

Did Application of Digested Sewage Sludge as Soil Amendment Alter the Abundances of Antibiotic Resistance Genes and Microbial Communities in Soil and Earthworm Gut?

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Research

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Abstract

Background

Digested sewage sludge has been widely applied as soil amendment for enhanced crop production. However, given that digested sludge is abundant with antibiotic resistance genes (ARGs) and antibiotic resistant bacteria, the impact of digested sludge amendment on the abundances of ARGs and microbial communities in soil and soil fauna (e.g., earthworms) remains largely unknown. In this study, the patterns of ARGs and microbial communities in soil and gut of earthworms after 80-days cultivation with digested sewage sludge amendment were investigated to gain insights into this impact.

Results

The results show that the digested sludge amendment increased the initial abundances of ARGs (e.g., tetA, tetQ, and sull) in soil. However, after 80-days cultivation, the absolute abundances of target ARGs decreased by 62.3–95.4%. The reduction in ARGs absolute abundances was further enhanced by 31.4–84.7% in the presence of earthworms. In contrast, the relative abundances of some ARGs (e.g., tetA, sull, and bla_{TEM-1}) in the gut of earthworms increased by 41–130 folds. The microbial community structure of soil was greatly altered because of the introduction of digested sewage sludge at initial, but it recovered to its original pattern after 80-days cultivation. This could be attributed to the gradual attenuation of anaerobic microorganisms under aerobic conditions in soil. In particular, the presence of earthworms further enhanced this phenomenon. The reduction of ARGs in the amended soils was likely attributed to microbial community shift based on redundancy analysis. Several bacterial families (e.g., *Saprospiraceae*, *Chitinophagaceae*, and *Rhodanobacteraceae*) were significantly correlated with the target ARGs.

Conclusions

Our results reveal that the enrichment of ARGs in soil caused by digested sludge-amendment would recover to their original levels before amendment, highlighting the contribution of earthworms to reducing the ARG abundances in amended soil via shifting the microbial community. However, we also found that the amended soil could increase ARGs abundance in the earthworm gut, which may enhance the potential risk of ARGs spread via food chain. These results may provide a new sight on the control of ARGs occurrence and dissemination in sludge-amended soil ecosystem with consideration of the impact of earthworms.

Background

Antibiotic resistance has become a potential risk for global public health [1, 2]. Municipal wastewater treatment plants (WWTPs) are regarded as hotspots of antibiotic resistance genes (ARGs) and multidrug-

resistant bacteria [3]. The conventional biological treatment processes generally can not effectively reduce ARGs, leading to abundant ARGs are present in sewage sludge ($10^8 - 10^{12}$ copies/g suspended solids) [3, 4]. Nowadays, sewage sludge has been widely applied as soil amendment for enhanced crop production [5]. Although elevated soil quality could be achieved after sewage sludge amendment, the introduction of ARGs from sewage sludge may increase the abundances of ARGs in soil and even in crops as well as enhance the horizontal gene transfer in soil [5–7], causing potential threats to human health. Therefore, the sewage sludge should be properly treated before application to agricultural soil.

Anaerobic digestion is a commonly applied pretreatment for sewage sludge to recover resources as well as reduce harmful organic matters and pathogenic bacteria [8, 9]. It can also reduce the abundances of ARGs in sewage sludge [8]. Thus, anaerobic digestion is a promising pretreatment technique for sewage sludge prior to land application to control the occurrence and spread of ARGs in soil. However, the fate of ARGs in digested sludge-amended soil has not been well investigated.

Soil is a huge and complex ecosystem, containing abundant microorganisms and soil fauna. The soil fauna can change the physicochemical properties of soil. For instance, earthworms, the predominant group of soil invertebrates in most soils, can change the permeability, humification depth, and oxygen concentration of soil via burrowing, ingesting, and excreting [10–12]. The microbial community in soil can also be altered by earthworms [13–15]. The shift of microbial community has been regarded as an important driver for ARGs evolution in various environments [6, 16]. Thus, earthworms may play an important role in affecting the fate of ARGs in the amended soils. Moreover, in the gut of soil fauna (e.g., earthworm and collembolan), abundant ARGs have been detected [17–20]. The introduction of environmental pollutants (e.g., antibiotics, fungicides, and nanoplastics) and soil amendment (e.g., manure) can increase the abundances of ARGs in the gut of soil fauna [19, 21–23]. As one of the most commonly applied soil amendment agents, the digested sludge with abundant ARGs would probably affect the abundances of ARGs in the gut of soil fauna, and thus affect the ARG abundances and microbial community in soils. Therefore, investigating the variations of ARGs and microbial communities in digested sludge-amended soil should take consideration of the role of earthworms. This still remains unknown and further studies are required.

In this work, soil was amended with digested sewage sludge in the presence of earthworms, and the variations of target ARG abundances and microbial communities in the amended soil and the gut of earthworms were determined to achieve the following objectives: (1) to investigate the fate of ARGs and variation of microbial community in digested sludge-amended soil under the effect of earthworms; (2) to evaluate the impact of digested sludge amendment on the evolution of ARGs and microbial community in the gut of earthworms. These results may illuminate the fate and spread of ARGs in digested sludge-amended soil ecosystems with considering the effect of earthworms.

