



Full length article

Field-based evidence for the prevalence of soil antibiotic resistomes under long-term antibiotic-free fertilization

Yuhao Fu^{a,b}, Fang Hu^{a,b,c}, Fang Wang^{a,b,*}, Min Xu^{a,b}, Zhongjun Jia^{b,d}, Wulf Amelung^{e,f}, Zhi Mei^{a,b}, Xiaozeng Han^{b,g,*}, Marko Virta^h, Xin Jiang^{a,b}, James M. Tiedjeⁱ

^a State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 211135, China

^b University of Chinese Academy of Sciences, Beijing 100049, China

^c Chongqing Changan Automobile Co., Ltd., Chongqing 400023, China

^d State Key Laboratory of Black Soils Conservation and Utilization, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, China

^e Agrosphere Institute (IBG-3), Forschungszentrum Jülich GmbH, Jülich 52428, Germany

^f Institute of Crop Science and Resource Conservation (INRES)-Soil Science and Soil Ecology, University of Bonn, Bonn, Germany

^g Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China

^h Department of Microbiology, University of Helsinki, Viikinkaari 9, 00014, Finland

ⁱ Center for Microbial Ecology, Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI 48824, United States

ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:

Antibiotic resistome
Long-term fertilization
Antibiotic-free manure
Bacterial community
Black soil

ABSTRACT

Growing evidence suggests that the use of manure containing residual antibiotics universally leads to an increase in soil antibiotic resistance genes (ARGs). However, there is limited understanding of the influence of long-term antibiotic-free fertilization and the differences between antibiotic-free manure and chemical fertilizer on soil ARGs. This study aimed to quantify the assembly patterns of the antibiotic resistome by in situ probing bacterial community and environmental variations in field soils that have been subjected to long-term exposure to chemical fertilizer and/or manure from animals without antibiotic amendments. Long-term fertilization slightly impacts the diversity of antibiotic resistomes, with 85.5 % of total ARGs and mobile genetic elements (MGEs) being common across all treatment types, while significantly increasing their abundances from 0.68 to a maximum of 0.90 copies/16S rRNA. The rise in ARG abundances was less pronounced when using antibiotic-free manure compared to chemical fertilizer, particularly for Rank II ARGs. However, when antibiotic-free manure and chemical fertilizer were combined, a significant increase in nutrients (such as available nitrogen and organic matter) and MGEs occurred, leading to the enrichment of soil microbial populations, especially in certain resistant species, and Rank I and II ARGs. Despite the influence of various factors like bacterial communities, soil properties, heavy metals, and MGEs, the MGEs had the most significant standardized effects on shaping ARGs through both direct and indirect pathways. Our findings indicate that while of antibiotic-free manure can lower the risk of antibiotic residues and promote sustainable farming practices, it may not fully eliminate the prevalence of ARGs, highlighting the need for more comprehensive strategies to address antibiotic resistance in agriculture rather than simply prohibiting the use of antibiotics.

1. Introduction

The escalating global challenge of antibiotic resistance, propelled by the rapid dissemination of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB), presents significant risks to both environmental and human health (Larsson & Flach, 2022; Wang et al., 2024). The spread of antibiotic resistance via horizontal gene transfer among bacteria, transmitting the resistome to resistant pathogens, may

constitute a global reservoir of ARGs (Jiang et al., 2017). Alarming, the rise in cases where antibiotic therapies are ineffective against resistant infections has been noted in various regions (UNEP, 2022). A staggering 1.27 million deaths were directly attributable to antibiotic-resistant infection in 2019 (Murray et al., 2022). Endorsed by the World Health Organization and numerous global health agencies, the spread of antibiotic resistance is an urgent issue that requires coordinated efforts to address effectively.

* Corresponding authors at: University of Chinese Academy of Sciences, Beijing 100049, China.

E-mail addresses: wangfang@issas.ac.cn, fangwang321@issas.ac.cn (F. Wang), xzhan@iga.ac.cn (X. Han).

<https://doi.org/10.1016/j.envint.2024.109202>

Received 30 May 2024; Received in revised form 4 December 2024; Accepted 9 December 2024

Available online 10 December 2024

0160-4120/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

The global consumption of antibiotics in livestock, driven by the increasing demand for animal proteins, is projected to surge from 63151 ± 1560 tons in 2010 to 105596 ± 3605 tons by 2030 (Van Boeckel et al., 2015). A significant proportion of these antibiotics, approximately 20 % to 90 %, excreted by livestock in urine and feces, remains unmetabolized and often contaminates agricultural soils through manure application (Hurst et al., 2019). A global survey revealing the difference between antibiotic-free manure and the commonly used manure found that conventional farms showed slightly higher levels of antimicrobial resistance (28 %) compared to antibiotic-free farms (18 %) (Ager et al., 2023). The sustained presence of antibiotics in the soil, often at low concentrations, can exert selection pressure to alter overall microbial community structure and elevate the level of ARGs across numerous species in the terrestrial microbiome (Shawver et al., 2021). Furthermore, ARGs can be easily propagated by mobile genetic elements (MGEs) between manure-borne bacteria and native soil microbiota, heightening the risk of spreading the antibiotic resistome from soil to various ecosystems (Gillings et al., 2015). The transmission of resistant bacteria and ARGs can amplify microbial threats to human health, such as direct contact with colonized or infected humans/animals as well as biological substances (e.g., blood, urine, and feces), and the consumption of contaminated food or derived food products via indirect pathways (Tiedje et al., 2023). Countries have taken markedly restrictive measures to govern the use of antimicrobials in livestock production and advocate organic farming (without antibiotic usage) to combat antibiotic resistance and protect public health. Using antibiotic-free amendments in agriculture might promote healthier soil ecosystems, minimize the selection pressure on ARG proliferation, enhance consumer safety, and foster research and innovation in sustainable agriculture (Bothe et al., 2024). However, research on antibiotic-free fertilization by profiling the evolution of ARGs that can provide evidence to compare the effects with antibiotic use in agriculture still remains scanty.

Notably, ARG enrichment in soils after manure input is not merely associated with antibiotic residues, in which a buildup of ARGs was not constructed as antibiotic-rich manure was applied (Wang et al., 2018). This indicates that many factors are involved in shaping the soil resistome, e.g., the boosting of microbial activity after the addition of labile carbon sources, variations in physicochemical properties after the input of animal manures, nutrient availability, contents of heavy metals, and biological competitive, predation or cooperative behaviors between native soil microorganisms and manure-borne bacteria (Liu et al., 2023b; Nguyen et al., 2023). Currently, literature about the increased prevalence of ARGs and ARB in soils after antibiotic-free manure input has been reported (Shawver et al., 2021; Udikovic-Kolic et al., 2014). However, to our knowledge, studies have merely followed with great interest the relatively short-term impacts, while historical effects on soil resistome after the recycled application of antibiotic-free manure remain poorly understood. Gaining insights into the role of antibiotic-free manure in ARG proliferation is crucial, as it will benefit to disentangle which factors can be critical management controls independent of antibiotic exposure, thereby offering niche-targeting solutions for ARG control in agricultural practices.

There is emerging evidence that organic manure, often containing a multitude of ARG-carrying bacteria, MGEs, and various carbon and nutrient sources, can alter soil resistome structures upon application (Jechalke et al., 2013). Research has shown that while the profile of exogenous ARGs in chemical fertilizers is minimal, their application can still lead to notable changes in ARG prevalence within soil environments. This alteration is likely attributable to changes in soil properties and the microbial community dynamics (Sun et al., 2020). Further investigation into the simultaneous use of animal manure and chemical fertilizers has shown a substantial increase in the pollution levels of ARGs in agricultural soil and plants (Xie et al., 2018). However, the understanding of soil antimicrobial resistance caused by the simultaneous use of antibiotic-free manure and chemical fertilizer is still

limited. Furthermore, the specific impacts of various shared factors such as MGEs, bacterial community, soil physicochemical properties and heavy metal contents on the soil antibiotic resistome within the context of long-term fertilization with antibiotic-free amendments remain largely unexplored.

Research indicates that using manure with antibiotics can increase soil ARGs. However, the impact of long-term antibiotic-free fertilization and the differences between antibiotic-free manure and chemical fertilizer on soil antibiotic resistance genes are not well understood. To fill this gap, we studied soil properties, heavy metals, bacterial communities, and ARGs in soils with different fertilization practices over 14 years. The primary aims of our work are to (1) delineate the overall structure of the soil antibiotic resistome under prolonged antibiotic-free fertilization conditions, (2) compare the differences in soil antibiotic resistome resulting from different fertilization treatments, and (3) explore the intricate interactions among multiple factors – such as bacterial community, soil heavy metals physicochemical properties, and MGEs – in driving the evolution of the soil antibiotic resistome. This study may advance our understanding of ARG patterns under long-term antibiotic-free fertilization, analyze the factors that influence the soil antibiotic resistome, and direct us towards developing risk mitigation strategies.

