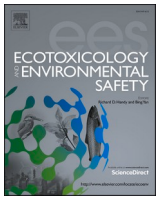
Ecotoxicology and Environmental Safety 227 (2021) 112946

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Potential role of organic matter in the transmission of antibiotic resistance genes in black soils

Sen Li [a,b], Junjie Liu [a], Qin Yao [a], Zhenhua Yu [a], Yansheng Li [a], Jian Jin [a], Xiaobing Liu [a], Guanghua Wang [a,*]

[a] Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China
[b] University of Chinese Academy of Science, Beijing 100049, China

ARTICLE INFO

Edited by: Dr G Liu

Keywords:
Mollisols
Soil resistome
Soil bacterial community
HT-PCR
Illumina MiSeq

ABSTRACT

The degradation of black soil is a serious problem with the decrease in soil organic matter (SOM) content in northeast China, and animal manure as a reservoir of antibiotic resistance genes (ARGs) is commonly amended into soil to sustain or increase the SOM content. However, the potential effect of SOM content on soil resistome remains unclear. Here, a soil microcosm experiment was established to explore the temporal succession of antibiotic resistance genes (ARGs) and bacterial communities in three black soils with distinct difference in SOM contents following application of poultry manure using high-throughput qPCR (HT-qPCR) and MiSeq sequencing. A total of 151 ARGs and 8 mobile genetic elements (MGEs) were detected across all samples. Relative abundance of ARGs negatively correlated with SOM content. Manure-derived ARGs had much higher diversity and absolute abundance in the low SOM soils. The ARG composition and bacterial community structure were significantly different in three soils. A random forest model showed that SOM content was a better predictor of ARG pattern than bacterial diversity and abundance. Structural equation modeling indicated that the negative effects of SOM content on ARG patterns was accomplished by the shift of bacterial communities such as the bacterial diversity and abundance. Our study demonstrated that SOM content could play an important role in the dissemination of ARGs originated from animal manures, these findings provide a possible strategy for the suppression of the spread of ARGs in black soils by increasing SOM content.

1. Introduction

Antibiotic resistance complicates the prevention and treatment of the infectiousness of pathogenetic bacteria between humans posing a serious challenge to public health (Aslam et al., 2018). Generally, antibiotic resistance encoded by antibiotic resistance genes (ARGs) is largely attributed to the improper use of antibiotics in animals and humans. However, the dissemination of worldwide ARGs and long-term persistence of resistant bacteria are also detected in the absence of selective pressure of antibiotics (Hu et al., 2018), suggesting that antibiotic resistance predates the discovery of antibiotics as well as the emergence of resistance genes-mediated antibiotic resistance may be associated with certain environmental factors. Previous studies have shown that several environmental factors, such as salinity (Zhang et al., 2019a, 2019b), organic carbon (Wan et al., 2019) and heavy metals (Dickinson et al., 2019), are important drivers of the spread of ARGs. Furthermore, soil bacterial community composition is correlated with ARGs content (Forsberg et al., 2014; Hu et al., 2018), and mediated by the soil environmental factors (Tripathi et al., 2018). Therefore, understanding how soil conditions drive the dissemination of antibiotic resistance is essential for making management strategies to control the spread of antibiotic resistance genes in the soils.

Black soil (Mollisol) is an important soil resource to ensure the national food safety of China, the total area of black soil region in northeast China, including three provinces of Heilongjiang, Jilin and Liaoning, as well as east part of Inner Mongolia, is approximately 109 million ha (Liu et al., 2021). However, due to the excessive reclamation and extensive use of black soils in this region, soil organic matter (SOM) content has prominent degradation (Wang et al., 2020). As core components for functional ecosystem, SOM not only play a role in the soil physical, chemical and biological processes, but also is a nutrient reservoir for plant growth and an energy substrate for soil organisms (Schmidt et al., 2011; Dhaliwal et al., 2019). Therefore, the study of SOM in black soils is advantageous to enrich the theoretical framework of soil health.

* Correspondence to: Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin 150081, China.
E-mail address: wanggh@iga.ac.cn (G. Wang).

https://doi.org/10.1016/j.ecoenv.2021.112946
Received 18 July 2021; Received in revised form 20 October 2021; Accepted 22 October 2021
Available online 26 October 2021

Animal manure is regarded as a vital reservoir of ARGs, and is commonly used to agricultural soil as an effective approach to sustain or increase SOM content (Udikovic-kolic et al., 2014; Ye et al., 2019). Application of animal manure not only increases the abundance and mobility of ARGs, but also introduces antibiotic-resistant bacteria and pathogens into agricultural soil (Chen et al., 2017). In addition, the diversity and abundance of ARGs have obvious differences in the different types of animal manure. For instance, swine and poultry manures have significantly higher ARGs diversity than the cattle manure (Han et al., 2018). Given that soil bacterial community is the dominant factor in the spread of ARGs, the resistance genes could be transferred from soil to plant microbiome by horizontal gene transfer (HGT) via the mobile genetic elements (MGEs), which is finally employed in human or animal consumption (Chen et al., 2017, 2018; Zhang et al., 2019a, 2019b). However, less is known about the transmission of the introduced resistance genes in black soil differing in SOM content.

To explore the effects of SOM content on the fate of ARGs in black soil, a microcosm experiment with three soils differing in SOM content was constructed. Each soil was treated with or without poultry manure, and the incubation experiment was performed for up to one month. Here, the abundance and diversity of ARGs in soil were estimated by the high-throughput quantitative PCR (HT-qPCR) containing 296 validated primer sets conferring resistance to most major classes of antibiotics and the bacterial communities were analyzed by Illumina MiSeq sequencing. The objectives of this study were to investigate the temporal changes of the ARGs in three black soils with different SOM contents and further to examine the role of SOMs in shaping the distribution of exogenous ARGs introduced by animal manure.

2. Materials and methods

2.1. Microcosm experiment setup and soil sampling

A commercial air-dried poultry manure was collected from a livestock organic fertilizer manufacture in Hulan District in August 2018, Harbin City, Heilongjiang Province, China. The manure was kept in the Lab until use. Black soils were sampled from three farmlands located in Beian (B), Hailun (H) and Lishu (L) with SOM content of 92.3, 51.7 and 20.3 g kg^{-1}, respectively, in April 2007 in northeast China. These soil samples were kept in several big plastic boxes in the Lab at the room temperature until use. The chemical properties of the soil and poultry manure were shown in Table S1. The microcosm experiment was conducted in March, 2019. Before adding poultry manure, air-dried soils were adjusted to moisture content 40% with sterilized ultrapure water. The soil was then pre-incubated in the dark condition at 25 °C for 3 weeks to retrieve the microbial activity. After the soil pre-incubation, soil microcosms were established in the sterile petri dishes (radius 8.8 cm, height 1.8 cm) with per dish containing 50 g soils (dry weight). The treatments were as follows: three control treatments with soil only (B, H and L) and three manure added treatments (BM, HM and LM) with the amendment of 10% dry poultry manure (w/w) (Millner et al., 2004). Each treatment had three replicates. The petri dishes were kept in an incubator at 25 °C under dark condition, and sterilized ultrapure water was added regularly to maintain the same soil moisture content about 40% based on the weighing method. Approximately 2 g soils were sampled from each dish with a sterilized toothpick at incubation days of 0, 15 and 30, and then placed into the 2 mL sterilized centrifuge tube and stored in the refrigerator at −20 °C until use.

