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Application of earthworm and silicon can alleviate antibiotic resistance in soil-Chinese cabbage system with ARGs contamination☆

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ABSTRACT

Organic fertilization is a major contributor to the spread of antibiotic resistance genes (ARGs) in the agroecosystem, which substantially increases the risk of ARGs acquisition and their transmission into human food chains. Earthworms are among the most vital soil faunas involved in the link between belowground and aboveground, and silicon is beneficial for soil health and plant stress resistance. This study aims to explore the effect of different amendment strategies (earthworm and/or silicon) and the related influencing factors on the alleviation of ARGs using high-throughput qPCR. The results showed that the application of earthworms and silicon fertilizers reduced the absolute abundance of ARGs in the rhizosphere soils, either singly or in combination. According to the structural equation model and random forest analysis, mobile genetic elements are the major factors enhancing ARGs transfers and the treatment affects ARGs in direct or indirect ways. Our results highlight the role of "rhizosphere effect" in alleviating antibiotic resistance and suggest that silicon fertilizers, together with the earthworms, can be considered as a sustainable and natural solution to mitigate high-risk ARGs spread in the soil-plant systems. Our findings provide guidance in formulating strategies for halting the spread of ARGs in the agroecosystem.

1. Introduction

Antibiotic resistance genes (ARGs) have been widely regarded as an emerging environmental pollution and a global threat to public health (Allen et al., 2010; Larsson and Flach, 2022; Zhu et al., 2017). Horizontal gene transfer (HGT) via conjugation promotes the rapid development and dissemination of ARGs in humans, animals, and various environmental media (Allen et al., 2010; Laxminarayan, 2022; Rodríguez-Beltrán et al., 2021). In recent years, there has been a growing recognition that environmental change is an important risk factor for human health, including the emergence of resistant pathogens and the spread of resistant bacteria (Bengtsson-Palme et al., 2018; Finley et al., 2013). More notably, antibiotic resistance (AR) and associated genes may still occur and be ubiquitous in environments without selection pressure due to HGT-mediated AR transmission (D'Costa et al., 2011; Holmes et al., 2016). Agroecosystems are important reservoirs and non-point sources of environmental antibiotic resistome. With the development of rapid molecular tools, there has been an enhanced understanding of the occurrence and distribution of ARGs in the environment via agricultural organic fertilization under both laboratory and field conditions, especially the fate of ARGs in the plant-soil system (Chen et al., 2017; Mei et al., 2021; Sun et al., 2021; Zhang et al., 2019). However, effective mitigation strategies for inhibiting the spread of ARGs in ARG-contaminated soil-plant systems have not been developed.

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Previous studies have frequently overlooked the remediation of ARG-contaminated soils and the risk grades of ARGs, while focusing on resistance mechanisms, antibiotic types, and treatment at the source of the emission (Wu et al., 2022; Zhang et al., 2021). Therefore, the "high-risk" resistance genes and the development of advanced remediation strategies demand more attention (Wang et al., 2022). Earthworms are hailed as ecosystems or soil engineers for their dedication to ecosystem services (Phillips et al., 2019), however, the role of earthworms in the spread of antibiotic resistance is still equivocal and controversial (Chao et al., 2019; Ding et al., 2019). Researchers have revealed that soil earthworms have decreased ARGs abundance in ciprofloxacin-amended soils (Pu et al., 2020). Recent studies have also shown that manure fertilization has increased the abundance of ARGs in earthworm gut microbiomes (Ding et al., 2019; Zhou et al., 2020). Meanwhile, researchers have also identified a variety of ARGs were identified in earthworm (*Metaphire guillelmi*) guts (Li et al., 2022). Silicon, the second most abundant element on earth (Epstein, 1994), is widely used in the field of preventing soil-borne disease (Song et al., 2016), as well as the prevention and remediation of heavy metal pollution (Das et al., 2021a; Das et al., 2021b; Song et al., 2021; Wang et al., 2020a). The application of silicon fertilizers would significantly improve crop yields while increasing the resistance of the crop to lodging and disease (Song et al., 2021). In addition, silicon fertilization alters the microbial community, thereby improving soil and food quality in the soil-plant system (Das et al., 2019; Samaddar et al., 2019). As a major carrier of ARGs, the changes in the microbial communities have influenced antibiotic resistome in the environment (Chen et al., 2019). Nonetheless, the effect of the silicon fertilizers amendment on soil ARGs has not been investigated in any ARG-contaminated soil-plant systems. Therefore, it is necessary to study the impact of earthworms and silicon fertilizers on mitigating the spread of ARGs in ARG-contaminated soil-plant systems.

In this study, we aimed to evaluate the effects of the earthworms and silicon fertilizers on the different risk grades of ARGs in ARG-contaminated soil-plant systems with the following three questions: (1) whether earthworms and silicon fertilizers can be beneficial for increasing the microbial diversity in ARG-contaminated soil-plant systems; (2) whether earthworms and silicon fertilizers can inhibit the spread of different risk grades of ARGs in ARG-contaminated soil-plant systems; (3) whether the intervention changes the key factors of ARGs spread. Our study provides novel insights into the understanding of the effects of the earthworms and silicon fertilizers on ARGs, mobile genetic elements (MGEs), potential pathogenic bacterium (PPBs), and the composition and functions of the bacterial community in ARG-contaminated soil-plant systems, which have important implications for preventing the spread of ARGs in agricultural production.

2. Materials and methods

2.1. Materials

The red soils (0 – 20 cm) used for pot cultures were collected from a local vegetable plot in Ningbo, China, which had not been amended with pig manure or other organic fertilizers in the past five years. Soils were air-dried, ground, and passed through a 2 mm mesh stainless steel screen, with the addition of pig manure at a ratio of 5% (w/w) (Xiao et al., 2021) as a base fertilizer for Chinese cabbage to mimic the management of actual farmland. The pig manure was sourced from an organic fertilizer factory (Huanying, Ningbo, China). Silicon fertilizer was purchased from an online shopping platform (Guilai, Taobao, China). Well-developed earthworms (*Eisenia fetida*) were purchased from an earthworm company (Wangjun, Nanjing, China). Earthworms were selected at the same stage and were therefore highly similar in anatomy and size. They were incubated in the untreated soil under laboratory conditions (20 ± 2 °C, ~70% relative humidity) for 14 days before the experiment. The earthworm species were identified by morphology and molecular biology as described in previous reports (Wang et al., 2020b; Yao et al., 2019). The seed of Chinese cabbage was purchased from an online shopping platform (Taobao, China). The physicochemical properties and elemental compositions of the soil (pretest-posttest), pig manure and silicon fertilizer are shown in Table S1.

