Does reduced usage of antibiotics in livestock production mitigate the spread of antibiotic resistance in soil, earthworm guts, and the phyllosphere?

Zhou, Shu-Yi-Dan; Zhu, Dong; Giles, Madeline; Daniell, Tim; Neilson, Roy; Yang, Xiao-Ru

Published in:
Environment International

DOI:
10.1016/j.envint.2019.105359

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Environment International 136 (2020) 105359

Contents lists available at ScienceDirect

Environment International

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

Does reduced usage of antibiotics in livestock production mitigate the spread of antibiotic resistance in soil, earthworm guts, and the phyllosphere?

Shu-yi-dan Zhou[a,b,1], Dong Zhu[a,b,1], Madeline Giles[c], Tim Daniell[d], Roy Neilson[c], Xiao-ru Yang[a,*]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China
[b] University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China
[c] Ecological Sciences, The James Hutton Institute, Dundee, DD2 5DA Scotland, UK
[d] Department of Animal and Plant Sciences, The University of Sheffield, Sheffield S10 2TN, UK

ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Antibiotic resistance genes
HT-qPCR
Gut resistome

ABSTRACT

The overuse of antibiotics in animal husbandry is widespread and believed to significantly contribute to the selection of antibiotic resistance genes (ARGs) in animals. Thus, there is a global drive to reduce antibiotic use in the agricultural sector. However, it has not been established whether a reduction in the use of antibiotics in livestock production would be effective in reducing the spread of ARGs. A microcosm approach was used to determine how the addition of manure with either reduced antibiotic levels or with typical antibiotic levels could affect the spread of antibiotic resistance genes between soil, earthworms and the phyllosphere. When compared to the control soil, earthworm and phyllosphere samples had the greater increase in ARG abundance in conventional manure treatments ($P < 0.05$). Reduced antibiotic manure also enriched the abundance of ARGs in the phyllosphere and soil but not earthworm guts when compared to the control ($P < 0.05$). In both soil and earthworm guts, the enrichment of ARGs was lower in reduced antibiotic manure than in conventional manure. This study has identified bacterial transfer through the soil-earthworm-phyllosphere system as a potential means to spread ARGs between habitats after fertilization with livestock derived manures.

1. Introduction

The overuse of antibiotics in livestock production is a global issue (Martinez, 2008; Zhu et al., 2013). In agriculture, antibiotics are typically used in animal husbandry to prevent bacterial infection and promote growth of livestock (Cheng et al., 2013; Nesme and Simonet, 2015). The incomplete metabolism of antibiotics in animal gut contributes to antibiotics being distributed into the wider environment through manures (Hao et al., 2008). As current practice drives the selection of ARGs in animals, it is recognized that antibiotic use needs to be reduced in livestock husbandry (Zhu et al., 2013). However, it is not known whether manures from farms where antibiotic application is reduced will lead to a concomitant reduction in the environmental spread of ARGs. A recent study has shown that manure from both farmed livestock and wild animals had diverse ARGs (Swift et al., 2019), suggesting that ARGs also exist in wild animals with no direct anthropogenic antibiotic input.

The distribution and abundance of ARGs in soils are believed to increase (Wang et al., 2017; Zhao et al., 2018b) with the use of animal manures as agricultural fertilizers (Rahman et al., 2018; Zhao et al., 2018a). Furthermore, this anthropogenic driven introduction of ARGs is not limited to soils as increased ARG abundance has been found on crops (Chen et al., 2018; O'Flaherty et al., 2018) used for human consumption (Marshall and Levy, 2011). While there is considerable published evidence on ARGs in the environment (Zhu et al., 2017), a holistic view of the complete soil, animal and plant system, is still required.

Earthworms, a key biological component of soils (Bartlett et al., 2010) have been shown to distribute ARGs through (Kotzerke et al., 2010). Earthworms also promote the growth of plants through the cycling of nutrient and organic compounds and alteration of the associated bacterial communities (Pelosi et al., 2014; Thakuria et al., 2010).

Conditions found in the guts of earthworms are harsh with the complex chemical and physical conditions exerting a selective pressure for specific microorganisms, which in turn has been shown to impact

* Corresponding author at: Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China.
E-mail address: xryang@iue.ac.cn (X.-r. Yang).
[1] Shu-yi-dan Zhou and Dong Zhu contributed equally to this paper.

https://doi.org/10.1016/j.envint.2019.105359
Received 9 September 2019; Received in revised form 11 November 2019; Accepted 22 November 2019
Available online 06 February 2020

the soil microbial community (Hu et al., 2018; Zhang and Schrader, 1993; Zhou et al., 2019a). Notwithstanding this, the composition of bacterial communities in soil and earthworm guts have been found to be similar (Drake and Horn, 2007). The role of earthworms in spreading antibiotic resistance should not be neglected as earthworms can acquire antibiotics directly from soils (Ding et al., 2019). For example, a recent study found that the application of sewage sludge and chicken manure to fields led to an increase in the abundance of ARGs in earthworms (Ding et al., 2019). However, it is not known how the use of livestock manure for fertilization affects the earthworm gut microbiome and subsequently antibiotic resistance in earthworms.

Leaves constitute the majority of aboveground plant biomass and are known to be inhabited by numerous microorganisms (Vorholt, 2012) as the surface area of leaves can provide a favorable habitat for microorganisms (Lindow and Brandl, 2003). Multiple factors affect plant bacterial communities including plant genotype, leaf-age, radiation and nutrient supply (Ikeda et al., 2011; Kadivar and Stapleton, 2003). Rain washing and air movement over deciduous plants provide the potential for microorganisms adhering to plant leaves to become mobile (Allen et al., 2010). Several studies have found the existence of ARGs in the phyllosphere of vegetables (Marti et al., 2013; Wu et al., 2018) and maize associated with the application of manure and other organic fertilizers (Chen et al., 2018; Zhang et al., 2019). Once ARGs are present, bacterial communities in the phyllosphere have been found to spread ARGs by mutation and horizontal gene transfer, leading the phyllosphere to be a reservoir of ARGs (Chen et al., 2019). Thus, it is critical to understand how ARGs spread between soil and the phyllosphere to gain better insight into the distribution of ARGs from cropping systems to the wider environment.