Methods

Digested sewage sludge, soil, and earthworms

Sewage sludge, which was obtained from a municipal WWTP in Hefei, China, was anaerobically digested at 37 °C for 60 days in the laboratory before dosed into the soil. The water content and pH of digested sewage sludge were $90.3\% \pm 2.0\%$ and 6.8 ± 0.2 , respectively. The raw soil was purchased from a nutritional soil Co., LTD (Jiangsu, China). The water content and pH of raw soil were $67.8\% \pm 3.0\%$ and 6.9 ± 0.2 , respectively. The water content was quantified by the difference in wet and dry weight. Other properties of soil and digested sludge were provided in the Supporting Information Table S1. Sludge and soil samples were mixed with deionized water at a weight/volume ratio of 1:25 prior to pH measurement by a pH meter (pHS-3C, Leici, China). *Eisenia foetida* (*E. foetida*), which was often applied in soil bioremediation [15], was chosen as the model earthworm in this work. Prior to the experiment, earthworms were precultured in the raw soil (without addition of digested sewage sludge) for 60 days to stabilize the microbial community in the gut of earthworms.

Experimental setup and sampling

After 60-day anaerobic digestion, the digested sludge was mixed uniformly with raw soil at a ratio of 1:2 (wet basis) to obtain the digested sludge-amended soil. Twenty earthworms (weight 4.74 ± 0.20 g, length 5.0 ± 0.4 cm) were inoculated into each plastic beaker (diameter 12 cm, height 18 cm) containing about 300 g amended soil (raw soil: digested sludge=2:1 wet basis). The amended soil without addition of earthworms was set up as the blank control group. All reactors were performed in triplicate and incubated in dark at room temperature (around 25°C). To maintain the water moisture, 10 mL deionized water was sprinkled into each reactor once a week. Before 80-day cultivation, the digested sludge, raw soil, and the sludge-amended soil (blank control group, defined as blank-0 soil) were collected before subsequent measurements.

After 80-day cultivation, soil from the blank control group (blank-80 soil) was homogeneously mixed and collected. For the reactors with earthworms, two types of soil, i.e., surface wormcast and residual soil, were collected to investigate the effects of earthworms on the variation of ARGs and microbial communities in soil. Since the wormcast excreted by earthworms is a rich source of micro- and macro-nutrients, it is often applied as bio-fertilizer in soil [15]. It is necessary to evaluate the ARGs abundance in the wormcast to determine the potential dissemination of ARGs in receiving soil and crops. Therefore, the the earthworms were then collected from each reactor; lastly, the residual soil was mixed homogeneously and sampled. The earthworms were rinsed by deionized water and wiped dry using tissue. Afterward, they were transformed into a clean Petri dishes and kept in the dark overnight to excrete feces. All feces and soil samples were stored at -20 °C prior to DNA extraction using a PowerSoil® DNA isolation kit (MoBio Laboratories, USA). The DNA concentration and purity were determined by spectrophotometry (NanoDrop ND 2000, Thermo, USA).

ARG Quantification by Quantitative Polymerase Chain Reaction (qPCR)

A total of eight target genes containing six ARGs (tetA, tetQ, tetW, sull, sulll, and bla_{TEM-1}), an integrase (intl1), and 16S rRNA gene were determined by qPCR using SYBR Premix Ex TaqII (Takara, Japan). These

six ARGs belong to three of the most widely distributed and highly abundant ARGs classes (tet, sul, and bla) in various environmental matrices to resist tetracycline, sulfonamide, and beta-lactam antibiotics, respectively. The *Int1* is an important mobile genetic element, which is an indicator of the horizontal gene transfer of ARGs. All qPCR assays were performed using a Roche LightCycler® 96 system (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequence, annealing temperature, amplicon size, and qPCR running conditions were used as described previously [24]. All samples were analyzed in duplicate in each qPCR run with a 6-point standard curve (amplification efficiencies 90-110%; $R^2 > 0.99$) and a negative control. The abundances of target genes were normalized per mass of dry solid (DS) and 16S rRNA gene copies to obtain absolute abundances (copies/g DS) and relative abundances (copies/16S rRNA gene copies), respectively.

16S rRNA Gene Sequencing and Data Analysis

All DNA samples were PCR-amplified with the universal primer set 341F and 806R to target the V3-V4 regions of 16S rRNA genes and then sequenced by the Illumina HiSeq PE250 platform (Novogene, China). The detailed analysis procedure has been described in our previous work [24]. Canoco 5.0 was used to perform principal coordinates analysis (PCoA) to evaluate the profiles of ARGs (based on relative abundance) and microbial communities based on Bray-Curtis distance. The significant difference of target genes between samples was assessed using one-way ANOVA. All statistical tests were considered significant at $p < 0.05$. RDA analysis was applied to figure out the correlation between microbial community and ARGs (based on relative abundance) using Canoco 5.0. Pearson correlation analysis among target genes was conducted using IBM SPSS Statistics 20 to identify their co-occurrence relevance. To visualize the correlations between target genes (based on relative abundance) and microbial taxa (based on family level), heatmap was performed in R (version 3.5.1).