2.2. DNA extraction

Total DNA of each sample was extracted from 0.5 g of fresh soils stored in the refrigerator using the FastDNA® Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA) following the manufacturer's instructions. After testing the concentration and quality of extracted DNA by the Thermo Scientific NanoDrop 2000 spectrophotometer (NanoDrop, USA), the DNA was diluted to 18 ng μl^{-1} using sterilized ultrapure water and stored at −20 °C for further analysis.

2.3. High-throughput quantitative PCR (HT-qPCR)

The SmartChip Real-time PCR (Wafergen Inc. USA) was used to perform the high-throughput quantitative PCR (HT-qPCR) of ARGs using 296 primer sets (Zhu et al., 2017). These primer sets included 283 primer sets targeting almost all major classes of ARGs, and 12 primer sets for MGEs (8 transposase genes and 4 integrase genes) and a primer set of the 16 S rRNA gene (Table S2). Amplification of each DNA sample was performed in triplicate with a non-template negative by the following thermal cycling: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s. The melting curve analysis was automatically created by Wafergen software. After that, each primer set with multiple melting peaks or amplification efficiencies beyond the range of 90%–110% were discarded. A threshold cycle (C_T) value of 31 was used as the detection limit, the positive detection had configured that the samples with three technical replicates below the detection limit. The relative copy number was calculated with $10^{(31\text{-}C_T)/(10/3)}$, where C_T is the threshold cycle. The absolute copy numbers were quantified with relative copy number and the absolute 16 S rRNA gene copy number generated by the LightCycler® 480 (Roche Applied Science). The normalized copy number of ARGs or MGEs was represented by the relative abundances of ARGs or MGEs multiplied 4.1, the average number of 16 S rRNA gene per bacterium based on the Ribosomal RNA Operon Copy Number Database (rrnDB version 4.3.3), to estimate the total ARGs in the total bacterial communities (Klappenbach et al., 2001).

2.4. Illumina MiSeq sequencing of soil bacterial community

The V4-V5 region of the bacterial 16 S rRNA gene was amplified using primer sets 515 F (5'-GTG CCA GCM GCC GCG GTA A-3') / 907 R (5'-CCG TCA ATT CCT TTG AGT TT-3') modified with a unique 6 nt barcode at the end of the forward primer to differentiate soil samples (Angenent et al., 2005). The 20 μl mixtures contained 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of forward primer (5 μM), 0.8 μl of reverse primer (5 μM), 0.4 μl of FastPfu polymerase, 0.2 μl of BSA, 1 μl of DNA template and 11.8 μl the sterile ultrapure water. The PCR reactions were conducted in ABI GeneAmp® 9700 platform with under the following thermal programs: initial denaturation at 95 °C for 3 min, followed by 30 cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s). Each sample was subjected to three PCR amplifications, the PCR products were pooled and purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and sequenced using Illumina MiSeq PE 250 at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Raw reads were assembled using Quantitative Insights Into Microbial Ecology (QIIME) software (version 1.9.0) after the elimination of low-quality sequences with a low average quality score (< 20) and short length (< 200 bp) (Caporaso et al., 2010). Subsequently, USEARCH software was applied to check and remove the chimera using the UCHIME algorithm (Edgar et al., 2011). According to the open reference method and the UCLUST algorithm, the high-quality sequences were clustered to Operational Taxonomic Units (OTUs) with a 97% sequence similarity threshold (Edgar, 2010). Taxonomic classification was processed using the Ribosomal Database Project (RDP) classifier at an 80% confidence threshold with SILVA database for the bacterial dataset (Quast et al., 2013). Mitochondrial, chloroplast, and singletons OTUs were removed from the final OTU data set. To preclude bias in the number of reads per sample, 25,627 sequences were randomly subsampled from each bacterial sample for subsequent community analysis. Raw sequences of bacterial communities were deposited in the NCBI Sequence Read Archive under accession number PRJNA666408.

2.5. Statistical analysis

A data organization was constructed on Microsoft Excel 2016. One-way ANOVA based on least significant difference (LSD, $P < 0.05$), two-way ANOVA and Pearson correlation analysis was determined by SPSS 21.0 (IBM, USA). Non-metric multidimensional scaling (NMDS) analysis, a similar analysis (ANOSIM), non-parametric MANOVA (Adonis), and multi-response permutation procedure (MRPP) based on the Bray-Cutis distance were performed using R software with the vegan and ggpubr packages (Kassambara, 2019; Oksanen et al., 2019). Procrustes analysis and Mantel test were conducted using vegan packages in R based on Bray-Curtis distance to test the correlation between ARGs and soil bacterial taxa. Line regression analysis was conducted with the ggplot2 package based on lm method in R (Wickham, 2016). The linear discriminant analysis (LDA) effect size (LEfSe) was conducted online (http:/huttenhower.sph.harvard.edu/galaxy/) to determine biomarker at multiple taxonomical levels using an alpha value of 0.01 and an LDA threshold score of > 3.0 (Segata et al., 2011).

A valid co-occurrence network was constructed by a robust correlation between ARGs and soil bacterial taxa if the spearman's correlation coefficient (ρ) was both > 0.8 and the *P*-value <0.01 (Chen et al., 2016). Co-occurrence networks were visualized by Gephi (v0.9.2) software using the *Frucherman Reingold* algorithm (Bastian et al., 2009). In the co-occurrence network, the edges were weighted by the correlation coefficient between the ARGs and bacterial taxa, and nodes were proportional to the number of connections. A classification random forest (RF) analysis was used to identify the main predictors for the ARGs contents in soil by the percentage increases in the mean square error (MSE) of variables as well as the significance of the importance of each predictor using the rfPermute package in R (Archer, 2021). Variables including MGEs, manure addition, Soil organic matter, bacterial abundance (16 S rRNA gene copies), and bacterial diversity (OTU richness) were filtered using the *vif* function in the R car package (Fox and Wersberg, 2019). Structural equation modeling (SEM) was constructed to test the direct and indirect effects of soil organic matter, manure addition, the abundance and diversity of bacteria (including 16 S rRNA gene copies and OTU richness, respectively), and MGEs, on the ARG patterns via the establishment of a priori model based on the known relationships among the factors influencing ARG patterns (Grace, 2006). All data were standardized using Z-scores importing into AMOS 21 (SPSS Inc., Chicago, USA) for the SEM construction based on maximum-likelihood method. The overall goodness of model fit was evaluated using the χ^2 test ($P > 0.05$), goodness-of-fit index (GFI > 0.90), akaike information criteria (AIC), and the root mean square error of approximation (RMSEA < 0.05).

3. Results

3.1. Diversity of antibiotic resistance genes

A total of 159 genes including 151 ARGs, 6 transposase genes, and 2 integron-integrase genes were detected in all soil samples (Table S4). The average number of ARGs and MGEs detected in each sample ranged from 7 (sample H15) to 124 (sample LM30), and the soil samples amended with poultry manure harbored more diverse ARGs and MGEs than the unamended soils (Fig. 1). Moreover, a significant decline trend in the number of ARGs was observed in B and H soils but not in L soil with the incubation time. In contrast, the number of ARGs was increased in all manured soils over the incubation time, especially in the LM treatment ($P < 0.05$). In addition, by using two-way ANOVA analysis, the number of ARGs was significantly influenced by the interaction between manure addition and incubation time ($P < 0.01$) (Fig. 1).