We hypothesize that there will be overlap in both bacterial community composition and ARG composition between soil, earthworm and phyllosphere samples and that the use of reduced antibiotic manure will lead to a reduced enrichment of the antibiotic resistome, when compared to conventional manures. Thus, in this study, our aims were to (1) compare the abundance and diversity of ARGs in microcosms fertilized with contrasting livestock derived manures (reduced and conventional antibiotic levels); (2) determine the composition of bacterial communities in soil, earthworm gut and phyllosphere; (3) explore the relationship between bacterial communities, soil properties, ARGs and mobile genetic elements (MGEs) within the soil-earthworm-phyllosphere system.

2. Material and methods

2.1. Soil and manure

Soil was collected from the top 20 cm of an agricultural field in Xiamen city, Fujian, China (24°64′N, 118°05′E). The soil type was a sandy loam and prior to sampling the field had been planted with lettuce. The field had not been fertilized using manure or any other organic fertilizer in the previous three years. Plant stubble, roots and soil macrofauna were removed, and the soil was sieved to 2 mm. Manure was collected from two different farms located locally, one representing manure from reduced antibiotic practice and another representing manure from conventional practice with typical levels of antibiotic burden. The concentration of C, N and key antibiotics in the different manures were listed in Table S2. At the completion of the experiment the physicochemical properties (Total N, total C, C/N ratio, NH_4^+-N Content, NO_3^--N Content, Clay, pH and electric conductivity) of the soil treated with both manures, and the control soil, were determined (Table S3). Methods used to determine the physicochemical properties were described as previously (Ding et al., 2019; Zheng et al., 2019).

2.2. Experimental design

Microcosms (15 cm diam × 23 cm height) were constructed from polyvinyl chloride pipes and filled with 3.5 kg dry weight soil. To balance the N content between manure treatments, soil was mixed with either 0.5% reduced antibiotic or 0.57% conventional manure before being packed into the microcosms. These manure additions led to a N content of 12.6 g N/m^2 which represented standard local agricultural practices. Soil moisture was adjusted to 60–70% of soil water holding capacity (WHC) and pre-incubated for 2 weeks (25 °C) before being planted with lettuce (Degl'Innoocenti et al., 2008; Fang et al., 2015). Each microcosm was planted with 3 *Lactuca sativa* seedlings which were grown in moist perlite for one week prior to transfer to the microcosms. Fifteen adult earthworms (*Eisenia foetida*) all of a similar size (5 cm, 0.3–0.5 g) were collected from a field of a *peri*-urban farm located at Ningbo (29°46′N, 121°20′E), China and added to the microcosms (Ding et al., 2019). Treatments consisted of a control (no fertilizer added to the microcosms), microcosms fertilized with conventional manure and microcosms fertilized with reduced antibiotic manure. Four replicate microcosms per treatment were established and once planted were left for 65 days. At the end of microcosm experiment, samples were destructively collected, lettuce plants were removed, and for each microcosm the soil was mixed and sub-sampled and 3 earthworms randomly were collected from the remaining soil prior to DNA extraction.

2.3. DNA extraction of soil, earthworm guts and phyllosphere

DNA extraction from *Lactuca sativa* phyllosphere samples followed Zhu et al. (2016) with pre-treatment of lettuce leaves according to Zhou et al. (2019b). Briefly, saline solution was filtered through a nylon gauze to remove large particles and then through a cellulose membrane (0.22 μm) to capture the bacterial community washed from the leaves. The cellulose membrane was cut into pieces and used for DNA extraction. Individual earthworms were washed with sterile deionized water five times before dissection. Sterile scissors were used to excise the gut and intestinal contents collected for DNA extraction. DNA was extracted from prepared soil, phyllosphere and earthworm gut samples using a FastDNA Spin Kit for Soil (MP Bio, USA) following the protocol provided by the manufacturer. A quality check and DNA quantification was conducted using a NanoDrop ND 1000 (Thermo Scientific, Waltham, MA).

2.4. Quantification of ARGs and MGEs

ARGs and MGEs were quantified using a High-throughput quantitative PCR method as described by Zhou et al. (2019a,b). The reaction system and primer sets used were as previously reported (Chen et al., 2018; Zhu et al., 2013). For each sample there were three technical replicates for every primer set. The threshold cycle was set as 31 to estimate the success of the amplification (Su et al., 2015). Amplification was only considered successful if all three technical replicates were positive. Relative and normalized gene copy number were calculated following the equation reported previously by Zhu et al. (2013).

2.5. Illumina sequencing and data analysis

Primer set 515F/907R was used to target the V4-V5 region of the 16S rRNA gene to determine the structure of the bacterial communities in soil, earthworm gut and phyllosphere samples (Turner et al., 1999). Standard PCR conditions followed Chen et al. (2018). Sample preparation for Illumina sequencing followed that previously reported (Zhu et al., 2018) and each sample had its own unique barcode to distinguish the sample (Rastogi et al., 2012). A Qubit 3.0 fluorimeter was used to quantify the concentration of the PCR products prior to sample normalization and pooling. An Illumina Hiseq2500 platform (Novogene, Tianjing, China) was used to sequence the prepared amplicon libraries.

Quantitative Insights Into Microbial Ecology QIIME (version 1.9.1) (Caporaso et al., 2010b) was used to analyse sequences, with only a

single OTU sequence discarded. Operational taxonomic unit (OTUs) similarity was set at 97% and OTU's determined using UCLUST (Edgar, 2010). One sequence of each OTU was used to align sequences with the alignment carried out using PyNAST (Caporaso et al., 2010a). The taxonomic identity and relative abundance of OTUs were assigned using the Ribosomal Database Project, which holds the Greengenes data base (Version 13.8) (Langille et al., 2013; McDonald et al., 2012).

2.6. Statistical analysis

Calculations on the raw data for means and standard errors were conducted in Excel 2016. SPSS (version 21) was used to perform Analysis of Variance (ANOVA) and Pearson Correlation Coefficient analysis with tests considered significant at $P < 0.05$. Inverse Simpson and Shannon index (for ARGs), Canonical Correlation Analysis (CCA), mantel test, Ordinary Least-Squares (OLS), Variation Partitioning Analysis (VPA) (Borcard et al., 1992), Procrustes test (Dixon, 2003) and Principal Coordinate Analysis (PCoA) were performed in R with the package "vegan" (Oksanen, 2018). Figures were created using "ggplot2" (Wickham et al., 2018) version 3.1. Bar charts were created using OriginLab 2018 and Venn charts created using online software "Venny 2.1.0" (http://bioinfogp.cnb.csic.es/tools/venny/). QIIME was used to calculate the phylogenetic diversity (PD whole tree analysis) of bacterial OTUs in soil, earthworm gut and phyllosphere samples.

