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Alleviated Antibiotic-Resistant Genes in the Rhizosphere of Agricultural Soils with Low Antibiotic Concentration

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ABSTRACT: The influence of the rhizosphere on the abundance and diversity of antibiotic resistance genes (ARGs) has been recognized but there is a lack of consensus because of broad ranges of plant species and antibiotic concentrations across different habitats and the elusive underlying mechanisms. Here, we profiled antibiotic concentrations and resistomes in the rhizosphere and bulk soils by cultivating 10 types of crops in manure-amended agricultural soils. Rhizosphere effects altered the antibiotic resistome structure, significantly increased the absolute abundance of the antibiotic resistome, and decreased their relative abundance, contrasting previous studies. Such plantation-driven variation in ARGs resulted from the boost of bacterial lineages with negative relationships with ARGs and the constraint of the potential ARG-hosts in the rhizosphere of plants cultivated in soils with low antibiotic concentrations as the selective pressure. This mechanism is not reported previously and deepens our understanding about the rhizosphere effects on ARGs.

KEYWORDS: *rhizosphere, antibiotic-resistant genes, manure, agricultural soil, low antibiotic concentration*

1. INTRODUCTION

Antibiotic resistance is ancient and naturally present in environmental bacteria. Diverse homologs of known resistance genes are widely distributed across environmental locales and are present at basal levels in all environments.[1−3] However, the risks of antibiotic resistance are rising because of the improper and excessive use of medical and veterinary antibiotics for the treatment of infectious diseases and animal growth.[4,5] China alone produces about 210,000 tons of antibiotics every year, of which 85% are used for livestock breeding.[6,7] These administered antibiotics are poorly absorbed by livestock, excreted as parent antibiotic compounds or their metabolites into urine and feces, and are often applied to agricultural soils as fertilizers, leading to increased antibiotic concentrations in the environment. The residual antibiotics exert a selective pressure that facilitates the propagation and dissemination of resistant bacteria.[1,3] In addition, the antibiotic resistance genes (ARGs) in manure can disperse into the indigenous bacteria that reside in agricultural soils via horizontal gene transfer (HGT), and can be transmitted into edible vegetables, inhaled or ingested by humans, and eventually acquired by clinically relevant pathogens.[8,9] This process is leading to a lack of effective antibiotic therapies available for life-threatening infections in many areas of the world, and also threatens the ecological environment. Thus, the emergence and dissemination of antibiotic resistance in manure-amended agricultural soils, as well as the underlying mechanism involved, are of great interest.

The soil rhizosphere is a unique microenvironment that possesses different physiochemical properties and microbial communities from those of bulk soils. Rhizosphere may have a special influence on the occurrence of antibiotic resistomes in manure-amended soils.[10] Results from several previous studies were inconsistent in terms of the profiles and relative abundances of ARGs between the rhizosphere and bulk soils. In pots receiving sulfadiazine manure, the decreased sulfadiazine concentration was explained by the degradation by rhizosphere microorganisms, which might result in the lower relative abundances of *sul* genes in the rhizospheres of maize and grass; however, the transferability of *sul* genes among rhizosphere microbes was enhanced because of the increasing nutrients and microbial populations.[4,11] Decreased trends in ARGs and dissipation of antibiotics resulting from the variation of microbial activity were also observed in the rhizosphere of lettuce, endive, and ryegrass planted in manure-amended soils.[10,12] However, other studies had the opposite opinion, reporting that rhizospheres of lettuce (*Lactuca sativa* L.), radish (*Raphanus sativus* L.), and broccoli did not affect the relative abundances of *sul*1, *erm*B, *tet*W, or *int*1 genes when planted in soils amended with manure containing antibiotics.[13] Alternatively, some studies found increased relative abundances of ARGs such as *tet*X genes in the rhizosphere compared to bulk soils.[12] These discrepant observations might be attributable to differences in environmental variables such as soil types, plant species, antibiotic contents, and ARG types which affect the proliferation and transfer of resistomes differently, leaving a significant gap to be filled to acquire

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comprehensive knowledge of the effects of the rhizosphere on ARG distribution and composition.

In this study, pot experiments with 10 plant species, including common grains, vegetables, and oil plants, were carried out to study the rhizosphere effects on ARGs in agricultural soils amended for 5 years with swine manure from intensive livestock farms. High-throughput quantitative polymerase chain reaction (HT-qPCR) targeting major classes of ARGs and mobile genetic elements (MGEs), illumina sequencing, and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) were employed to explore bacterial communities and antibiotic resistomes in both rhizosphere and bulk soils. The study aimed to investigate the effects of the rhizosphere on the abundance and composition of the antibiotic resistome, and to unravel the underlying mechanisms. These findings will contribute to a more comprehensive and accurate evaluation of rhizosphere effects on the proliferation of the antibiotic resistome in agricultural soils.

2. MATERIALS AND METHODS

2.1. Soil Collection and Experimental Setup. Soils were collected from a vegetable field close to a pig farm located in Yunfu, Guangdong, China (22°42′51″ N, 112°14′22″ E). The pH value and total carbon content of the loamy soils were 6.03 and 1.51%, respectively. The total concentration of antibiotics including 22 compounds was 830.21 ng g^{-1} (Table S1). Soils were air-dried to a moisture content of 20%, sieved through a 2 mm mesh, and packed into individual ceramic pots. The plants used in this study included *Zea mays* L. (maize), *Oryza sativa* L. (rice), *Glycine max* L. (soybean), *Arachis hypogaea* L. (peanut), *Helianthus annuus* L. (sunflower), *Lolium perenne* L. (ryegrass), *Lycopersicon esculentum* Mill. (tomato), *Cucumis sativus* L. (cucumber), *Brassica napus* L. (rape), and *L. sativa* L. (lettuce). The seeds were purchased from Guangdong Academy of Agricultural Sciences, Guangdong Province, China. After sterilizing with 1% (w/v) NaClO for 5 min, the selected seeds were washed, imbibed with distilled water for 16 h, and incubated on moistened filter papers till germination.

