pressures, such as heavy metal,[12] antibiotic,[13] and nanoparticle[14] pollution, have conditionally caused ARGs to further accumulation in soil organisms, but little is known about how ecological phenomena impact ARG transmission in soil ecosystems. For example, prey-predator relationships are a mainstay in soil ecosystems.[15−17] Studies have shown that antibiotics[18] and nanoparticles[19] can be transported through soil food chains as well as microorganisms moving across trophic networks, interacting with their hosts and with each other.[20,21] However, the specific transfer of ARGs from prey to predators in food chains has not been studied heavily, especially in the presence of other pollutants such as pesticides.

Pesticides are used extensively in soil ecosystems to control pests and to prevent crops from invasive infections,[22−25] and there is evidence that they or their breakdown products contribute to the acquisition and spread of antibiotic resistance.[26,27] Different mechanisms have been proposed. For example, pesticide exposures may modulate susceptibility of certain microorganisms to antibiotics and may accelerate rates of horizontal ARG transfer of mobile genetic element (MGEs).[28,29] Alternatively, pesticides might exert selective pressure against sensitive strains, thereby altering microbial community levels of resistance to antibiotics.[30] For instance, carbendazim has been shown to increase soil bacterial community resistance to chlortetracycline by elevating the abundance of certain bacterial genera.[31] However, microbial

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selection and ARG prevalence under pesticide exposure in soil fauna or their food chains still remain elusive.

Here we studied the impact of zinc thiazole (ZT) exposure on gut resistomes and microbiomes in collembolans and a related model food chain, comprised of the parthenogenetic collembolan, *Folsomia candida*, as the prey, and the predatory mite, *Hypoaspis aculeifer*, a typical soil predator of collembolans. ZT was chosen because of its high efficiency and low toxicity,[32] and it has been shown to influence soil microbial activity.[33−35] The collembolan-predatory mite prey-predator pair was chosen because this model is often used in ecotoxicity tests.[36−41] High-throughput quantitative PCR (HT-qPCR) and 16S Illumina sequencing were used to quantify the gut resistomes and microbiomes, respectively. Here we did the following: 1) quantified changes in gut microbiomes and resistomes of collembolans under different ZT pressures; 2) examined how the consumption of ZT-exposed collembolans impacted gut microbiomes and resistomes of the predatory mites; and 3) disentangled possible drivers of ARGs in this food chain. We hypothesize that 1) ZT-exposure will alter the microbiomes and resistomes of collembolan guts; and 2) ARGs and MGEs may transfer from collembolan microbiomes to predatory mite microbiomes via predation.

2. MATERIALS AND METHODS

2.1. Test Animals and Pesticide-Contaminated Food. The soil collembolans (*F. candida*) and predatory mites (*H. aculeifer*) were generously provided by Aarhus University in Denmark and subsequently cultured in our laboratory for over six years. Individual organisms were reared in Petri dishes (90 × 13 mm) on a moist layer containing activated charcoal, plaster of Paris (Usg Boral, China, made from a mixture of extremely fine calcined gypsum, wood fiber, and other additives), and ultrapure water in a mass ratio of 1:8:8 in a climate chamber in the dark at 20 ± 1 °C and 70% humidity. The feed for the collembolans was dried Baker's yeast (Angel Yeast Co., Ltd., Yichang, Hubei, China), fed twice per week. Juvenile *F. candida* were then intermittently fed to the *H. aculeifer*. Ultrapure water was added to maintain the moisture content when it was necessary. To minimize individual variation, age-synchronized *F. candida* (10−12 days old) and *H. aculeifer* (33−35 days old) were used across the study, according to standard methods of the Organization for Economic Cooperation and Development (OECD).[42,43] More details on test animal culturing and synchronizing were described in previous studies.[37,44]

ZT-contaminated feed for *F. candida* was obtained by mixing the ground yeast with a series of ZT solutions (2-amino-5-mercapto-1,3,4-thiadiazole zinc, Alta Scientific Co., Ltd., Tianjin, China). The food was mixed carefully and thoroughly, freeze-dried, ground, and stored at 4 °C before use. The same volume of deionized water was mixed with uncontaminated yeast for the control group.

2.2. Experimental Design. To quantify the effects of different ZT exposures on the microbiomes and resistomes of the collembolans and their predators, two experiments were setup based on single-species and two-species toxicity testing, respectively. Given no previous studies assessed ZT toxicity in collembolans, we set up a concentration gradient of 0, 0.1, 1, 10, 100, 1000, and 10,000 μg ZT kg^{-1} yeast (CK, A, B, C, D, E, F group) according to OECD guidelines for assessing the effects of chemicals.[43] Seven experimental treatments were employed based on ZT exposures, including four replicates of each treatment and the control, providing a total of 28 samples. For each replicate, 20 synchronized individuals were transferred into a Petri dish with a culture mixture. To ensure ample food supply, enough ZT-spiked or control yeasts were added, respectively, and any unused "old" food was removed twice per week. ZT exposure tests on *F. candida* was performed over 4 weeks in a climate chamber (dark at 20 ± 1 °C, 70% humidity). The number of adults and juveniles was counted in each replicate dish at the end of the incubation, and then all the adults were harvested for DNA extraction. No significant changes in reproduction rates or mortality of the collembolans were seen over the experiments ($P > 0.05$) (Figure S1a).

Based on early data from our single-species test and results from previous studies,[32,33] three further treatments were set up (0, 0.1, and 10 mg ZT kg^{-1} yeast; CK, L, H, respectively), with five replicates per treatment. First, 250 age-synchronized collembolans were exposed orally to ZT in Petri dishes. Two weeks later, ZT-exposed collembolans in each Petri dish were collected, with 200 individuals being randomly collected as prey for predatory mites in new Petri dishes. The remaining collembolans were used for DNA extraction. Eight synchronized predatory mites then were added to each Petri dish (including 200 collembolans) and incubated for another 2 weeks. During these 2 weeks, the mites were fed with ZT pre-exposed prey but were not directly exposed to ZT itself. This was to ensure that any observed changes in mite resistomes were only due to the consumption of ZT-treated collembolans, not ZT itself. After incubation, the numbers of collembolans and predatory mites were counted, and predatory mites were collected for DNA extraction. No significant differences in the mortality of the collembolans or predatory mites were observed over the experiment ($P > 0.05$) (Figure S1b).