2.2. Experimental setup and sample collection

The 14-day-old Chinese cabbage seedlings were transplanted with three seedlings per pot (height, 20 cm; diameter, 29 cm; 2.5 kg of dry soil per pot). The pot experiment was carried out in a greenhouse with natural illumination and humidity at a temperature of 25 – 30 °C. There were four amendment strategies: soils with (1) no treatment (bulk soil, BS; rhizosphere soil, RS; phyllosphere, P), (2) 5% silicon fertilizers (bulk soil, BS + S; rhizosphere soil, RS + S; phyllosphere, P + S), (3) 5% silicon fertilizers and 10 earthworms (bulk soil, BS + SE; rhizosphere soil, RS + SE; phyllosphere, P + SE), (4) 10 earthworms (bulk soil, BS + E; rhizosphere soil, RS + E; phyllosphere, P + E). Specific treatment strategies are shown in Figure S1A and Table S2. Each treatment was carried out in four repeats. The pots were artificially irrigated with deionized water every two days, to maintain constant soil humidity throughout the experimental period (70% of maximum moisture content).

At the end of the pot experiment (60 days after Chinese cabbage transplanting), the cabbage plants were harvested from the soil. The large soil aggregates were removed by vigorously shaking the roots, and the soil adhering to the roots was collected as the rhizosphere soil sample. After shaking the roots, the larger soil aggregates were collected and thoroughly mixed in a beaker, which was regarded as the bulk soil. All samples were kept in aseptic plastic bags and transported to the laboratory in ice boxes (Mcpherson et al., 2018).

2.3. DNA extraction, amplicon sequencing, and data processing

The preprocessing of the rhizosphere soil and phyllosphere samples was completed within 24 h of harvesting. Details are presented in Figure S1B (Durán et al., 2018; Mcpherson et al., 2018). After pretreatment, the DNA of all samples was extracted with an extraction kit (FastDNA Spin Kit for Soil (MP Biomedical, CA)). The DNA concentration was measured using a Qubit™ 3.0 fluorometer (Thermo Fisher Scientific Inc, Waltham, USA). Then, all DNA samples were diluted to a concentration of 20 ng μL^{-1} and stored at −80 °C until use.

The bacterial 16S ribosomal RNA (16S rRNA) gene (V4 – V5 hypervariable regions) was amplified using primers in a thermocycler PCR system (Table S3). Detailed steps can be found in the previous work in the series (Table S4) (Chen et al., 2017; Xiao et al., 2021). Finally, the sample of the bacterial 16S rRNA gene was submitted to MICROANALY (Hefei, China) for sequencing. The raw sequence data obtained were deposited in the National Genomics Data Center (Members, 2021) at the Genome Sequence Archive (Wang et al., 2017). The accession number is CRA005795 (https://ngdc.cncb.ac.cn/gsa).

Double-end amplicon reads were joined (fastq_mergepairs, VSEARCH v2.17.0_linux_x86_64, default), primers were then cut off, and a quality filter was performed (fastx_filter and fastq_stripleft, VSEARCH). The filtered reads were dereplicated (derep_fulllength, VSEARCH), sorted by copy number (minuniquesize is 20), and denoised using the UNOISE3 (USEARCH v11) at ~100% sequence identity (Edgar, 2017). Chimera detection and removal were conducted by VSEARCH (uchime_ref, VSEARCH) on the denoised reads. All retained zero-radius operational taxonomic units (zOTUs) were aligned to Silva (Silva 138, https://github.com/jameslz/protocols/blob/master/How-to-generate-USEARCH-compatible-SILVA138-fasta.md) using the R script. The non-aligned, plastid, and non-bacteria were removed. Then the zOTU was annotated by VSEARCH (sintax, VSEARCH) with taxonomic classification. The remaining sequences were used to create a zOTU table (usearch_global 97%, VSEARCH). Using the Basic Local Alignment

Search Tool (BLAST) (version 2.12.0) with E-value $<1 \times 10^{-5}$, we aligned high-quality sequences with the reference database to identify the PPBs. The reference database was obtained from the bacterial pathogen database of 16S rRNA sequences. Specific details can be referred to our previous studies (Chen et al., 2016; Yang et al., 2020).

2.4. High-throughput qPCR

ARGs and MGEs were checked via the SmartChip Real-Time PCR System (WaferGen Inc., Fremont, CA, USA). All high-throughput qPCR (HT-qPCR) experiments contained three technical repetitions. Gene-specific primer sets used for different sets of genes linked to antibiotic resistome are shown in Table S5. In total, we included 16S rRNA genes, 7 taxonomic genes, 57 MGEs (including 3 integrase genes, 11 plasmids, 10 transposase genes, 9 insertional sequences, and 24 MGEs), and 319 ARGs in the analysis. The ARG risk grades were classified according to host pathogenicity, gene mobility, and human-associated enrichment (Zhang et al., 2021). The risk grades were classified as Rank I ARGs (52, very high risk), Rank II ARGs (9), Rank III ARGs (38), Rank IV ARGs (74, very low risk) and Unassessed (146). Further details of the data processing algorithm and experimental protocol can be found in previous studies (Xiao et al., 2021; Yang et al., 2020).

2.5. Statistical analyses

All raw data were processed using SPSS Statistics 22 (SPSS Inc., Chicago, USA) and Excel(Microsoft Office Professional Plus, 2019) for statistical and mathematical analysis. The distribution of bacteria and ARGs among the samples was investigated using principal coordinates analysis (PCoA), constrained PCoA (CPCoA), and non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distance. The structural equation model (SEM) was used to assess the indirect and direct effects of sample type, treatment, MGEs, bacterial richness, bacterial abundance, the abundance of PPBs, and the richness of PPBs on the profiles of ARG (Rank I ARG and Rank II ARG). The main microbial predictors for the ARG profiles in the soil-plant system were identified by a random forest (RF) analysis (Jiao et al., 2018). The above analytical processes employed the R package "amplicon" (https://github.com/microbiota/amplicon), and "vegan" (Oksanen et al., 2013). Heatmaps were generated to show the relative abundance of the PPBs with the "pheatmap" (https://github.com/fw1121/pheatmap) package, while the ordinary least squares (OLS) analyses and RF was drawn with the