2. Materials and methods

2.1. Sampling sites and study design

The field experiment, initiated in 2003, was performed at the Hailun Agro-ecosystem Experimental Station, Chinese Academy of Sciences, located in Heilongjiang province, China ($47^{\circ} 26' N$, $126^{\circ} 38' E$). The site features typical black soils and a temperate continental monsoon climate, characterized by an average annual rainfall of 500–600 mm, a mean annual air temperature of $1.5^{\circ} C$, and a mean annual sunshine duration ranging from 2600 to 2800 h (Lu et al., 2020). The soil, classified as Chernozems according to the World Soil Reference Base and as Mollisols according to the USDA soil Taxonomy, has a silty clay texture with high organic matter content (Sheng et al., 2020).

Four distinct fertilization treatments were set across the farmland, each with three replicates: 1) control (no fertilizer), 2) chemical fertilizer (CF), 3) recycled organic manure without antibiotics (ROM), and 4) a combination of chemical fertilizer and recycled organic manure (CF&ROM). The specific application rates for chemical fertilizers and recycled organic manure are detailed in Table S1. Although fertilizers are devoid of antibiotics by nature, their utilization can nevertheless exert an influence on the behavior and spread of ARGs within the soil. Herein, the inclusion of chemical fertilization empowers us to draw comparisons between the effects of antibiotic-free manure on its own and the cumulative consequences that emerge from the simultaneous application of fertilizers and antibiotic-free manure. The ROM originated from 80 % of the harvested grains and shredded straw from the experimental plots, which were fed to pigs and used as bedding, respectively. The decomposed mix of manure and residues was then recycled as ROM and applied annually to the plots (Yan et al., 2019). Notably, these pigs were reared in a hygienic and low-intensity setting without antibiotics, underwent regular vaccination to prevent various disease challenges and pathogens, and were closely monitored daily by animal husbandry staff and veterinarians within this system. It cannot be excluded that the gut of the animals still included some heavy metals and disinfectant-mediated ARGs, but since this is common practice in pig farming, we did not try to eliminate other factors typical for pig farming. Intensive tillage was used in these four treatments under a 3-year crop rotation pattern (wheat (*Triticum aestivum* L.) – maize (*Zea mays* L.) – soybean (*Glycine max* (L.) Merrill.)) (You et al., 2019). All treatments were practiced with the same crop each year. Seven cores were collected in 2017 from the topsoil (0–20 cm) of each plot and bulked as one replicate. The collected soil was air-dried, manually

ground, and sieved through a 2-mm mesh. Subsamples were preserved at 4 °C for physicochemical analysis and −80 °C for DNA extraction.

2.2. Soil physicochemical properties

The soil–water ratio at 1:2.5 was used to determine soil pH by a Mettler Toledo pH meter (S220, Switzerland). Organic carbon and total nitrogen were measured by a TOC-/TN_b Analyzer (Multi N/C 3100, Jena, Germany) (Wang et al., 2022a). Available nitrogen, phosphorus, and potassium were determined by the alkali hydrolysis diffusion method, NaHCO₃ extraction/Mo-Sb colorimetric method, and CH₃COONH₄ extraction/flame photometer method, respectively (Li et al., 2019; Tian et al., 2019). The determination of cation exchange capacity extracted by CH₃COONH₄ (1 M, pH 7.0) was conducted using an atomic absorption spectroscopy (Nel et al., 2022). Bioavailable heavy metals represent those active portions that easily pose selection pressure on soil microbiota to increase the prevalence of ARGs (Fu et al., 2021). Bioavailable heavy metals (Table S2) including lead (Pb), chromium (Cr), zinc (Zn), cadmium (Cd), nickel (Ni), copper (Cu), and arsenic (As) from fine samples (passed through a 0.15-mm sieve) were extracted by HCl (0.1 M) for 1.5 h shake at 25 °C (Wang et al., 2021a). The concentrations except for As were all determined by a 700x High-Performance Liquid Chromatography combined with Inductive Coupling Plasma Mass Spectrometry (HPLC-ICP-MS, Agilent Co., USA), and As was measured by a 230E Atomic Fluorescence Spectroscopy (AFS-3100, Beijing Kechuang Haiguang Instrument Co., China).

2.3. High-throughput quantitative PCR of ARGs

Microbial DNA was extracted from 0.5 g of soil using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) and quantified with a Qubit 3.0 fluorometer (Life Technologies, Eugene, OR, USA). A total of 384 valid primer sets (Table S3), covering 325 ARGs, 49 MGEs, 8 taxonomies, and 2 16S rRNA genes, were used for resistome analysis via the Takara SmartChip Real-time qPCR system (Stedtfeld et al., 2018). Each SmartChip nano-well with 100 nL reaction mixture was composed of 50 nL 2 × LightCycler 480 SYBR Green I Master, 5 nL 0.5 μM forward/reverse primer, 10 nL 20 ng/μL DNA template, and 30 nL nuclease-free sterile water. Three technical duplicates and corresponding DNA-free controls for each sample were conducted. Conditions of the qPCR reaction started from enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s, concluding with a melting curve analysis (Fu et al., 2022). Data of positive detections met the criteria of two or more replicates, a threshold cycle (Ct) > 30, and an amplification efficiency of 90 %–110 %, which could be used for further analysis (An et al., 2018; Wang et al., 2018). Gene copy number can be calculated by $10^{(30-Ct)/(10/3)}$, and the relative abundance of ARGs was calculated by ARG copy number / 16S rRNA gene copy number (Liu et al., 2022). Fold change (FC) used to compare the ARG enrichment between different treatments was calculated by $1) \Delta Ct = Ct(ARG) - Ct(16S\ rRNA)$, $\Delta\Delta Ct = \Delta Ct(target) - \Delta Ct(reference)$, and $FC = 2^{(-\Delta\Delta Ct)}$, where Ct was the threshold cycle, target refers to the treatment of CF, ROM or CF&ROM, and reference refers to the control (Fu et al., 2022).

The World Health Organization has prioritized certain ARGs due to their elevated risk profiles. To evaluate the potential threat of ARGs to human health, it takes into account factors such as human exposure, pathogenicity, and gene transferability (Zhang et al., 2021), and classifies resistance genes into four levels of risk: significant risk (ARGs associated with humans, pathogenic, and mobile, Rank I level), high risk (ARGs associated with humans, pathogenic, and non-mobile, Rank II level), general risk (ARGs associated with humans and non-pathogenic, Rank III level), and low risk (ARGs not linked to humans, Rank IV level). The highly concerned ARGs, i.e., Rank I and II levels, are listed in Table S4.

2.4. Miseq Illumina sequencing of 16S rRNA gene

The bacterial community was characterized by sequencing the V4-V5 region of bacterial 16S rRNA gene with primers 515F (5'-GTGCCAGCMGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTT-3'). This process was conducted on the Illumina HiSeq2500 platform (Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China). The sequencing protocol involved an initial denaturation at 95 °C for 3 min, followed by 27 amplification cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s) and a final extension at 72 °C for 10 min. Sequencing reads can be trimmed to acquire the high-quality sequence. Filter bases in reads with a quality value < 20 using a 10 bp sliding window to trim the ends if the average quality is < 20; remove reads shorter than 50 bp or containing N bases; merge paired-end reads with a minimum overlap of 10 bp while allowing a maximum mismatch ratio of 0.2 in the overlapping region to screen out non-matching sequences; distinguish samples based on their barcodes and primers, ensuring that barcodes have 0 mismatches and allowing a maximum of 2 mismatches for primers while adjusting sequence direction accordingly. The refined data were processed by Quantitative Insights Into Microbial Ecology (QIIME) and clustered into operational taxonomic units (OTUs) at the level of 97 % sequence similarity using the UCLUST algorithm (Li et al., 2024). For taxonomic annotation of representative OTUs, the Ribosomal Database Project (RDP) classifier was employed, referencing the SILVA 16S rRNA gene database at a 70 % confidence level (Cole et al., 2009). Raw sequencing reads were publicly available under the accession number PRJNA971109 in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database.

2.5. Statistical analysis

The data, presented as means ± standard deviation, were processed using Microsoft Excel 2016. Differences between fertilization treatments were analyzed using one-way analysis of variance (ANOVA) at a significance level of $P < 0.05$, conducted with SPSS Statistics 22.0. Comparisons of ARG enrichments relative to the control and the profiles of soil microbiota across different treatments were conducted using R version 3.4.1 with the “pheatmap” package (Xu et al., 2023). Non-metric multidimensional scaling (NMDS) was utilized to showcase the bacterial community profile, while principal coordinate analysis (PCoA) depicted the overall distribution patterns of ARGs. The Mantel test compares the correlation between two distance matrices from the same samples. A Mantel test using the Bray-Curtis algorithm was performed to assess the relationships between heavy metals, nutrient factors, bacterial community, MGEs and ARGs. Network analysis, visualizing the interactions among ARGs, MGEs, and bacterial taxa, was executed by Gephi 0.9.2, employing the Spearman correlation coefficient ($\rho > 0.8$) and significance levels ($P < 0.05$) derived from Cytoscape v3.8.2. Shared ARGs and MGEs among the control, CF, ROM, and CF&ROM treatments were represented through a Venn diagram (<https://bioinformatics.psb.ugent.be/webtools/Venn/>) using an online tool of Bioinformatics & Systems Biology. The partial least-squares path modeling (PLS-PM) is a robust statistical technique used to analyze interactive relationships between observed and latent variables, commonly utilized for explaining and predicting relationships in complex data sets. Herein, the PLS-PM was applied to dissect the direct, indirect, and interactive effects among bacterial communities, soil properties, heavy metals, MGEs, and ARGs using R with the “plsppm” package (Liao et al., 2018).