Results

Variations of ARGs and *intl1*

Seven target genes, including six ARGs (*tetA*, *tetQ*, *tetW*, *sull*, *sulll*, and *bla_{TEM-1}*), and *intl1*, were detected to evaluate the effect of digested sludge-amendment on the abundances of ARGs in soils. The absolute and relative abundances of ARGs and *intl1* in digested sludge were 0.7-2.2 and 0.2-1.6 logs higher than those of raw soil, respectively (Fig. 1a and 1b). After mixed the raw soil and digested sludge (1:2 wt/wt wet basis), the absolute and relative abundances of target genes (i.e., six ARGs and *intl1*) in the amended soil (blank-0 soil) were 2.8×10^5 - 3.3×10^8 copies/g DS and 2.1×10^{-4} - 4.8×10^{-1} copies/16S rRNA gene copies, respectively (Fig. 1), which were 1.3-20.4 and 1.6-16.2 folds higher than those in the raw soil, respectively (Fig. 1).

After 80-days cultivation, the absolute and relative abundances of various ARGs in the amended soil without earthworms (blank-80 soil) decreased to 1.1×10^4 - 7.7×10^7 copies/g DS and 4.3×10^{-5} - 3.0×10^{-1} copies/16S rRNA gene copies, respectively (Fig. 1a and 1b), which were 4.6%-35.2% and 12.2%-62.5% of

those in the blank-0 soil, respectively (Fig. 1). The presence of earthworms further enhanced the reduction of most target genes (except tetW). After 80-day cultivation, the absolute abundances of ARGs in surface wormcast and residual soil (with earthworms) decreased to 1.7×10^3 - 2.4×10^7 and 4.1×10^3 - 4.4×10^7 copies/g DS, respectively. Those were 15.3%-68.5% and 37.2%-88.3% of those in the blank-80 soil (without earthworms), respectively. Furthermore, the absolute abundances of ARGs (except tetW) in surface wormcast were even 6.1%-37.5% of those in the raw soil (without digested sludge amendment) (Fig. 1). For the relative abundances, enhanced reduction of ARGs was also achieved by earthworms (Fig. 1b and 1b). The relative abundances of ARGs (i.e., tetA, tetQ, sull, sulll, and bla_{TEM-1}) in surface wormcast (with earthworms) were 28.7%-95.4% of those in blank-80 soil (Fig. 1b). In contrast, the absolute and relative abundances of tetW in surface wormcast increased by 51.5% and 184.4%, respectively, compared with those in the blank-80 soil.

The abundances of ARGs and 16S rRNA gene in the gut of earthworms were also determined. After cultivation in the digested sludge-amended soil for 80 days, the absolute abundance of 16S rRNA gene in the gut of earthworms decreased by about 0.8 log (Fig. 1c). The absolute and relative abundances of tetA, sull, sulll, bla_{TEM-1}, and intl1 in earthworm gut after cultivation increased by 0.8-1.3 and 1.6-2.1 logs, respectively. In contrast, the absolute abundances of tetQ and tetW decreased by 2.1 and 0.9 logs, and their relative abundances decreased by 1.3 and 0.1 logs, respectively (Fig. 1c).

To reveal the similarity of gene pattern among different samples, PCoA analysis was performed based on the relative abundances of target genes. As shown in Fig. 2a, the digested sludge-amended soil samples (i.e., surface wormcast and residual soil) after 80-days cultivation in the presence of earthworms were distinctly separated from the blank-0 soil and blank-80 soil (without earthworms), further demonstrating that earthworms could change the abundance of ARGs and thus alter the gene pattern of soils. Meanwhile, the gene patterns of earthworm gut were greatly altered by digested sludge amendment after 80-days cultivation given that they were distinctly separated from those in the gut of earthworm before cultivation (Fig. 2b).

Composition of Microbial Community

As shown in Fig. 3, the microbial community of digested sludge was different from that of raw soil. Higher abundances of *Clostridia* and *Bacteroidia* were found in the digested sludge. After introducing digested sludge, the abundances of anaerobic microbes (e.g., *Bacteroidia* and *Clostridia*) increased in the digested sludge-amended soil (blank-0 soil) compared with those in the raw soil (Fig. 3). At the phylum level, the dominated phyla in the blank-0 soil were *Proteobacteria* (occupying 52.8%), *Bacteroidetes* (15.9%), *Actinobacteria* (6.6%), and *Firmicutes* (6.3%), which occupied 81.6% of the total microbial abundance (Fig. 3a). At the class level, the dominated classes in the blank-0 soil were *Gammaproteobacteria* (26.6%), *Alphaproteobacteria* (17.2%), *Bacteroidia* (15.0%), *Deltaproteobacteria* (9.0%), and *Clostridia* (5.5%), occupying 73.3% of the total microbial abundance (Fig. 3b). However, 80-day cultivation greatly decreased the relative abundances of anaerobic microbes and enriched aerobic microbes in soils (Fig. 3a and Fig. 3b). For example, at the class level, the relative abundances of