Each ceramic pot was filled with 1000 g of soil and planted with the prepared seedlings. Unplanted pots were also prepared as controls to investigate the effects of planting on the microbiota in manure-amended agricultural soils. All treatments were performed in quadruplicate. All pots were incubated in a greenhouse with natural illumination and humidity and temperatures of 35 °C during the day and 28 °C at night. During the cultivation period (45 days), deionized water was sprayed every day to compensate for water loss and to maintain the soil moisture at 60% of its water-holding capacity.

2.2. Sample Collection. After 45 days, soils were collected for chemical and biological analyses. Bulk soils were directly collected from unplanted pots. For rhizosphere samples, all soils in each pot were gently removed together with the plants, and the soil fraction strongly adhering to the roots (<2 mm from roots) was collected. All soils were immediately stored at −20 °C until further analysis.

2.3. DNA Extraction, 16S rRNA Gene Sequencing, and Data Processing. DNAs from all soil samples were extracted using a Powersoil Extraction Kit (MO BIO Laboratories, USA) as previously described.[14] To characterize bacterial community (BC) structures and compositions, the V4 hypervariable region of bacterial 16S rRNA genes was then amplified from the extracted DNA using universal primer set of 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The unique 12-bp barcode was added to the reverse primer to discriminate the amplicons of different samples. The amplicons of each treatment were combined and then sent to Beijing Genomics Institute (Shenzhen, China) for sequencing (Miseq PE250., Illumina, San Diego, CA, USA) as previously described.[14] The sequences were archived at the NCBI under the accession number SRR6998931 (BioProject ID: PRJNA449235).

Raw sequence data were demultiplexed, quality-filtered, grouped, and annotated following the analysis examples of Mothur and Quantitative Insights into Microbial Ecology 2 (QIIME2) with some modification as our previous study.[14] Briefly, reads with low quality and the singleton sequences were removed. After picking out operational taxonomic units (OTUs) with 97% similarity, we chose the representative sequence set and discarded the chimeric sequences. The OTUs were then normalized according to the sample with minimum number of sequences for further analysis. The phylotype information was identified based on Greengenes 13.5 database using "q2-phylogeny" on https://docs.qiime2.org/2019.10/tutorials/phylogeny/. Rarefaction curves were generated to describe the alpha-diversity and to compare the level of bacterial OTU diversity. The beta-diversity of different samples was compared using principal coordinate analysis (PCoA) based on the Bray−Curtis distance. PICURST was employed to predict the functional genes in soils.[15] The relative abundance of each taxonomic and functional gene was estimated by comparing the number of sequences classified as the specific taxon to the total number of sequences in the individual sample.

2.4. Antibiotic Resistome Detecting by HT-qPCR. HT-qPCR was employed to investigate the abundance and diversity of ARGs and MGEs.[16] The 35 used primer sets targeted resistance genes for major classes of antibiotics (30 primer sets), two transposase genes (*Tnp*A and *Tp*614), two of the universal class 1 and 2 integron integrase gene (*int*1 and 2), one of the clinical class 1 integron-integrase gene (*cint*1), and 16S rRNA genes (Table S2). All DNA samples were diluted to 20 ng μL^{-1} using sterile water and amplified in triplicate for each primer set with a SmartChip Real-time PCR system (Wafergen, Fremont, CA) in 100 nL reaction volumes with the detection limit of 31 cycles as the threshold cycle. Negative controls without template were included. The HT-qPCR data were analyzed using the SmartChip qPCR software (V 2.7.0.1). Reactions with poor melting curves or amplification efficiencies beyond 90−110% were discarded. Retained reactions had both a $C_T < 31$ and three positive replicates. The relative copy numbers of target genes were determined using the following equation: $CN_R = 10^{(31-C_T)/(10/3)}$, where C_T refers to threshold cycle and CN_R represents the relative copy numbers of target genes (ARG copy number/16S rRNA gene copy number). The absolute abundance of ARGs and MGEs was calculated as follows: $CN_A = CN_R \times CN_{16S}$, where CN_A and CN_{16S} refers to the absolute abundance of ARGs (or MGEs) and 16S rRNA genes, respectively. The diversity of antibiotic resistomes in different soils was compared using PCoA based on the Bray−Curtis distance. The contribution of BC and MGEs to the variance of ARGs was analyzed by using redundancy analysis (RDA) and partial RDA.

The absolute abundance of 16S rRNA gene was quantified separately using standard curves with an ABI 7500 system. Each 20 μL of the qPCR mixture consisted of 10 μL of 2× SYBR Green I Master, 1 μL of bovine serum albumin (0.5 $\mu g\ \mu L^{-1}$), 1 μL of each primer (1 μM), 1 μL of template DNA (5 ng μL^{-1}), and 6 μL of nuclease-free PCR-grade water. A standard control containing plasmids with cloned and sequenced 16S rRNA gene fragment (1.18×10^{10} copies per microliter) was used to generate an eight-point calibration curve from 10-fold dilutions for calculation. All qPCRs were performed in technical triplicates with template-free negative controls.

2.5. Network Construction. The co-occurrence ecological networks were constructed to show the correlation between ARGs, MGEs, and bacterial communities via online Molecular Ecological Network Analyses (MENA) pipeline (http://ieg2.ou.edu/MENA)[17] and visualized by Cytoscape. The relationship was tested via Pearson correlation analysis and considered as significant at $p < 0.05$. A random matrix theory-based approach was performed to generate the threshold.