The detected ARGs potentially conferred resistance to 8 major classes of antibiotic (Fig. 2a), and contained 3 antibiotic mechanisms (Fig. 2b). The aminoglycoside and MLSB were the higher resistance classes in the manured soils than those in the control, but multidrug, beta-lactams and vancomycin showed in the opposite trend. In addition, genes conferring resistance to sulfonamide were not detected in the control soils (Fig. 2a). Of the mechanisms of resistance, antibiotic deactivate and efflux pump were the 2 dominant resistance mechanisms in all soils, but the efflux pump was the higher resistance mechanism in the control soils, and antibiotic deactivate and cellular protection were higher in the manured soils (Fig. 2b).

3.2. Temporal changes in the abundance of antibiotic resistance genes

Poultry manure amendment significantly increased the absolute abundance and relative copy number of ARGs and MGEs ($P < 0.05$)

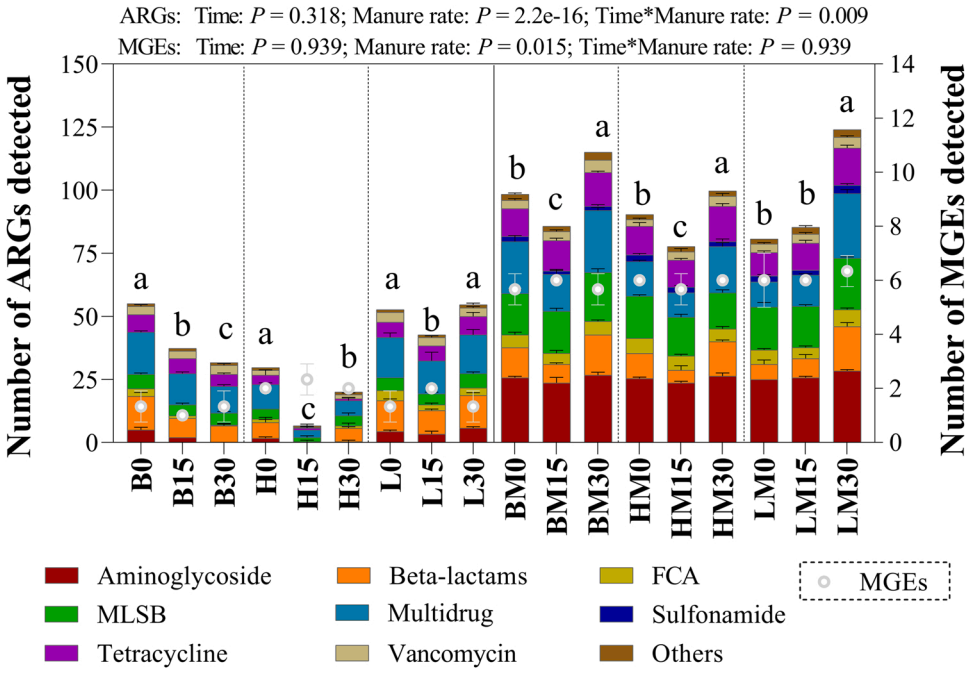


Fig. 1. The average numbers of ARGs and MGEs detected in the soil samples under different incubation time. Abbreviations of B, H and L represent the soils collected from Beian, Hailun and Lishu, respectively; Abbreviation of M indicates the soils added with the poultry manure; 0, 15 and 30 represent the soil samples collected at the incubation times of 0, 15 and 30 days. Error bars represent standard deviation. Diverse lowercase letters above box and bar plots illustrated significant differences among treatments in three black soils.

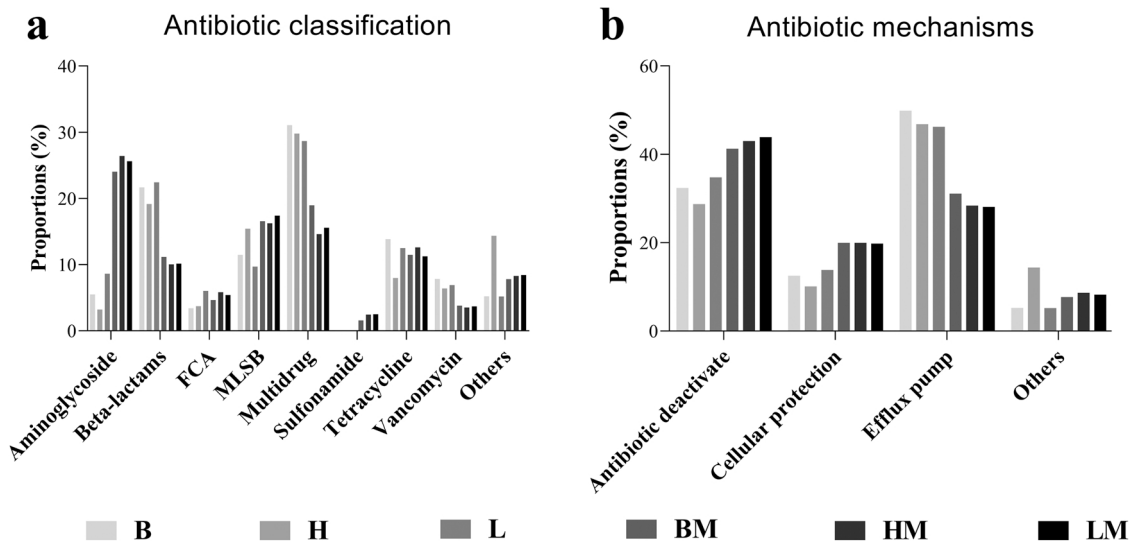


Fig. 2. Proportion of different antibiotic groups (a) and mechanisms (b) of ARGs detected in different soil treatments. FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance; B, H and L represent the soils collected from Beian, Hailun and Lishu, respectively; and M indicates the soils added with the poultry manure.