2.3. DNA Extraction. Before DNA extraction, the collembolans and predatory mites were first washed with 2% sodium hypochlorite solution for 10 s, rinsed four times in fresh phosphate-buffered saline (PBS), and five times with sterile ultrapure water to minimize surface microbial contamination. This stringent washing process was to ensure that the DNA was from bacteria within the invertebrates, predominantly from the guts. DNA extraction was carried out using the DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's instructions. The purity of the extracted DNA was determined with a NanoDrop 2000. The DNA solution was stored at −20 °C until use.

2.4. High Throughput Quantitative PCR (HT-qPCR). The abundance and composition of ARGs and MGEs in gut microbiomes were determined using 384 primer pairs based on methods in previous studies.[45,46] HT-qPCR was performed as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, and 60 °C for 30 s. The data obtained were analyzed using SmartChip qPCR software (version 2.7.0.1, WaferGen Biosystems, Inc.; Takara Bio.), and the threshold cycle was 31. The gene copy numbers of ARGs were calculated based on previous studies.[47,48] The relative abundance of ARGs was the ratio of the ARG copy numbers to the 16S rRNA gene copy numbers.

2.5. Amplification, High Throughput Sequencing, and Bioinformatics Analysis. To characterize bacterial communities, the primers 515F/806R were used to target the V4 regions of the 16S rRNA gene. DNA templates were amplified as previously described.[49] Purified amplification products were sequenced using Illumina Novaseq platform (Meiji Biological Medicine Co., Ltd., Shanghai, China). The raw data were analyzed by Quantitative Insights Into Microbial

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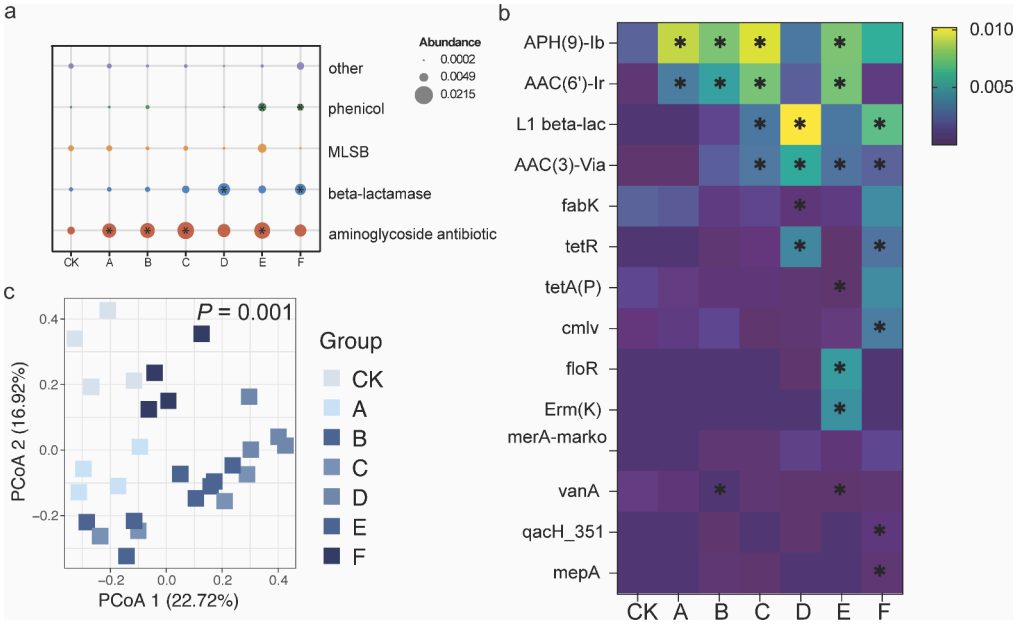

Figure 1. ARG profiles in collembolan microbiomes. (a) Bubble plot revealing the abundance of ARG drug classes (mean ± SE, $n = 4$). Asterisks represent the level of significance lower than 0.05. (b) Heatmap describing enrichment of ARG abundance of collembolan guts in the treated groups compared with control groups (mean, $n = 4$). Asterisks represent where the level of significance is lower than 0.05. (c) Principal coordinates analysis (PCoA) of the abundance of ARGs based on Bray−Curtis distances. The sample variation explained by the first two PCoA axes is given in parentheses.

Ecology version 2 (QIIME2) (version 2021.11)[50] following the online instructions. After removing the adapter, primers, and low-quality sequences, DADA2[51] was used to cluster amplicon sequence variants (ASVs) at 100% similarity. The SILVA database (version 138)[52] was used to identify bacterial taxonomic assignment.

ASVs belonging to the endosymbiont *Wolbachia* genus were excluded from the data set according to previous studies[49] due to their potential to dominate sequencing reads, which may mask the presence of less abundant ASVs and lead to an underrepresentation of diversity in further analysis. Data were rarefied to the minimum read count across all samples to correct for uneven sampling. For the estimation of potential pathogens, the high-quality sequences were blasted against reference sequences from multiple bacterial pathogen detection (MBPD) database[53] with an *E*-value < 1×10^{-10} and a sequence identity threshold > 99% which was reported previously.[54−56] The alpha-diversity of gut microbial communities were estimated by the Shannon index using the ASVs of the observed species. Beta-diversities were examined using principal coordinate analysis (PCoA) based on the Bray−Curtis distance matrices.

2.6. Statistical Analysis. Data are reported as the mean values ($n = 4$ in the single-species test, and $n = 5$ in the food chain test) ± standard errors (SE). The relative abundance of bacterial taxa or ARG classification was calculated using the R package *tidyverse*.[57] Data normality was first determined. Differences among treatments were determined via analysis of variance (ANOVAs) or the nonparametric Kruskal−Wallis test in the R package *vegan* or *nparcomp*. The index of community resistance (Rs) was calculated according to a previous study by comparing the alpha-diversity between control groups and treated groups to determine the relative consistency of microbial communities.[58] The Adonis test, distance-based redundancy analysis (db-RDA), the Procrustes test, and the Mantel test were conducted in the R package *vegan*. Hellinger-transformed relative abundance data was used in db-RDA.

Stackplots and bubble diagrams were generated using the *ggplot2* package of R.[59] The *pheatmap* package of R[60] was used to draw the heatmap, whereas the *ggtern* package[61] was used to draw ternary plots. Venn diagrams were constructed using *jvenn*.[62] Sankey diagrams illustrating composition profiles of potential pathogens were conducted using *networkD*.[63] SourceTracker model,[64] based on Bayesian algorithms, was used to predict the composition ratio of the target predator samples from source prey samples.