2.6. Antibiotics Analysis. Twenty-two antibiotics covering sulfonamide, quinolone, tetracycline, chloramphenicols, and macrolide were selected as target compounds in the study. Solid phase

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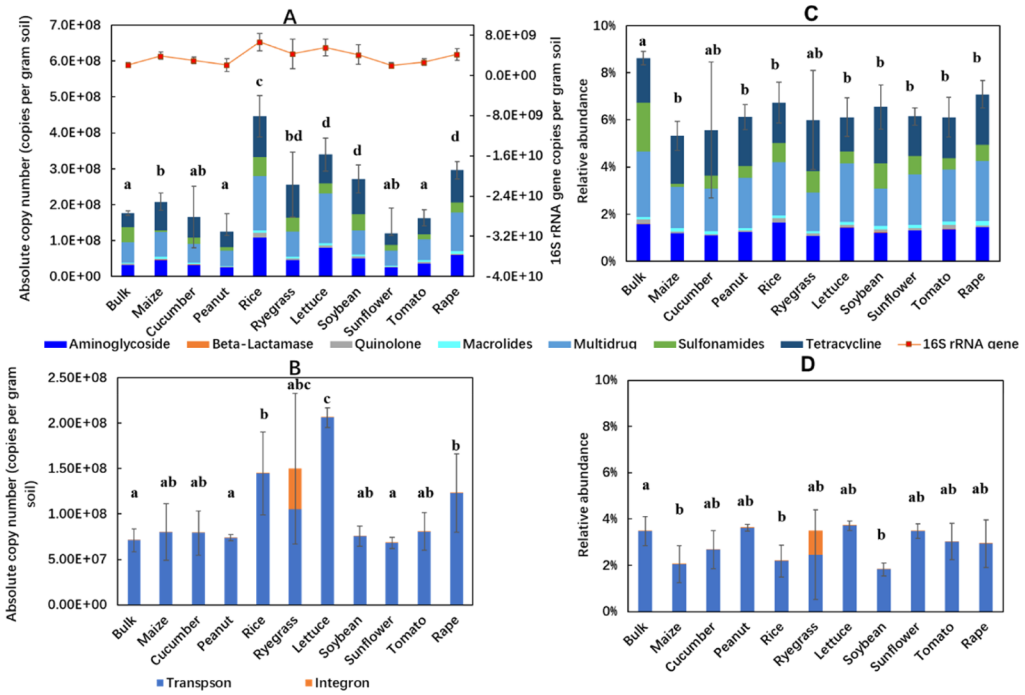

Figure 1. Abundance of antibiotic resistomes in bulk soils and the rhizosphere of different plant species. (A) Absolute abundance of ARGs. (B) Absolute abundance of MGEs. (C) Relative abundance of ARGs. (D) Relative abundance of MGEs. Bars with different case letters refer to significant differences between treatments at $p = 0.05$ level, where the same letter indicates no significant difference.

extraction and an Agilent 1290 ultra-high-performance liquid chromatography coupled with Agilent 6460 triple quadrupole mass spectrometry were employed to analyze the antibiotic contents in soils according to the methods reported previously.[14] Detailed information about the antibiotics was listed in the Supporting Information (Table S1).

2.7. Statistical Analysis. Statistical analysis was carried out by using SPSS and R with package "vegan". One-way analysis of variance (ANOVA) and the least significant difference test were applied to analyze the statistically significant differences ($p < 0.05$) on the abundance of ARGs, MGEs, specific bacterial taxa, and functional genes among different treatments. PCoA was performed in R to show the profiles of ARGs and microbial community derived from each treatment. The contributions of BC, antibiotics, and MGEs to the variance of ARGs were evaluated by RDA and partial RDA. R was used to perform the Mantel and Procrustes test for correlation analysis between ARGs and bacterial communities and antibiotics.

3. RESULTS

3.1. Rhizosphere Antibiotic Concentrations. Among 22 commonly used antibiotics belonging to five classes (sulfonamides, quinolones, tetracyclines, aminoglycosides, and macrolides), only 10 were detected in soils (Figure S1). Their individual contents ranged from below the limit of detection to 355.3 ng g^{-1}, with oxytetracycline having the highest concentration in all samples (Figure S1). The total amounts of antibiotics varied within the scope of 482.2−826.9 ng g^{-1}. Bulk soils possessed the highest antibiotic concentrations, and the rhizosphere of rape had the lowest concentration (Figure S1). The total rhizosphere antibiotic concentrations were 647.8, 591.7, 706.4, 482.2, 787.2, 763.5, 577.5, 648.9, 724.2, and 601.6 ng g^{-1} for the rhizospheres of maize, rice, ryegrass, rape, tomato, soybean, cucumber, lettuce, peanut, and sunflower, respectively. Besides, the antibiotic concentration detected in soils of this study was much lower than the MIC provided by Clinical and Laboratory Standards Institute, USA (Table S3).

3.2. Microbiomes across Bulk Soils and Rhizosphere. After assembling and quality filtering, a total of 1,762,341 high-quality sequences were obtained from all 44 soil samples, ranging from 16,887 to 64,769 sequences per sample (mean, 40,053). These sequences were clustered into 1,433,388 OTUs at a 3% dissimilarity cutoff, ranging from 15,177 OTUs (ryegrass) to 52,100 OTUs (cucumber), with a mean value of 32,577 OTUs. The microbial communities were classified into 30 phyla using the RDP database; the 10 most abundant phyla were Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes, Planctomycetes, Bacteroidetes, Gemmatimonadetes, Cyanobacteria, and Verrucomicrobia (Figure S2). Significant differences in bacterial taxa occurred between bulk soils and the rhizosphere according to the results obtained by ANOVA. Bacterial phyla OP3, Planctomycetes, Proteobacteria, SR1, Spirochaetes, TM6, TM7, Tenericutes, Verrucomicrobia, WPS-2, WS2, WS3, and WS4 were markedly enriched in the rhizospheres ($p < 0.05$), whereas Actinobacteria and Gemmatimonadetes were higher in bulk soils ($p < 0.05$). Rarefaction curves for OTUs showed that planting decreased the alpha diversity of soil bacteria, whereas bulk soils harbored the most diverse OTUs (Figure S3). The number of OTUs plateaued at a sequence depth of 8000, indicating that the sequencing depth in the present study was sufficient to target all of the soil microbes.