3. Results

3.1. Composition and diversity of ARGs in soil, earthworm and phyllosphere

Across all soil, earthworm gut and phyllosphere samples, 152 ARGs and 10 MGEs were detected from the 194 ARGs and 11 MGEs targets tested using HT-qPCR (Table S1). The number of the major classes of ARGs (Aminoglycoside, Beta Lactamase, Chloramphenicol, Macrolides, Lincosamides, and Streptogramin B (MLSB), Multidrug, Tetracycline, Sulfonamide, Vancomycin and Other unknown) ranged from 22 to 69, while the number of MGEs (Transposase and integron) ranged from 2 to 5.

The total normalized abundance of ARGs in soil (0.21 copy/cell) was significantly higher than the phyllosphere (0.07 copy/cell) or earthworm guts (0.12 copy/cell) ($P < 0.001$, ANOVA). ARG diversity (Inverse Simpson index) of soil (13.3) and earthworm gut (15.9) was significantly higher than the phyllsophere (5.6) ($P < 0.05$, ANOVA) (Fig. S1). The structure of ARGs in soil and earthworm gut samples were similar, but significantly different ($P < 0.05$, PERMANOVA) to those in phyllosphere samples (Fig. 1). Phyllosphere ARGs were well separated into 3 groups, representing control, and the two contrasting manure treatments (Fig. 1).

3.2. Effects of manure on ARGs

In soil, the application of manures significantly increased the abundance of ARGs when compared to the control ($P < 0.05$, ANOVA) with conventional manure leading to a greater ARG enrichment (35% increase) than reduced antibiotic manure (Fig. 2). In contrast to soils, earthworm guts exposed to the conventional manure treatment had a higher ARG enrichment than the reduced antibiotic manure treatment ($P < 0.05$, ANOVA) but not the control. In the phyllosphere, normalized abundance of ARGs was significantly higher in both manure treatments than the control ($P < 0.05$, ANOVA) (Fig. 2).

In soil samples treated with conventional antibiotic manure the abundance of Aminoglycoside resistance genes was significantly enriched when compared to the control and reduced antibiotic manure treatments ($P < 0.05$ ANOVA, Fig. S2). Phyllosphere samples from both manure treatments had a significantly increased abundance of Beta lactamase resistance genes compared to the control ($P < 0.05$ ANOVA, Fig. S2). The multidrug gene was significantly enriched in the

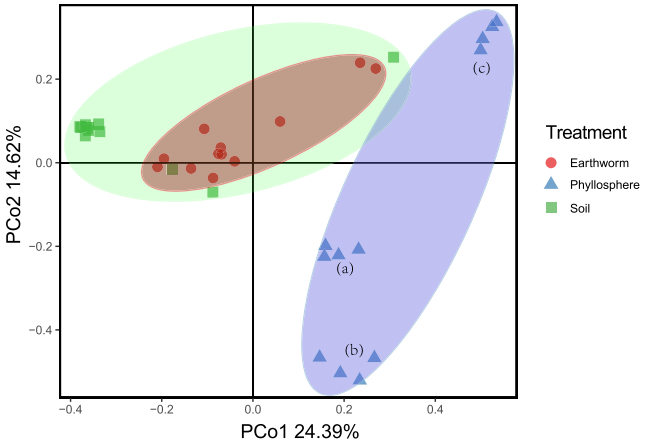

Fig. 1. Principal coordinates analysis (PCoA) of ARGs in phyllosphere, soil and earthworm gut samples (n = 36). Different colors indicate the different habitats. Group (a) represents the control, (b) represents the reduced antibiotic manure treatment and group (c) represents the conventional manure treatments of the sampled phyllosphere.

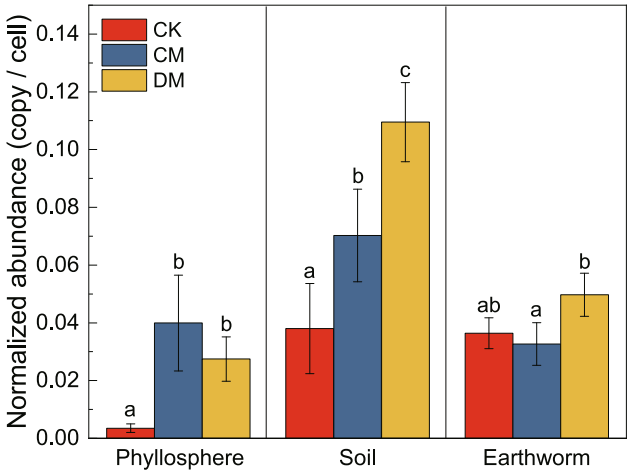

Fig. 2. Normalized abundance of ARGs in phyllosphere, soil and earthworm gut samples. Different letters indicate significant differences between treatments (control (CK), reduced antibiotic manure (CM) and conventional manure (DM)), at the $P < 0.05$ level (Tukey s-b, ANOVA). Error bars are Standard Errors.

reduced antibiotic manure treatment in both phyllosphere and soil ($P < 0.05$ ANOVA, Fig. S2) and the conventional manure treatment in soil and earthworm samples ($P < 0.05$ ANOVA, Fig. S2). Significant enrichment of the MLSB resistance gene class occurred in the conventional manure treatment in both phyllosphere and soil ($P < 0.001$ ANOVA, Fig. S2). Abundance of Tetracycline resistance genes was enriched in the conventional manure treatment in the phyllopshere, soil and earthworms, while in reduced antibiotic manure samples it was only enriched in earthworm guts ($P < 0.05$ ANOVA, Fig. S2).

3.3. Characterization of bacterial communities

A total of 1,440,543 high-quality sequences were detected across all samples with sequences per sample ranging from 8374 to 61,557. A total of 36,697 OTUs were obtained using a 97% similarity cutoff. Four dominant phyla Acidobacteria (7.0%), Actinobacteria (10.1%), Firmicutes (20.5%) and Proteobacteria (48.7%) were observed in all samples (Fig. 3A).