3. Results

3.1. Response of ARGs in the fertilized soil

A total of 159 targeted genes out of 384 primer sets were detected among all soil samples. Different fertilizers did not significantly affect the treatments ($P > 0.05$) in the diversity of ARGs where the control

harbored ARGs and MGEs (138), similar to the treatments of CF (147), ROM (142), and CF&ROM (143) (Fig. 1A). Notably, a significant portion (136 out of the total ARGs and MGEs) was shared across the control, CF, ROM, and CF&ROM treatments, accounting for 85.5 % of the total detected genes (Fig. S1). Contrasting with diversity, significant differences were observed in the total relative abundances of ARGs and MGEs across the treatments ($P < 0.05$), with an increase in relative abundances from 0.68 to 0.90 copies/16S rRNA after fertilization (Fig. 1B). Substantial ARGs were detected in antibiotic-free manure, showing higher diversity than soil samples. ARGs in antibiotic-free manure are present at a level of 0.46 copies/16S rRNA, much lower than in fertilized samples (i.e., CF, ROM and CF&ROM treatments). In contrast, the abundance of MGEs in antibiotic-free manure is significantly higher than in fertilized soil samples. Among the detected resistance genes, aminoglycoside resistance genes and MGEs were predominant in both diversity and abundance of all soil and manure samples (Fig. 1).

3.2. Enrichment of ARGs in soil under different fertilization treatments

Fold change analysis of shared ARGs and MGEs among all treatments indicated an enrichment range from 0.37 to 4.44-fold following the application of chemical fertilizer and/or recycled antibiotic-free manure

(Fig. 2). The number of significantly enriched target genes varied, with fold values > 1 , ranging from 97 to 123. The CF&ROM treatment generally showed higher enrichment in the number and fold of ARGs and MGEs compared to the CF and ROM treatments alone (Fig. 2). The heatmap analysis revealed a notable enrichment of certain resistance genes, predominantly focused on multidrug resistance genes and MGEs like *mdtE/yhiU*, *emrD*, *IS1133*, and *int1-a*, highlighting their significant role in the dissemination of ARGs.

3.3. Identification of high-risk ARGs in different fertilized soil

Among the 159 ARGs detected from all soil samples, 47 ARGs were identified as high-priority ARGs i.e., Rank I and II levels, with the total relative abundances from 0.12 to 0.19 copies/16S rRNA (Fig. 3). Here, sampling was performed 14 years after experiment start. The soil under long-term fertilization had an increase in the relative abundance of both Rank I and II ARGs. The increase in ARGs was lower in ROM treatment when compared to CF treatment, especially in Rank II ARGs. However, combining chemical fertilizer with recycled organic manure (i.e., CF&ROM treatment) significantly increased the proliferation of high-risk ARGs more than using a single fertilizer treatment (CF or ROM treatment). Among those treatments, genes resistance to aminoglycoside

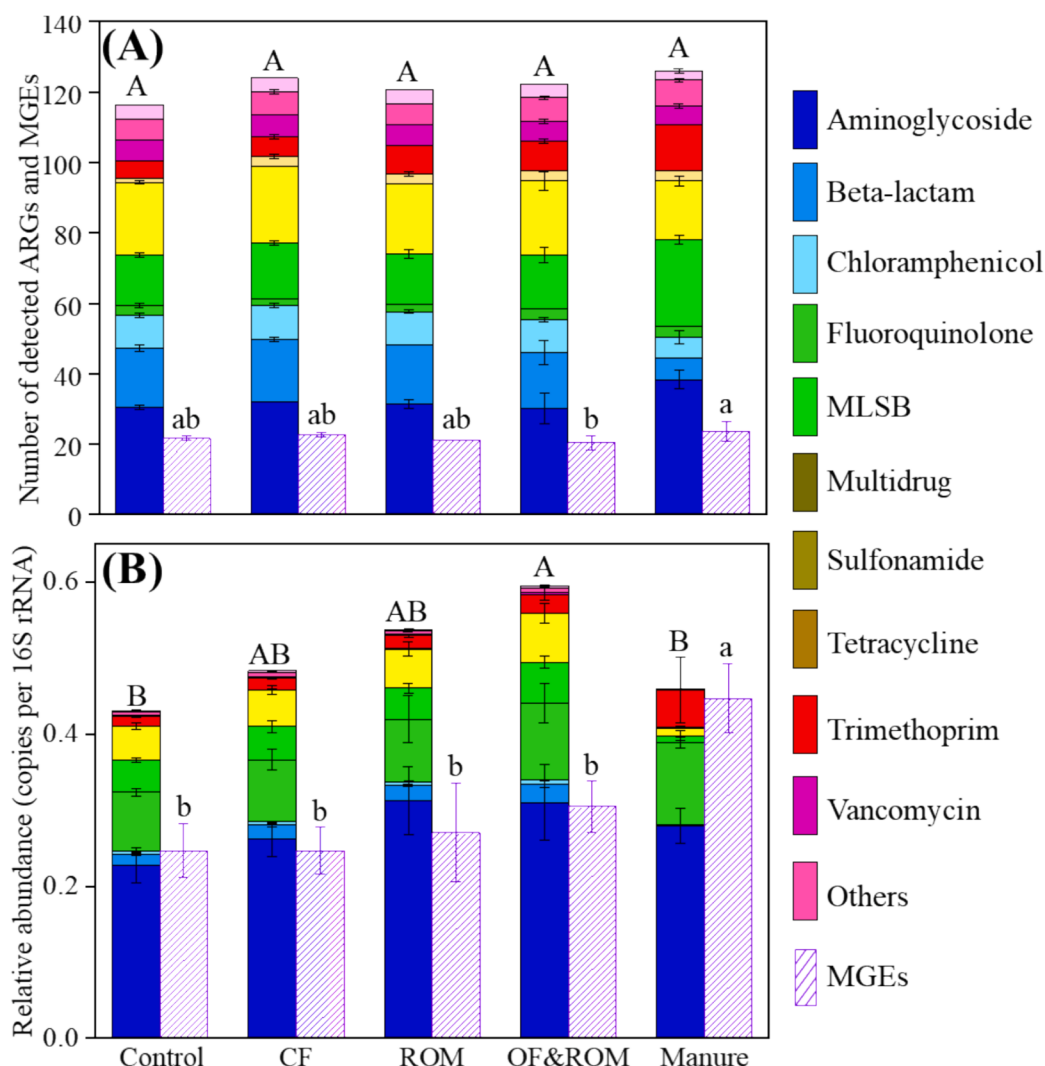


Fig. 1. (A) The number of detected and (B) relative abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in the soil applied with no fertilizer (Control), chemical fertilizer (CF), recycled organic manure (ROM) and chemical fertilizer plus recycled organic manure (CF&ROM), respectively. Manure treatment is recycled organic manure without antibiotics. Values with lowercase letters in the same column indicate significant differences at $P < 0.05$. MLSB, macrolide-lincosamide-streptogramin B.