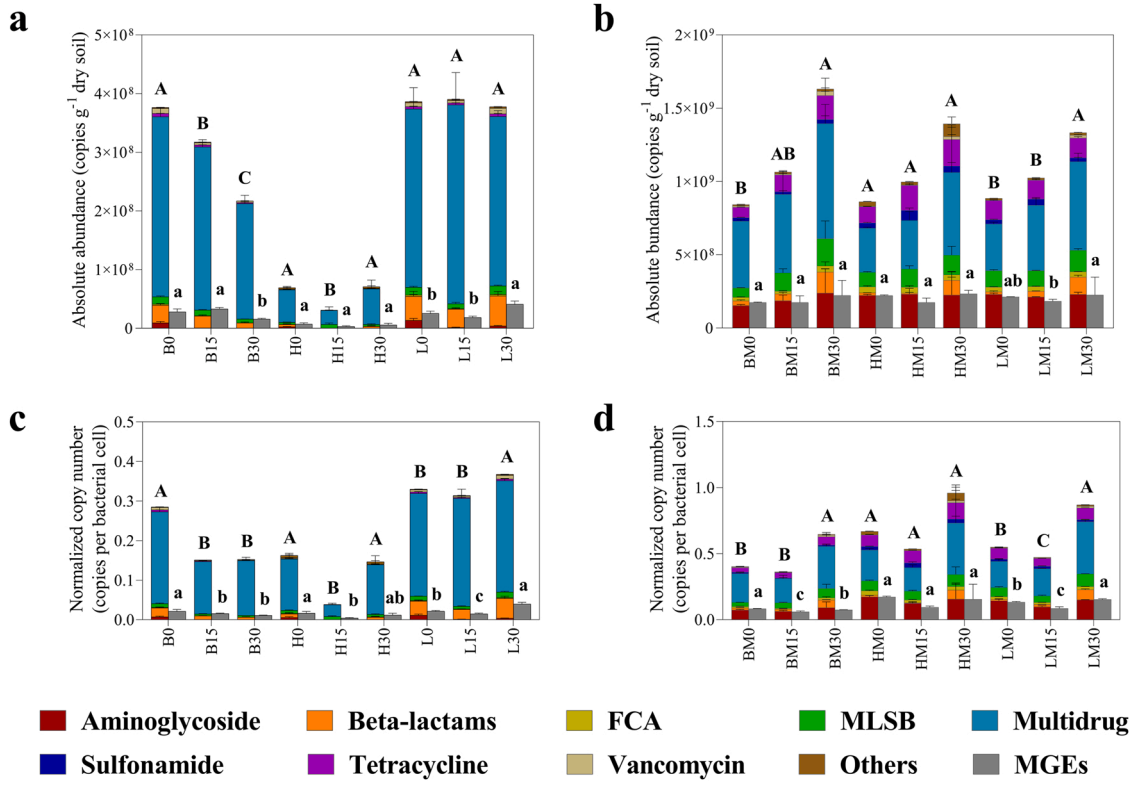


Fig. 3. Temporal changes of the absolute abundances of ARGs and MGEs detected in the untreated (a) and manured soils (b). Temporary changes of the normalized copy number of ARGs and MGEs detected in the untreated (c) and manured soils (d). Diverse capital and lowercase letters illustrated significant differences of abundance of ARGs and MGEs among treatments within individual location, respectively (test: ANOVA; $P < 0.05$); B, H and L represent the soils collected from Beian, Hailun and Lishu, respectively; Abbreviation of M indicates the soils added with the poultry manure; 0, 15 and 30 represent the soil samples collected at the incubation times of 0, 15 and 30 days; Error bars represent standard deviation.

(Fig. 3). For the control soils, the absolute abundances of the ARGs and MGEs decreased over time in B, and the absolute abundance of MGEs in L significantly increased ($P < 0.05$), but no significant changes were observed in the absolute abundance of ARGs and MGEs in H (Fig. 3a). For the manured soils, the absolute abundance of ARGs was significantly increased in BM and LM but not in HM with the incubation time ($P < 0.05$) (Fig. 3b). The relative copy number of ARGs and MGEs had similar changing patterns with absolute abundance (Fig. 3c and d). The NMDS ordination based on the Bray-Curtis dissimilarity matrices revealed that the treatments with poultry manure had a clear divergence from the untreated samples (ANOSIM test, $P < 0.001$) with a more aggregation in the manured treatments among three soils (Fig. 4a). In

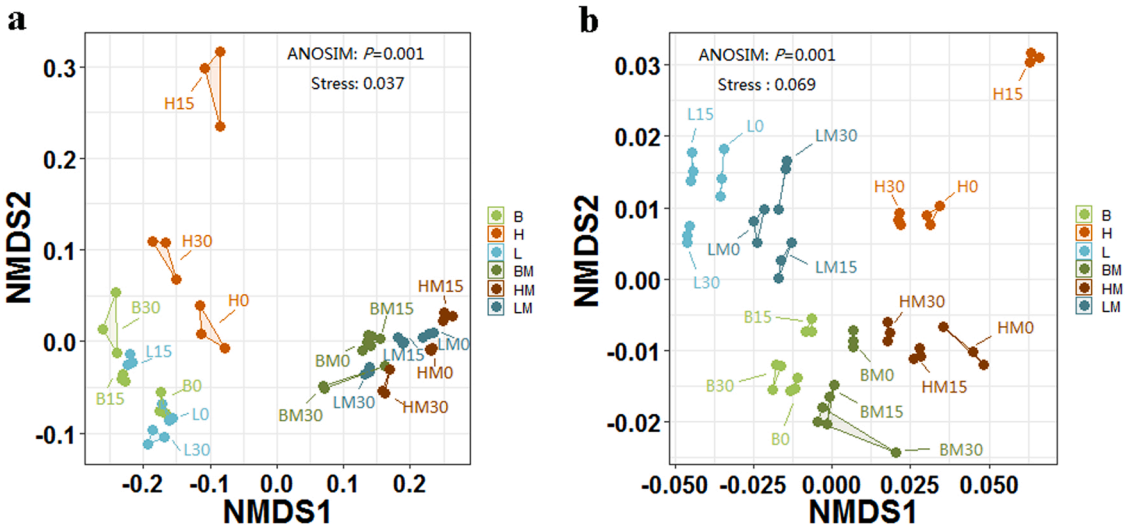


Fig. 4. NMDS plot calculated with relative abundance of ARGs and bacterial community based on Bray-Curtis distance showing the temporary changes of the distribution patterns of ARGs (a) and bacterial community (b) in all soils. Abbreviations of B, H and L represent the soils collected from Beian, Hailun and Lishu, respectively; Abbreviation of M indicates the soils added with the poultry manure; 0, 15 and 30 represent the soil samples collected at the incubation times of 0, 15 and 30 days.

addition, the treatments among three soils with or without adding manure were also separated significantly from each other with an exception between HM and LM (Fig. 4a; Table S3).

3.3. Analysis of shared resistance genes

A total of 36 (including 2 MGEs: *intl-1 (clinic)* and *tnpA-02*) and 118 resistance genes (including 6 MGEs: *intl-1(clinic)*, *intl-1LC*, *tnpA-02*, *tnpA-04*, *tnpA-05* and *tnpA-07*) were shared among control soils and manured soils, respectively (Table S4). Classification showed that these genes were mostly conferred to multidrug and aminoglycoside resistance classes (Table S4). In the control soils, the number of unique genes was 4, 2, and 11 for B, H, and L, respectively. The unique genes increased to 6, 4, and 9 for BM, HM, and LM, respectively, when poultry manure was applied. The shared genes between B and L were 29 (28 ARGs+ *tnpA-01*) which conferred to aminoglycoside, beta-lactams, FCA, multidrug, MLSB, tetracycline, and vancomycin. Three genes (*tetPA*, *acrA-01*, and *tnp-04*) were shared between H and L, but none of the genes shared between B and H (Table S4). In the manured samples, there were 13, 4, and 3 genes shared between LM and BM, between BM and HM, and between LM and HM, respectively (Table S4). By comparing 118 genes shared in manured samples with all genes in the control, 48 unique genes exclusively introduced through adding poultry manure were confirmed by comparing the detected ARGs in soils with or without manure (Table S5). Among 48 unique genes, the absolute abundance of most genes in LM did not vary significantly during the incubation, and the higher absolute abundances of genes were observed in LM than that in BM and HM with a few exceptions (Table S5).