In soil samples, relative abundance of Acidobacteria and Chloroflexi increased after application of both manures ($P < 0.05$, ANOVA) but

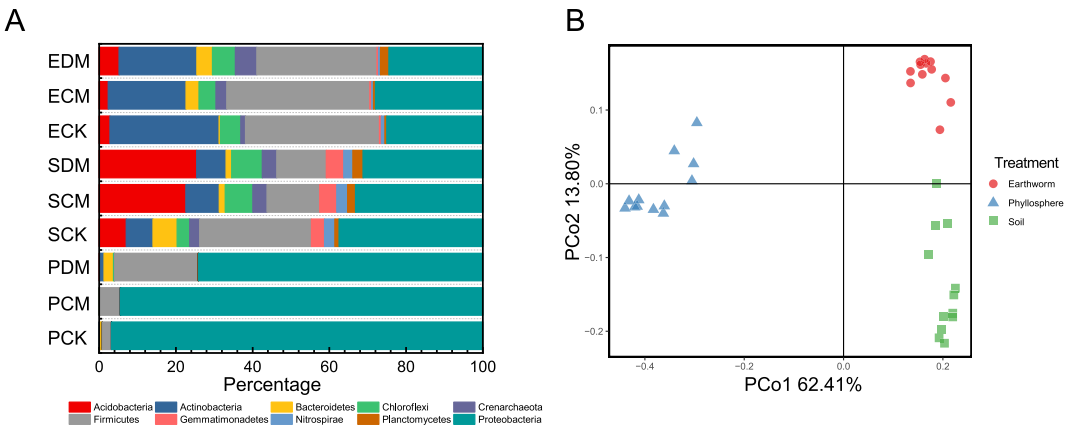

Fig. 3. (A) Percentage of dominant phyla (> 1%) in all samples. Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Crenarchaeota, Firmicutes, Gemmatimonade, Nitrospirae, Planctomycetes, Proteobacteria are considered the dominated phylum in all samples and treatments: control (CK), reduced antibiotic manure (CM), conventional manure (DM), phyllosphere (P), soil (S) and the earthworm gut (E). (B) Principal coordinates analysis (PCoA) of OTUs in phyllosphere, soil and earthworm gut samples.

lowered the relative abundance of Firmicutes ($P < 0.05$, ANOVA) and Bacteroidetes ($P < 0.001$, ANOVA) when compared to the control (Fig. 3A). In earthworm guts, the relative abundance of Actinobacteria decreased ($P < 0.05$, ANOVA) in both manure treatments whereas Planctomycetes increased under the conventional manure treatment ($P < 0.05$, ANOVA, Fig. 3A). In the phyllosphere, Proteobacteria (96.5%) was the dominant phylum in the control treatment whereas in the conventional manure treatment Proteobacteria significantly decreased ($P < 0.001$, ANOVA) compared to both control and reduced antibiotic manure treatments.

Similar to ARGs, phyllosphere bacterial communities were clearly clustered into 3 groups, with phyllosphere separating from soil and earthworm in dimension 1 and soil separating from earthworm in dimension 2 (Fig. 3B). Phylogenetic diversity of bacterial communities ranked as follows: soil > earthworm > phyllosphere ($P < 0.05$ ANOVA, Fig. S3). In contrast to ARGs, bacterial communities from soil and earthworm gut samples also separated from each other (Fig. S4).

3.4. Relationship between bacterial communities, ARGs, MGEs and soil properties

Samples from soil, earthworm guts and the phyllosphere shared 66% of the detected ARGs and MGEs, with the fewest unique classes of ARGs in earthworm gut samples (8.7% unique ARGs) and the greatest in phyllosphere samples (14.9% unique ARGs) (Fig. 4A). A total of 1178 OTUs were shared across samples (Fig. 4B). Soil and earthworm gut samples shared the most OTUs (32.7% of total OTUs). Phyllosphere samples had the fewest unique OTUs (7.2% of total OTUs) compared to soil (34.6% of total OTUs) or earthworm guts (22.7% of total OTUs). Multidrug gene and Vacomycin genes were the most and least shared ARGs, respectively (Fig. 4C). At the family level, shared OTUs included Aeromonadaceae, Bacillaceae, Enterobacteriaceae, Micrococcaceae, Paenibacillaceae, Phyllobacteriaceae, Pseudomonadaceae, Rhizobiaceae (Fig. 4D). Pearson Correlation Coefficient analysis showed that each detected bacterial family had a strong correlation to different classes of ARGs (Table S4). For example, Aminoglycoside resistance genes had a significant ($P < 0.05$) positive correlation with Phyllobacteriaceae but a negative correlation with Aeromonadaceae. Beta lactamase resistance genes had significant ($P < 0.01$) positive correlations with most of the shared OTUs and Chloramphenicol and Vancomycin resistance genes had positive correlations with Phyllobacteriace ($P < 0.05$). A significant negative correlation was found between Tetracycline resistance genes and Aeromonadaceae ($P < 0.01$), and between Tetracycline resistance genes and Rhizobiaceae ($P < 0.05$) (Table S4).

A total of 64 shared ARGs and 23 shared OTUs, from all detected ARGs and OTUs, were selected for Procrustes analysis. A significant correlation was found between the composition of ARGs and bacterial communities (Procrustes sum of squares $M^2 = 0.2882$, r = 0.7178, $P < 0.001$, 999 free premutation) (Fig. 5A). Canonical Correlation Analysis (CCA) indicated that ARG composition across all samples was significantly correlated to Proteobactia ($P < 0.001$ $R^2 = 0.8295$), Firmicutes ($P < 0.001$ $R^2 = 0.4609$), Actinobacteria ($P < 0.001$ $R^2 = 0.2949$), Acidobacteria ($P < 0.001$ $R^2 = 0.5485$) and MGEs ($P = 0.011$ $R^2 = 0.2712$) (Fig. S4).

Variation partitioning analysis (VPA) showed that bacterial communities, physicochemical properties and MGEs accounted for 39.4%, 22.0% and 15.1% of the variation in ARG abundance, respectively (Fig. 5B).