5

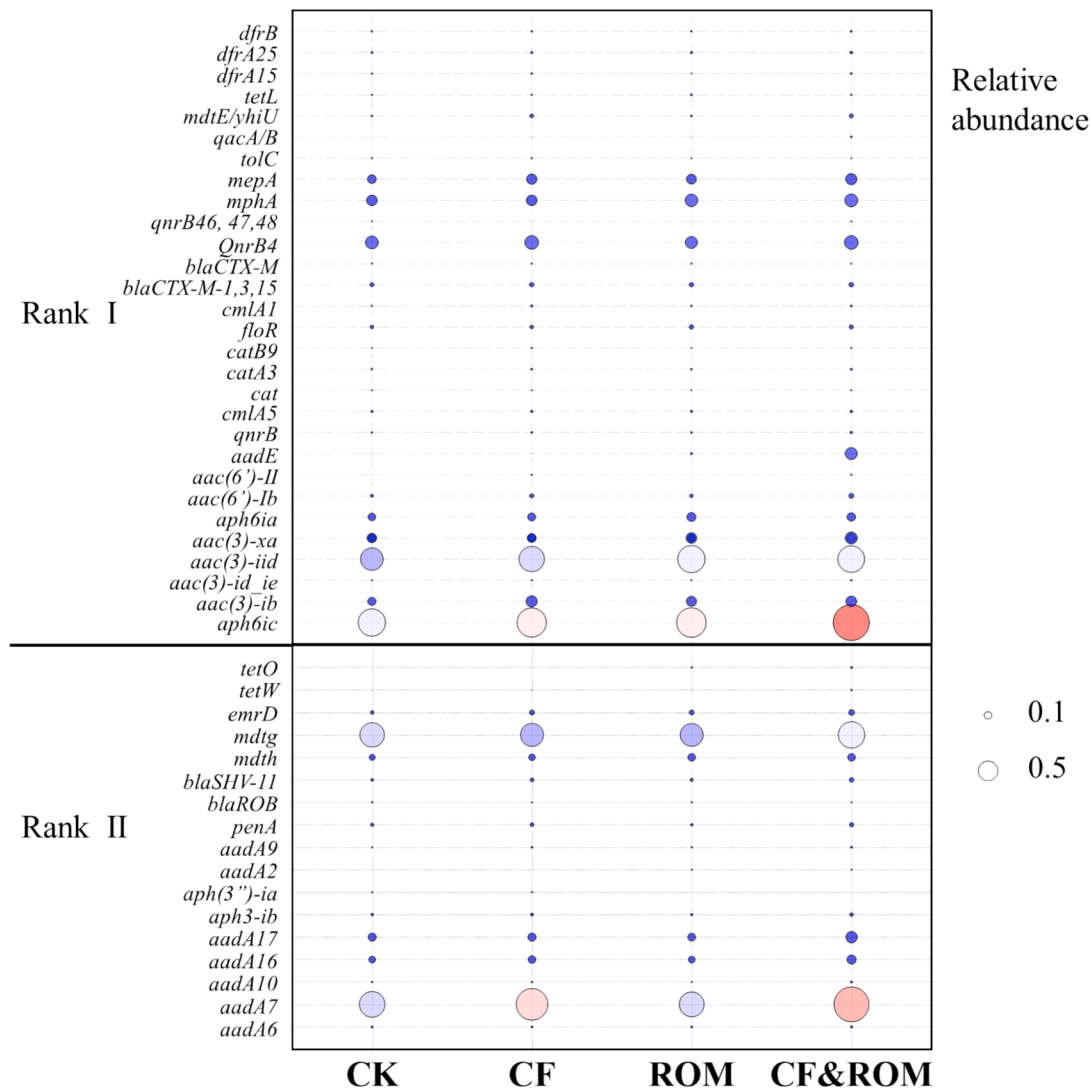


Fig. 3. Relative abundance of risk genes (Rank I high-risk and Rank II secondary high-risk genes) in the soil applied with no fertilizer (Control), chemical fertilizer (CF), recycled organic manure (ROM) and chemical fertilizer plus recycled organic manure (CF&ROM), respectively.

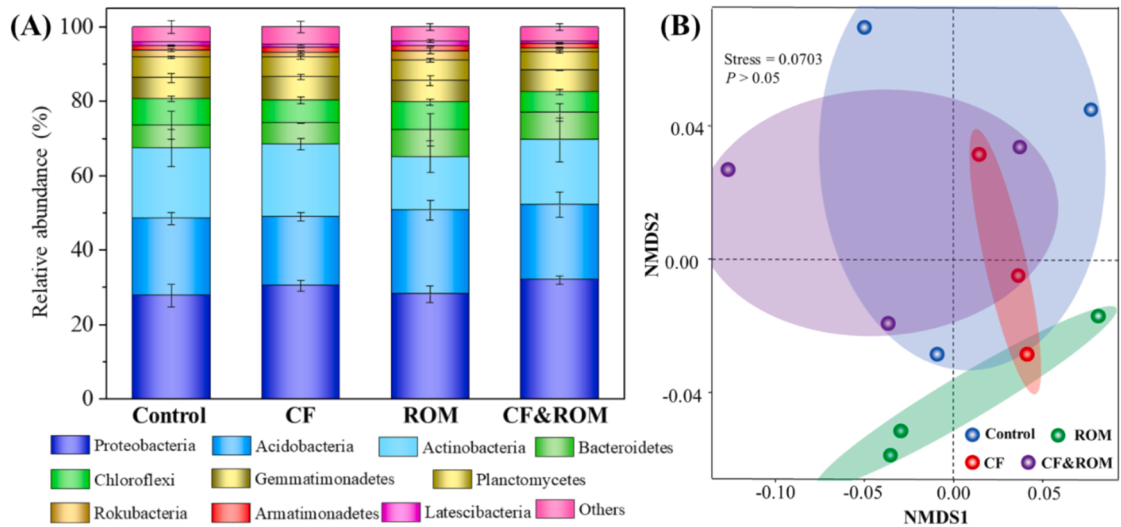


Fig. 4. (A) Bacterial community compositions at the phylum level in the control, chemical fertilizer (CF), recycled organic manure (ROM), and chemical fertilizer plus recycled organic manure (CF&ROM) treatments. (B) Non-metric multidimensional scaling (NMDS) analysis based on the Bray-Curtis distance showing the dissimilarity of the bacterial community.

(Table S5). This indicates significant differences in microbial biomass due to various fertilization practices. Analysis of high-quality sequences resulted in 3164 OTUs from 12 samples, with a total of 217,020 clean reads. Fertilization treatment has not caused dramatic change in the diversity of bacterial community at the phylum level, wherein the most abundant phyla were still Proteobacteria, Acidobacteria, and Actinobacteria, comprising 67.6 % to 73.1 % of the total bacterial OTUs (Fig. 4A). At the family level, over 40 % of the top 50 abundant families displayed considerable variation in bacterial abundance (Fig. S3). The alpha-diversity indices (i.e., Chao1 estimator, Ace estimator, Shannon diversity, and Simpson index) indicated an increase in bacterial

richness, particularly with the use of exogenous organic manure, whereas chemical fertilizer application did not have a similar effect (Table S5). Despite different fertilization treatments, the diversity of soil microbiota, as indicated by Shannon and Simpson indices, remained consistent. NMDS analysis reflected the similarity among bacterial communities across different treatments, revealing limited differences (Fig. 4B).