Data of the top 50 abundant potential pathogen species and top 50 abundant ARGs including MGEs were used to construct a co-occurrence network by package *edgeR*,[65] and Spearman coefficients were calculated and adjusted using *Hmisc* package of R.[66] Correlations were considered statistically significant if the absolute value of Spearman correlation coefficients was greater than 0.7 and the fdr-adjusted *p*-value was less than 0.05. The network was visualized in Gephi 0.10.1 with the dual circulate layout.[67]

A structural equation model (SEM) was constructed to assess the direct and indirect effects of 1) alpha-diversity and community structure of prey gut microbiomes; 2) relative abundance of MGEs and ARGs in prey guts; 3) alpha-diversity and community structure of predator microbiomes; and 4) relative abundance of MGEs on the total relative abundance of ARGs in predator guts, using AMOS 26.0.0.0 (SPSS Inc., Chicago) based on maximum-likelihood estimation. For gut bacterial communities, the value of principal coordinate axis 1 from PCoA results was used to represent the community structure. The overall model fit was evaluated with the goodness-of-fit index (GFI), the Bentler comparative fit index (CFI), and root-mean-square error of approximation (RMSEA).

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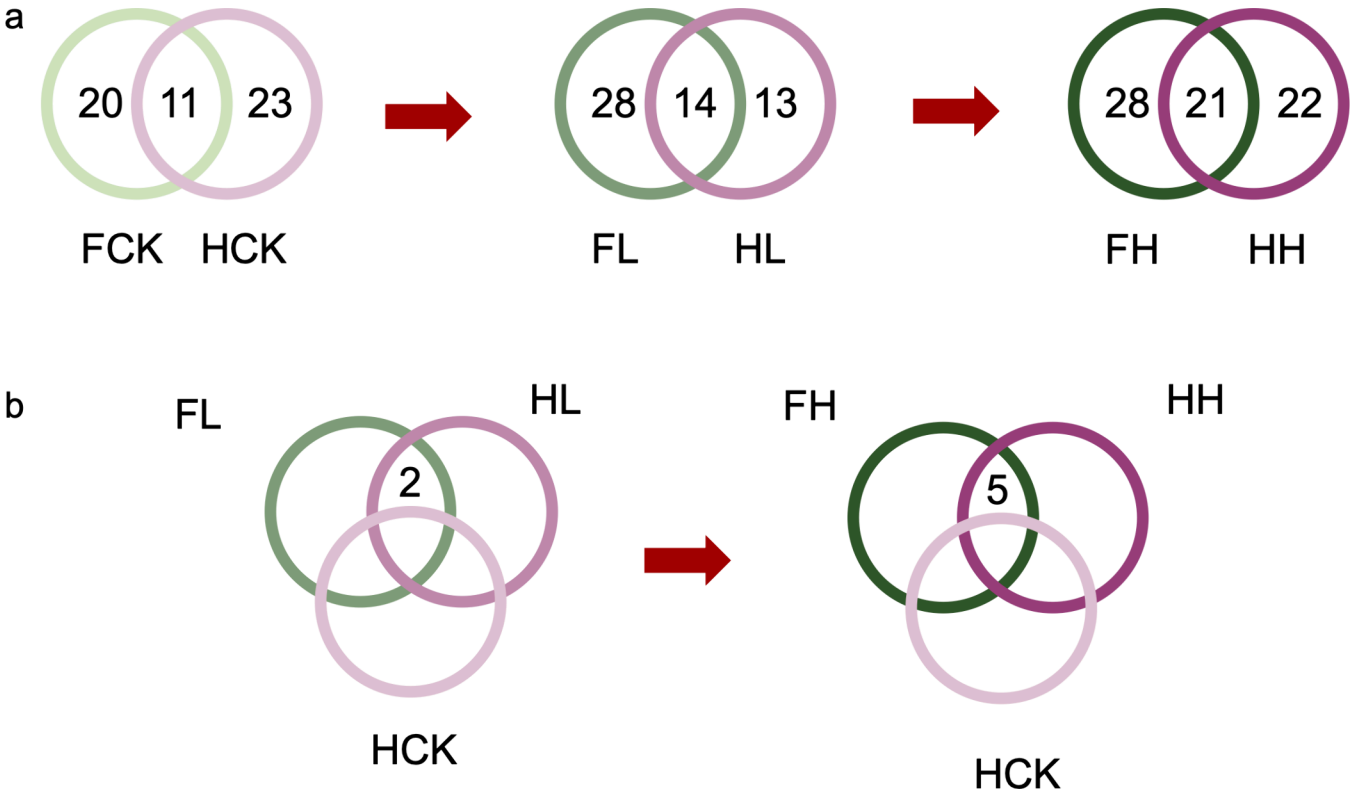

Figure 2. Venn diagrams revealing (a) the shared ARGs between trophic levels and (b) the unique ARGs shared between treated prey collembolan and predatory mite groups while excluded from the control mite group.

3. RESULTS

3.1. Effects of ZT on Resistomes and Microbiomes of Collembolans. In general, 91 ARGs and 20 MGEs were detected in the collembolan gut samples. An increasing number of ARGs was observed with increasing ZT concentration, though not statistically significant (Figure S2a). These ARGs confer resistance to most major classes of antibiotics, including aminoglycoside (42.85%), tetracycline (15.45%), beta-lactamase (14.64%), and others (Figure S2b). High-dose ZT (in E&F groups) significantly increased the total relative abundance of ARGs (Figure S2b). ZT exposure significantly increased the relative abundance of aminoglycoside and beta-lactamase ARGs in collembolan guts (Figure 1a). *APH(9)-Ib*, *AAC(6')-Ir*, *L1 beta-lac*, and other selected ARGs were enriched significantly (Figure 1b). PERMANOVA results indicated that collembolan gut resistomes significantly differed among controls and ZT treated groups ($P = 0.001$) (Figure 1c).

At least 11782 sequences per sample were obtained, and 266 bacterial ASVs were identified across all samples. Bacteroidota, Proteobacteria, Firmicutes, and Actinobacteriota were the four dominant phyla in collembolan gut samples (Figure S3a). Some phyla showed significant changes in the relative abundance under ZT treatments. For example, the relative abundance of Bacteroidota increased significantly in the C and D groups when compared to control (Figure S3a). A significant decrease in the Shannon index was seen in the C, D, and F groups compared to control (CK) (Figure S3b). The PCoA based on the Bray−Curtis dissimilarity indicated that ZT exposure significantly altered gut microbial communities (PERMANOVA, $P < 0.05$) (Figure S3c).