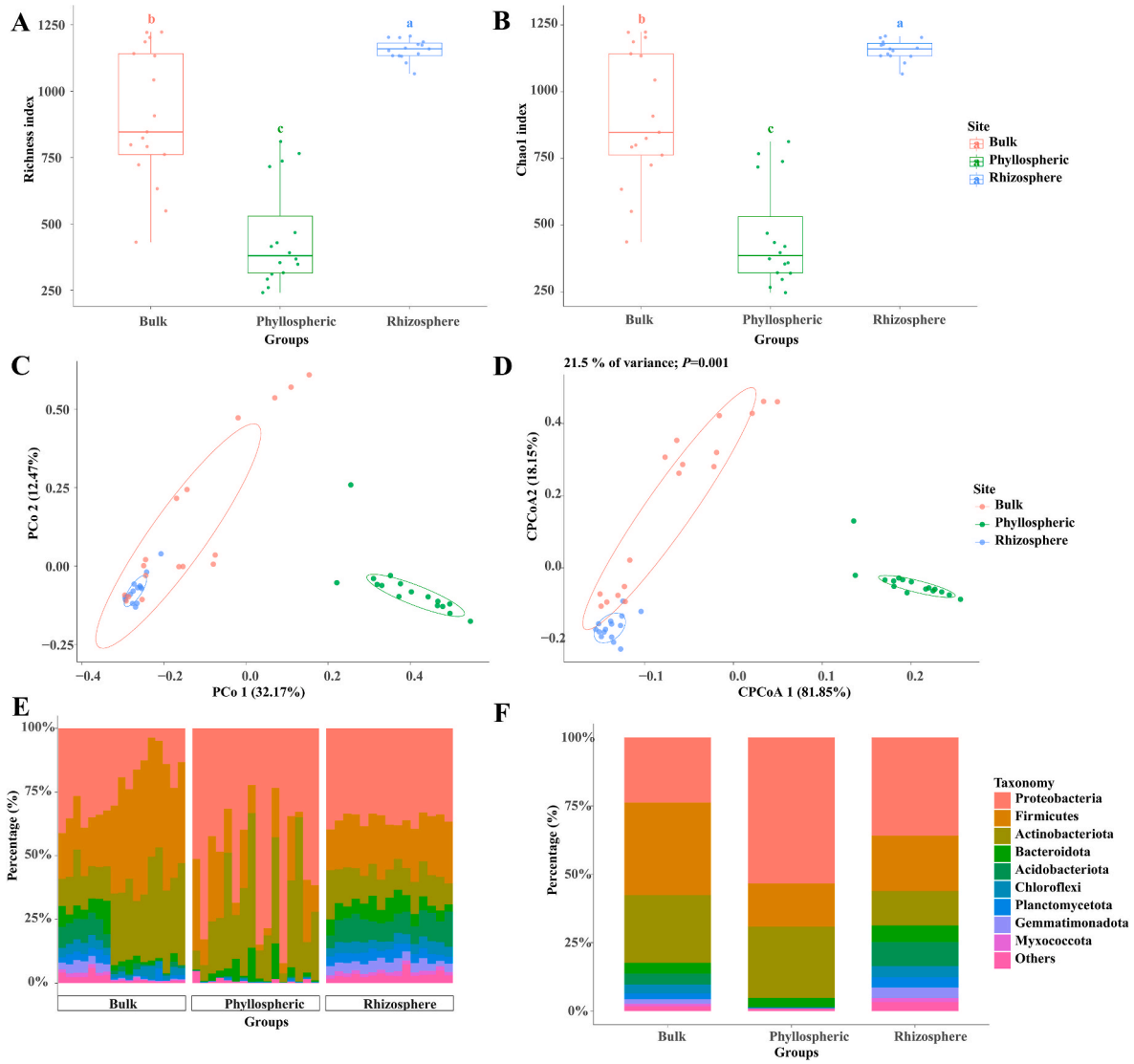


Fig. 1. Alpha indexes (A, Richness index; B, Chao1 index) of bacterial communities in different sample types. Principal coordinate analysis (C) and constrained principal coordinate analysis (D) of Bray–Curtis dissimilarity on community composition in different sample types. Taxonomic classification of bacterial DNA sequences from different sample types at the phylum level (E and F).

"ggplot2" (Villanueva and Chen, 2019) and "randomForest" (Liaw and Wiener, 2002) (version 4.6–14) package in R (version 3.6.3) (Team, 2011). SEM analysis was performed using Amos Graphics 21.0 software (IBM), and the figure was plotted Adobe Illustrator 2022 software (Adobe) (Li et al., 2022).

3. Results

3.1. Bacteria compositions in soil and Chinese cabbage

A total of 1,949,148 filtered reads were acquired from soil and phyllosphere samples, ranging from 8350 to 159,498 reads for collected samples. All reads were denoised into 4156 zOTUs at a threshold of ~100%. Proteobacteria (3.63% – 92.2%), Actinobacteria (3.43% – 64.5%), Firmicutes (2.11% – 52.6%), and Bacteroidetes (0.08% – 14%) dominated the bulk soil, rhizosphere soil, and phyllosphere (Fig. 1 E and F). Regardless of the treatment, alpha diversity of the bacterial community was higher in rhizosphere soil, followed by bulk soil and phyllosphere (Fig. 1 A and B). But in more detailed subgroups of sample types, the law was not found in a single application of silicon fertilizers or earthworms (Fig. 2 A and B). Among the phylum, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Planctomycetota, Nitrospirota, Gemmatimonadota and Chloroflexi had significantly changed in rhizosphere soil as well as phyllosphere in response to earthworm-silicon fertilization (Fig. 1 E and B). At the genus level, the relative abundance bubble heatmap showed the top 110 genera among all groups. *Bacillus*, *Amylibacter* and *Streptococcus* dominated all samples (Figure S2).

PCoA and CPCoA were employed to examine the beta diversity among the bacteria of various treatments (i.e., no treatment (BS, RS, P)), silicon fertilizers only (BS + S, RS + S, P + S), with silicon fertilizers and earthworms (BS + SE, RS + SE, P + SE), and earthworms only (BS + E, RS + E, P + E)) (Fig. 2 C – H) and different sample types (i.e., bulk soil (BS), rhizosphere soil (RS), and phyllosphere (P)) (Fig. 2 C and D) using distance based on Bray-Curtis dissimilarity calculated at the zOTU level. The first two principal coordinates (PCo1 and PCo2, CPCo1 and CPCo2) represented 44.64% (Fig. 1 C), 70.46% (Fig. 2 C), 40.32% (Fig. 2 D), 33.06% (Fig. 2 E) of the variation and 30.6% (Fig. 2 F), 26.6% (Fig. 2 G),