A PCoA score plot based on the Bray−Curtis distance illustrated the overall distribution patterns of bacterial communities in the rhizospheres and bulk soils (Figure S4A). At the OTU level, the first principal coordinate (PCoA1) and the second principal coordinate (PCoA2) explained 36.80 and 25.10% of the total variance, respectively. Planting altered the overall patterns of bacterial composition in soils, proved by the separation of bacterial communities in bulk

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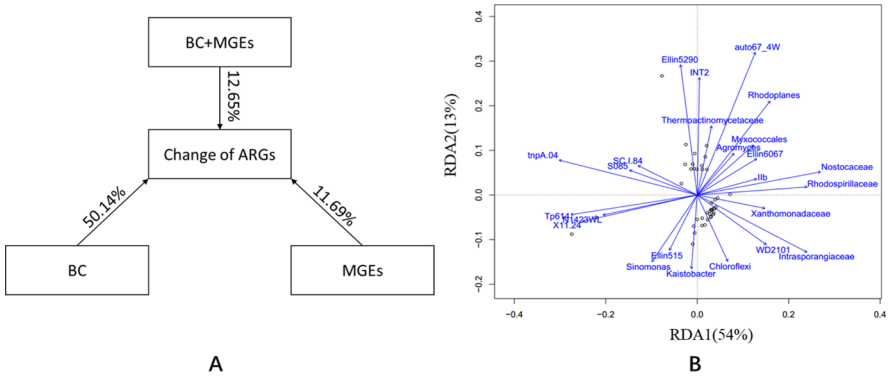

Figure 2. Driving forces for the ARGs variation in the rhizosphere. (A) Partial RDA explaining the contribution of BC and MGEs to the variance of ARGs. (B) RDA of the relationship between ARGs, MGEs, and major microbial phyla (>1% in any sample).

soils from the rhizosphere. Additionally, bacterial communities from the rhizospheres of sunflower and cucumber were clustered together and separated from the rest, suggesting the rhizosphere effects of different plants on the variation in BC.

3.3. Absolute and Relative Abundance of ARGs. In total, 28 ARGs were detected in bulk soils and the rhizosphere, including genes encoding resistance to aminoglycosides (3), beta-lactamases (2), quinolones (5), macrolides (4), sulfonamides (3), tetracyclines (9), and multiple drugs (2) (Figure S5). The number of detected ARGs in rhizospheres varied from 25 (sunflower) to 28 (rape) with the mean value of 26.1 (Figure S5), which was slightly higher than the 26 ARGs detected in bulk soils but showed no significant difference.

The absolute abundance of ARGs in all soils changed from 0.51×10^8 to 5.03×10^8 copies g^{-1} soil (Figure 1A,B). The amount of ARGs in bulk soils (1.77×10^8 copies g^{-1} soil) was significantly lower than treatments planted with maize (2.09×10^8 copies g^{-1} soil), rice (4.46×10^8 copies g^{-1} soil), ryegrass (2.56×10^8 copies g^{-1} soil), lettuce (3.40×10^8 copies g^{-1} soil), soybean (2.72×10^8 copies g^{-1} soil), or rape (2.96×10^8 copies g^{-1} soil) (Figure 1A). Similarly, the absolute abundance of 16S rRNA genes in bulk soils (2.05×10^9 copies g^{-1} soil) was also significantly lower than rhizosphere soils, and the rhizosphere of rice had the highest 16S rRNA gene absolute abundance (6.63×10^9 copies g^{-1} soil). The increase of the 16S rRNA genes in the rhizosphere followed a similar trend of ARGs (Figure 1A). For all samples, a significant positive correlation was observed between the absolute abundances of 16S rRNA genes and ARGs ($p < 0.01$, $r = 0.951$).

In order to evaluate the relative abundance of ARGs in the total BC, the absolute abundance of ARGs was normalized to that of 16S rRNA genes (Figure 1C,D). In the present study, the relative abundance of ARGs fell in the scope of 3.81−8.90%. Bulk soils harbored the highest relative abundance of ARGs (8.90%), whereas they were significantly lower in the rhizosphere ($p < 0.05$) (Figure 1C). Additionally, the PCoA score plot suggested a significant segregation of ARG compositions between bulk soils and the rhizosphere (Figure S4B).

3.4. Absolute and Relative Abundance of MGEs. In addition to ARGs, the abundances of MGEs were also studied because of their important roles in spreading ARGs via HGT. Of five target MGEs, including two transposase genes (*TnpA*04 and *Tp*614), two integrase genes (*int*1 and *int*2), and one clinical integrase gene (*Cint*1), two (sunflower) to five (ryegrass) were detected in the soils (Figure S5).

The absolute abundance of MGEs was 7.11×10^7 copies g^{-1} in bulk soils, and changed from 6.84×10^7 to 2.06×10^8 copies g^{-1} in the rhizosphere (Figure 1B). MGEs in the rhizospheres of rice, lettuce, and rape were 1.45×10^8, 2.06×10^8, and 1.23×10^8 copies g^{-1}, respectively, higher than those in bulk soils. Notably, the absolute abundance of MGEs was significantly lower than that of ARGs for each treatment. The MGEs and ARGs in all soils remained within 6.80×10^7 to 2.1×10^8 copies g^{-1}, and 1.21×10^8 to 4.46×10^8 copies g^{-1} (average amount), respectively (Figure 1A,B). The relative abundance of MGEs in the rhizosphere was within the range of 1.50−5.40% (Figure 1D), which was significantly lower ($p < 0.05$) for maize (2.00%), rice (2.10%), and soybean (1.80%) compared with bulk soils (3.50%).

3.5. Relationship between ARGs and MGEs. A significant positive relationship was observed between the absolute abundance of MGEs and ARGs (Table S4). Transposase was significantly correlated with the total amount of ARGs ($p = 0.039$, $r = 0.336$) and ARGs resisting multiple drugs ($p = 0.012$, $r = 0.402$) and aminoglycosides ($p = 0.043$, $r = 0.330$). *Tnp*A significantly and positively connected with ARGs resisting beta-lactamases ($p = 0.032$, $r = 0.348$), whereas *Tp*614 had a positive correlation with ARGs resisting tetracyclines ($p = 0.029$, $r = 0.355$). Additionally, a significantly positive association was observed between ARGs resisting tetracycline and the total amount of integrase genes ($p = 0.022$, $r = 0.305$) or *cint*1 ($p = 0.039$, $r = 0.295$). *Int*1 was positively relevant to ARGs resisting macrolides ($p = 0.006$, $r = 0.440$). These results hinted that the transfer of specific ARGs was dependent on the types of MGEs, potentially owing to their genetic co-occurrence. Thus, the types of transposase and integrase can affect the variance and diversity of ARGs in soils.

