Environmental Science & Technology

Article

Cite This: Environ. Sci. Technol. 2019, 53, 7770−7781

pubs.acs.org/est

Trophic Transfer of Antibiotic Resistance Genes in a Soil Detritus Food Chain

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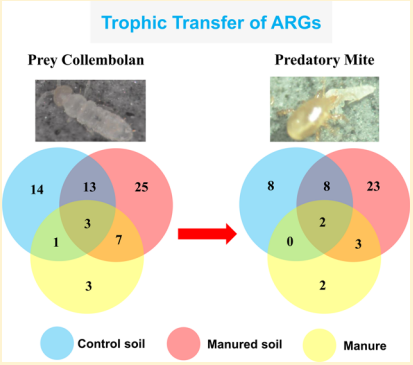
Supporting Information

ABSTRACT: The presence and spread of antibiotic resistance genes (ARGs) are causing substantial global public concern; however, the dispersal of ARGs in the food chain is poorly understood. Here, we experimented with a soil collembolan (*Folsomia candida*)-predatory mite (*Hypoaspis aculeifer*) model food chain to study trophic transfer of ARGs in a manure-contaminated soil ecosystem. Our results showed that manure amendment of soil could significantly increase ARGs in the soil collembolan microbiome. With the ARGs in the prey collembolan microbiome increasing, an increase in ARGs in the predatory mite microbiome was also observed, especially for three high abundant ARGs (*blaSHV*, *fosX* and *aph6ia*). Three unique ARGs were transferred into the microbiome of the predatory mite from manure amended soil via the prey collembolan (*aac(6′)-lb(akaaacA4)*, *yidY_mdtL* and *tolC*). Manure amendment altered the composition and structure and reduced the diversity of the microbiomes of the prey collembolan and the predatory mite. We further reveal that bacterial communities and mobile genetic elements were two important drivers for the trophic transfer of ARGs, not just for ARGs distribution in the samples. These findings suggest that the importance of food chain transmission of ARGs for the dispersal of resistance genes in soil ecosystems may be underestimated.



INTRODUCTION

Antibiotic resistance has only been claimed to be ancient, while not proven.[1,2] Recently, the overuse of antibiotics in medicine and animal husbandry has increased the abundance of antibiotic resistance genes (ARGs) in human-associated environments at an unprecedented rate.[3,4] The appearance of ARGs in medically relevant strains is causing the global health crisis, because the increase in antibiotic-resistant pathogens is making it more difficult to treat infectious diseases.[5,6] Numerous studies have identified the number and abundance of environmental ARGs (e.g., in the air,[7] phyllosphere,[8] water,[9] soil,[10] animal gut[11]). These studies indicate that ARGs are continuing to spread through the environment. For instance, the transfer of antibiotic resistant plasmids from chickens to humans was identified in a very early study.[12] Recently, many studies have also illustrated the spread of ARGs from the soil to the phyllosphere of vegetables[13] and from wastewater treatment plants to arable land.[14] Additionally, bacteria have been shown to acquire antibiotic resistance from each other via horizontal gene transfer (HGT).[15,16] A large number of ARGs have also been detected in the gut microbiome of individual animal species, e.g. collembolan,[3,17,18] honey bee,[11] fish,[19] baboon[20] and house fly,[21] which suggests that these animals may act as reservoirs and transportation systems for ARGs.[22,23] However, knowledge of the transmission of ARGs within natural food webs and ecosystems remains limited. And the movement of ARGs may be substantially influenced by trophic relationships within food webs.

Numerous studies have shown that manure can enhance the abundance and diversity of ARGs and contribute to the dissemination of ARGs within affected environments (e.g., soil,[24] rhizosphere[25] and phyllosphere[26]). In a recent study, a relationship was observed between manure addition to soil and the ARGs in the soil collembolan microbiome,[3] suggesting that manure may influence the ARG suite in the microbiomes of soil fauna more generally. Manure entering soil ecosystems is preferentially ingested by soil fauna (e.g., collembolans,

Received: January 11, 2019
Revised: April 25, 2019
Accepted: June 4, 2019
Published: June 4, 2019

DOI: 10.1021/acs.est.9b00214
Environ. Sci. Technol. 2019, 53, 7770−7781

enchytraeids and earthworms),[27,28] potentially leading to an increase in ARGs in the microbiomes of that fauna,[29] because it has been identified that manure can carry a large number of ARGs and antibiotics,[10,28] depending on the source, manure additions may exert a selection pressure on environmental bacteria[4,6] and therefore on soil food webs.

Collembolans and predatory mites are the two most abundant soil microarthropods in natural ecosystems, occupying key trophic positions in the soil food web and playing an important role in soil ecological processes (e.g., decomposition of litter and carbon−nitrogen cycling).[30−33] Various foods can be ingested by soil collembolans such as manure, litter, bacteria and fungi,[34,35] and collembolans are important prey for predatory mites.[31,36] A large number of microorganisms including pathogenic microbiota routinely colonize soil collembolans and predatory mites.[37−40] Previous studies have shown diverse ARGs in the microbiome of soil collembolans from field soils,[3] and that oral antibiotic exposure can significantly enhance the abundance and diversity of ARGs in the gut microbiome of collembolans.[17] Mite predation on collembolans may result in trophic transfer of ARGs enhancing the chance that the pathogenic microbiota of predatory mites may obtain antibiotic resistance genes.[3] However, soil fauna resistome is poorly understood, and no experiment has yet revealed the abundance and diversity of ARGs in the microbiome of predatory mites and any effects of manure on trophic transfer of ARGs within the soil food chain. The term resistome indicates all antibiotic resistance in our study.

In the present study, we used high-throughput quantitative PCR (HT-qPCR) to screen for potential resistance determinants. Our aims were to (1) characterize the composition of ARGs and mobile genetic elements (MGEs) in the microbiomes of the prey collembolan and predatory mite, (2) reveal the effects of manure on ARG and MGE shifts in the prey collembolan and predatory mite microbiomes, (3) identify any trophic transfer of ARGs, (4) investigate the change in microbiome due to the addition of manure using next generation sequencing of 16S rRNA genes, and (5) explore the relationship between the bacterial community and the MGE and the ARG profile in the soil detritus food chain. We hypothesized that (1) manure can enhance the abundance and diversity of ARGs in the microbiome of prey collembolans when ARGs and antibiotics are abundant in the manure,[10] and (2) because collembolans act as a reservoir and transportation system for ARGs in soil ecosystems,[3] ARGs can be transferred from manure to the microbiome of a predatory mite via collembolan predation.

MATERIALS AND METHODS

Manure, Soil and Animals. Swine manure commonly applied to the arable land was purchased from a Biofertilizer co., LTD (Zhejiang, China). We collected soils (sandy loam) of 0−20 cm depth from an arable land site (29° 48′N, 121° 23′E; Zhejiang, China) after harvest of a *Brassica chinensis* crop. After the collected soil was air-dried in the shade, we carefully removed root pieces, large stones and other allogenic materials. Then the manure and soil were sieved (2 mm). We divided the soil into two parts. One part was mixed with the manure (5 g manure per kg dry soil based on local agronomic practice) to obtain manure-amended soil (Manured soil), the second part was not amended with manure to be used as a control (Control soil). The water content of the soils was adjusted to 60% of field capacity (17 g water per 100 g soil), and the soils were preincubated for 2 weeks before use. The physicochemical properties and basic characteristics of the ARG and bacterial communities of the manure and soil are summarized in Tables S1, S2 and S3 and Figures S1, S2, S3 and S4.