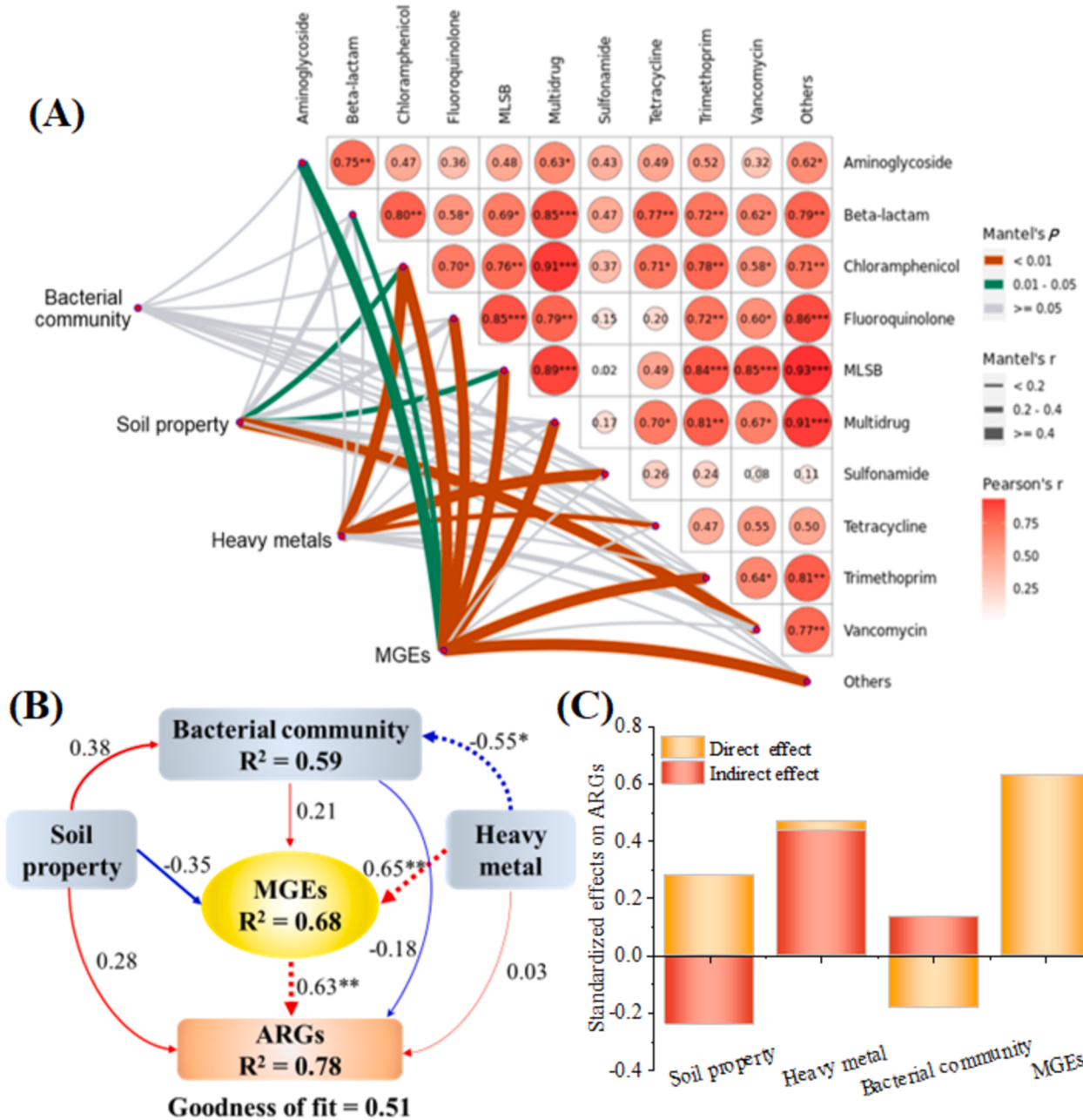


Fig. 5. (A) Mantel test exploring the relationship of the bacterial community, soil property, heavy metal, mobile genetic elements (MGEs), and antibiotic resistance genes (ARGs). Edge width corresponds to Mantel's r correlation, and edge color denotes the statistical significance (Mantel's P) based on 999 permutations. The color gradient indicates Spearman's rank correlation coefficients. (B) Partial least squares path modeling analysis among soil property, heavy metal, bacterial community, ARGs, and MGEs. Numbers adjacent to the arrows are path coefficients (λ), and the width of the arrows is proportional to the strength of the path coefficients. Red and blue arrows indicate positive and negative relationships, respectively. Continuous and dashed arrows indicate nonsignificant and significant relationships, respectively. (C) Standardized effects (direct and indirect effects) on ARGs were derived from the partial least squares path modeling. Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). MLSB, macrolide-lincosamide-streptogramin B.

3.5. Co-occurrence and correlation between MGEs, bacterial community, and ARGs

Co-occurrence profiles of ARGs, MGEs, and bacterial taxa were exhibited by network analysis with the parameters of 389 edges, 117 nodes, and 0.52 modularity index (Fig. S2). Dominant components in the network included aminoglycoside resistance genes (26 nodes), MGEs (20 nodes), and multidrug resistance genes (15 nodes). Proteobacteria, Latescibacteria, and Rokubacteria were identified as potential hosts in these co-occurrence patterns. The Mantel test assessed the influence of bacterial taxa, soil properties, heavy metals, and MGEs on ARG variations. A significant linear relationship was not observed between the bacterial community and ARGs ($P > 0.05$) across all resistance gene types (Fig. 5A, Table S6). However, a positive correlation was found between the relative abundance of MGEs and ARGs, including resistance to aminoglycoside ($P < 0.05$), beta-lactam ($P < 0.05$), chloramphenicol ($P < 0.01$), fluoroquinolone ($P < 0.01$), multidrug ($P < 0.01$), trimethoprim ($P < 0.01$), macrolide-lincosamide-streptogramin B (MLSB) ($P < 0.01$), and others ($P < 0.01$) (Fig. S4, Table S6). PCoA revealed that the first two principal components accounted for 83.3 % of the total variance based on Bray-Curtis distance, showing a notable contrast in ARG patterns between the CF treatment and those involving ROM and CF&ROM (Fig. S5). This suggests that the application of antibiotic-free manure might lead to a distinct shift in the ARG cluster. Soil properties and heavy metals also influenced ARG profiles, particularly affecting specific ARG subtypes like chloramphenicol, MLSB, sulfonamide, tetracycline, and vancomycin (Fig. 5A, Table S6).

The PLS-PM analysis further elucidated the interactive relationships between ARGs and various factors. Soil property was directly related to ARG abundance ($\lambda = 0.28$) and indirectly influenced ARG profiles through the interactions with the bacterial community ($\lambda = 0.38$) and MGEs ($\lambda = -0.35$) (Fig. 5B). Heavy metals had a minor direct impact on ARG abundance ($\lambda = 0.03$), but significantly influenced the bacterial community ($P < 0.05$, $\lambda = -0.55$) and MGEs ($P < 0.01$, $\lambda = 0.65$), thereby indirectly affecting ARG abundances. The bacterial community, as a vector for ARG dissemination, was impacted by multiple factors, contributing to ARG shifts both directly ($\lambda = -0.18$) and indirectly through MGEs ($\lambda = 0.21$). Consistent with the Mantel test results, MGEs under different factors positively influenced ARG profiles ($P < 0.01$, $\lambda = 0.63$) (Fig. 5B). The standardized effects from PLS-PM analysis indicated that MGEs were the primary drivers shaping ARG structure, followed by heavy metals (strong impact) and soil properties (weak impact). Contrarily, the bacterial community showed an overall negative effect on ARG prevalence (Fig. 5C).

4. Discussion

4.1. Impacts of long-term fertilization on soil antibiotic resistome

The control soil, which had not received any fertilizer application for approximately 14 years before sampling, exhibited 138 resistance genes in diversity and a relative abundance of 0.68 copies/16S rRNA (Fig. 1). Soil inherently harbors diverse intrinsic ARGs predating the use of antibiotics (Wang et al., 2021b). Agricultural fertilization is widely known to modify both the natural and introduced soil antibiotic resistomes through a process of (co)selection, involving agents such as antibiotics, metals, available carbon, microplastics, and other co-selectants (Jechalke et al., 2014; Lima et al., 2020; Zhu et al., 2022). The application of chemical fertilizer and/or recycled antibiotic-free manure did not significantly alter the total count of detected ARGs and MGEs, with the majority being common across all treatment types (Figs. 1, S1). However, ongoing use of these fertilizers significantly increased the abundance of ARGs and MGEs, resulting in several-fold enrichment compared to the control (Figs. 1, 2). Even though there were no significant differences between individual fertilizer or manure treatments and the control group, it is important to note that both chemical fertilizers

and antibiotic-free manure treatments did result in an increased total abundance of ARGs (Fig. 1). The increase in total ARG abundance after fertilization could indicate a response to nutrient inputs or shifts in microbial community structures that may not be immediately evident in terms of diversity. While these treatments may not directly affect the diversity of ARGs, they could influence the overall abundance in ways that could have implications for soil health and microbial dynamics. Factors such as nutrient availability, microbial community shifts, and long-term effects of ARG cycling may play a role in these observations.

Long-term fertilization can result in nutrient buildup in the soil, promoting the growth of specific microbial populations carrying ARGs (Liu et al., 2023a). While fertilization may enhance the proliferation of ARG-carrying microorganisms, it hardly changed the overall microbial community composition (Table S5), indicating that certain species may thrive under favorable conditions, but the diversity of the entire microbial community remains relatively constant. It has been suggested that the rise in antibiotic resistomes in soil amended with antibiotic-free cow manure might be linked to the proliferation of certain resistant species, rather than the introduction of ARB from manure (Udikovc-Kolic et al., 2014).

In contrast to the relative stability in ARG diversity, changes in nutrient conditions due to fertilization might be responsible for the observed increases in ARG abundance (Xu et al., 2024). A previous study reported that adding 100–200 mg/kg of ammonium nitrogen fertilizer significantly increased the prevalence of ARGs in the soil; the maximum increase in relative abundances of *bla*TEM-1, *cml*A, *str*, *sul*1, *tet*O, and *trp*A-4 were 5.99, 10.62, 1.99, 5.38, 3.97 and 2.36 folds, respectively, compared to the control treatment (Sun et al., 2020). Therefore, long-term application of fertilizers (here: 14 years) can support the growth of certain resistant bacteria that are well adapted to the fertilized conditions, leading to an increase in their abundance without a corresponding increase in diversity. The lack of new genetic material from less resistant strains could result in the dominance of the existing resistant population over time.

4.2. Impacts of different fertilization on soil antibiotic resistome

Examination of the different fertilizer types reveals that the application of ROM fertilizer is more likely to result in ARG accumulation and enrichment compared to the CF treatment (Figs. 1, 2). Manure improves soil fertility by altering its physical and chemical characteristics, such as structure, water content, pH levels, nutrient accessibility, and microbial relationships (Pérez-Valera et al., 2019). ROM fertilizer often contains large populations of ARG-carrying bacteria and is rich in nutrients conducive to keystone microbial cluster across aggregates (Fig. 1), potentially facilitating the spread of ARGs in agricultural fields. Additionally, as a significantly great abundance of MGEs were detected on the ROM fertilizer, the potential interaction between the soil microbiota using MGEs as vectors may be enhanced, thus prompting resistance gene transmission through possible horizontal gene transfer pathway (Liu et al., 2024). Here, the addition of ROM and chemical fertilizers showed the highest impact on ARG abundances (Figs. 1, 3). In line with other research findings (Guo et al., 2023; Xie et al., 2018), the rise in ARG levels in the CF&ROM treatment can be linked to the higher bacterial biomass (as indicated by the abundance of 16S rRNA genes in Table S5) and the elevated nitrogen levels (Table 1). It is important to acknowledge that while this study did not specifically examine the impact of fertilizer application on ARG prevalence, the notable increase in ARG abundance in the CF&ROM group warrants further detailed investigation in future studies. This finding holds significant implications for addressing the challenge of balancing plant growth requirements with the rapid proliferation of ARGs.