3.4. Changes of soil bacterial community

A total of 1,383,858 high-quality sequences were obtained from all 54 samples, which were clustered into 7293 operational taxonomic units (OTUs) at 97% sequence identity. Soil bacterial communities were dominated by the phylum *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Chloroflexi*, and *Acidobacteria*, accounting for 80% of the total bacterial sequences (Fig. S1). Applying manure increased the alpha-diversity such as OTU richness, Shannon diversity, Chao1 richness, and Phylogenetic diversity of B and H, but had little effect on the α-diversity of L (Fig. S2). The α-diversity was higher in L than that in B, which was followed by H. The NMDS analysis based on the Bray-Curtis showed that the soil bacterial communities of each manured soils clearly separated from the corresponding unmanured control soils throughout the incubation (Fig. 4b). Procrustes analysis ($M^2 = 0.853$, $P < 0.001$) and Mantel test ($r = 0.334$, $P < 0.01$) showed that ARGs profiles were significantly correlated with soil bacterial community (Fig. 5). LEfSe analysis showed the significant differences of bacterial taxa among treatments at multiple taxonomic levels (Fig. S3). *Gemmatimonadota* and *Actinobacteriota* were markedly enriched in the B, *Firmicutes* was confirmed to be richer in the H, and the significantly enriched phyla in L were *Acidobacteriota* and *Chloroflexi* (Fig. S3). When soils were amended with poultry manure, the biomarkers were obviously changed among three black soils (Fig. S3).

3.5. Co-occurrence network of ARGs, MGEs, and bacterial taxa

The co-occurrence pattern of ARGs, MGEs, and bacterial communities were further explored by network analysis, which was identified

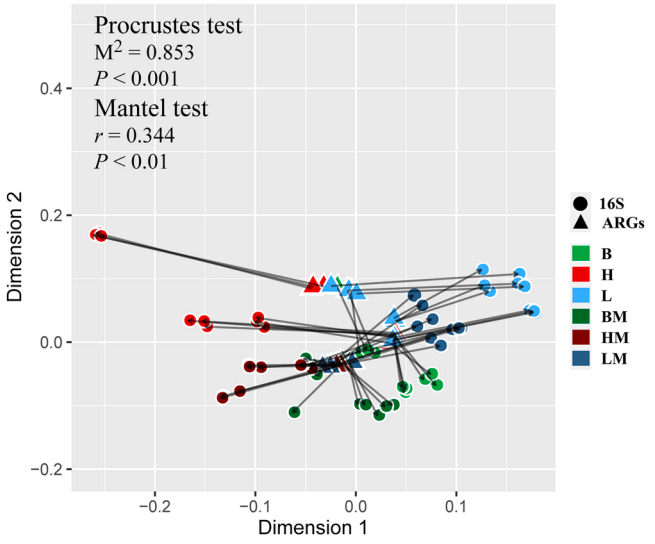


Fig. 5. Procrustes analysis shows correlation of ARG profiles and bacterial community based on Bray-Curtis dissimilarity matrices. B, H and L represent the soils collected from Beian, Hailun and Lishu, respectively; Abbreviation of M indicates the soils added with the poultry manure.

with strong and significant correlations (Spearman's $\rho > 0.8$, $P < 0.01$). Manure application increased the number of nodes and edges of the network, and the highest number of nodes and edges were observed in LM, followed by HM and BM (Fig. S4).

3.6. Correlation among SOM content, bacterial community, and ARG abundances

Pearson correlation analysis showed a significantly positive correlation between the relative abundance of ARGs and relative abundance of MGEs in the control ($r = 0.569$, $P = 0.002$) and manured samples ($r = 0.801$, $P < 0.001$) (Fig. S5a). SOM content had a significantly positive correlation with the absolute abundance of 16 S rRNA gene ($r = 0.450$, $P = 0.018$ for control soils; $r = 0.5826$, $P = 0.001$ for manured soils) (Fig. S5b), and had a significantly negative correlation with relative abundance of total ARGs in all soil samples ($r = -0.492$, $P = 0.009$ for control soils; $r = -0.552$, $P = 0.003$ for manured soils) (Fig. S5c). However, the relative abundance of total ARGs only had a significantly negative correlation with the absolute abundance of 16 S rRNA gene in the manured samples ($r = -0.680$, $P < 0.001$) (Fig. S5d). In contrast, Shannon's diversity and OTU richness were highly and positively correlated with the ARGs abundance in the control soils ($r = 0.840$, $P < 0.001$; $r = 0.856$, $P < 0.001$) (Fig. S5e and f). Further analyses showed that OTU richness was correlated negatively with organic matter ($r = -0.531$, $P = 0.004$ for control soils; $r = -0.565$, $P = 0.002$ for manured soils), and positively with Shannon's diversity in all soil samples ($r = 0.945$, $P < 0.001$ for control soils; $r = 0.957$, $P < 0.001$ for manured soils) (Fig. S5g and h). Besides, the absolute abundance of 23 genes within 48 genes introduced by adding manure, including 22 ARGs (9 aminoglycoside genes, 7 MLSB genes, 2 tetracycline genes and one gene separately for sulfonamide, FCA, multidrug and streptothricin) and an integrase gene, had significantly negative correlations with SOM content ($P < 0.05$) (Table S6). The left 25 introduced genes did not show any significant relationships with SOM content ($P > 0.05$) (Table S6).

3.7. Potential drivers of ARG profiles in soil

To illustrate the potential drivers of ARG patterns in soils, we identified the main predictors for the ARG profiles using random forest (RF) analysis (Fig. 6). MGEs was found to be the most important variable for predicting the soil ARG profiles followed by manure addition. In addition, Soil organic matter was more important than soil bacterial communities in predicting the ARG patterns (Fig. 6).

A SEM was further applied to qualify the direct and indirect effects of manure addition, soil organic matter, bacterial abundance, bacterial diversity, and MGEs on the ARG profiles (Fig. 7). All variables explained 83% of the variance of ARGs. Manure addition could directly affect the abundance of ARGs, and indirectly affect ARG abundance by strongly influencing MGE abundance. Bacterial diversity and MGEs showed a strong and positive effect on the abundance of ARGs. Bacterial abundance had no significant impact on ARGs, but indirectly affected ARG patterns by strongly and negatively affecting MGE abundance. Soil organic matter affected the ARG profiles via its effect on the bacterial diversity and bacterial abundance directly or the abundance of MGEs indirectly. Consistent with the correlation analysis (Fig. S5), soil organic matter and manure addition had a significantly negative and positive effect on bacterial diversity and bacterial abundance, respectively. Furthermore, standardized total effects from SEM indicate manure addition, soil organic matter, bacterial diversity, bacterial abundance, and MGEs had strong indirect and direct effects on ARG profiles.

4. Discussion

4.1. Poultry manure application impacts the profile of antibiotic resistome

Applying animal manure is one of the most common measures to improve soil fertility. However, since animal manure is an important reservoir of ARGs, the potential ecological risk of application of manure into soils cannot be ignored (Udikovic-kolic et al., 2014). In this microcosm experiment, although the three soils were kept under dry condition for more than 7 years, we still detected varied numbers of ARGs among three soils with different incubation times, suggesting that ARGs in soils are very stable and resistant to degradation for a long time (Fig. 1). Antibiotic resistance is ancient, ARGs predate the utilization of antibiotics (D'Costa et al., 2011). This is the reason why the incubation time did not impact the number of ARGs and MGEs. Even in the absence of selective pressure caused by antibiotic, manure application could impact the diversity and potential mobility of ARGs (Hu et al., 2016). Consistent with previous studies (Han et al., 2018), the number of ARGs and MGEs in three manured soils were significantly higher than those in unmanured soils (Fig. 1), indicating that manure application had the effects on the spread of ARGs. In addition, we found that the temporal succession of ARGs in L had a more relatively stable trend than in B and H through the whole incubation period, and there was a quickly growing trend in LM after 15 days of incubation (Fig. 1). We speculated the reason for this trend difference may be related to soil properties and soil microorganisms.