The model soil collembolan (*Folsomia candida*) and predatory mite (*Hypoaspis aculeifer*) (originally obtained from Aarhus University, Denmark) were used in our study to construct a model soil detritus food chain. We have reared these species for more than seven years, and they are maintained on Petri dishes covered with a layer of a mixture of activated carbon and moist plaster of Paris (1:9 w/w). More details on culturing and synchronizing of these animals are described in previous studies.[30,31] The 10−12 day-old collembolans and 33−35 day-old female predatory mites were used in the current study.

Experimental Design. Our study included two treatments (Control soil and Manured soil), and each treatment included three replicates. The exposure of collembolans to the manure was conducted in the soil system. 300 age-synchronized collembolans (10−12 days) were transferred into plastic containers (9 cm high, inner diameter 6 cm) with 60 g soil. Soil water was replenished twice a week with ultrapure water. After 2 weeks of incubation, all collembolans in each container were extracted by water floating. On the basis of the body size of the collembolan, we isolated the introduced collembolans from any juveniles produced. Then, 250 introduced collembolans were placed on 9 cm Petri dishes with a layer (thickness: 0.5 cm) of the mixture of activated carbon and moist plaster of Paris (1:9 w/w), and the surplus collembolans were used to extract DNA. Ten synchronized predatory mites (33−35 days) were added into each Petri dish and incubated for 2 weeks. In this step, the predatory mite was not directly exposed to the manure-amended soil, but was cultured in the cleaned Petri dish by adding the prey collembolan with an altered resistome, which ensured that the change in the predatory mite resistome was due to direct feeding on the prey collembolan with an altered resistome. After 2 weeks, we identified the introduced predatory mites according to their body size and used them to isolate DNA. In addition, we determined the mortality of the collembolan and mite during the study and found that the manure has no significant impact on the mortality of the collembolan and mite.

DNA Extraction. Three collembolans or predatory mites were used to isolate DNA from each sample. Before DNA extraction, we used 0.5% sodium hypochlorite to wash collembolans and predatory mites three times, and then sterile water was used to wash them five times at 4 °C. We used a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) to extract DNA of collembolans and predatory mites. In brief, a microelectric tissue homogenizer was used to homogenize collembolans and predatory mites in sterile 1.5 mL centrifuge tubes. After homogenization, we added 180 μL of tissue lysis buffer and 20 μL of proteinase K (>600 mAU mL^{-1}, QIAGEN, Hilden, Germany) into each centrifuge tube, which was vortexed for 2 min. The centrifuge tube was incubated at 56 °C in a water bath lasting 10 h. Other extraction processes followed the kit manufacturer's instructions. Finally, we used 100 μL of elution buffer to elute the isolated collembolan and predatory mite DNA, and then the DNA obtained was kept at −20 °C until further use.

High-Throughput Quantitative PCR. We used a total of 296 primer sets (Table S4) to determine ARG profiles in the

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Environ. Sci. Technol. 2019, 53, 7770−7781

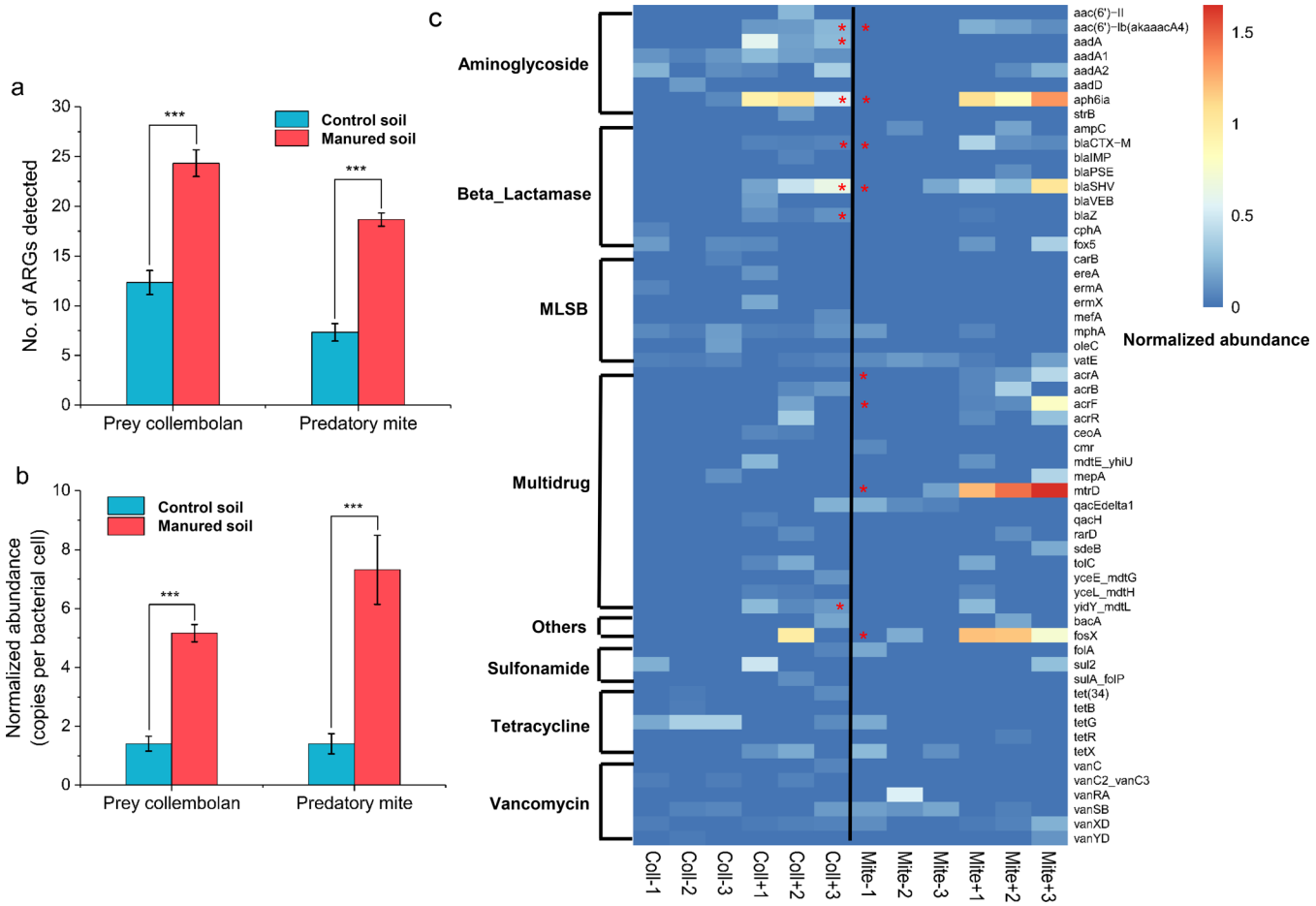

Figure 1. Characterization of antibiotic resistance genes (ARGs) in the microbiome of a model soil food chain. The detected number (a) and normalized abundance (b) of ARGs (n = 3; Mean ± SE) in the microbiome of the prey collembolan and predatory mite. A t-test was used to compare the difference within species between control soil and manured soil treatments (Significant level: 0.05). (c) The heatmap revealed the normalized abundance of every ARG in all samples. On the basis of the antibiotics they resist, we classified ARGs into eight categories (aminoglycoside, β-lactamase, macrolide-lincosamide-streptogramin B (MLSB), multidrug, others, sulfonamide, tetracycline, vancomycin). Coll−, prey collembolan in the control soil treatment; Coll+, prey collembolan in the manured soil treatment; Mite−, predatory mite in the control soil treatment; Mite+, predatory mite in the manured soil treatment. The red asterisk to the left of the black line indicates a significant difference between collembolans of the control soil treatment and the manured soil treatment. The red asterisk to the right of the black line indicates a significant difference between predatory mites of the control soil treatment and the manured soil treatment. The "*" indicates $P < 0.05$, and the "***" indicates $P < 0.001$.