4.3. Multiple factors contributing to shaping soil ARG profiles

Manure from antibiotic-treated animals is widely used in agriculture,

Table 1

Soil physicochemical properties under long-term different fertilization. Different letters represent significant differences by Duncan's multiple-range test at $P < 0.05$. Control, no fertilizer applied; CF, nitrogen and phosphorus fertilizers plus potassium fertilizer; ROM, recycled organic manure consisting of the decomposed, mixed manure and residues; CF&ROM, nitrogen, phosphorus, and potassium fertilizers plus recycled organic manure.

Treatments	pH	Organic carbon (g/kg)	Total N (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)	CEC (cmol/kg)
Control	6.36 ± 0.08a	45.66 ± 2.15b	2.21 ± 0.15b	254.60 ± 0.00b	8.21 ± 0.54d	200.83 ± 1.44c	37.22 ± 0.42a
CF	5.97 ± 0.07b	50.45 ± 2.50ab	2.48 ± 0.19ab	259.70 ± 8.49b	16.77 ± 1.46b	223.33 ± 5.20b	36.17 ± 0.59ab
ROM	6.30 ± 0.07a	50.97 ± 3.55ab	2.51 ± 0.22ab	254.80 ± 16.97b	10.50 ± 1.23c	205.83 ± 6.29c	35.83 ± 0.20b
CF&ROM	6.11 ± 0.10b	55.85 ± 4.22a	2.65 ± 0.22a	294.00 ± 14.70a	23.30 ± 1.04a	250.83 ± 12.58a	36.71 ± 1.00ab

driven by the rising costs of chemical fertilizers, concerns about soil compaction and nutrient imbalance, and the urgent need for waste disposal (Lin et al., 2016). However, the application of such manure has raised concerns due to the bloom of ARB and ARGs in soil, attributed to the sustained selection pressures from residual antibiotics (Xie et al., 2018). Our study delves into additional potential pathways for ARG spread by the application of antibiotic-free manure. Fertilization effectively improved the soil physicochemical conditions, such as soil organic carbon and available nitrogen (Table 1), but increased the risk of ARG dissemination, significantly associated with the gene resistance to chloramphenicol ($P < 0.05$), MLSB ($P < 0.05$), and vancomycin ($P < 0.05$) (Fig. 5A, Table S6). Elevated nitrogen levels post-fertilization may favor ARG evolution in certain organisms (e.g., copiotrophs), possibly altering the soil bacterial community's composition and, consequently, the soil antibiotic resistome (Forsberg et al., 2014; Wang et al., 2022b).

Heavy metals were commonly recognized as a selective pressure for metal-resistance bacteria, and also frequently associated with ARGs by the mechanism of co-resistance, cross-resistance, and co-regulation (Zhao et al., 2020). Unlike antibiotics, which degrade, heavy metals can persist in the environment, exerting long-term selective pressures on soil microbiota (Song et al., 2017). Consistent with our findings, available heavy metals significantly correlated with the abundance of certain ARGs (Fig. 5A), primarily driving ARG proliferation by applying sustained pressures on bacterial communities and MGEs (Fig. 5B, 5C). However, long-term use of different fertilizers did not significantly alter the concentration of available heavy metals among these treatments (Table S2), suggesting that while heavy metals influence ARG occurrence, similar concentrations may not dominate ARG change patterns.

The roles of bacterial phylogeny and MGEs in shaping the soil resistome are significant but challenging to quantify (Ellabaan et al., 2021; Forsberg et al., 2014). In the present study, MGEs had a greater impact on ARG prevalence than bacterial communities (Fig. 5). A strong positive correlation between MGEs and ARGs ($r = 0.75$, $P < 0.01$) underscores the pivotal role of MGEs in ARG dissemination (Fig. S4). Network analysis and Mantel test further confirmed the close association between multiple MGEs (i.e., *IS1133*, *IS1247*, *int1-a*, *ISCR1*, *ISSm2*, *orf39-IS26*, *cro*, *int13*, *int11F165*, *EAE_05855*, *Inc11*, *IS1111*, *IS630*, *trb-C*, *IS6100*, *IS21-ISAs29*, *ISEcp1*, *IncN_rep*, *IS3* and *IncP_oriT*) and various classes of ARGs (i.e., aminoglycoside, beta-lactam, chloramphenicol, fluoroquinolone, MLSB, multidrug, trimethoprim and others) (Figs. S2, 5A), suggesting that MGEs were the primary determinants in structuring ARG profiles. The PLS-PM analysis further revealed that MGEs are key in multiple factors shaping ARG patterns, both directly and indirectly (Fig. 5B, 5C). The network analysis also notes that members of the bacterial community are closely linked to ARGs. Native soil bacteria like Proteobacteria, Latescibacteria, and Rokubacteria, associated with various ARG classes, potentially act as hosts (Fig. S2). Correlation analysis in statistics may not definitively identify hosts of ARGs. More advanced methods like metagenomic sequencing, emulsion paired isolation and concatenation PCR, fluorescence-activated cell sorting, and genomic cross-linking will aid in determining ARG-host relationships in various environments in the future.

Fertilization does not only alter soil abiotic properties but also increases the potential risks of ARG dissemination due to the proliferation

of the bacterial community (Table S5). This proliferation, coupled with improved living environments, may facilitate the transfer of genetic materials within and across different species (Han et al., 2018). Both vertical gene transfer and horizontal gene transfer mediated by MGEs during natural microbial succession significantly influence ARG reservoir dynamics (Wang et al., 2021c). The increase in ARG concentrations may be partially attributed to the increased bacterial populations post-fertilization. However, bacteria with a broad range and non-targetability are capable of randomly receiving ARGs via vertical/horizontal gene transfer to be potential hosts, possibly explaining the relatively low correlation via bacterial taxonomy to diffuse ARGs as compared to MGEs (Xie et al., 2022).

Antibiotic-free manure, sourced from animals not treated with antibiotics, is a safer and more sustainable option for soil health and aligns with consumer preferences for products raised without antibiotics. While some risks regarding antibiotic resistance still exist, sourcing manure from antibiotic-free animals reduces the risk of antibiotic residues and supports sustainable farming practices. Furthermore, efforts to mitigate the dissemination of resistance should prioritize minimizing the proliferation of ARGs and ARB. To address ARG pollution in a cost-effective manner that aligns with other environmental benefits, the following approaches may be considered: (1) the application of treated fertilizers that have a reduced initial concentration of ARGs and ARB; (2) precision fertilization to strike a balance between promoting plant growth and mitigating the potential risks associated with ARG and ARB proliferation (Sanz et al., 2022); (3) the removal of potential selection agents that could enhance the likelihood of gene transfer; and 4) the use of carbon-based materials, such as biochar and hydrochar, as soil amendments to control ARG pollution (Fu et al., 2024). Active implementation of these mitigation practices is essential to suppress the spread of resistance genes, particularly in complex field environments.

5. Conclusions

In conclusion, long-term use of chemical fertilizer and/or recycled antibiotic-free manure does not significantly alter ARG diversity in soil, but it does lead to a notable increase in ARG abundance. Examination of the different fertilization treatment shows a distinct shift in the ARG cluster, with antibiotic-free manure introducing more bacterial biomass, MGEs, and available nitrogen that can influence ARG levels compared to chemical fertilizer. Followed by Mantel and PLS-PM analyses on interactive relationships between bacterial communities, soil properties, heavy metals, MGEs, and ARGs, MGEs are confirmed as crucial vectors and primary determinants in structuring soil ARG profiles from both direct and indirect pathways. While using antibiotic-free manure can reduce the risk of antibiotic residues and support sustainable farming practices, it may not completely eliminate ARG prevalence. Future soil management strategies to address antibiotic resistance should consider the broader context of manure management.

Author contributions.

Yuhao Fu: Investigation, Methodology, Software, Formal analysis, Visualization, Validation, Data curation, Writing – original draft. Fang Hu: Investigation, Methodology, Software, Formal analysis, Writing – original draft. Fang Wang: Funding acquisition, Conceptualization,

Methodology, Validation, Supervision, Writing – review & editing. **Min Xu:** Investigation, Methodology. **Zhongjun Jia:** Writing – review & editing. **Wulf Amelung:** Writing – review & editing. **Zhi Mei:** Investigation, Methodology. **Xiaozeng Han:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Marko Virta:** Writing – review & editing. **Xin Jiang:** Supervision, Writing – review & editing. **James M. Tiedje:** Supervision, Writing – review & editing.

CRedit authorship contribution statement

Yuhao Fu: Writing – original draft, Methodology, Data curation. **Fang Hu:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Fang Wang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Min Xu:** Formal analysis, Data curation. **Zhongjun Jia:** Writing – review & editing, Validation, Supervision. **Wulf Amelung:** Writing – review & editing, Supervision, Conceptualization. **Zhi Mei:** Software, Methodology, Investigation. **Xiaozeng Han:** Writing – review & editing, Supervision, Resources, Conceptualization. **Marko Virta:** Writing – review & editing. **Xin Jiang:** Writing – review & editing, Supervision, Conceptualization. **James M. Tiedje:** Writing – review & editing, Supervision, Conceptualization.