Procrustes analysis showed a strong positive correlation between bacterial community composition and ARGs in the collembolan gut ($M^2 = 5.63$, $P < 0.01$) (Figure S4a). Specifically, microbial community composition was significantly associated with ARG profiles based on db-RDA analysis ($P < 0.05$) (Figure S4b). Among resident bacteria, Sphingobacteriaceae, Bacillaceae, and Xanthomonadaceae were families that had strongest links with ARG profiles (Figure S4b). The relative abundance of MGEs was also significantly associated with ARG profiles ($P < 0.05$).

3.2. Resistome Distribution along the Model Food Chain. 62 ARGs and 16 MGEs were detected in prey collembolan gut microbiomes, whereas 65 ARGs and 14 MGEs were found in the predatory mite microbiomes. 32 ARGs and six MGEs were shared between the collembolan and mite samples, accounting for 32.30% and 24% of the total number of ARGs and MGEs detected, respectively. Venn plots show that exposure to ZT increased the number of shared ARGs between collembolan and mite gut samples (Figure 2a). Shared genes were mainly within the aminoglycoside and multidrug (i.e., nonspecific ARGs) resistance classes. Eleven ARGs were shared between FCK and HCK groups, whereas 14 were shared between the FL and HL groups, and 21 were shared between the FH and HH groups. Of particular interest are ARGs that shared between the ZT-exposed prey and predators that were not present in the control predator group. Two unique genes (*aacA43*, *mel_1*) were found in 0.1 ppm ZT-exposed prey-predator pair, and five unique genes (*aacA43*, *cmx*, *tetG*, *vanA*, *vatA*) were found in the 10 ppm ZT-exposed prey-predator pair (Figure 2b).

Among these genes, ternary plots showed that different classes of ARGs were enriched in collembolan or predatory

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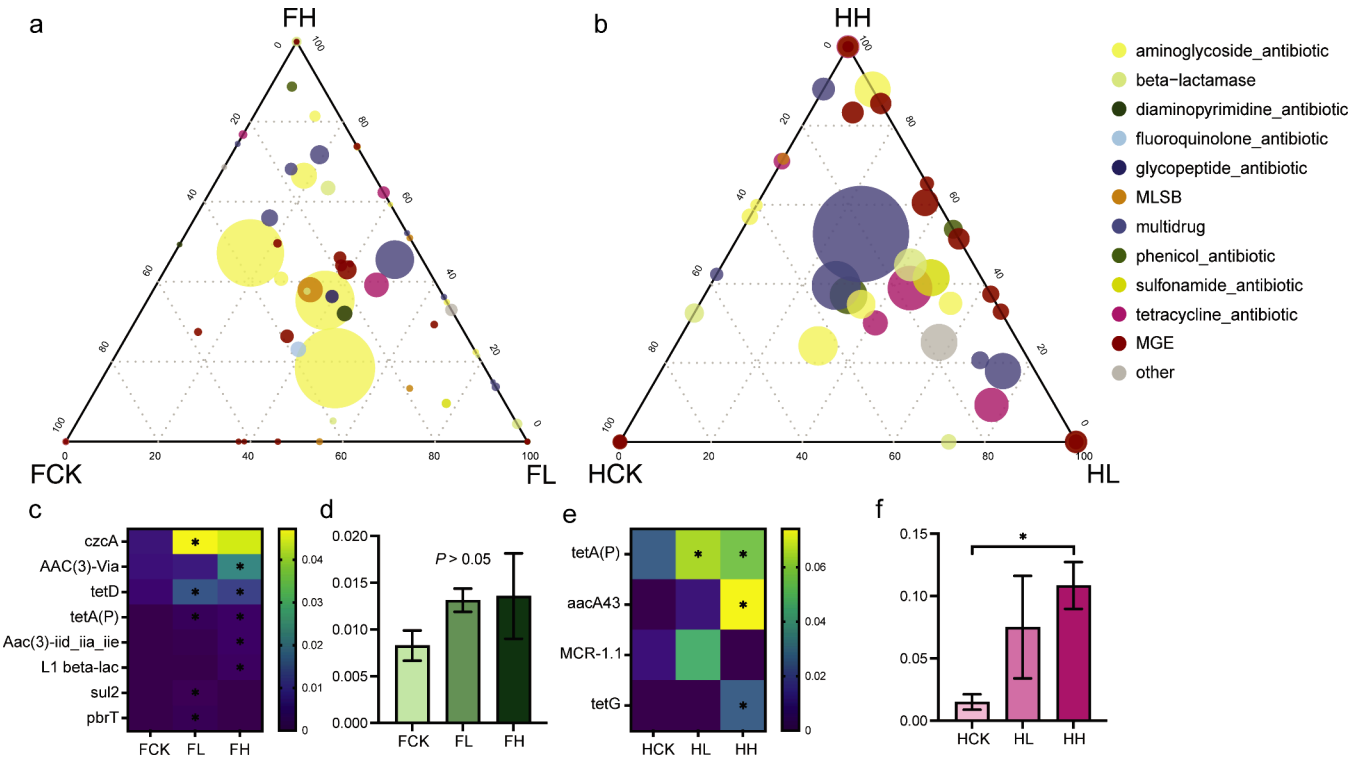

Figure 3. Ternary plots of ARGs and MGEs detected in (a) collembolan and (b) predatory mite gut microbiomes, respectively. Each symbol represents a single ARG or MGE. The size of each symbol represents its relative abundance and color its drug class. The position of each symbol indicates the contribution of each ARG or MGE to total gut resistome abundances (mean, $n = 5$) in control organisms (CK), and organisms under low (L) and high (H) zinc-thiazole exposures. Heatmaps describing the abundance of selected ARGs in (c) collembolan and (e) predatory mite and (mean, $n = 5$) gut microbiomes are shown. Total abundances of MGEs in (d) collembolan and (f) predatory mite (mean, $n = 5$) gut microbiomes are also provided.

mite guts (Figure 3a,b). Aminoglycoside ARGs were the most abundant in collembolan samples, whereas multidrug ARGs were most prevalent in mite samples. Compared with the control group, the abundance of *czcA*, *AAC(3)-Via*, *tetD*, *tetA(P)*, *Aac(3)-iid_iia_iie*, *L1 beta-lac*, *sul2*, and *pbrT* were significantly enhanced in ZT-exposed collembolan guts (Figure 3c), whereas *tetA(P)*, *aacA43*, and *tetG* were selected in mite guts (Figure 3e). PCoA and PERMAVONA results showed that distribution patterns of ARGs in collembolan guts were significantly different among different ZT treatments ($P < 0.05$) (Figure S5). Although individual MGE relative abundances did not significantly change with ZT exposure ($P > 0.05$), the total abundance of MGEs in mite gut samples increased after consuming ZT-exposed collembolans (Figure 3f). For example, HH MGE abundances were significantly higher than those of the HCK group ($P < 0.05$). Notably, the abundance of total MGEs was about 5.00- and 7.23-fold higher in the HL and HH groups, respectively, compared to the control group.