microbiome of the prey collembolan and the predatory mite. All major classes of antibiotic resistance genes (285), 10 genes of MGEs and one 16S rRNA gene were included in these primer sets. The Wafergen SmartChip Real-time PCR system was selected to conduct the high-throughput quantitative PCR (HT-qPCR). Each sample was detected three times (three technical replicates), and the sterile water was also determined as the negative control, which ensured the validly of result. A standard mixed plasmid containing multiple target genes was used as the positive control. The system and condition of the HT-qPCR were consistent with previous studies.[3,17] We used the SmartChip qPCR software (V 2.7.0.1, WaferGen Biosystems, Inc.; Takara Bio.) to analyze the data obtained. Only when the results of three technical replicates were all positive, amplification efficiency was between 1.8 and 2.2 and r^2 was >0.99, the data from amplification was adopted. Considering the PCR bias, an amplification efficiency calibration formula was used to minimize the error before further data analysis. More details of the formula are described in a previous study.[3] The threshold cycle was set at 31 as a detection limit for amplification.[18] We used a normalized copy number of ARGs per bacterial cell to represent the abundance of ARGs detected in samples, which was calculated based on previous studies.[3,8] This was used because the normalized abundance can minimize the error produced by the differences of 16S rRNA gene abundance in different types of samples.[3]

DNA Amplification, Library Preparation, Sequencing and Bioinformatics Analysis. The amplicons of the V4 region of bacterial 16S rRNA genes were generated by using the primer 515F (GTGCCAGCMGCCGCGG) and 806R (GGACTACNVGGGTWTCTAA) with unique barcodes. The system and condition of amplification and purification of PCR products were consistent with a previous study.[41] The Qubit 3.0 fluorimeter (Invitrogen) was used to determine the concentration of PCR purified products in all samples. We pooled 24 samples of equal DNA content to a library, which was sequenced at the MiSeq 300 instrument with illumina MiSeq Kit v2 (Read length: 2 × 300 bp; Meiji biological medicine co., LTD, Shanghai, China). All high-throughout

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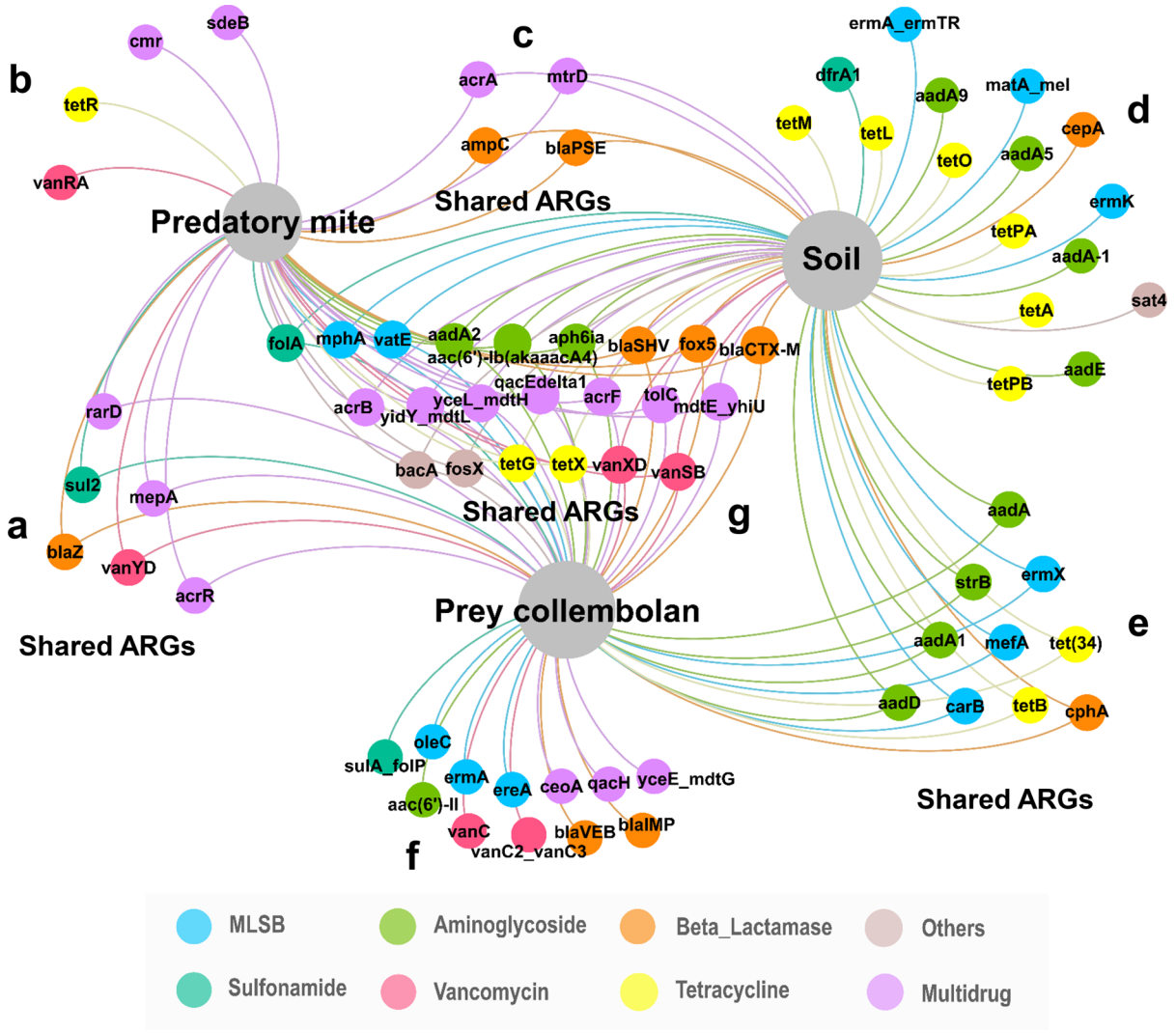

Figure 2. Bipartite network analysis revealing shared and unique ARGs between the microbiomes of the soil, prey collembolans and predatory mites. In the bipartite network analysis, we did not differentiate samples from the control soil treatment or manured soil treatment. For example, for prey collembolan, samples from the control soil treatment and manured soil treatment were both included. The meanings of the letters are (a) 6 ARGs shared between prey collembolan and predatory mite; (b) 4 ARGs unique to the predatory mite; (c) 4 ARGs shared between the predatory mite and soil; (d) 16 ARGs unique to the soil; (e) 10 ARGs shared between the soil and prey collembolan; (f) 12 ARGs unique to the prey collembolan; (g) 22 ARGs were found in all samples. On the basis of the antibiotics they resist, we classified ARGs into eight categories (aminoglycoside, β-lactamase, macrolide-lincosamide-streptogramin B (MLSB), multidrug, others, sulfonamide, tetracycline, vancomycin), and the same category was labeled with the same color.

sequence data are deposited in the NCBI Sequence Read Archive under the accession number SRP116006.

The Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) was selected to perform the high-throughput 16S sequencing data analysis based on the online instructions.[42] In short, first, we merged paired-end reads, removed primer sequences and filtered the reads via Phred quality scores to obtain clean reads of samples. Then, the chimeras were removed by using usearch quality filter in the QIIME, and the operational taxonomic units (OTUs) were clustered based on the 3% sequence difference by *de novo* OTU picking.[43] We discarded singletons, and OTUs were represented by the most abundant sequence of each cluster. We used the Greengenes 13.8 reference database to align the representative sequences via the PyNAST and to assign taxonomic status of OTUs by the RDP Classifier 2.2.[44−46] The Greengenes lanemask was selected to filter the alignment to remove these positions that are not needed in building phylogenetic trees. A phylogenetic tree was built by using the FastTree algorithm[47] for further downstream analysis. The bacterial alpha diversity was presented using the Shannon index, which was calculated from the relative abundance of OTUs. We used the Adonis test to reveal the difference in bacterial community patterns (significant level: 0.05) and principal coordinate analysis (PCoA) to show the distribution of the bacterial communities of different samples based on the Bray−Curtis distance.

Statistical Processing. The data of ARGs and bacterial communities was presented via mean ± standard error (SE), which was calculated by using the Microsoft Excel. Significant differences between treatments were compared using *t*-test in the SPSS v20.0. The significance level was set at 0.05. Bonferroni-corrected was applied in the multiple test. The R software with version 3.4.3 was used in this study. The heatmap was used to display the normalized abundance of

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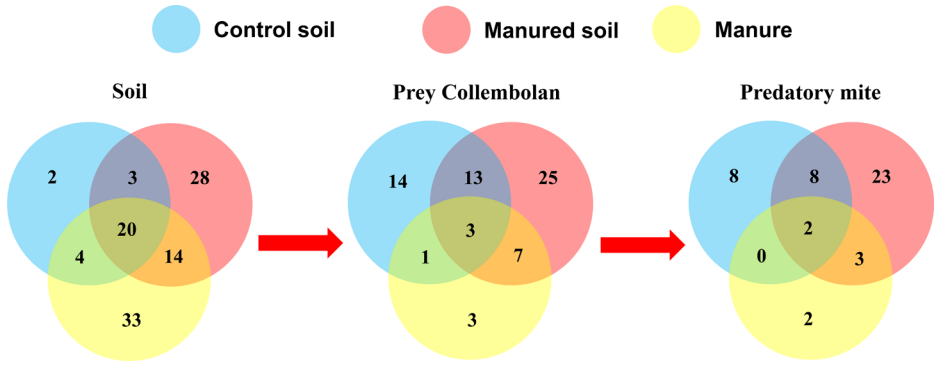

Figure 3. Venn diagram depicting the number of unique and shared ARGs between ARGs in manure and ARGs in samples from the control soil treatment and samples from the manured soil treatment, and showing the transfer of ARGs number in a model soil food chain from manure to a predatory mite via soil and a prey collembolan.

ARGs in all samples, which was produced by the pheatmap package within R.[48] The Gephi with version V 0.9.2 was selected to perform the bipartite network analysis, showing the shared and unique ARGs between the microbiomes of soil, prey collembolan and predatory mite.[3] We used the online version of Venny 2.1 to produce the Venn diagram.[18] The heatmap of the bacterial community at the genus level was drawn in Microsoft Excel. We used the EcoSimR NulModels for Ecology within R to calculate the checkerboard scores (C-scores) of the assembly of the shared ARGs among all sample types in the model soil food chain.[41,49] The proportion of ARG pairs was measured in the C-score. We used 5000 random permutations of the same data set to generate the score distribution of a simulated metric. By comparing with the score distribution, we could test the rule of ARGs community assembly in the model soil food chain. The null hypothesis indicated random ARGs assortment. The vegan 2.3-1 package within R was used to conduct the Procrustes, Adonis, Anosim and Mantel tests and partial redundancy and nonmetric multidimensional scaling (NMDS) analysis.[3] We used the ggalluvial package within R to draw the alluvial diagram of bacterial composition at the phylum level.[50] Other graphics were produced by OriginPro 9.1.

RESULTS

Characterization of Antibiotic Resistance Genes. A total of 58 ARGs and 5 MGEs were detected in the microbiomes of all prey collembolan and predatory mite samples. According to the antibiotic resistance of ARGs, the detected ARGs can be divided into eight categories (aminoglycoside, β-lactamase, macrolide-lincosamide-streptogramin B (MLSB), multidrug, others, sulfonamide, tetracycline, vancomycin). The addition of swine manure significantly increased the number of ARGs detected in the microbiome of the prey collembolan (t-test, $t = 6.69$, df = 4, $P = 0.003$) and predatory mite (t-test, $t = 10.25$, df = 4, $P < 0.001$) compared to those in the control, respectively (Figure 1a). The normalized abundance of ARGs in the microbiomes of the prey collembolan (t-test, $t = 9.64$, df = 4, $P < 0.001$) and predatory mite (t-test, $t = 4.85$, df = 4, $P = 0.008$) in the manured soil treatment were approximately 3.6 and 5.2 times higher than those in the control (Figure 1b), respectively. At the same time, the addition of manure also significantly increased the number and abundance of ARGs in the soil microbiome (Figure S1; t-test, $t < 3.61$, df = 4, $P < 0.05$). Compared with the control, higher abundant MGEs were observed in the microbiomes of collembolan and predatory mite in the manured soil treatment (Table S3; t-test, $t < 3.35$, df = 4, $P < 0.05$). With the ARGs in the prey collembolan microbiome increasing, an increase of ARGs in the predatory mite microbiome was also observed. The normalized abundance of most ARGs was enhanced in the manure-treated prey collembolan or predatory mite compared to those in the control, as shown in the heatmap, especially for aminoglycoside, β-lactamase, multidrug and others (Figure 1c). Notably, we identified three abundant ARGs (*blaCTX-M*, *blaSHV* and *aph6ia*), and their normalized abundance was significantly increased in the microbiomes of the prey collembolan and predatory mite in the manured soil treatment (t-test, $t = 4.12$, df = 4, $P < 0.05$). The ARG distributions of the same treatments are clustered and different from other treatments according to the nonmetric multidimensional scaling analysis (stress = 0.086; Figure S5). Along with the NMDS1 axis, the ARGs profiles of samples in the manured soil treatment were separated from the samples in the control. In the dimension 2 (NMDS2), collembolan ARGs profiles were clustered separately from the predatory mite. The Anosim and Adonis tests further indicate that the distribution patterns of ARGs from different treatments are significantly different ($R = 0.821$, $P = 0.001$; Table S5).