Bacteroidia and *Clostridia* in the blank-80 soil (without earthworm) decreased from 15.0% to 8.8% and from 5.5% to 1.6% after 80-days cultivation, respectively. Meanwhile, the relative abundances of aerobic microbes in blank-80 soil showed increasing trends. For example, the abundance *Acidimicrobiia* increased from 2.9% to 4.0%. Under the effect of earthworms, the variation of microbial community was further amplified (Fig. 3a and 3b). At the phylum level, the relative abundance of *Actinobacteria* in surface wormcast were greatly changed by earthworms, which increased by 175.6% compared to that in the blank-80 soil (without earthworms). However, the abundance of *Bacteroidia* in surface wormcast decreased by 37.0%.

The variation of the microbial community in the gut of earthworms was also observed (Fig. 3a). At the class level, the relative abundances of *Acidimicrobiia* in the gut of earthworms decreased by 70.6%. In contrast, the relative abundances of *Clostridia* and *Bacteroidia* increased by 409.4% and 79.8% in the gut of earthworms after cultivation, respectively. At the family level, significant attenuation of *Saprospiraceae*, *Sneathiellaceae*, and *Rhodanobacteraceae* in soils as well as *Rhodocyclaceae*, *Microscillaceae*, and *Nitrosomonadaceae* in the gut of earthworms after cultivation were observed (Fig. 3c). The abundances of *Sandaracinaceae* and *Haliangiaceae* in soil as well as *Rhizobiaceae* in the earthworm gut were all enriched after cultivation.

PCoA analysis shows that the microbial community patterns of the amended soils with and without earthworm after 80-days cultivation were separated from that of the blank-0 soil (Fig. 4). In addition, the samples of earthworm guts show a distinct difference from all soil samples. The surface wormcast and residual soil samples were clustered, and they were separated from other samples.

Relationships between ARGs and microbial communities

As shown in Table 1, significant correlations between two target ARGs (i.e., tetA, and sullI) and intI1 were observed based on Spearman's correlation analysis. The relationships between microbial communities and targeted ARGs were assessed using redundancy analysis (RDA). A total of 93.9% variance of ARGs could be explained by selected variables (Fig. 5). In particular, Three phyla (*Firmicutes*, *Bacteroidetes*, and *Acidobacteria*) exhibited positive relationships with ARGs in the blank-0 soil. *Proteobacteria* showed a significantly positive correlation with the ARGs abundance in the blank-80 soil and the raw soil. Moreover, *Actinobacteria* and *Chloroflexi* were positively related to the surface wormcast samples.

To further investigate the potential host bacteria of the ARGs, the quantitative correlation between the target ARGs and 29 bacterial taxa at the family level were described by the heatmap (Fig. 6). The significant positive correlations between three families (i.e., *Rhodanobacteraceae*, *Saprospiraceae*, and *Chitinophagaceae*) and two ARGs (i.e., tetQ and sullI) were observed ($R > 0.8$, $P < 0.01$). The potential hosts for intI1, sull, and bla_{TEM-1} were identified as *Saprospiraceae*, *Chitinophagaceae*, and unidentified *Clostridiales*, respectively. In particular, *Saprospiraceae* and *Chitinophagaceae* were also identified as potential multi-ARG hosts (Fig. 6).

Discussion

Digested sludge amendment and earthworms shifted microbial communities in soil As one of the most widely distributed bacteria [25], *Proteobacteria* was the dominated phyla, occupying more than 40% in the microbiome of digested sludge and soil, followed by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* (Fig. 3). A significant difference in microbial community of digested sludge-amended soil before 80-day cultivation (blank-0 soil) was found compared to that of raw soil, which was caused by digested sludge amendment. For instance, the higher abundance of *Bacteroidetes* and *Firmicutes* and lower abundances of *Proteobacteria* and *Actinobacteria* were found in the blank-0 soil compared to those in the raw soil. A possible explanation was that introducing the digestion sludge effectively decreased the abundances of aerobes (e.g., *Acidimicrobiia*) and enriched anaerobes (e.g., *Bacteroidia*) in the blank-0 soil. However, the abundances of anaerobes would be greatly reduced in the blank-0 soil after 80 days' cultivation due to the high oxygen concentration in soil, and finally the microbial pattern was similar to that of raw soil (Fig. 4). This indicates that the microbial community shift in blank-0 soil caused by digested sludge addition would not persist.