3.3. Microbiome Changes in Soil Food Chain (Including Pathogens). Overall, 389 ASVs were found in collembolan gut samples, and 4740 ASVs were found in the mite samples, with 181 ASVs shared between two sample sets. The phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidota were the four dominant bacterial taxa found in both collembolan and mite microbiomes. Specifically, Bacteroidota was the most prevailing phylum in the collembolan microbiomes (Figure S6a), in accordance with our previous results, whereas Firmicutes was most abundant in the predatory mites (Figure S6b). ZT exposure significantly altered the relative abundance of Actinobacteriota, Acidobacteriota, and Bdellovibrionota in the microbiomes of collembolan in ZT-treated groups compared to the control group ($P < 0.05$), whereas the relative abundance of Actinobacteriota, Chloroflexi, and Verrucomicrobiota were significantly different in the microbiomes of mites that consumed ZT-exposed collembolans (compared with the control; $P < 0.05$). In terms of diversity, a significant decrease in the Shannon index was seen in the collembolan FH group compared to the control group (FCK) (Figure S6c). No significant changes were seen in equivalent predatory mites (Figure S6e). We calculated the Rs index of the collembolan and mites gut communities, but no significant changes in Rs were seen with ZT exposures ($P > 0.05$) (Figure S6d,f). Conversely, PCoA and PERMANOVA analysis showed significant differences between collembolan and mite gut microbiomes ($P < 0.05$) (Figure S6g). Overall, however, the bacterial gut community structure of collembolans and predatory mites did not show significant differences across treatments (both $P > 0.05$) (Figure S6h,i).

Fifty-two bacterial species were flagged as possible pathogens in collembolan microbiomes, whereas 174 putative pathogens were found in predatory mite microbiomes. Sankey plots were developed, and the 10 most abundant putative pathogenic species and their classification of families and disease types (including animal, zoonotic, and plant disease types) were identified in the collembolan and mite samples versus treatment (Figure S7a,b). In the collembolan microbiomes, the abundance of species *Brevundimonas diminuta* was significantly enriched in FL treatment ($P < 0.05$), and

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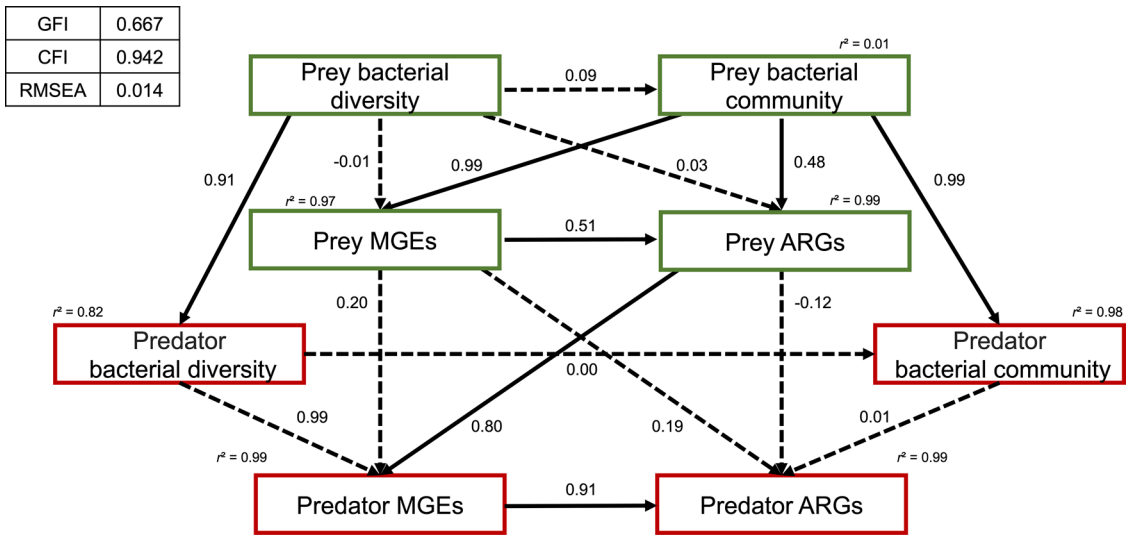

Figure 4. Structural equation models (SEM) showing the standardized effects of the variables concerned on the changes of antibiotic resistomes in this soil food chain. The goodness-of-fit index and Bentler comparative fit index indicate the goodness-of-fit of the models to the original data. The solid lines mean a direct effect, and the dashed lines mean an indirect effect. Numbers at arrows are standardized path coefficients. R^2 indicates the proportion of variance explained.