Ordinary least-squares (OLS) regression revealed that MGE abundance was linearly and positively correlated with ARG abundance ($P = 0.0001$, $R^2 = 0.5052$) (Fig. 6). Pearson correlation analysis (Table S6) showed positive and significant correlations between MGEs and Aminoglycoside, MLSB, Multidrug and Tetracycline resistance genes ($P < 0.01$) and between MGEs and Vancomycin resistance genes ($P < 0.05$). Also, a negative correlation was found between MGEs and Beta lactamase resistance genes ($P < 0.05$) while positive correlations were found between Beta Lactamase resistance genes and five of the most dominant microbial families ($P < 0.01$, Table S4).

Total nitrogen ($P < 0.05$), total carbon ($P < 0.05$), C:N ratio ($P < 0.05$) and pH ($P < 0.01$) had positive and significant correlations with ARGs (Pearson correlation analysis, Table S5), whereas NO_3-N concentrations ($P < 0.01$) and clay content had a negative correlation ($P < 0.05$) with ARGs.

4. Discussion

4.1. Antibiotic resistome in the soil, earthworm and phyllosphere

Previous studies have found a correlation between ARGs found in the phyllosphere of arable corps such as maize, brassica and lettuce and those in associated soils, as well as between soils and the guts of earthworms (Ding et al., 2019; Zhu et al., 2016). This study has built on these findings by exploring the distribution of ARGs between three habitats (soil, guts of earthworms and the phyllosphere) from a single (soil-earthworm-phyllosphere) system.

Lactuca sativa was chosen for this study as it is a globally used salad vegetable (Baslam et al., 2013) and consequently a potentially important pathway of ARGs to humans. The composition of ARGs in the phyllosphere of *Lactuca sativa* differed to those in soil and earthworm

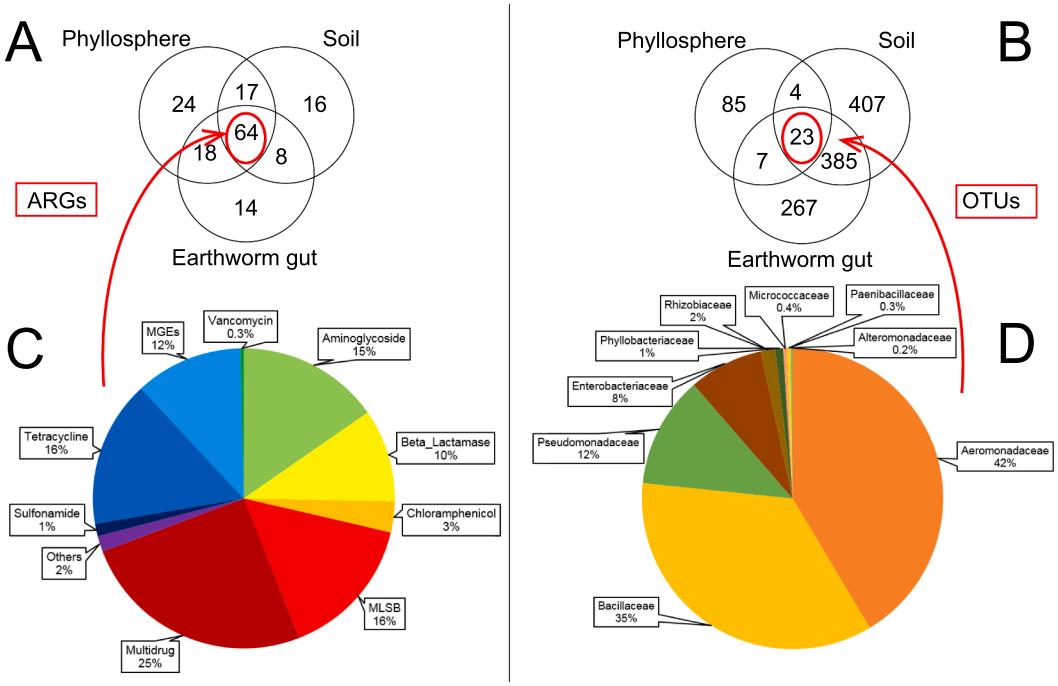

Fig. 4. Venn diagram showing shared ARGs (A) and shared OTUs (B) at the family level among phyllosphere, soil and earthworm gut samples. The percentage of each class of ARGs (C) and shared OTUs (D) at the family level are listed in the pie-charts. Aeromonadaceae, Bacillaceae, Enterobacteriaceae, Micrococcaceae, Paenibacillaceae, Phyllobacteriaceae, Pseudomonadaceae, Rhizobiaceae are shared OTUs detected across the three habitats. There are nine classes of antibiotic: Aminoglycoside, Beta Lactamase, Chloramphenicol, MLSB, Multidrug, Tetracycline, Sulfonamide, Vancomycin, Other unknown and MGEs that are shared between habitats.

guts, however ARGs were similar between soil and earthworm gut samples. Soil and earthworm guts samples also had a higher diversity of ARGs than that in phyllosphere samples ($P < 0.05$ ANOVA). These results were not unexpected as when compared to soils the phyllosphere represents a temporally variable and oligotrophic habitat (Vorholt, 2012). Additionally, the phylogenetic diversity of bacterial communities in earthworm's guts was significantly lower than that in soil ($P < 0.05$ ANOVA), which was consistent with previous studies (Zhou et al., 2019a). The number of unique ARGs between soil, earthworm and phyllosphere samples accounted for 9.9%, 8.7% and 14.9% of total ARGs in each of these groups, respectively. Whereas, unique OTUs (> 1%) represented 7.2%, 34.5% and 22.7% of the total OTUs in each sample group, which suggests that the level of shared OTUs is considerable.

Although differences in both ARG and OTU composition were found in soil, earthworm guts and the phyllosphere, the number of shared ARGs and OTUs was still considerable (66% and 36%, respectively). The strong correlation between shared ARGs and OTUs (r = 0.7178, $P < 0.001$ Mantel test) indicated that samples with similar ARG profiles also had similar OTU profiles. Movement of microbiota (OTUs) may play an important role in determining the movement of ARGs between habitats (Zhu et al., 2017) and these results suggest a possible pathway for ARGs to move between soil, earthworm guts and the phyllosphere. It has been reported that phyllosphere and soil bacterial communities can be similar (Afzal et al., 2014; Beattie and Lindow, 1999; Fang et al., 2015), and this similarity may be a result of the movement of microorganisms between these two habitats. Furthermore, airborne bacteria may also affect the bacterial structure of