3.6. Factors Affecting ARG Variation. To evaluate the effects of multiple factors on the profiles of ARGs in bulk soils and the rhizosphere, RDA was conducted, and the results suggested that microbes and MGEs explained 74.48% of the total variance (Figure 2A), accounting for 50.14 and 11.69%, respectively. Two transposases (*TnpA*04 and *Tp*614) and six bacterial lineages (genera *Ellin*5259, *N1423WL*, *Kaistobacter*, *anto 67_4W*, *X11.24*, and *Sinomonas*) represented the predominant contribution (Figure 2B). The results of Mantel tests showed significant correlations between the composition or structure of ARGs and the BC ($r = 0.34$, $p = 0.026$, 999 permutations). Procrustes analysis further validated that ARG profiles were significantly relevant to the BC (sum of squares = 0.39, $r = 0.7789$, $p = 0.005$, 999 permutations) based on Bray−Curtis dissimilarity metrics.

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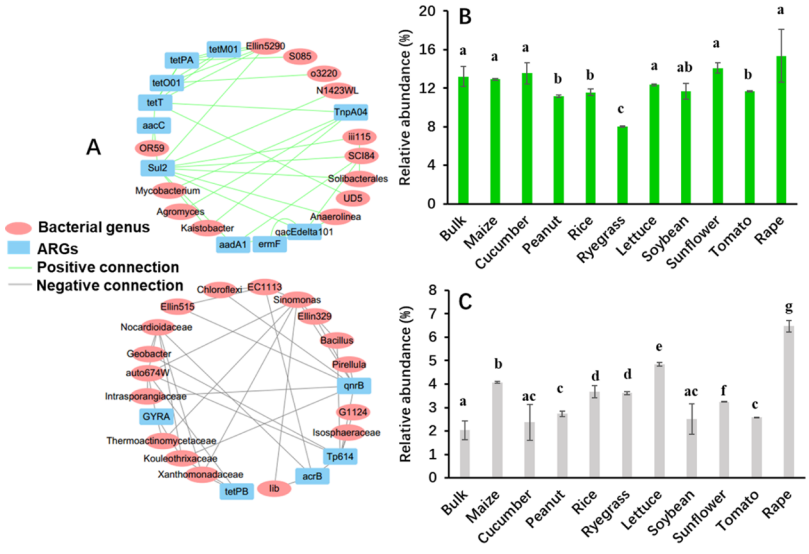

Figure 3. Co-occurrence of the ARGs and microbial taxa. (A) Co-occurrence network showing the relationships between ARGs and bacteria. (B) Relative abundance of potential ARG-hosts. (C) Relative abundance of microbes possessing negative relationships with ARGs. Bars with different case letters refer to significant differences between treatments at $p = 0.05$ level, where the same letter indicates no significant difference.

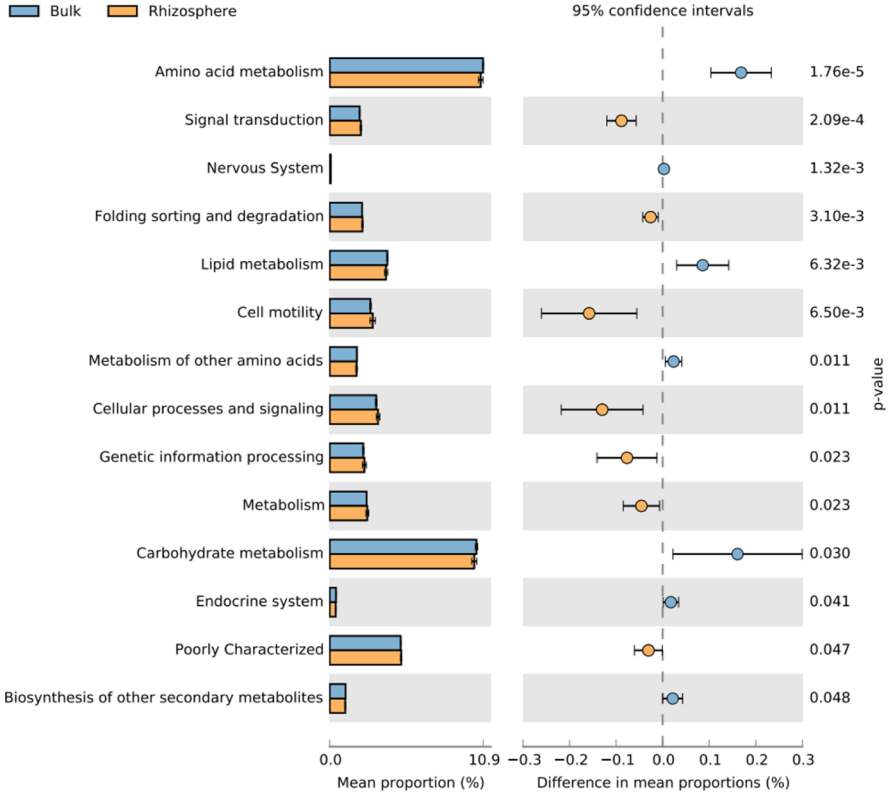

Figure 4. Differences in functional traits between bulk soils and the rhizosphere. The functional traits were predicted by PICRUSt based on 16S rRNA sequences.

The influence of antibiotic concentration on the ARG abundance was analyzed (Table S5), and the total antibiotic concentration was observed to be positively associated with the relative abundance of ARGs resisting quinolone ($r = 0.562$, $p = 0.036$). The concentration of total antibiotics and sulfonamides significantly and negatively connected with the absolute abundance of ARGs resisting beta-lactamase ($r = -0.651$, $p = 0.030$) and marcolides ($r = -0.646$, $p = 0.032$), respectively. However, no significant relationship between ARGs and antibiotic concentration occurred from RDA, Mantel tests, and Procrustes analysis, shedding light on the little impacts of antibiotic concentration on ARGs in this study.