Manure application increased the number of ARGs conferring to aminoglycoside and MLSB, especially for sulfonamide (Fig. 2a), and changed the resistance mechanisms that accounted for the largest proportion from the efflux pump to antibiotic deactivate (Fig. 2b), suggesting that animal manure is the reservoir of ARGs even from antibiotic-free animals (Hu et al., 2016). Diverse ARGs were detected

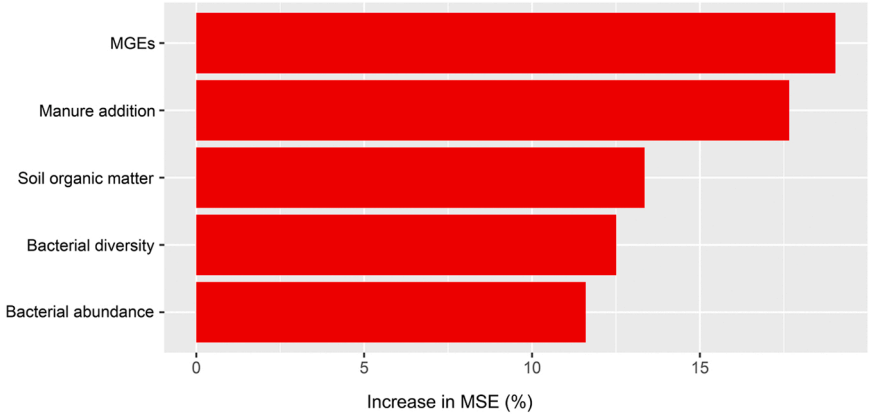


Fig. 6. Potential drivers of ARG patterns in black soils. Random forest result shows the mean predictor importance of variations on ARG patters with the percentage increases in the MSE (mean squared error). Red bars indicate the significant effects, $P < 0.05$.

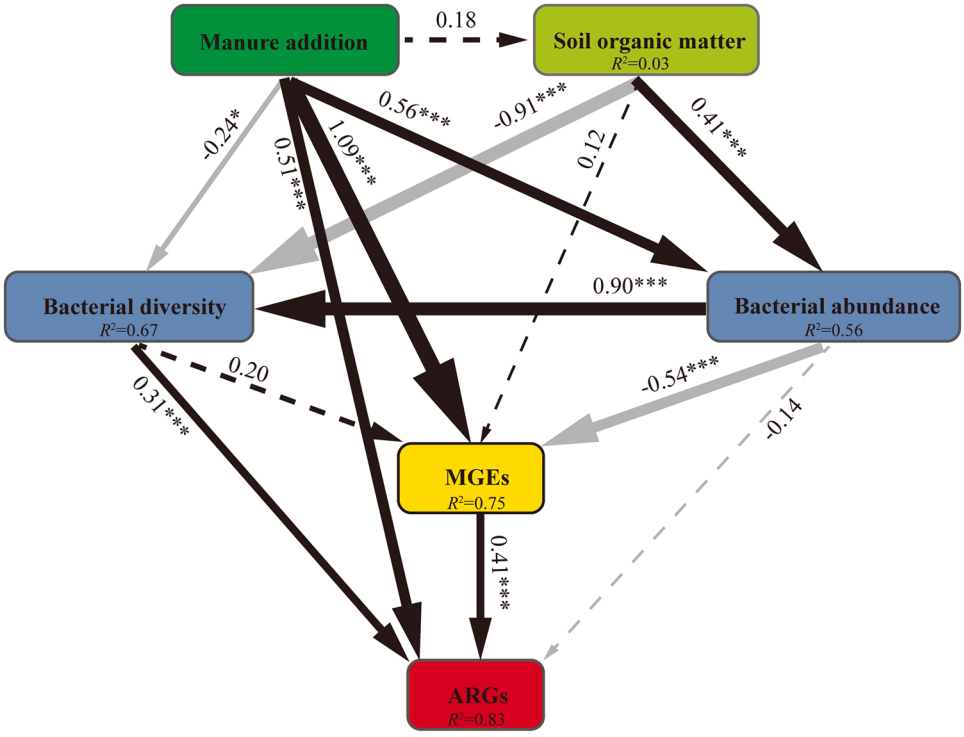


Fig. 7. Structural equation modeling (SEM) for clarifying the direct and indirect effects of manure addition, soil organic matter, bacterial diversity, bacterial abundance and MGEs on the profiles of ARGs. According to the goodness-of-fit statistics, the hypothetical model fits our data well: $\chi^2 = 0.06$, $P = 0.80$, df $= 1$, GFI $= 1.00$, AIC $= 40.06$, RMSEA $= 0.00$. The Black and grey arrows show positive and negative relationships, respectively. Solid and dashed arrows represent significant and non-significant relationships, respectively. Numbers adjacent to the arrows are standardized path coefficients, and the width of arrows is proportional to the strength of the path coefficients. R^2 indicate the proportion of variance explained by the variable. Significant path coefficients are displayed by $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Standardized total effects of the factors on the diversity of ARGs were calculated by the sum of direct plus indirect effect on the basis of the SEM.

in the animal manure which resulted in the changes of the primal types of ARGs (Chen et al., 2017). The source of animal manure could impact soil resistome, poultry manure harbours the highest diversity and abundance of ARGs (Han et al., 2018). The difference in the manure source could be attributed to the veterinary antibiotics used and gut resistome of animals. Additionally, we found that manure application increased the number of ARGs shared among three black soils, and significantly changed ARGs compositions (Fig. 4; Tables S3 and S4). These results further highlighted that the potential threat of resistance genes mainly came from animal manure application to farmland soils.

4.2. Low level of SOM boosting the spread of ARGs

Soil physicochemical properties have been recently documented as the nonnegligible factor in driving the dissemination of ARGs (Hu et al., 2018; Wan et al., 2019). SOM is one of the important soil properties in the respect of soil biological processes, soil quality, and sustainable agricultural production (Schmidt et al., 2011; Raphael et al., 2016). Although some SOM compounds like low-molecular-weight dissolved organic carbon decomposes readily, other SOM with recalcitrant structure could persist for a long time (Krull et al., 2006; Jones and Murphy, 2007). Since the SOM is the core elements of soil environments, in this study, we focused on the revealing the influence of SOM content on the dissemination of ARGs.

In this study, we found that SOM content was negatively correlated with the relative abundance of ARGs (Fig. S5c), and the 23 of introduced ARGs by manure application also had a significantly negative correlation with SOM content (Table S6). This finding supported the previous discovery that the richness of ARGs was negatively correlated with the total organic carbon content (Wan et al., 2019; Zhang et al., 2019a, 2019b). Additionally, the composition of ARGs was significantly influenced by SOM no matter whether the manure amended or not (Fig. 4; Table S3), these findings further emphasized that SOM content acted as a critical environmental factor in driving the abundance and composition of ARGs.