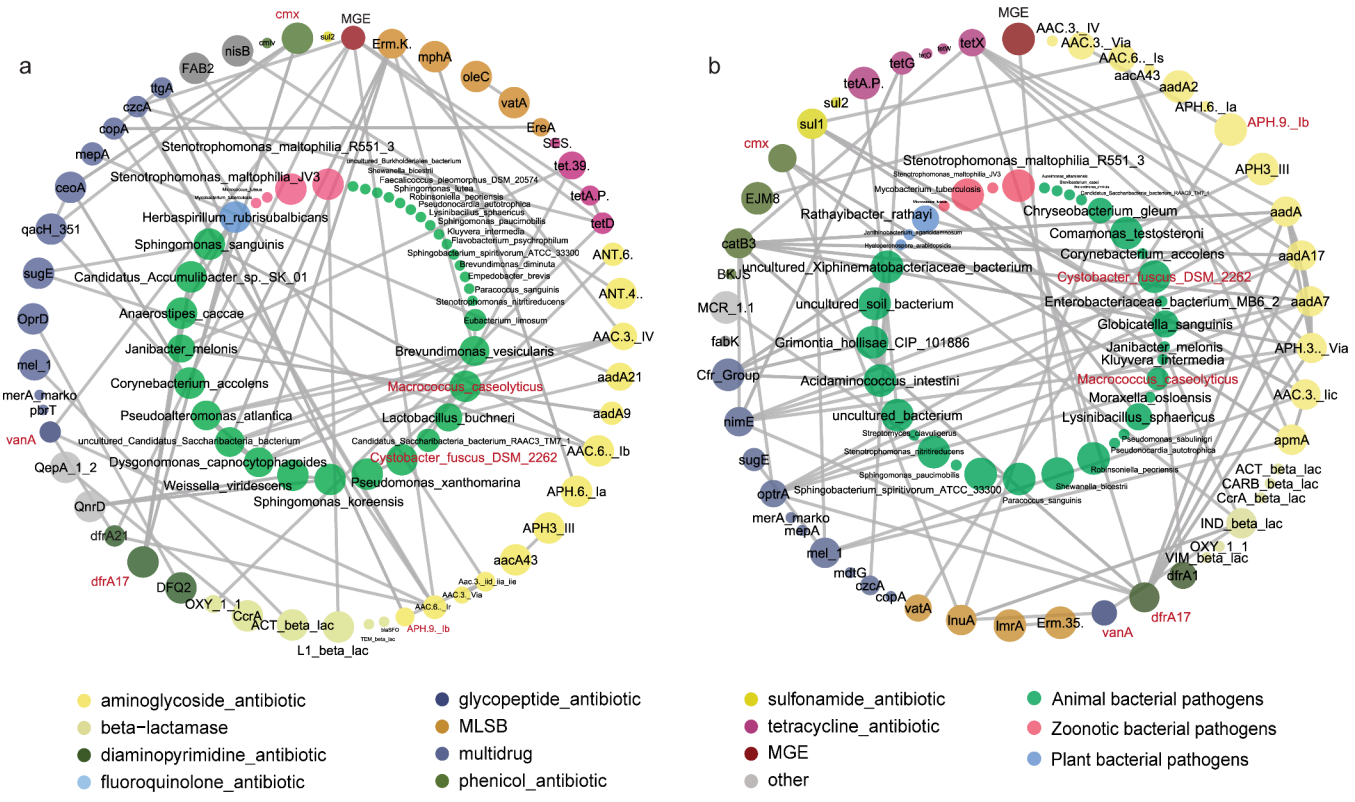

Figure 5. Network analysis showing interactions between potential pathogens and resistomes in (a) collembolans and (b) predatory mites. Nodes are colored by drug classes of ARGs and pathogen types. The node size represents the harmonic centrality degree. Lines connecting nodes (edges) represent strong corelation between ARGs, MGEs, and pathogenic species ($r > 0.7$, $P < 0.05$). Prevalent shared nodes between two networks are highlighted in red font.

abundance of *Sphingobacterium spiritivorum ATCC 33300* was significantly selected in the FH treatment ($P < 0.05$). No significant changes in abundance of potential pathogens were seen in mites that consumed ZT-exposed collembolans. However, SourceTracker analysis then was used to assess the relative contribution of pathogens from ZT-exposed collembolan gut samples to pathogens in the mite guts. Although not statistically significant, collembolans contribution to possible pathogens in the predatory mites increased with increased ZT pre-exposure (Figure S7c,d).

3.4. Potential Drivers of ARG Transmission in Soil Fauna Microbiomes. SEM was performed to estimate indirect and direct relationships among gut microbiomes and resistomes in our model prey-predator food chain relative to

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ARG transmission. The goodness-of-fit index (GFI) and Bentler comparative fit index (CFI) were used to assess the quality fit of the model to the original data. Modeling revealed that 99% of variance in ARG data in mite samples was explained by factors in the model ($P < 0.05$) (Figure 4). For collembolan gut samples, the bacterial community structure (*pco*1) showed strong positive associations with the relative abundance of ARGs ($\beta = 0.48$, $P < 0.01$) and MGEs ($\beta = 0.99$, $P < 0.001$). Further, MGE abundance had a significant positive association with ARG abundance ($\beta = 0.51$, $P < 0.01$). For mite gut samples, positive significant associations between MGE and ARG abundance also were observed ($\beta = 0.91$, $P < 0.05$). According to SEM, the bacterial diversity and community structure of the mite microbiomes were significantly influenced by consuming collembolans that were previously exposed to ZT ($\beta = 0.91$, $P < 0.001$ and $\beta = 0.99$, $P < 0.001$, respectively). A significant positive correlation also was seen between ARGs in collembolan microbiomes and MGEs in the mites ($\beta = 0.80$, $P < 0.001$), indicating potential ARG transfer within this prey-predator interaction.

3.5. Co-occurrence Networks between Pathogens and Resistomes. To examine the potential antibiotic-resistant pathogens in this food chain, co-occurrence networks were developed based on Spearman correlation coefficients on the abundance of gut microbiomes, ARGs, and MGEs in the collembolans and mites. In the collembolan network, 88 nodes and 98 edges were identified, whereas in the predatory mite network, 87 nodes and 110 edges were found (Figure 5a,b). In both networks, ARG nodes were mostly classified as aminoglycoside resistant genes, and pathogen nodes were primarily classified as animal pathogens. Among the 44 nodes shared between the two networks, six nodes exhibited a degree greater than two, implying connections with more than two other nodes. Within these six nodes, four were classified as ARGs (*dfrA17*, *cmx*, *vanA*, and *APH(9)Ib*), while the remaining two were categorized as pathogens (*Cystobacter fuscus DSM 2262* and *Macrococcus caseolyticus*). Further, in the collembolan network, 24 edges were noted that strongly connected ARGs and pathogens, with five possible pathogens (*Corynebacterium accolens*, *Dysgonomonas capnocytophagoides*, *Janibacter melonis*, *Sphingomonas sanguinis*, and *uncultured Candidatus Saccharibacteria bacterium*) being associated with nodes belonging to multiple drug classes (Figure 5a). MGEs were highly corelated with ARGs, especially aminoglycoside, multidrug, and tetracycline antibiotic resistant classes. In contrast, in the mite gut network, only 12 edges significantly connected ARGs and pathogens, with one pathogenic species (*Chryseobacterium gleum*) being connected to nodes attributed to multiple drug classes (Figure 5b).