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.

Acknowledgments

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA28030501), the National Natural Science Foundation of China (41991333, 42307048), the Youth Fund of the Natural Science Foundation of Jiangsu, China (BK20231099), the Institute of Soil Science, Chinese Academy of Sciences (ISSAS2419), Jiangsu Funding Program for Excellent Postdoctoral Talent (2022ZB460), International Atomic Energy Agency Coordinated Research Project (D15022), Chinese Academy of Sciences President's International Fellowship Initiative (2024DC0009), and the Center for Health Impacts of Agriculture (CHIA) of Michigan State University. Fang Wang was partly supported by the fellowship of Alexander von Humboldt for experienced researchers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2024.109202>.

Data availability

Data will be made available on request.

References

- Ager, E.O., Carvalho, T., Silva, E.M., Ricke, S.C., Hite, J.L., 2023. Global trends in antimicrobial resistance on organic and conventional farms. *Sci. Rep.* 13 (1), 22608.
- An, X.L., Su, J.Q., Li, B., Ouyang, W.Y., Zhao, Y., Chen, Q.L., Cui, L., Chen, H., Gillings, M.R., Zhang, T., Zhu, Y.G., 2018. Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR. *Environ. Int.* 117, 146–153.
- Bothe, H., Kamble, L., Bothe, S., 2024. Precision Farming: A New Era of Antibiotic-Free Agriculture. *International Journal of Agricultural Sciences and Technology* 4, 13–17.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., Tiedje, J.M., 2009. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res.* 37, D141–D145.
- Ellabaan, M.M.H., Munck, C., Porse, A., Imamovic, L., Sommer, M.O.A., 2021. Forecasting the dissemination of antibiotic resistance genes across bacterial genomes. *Nat. Commun.* 12 (1), 2435.
- Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. *Nature* 509 (7502), 612–616.
- Fu, Y., Jia, M., Wang, F., Wang, Z., Mei, Z., Bian, Y., Jiang, X., Virta, M., Tiedje, J.M., 2021. Strategy for mitigating antibiotic resistance by biochar and hyperaccumulators in cadmium and oxytetracycline co-contaminated soil. *Environ. Sci. Tech.* 55 (24), 16369–16378.
- Fu, Y., Wang, F., Wang, Z., Mei, Z., Jiang, X., Schäffer, A., Virta, M., Tiedje, J.M., 2022. Application of magnetic biochar/quaternary phosphonium salt to combat the antibiotic resistome in livestock wastewater. *Sci. Total Environ.* 811, 151386.
- Fu, Y., Wang, F., Xiang, L., Harindintwali, J.D., Elsner, M., Amelung, W., Kueppers, S., Jiang, X., Virta, M., Sillanpää, M., 2024. Combating antibiotic resistance in the human-impacted environment with carbon-based materials: Applications and challenges. *Crit. Rev. Environ. Sci. Technol.* 54 (9), 699–721.
- Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* 9 (6), 1269–1279.
- Guo, Y., Qiu, T., Gao, M., Ru, S., Gao, H., Wang, X., 2023. Does increasing the organic fertilizer application rate always boost the antibiotic resistance level in agricultural soils? *Environ. Pollut.* 322, 121251.
- Han, X.M., Hu, H.W., Chen, Q.L., Yang, L.Y., Li, H.L., Zhu, Y.G., Li, X.Z., Ma, Y.B., 2018. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102.
- Hurst, J.J., Oliver, J.P., Schueler, J., Gooch, C., Lansing, S., Crossette, E., Wigginton, K., Raskin, L., Aga, D.S., Sassoubre, L.M., 2019. Trends in antimicrobial resistance genes in manure blend pits and long-term storage across dairy farms with comparisons to antimicrobial usage and residual concentrations. *Environ. Sci. Tech.* 53 (5), 2405–2415.
- Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krögerrecklenfort, E., Brandes, N., Nordwig, M., Ding, G.C., Siemens, J., Heuer, H., Smalla, K., 2013. Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. *Appl. Environ. Microbiol.* 79 (5), 1704–1711.
- Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of veterinary antibiotics in soil. *Trends Microbiol.* 22 (9), 536–545.
- Jiang, X., Ellabaan, M.M.H., Charusanti, P., Munck, C., Blin, K., Tong, Y., Weber, T., Sommer, M.O.A., Lee, S.Y., 2017. Dissemination of antibiotic resistance genes from antibiotic producers to pathogens. *Nat. Commun.* 8 (1), 15784.
- Larsson, D.G.J., Flach, C.F., 2022. Antibiotic resistance in the environment. *Nat. Rev. Microbiol.* 20 (5), 257–269.
- Li, Y., Fang, F., Wei, J., Wu, X., Cui, R., Li, G., Zheng, F., Tan, D., 2019. Humic acid fertilizer improved soil properties and soil microbial diversity of continuous cropping peanut: A three-year experiment. *Sci. Rep.* 9 (1), 12014.
- Li, C., Gillings, M.R., Zhang, C., Chen, Q., Zhu, D., Wang, J., Zhao, K., Xu, Q., Leung, P.H., Li, X., Liu, J., Jin, L., 2024. Ecology and risks of the global plastisphere as a newly expanding microbial habitat. *The Innovation* 5 (1), 100543.
- Liao, H., Lu, X., Rensing, C., Friman, V.P., Geisen, S., Chen, Z., Yu, Z., Wei, Z., Zhou, S., Zhu, Y., 2018. Hyperthermophilic composting accelerates the removal of antibiotic resistance genes and mobile genetic elements in sewage sludge. *Environ. Sci. Tech.* 52 (1), 266–276.
- Lima, T., Domingues, S., Da Silva, G.J., 2020. Manure as a potential hotspot for antibiotic resistance dissemination by horizontal gene transfer events. *Veterinary Sciences* 7 (3).
- Lin, H., Sun, W., Zhang, Z., Chapman, S.J., Freitag, T.E., Fu, J., Zhang, X., Ma, J., 2016. Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy–upland rotation system. *Environ. Pollut.* 211, 332–337.
- Liu, W., Cheng, Y., Guo, J., Duan, Y., Wang, S., Xu, Q., Liu, M., Xue, C., Guo, S., Shen, Q., Ling, N., 2022. Long-term manure inputs induce a deep selection on agroecosystem soil antibiotic resistome. *J. Hazard. Mater.* 436, 129163.
- Liu, K., Li, P., Li, G., Ma, X., Liu, M., Liu, J., Wu, M., Li, Z., 2023a. Long-term fertilization promotes soil organic nitrogen accumulation by increasing the abundance of keystone microbial cluster across aggregates. *Appl. Soil Ecol.* 192, 105086.
- Liu, Z.T., Ma, R.A., Zhu, D., Konstantinidis, K.T., Zhu, Y.G., Zhang, S.Y., 2024. Organic fertilization co-selects genetically linked antibiotic and metal(loid) resistance genes in global soil microbiome. *Nat. Commun.* 15 (1), 5168.
- Liu, Z., Zhao, Y., Zhang, B., Wang, J., Zhu, L., Hu, B., 2023b. Deterministic Effect of pH on Shaping Soil Resistome Revealed by Metagenomic Analysis. *Environ. Sci. Tech.* 57 (2), 985–996.
- Lu, X., Mahdi, A.K., Han, X., Chen, X., Yan, J., Biswas, A., Zou, W., 2020. Long-term application of fertilizer and manures affect P fractions in Mollisol. *Sci. Rep.* 10 (1).
- Murray, C.J.L., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Achalapong, S., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Babin, F.-X., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Berkley, J.A., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalho, C., Castaneda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiruchit, S., Chowdhury, F., Clotaire Donatien, R., Cook, A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P.J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Duong Bich, T., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garcia, C., Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Hsia, Y., Iregbu, K.C., Jacobs, J.,