Shared and Unique Resistome between Soil, Prey Collembolan and Predatory Mite. The bipartite network analysis reveals that 22 ARGs are shared among soil, prey collembolan and predatory mite (Figure 2), which include eight categories. The number of multidrug ARGs (7) was the highest among the shared genes. The *blaSHV*, *fosX* and *aph6ia* had the highest normalized abundance among shared ARGs. With the abundance of *blaSHV*, *fosX* and *aph6ia* in the microbiome of prey collembolan increasing, their abundance also significantly increased in the predatory mite (t test, $t < 3.26$, df = 4, $P < 0.05$). Overall, the shared genes occupied 29.7% of all ARGs detected. Similar to microbial community analysis, we further used the C-score to assess the rule of the shared ARGs community assembly. In this analysis, the null hypothesis indicated random ARGs assortment. Our result that the C-score of ARGs is not included in the score distribution of a simulated metric rejected the null hypothesis, indicating that the assembly of shared ARGs is not random assortment in the model soil food chain (Figure S6). Six shared ARGs (*acrR*, *vanYD*, *mepA*, *blaZ*, *sul2* and *rarD*) were identified between prey collembolan and predatory mite (Figure 2a), and 10 shared ARGs were found between the soil and prey

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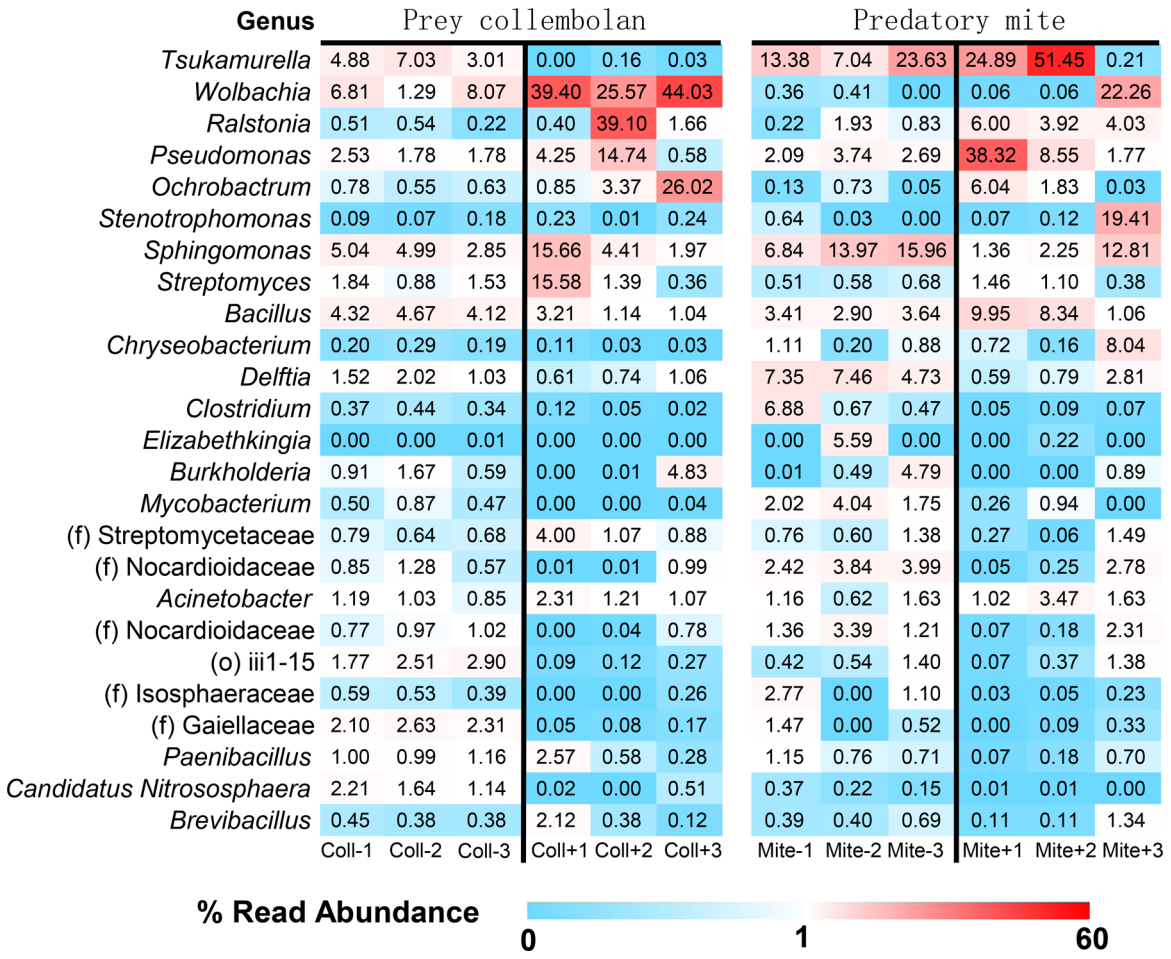

Figure 4. Composition and abundance of the microbiomes of the prey collembolan and predatory mite. Heatmap showing the 25 most abundant genera (>2% of the highest abundance of reads in all used samples) detected in the microbiome of a model soil food chain. If the name of the genus was not available, higher possible taxonomic classification was used. Coll−, prey collembolan in the control soil treatment; Coll+, prey collembolan in the manured soil treatment; Mite−, predatory mite in the control soil treatment; Mite+, predatory mite in the manured soil treatment.

collembolan (Figure 2e). The most unique ARGs (16) were observed in the soil microbiome (Figure 2d). Compared with the predatory mite (43), more ARGs are found in the microbiome of the prey collembolan (58). Moreover, it is the number of different ARGs that is different in their microbiome. Only four ARGs (*vanRA*, *tetR*, *cmr* and *sdeB*) were unique to the predatory mite (Figure 2b).

Venn diagrams reveal three unique ARGs (*aac(6′)-lb-(akaaacA4)*, *yidY_mdtL* and *tolC*) were transferred into the microbiome of the predatory mite from swine manure via the soil-prey collembolan−predatory mite food chain (Figure 3). 38 ARGs were shared between the soil and manure, and 14 ARGs were only shared between the manure and the soil amended with manure. Seven of those 14 ARGs were detected in the microbiome of the prey collembolan in the manured soil treatment, but not observed in the prey collembolan in the control. In the manured soil, a high number of ARGs pop up that are found in neither the control soil nor the supplemented manure (Figure 3). The addition of manure increased the number of unique ARGs in the microbiome of the soil, prey collembolans and predatory mites compared to the control.