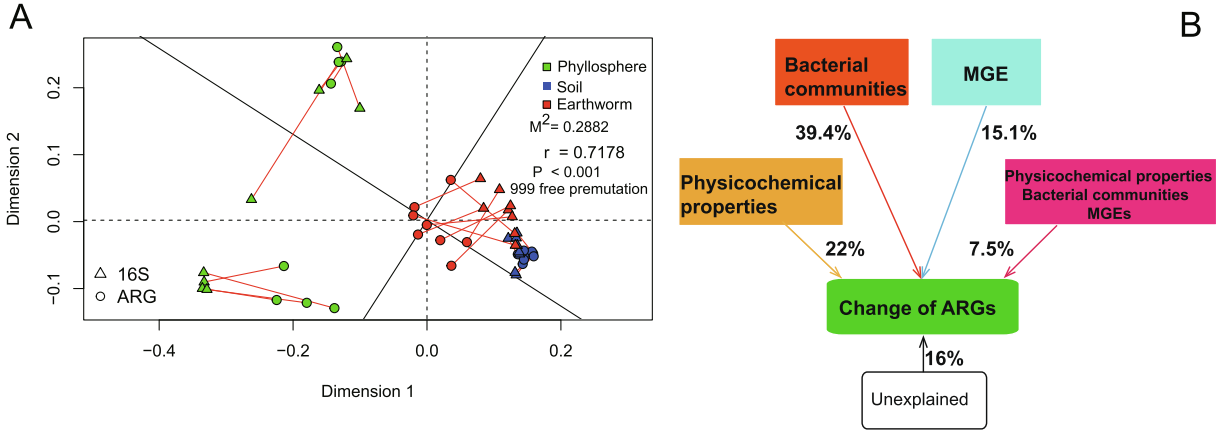

Fig. 5. (A) Procrustes analysis between shared OTUs and shared ARGs among phyllosphere, soil and earthworm gut samples after organic fertilization (Procrustes sum of squares $M^2 = 0.2882$, $r = 0.7178$, $P < 0.001$, 999 free premutation). Triangles and circles represent OTUs and ARGs respectively. (B) Variation partitioning analysis (VPA) showing that the contribution of bacterial communities, MGEs and physicochemical properties to changes in ARGs are 39.4%, 15.1% and 22.0% respectively. The coefficient of these three factors is 7.5%. Unexplained factors represent 16% of the total variance.

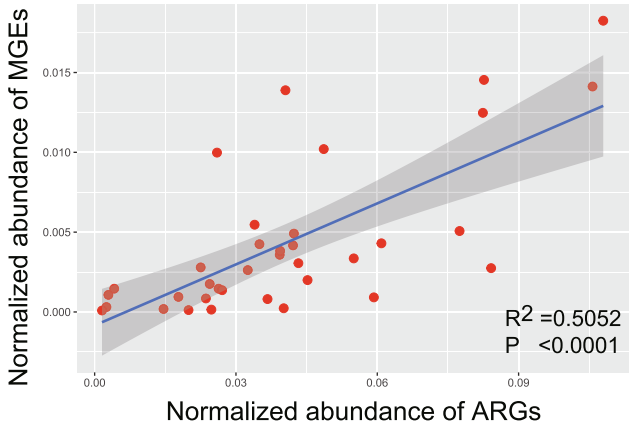

Fig. 6. Ordinary least-squares (OLS) analysis between ARGs and MGEs. A positive correlation between ARGs and MGEs is shown ($P < 0.001$ $R^2 = 0.5052$).

phyllosphere and soil (Bulgarelli et al., 2013). Water movement through rainfall or irrigation and air motion due to wind action as well as leaf fall due to senescence may lead to the movement of bacterial communities between the phyllosphere and soil and have a concomitant impact on the flow of ARGs (Vorholt, 2012; Williams and Marco, 2014). There is therefore potential for ARGs to disperse into the belowground soil food chain. It is well established that ingestion of soil by earthworms drives dispersion of bacteria (Drake and Horn, 2007) and strong correlations have been found between bacterial communities in soil and earthworm guts (Horn et al., 2003; Parle, 1963).

4.2. Effects of manures on ARGs

The addition of pig manure to soil led to an enrichment of ARGs across soil, earthworm guts and the phyllosphere. In contrast with previous studies that focused on either the phyllosphere and soil (Fang et al., 2015; Wang et al., 2015) or soil biota and soil (Ding et al., 2019), this study found that ARGs increased simultaneously across all three of these habitats after manure application. Since the potential correlation among soil, gut of earthworm and phyllosphere, the results indicated the manure application may affect not only the ARGs of soil itself, but also the soil-related circumstances by movement of microorganisms. Besides, the manure fertilizations may accelerate the distribution of ARGs into environment through agriculture activities, which should blame to human activities (Marti et al., 2013).

Human activities have been considered responsible for the movement of ARGs both into and across the environments (Zhu et al., 2017). Conventional manure enriched the abundance of ARGs across all samples whereas reduced antibiotic manure only enriched ARGs in soils and the phyllosphere ($P < 0.05$, ANOVA). This is consistent with previous studies that described enrichment of ARGs in soil and phyllosphere habitats through organic fertilizer amendment to soils (Kumar et al., 2005; Udikovic-Kolic et al., 2014). While reduced antibiotic manure increased the abundance of ARGs compared to the phyllosphere and soil controls, conventional manure enriched ARGs to a greater extent than reduced antibiotic manure in soil and earthworm gut samples ($P < 0.05$, ANOVA). Therefore, reducing the antibiotic burden, by using manure with a reduced antibiotic burden rather than conventional manure, may lower the enrichment of the resistome in both soils and earthworms though a risk of ARG dispersal remains. Such risk could be mitigated by the use of composting as a pretreatment of organic fertilizers in order to remove residual antibiotics. Combining the application of manures with non-animal derived organic amendments such as compost and biochar may also mitigate the increases in the antibiotic resistome (Cui et al., 2016; Dolliver et al., 2008; Su et al., 2015; Teixido et al., 2013).

5. Conclusions

This study used a HT-qPCR approach to concurrently quantify resistome profiles in soil, earthworm gut and phyllosphere samples. Although ARG and OTU profiles differed between soil, earthworm guts and the phyllosphere, a proportion of ARGs and OTUs were shared. Application of conventional manure (current antibiotic practice) increased ARG abundance compared to manure with a reduced antibiotic burden in both soil and earthworm guts. Bacterial communities, the physicochemical properties of soil and MGEs were main drivers of ARG profiles, suggesting that a complex mix of factors support the dispersal and subsequent distribution of ARGs.