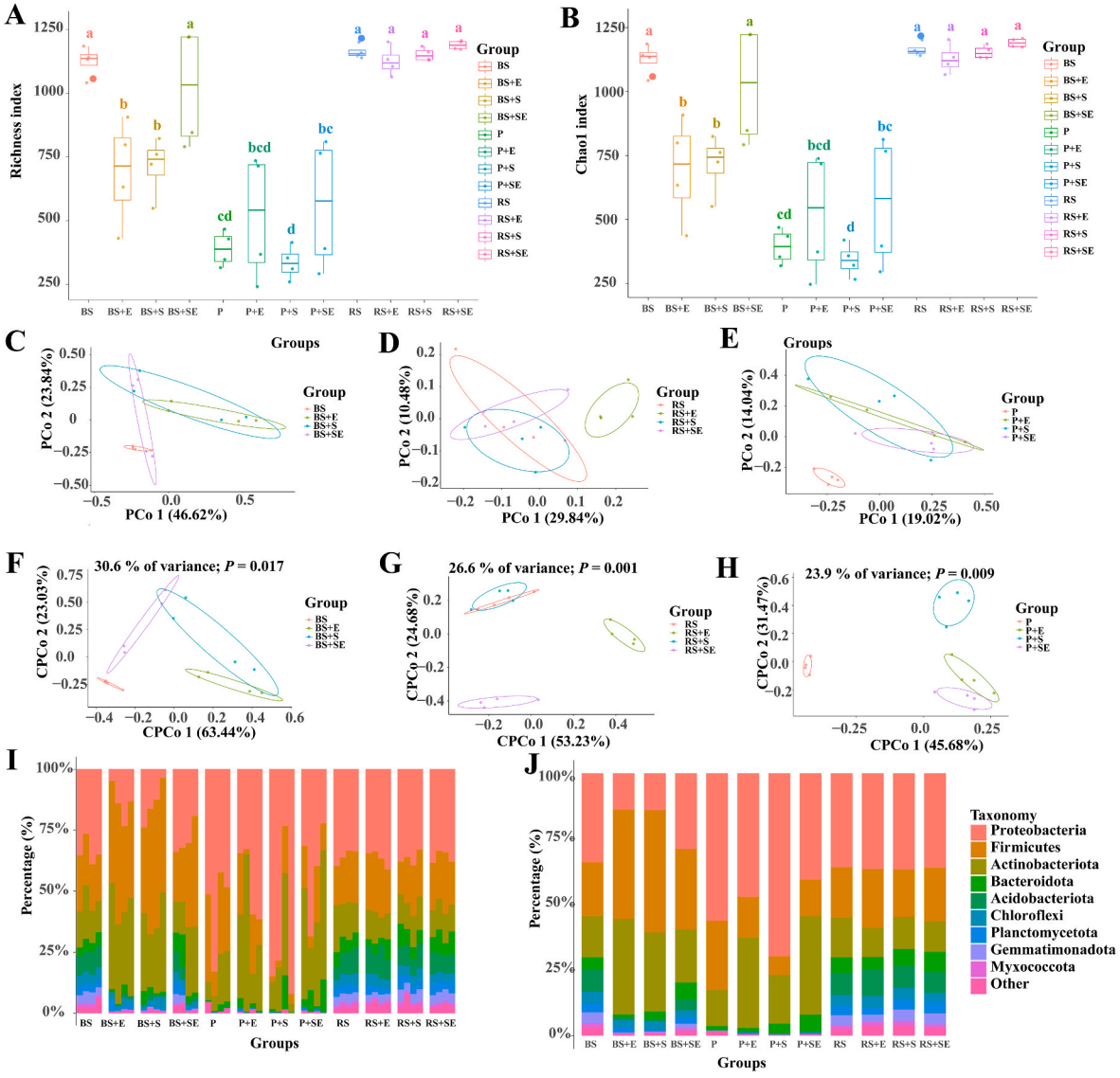


Fig. 2. The alpha index (A, Richness index; B, Chao1 index) of bacterial communities in different types of samples with various amendment strategies. Principal coordinate analysis (C, Bulk soil; D, Rhizosphere; E, Phyllosphere) and constrained principal coordinate analysis (F, Bulk soil; G, Rhizosphere; H, Phyllosphere) of Bray-Curtis dissimilarity on community composition in different types of samples with various amendment strategies. Taxa composition at the phylum level among the different types of samples with various amendment strategies (I and J).

23.9% (Fig. 2 H) of the total variance ($P < 0.05$ (Fig. 2 F), $P < 0.001$ (Fig. 2 G), $P < 0.01$ (Fig. 2 H)) in different sample types, respectively. All three treatments resulted in significant changes in bacterial compositions compared to that in no treatment, despite some overlaps of the microbiomes in some samples (Fig. 2C–H).

3.2. Antibiotic resistome in the soil-Chinese cabbage system

A total of 4 – 86 ARGs were detected for each sample, with the earthworm-amended phyllosphere sample (P + E) harboring the least diverse ARGs. Aminoglycoside, macrolide-lincosamide-streptogramin B (MLSB), beta-lactam, fluoroquinolone, and multidrug-resistance (MDR) genes were the most abundant ARG categories across all the samples (Fig. 3). In the bulk soil, compared with that in the BS group, the number of detected Rank I ARG decreased in the BS + E group, and increased in the BS + S and BS + SE group. In the rhizosphere soil, compared with that in the RS group, the number of detected Rank I ARG decreased in the RS + E and RS + SE groups and increased in the RS + SE group. In the phyllosphere, however, the P group displayed a higher number of detected Rank I ARG compared to the P + E, P + S and P + SE groups (Fig. 3). A total of 34 MGEs were identified, of which 17.7% were insertion sequences, 17.7% were transposase genes, 14.7% were plasmid, 5.9% were integrase genes and 44% were other MGEs, implying that MGEs could play important roles in the ARG development. (Figure S3).

For bulk soil, rhizosphere soil, and phyllosphere, the absolute abundance of ARGs ranged from 6.93×10^7 to 3.71×10^{10} copies g^{-1} dry solid and the absolute abundance of MGEs ranged from 4.00×10^6 to 1.28×10^{10} copies g^{-1} dry solid (Figure S4). For different ARGs risk grades, Rank I ARG absolute abundances ranged from 3.74×10^6 to 1.65×10^{10} copies g^{-1} dry solid, Rank II ARG absolute abundances ranged from 0 to 1.19×10^9 copies g^{-1} dry solid, Rank III ARG absolute abundances ranged from 0 to 7.34×10^8 copies g^{-1} dry solid, Rank IV ARG absolute abundances ranged from 2.76×10^6 to 1.10×10^{10} copies g^{-1} dry solid and Unassessed ARG absolute abundances ranged from 1.81×10^7 to 7.58×10^9 copies g^{-1} dry solid (Fig. 4). Both single and combined applications of silicon fertilizers and earthworms had significantly reduced the absolute copy number of ARGs (both the risk ARG categories and total ARGs absolute abundance) compared to the unamended rhizosphere soil, whereas the opposite trend was found in bulk soil groups. As for the phyllosphere, the absolute copy number of all ARGs and Rank I ARGs decreased significantly in all treatment groups (Fig. 4 and S4). PERMANOVA analyses revealed significant differences between the unamended group and amended group, showing that both single and combined applications of silicon fertilizers and earthworms had significantly changed the ARG profiles of bulk soil, rhizosphere soil, and phyllosphere ($P < 0.01$) (Figure S5). Meanwhile, we also found that the profile of ARGs in soil and phyllosphere presented significant differences ($P < 0.05$) (Figures S6 A and B). Utilizing one-variable linear regression, we identified the relationship between the absolute copy number of MGEs and different risk grades of ARGs (including Rank I ARGs, Rank II ARGs, Rank III ARGs, Rank IV ARGs and Unassessed). There were linear positive relationships among the absolute copy number of MGEs and Rank I ARGs ($R^2 = 0.762$, $P < 0.001$), Rank II ARGs ($R^2 = 0.786$, $P < 0.001$), Rank III ARGs ($R^2 = 0.93$, $P < 0.01$), Rank IV ARGs ($R^2 = 0.913$, $P < 0.001$), and Unassessed ($R^2 = 0.757$, $P < 0.001$) (Fig. 5).