In this study, earthworms could further affect the microbial communities in the digested sludge amended soil. A significant difference in the microbial pattern was observed between soils with (e.g., the surface wormcast and residual soil) and without (the blank control soil) earthworms (Fig. 3 and 4). This result was consistent with previous studies [12, 13, 15, 29], in which earthworms could hugely alter the biological and physicochemical properties of soil via burrowing, ingesting, and excreting, contributing to the shift of microbial communities in soil. For instance, increased oxygen concentration and humification depth in soil by earthworms [11, 30, 31] would promote the enrichment of aerobic *Acidimicrobiia* and inhibit the growth of anaerobic *Bacteroidia* in both surface wormcast and residual soil (Fig. 3a). In contrast, due to the particular anaerobic environment in the gut of earthworms, anaerobic bacteria introduced from digested sludge could be greatly enriched in the gut of earthworms compared to that before cultivation (Fig. 3a).

Digested sludge amendment did not increase the abundances of ARGs in soil after 80-days cultivation in the presence of earthworms

Digested sludge amendment greatly increased the abundances of ARGs in the digested sludge-amended soil (blank-0 soil) compared to those in the raw soil (Fig. 1). However, after 80-days cultivation, the absolute abundances of all target ARGs in the amended soil without earthworms (blank-80 soil) were reduced by 65%-95% compared with those in the blank-0 soil (Fig. 1a). Furthermore, the ARGs absolute abundances in blank-80 soil were even closed to those in raw soil. (Fig. 1a). This indicates that the increased ARGs in the amended soil caused by digested sludge were temporary and did not significantly influence the ARG abundances in soil after cultivation. This result was different from previous studies, in which amendment of sewage sludge permanently increased the abundances of ARGs in amended soils and on harvested vegetables due to the introduction of ARGs and colonization of ARB from sewage sludge [5-7, 32]. This might be due to that anaerobic digestion pretreatment of sewage sludge could

reduce ARGs abundances and their dissemination [11-13]. Besides, the proliferation of anaerobes in the digested sludge amend soil was greatly inhibited during the cultivation period, further impeding the colonization of antibiotic resistant bacteria from digested sludge in the amended soil. For example, oxygen exposure in soil had adverse effects on the obligate anaerobes, which were important ARGs hosts (e.g., *Bacteroidia*) in digested sludge, leading to the reduction of ARGs abundances in the digested sludge-amended soil [33, 34]. Therefore, anaerobic digestion pretreatment of sewage sludge prior to soil amendment may help mitigate the spread and dissemination of ARGs in the soil.

In the presence of earthworms, much lower abundances of target ARGs (except tetW) were found in surface wormcast and residual soil compared with those in the blank control soil and raw soil (Fig. 1), suggesting that earthworms had a positive effect on ARGs reduction in the digested sludge-amended soils. This could be explained by the following reasons. First, the amended soil was ingested and digested by earthworms, and the organic matters of the soil were degraded and transformed by the anaerobic and acidic digestive system of earthworms [12, 30]. The mucus released from earthworms could promote the degradation of organic matters [11]. ARGs, as one form of organic matter, might be degraded and utilized by the gut microbiome of earthworms [30, 35]. Second, earthworms could change ARGs abundances by shifting the microbial communities of soil [15, 33, 34, 36], reducing the abundances of potential ARGs hosts, such as obligate anaerobes *Bacteroidia* and *Clostridia* (Fig. 3 and Fig. 4).

Digested sludge amendment increased ARGs abundances in the gut of earthworms

Although earthworms contributed to the reduction of ARGs in soil, the relative abundances of most target ARGs in the gut of earthworms were greatly increased after 80-days cultivation (Fig. 1c). For instance, the relative abundances of tetA, sulII, intII, sulI, and bla_{TEM-1} increased by 41-130 folds after cultivation (Fig. 1c). Similar results had been reported in previous study that manure amendment enhanced the ARGs abundances in the gut of another common soil arthropod, such as collembolan [17]. PCoA analysis shows that the gene pattern of ARGs in the gut of earthworms after cultivation was similar to that of surface wormcast and residual soil rather than its initial gut gene pattern before 80-days cultivation (Fig. 2b). This was probably because the particular anaerobic and acidic environment of earthworm gut promoted the colonization and enrichment of some microorganisms (e.g., *Bacteroidia* and *Clostridia*, which were important ARGs hosts in the soil [33, 34]) in the gut of earthworms. These implied that the dietary composition could affect the abundances of ARGs in the gut of soil fauna [37, 38]. Although the abundances of ARGs in the gut of earthworms greatly increased after cultivation in the digested sludge-amended soil, the wormcasts did not enhance the ARGs abundances in soil (Fig. 1). This was probably because wormcasts would be exposed to higher concentration of oxygen after being excreted to soil (especially soil surface) by earthworms [39]. The oxygen in soil would alter the microbial community and facilitate the attenuation ARGs in wormcasts [15, 30, 35].

Significance of This Study

Activated sludge process is the widely applied biological wastewater treatment process. However, the treatment and disposal of massive sewage sludge have become a difficult and imminent problem. Since sewage sludge often contains abundant toxic pollutants (e.g., heavy metals and organic pollutants), ARGs, and various pathogens [3-5, 40-42], improper disposal of sewage sludge will bring severe environmental risks, such as ARG spread. Application of sewage sludge to agricultural soil is a common way of sewage sludge disposal, but this would greatly increase ARGs abundances in soil and crops, posing potential risks to public health [5-7]. This study shows that the enrichment of ARGs caused by dosing digested sewage sludge was temporary in soil, and they could recover to their original levels before sludge addition. Thus, our findings prove that anaerobic digestion can serve as a promising pretreatment for sewage sludge prior to soil amendment.