- Jarovsky, D., Javanmardi, F., Jenney, A.W.J., Khorana, M., Khusuwan, S., Kissoon, N., Kobeissi, E., Kostyanov, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H. H., Lim, C., Lim, K., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J., Manoharan, A., Marks, F., May, J., Mayxay, M., Mturi, N., et al., 2022. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* 399 (10325), 629–655.
- Nel, T., Clarke, C.E., Hardie, A.G., 2022. Evaluation of simple and multivariate linear regression models for exchangeable base cation conversion between seven measurement techniques on South African soils. *Geoderma Reg.* 30, e00571.
- Nguyen, T.B.A., Bonkowski, M., Dumack, K., Chen, Q.L., He, J.Z., Hu, H.W., 2023. Protistan predation selects for antibiotic resistance in soil bacterial communities. *ISME J.* 17 (12), 2182–2189.
- Pérez-Valera, E., Kyselková, M., Ahmed, E., Sladecek, F.X.J., Goberna, M., Elhottová, D., 2019. Native soil microorganisms hinder the soil enrichment with antibiotic resistance genes following manure applications. *Sci. Rep.* 9 (1), 6760.
- Sanz, C., Casado, M., Navarro-Martin, L., Cañameras, N., Carazo, N., Matamoros, V., Bayona, J.M., Piña, B., 2022. Implications of the use of organic fertilizers for antibiotic resistance gene distribution in agricultural soils and fresh food products. A plot-scale study. *Sci. Total Environ.* 815, 151973.
- Shawver, S., Wepking, C., Ishii, S., Strickland, M.S., Badgley, B.D., 2021. Application of manure from cattle administered antibiotics has sustained multi-year impacts on soil resistome and microbial community structure. *Soil Biol. Biochem.* 157, 108252.
- Sheng, M., Han, X., Zhang, Y., Long, J., Li, N., 2020. 31-year contrasting agricultural managements affect the distribution of organic carbon in aggregate-sized fractions of a Mollisol. *Sci. Rep.* 10 (1), 9041.
- Song, J., Rensing, C., Holm, P.E., Virta, M., Brandt, K.K., 2017. Comparison of metals and tetracycline as selective agents for development of tetracycline resistant bacterial communities in agricultural soil. *Environ. Sci. Tech.* 51 (5), 3040–3047.
- Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., Gunturu, S., Tift, L., Wang, F., Howe, A., Chai, B., Yin, D., Cole, J.R., Tiedje, J.M., Hashsham, S.A., 2018. Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol. Ecol.* 94 (9), f1y130.
- Sun, S., Lu, C., Liu, J., Williams, M.A., Yang, Z., Gao, Y., Hu, X., 2020. Antibiotic resistance gene abundance and bacterial community structure in soils altered by Ammonium and Nitrate Concentrations. *Soil Biol. Biochem.* 149, 107965.
- Tian, L., Zhao, L., Wu, X., Hu, G., Fang, H., Zhao, Y., Sheng, Y., Chen, J., Wu, J., Li, W., Ping, C.L., Pang, Q., Liu, Y., Shi, W., Wu, T., Zhang, X., 2019. Variations in soil nutrient availability across Tibetan grassland from the 1980s to 2010s. *Geoderma* 338, 197–205.
- Tiedje, J.M., Fu, Y., Mei, Z., Schäffer, A., Dou, Q., Amelung, W., Elsner, M., Adu-Gyamfi, J., Heng, L., Virta, M., Jiang, X., Smidt, H., Topp, E., Wang, F., 2023. Antibiotic resistance genes in food production systems support One Health opinions. *Curr. Opin. Environ. Sci. Health* 34, 100492.
- Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl. Acad. Sci.* 111 (42), 15202–15207.
- UNEP. 2022. Antimicrobial resistance: a global threat. <https://www.unep.org/explore-topics/chemicals-waste/what-we-do/emerging-issues/antimicrobial-resistance-global-threat>.
- Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant, A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci.* 112 (18), 5649–5654.
- Wang, F., Xu, M., Stedtfeld, R.D., Sheng, H., Fan, J., Liu, M., Chai, B., Soares de Carvalho, T., Li, H., Li, Z., Hashsham, S.A., Tiedje, J.M., 2018. Long-term effect of different fertilization and cropping systems on the soil antibiotic resistome. *Environ. Sci. Tech.* 52 (22), 13037–13046.
- Wang, F., Fu, Y.H., Sheng, H.J., Topp, E., Jiang, X., Zhu, Y.G., Tiedje, J.M., 2021b. Antibiotic resistance in the soil ecosystem: A One Health perspective. *Curr. Opin. Environ. Sci. Health* 20, 100230.
- Wang, H., Hou, L., Liu, Y., Liu, K., Zhang, L., Huang, F., Wang, L., Rashid, A., Hu, A., Yu, C., 2021c. Horizontal and vertical gene transfer drive sediment antibiotic resistome in an urban lagoon system. *J. Environ. Sci.* 102, 11–23.
- Wang, S., Sun, P., Zhang, G., Gray, N., Dolfing, J., Esquivel-Elizondo, S., Peñuelas, J., Wu, Y., 2022a. Contribution of periphytic biofilm of paddy soils to carbon dioxide fixation and methane emissions. *The Innovation* 3 (1), 100192.
- Wang, T., Sun, S., Xu, Y., Waigi, M.G., Odinga, E.S., Vasilyeva, G.K., Gao, Y., Hu, X., 2022b. Nitrogen regulates the distribution of antibiotic resistance genes in the soil–vegetable system. *Front. Microbiol.* 13.
- Wang, F., Xiang, L., Sze-Yin Leung, K., Elsner, M., Zhang, Y., Guo, Y., Pan, B., Sun, H., An, T., Ying, G., Brooks, B.W., Hou, D., Helbling, D.E., Sun, J., Qiu, H., Vogel, T.M., Zhang, W., Gao, Y., Simpson, M.J., Luo, Y., Chang, S.X., Su, G., Wong, B.M., Fu, T.M., Zhu, D., Jobst, K.J., Ge, C., Coulon, F., Harindintwali, J.D., Zeng, X., Wang, H., Fu, Y., Wei, Z., Lohmann, R., Chen, C., Song, Y., Sanchez-Cid, C., Wang, Y., El-Naggar, A., Yao, Y., Huang, Y., Cheuk-Fung Law, J., Gu, C., Shen, H., Gao, Y., Qin, C., Li, H., Zhang, T., Corcoll, N., Liu, M., Alessi, D.S., Li, H., Brandt, K.K., Pico, Y., Gu, C., Guo, J., Su, J., Corvini, P., Ye, M., Rocha-Santos, T., He, H., Yang, Y., Tong, M., Zhang, W., Suanon, F., Brahushi, F., Wang, Z., Hashsham, S.A., Virta, M., Yuan, Q., Jiang, G., Tremblay, L.A., Bu, Q., Wu, J., Peijnenburg, W., Topp, E., Cao, X., Jiang, X., Zheng, M., Zhang, T., Luo, Y., Zhu, L., Li, X., Barceló, D., Chen, J., Xing, B., Amelung, W., Cai, Z., Naidu, R., Shen, Q., Pawliszyn, J., Zhu, Y.G., Schaeffer, A., Rillig, M.C., Wu, F., Yu, G., Tiedje, J.M., 2024. Emerging Contaminants: A One Health Perspective. *The Innovation* 100612.
- Wang, A., Zou, D., Zeng, X., Chen, B., Zheng, X., Li, L., Zhang, L., Xiao, Z., Wang, H., 2021a. Speciation and environmental risk of heavy metals in biochars produced by pyrolysis of chicken manure and water-washed swine manure. *Sci. Rep.* 11 (1), 11994.
- Xie, W.Y., Yuan, S.T., Xu, M.G., Yang, X.P., Shen, Q.R., Zhang, W.W., Su, J.Q., Zhao, F.J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. *Soil Biol. Biochem.* 122, 111–119.
- Xie, W.Y., Wang, Y.T., Yuan, J., Hong, W.D., Niu, G.Q., Zou, X., Yang, X.P., Shen, Q., Zhao, F.J., 2022. Prevalent and highly mobile antibiotic resistance genes in commercial organic fertilizers. *Environ. Int.* 162, 107157.
- Xu, S., Zhan, L., Tang, W., Wang, Q., Dai, Z., Zhou, L., Feng, T., Chen, M., Wu, T., Hu, E., Yu, G., 2023. MicrobiotaProcess: A comprehensive R package for deep mining microbiome. *The Innovation* 4 (2), 100388.
- Xu, Y., Zhang, D., Li, H., Ye, H., Bai, M., Jiang, G., Li, X., 2024. Unraveling the determinants of antibiotic resistance evolution in farmland under fertilizations. *J. Hazard. Mater.* 474, 134802.
- Yan, J., Han, X., Chen, X., Lu, X., Chen, W., Wang, E., Zou, W., Zhang, Z., 2019. Effects of long-term fertilization strategies on soil productivity and soybean rhizobial diversity in a Chinese Mollisol. *Pedosphere* 29 (6), 784–793.
- You, M., Han, X., Chen, X., Yan, J., Li, N., Zou, W., Lu, X., Li, Y., Horwath, W.R., 2019. Effect of reduction of aggregate size on the priming effect in a Mollisol under different soil managements. *Eur. J. Soil Sci.* 70 (4), 765–775.
- Zhang, A.N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L.G., van Loosdrecht, M.C.M., Topp, E., Gillings, M.R., Hanage, W.P., Tiedje, J.M., Moniz, K., Alm, E.J., Zhang, T., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nat. Commun.* 12 (1), 4765.
- Zhao, X., Shen, J.P., Zhang, L.M., Du, S., Hu, H.W., He, J.Z., 2020. Arsenic and cadmium as predominant factors shaping the distribution patterns of antibiotic resistance genes in polluted paddy soils. *J. Hazard. Mater.* 389, 121838.
- Zhu, D., Ma, J., Li, G., Rillig, M.C., Zhu, Y.G., 2022. Soil plastspheres as hotspots of antibiotic resistance genes and potential pathogens. *ISME J.* 16 (2), 521–532.