The ecological network of ARGs, MGEs, and BC further revealed the factors affecting the variance of ARGs (Figure 3A). Twelve bacterial genera were significantly and positively linked to ARGs. Some ARGs subtypes were shared by more than one genus. For example, both *Anaerolinea* and *SC-I-84* showed positive relationships with the *qacEdelta1*-01 gene, and

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the *sul2* gene was significantly and positively relevant to the genera *Anaerolinea, Solibacter, Kaistobacter, N1423WL*, and *iii1-5*. In addition, positive connections existed between the *tetT* gene and *Ellin5290*, *OR-59*, and *o32-20* (Figure 3A). Among the potential ARG hosts, only the relevance between *Kaistobacter* and *sul2* was verified previously.[16] There were also some negative interactions between ARGs and bacteria affiliated with 17 genera, such as unclassified class *EC1113*, Chloroflexi, *Ellin515*, *Sinomonas*, *Bacillus*, *Ellin329*, *Geobacter*, *auto674W*, Nocardioidaceae, Intrasporangiaceae, Thermoactinomycetaceae, Kouleothrixaceae, Xanthomonadaceae, *Lib*, *G1124*, Isosphaeraceae, and *Pirellula*. It is worth noting that the relative abundances of all the potential ARG hosts were lower in the rhizospheres (12.24%) than bulk soils (13.21%), and the significant difference ($p < 0.05$) occurred for the treatments planted with lettuce, peanut, rice, and ryegrass (Figure 3B). Nevertheless, the relative abundance of those showing negative relationships with ARGs increased in the rhizospheres (3.61%) compared with bulk soils (2.03%), and the relative abundance in bulk soils was significantly lower than that in the rhizosphere of maize, peanut, rice, ryegrass, lettuce, soybean, sunflower, tomato, and rape ($p < 0.05$, Figure 3C). In addition, the relative abundance of ARGs negatively connected with the concentration of sulfonamides ($r = -0.662$, $p = 0.027$) and total antibiotics ($r = -0.714$, $p = 0.014$). Such a difference in the relative abundance between the potential ARG hosts and ARG-free bacteria gave a clue that ARG-carrying microbes cannot compete with the antibiotic susceptible microbes in the rhizosphere with alleviated antibiotics and rich nutrients, possibly attributing to their life burden of carrying ARGs,[18,19] and different functions to metabolize the root exudates (Table S5).[18,20,21]

Besides, we acquired the significant connection between MGEs and BC (Figure S6). *Tnp*A positively associated with genus *Agromyces*, *Mycobacterium*, and *Kaistobacter*. *Tp*614 had a negative relationship with the genus *G1124*, *Bacillus*, Isosphaeraceae, *Geobacter*, and *auto674W*. The relative abundance of the microbes being negatively related to MGEs was lower in bulk soil than the rhizosphere ($p < 0.05$, Figure S6A). For the bacterial lineages showing positive relevance with MGEs, their relative abundance was higher in the bulk soil compared with the rhizosphere of cucumber, ryegrass, soybean, and tomato ($p < 0.05$, Figure S6B). These results together with the change of microbes correlating with ARGs might elucidate that planting altered the microbial community, inhibited the lineages possessing MGEs and the growth of ARG hosts, making for the decrease of ARG transfer among species and their relative abundance in the rhizosphere.

3.7. Functional Traits of Soil Microbiota. The rhizosphere effect can affect the abundance and structure of microbes with specific functions by altering the soil physiochemical properties, consequently inducing the change of ARGs. Hence, the functional traits and their connection with the abundance of ARG-hosts and those negatively related to ARGs were studied (Figure 4 and Table S6). The functional profiles of bacterial communities present in bulk soils and rhizospheres were predicted based on 16S rRNA gene sequencing using PICRUSt coupled with the Kyoto Encyclopedia of Genes and Genomes. PICRUSt results showed a significant difference in functional traits between the rhizosphere and bulk soils, including genes related to the amino acid metabolism ($p = 1.76 \times 10^{-5}$), biosynthesis of other secondary metabolites ($p = 0.048$), carbohydrate metabolism ($p = 0.030$), cell motility ($p = 6.50 \times 10^{-3}$), cellular processes and signaling ($p = 0.011$), the endocrine system ($p = 0.041$), folding sorting and degradation ($p = 3.10 \times 10^{-3}$), genetic information processing ($p = 0.023$), lipid metabolism ($p = 6.32 \times 10^{-3}$), metabolism ($p = 0.023$), metabolism of other amino acids ($p = 0.011$), and signal transduction ($p = 2.09 \times 10^{-4}$) (Figure 4). Additionally, the average abundances of genes associated with cell motility; cellular processes and signaling; folding, sorting, and degradation; genetic information processing; metabolism; and signal transduction were higher in the rhizosphere than in bulk soils (Figure 4). These changes suggested significant differences of physical properties and substance metabolism driven by plants between bulk soils and the rhizosphere. Additionally, the correlation analysis showed a negative correlation between the relative abundance of ARG hosts and the genes related to metabolism ($r = -0.662$, $p = 0.027$), and the relative abundance of ARG-free microbes negatively correlated with the abundance of genes responsible for biosynthesis of other secondary metabolites ($r = -0.622$, $p = 0.041$), and signaling molecules and interaction ($r = -0.615$, $p = 0.044$). All these results hinted that the metabolisms of plant species facilitated the functional profiles of BC within the rhizosphere, resulting in diverse changes in the relative abundance of microbes negatively linked with ARGs or priorly adapting the rhizosphere and the decrease of ARG hosts. The more accurate information about the genes being responsible for the specific metabolism and the rhizosphere effect on these genes will be further explored in the future work by using metagenomic analysis.

4. DISCUSSION

It is well known that planting can change soil physiochemical properties, consequently shaping the diversity, abundance, and composition of microflora in the rhizosphere.[22] As a component of microbiota, antibiotic resistomes in soils are also affected by the rhizosphere.[10,11,13,21,23−25] However, there have been discrepant findings on the influence of planting on the relative abundance of ARGs. As comparisons were challenging because of the broad range of biotic and abiotic conditions such as plant species and antibiotic concentrations, the underlying mechanism has yet to be elucidated. Studies covering a wide range of plant cultivars are necessary to explore the universal mechanisms across different rhizosphere habitats. Our work comprehensively investigated the abundance and profiles of 35 genes related to antibiotic resistance/mobility and 22 antibiotics encompassing six antibiotic classes in the rhizospheres of 10 plant species. Deeper insight into the effects of the rhizosphere on the antibiotic resistome was obtained by analyzing the structure and functional traits of the bacterial lineages, and the co-occurrence of ARGs and rhizosphere bacteria.