Besides, earthworms were found to play an important role in further reducing ARGs abundances in the soil. The particular physiological behavior and gut environment of earthworms significantly altered the microbial community of soil and reduced ARGs abundances [11-14, 30-32]. Earthworms also have excellent performance in environmental bioremediation, such as heavy metals and organic pollutants [43-46]. Some of these environmental pollutants may contribute to the horizontal transfer of ARGs [47, 48]. Therefore, vermicomposting may provide a feasible bioremediation strategy for polluted soils and excess sludge, achieving simultaneous removal of pollutants and reduction of ARGs. However, it should be noticed that although earthworms could enhance ARGs reduction in soils, the increased ARGs abundances in the gut of earthworms may pose potential risks of ARGs dissemination via food chain (e.g., to fish and poultry). Further studies are still required to comprehensively evaluate the potential impacts of earthworms in ARGs control.

Conclusions

This study shows the fate of ARGs and microbial communities in digested sludge-amended soil in the presence of earthworms. The augment of ARGs abundances in soil after digested sludge-amendment decreased to its original level before amendment due to the attenuation of ARG microbial hosts. Earthworms exhibited further reduction in ARGs abundance in the amended soil via shifting the microbial community of soil. However, increased ARGs abundances were observed in the gut of earthworms and further studies are required to evaluate the potential risks of ARGs spread via food chain (e.g., from earthworms to fish and poultry). These results could help to understand the fate and spread of ARGs in soil ecosystem containing microbiome and soil fauna (e.g., earthworms) and provide an alternative for ARGs control via bioremediation using earthworms.

Supplementary Information

Additional file 1: Table S1, the main characteristics of digested sludge and different soil samples.

Declarations

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Authors' contributions

LY designed the research. ZHL and WS performed the experiments. ZHL analyzed the data. ZHL, YL, and GPS wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The raw reads of 16S rRNA sequencing in format of .fastq have been deposited in the Sequence Read Archive (SRA) database (submission number SUB7769833).

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Spearman’s correlation coefficients between ARGs and intI1 in the soil.

	bla _{TEM-1}	tetQ	intI1	tetW	sulI	sulII
tetA	0.810*	0.381	0.714*	0.833*	0.833*	0.762*
bla _{TEM-1}		0.429	0.500	0.714*	0.905**	0.905**
tetQ			0.500	0.310	0.524	0.690
intI1				0.452	0.595	0.714*
tetW					0.857**	0.595
sulI						0.881**