4. DISCUSSION

4.1. ZT Exposure Enhances the Relative Abundance of ARGs in Collembolan Gut Microbiomes. Pesticides have been increasingly applied and are frequently detected in soils, which can cause stress in the resident microbial communities. However, since no previous study had assessed ecotoxicity effects on collembolans with ZT exposures, the collembolans were exposed to low to high ZT doses, with consideration of previous studies.[32,33,68] No significant changes in mortality or reproduction were seen in the collembolans, whereas ARG selection and the diversity and composition of their gut microbiomes were sensitive to the ZT exposure level. Increased ZT exposures significantly increased the abundance of certain ARGs in the collembolan microbiomes, as we hypothesized. This is similar to what was observed in earthworm guts in a recent study, with some ARGs increasing by 4.5−6.3 times under 1.0 mg/kg ZT exposure compared to control organisms.[69] Their and our results hint that ZT exposure may generally contribute to enrichment of ARGs in soil fauna gut microbiomes. Our results also showed an increasing in number of ARGs and MGEs in collembolan gut microbiomes with the development of the host, which may due to community changes in gut microbiomes according to a previous study.[70] Meanwhile, bacterial diversity declined in the collembolan microbiomes upon ZT exposure, which is consistent with other studies on invertebrate gut microbiomes.[45] A recent study showed that ZT can enrich specific metabolism pathways such as citrate cycles in gut microbiomes of soil invertebrates.[69] Although ZT is recognized for its high efficiency and low toxicity, it may inadvertently alter the community composition and function of gut microbiomes, further leading to changes in the abundance of ARGs within the microbial communities. Moreover, changes in MGE abundances (including plasmids and integrons) and the bacterial community composition and structure both statistically correlated ARG enrichment in collembolan gut samples. Previous work has highlighted the crucial role of MGEs in altering ARG profiles,[71] supporting the idea that horizontal gene transfer is a key mechanism in ARG enrichment under ZT pressure. In short, our results show that collembolan gut microbiomes become enriched with ARGs under ZT-pressure, with MGEs potentially mediating antibiotic resistance selection.

4.2. Trophic Transfer of ARGs in the Food Chain. Given that collembolan microbiomes became enriched with ARGs upon ZT exposure, gut resistomes in the collembolans and their mite predators were examined in the trophic chain. As background, intrinsic antibiotic resistomes existed in control organisms in our study, consistent with previous studies on wild bumblebees[72] and mites,[73] confirming antibiotic resistance exists everywhere and has origins from environments.[74] However, the intrinsic composition of ARGs was significantly different between collembolans and predatory mites. Previous soil food web studies also have shown a robust selectivity for ARGs among species,[40] suggesting the selection for ARGs in diverse gut environments. Different genes were enriched in collembolans and predatory mites under ZT pressure in our study, which may be attributed to their intrinsic differences in the resistomes and microbiomes. Additionally, these differences could result in varying strategies to cope with pressures, leading to distinct adaptations and gene enrichment patterns.

Here we found that many unique ARGs were selected in the ZT-treated collembolans and predatory mites but excluded by control predatory mites (Figure 2b). This suggests some ARGs in predatory mites may origin from ARGs in collembolans, further indicating a potential transfer of ARGs from the collembolans to the predatory mites along with predation, showing antibiotic resistance may occur via trophic transfer in soil food webs. There are two potential mechanistic explanations for the transfer and selection of ARGs between prey and predator. First, when prey gut bacteria carrying ARGs are consumed by predators, they may be selected and survive in the predator gut, although the specific driver of this selection remains unclear under ZT pressure. Second, horizontal transfer of ARGs may be facilitated under ZT stress, as the gut bacteria

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of mites acquire MGEs and/or ARGs from their prey organisms, thereby contributing to the development of resistomes in the predators.[75−77] To better understand the role of internal ZT selection in both gut microbiomes, measuring the internal concentrations of ZT in both prey and predators would provide valuable insights.

Some ARGs, such as *tetA(P)*, *aacA43*, and *tetG*, were significantly enriched in mites that consumed ZT-exposed collembolans. *AacA43* is of particular interest because it codes for a gene cassette that confers clinically relevant resistance to kanamycin, tobramycin, and some aminoglycosides.[78] *AacA43* was noted previously as important in another food chain study on lettuce and snails,[79] suggesting that trophic transfer of ARGs via this gene cassette may be important in soil food chains.

Abundant multidrug resistance genes were detected and enriched in predatory mite microbiomes, possibly from the prey collembolans. Multidrug efflux pump has been reported being able to extrude various toxic compounds including conventional antibiotics, pesticides, and heavy metals.[80,81] In general, multidrug resistance is primarily associated with nonspecific resistance mechanisms. However, it suggests that genes conferring broad-spectrum resistance are being selected, which increases the potential for resistance to develop at higher trophic levels. Invertebrates at higher trophic levels have been reported to possess higher microbial diversity and participate in a broader range of activities.[40] The altered and expanded resistomes of predators suggest that the transmission of ARGs through the soil food chain contributes to resistance spread to higher trophic levels, and our research indicates that this process is amplified under pesticide pressure. Thus, resistomes may further transfer into higher trophic levels in the food web along with predation and may spread to diverse ecosystems accompanied by invertebrate behavior or activities.

4.3. Possible Drivers of ARG Transfer. Here we show a plausible track for the enrichment and trophic transfer of ARGs in our model food chain (Figure 4). The SEM we constructed showed that the abundance of ARGs found in the mite guts was strongly associated with the abundance of MGEs, bacterial diversity, and community structure. MGEs were suggested as a significant factor and possible mediator in the trophic transfer of ARGs in our model food chain, which is consistent with other studies.[82] Generally, ARGs within environmental microbiomes often correlate with MGEs, such as integrons, transposons, and plasmids, which is believed to be an important mechanism in antibiotic resistance transmission in ecosystems.[83,84] Our results suggest that ARG spread to predatory mites may not be directly promoted by ARGs in ZT-exposed collembolans but rather mediated by MGEs in predatory mites. Work on fungicides have shown a similar mechanism; i.e., the fungicide serves as a coselector of ARGs in original hosts, but elevated abundance is driven by MGEs and horizontal gene transfer in subsequent hosts.[26] Despite the absence of concrete evidence of MGE transmission in metagenome-assembled genomes, our findings indicate that the exchange of ARGs via MGEs likely occurs frequently in soil food chains.