Composition and Diversity of the Bacterial Community. We identified 315 875 high-quality sequences from all samples of the prey collembolan and predatory mite, and at least 18 164 reads were obtained in each sample. Overall, 10 593 OTUs were clustered at 97% similarity across all prey collembolan and predatory mite samples. The prey collembolan includes 9195 OTUs, and 3631 OTUs were identified in the microbiome of the predatory mite. 61.5% of all OTUs of the predatory mite are shared with the prey collembolan. The phyla Proteobacteria and Actinobacteria are the two dominant bacterial taxa in the microbiomes of the prey collembolan (occupying 60.4 ± 10.1% and 14.5 ± 3.2%) and the predatory mite (occupying 46.3 ± 6.3% and 30.8 ± 6.5%), respectively (Figure S7). The read abundance of Proteobacteria was significantly enhanced in the microbiome of the prey collembolan in the manured soil treatment compared to that in the control, by 110% (*t*-test, $t = 5.68$, df = 4, $P = 0.005$), but no significant change was observed in the predatory mite. The addition of swine manure significantly reduced the read abundance of Firmicutes and Acidobacteria in the microbiome of the prey collembolan, by 61% and 93%, respectively (*t*-test, $t < 3.58$, df = 4, $P < 0.05$). Although there is no significant difference, the read abundance of Actinobacteria, Firmicutes and Acidobacteria were reduced in the predatory mite in the manured soil treatment. At the genus level, *Wolbachia* (20.9 ± 7.4%) was the dominant microorganism in the microbiome of the prey collembolan, and the predatory mite microbiome was dominated by *Tsukamurella* (20.1 ± 7.4%) (Figure 4). The endosymbionts *Wolbachia* were strikingly overrepresented in

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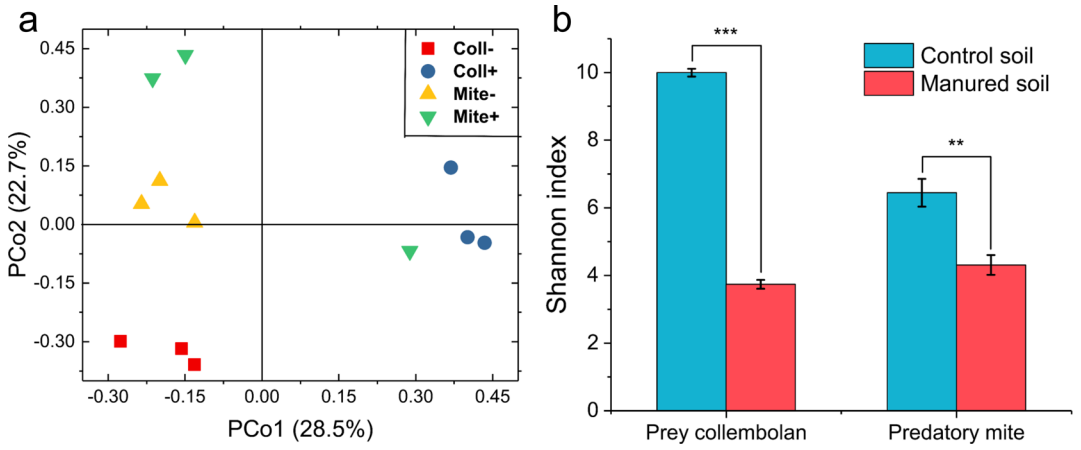

Figure 5. (a) Principal coordinates analysis (PCoA) depicting the distribution pattern of bacterial communities of the prey collembolan and predatory mite based on the Bray−Curtis distance of OTU in a model soil food chain. Interpretation of the variation is listed in parentheses by the PCoA axes. Coll−, prey collembolan in the control soil treatment; Coll+, prey collembolan in the manured soil treatment; Mite−, predatory mite in the control soil treatment; Mite+, predatory mite in the manured soil treatment. (b) The Shannon index (n = 3; Mean ± SE) of bacterial communities of the prey collembolan and predatory mite at a sequencing depth of 18 164. A t-test was used to compare the difference between control soil and manured soil treatments for the same soil animal species (significant level: 0.05). The "***" indicates $P < 0.001$, and "**" indicates $P < 0.01$.

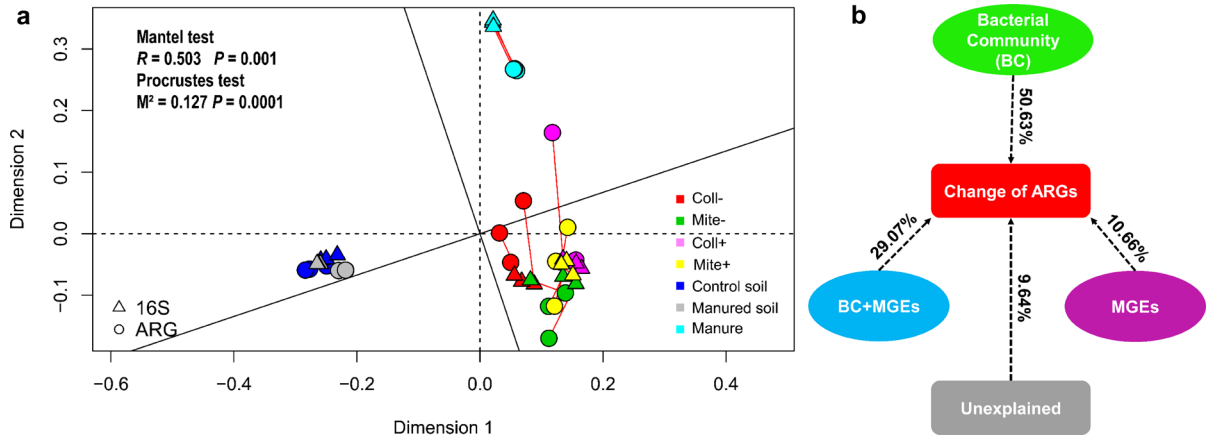

Figure 6. (a) Procrustes analysis depicting the significant correlation between antibiotic resistance gene (ARG) profiles and composition of the bacterial community (the relative abundance of 16S rRNA gene OTU data) based on Bray−Curtis dissimilarity metrics (sum of squares M^2 = 0.127, P = 0.0001, 9999 permutations). The triangles represent the composition of the bacterial community, the circles indicate ARG profiles, and the colors of squares indicate different samples. Coll−, prey collembolan in the control soil treatment; Coll+, prey collembolan in the manured soil treatment; Mite−, predatory mite in the control soil treatment; Mite+, predatory mite in the manured soil treatment. The relationship between ARGs and bacterial communities is also determined by the Mantel test using the Bray−Curtis distance. (b) Partial redundancy analysis (pRDA) revealing the contribution of bacterial communities (BC; genus level) and mobile genetic elements (MGEs) to the change in ARGs in all samples.

the collembolan after manure treatment, by 574% (t-test, t = 5.22, df = 4, P = 0.006). Most of the other genera seemed to be more randomly distributed between treatments and hosts. For example, *Tsukamurella* might be relatively less abundant in collembolan samples (0.06 ± 0.05%) in the manured soil treatment compared to the control (4.97 ± 2.01%) (t-test, t = 4.23, df = 4, P = 0.013), but in the predatory mite, there was no clear pattern (t-test, t = 0.70, df = 4, P = 0.525) since they had a very low relative abundance in a sample (mite+3). The prey collembolan and predatory mite have more similar microbiomes when they come from the same soil treatment. High read abundance of genera were shared between all samples.

A significant difference was observed between the distribution patterns of the microbiomes of soil, manure and fauna (Figure S8; Adonis test, $P < 0.001$). Principal coordinates analysis shows that the bacterial community patterns of the prey collembolan in the control soil treatment are clustered and separated from those in the manured soil treatment along the PCo1 axis (explaining 28.5% of the variation; Figure 5). The Adonis test further indicates that the distribution patterns of bacterial communities of the prey collembolan and predatory mite show a significant difference between different manure treatments ($P < 0.01$; Table S5). In addition, the addition of manure also significantly altered the bacterial community composition of the soil (Figure S4; Adonis test, $P < 0.05$). The addition of swine manure significantly reduced the Shannon index of diversity in the prey collembolan and predatory mite microbiome compared to that in the control, by 62.6% and 33.1%, respectively (Figure 5b; t-test, $t < 4.24$, df = 4, $P < 0.05$).