Significant difference was indicated at * $P < 0.05$ or ** $P < 0.01$ levels

Figures

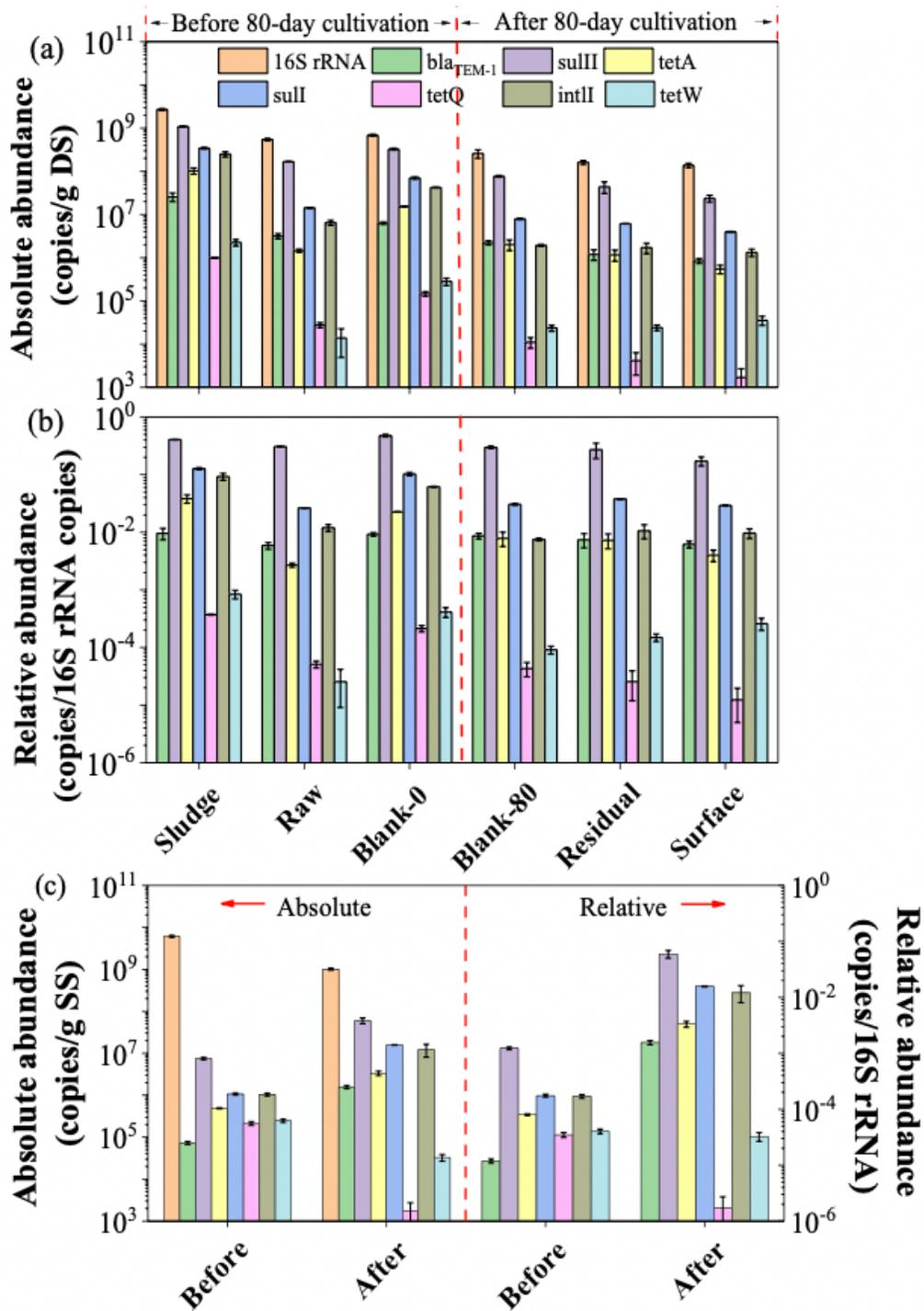


Figure 1

Variation of ARGs in soils and gut of earthworms before and after 80-day cultivation (a) Absolute and (b) relative abundances of target genes in soils before and after 80-day cultivation; (c) gene abundances in the gut of earthworms before and after 80-day cultivation. "Sludge", "Raw", and "Blank-0" represented the digested sludge, raw soil, and amended soil before 80-day cultivation, respectively; "Blank-80", "Surface", and "Residual" represented amended soil (without earthworms), surface wormcast, and residual soil (with

earthworms) after 80-day cultivation, respectively; “Before” and “After” represented the gut of earthworms before and after 80-day cultivation, respectively.