Microbial interaction is the foundation of microbial ecosystems, which strongly impact the composition, biodiversity, and stability of soil microbial community (Faust and Raes, 2012). Under a high nutritional condition, an extensive microbial growth leads to strongly negative interactions between species, which can decrease the stability of the microbial community and result in an acute loss of biodiversity (Conley et al., 2009). According to the stress gradient hypothesis, positive interactions (facilitation) should be more common in stressful environments, compared with benign environments where negative interactions (competition) should be more common in a microbial community (Hammarlund and Harcombe, 2019). Accordingly, in the low level of SOM content soils, such as L soil in this study, we speculated that a bacterium slowed down its growth and did not thoroughly inhibit other bacterial growth, which mainly manifested as lower bacterial abundance and higher bacterial diversity (Figs. S2 and S5b). Under the circumstance, soil bacterial communities tended to be stable and symbiotic, the exchange of material and energy between bacterial species became more frequent so that the ARGs were spread widely.

4.3. SOM content impacts the variation of MGEs

In this study, we found that the relative abundance of MGEs had a significantly positive correlation with that of ARGs in soils (Fig. S5A), which was consistent with the findings of the application of biochar in manured soils (Chen et al., 2018). MGEs such as integron, transposons, plasmids, gene cassettes, and bacteriophages play an important role in the transfer of resistance genes among the microorganisms by horizontal gene transfer-conjugation, transformation, and transduction (Partridge et al., 2009; Aminov, 2011). Integrons, including three core features: an integron-integrase gene (*intI*), a recombination site (*attI*), and a promoter (Pc), are a common genetic element of bacterial genomes, providing a mechanism for capturing and expressing exogenous genes (Gillings, 2014). According to the phylogeny of integrase genes, integrons are classified into different groups (mostly class 1, 2, and 3). Thereinto, class 1 integrons are most prevalent among resistant bacteria, and are considered as the pivotal agents in the dissemination of ARGs, which are associated with Tn402- like transposons carrying *qacEΔ1* cassette and *sul1* genes in the 3' conserved segment, encoding resistance to disinfectants and sulfonamides, respectively (Gillings, 2014).

Moreover, class 1 integron-integrase gene (*intI1*), a potential marker for anthropogenic pollution, is often detected in various environments (Gillings et al., 2015; Han et al., 2018). Previous studies showed that gene cassettes *aadA* and *drfA* commonly captured by class 1 integrons were detected in the multidrug-resistant bacteria, and diverse gene cassettes, such as *aac*, *sat*, *str* were also observed in the gene cassette arrays conferring resistance to antibiotics (Binh et al., 2009; Partridge et al., 2009; Xu et al., 2009). In this study, we found that absolute abundances of class 1 integron gene *intIl-LC1* and 8 gene cassettes (*aadA1*, *aadA-01*, *aadA2–02*, *aadA2–03*, *aadA5–01*, *str*, *dfrA1*, and *qacEdeltal-01*) were negatively correlated with SOM content (Table S6), suggesting that SOM content plays an important role in the dissemination of ARGs by affecting the absolute abundance of MGEs in soils.

4.4. SOM content as an important driver in the spread of ARGs

Evidence showed that microorganisms derived from manure could boost the level of ARGs owing to the supplement of antibiotic-resistant bacteria in soil, and in contrast, the indigenous soil microorganisms could inhibit the prevalence of ARGs from manure to soil environment (Chen et al., 2017; Han et al., 2018). Procrustes analysis and mantel test revealed that ARGs profiles had a significant relationship with soil bacterial community compositions (Fig. 5). This result is in line with the previous studies that the phylogenetic and taxonomic composition of bacterial community is the primary determinant of soil ARG content (Forsberg et al., 2014). Thus, soil bacterial community plays a key role in the regulation of antibiotic resistome (Hu et al., 2018).

It is well known that SOM affects soil microbial abundance, diversity and community composition (Hu et al., 2014). In this study, we found that soil bacterial diversity and composition showed significant temporal changes in black soil with different SOM content (Figs. S1 and S2). Furthermore, SOM content was negatively correlated with the relative abundance of ARGs and OTU richness, and positively correlated with the absolute abundance of bacteria across all soils (Fig. S5b, c and h). In addition, the relationships between ARGs and bacterial genera became more complex in the manured soils from high-level SOM to low-level SOM contents (Fig. S4), suggesting that applying animal manure to the low SOM content soils could be easier to accelerate the spread of ARGs than the high SOM content soils. These results were further confirmed by random forest analysis and structural equation modeling (Figs. 6 and 7), which indicated that SOM content indirectly affected ARGs through its strongly positive and negative impacts on bacterial abundance and bacterial diversity, respectively. Overall, less of SOM content could lead to the low quantity and high diversity of soil bacteria, which impairs the inhibition of inherent bacteria to manured bacteria, more conducive to the dissemination of ARGs from manure application.

5. Conclusion

In this microcosm experiment, we explored the changes of antibiotic resistome in response to manure application in the three black soils with significantly different contents of SOM. Our results indicated that SOM content plays an important role in driving the distribution of ARGs in black soils. The soil with low level of SOM can foster the spread of ARGs contained in the animal manure through its effects on the diversity and abundance of soil bacterial community increasing the level of MGEs, which highlighted that the strategy of increasing SOM content via alternative ways, such as the amendment of crop residues into soils or the composting manure with low ARGs content would be very important for suppressing the dissemination of AGRs in black soils.

CRediT authorship contribution statement

Sen Li: Writing – original draft, Conceptualization, Methodology, Software, Data visualization. **Qin Yao:** Supervision. **Junjie Liu:** Supervision. **Yu Zhenhua:** Supervision. **Yansheng Li:** Supervision. **Jian Jin:** Supervision. **Xiaobing Liu:** Supervision. **Guanghua Wang:** Writing – review & editing.

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.

Acknowledgements

This research was financially supported by the Natural Science Foundation of Heilongjiang Province (ZD2018009) and the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA23060503).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112946.