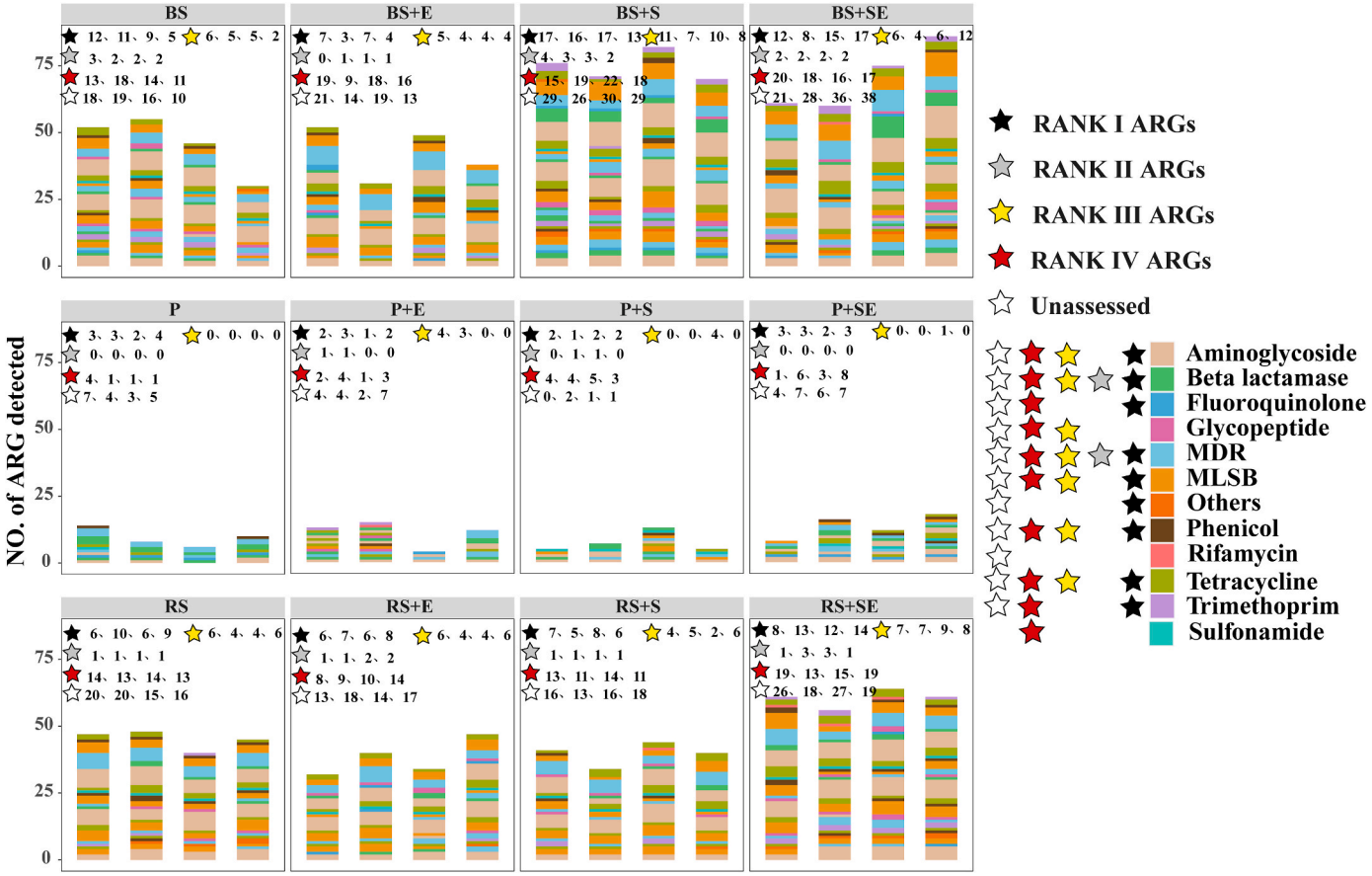


Fig. 3. Detected number of different risk grades of ARGs of different sample types (BS, Bulk soil; RS, Rhizosphere soil; P, Phyllosphere) with different amendment strategies (S, Silicon fertilizer; E, Earthworm; SE, Silicon Fertilizer + earthworm).

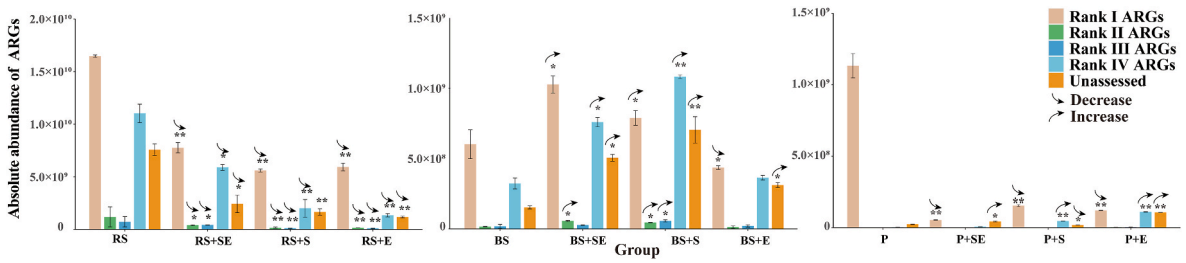


Fig. 4. The absolute copy number of different risk grades of ARGs. The data and error bars reflect mean ± standard error mean (n = 4) (*: Correlations significant at $P < 0.05$; **: Correlations significant at $P < 0.01$).