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

We are particularly grateful to Professor Yong-Guan Zhu for supervising this work. This work was funded by the National Key Research and Development Program of China-International collaborative project from Ministry of Science and Technology (2017YFE0107300), the National Natural Science Foundation of China (41571130063) and the K.C. Wong Education Foundation. This work was in part supported by the Natural Environment Research Council grant number NE/N00745X/1. The James Hutton Institute received financial support from Scottish Government Rural and Environment Science and Analytical Services (RESA).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105359.

References

Afzal, M., Khan, Q.M., Sessitsch, A., 2014. Endophytic bacteria: Prospects and applications for the phytoremediation of organic pollutants. Chemosphere 117, 232–242.
Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., Handelsman, J., 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8, 251–259.
Bartlett, M.D., Briones, M.J.I., Neilson, R., Schmidt, O., Spurgeon, D., Creamer, R.E., 2010. A critical review of current methods in earthworm ecology: from individuals to populations. Eur. J. Soil Biol. 46, 67–73.
Baslam, M., Morales, F., Garmendia, I., Goicoechea, N., 2013. Nutritional quality of outer and inner leaves of green and red pigmented lettuces (*Lactuca sativa L.*) consumed as salads. Sci. Hortic. 151, 103–111.
Beattie, G.A., Lindow, S.E., 1999. Bacterial colonization of leaves: a spectrum of strategies. Phytopathology 89, 353–359.
Borcard, D., Legendre, P., Drapeau, P., 1992. Partialling out the spatial component of ecological variation. Ecology 73, 1045–1055.
Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E.V.L., Schulze-Lefert, P., 2013. Structure and functions of the bacterial microbiota of plants. In: In: Merchant, S.S. (Ed.), Annual Review of Plant Biology, vol. 64. pp. 807–838.
Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R., 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266–267.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010b. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
Chen, Q.-L., Cui, H.-L., Su, J.-Q., Penuelas, J., Zhu, Y.-G., 2019. Antibiotic resistomes in plant microbiomes. Trends Plant Sci. 24, 530–541.
Chen, Q.L., An, X.L., Zheng, B.X., Ma, Y.B., Su, J.Q., 2018. Long-term organic fertilization increased antibiotic resistome in phyllosphere of maize. Sci. Total Environ. 645, 1230–1237.
Cheng, W., Chen, H., Su, C., Yan, S., 2013. Abundance and persistence of antibiotic resistance genes in livestock farms: a comprehensive investigation in eastern China. Environ. Int. 61, 1–7.