References

Aminov, R.I., 2011. Horizontal gene exchange in environmental microbiota. Front. Microbiol. 2, 158.
Angenent, L.T., Kelley, S.T., Amand, A.S., Pace, N.R., Hernandez, M.T., 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc. Natl. Acad. Sci. U.S.A. 02, 4860–4865.
Archer, E. , 2021. rfPermute: Estimate permutation p-values for random forest importance metrics (R package version 2.2).
Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Nisar, M. A., Alvi, R.F., Aslam, M.A., Qamar, M.U., Salamat, M.K.F., Baloch, Z., 2018. Antibiotc resistance: a rundown of global crisis. Infect. Drug. Resist. 11, 1645–1658.
Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: an open source software for exploring and manipulating networks. ICWSM 8, 361–362.
Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2009. Diverse *aadA* gene cassettes on class 1 integrons introduced into soil via spread manure. Res. Microbiol. 160, 427–433.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of highthroughput community sequencing data. Nat. Methods 7, 335–336.
Chen, Q.L., An, X.L., Li, H., Zhu, Y.G., Su, J.Q., Cui, L., 2017. Do manure-borne or indigenous soil microorganisms influence the spread of antibiotic resistance genes in manured soil? Soil Biol. Biochem. 114, 229–237.
Chen, Q.L., Fan, X.T., Zhu, D., An, X.L., Su, J.Q., Cui, L., 2018. Effect of biochar amendment on the alleviation of antibiotic resistance in soil and phyllosphere of *Brassica chinensis* L. Soil Biol. Biochem. 119, 74–82.
Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., Lancelot, C., Likens, G.E., 2009. Controlling eutrophication: nitrogen and phosphorus. Science 323 (5917), 1014–1015.
D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461.
Dhaliwal, S.S., Nareshl, R.K., Mandal, A., Singh, R., Dhaliwal, M.K., 2019. Dynamics and transformations of micronutrients in agricultural soils as influenced by organic matter build-up: a review. Environ. Sust. Indic. 1–2, 100007.
Dickinson, A.W., Power, A., Hansen, M.G., Brandt, K.K., Piliposian, G., Appleby, P., O'Neill, P.A., Jones, R.T., Sierocinski, P., Koskella, B., Vos, M., 2019. Heavy metal pollution and co-selection for antibiotic resistance: a microbial palaeontology approach. Environ. Int. 132, 105–117.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nat. Rev. Micro 10, 538–550.
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, 612–616.
Fox, J., Wersberg, S., 2019. An R Companion to Applied Regression, third ed. Sage, California.
Gillings, M.R., 2014. Integrons: past, present, and future. Microbiol. Mol. Biol. Rev. 78, 257–277.
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, 1269–1279.
Grace, J.B., 2006. Structural Equation Modeling and Natural Systems. Cambridge University Press.

Hammarlund, S.P., Harcombe, W.R., 2019. Refining the stress gradient hypothesis in a microbial community. Proc. Natl. Acad. Sci. U.S.A. 116, 201910420–201915762.
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.
Hu, H.W., Han, X.M., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Temporal changes of antibiotic-resistance genes and bacterial communities in two contrasting soils treated with cattle manure. FEMS Microbiol. Ecol. 92, fiv169.
Hu, H.W., Wang, J.T., Singh, B.K., Liu, Y.R., Chen, Y.L., Zhang, Y.J., He, J.Z., 2018. Diversity of herbaceous plants and bacterial communities regulates soil resistome across forest biomes. Environ. Microbiol. 20, 3186–3200.
Hu, Y.J., Xiang, D., Veresoglou, S.D., Chen, F., Chen, Y.L., Hao, Z.P., Zhang, X., Chen, B. D., 2014. Soil organic carbon and soil structure are driving microbial abundance and community composition across the arid and semi-arid grasslands in northern China. Soil Biol. Biochem. 77, 51–57.
Jones, D.L., Murphy, D.V., 2007. Microbial response time to sugar and amino acid additions to soil. Soil Biol. Biochem. 39, 2178–2182.
Kassambara, A. , 2019. 'ggplot2' Based Publication Ready Plots. R Package version 0.2.4.
Klappenbach, J.A., Saxman, P.R., Cole, J.R., Schmidt, T.M., 2001. rrndb: the rhibosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184.
Krull, E.S., Swanston, C.W., Skjemstad, J.O., McGowan, J.A., 2006. Importance of charcoal in determining the age and chemistry of organic carbon in surface soils. J. Geophys. Res. 111, G04001.
Liu, B.Y., Zhang, G.L., Xie, Y., Shen, B., Gu, Z.J., Ding, Y.Y., 2021. Delineating the black soil region and typical black soil region of northeastern China (in Chinese with English abstract). Chin. Sci. Bull. 66, 96–106.
Millner, P.D., Ringer, C.E., Maas, J.L., 2004. Suppression of strawberry root disease with animal manure composts. Compost. Sci. Util. 12, 298–307.
Oksanen, J. , Blanchet, F.G. , Friendly, M. , Kindt, R. , Legendre, P. , McGlinn, D. , Minchin, P.R. , O'Hara, R.B. , Simpson, G.L. , Solymos, P. , Stevens, M.H.H. , Szoecs, E. , Wagner, H. , 2019. Vegan: Community Ecology Package. R package version 2.2–5.
Partridge, S.R., Tsafnat, G., Coiera, E., Iredell, J.R., 2009. Gene cassettes and cassette arrays in mobile resistance integrons. FEMS Microbiol. Rev. 33, 757–784.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596.
Raphael, J.P.A., Calonego, J.C., Milori, D.M.B.P., Rosolem, C.A., 2016. Soil organic matter in crop rotations under no-till. Soil Till. Res. 155, 45–53.
Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., Kleber, M., Kogel-Knabner, I., Lehmann, J., Manning, D.A., Nannipieri, P., Rasse, D. P., Weiner, S., Trumbore, S.E., 2011. Persistence of soil organic matter as an ecosystem property. Nature 478, 49–56.
Segata, N., Izard, J., Waldon, L., Gevers, D., Miropolsky, L., Garrett, W.S., Hutternhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.
Tripathi, B.M., Stegen, J.C., Kim, M., Dong, K., Adams, J.M., Lee, Y.K., 2018. Soil pH mediates the balance between stochastic and deterministic assembly of bacteria. ISME J. 12, 1072–1083.
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. USA 111, 15202–15207.
Wan, K., Zhang, M.L., Ye, C.S., Lin, W.F., Guo, L.Z., Chen, S., Yu, X., 2019. Organic carbon: an overlooked factor that determines the antibiotic resistome in drinking water sand filter biofilm. Environ. Int. 125, 117–124.
Wang, S.C., Zhao, Y.W., Wang, J.Z., Gao, J.J., Zhu, P., Cui, X.A., Xu, M.G., Zhou, B.K., Lu, C.A., 2020. Estimation of soil organic carbon losses and counter approaches from organic materials in black soils of northeastern China. J. Soil. Sediment. 20, 1241–1252.
Wickham, H. , 2016. Create elegant data visualization using the grammar of graphics. R package version 3.2.1.
Xu, H.X., Su, Z.L., Wang, S.J., Dai, X.L., Chen, J.G., Kong, F.Z., Li, Y.Z., Peng, S.F., Shao, Q.X., Lu, L.W., Ezaki, T., 2009. Four novel resistance integron gene-cassette occurrences in bacterial isolates from zhenjiang, China. Curr. Microbiol. 59, 113–117.
Ye, G.P., Lin, Y.X., Kuzyakov, Y., Liu, D.Y., Luo, J.F., Lindsey, S., Wang, W.J., Fan, J.B., Ding, W.X., 2019. Manure over crop residues increases soil organic matter but decreases microbial necromass relative contribution in upland Ultisols: Results of a 27-year field experiment. Soil Biol. Biochem. 134, 15–24.
Zhang, Y.J., Hu, H.W., Chen, Q.L., Singh, B.K., Yan, H., Chen, D.L., He, J.Z., 2019a. Transfer of antibiotic resistance from manure amended soils to vegetable microbiomes. Environ. Int. 130, 104912.
Zhang, Y.J., Hu, H.W., Yan, H., Wang, J.T., Lam, S.K., Chen, Q.L., Chen, D.L., He, J.Z., 2019b. Salinity as a predominant factor modulating the distribution patterns of antibiotic resistance genes in ocean and river beach soils. Sci. Total. Environ. 668, 193–203.
Zhu, Y.G., Zhao, Y., Li, B., Huang, C.L., Zhang, S.Y., Yu, S., Chen, Y.S., Zhang, T., Gillings, M.R., Su, J.Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 2, 16270.