The variation in ARG hosts and the positive association between ARGs and MGEs together hinted that specific ARGs were transferred among species by HGT in soils amended with manure for a long time. Transpose and integron are moved from donor cells to recipient cells during the process of HGT and involved in the mobility of genes and rearrangement of plasmids and chromosomes,[26] playing a key role in microbial evolution to adapt to the ever-changing environment.[27] These MGEs were little related to ARGs in the pristine environments devoid of human activities, such as soils from Tibetan.[28] With the selection pressure of antibiotics from human activities,

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ARGs were flanked by transpose and integron and transferred between species, arousing the intense connections between ARGs and MGEs and the sharing of some ARGs among different species.[3,23,29] For example, Tn3 transposon was reported to carry antimicrobial passenger genes, recruit mobile integrons, and promote the exchanges of gene cassettes; it flanked tetracycline and β-lactamase under antibiotic exposure.[29] The results of this study showed that *Tnp*A, *Cint*1, and *int*1 exhibited significantly positive links with ARGs resisting beta-lactamases, tetracycline, and macrolides, indicating that these ARGs might be flanked by these MGEs and transferred among species. Previous studies also observed that *Tnp*A and *int*1 significantly correlated with β-lactamase, aminoglycoside, multidrug, sulfonamide, and tetracycline resistance genes in the waterbodies[16] and soils.[30] *Cint*1 was reported to co-occur with ARGs resisting tetracycline and macrolides in the sediments of estuaries.[2] The transfer of these ARGs led to the variety of ARG hosts in the human-affected environments, consistent with our findings that *tetT* was significantly related to bacteria affiliated to phylum Acidobacteria, Gemmatimonadetes, Verrucomicrobia. Tetracycline resistance genes in the digested residues from biogas plants were also reported to be harbored by genus Peptostreptococcaceae and *Butyrivibrio*.[31] However, the opportunity of ARG transfer among species depended on the gene types, and some ARGs were limited to few hosts.[32] Hence, the structure and abundance of ARGs in the environments were influenced by integrons and transponse via HGT.

In all treatments, the absolute abundance of ARGs and MGEs in the rhizosphere was higher than or equal to that in bulk soils, consistent with the trends of 16S rRNA genes (Figure 1A,B). The enhanced absolute abundance of ARGs and MGEs was previously observed in the rhizosphere of *Brassica chinensis* L.[8,33] These observations might be attributable to the improved habitats in the rhizosphere because of the abundant nutrients excreted by roots and better air permeability and porosity caused by root growth and embedding in soils.[34] These conditions likely promote the growth of microbes, including but not limited to ARG-hosting bacteria, consequently increasing the number of 16S rRNA genes, ARGs, and MGEs. In addition, the influence of planting on the abundance of ARGs depended on the ARG type. Here, the abundances of ARGs encoding resistance to tetracycline, multiple drugs, and aminoglycoside were higher in the rhizosphere of rice than in bulk soils, whereas ARGs resisting quinolone and macrolides were scarcely affected by the presence of rice roots (Figure 1A,B). These findings were in agreement with a previous report that lettuce enhanced the abundance of *tet*A, *tet*X, *tet*BP, and *tet*C genes in soils, but not *sul*1, *sul*2, or *int*1 genes.[12] Additionally, plant cultivars affected the abundance of ARGs to different extents. The increasing trends of the absolute abundance of total ARGs in the rhizosphere varied across plant species, and was higher in the rhizospheres of rice, lettuce, and rape than in those of other plants (Figure 1A). Previous studies have demonstrated an increasing absolute abundance of ARGs in the rhizospheres of lettuce, *Brassica*, endive, ryegrass, and ginger.[9,12,25,35,36] These similar phenomena are possibly explained by the varying abiotic and biotic properties among the rhizospheres of different plant species, which were selected for specific resistant microbes and influenced the HGT frequency.[4,12,21,34]

In addition to the absolute abundance, attenuation of the relative abundance of ARGs occurred in the rhizospheres of the studied plants. This observation and the results of previous studies (Table S7) showed the complexity of the effects of plantation on the relative abundance of ARGs, with ARG dissipation,[4,10−12] invariability,[13] and enhancement[8,24,25,33] all demonstrated. The relative abundance of ARGs in our work was decreased in the rhizosphere, which was in line with several previous studies on soils amended with low levels of antibiotics (<2 mg kg^{-1}).[4,10−12] However, contrary to our findings, the relative abundances of *tet*C, *tet*A, *tet*G, *tet*W, *tet*X, *sul*1, *sul*2, and *int*1 genes in the rhizospheres of cucumber,[24] rape (*B. chinensis* L.),[8,33] and pepper[25] amended with manure or wastewater containing high antibiotic concentrations were higher than in bulk soils (Table S7). These differences indicated the important role of antibiotic concentration on the ARGs abundance in the rhizosphere. Furthermore, our results illustrated that the effect of the rhizosphere on the relative abundance of ARGs also depended on the ARG type and plant cultivar (Figure 1C). This is evidenced by a previous study showing that planting decreased the *tet*W and *tet*BP genes to different extents.[12] In addition, the relative abundances of some ARGs exhibited different decreasing patterns across plant species; for example, the decrease in the relative abundance of ARGs resisting sulfonamides varied greatly among the rhizospheres of 10 plant species (Figure 1C), and the relative abundance of *tet*BP was lower in the rhizosphere of endive than lettuce.[4,12]