Relationship between the Antibiotic Resistome and Bacterial Community and MGEs. A significant correlation between matrixes of ARG profiles and bacterial communities

DOI: 10.1021/acs.est.9b00214
Environ. Sci. Technol. 2019, 53, 7770−7781

was observed in all samples using the mantel test (Figure 6; R = 0.503, P = 0.001). Procrustes analysis further indicates that the distribution patterns of ARGs and the bacterial community are clustered by type of sample and treatment, and ARG profiles are significantly related with the composition of the bacterial community (Figure 6; M^2 = 0.127, P = 0.0001). Partial redundancy analysis also reveals that 90.36% of total ARGs variation can be explained by using the data from the bacterial community and MGEs (Figure 6b). The bacterial community contributes 50.63% of total variation, and the MGEs explain 10.66% of total variation in the ARG profiles shift. Linear regression also shows a significant positive relationship between the abundance of MGEs and ARGs (R^2 = 0.815, P < 0.001; Figure S9).

DISCUSSION

In this study, although we only performed three replication due to the complexity of soil food chain operations, low coefficient of variation in each treatment ensured the validity of the experimental results (Figures 1 and 5). Moreover, significant statistical results also confirmed solidly trophic transfer of ARGs in our model food chain. The collembolan−predatory mite food chain used in our study can be commonly found in the real soil ecosystem, and the collembolan and mite occupy central trophic levels in real soil food webs. In addition, the food chain system was likely unstable when it was composed of the animal recently collected from the field. To obtain reliable and repeatable results, the continuously reared organisms are a good choice to construct a model food chain. In this study, we focused on the unsolved question that whether ARGs can be transferred in the soil food chain, and the model food chain could satisfy our need. At the same time, the exposure of collembolans to the manure was conducted in the soil system, and the ratio of collembolan and predatory mite was based on the real soil food web. These all ensured that our laboratory study could reflect the real soil food web to some extent and confirm the aim of our study.

In the present study, putative resistance genes were investigated by using HT-qPCR, and not all genes detected had been proven to provide resistance.[1] Many of the investigated genes are inherent to sensitive bacteria where they do not provide resistance, and the rate of false positive calling of actual resistance genes should always be considered very high with these types of studies. However, these facts do not affect our ability to answer the questions of our study. Our results showed that manure amendment of soil could increase the number and abundance of ARGs in the soil collembolan microbiome. With the ARGs in the prey collembolan microbiome increasing, an increase in ARGs in the predatory mite microbiome was also observed. We further identified that three unique ARGs were transferred into the microbiome of the predatory mite from manure-amended soil via the prey collembolan. These results all demonstrated our hypotheses, and more attention should be focused on trophic transfer of ARGs in the soil food web in further study.

Manure Enhances ARGs. As we hypothesized, addition of manure significantly increases the number and abundance of ARGs in the microbiome of the soil collembolan, suggesting that the manure may make an important contribution to the occurrence and spread of ARGs in the soil fauna microbiome. Three possible reasons are given to explain the result. (1) Our results indicate that the manure used contained abundant ARGs (Table S2). These ARGs may directly enter the microbiome of the soil collembolan by ingestion. (2) Multiple antibiotics were also detected in the used manure (Table S1). This suggests that collembolans feeding on the manure will be exposed to multiple antibiotics which may increase the ARGs in the microbiome of the collembolan, because previous studies have shown that the concentration of antibiotics detected in our test can cause an increase in ARGs in natural environments.[4,51] (3) Manure addition significantly increased the number and abundance of ARGs in the soil microbiome (Figure S1), which might contribute indirectly to the increase in ARGs in the microbiome of the collembolan by the exchange of microbiota carrying ARGs between soil and collembolan.[23] Since collembolans are usually colonized with many pathogenic microbiota,[39,40,52] the increase in ARGs in the collembolan microbiome may enhance the occurrence of resistant pathogenic microbiota in soil ecosystems. In our study, more aminoglycoside, β-lactamase, multidrug and others genes (not classified according to their antibiotic resistance) are observed in the manure-treated collembolan microbiome compared to the control, indicating that these genes are more readily transferred to the collembolan microbiome from the manure. With the activity of the collembolan, genes may be more easily dispersed in soil ecosystems. Considering that these genes are commonly found in ARG-contaminated soils[10,53] and there is a huge demand in the human and veterinary medicine[54] for the antibiotics they are resistant to, more focus may be needed to understand the spread of these ARGs by soil fauna in soil ecosystems.

In the present study, low abundance of β-lactam resistance genes were observed in the microbiome of collembolan, which suggested β-lactam biosynthetic gene might be not over-represented in the collembolan, because collembolans have recently been shown to include antibiotic biosynthetic gene clusters in their genome.[55,56] More specifically, β-lactam biosynthetic gene clusters were shown in the genome of *F. candida* and actively transcribed in the gut.[56] The functions of these gene products remain elusive, but it is likely that they are produced to interfere with the microbiome and the occurrence of ARGs.

Trophic Transfer of ARGs. In our study, the trophic transfer of ARGs is identified for the first time in the microbiomes throughout the soil collembolan−predatory mite food chain, indicating that the dispersal of ARGs in soil food webs is occurring, and can be enhanced by application of manure. This is easy to understand, because after soil collembolans are consumed by predators, their microbiota usually remain in the gut of the predator for some time.[57] A part of the microbiota with these ARGs may remain and horizontal transfer of ARGs from one bacterium to another may occur in the gut of the predator,[16,58] which will contribute to trophic transfer of ARGs in the food chain. The transfer of ARGs may have two opposite effects on the host animal. On the one hand, when ARGs are transferred into pathogens, they may be harmful for the host animal due to increasing the resistance of pathogens. On the other hand, animal-beneficial bacteria carrying these ARGs may enhance the host resistance and prevent their loss in polluted agricultural landscapes, such as where manure is applied. However, to our knowledge, so far no study has explored the effects of ARGs on the health of collembolan or mites. Approximately 29.7% of all detected ARGs were shared between the soil, prey collembolan and predatory mite (Figure 2), suggesting that these genes have a potential chance of transfer into the prey collembolan−