4.4. Microbiomes in the Soil Food Chain. Invertebrates are diverse microbial repositories within soil ecosystems, and gut microbiomes in higher trophic level organisms can be unique and especially diverse. However, the gut microbial diversity and community structure of predators, such as in mites, are significantly influenced by their prey, collembolans here. These findings are consistent with previous work, where predators have been observed to obtain microorganisms from their prey through general activity, ingesting organisms at lower trophic levels.[85,86] Though microbial composition and function in an individual gut can be resilient to perturbations under environmental pressures,[87] Rs of the microbiomes in our model food chain were calculated, and it was found that neither the collembolan nor the mite guts changed significantly under ZT exposure. During host development, gut microbial community and composition in some species have been reported to be constantly changing with host development.[88,89] Our results showed both similarities and differences in the microbial composition of 14- and 28-day-old collembolans; thus, further work is needed to examine the temporal dynamics of gut microbiomes in soil invertebrates.

4.5. Potential Pathogen Hosts of ARGs. Bacterial pathogens are a central concern in human and ecosystem health.[90] Therefore, their promulgation in any ecosystem is a concern, especially zoonotic pathogens, which can mutually impact humans and animals. Previous work has shown that the collembolan gut can be colonized with pathogens.[91,92] We found *Brevundimonas diminuta* and *Sphingobacterium spiritivorum ATCC 33300* were significantly enriched in ZT-exposed collembolans, which is consistent with recent work in honeybees[93] that showed enrichment of opportunistic bacterial pathogens can occur in their gut microbiomes under environmental stress. Prior studies noted that pesticide pressure might promote infectious disease in nontarget animals,[94] including zoonotic strains that could influence human health.[95] Though no significant increases in potential pathogens were observed in our predatory mite guts, SourceTracker analysis showed that the proportion of pathogenic origin from collembolans increased with the ZT concentration increasing. This observation suggests that pesticide exposure and pressure may accelerate pathogen acquisition at higher trophic levels.

The co-occurrence of potential pathogens and ARGs (Figure 5) showed that ZT pre-exposure can enrich potential resistant pathogens. Specifically, a wide range of potential pathogenic hosts of ARGs were identified in our results, and they exhibited different patterns between collembolan and predatory mite networks, including a range of shared nodes between networks including ARGs and potential pathogens. For example, shared dfrA17 and cmx genes were found in both networks and were related to a range of different pathogens. These diverse genes, classified as diverse drug resistance types, have been reported in hundreds of pathogens,[96,97] suggesting a potential risk for further transmission if they enter higher trophic levels. Shared nodes suggest consistency of resistomes and pathogens between adjacent trophic levels.

However, we did not find shared edges between collembolan and predatory mite networks, which may be due to intrinsic differences in their gut microbiomes, even though some pathogens are related to multiple ARGs or MGEs in both networks. For instance, *Corynebacterium accolens* showed a strong corelation with *ceoA*, *qacH_351* and *cmx* genes in the collembolan network, and *Chryseobacterium gleum*, a well-recognized human pathogen, showed strong corelation with *IND_beta_lac* and *lnuA* genes in the mite network. These genes represent different drug resistance types, which means that these pathogens may carry multiple types of ARGs. This finding is consistent with recent studies indicating that *Chryseobacterium* species are resistant to several antimicrobials

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and can occur widely in environmental, food, and water sources.[98,99] The simultaneous emergence of these pathogens suggests their potential transmission and colonization in the guts of invertebrates. To address this potential risk, efforts should be focused on identifying the pathogenic hosts that carry ARGs and MGEs, which facilitate their spread. These pathogens may "infect" various organisms across different food chains in diverse environments,[100] posing a significant threat of transmission and spread through soil ecosystems. Future studies are encouraged to adopt cultivation-based approaches to explore the phenotypic differences between trophic levels before and after exposure to ZT, particularly in comparison between matched pathogens harboring distinct MGE or ARGs.

Overall, ZT exposure enriched not only the number and abundance of ARGs in exposed collembolan guts but also those in the gut of predatory mites that were not directly exposed to ZT. This enrichment was found to include potential pathogens in the guts of both collembolans and predatory mites, indicating the potential for trophic transfer of ARGs and pathogens among soil invertebrates. Potential ARG transmission and selection were closely associated with MGEs, and bacterial community diversity and composition, both possible drivers of trophic transfer of ARGs through the collembolan-predatory mite food chain. Our results have enhanced our understanding of trophic transfer of ARGs in the soil food chain. These findings draw attention to the spread of ARGs and potential pathogens within soil food webs and emphasize previously overlooked risks associated with pesticide usage in soil ecosystems. However, more studies are necessary to further identify the specific microorganisms that carry ARGs and gain deeper insights into the mechanisms of resistance and pathogenic trophic transfer. Detailed analysis of metagenome-assembled genomes and phenotypic changes in the gut microbiomes of soil invertebrates could further allow us to pinpoint the microbial hosts of ARGs and elucidate the potential pathways of MGE-mediated spreading. Given the widespread presence of collembolans and predatory mites as keystone species in soil ecosystems, it is imperative to conduct further research to evaluate the broader risks of resistome trophic transfer within ecosystems. The potential impacts on public health also warrant a comprehensive exploration.

■ ASSOCIATED CONTENT

Data Availability Statement

The raw sequencing data are available at the NCBI Sequence Read Archive with the Bioproject ID PRJNA1140252. The authors declare that the other main data supporting the findings of this study are available within this Article and in the Supporting Information files. Extra data supporting the findings of this study are available from the corresponding author upon reasonable request. Custom codes for all analyses are available from the corresponding authors upon request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.4c07822.

The numbers of collembolans and predatory mites after exposure (Figure S1); dose effect of ZT on the shifts of number and relative abundance of ARGs in collembolan gut microbiomes (Figure S2); microbial composition, diversity, and community structure in collembolan microbiomes (Figure S3); factors influencing the variation in relative abundance of ARGs in gut microbiomes (Figure S4); community structure of ARGs in the microbiomes of collembolans and predatory mites (Figure S5); microbial composition, diversity, and community structure in prey collembolans and predatory mites (Figure S6); taxonomy classification of potential pathogens identified in collembolan guts and predatory mite guts with SourceTracker analysis of contributions of collembolan gut pathogen communities to predatory mite gut pathogen communities (Figure S7) (PDF)

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Author Contributions

M.Q. conceived and designed the experiments. Y.-F.W. and Z.-L.L. carried out the experiments. Y.-F.W., D.Z., and Z.-L.L. analyzed the data. Z.-L.L. prepared the figures and wrote the paper. Y.-F.W., D.Z., Y.-G.Z., M.Q., D.G., and M.Q.-B. reviewed and commented on the paper. All authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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