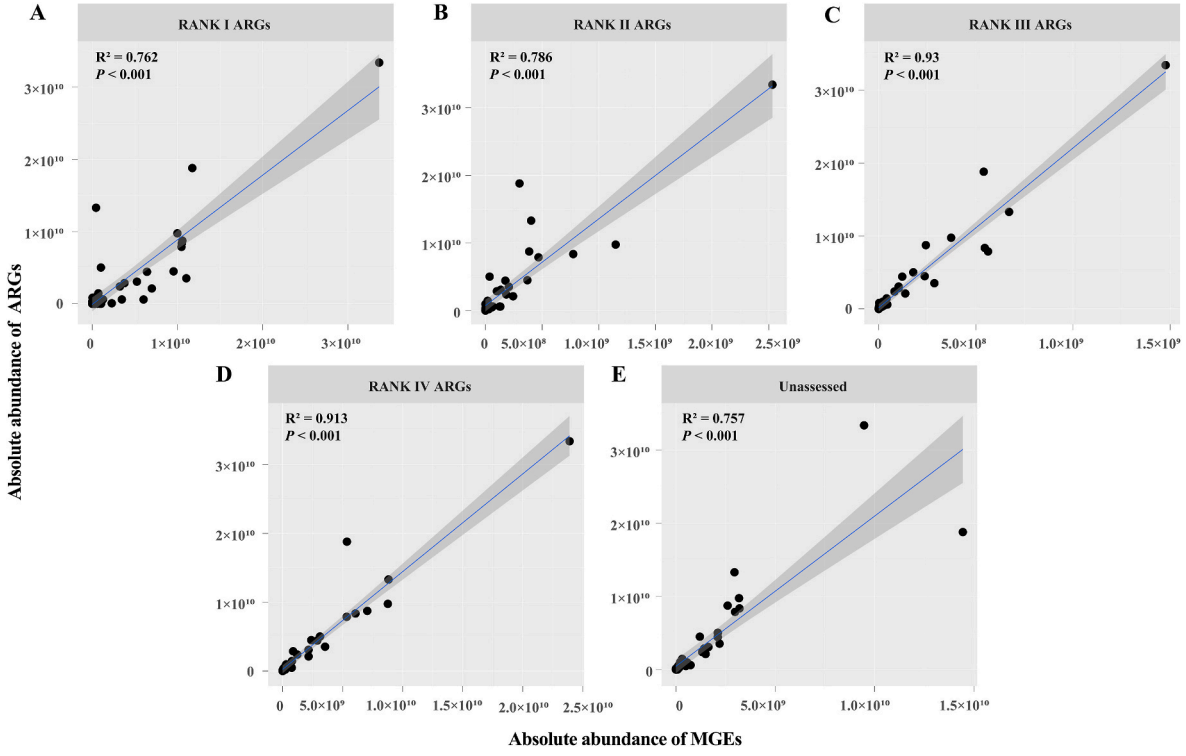


Fig. 5. OLS regression showing the correlation between the total absolute copy number of MGEs and that of Rank I ARGs, Rank II ARGs, Rank III ARGs, Rank IV ARGs and Unassessed.

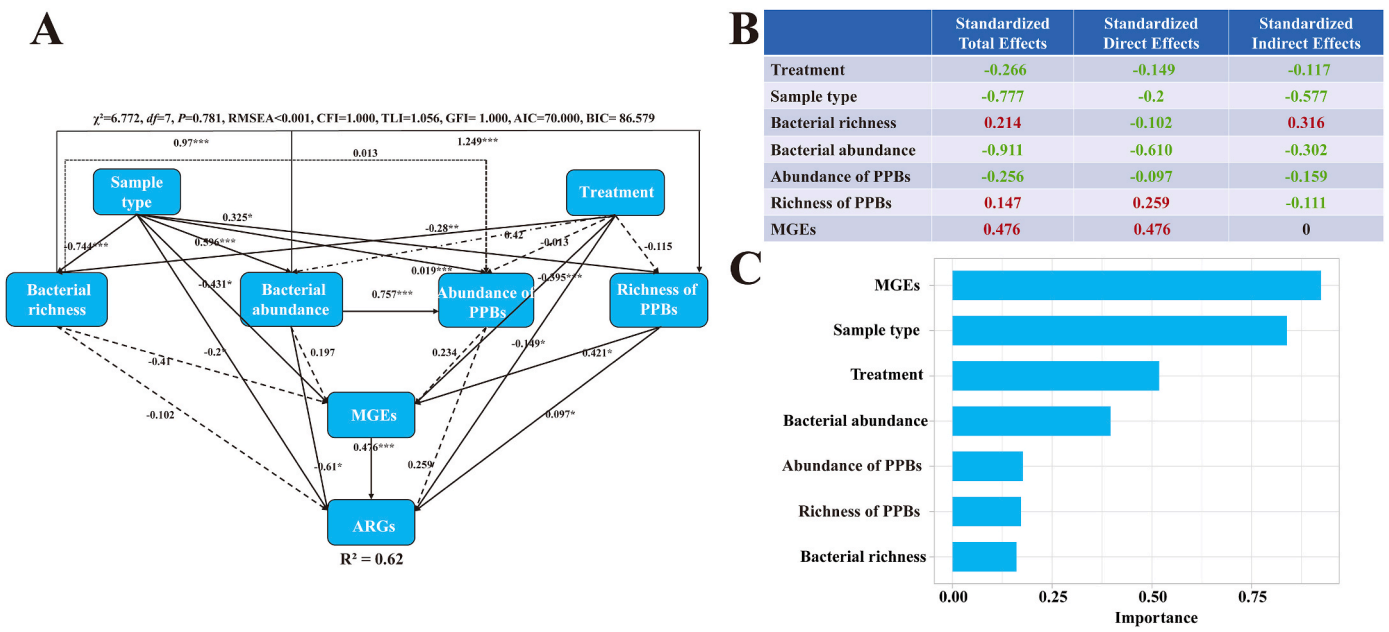
3.3. PPBs in the soil-Chinese cabbage system

In all samples, approximately 6.13% (93) of zOTUs were classified into 25 PPBs (Table S6). The heatmap depicted the relative abundance of the PPBs across all samples (Figure S7). The colors in the heatmap indicated the relative abundance of PPBs (in blue: increased; in red: decreased). In the phyllosphere, the main PPBs were *Enterobacter_cloacae_Nr_3_Y17665_1* and *Pseudomonas_mendocina_ymp_NC_009439_1*, followed by *Bartonella_grahamii_as4aup_NC_012846_1*. In the rhizosphere soil, the main PPBs were *Bartonella_grahamii_as4aup_NC_012846_1* and *Listeria_monocytogenes_strain_L312_NC_018642_1*, followed by *Mycobacterium_smegmatis_JS623_NC_019966_1*. In the bulk soil, the main PPBs were *Bacillus_cytotoxicus_NVH_39198_NC_009674_1* and *Pseudomonas_fluorescens_SBW25_NC_012660*, followed by *Pseudomonas_syringae_pv_syringae_B728a_NC_007005_1*.