DOI: 10.1021/acs.est.9b00214
Environ. Sci. Technol. 2019, 53, 7770−7781

predatory mite food chain from soils. Multidrug genes account for 32% of these genes, which is not surprising, because a previous study has indicated that multidrug efflux pumps are native to all bacteria—even the one sensitive to antibiotics.[1] Therefore, their presence did not indicate the presence of resistant bacteria. To evaluate the risk of ARGs for soil ecosystem health, further study may need making the efforts to check for actual resistant bacteria. Our results showed that the *blaSHV* (*β*-lactamase), *fosX* (others) and *aph6ia* (aminoglycoside) had the highest normalized abundance among these shared ARGs, and their trophic transfer occurred in the model soil food chain. This suggested that these ARGs may cause a greater risk for soil ecosystem health compared to the dispersal of low abundant resistance genes. We further find that the assembly of these shared genes is not neutral, suggesting that the soil food chain may influence selection in the transfer of ARGs. Selection may be related to the unique niches of different soil fauna, because different niches are generally inhabited by different microorganisms,[41] and ARGs are usually associated with microbes.[3,59,60] Noteworthily, in the manured soil, a high number of ARGs pop up that arefound in neither the control soil nor the supplemented manure. These ARGs may mainly originate from soil, because the addition of manure altered soil microbial community and might produce a pressure for soil bacterial community due to commonly occurring toxic compounds (e.g., antibiotics and heavy metals). Of course, the detection limit might also be a reason, because manure might enhance the abundance of "native" ARGs which might be not detected due to low abundance before the addition of manure. In the present study, three unique ARGs are identified, which are transferred into the microbiome of the predatory mite from the manure via the prey collembolan. This is consistent with our hypothesis, and the soil collembolan—predatory mite food chain may act as a reservoir and transportation system for ARGs in soil ecosystems. Thus, food chain transmission of resistance genes is a potentially important pathway for the dispersal of ARGs, especially in ARGs-contaminated soil ecosystems. These results also suggested that when the collembolan and mite are preyed by other animals (e.g., spider and bird), the ARGs may also transfer to animals of a second trophic level. With the collembolan and mite moving, these ARGs may also be transferred into plants. By consuming the plant and direct or indirect contacting with these animals, these ARGs may have implications for human health. In this study, many unique ARGs were observed in microbiomes of soil, prey collembolan and predatory mite. The ARGs unique to soil might be genes naturally inherent to soil bacteria that cannot live in the studied mite/collembolan microbiomes. Likewise, the ARGs unique to mites and collembolans might be part of the core genome of bacteria that obligately (or facultatively) live in the microbiomes of these animals.

Change in Bacterial Community. In our study, Proteobacteria and Actinobacteria were two dominant phyla in the microbiome of collembolan *F. candida*, which was different from a previous study[55] that Firmicutes and Gammaproteobacteria were confirmed in the core microbiota of collembolan. This may be because the different of cultivation environment leads to this difference. Our collembolans were obtained from the soil system amended with the manure, and the previous study was conducted in the plate system. Similar results have also been observed in our previous study.[52] Our results show that addition of manure significantly alters the composition and structure of the bacterial communities of the prey collembolan and predatory mite, because bacteria are an important part of the feeding ecology of the Collembola, and the addition of manure changed the diet of the soil fauna by changing the bacterial community. In addition, in our study, microbial communities show a significant difference between soils in the control and manured treatments. Diet and environmental factors all have important effects on changes in animal microbiomes.[41,61,62] The change in the predatory mite microbiome may be due to the microbiome of the prey collembolan shift. Because, for the predatory mite, the difference between different treatments was mainly from prey collembolans offered. The diversity of the bacterial community in the prey collembolan and predatory mite food chain is significantly reduced with the addition of manure. Collembolans were likely attracted to the manure as a food source which reduced the diversity of food types ingested by the collembolans, and the diversity of food has a relationship with the diversity of the gut microbiota.[63] Moreover, the swine manure has different physicochemical properties than the soil or contains some compounds toxic to the soil-inherent bacteria, thereby reducing the microbial diversity, which is also an important reason for the change of collembolan microbiome. Our results also showed that the endosymbionts *Wolbachia* were strikingly overrepresented in the collembolan after manure treatment. It is well established that *Wolbachia* has an important contribution to the fitness and reproduction of arthropod.[64] Therefore, the manure might affect the health of collembolan by altering the abundance of *Wolbachia*.

Contribution of the Bacterial Community and MGEs to the Change in ARGs. A significant correlation between bacterial communities and ARG profiles is observed in our study, and bacterial communities can explain 50.63% of all ARGs variation. This implies that bacterial communities are a dominant driver of the change in ARGs in the microbiomes throughout the soil collembolan—predatory mite food chain. Numerous previous studies also support this assumption.[3,8] MGEs are usually known to have an important contribution to the horizontal transfer of ARGs between bacteria.[16,58] Our results reveal that high abundance of MGEs was detected in the microbiomes of the prey collembolan and predatory mite in the manured soil treatment, and the relative abundance of MGEs was significantly correlated with the abundance of ARGs. The pRDA analysis also shows that 10.66% of all ARGs variation can be explained by the MGEs, which is higher than that of previous studies in the soil[65] and phyllosphere.[8] This indicates that MGEs play an important role in the ARGs shift in the prey collembolan and predatory mite. Therefore, bacterial communities and MGEs are two important drivers of trophic transfer of ARGs in the microbiomes throughout the soil collembolan—predatory mite food chain.

In summary, the trophic transfer of ARGs in the microbiomes of the prey collembolan and predatory mite has been observed in our study. The addition of manure to soil significantly increased the abundance and diversity of ARGs in the microbiome of the prey collembolan. The 22 ARGs identified have the potential to migrate in microbial communities throughout the soil food chain, and the assembly of these genes is not random. We also found that three unique ARGs can be transferred from manure into the microbiome of a predatory mite via collembolan predation. Manure can alter the composition and structure of the bacterial community and reduce the diversity in the microbiomes of the prey

DOI: 10.1021/acs.est.9b00214
Environ. Sci. Technol. 2019, 53, 7770−7781

collembolan and predatory mite. Bacterial communities and MGEs are two important drivers of the trophic transfer of ARGs in microbial communities throughout the soil collembolan−predatory mite food chain. These findings contribute to our knowledge of trophic transfer of ARGs in the soil detritus food chain and will direct our attention to the spread and dissemination of resistance genes in the soil food web.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b00214.

Table S1: The basic properties (n = 3; Mean ± SD) of the swine manure and soil used in the experiment. Table S2: The normalized abundance (n = 3; Mean ± SE) of antibiotic resistance genes in the swine manure used. Table S3: The normalized abundance (n = 3; Mean ± SE) of MGEs in the microbiomes of collembolan, predatory mite, soil and manure. Table S4: Information on the 296 genes detected in the gene chip. Table S5: Analysis of the Adonis test revealing the difference of ARGs and bacterial community patterns between different samples. Figure S1: The characterization of ARGs in the soil microbiome. Figure S2: The heatmap revealing the relative abundance of every ARG in all samples. Figure S3: Composition and abundance of the microbiomes of manure and soil. Figure S4: (a) PCoA depicting the distribution pattern of soil bacterial communities based on the Bray−Curtis distance for OTUs. (b) The Shannon index (n = 3; Mean ± SE) of soil bacterial communities at a sequencing depth of 18 164. Figure S5: Nonmetric multidimensional scaling analysis depicting the differences in ARG profiles between different samples in the model soil food chain. Figure S6. The assembly of the shared ARGs in the model soil food chain. Figure S7: Alluvial diagram showing the composition (n = 3; Mean) of the microbiomes, at the phylum level, of the prey collembolan and predatory mite. Figure S8: PCoA depicting the distribution pattern of bacterial communities based on the Bray−Curtis distance for OTUs in all samples. Figure S9: Linear regression between relative abundance of MGEs and ARGs. (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by the National Natural Science Foundation of China (41571130063), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15020302 and XDB15020402) and the National Key Research and Development Program of the China-International collaborative project from the Ministry of Science and Technology (Grant No. 2017YFE0107300).

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DOI: 10.1021/acs.est.9b00214
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