The plant cultivar is an important factor in the effect of the rhizosphere on ARG levels, which includes the specific physical properties and the differences in substance metabolism among the plant species growing in same soil. Both our and previous studies prove that the change of ARG relative abundance relied on the plant species.[4] There are three possible explanations for such species-specific rhizosphere effects. First, the antibiotic reduction in the rhizosphere differed among plant species because of the variation in their ability of taking up and transforming antibiotics, giving rise to inconsistent selection pressure on soil microbiota (Figure S1).[37] Second, members of the soil microbial community, including ARG-carrying bacteria, were distinctly affected by different plant species because of species-specific factors such as root exudates, the uptake, and metabolism of nutrients in the soils.[38] Plant roots can absorb various nutrient elements and release exudates composed of various compounds, which differed across plant species, developing the plant-specific microenvironment and then microbial communities in the rhizosphere as soil bacteria responded differently to the compositions of root exudates and the nutrients in the habitat (Figures 4 and S7).[22] In the rhizosphere of a specific plant cultivar, microbes or ARG-hosting bacteria with the preference to utilize root exudates and adapt to the nutrition environment were favored, resulting in the variation of the abundance of ARG-carrying bacteria across the rhizospheres of different plant species.[22,39] Although ARGs can be dispersed among different microbial species by HGT,[27,40] certain ARGs were limited to several microbes with close taxonomic relationships, and thus the antibiotic resistome structure was reported to closely correlate with microbial phylogenetic and taxonomic structure in the rhizosphere.[23,40,41] In addition, the specific physical characteristics shaped by roots also performed as a selection pressure to affect the structure and abundance of the potential ARG hosts, such as air permeability, soil moisture, and soil aggregate structure.[39,42−46] Third, the frequency of HGT was dependent on the plant species. Previous studies have demonstrated

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species-specific rates of conjugal transfer in the rhizospheres of several different plants, potentially influenced by the density and distribution pattern of donor and recipient cells in the rhizosphere.[4,34] For example, the transfer ratio in the pea rhizosphere was 10 times higher than that in the barley rhizosphere because of the higher donor density and more ubiquitous distribution of donors and transconjugants in the pea rhizosphere.[21]

In addition to the plant species, the varying effects of the rhizosphere on soil antibiotic resistomes might attribute to the relationship between the concentration of the bioavailable antibiotic in the studied microenvironment and the minimal inhibition concentration (MIC) (Figure S7). Bioavailable antibiotics of different concentrations showed distinct impacts on the growth of susceptible and resistant bacteria.[18,47] More precisely, the growth rate of susceptible bacteria was negatively related to the bioavailable antibiotic concentration when it was below the MIC and reduced to zero when reaching the MIC, because the susceptible bacteria were either in a state of dormancy or killed by the antibiotics.[18,48,49] For ARG-carrying bacteria, their growth remained satisfactory in bioavailable antibiotic concentrations below the MIC and showed negative relationships with bioavailable antibiotic concentrations above the MIC.[47] As microbes are the carriers of ARGs, variation in the relative abundance of ARGs in the microbial community depends on the competitive advantages between antibiotic-susceptible bacteria and resistant bacteria (ARG hosts). The results of the present study suggested a relationship between antibiotic-susceptible bacteria and ARG hosts in the rhizosphere and its surroundings, as illustrated in Figure S7. In the rhizosphere, plants reduced the amounts of bioavailable antibiotic in the rhizosphere via plant uptake, plant metabolism, and root exudates,[36] altering the competition dynamics in soils and allowing the growth of susceptible bacteria to outcompete ARG hosts under subinhibitory antibiotic concentrations (Figure 3A). Additionally, the susceptible bacteria with no ARGs have a lower survival burden[18,19] and possess growth advantages in the rhizosphere with rich nutrients and ameliorative soil physiochemical properties as compared to ARG-hosting bacteria, especially in soils with antibiotic contents far below the MIC.[18,20,21] Thus, the potential ARG hosts had lower relative abundance, and the total microbial populations possessed negative relationships with ARGs flourish (Figure 3B). Hence, susceptible bacteria might have competitive advantages over ARG hosts in the rhizosphere, bringing about a decreasing relative abundance of ARGs. This revealed the mechanism underlying the decreasing relative abundance of potential ARG hosts and ARGs in the rhizosphere of this work and the previous studies with low antibiotic content, where the antibiotic concentration was lower than the MIC (Table S3). Alternatively, when bioavailable antibiotic concentrations were above the MIC, ARG-hosting bacteria were more active in soils, possessing a competitive growth advantage and higher growth rates in the rhizosphere because of the lower antibiotic levels and the enhanced nutrient supply from root exudates.[4,48,49] In contrast, the susceptible bacteria might still be dormant, thus increasing the relative abundance of ARGs in the rhizosphere, as the observation reported by studies with antibiotic concentration higher than the MIC. Accordingly, the bioavailable antibiotic concentration is another key determinant in the effect of the rhizosphere on the abundance and composition of antibiotic resistomes and ARG hosts, and requires more study in the future.

The rhizosphere effects on the antibiotic resistomes are complex and affected by various abiotic and biotic factors.[42,50] Aside from the plant cultivar and antibiotic content, other variables, including the soil properties, root growth, exudate production, and growth stage, might affect the soil microbial community structures and functions, the stimulation of microbial activities, and the transferability of resistance genes, thereby affecting the abundance, composition, and distribution of ARGs. Individual soils, with differences in compositions of soil aggregates, pH, ion types, and organic matter contents, can thus affect the structure of ARG hosts and susceptible bacteria in the rhizosphere by changing the antibiotic concentration trends,[50,51] providing unique habitats, or affecting the plant root function and exudates in a soil-specific manner.[39,52] The soil type has been reported to greatly influence the fate of ARG-hosting bacteria after land application of sludge compost[53] as well as the occurrence and distribution of ARGs in natural environments.[54] The composition of root exudates and the root physiological properties were strongly affected by the plant species and developmental stage, which in turn can change the rhizosphere.[39,42−46] Therefore, the effect of the rhizosphere on the resistome in soil should be further studied to consider the effect of a broad range of environmental factors.

In summary, the results of our study have revealed that planting affected ARG profiles, enhanced their absolute abundance, and reduced their relative abundance in agricultural soils with low antibiotic concentrations. The effect of the rhizosphere on ARG abundance was influenced by not only plant cultivars but also antibiotic concentration, which has not been reported previously. The contrasting results among current studies on the effects of the rhizosphere on ARGs emphasized the complexity of the rhizosphere and the difficulty in evaluating changes in the resistome. As multiple factors can affect the behavior of ARGs in the rhizosphere, extensive effort is needed to understand the mechanisms of the effect of the rhizosphere on ARGs in soils.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b06634.

Additional experimental data and results (PDF)

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Notes

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