3.4. Effects of multiple factors on antibiotic resistome

The structural equation model (SEM) was used to assess the indirect and direct effects of sample type, treatment, MGEs, bacterial richness, bacterial abundance, the abundance of PPBs, and the richness of PPBs on the profiles of ARG (Rank I ARG and Rank II ARG). Sample type directly impacted the absolute copy number of ARGs (Rank I ARGs and Rank II ARGs) (λ = -0.2*, $P < 0.05$), MGE (λ = -0.431*, $P < 0.05$), bacterial richness (λ = -0.744***, $P < 0.001$), bacterial abundance (λ = 0.596***, $P < 0.001$), abundance of PPBs (λ = 0.019***, $P < 0.001$), richness of PPBs (λ = 0.325*, $P < 0.05$) and indirectly impacted the absolute copy number of ARGs (Rank I ARGs and Rank II ARGs) by affecting the absolute copy number of MGEs (λ = -0.431*, $P < 0.05$), bacterial abundance (λ = 0.596***, $P < 0.001$) and richness of PPBs (λ = 0.325*, $P < 0.05$). Treatment directly impacted the absolute copy number of ARGs (Rank I ARGs and Rank II ARGs) (λ = -0.149*, $P < 0.05$), MGE (λ = -0.395***, $P < 0.001$), bacterial richness (λ = -0.28**, $P < 0.01$) and indirectly impacted the absolute copy number of ARGs (Rank I ARGs and Rank II ARGs) by affecting the absolute copy number of MGEs (λ = -0.395***, $P < 0.001$) (Fig. 6 A). We found a significant positive correlation between the absolute copy number of ARGs (Rank I ARGs and Rank II ARGs) and MGEs. This result showed that the MGEs played critical roles in the spread of ARGs.

To examine the effects of stimulus intensity on ARG patterns, we evaluated the driving capability of ARGs based on standardized direct



	Standardized Total Effects	Standardized Direct Effects	Standardized Indirect Effects
Treatment	-0.266	-0.149	-0.117
Sample type	-0.777	-0.2	-0.577
Bacterial richness	0.214	-0.102	0.316
Bacterial abundance	-0.911	-0.610	-0.302
Abundance of PPBs	-0.256	-0.097	-0.159
Richness of PPBs	0.147	0.259	-0.111
MGEs	0.476	0.476	0

Fig. 6. SEMs revealing the direct and indirect effects of different factors (Sample types, Abundance of PPBs, Richness of PPBs, and MGEs) on the ARG patterns with different amendment strategies (A, Silicon fertilizer + Earthworm; B, Silicon fertilizer; C, Earthworm). Standardized effects (Black, Indirect effects; Red, Direct effects) of various factors on ARGs derived from the SEMs (D, Silicon fertilizer + Earthworm; E, Silicon fertilizer; F, Earthworm). Variables with importance in the trained RF model (G, Silicon fertilizer + Earthworm; H, Silicon fertilizer; I, Earthworm).

and indirect effects in different groups. In all treatments, for the total effects (indirect effect plus direct effect), bacterial abundance (negative factor) was the predominant factor in shaping ARG profiles, followed by sample type (negative factor) and MGEs (positive factor) (Fig. 6 B). For the RF model of all samples based on all variables, we found that MGEs were the most important variable (92.4%) among all the explanatory variables, followed by the sample type (83.8%) and treatment (51.8%) (Fig. 6 C). We then performed an RF analysis on different amendment strategies. We found that in the S + E and E groups, the most important variable was MGEs with a contribution of 81.0% and 68.4%, respectively (Figures S8 A and C). However, in the S group, MGEs were not the most important variable but their contribution was ranked third (Figure S8 B).

4. Discussion

4.1. Silicon fertilizers significantly alleviated the spread of ARGs

Silicon fertilizers application significantly decreased not only the absolute copy number of the ARGs (both the risk ARG categories and total ARGs absolute abundance) in rhizosphere soil but also the detected number of ARGs ($P < 0.05$; Figs. 3 and 4). It is likely that (1) silicon fertilizers increased soil labile carbon (e.g., dissolved organic matter), capillary porosity, total soil porosity, and improved soil physicochemical properties (Khan et al., 2021), consequently altering the soil microbiome associated with antibiotic resistance; (2) silicon fertilizers in the soil promoted the growth of plants, thus affecting the structure and diversity of the soil microbial community (Li et al., 2019); (3) adsorption capacity of hydrocarbons greatly enhanced due to the silicon fertilizer structure (Bryk et al., 2020). While previous studies have reported that the soil microbial community structure and composition were significantly different between untreated and silicon fertilizer treatments, they failed to evaluated the role of silicon fertilizers on the restraint of ARG profiles (Das et al., 2019; Das et al., 2021a; Dykes et al., 2021; Li et al., 2019). It was also found that the microbial activity increased with silicon addition which resulted in a shift of the microbial community structure in soil with tomatoes (*Solanum lycopersicum* L.) planted (Wang et al., 2013). There was little research focused on the effects of silicon fertilizers on ARG profiles in soil. Therefore, further research is needed to fill this knowledge gap.

4.2. Earthworms significantly alleviated the spread of ARGs

Our results further showed that the presence of earthworms not only modified the structure and composition of the bacterial community but also influenced the ARGs distribution in soil and phyllosphere. That may be because the burrowing activities of earthworms can indirectly and directly affect the microbial community via modifying the soil habitat. A previous study revealed that the material earthworm ingested was a soil-organic material mixture, especially organic-rich materials, e.g., organic polymers derived from bacteria, fungi, protozoa, and plants (Tiunov and Scheu, 1999). Earthworms also promoted the formation of soil aggregates by stabilizing soil particles, increasing soil porosity, enhancing soil air circulation, and improving soil infiltration rate. Thus, earthworms have fundamentally altered the physicochemical properties of the soil and habitats of plants and microbiomes (Blakemore and Hochkirch, 2017; Fierer, 2019; Gong et al., 2018). Earthworms are textbook cases of organisms that stimulate soil nitrogen mineralization and decomposition (Blume-Werry et al., 2020), thereby stimulating plant growth (Van Groenigen et al., 2014) and microbial processes (Gong et al., 2018). It is thus predictable that effects brought by earthworms could alter the distribution of ARGs. In this study, the SEM also showed that treatment directly affected ARGs (Rank I ARGs and Rank II ARGs) and MGEs. ARG-contaminated soil-plant ecosystems can be alleviated by a natural solution with the burrowing activities of earthworms (Zhou et al., 2020; Zhu et al., 2020). There is also a possibility that less exposure to antibiotics may result in the weakening of selection pressure. Previous research has shown that earthworms can reduce residual antibiotics in the soil, thereby further reducing antibiotic resistance (Pu et al., 2020).