Cui, E.P., Wu, Y., Zuo, Y.R., Chen, H., 2016. Effect of different biochars on antibiotic resistance genes and bacterial community during chicken manure composting. Bioresour. Technol. 203, 11–17.
Degl'Innoocenti, E., Pardossi, A., Tattini, M., Guidi, L., 2008. Phenolic compounds and antioxidant power in minimally processed salad. J. Food Biochem. 32, 642–653.
Ding, J., Zhu, D., Hong, B., Wang, H.T., Li, G., Ma, Y.B., Tang, Y.T., Chen, Q.L., 2019. Long-term application of organic fertilization causes the accumulation of antibiotic resistome in earthworm gut microbiota. Environ. Int. 124, 145–152.
Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930.
Dolliver, H., Gupta, S., Noll, S., 2008. Antibiotic degradation during manure composting. J. Environ. Qual. 37, 1245–1253.
Drake, H.L., Horn, M.A., 2007. As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. Annu. Rev. Microbiol. 61, 169–189.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
Fang, H., Wang, H.F., Cai, L., Yu, Y.L., 2015. Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. Environ. Sci. Technol. 49, 1095–1104.
Wickham, Hadley, Chang, Winston, Henry, Lionel, Pedersen, Thomas Lin, Takahashi, Kohske, Wilke, Claus, Woo, Kara, Yutani, Hiroaki, RStudio, 2018. ggplot2: Create elegant data visualisations using the grammar of graphics. https://CRAN.R-project.org/package=ggplot2.
Hao, X.H., Liu, S.L., Wu, J.S., Hu, R.G., Tong, C.L., Su, Y.Y., 2008. Effect of long-term application of inorganic fertilizer and organic amendments on soil organic matter and microbial biomass in three subtropical paddy soils. Nutr. Cycl. Agroecosyst. 81, 17–24.
Horn, M.A., Schramm, A., Drake, H.L., 2003. The earthworm gut: an ideal habitat for ingested N2O-producing microorganisms. Appl. Environ. Microbiol. 69, 1662–1669.
Hu, L., Xia, M., Lin, X.H., Xu, C.C., Li, W., Wang, J., Zeng, R.S., Song, Y.Y., 2018. Earthworm gut bacteria increase silicon bioavailability and acquisition by maize. Soil Biol. Biochem. 125, 215–221.
Ikeda, S., Anda, M., Inaba, S., Eda, S., Sato, S., Sasaki, K., Tabata, S., Mitsui, H., Sato, T., Shinano, T., Minamisawa, K., 2011. Autoregulation of nodulation interferes with impacts of nitrogen fertilization levels on the leaf-associated bacterial community in soybeans. Appl. Environ. Microbiol. 77, 1973–1980.
Oksanen, Jari, Blanchet, F. Guillaume, Friendly, Michael, Kindt, Roeland, Legendre, Pierre, McGlinn, Dan, Minchin, Peter R., O'Hara, R.B., Simpson, Gavin L., Solymos, Peter, Stevens, M. Henry H., Szoecs, Eduard, Wagner, Helene, 2018. Vegan: Community Ecology Package. R package version 2.5-3. https://CRAN.R-project.org/package=vegan.
Kadivar, H., Stapleton, A.E., 2003. Ultraviolet radiation alters maize phyllosphere bacterial diversity. Microb. Ecol. 45, 353–361.
Kotzerke, A., Klemer, S., Kleineidam, K., Horn, M.A., Drake, H.L., Schloter, M., Wilke, B.M., 2010. Manure contaminated with the antibiotic sulfadiazine impairs the abundance of nirK- and nirS-type denitrifiers in the gut of the earthworm *Eisenia fetida*. Biol. Fertil. Soils 415–418.
Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., Rosen, C.J., 2005. Antibiotic uptake by plants from soil fertilized with animal manure. J. Environ. Qual. 34, 2082–2085.
Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821.
Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl Environ Microbiol 69, 1875–1883.
Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718–733.
Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Topp, E., 2013. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of detection of antibiotic resistance genes in soil and on vegetables at harvest. Appl. Environ. Microbiol. 79, 5701–5709.
Martinez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321, 365–367.
McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618.
Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930.
O'Flaherty, E., Solimini, A.G., Pantanella, F., De Giusti, M., Cummins, E., 2018. Human exposure to antibiotic resistant-*Escherichia coli* through irrigated lettuce. Environ. Int. 122, 270–280.
Parle, J.N., 1963. Micro-organisms in intestines of earthworms. J. Gen. Microbiol. 31, 1–11.
Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., Vandenbulcke, F., 2014. Pesticides and earthworms. A review. Agron. Sustain. Dev. 34, 199–228.
Rahman, M.M., Shan, J., Yang, P., Shang, X., Xia, Y., Yan, X., 2018. Effects of long-term pig manure application on antibiotics, abundance of antibiotic resistance genes (ARGs), anammox and denitrification rates in paddy soils. Environ. Pollut. 240, 368–377.
Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L., Leveau, J.H.J., 2012. Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J. 6, 1812–1822.
Su, J.-Q., Wei, B., Ou-Yang, W.-Y., Huang, F.-Y., Zhao, Y., Xu, H.-J., Zhu, Y.-G., 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ. Sci. Technol. 49, 7356–7363.
Swift, B.M.C., Bennett, M., Waller, K., Dodd, C., Murray, A., Gomes, R.L., Humphreys, B., Hobman, J.L., Jones, M.A., Whitlock, S.E., Mitchell, L.J., Lennon, R.J., Arnold, K.E., 2019. Anthropogenic environmental drivers of antimicrobial resistance in wildlife. Sci. Total Environ. 649, 12–20.
Teixido, M., Hurtado, C., Pignatello, J.J., Beltran, J.L., Granados, M., Peccia, J., 2013. Predicting contaminant adsorption in black carbon (biochar)-amended soil for the veterinary antimicrobial sulfamethazine. Environ. Sci. Technol. 47, 6197–6205.
Thakuria, D., Schmidt, O., Finan, D., Egan, D., Doohan, F.M., 2010. Gut wall bacteria of earthworms: a natural selection process. ISME J. 4, 357–366.
Turner, S., Pryer, K.M., Miao, V.P.W., Palmer, J.D., 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small submit rRNA sequence analysis. J. Eukaryot. Microbiol. 46, 327–338.
Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. PNAS 111, 15202–15207.
Vorholt, J.A., 2012. Microbial life in the phyllosphere. Nat. Rev. Microbiol. 10, 828–840.
Wang, F.H., Qiao, M., Chen, Z., Su, J.Q., Zhu, Y.G., 2015. Antibiotic resistance genes in manure-amended soil and vegetables at harvest. J. Hazard. Mater. 299, 215–221.
Wang, H., Sangwan, N., Li, H.Y., Su, J.Q., Oyang, W.Y., Zhang, Z.J., Gilbert, J.A., Zhu, Y.G., Ping, F., Zhang, H.L., 2017. The antibiotic resistome of swine manure is significantly altered by association with the *Musca domestica* larvae gut microbiome. ISME J. 11, 100–111.
Williams, T.R., Marco, M.L., 2014. Phyllosphere microbiota composition and microbial community transplantation on lettuce plants grown indoors. Mbio 5 01564–01514.
Wu, S., Huang, J.H., Wu, Q.P., Zhang, F., Zhang, J.M., Lei, T., Chen, M.T., Ding, Y., Xue, L., 2018. Prevalence and characterization of staphylococcus aureus isolated from retail vegetables in China. Front. Microbiol. 9.
Zhang, H., Schrader, S., 1993. Earthworm effects on selected physical and chemical-properties of soil aggregates. Biol. Fertil. Soils 15, 229–234.
Zhang, Y.-J., Hu, H.-W., Chen, Q.-L., Singh, B.K., Yan, H., Chen, D., He, J.-Z., 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environ. Int. 130, 104912.
Zhao, X., Wang, J., Zhu, L., Wang, J., 2018a. Field-based evidence for enrichment of antibiotic resistance genes and mobile genetic elements in manure-amended vegetable soils. Sci. Total Environ. 654, 906–913.
Zhao, Y., Su, J.Q., An, X.L., Huang, F.Y., Rensing, C., Brandt, K.K., Zhu, Y.-G., 2018b. Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. Sci. Total Environ. 621, 1224–1232.
Zheng, F., Zhu, D., Giles, M., Daniell, T., Neilson, R., Zhu, Y.-G., Yang, X.R., 2019. Mineral and organic fertilization alters the microbiome of a soil nematode Dorylaimus stagnalis and its resistome. Sci. Total Environ. 680, 70–78.
Zhou, G.W., Yang, X.R., Sun, A.Q., Li, H., Lassen, S.B., Zheng, B.X., Zhu, Y.-G., 2019a. Mobile incubator for Iron(III) reduction in the gut of the soil-feeding earthworm *pheretima guillelmi* and interaction with denitrification. Environ. Sci. Technol. 53, 4215–4223.
Zhou, S.-Y.-D., Zhu, D., Giles, M., Yang, X.-R., Daniell, T., Neilson, R., Zhu, Y.-G., 2019b. Phyllosphere of staple crops under pig manure fertilization, a reservoir of antibiotic resistance genes. Environ. Pollut. 252, 227–235.
Zhu, B., Chen, Q., Chen, S., Zhu, Y.-G., 2016. Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced? Environ. Int. 98, 152–159.
Zhu, D., Zheng, F., Chen, Q.L., Yang, X.R., Christie, P., Ke, X., Zhu, Y.-G., 2018. Exposure of a soil collembolan to Ag nanoparticles and AgNO3 disturbs its associated microbiota and lowers the incidence of antibiotic resistance genes in the gut. Environ. Sci. Technol. 52, 12748–12756.
Zhu, Y.-G., Johnson, T.A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. 110, 3435–3440.
Zhu, Y.-G., Gillings, M., Simonet, P., Stekel, D., Banwart, S., Penuelas, J., 2017. Microbial mass movements. Science 357, 1099–1100.