4.3. Combined applications of earthworms and silicon fertilizers significantly alleviated the spread of ARGs

In our study, the combined applications of earthworms and silicon fertilizers could significantly reduce ARGs (including Rank I ARGs, Rank II ARGs, Rank III ARGs, Rank IV ARGs, and total ARGs absolute

abundance) in the phyllosphere and rhizosphere soil ($P < 0.05$). The absolute copy numbers of ARGs (both the risk ARG categories and total ARGs absolute abundance) and MGEs in the phyllosphere were the lowest in all treatments compared to those in the rhizosphere and bulk soil. In addition, we also found that the absolute copy number of ARGs (both the Rank I ARGs, Rank II ARGs, Rank III ARGs, Rank IV ARGs, and total ARGs absolute abundance) was significantly higher in all phyllosphere groups compared with the P + SE group. This indicated that silicon fertilizers could collaborate with earthworms to alleviate the antibiotic resistance crisis. Silicon plays an important role in plants resisting stress. Nevertheless, the soil has little available silicon. Earthworms have shown the ability to improve the bioavailability and mobility of silicon in soils through burrowing activities (Hu et al., 2018). Changes in effective silicon could alter the metabolic pathway and metabolite synthesis of the plant. It has been suggested that the treatment with earthworms in combination with silica nanomaterials exposure resulted in a more significant increase of maize root metabolites (e.g., small molecule organic acids, sugar, and amino acids) (Ma et al., 2021). The triple effects of silicon fertilizers, earthworm activities, and enhanced root metabolic activity could have caused a more intense shift in bacterial community composition, resulting in changes in ARGs profile.

4.4. Factors regulating the ARG profiles in soil-Chinese cabbage system

RF models indicated that MGEs were the most important driving factor of ARGs in all experimental groups (Fig. 6 C). The results of RF further supported the key role of MGE in influencing the ARG profiles. Although MGEs were not the most important factors in all experimental groups, they still played an important role in all of them (Figure S8). There are plenty of evidences that show MGEs may speed up the horizontal transfer of ARGs between bacterial cells (Ellabaan et al., 2021). MGEs (e.g., transposons, integrases, and recombinases) that tend to contribute to the initial mobilization were able to capture ARGs from host genomic DNA and horizontally transfer them to other bacteria by phage or plasmid (Ellabaan et al., 2021; Jiang et al., 2017). ARGs (different ARGs risk grades) showed strong positive correlations with MGEs calculated with the OLS regression method (Fig. 5). These results also verified the key role of the MGEs in ARG profiles formation during the pot experiment. Furthermore, network and correlation analysis demonstrated that MGEs were the decisive factors in ARGs proliferation (Figure S9). The interpretation of our experiment is also substantiated by the results of SEM. We have demonstrated that multiple factors could also affect ARGs proliferation indirectly or directly via MGEs. These findings consolidated the inference that MGEs contributed to ARGs proliferation. While our series of analytical methods may not be sufficient to offer straightforward evidence, various analytical approaches (e.g., metagenomic and HT-qPCR) could help to clarify the transmission mechanisms for ARGs. We used RF model to further assess the effects of different factors on MGE profiles. It was found that the application of earthworms and/or silicon fertilizers changed MGE profiles, which further demonstrated that the application of earthworms and/or silicon fertilizers could also influence the ARG profiles by affecting the MGEs (Figure S10).

Additionally, different amendment strategies stimulation also significantly increased the absolute copy number of ARGs in bulk soil. Obviously, amendments led to an improvement in plant growth and the changes in the microbial community composition of the microhabitats investigated. Among them, the absolute copy number of ARGs (both the risk ARG categories and total ARGs absolute abundance) in bulk soil was mainly affected by silicon fertilizers or earthworms and silicon fertilizers when the "rhizosphere effect" was small, and it (including Rank II ARGs, Rank III ARGs, Rank IV ARGs and total ARGs absolute abundance) did not change significantly in the BS + E group. This could be related to the "rhizosphere effect", in which physical, chemical, and biological properties of the rhizosphere soils changed due to root exudates and rhizodeposition. Influences of the "rhizosphere effect" displayed a tendency of "rhizosphere soil" > "near-rhizosphere soil" > "bulk soil" (Wang et al., 2020c). Meanwhile, due to the use of a large experimental pot and the relatively small plants, "rhizosphere effect" might amplify processing and thus increasing variance between rhizosphere and bulk soil. This was also reflected in the great significance of "rhizosphere effect" in alleviating the antibiotic-resistant crisis.

5. Conclusions

In summary, this research revealed that antibiotic resistance pollution can be mitigated by introducing silicon fertilizers into the soil-Chinese cabbage system. Moreover, the reintroduction of earthworms further promoted ARGs mitigation. Earthworms and silicon fertilizers decreased the absolute abundance of ARGs (including Rank I ARGs and total ARGs absolute abundance) in the microbiomes of the phyllosphere and rhizosphere soil, either singly or in combination. Our study collectively indicated that a combination of earthworms and silicon fertilizers can be used to mitigate ARGs (including Rank I ARGs and total ARGs absolute abundance) transfer in the soil-Chinese cabbage system. Significant differences in ARG profiles between bulk and rhizosphere soil suggested the essential role of the rhizosphere in alleviating antibiotic resistance in ARG-contaminated soil-plant systems. The occurrence and dissemination of ARGs (Rank I ARGs and Rank II ARGs) in ARG-contaminated soil-plant systems were modulated by treatment (amendment strategy), sample type, bacterial abundance, richness of PPBs and MGEs via both indirect and direct pathways. Thereinto, the spread of ARGs in the ARG-contaminated soil-plant system was mainly governed by MGEs. Our findings provided sufficient evidence that combining earthworms and silicon fertilizers can be used for the alleviation of the antibiotic resistance crisis in food production systems. To mitigate the spread of ARGs, particularly high-risk ARGs, future studies should be conducted to investigate the efficacy of different amendment strategies (e.g., novel materials, plant genotypes, soil types, and soil faunas) under field conditions.

Authorship contributions

Conception and design of study: Z. F. Xiao, G. Li; acquisition of data: Z. F. Xiao, R. X. Han; analysis and/or interpretation of data: Z. F. Xiao, R. X. Han, J. Y. Zhao, G. Li; Drafting the manuscript: Z. F. Xiao, Y. Zhao, G. Li, Z. Zhu, Q. L. Chen; revising the manuscript critically for important intellectual content: Z. F. Xiao, Y. Zhao, G. Li, Z. Zhu, Q. L. Chen, R. X. Han, J. Q. Su, Y. G. Zhu; Approval of the version of the manuscript to be published: Zufei Xiao, Ruixia Han, Jianqiang Su, Zhe Zhu, Yi Zhao, Qinglin Chen, Junyi Zhao, Gang Li, Yong-Guan Zhu.

Declaration of competing interest

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

Data availability

I have shared the link to my data at the manuscript file.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2022.120900.

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Environmental Pollution*.

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