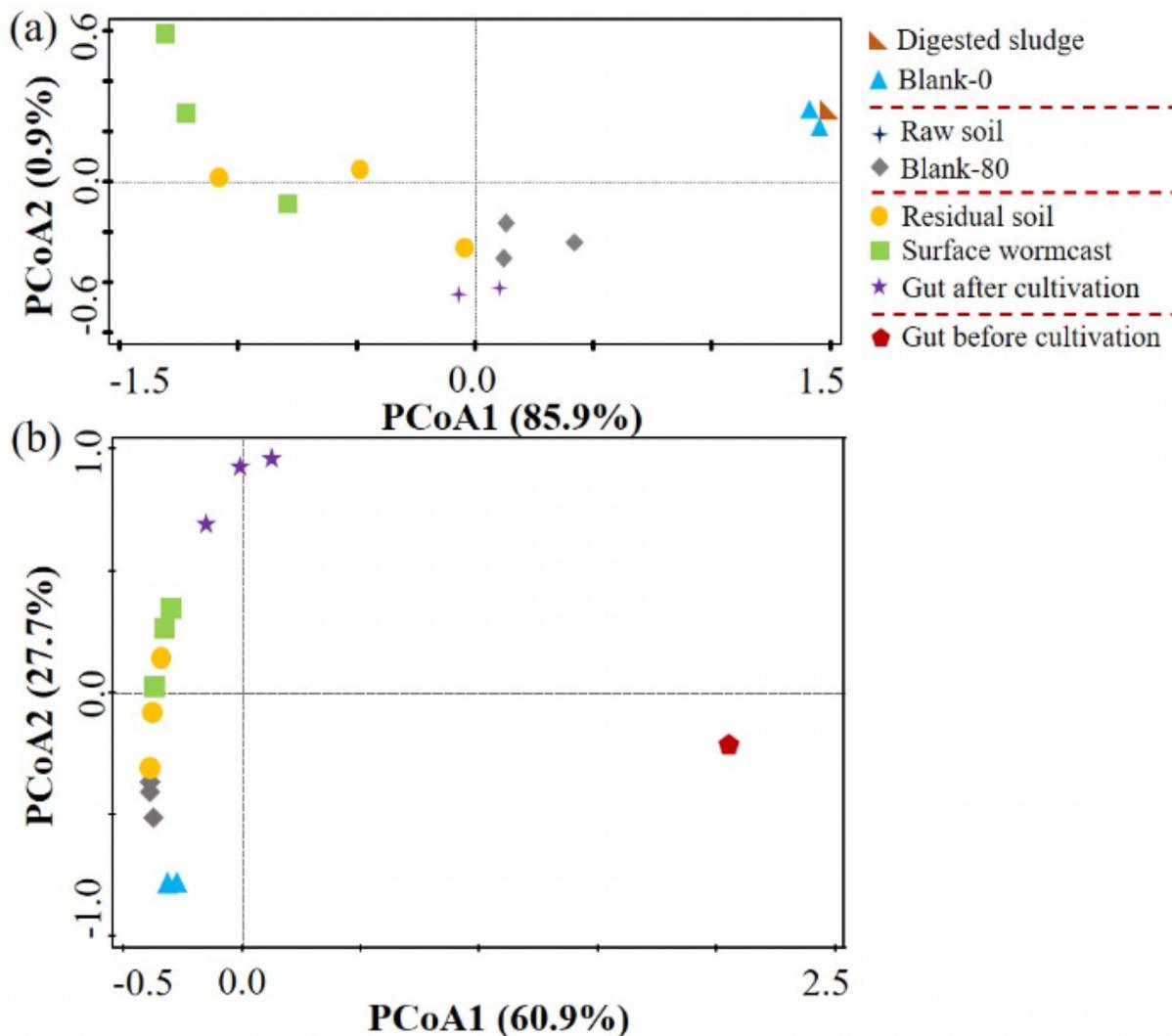


Figure 2

Differences in relative abundance of ARGs between soil and gut samples. PCoA analysis based on the relative abundance of ARGs showing the overall gene pattern in a soil samples, and b soil and earthworm gut samples before and after 80-day cultivation. “Blank-0” and “Blank-80” represented the sludge-amended soil (without earthworms) before and after 80-day cultivation, respectively.

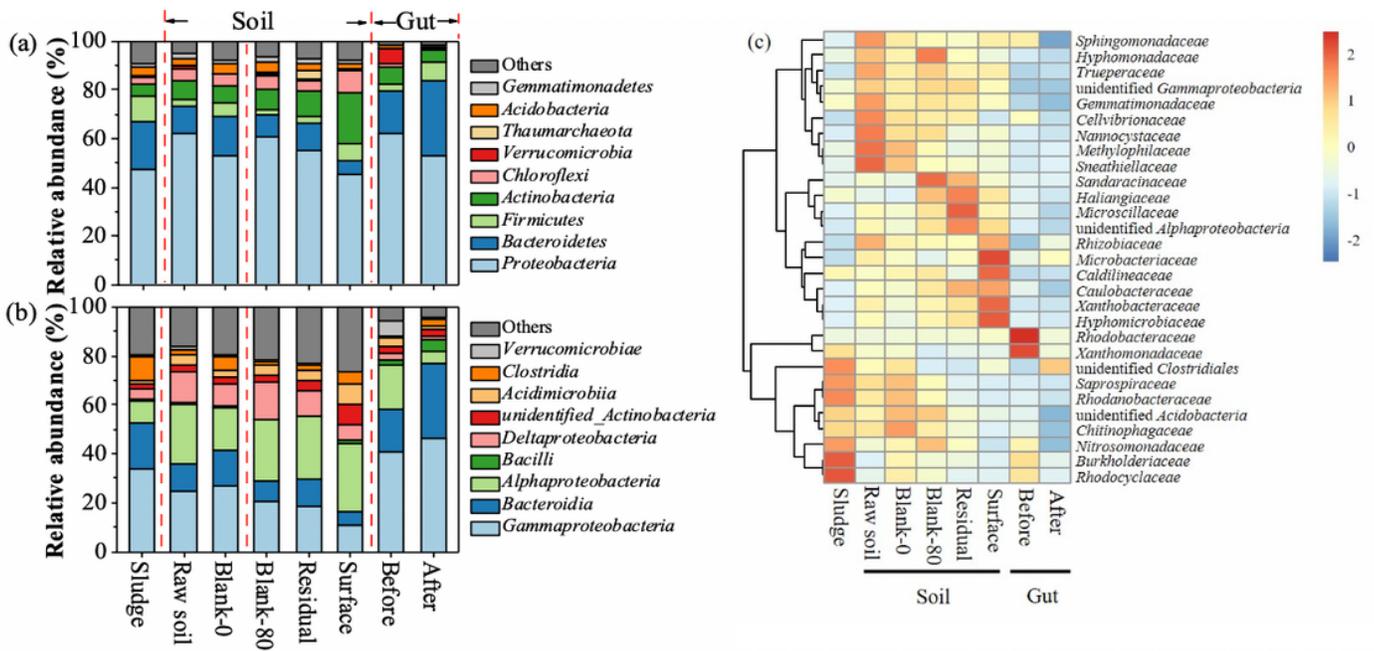


Figure 3

Changes in microbial community of soil and earthworm gut at a phylum and b class levels. c Heatmap describing the shift of microbial communities at the family level. “Sludge”, “Raw”, and “Blank-0” represented the digested sludge, raw soil, and amended soil before 80-day cultivation, respectively; “Blank-80”, “Surface”, and “Residual” represented amended soil (without earthworms) after 80-day cultivation, surface wormcast, and residual soil (with earthworms), respectively. “Before” and “After” represented the gut of earthworms before and after 80-day cultivation, respectively.

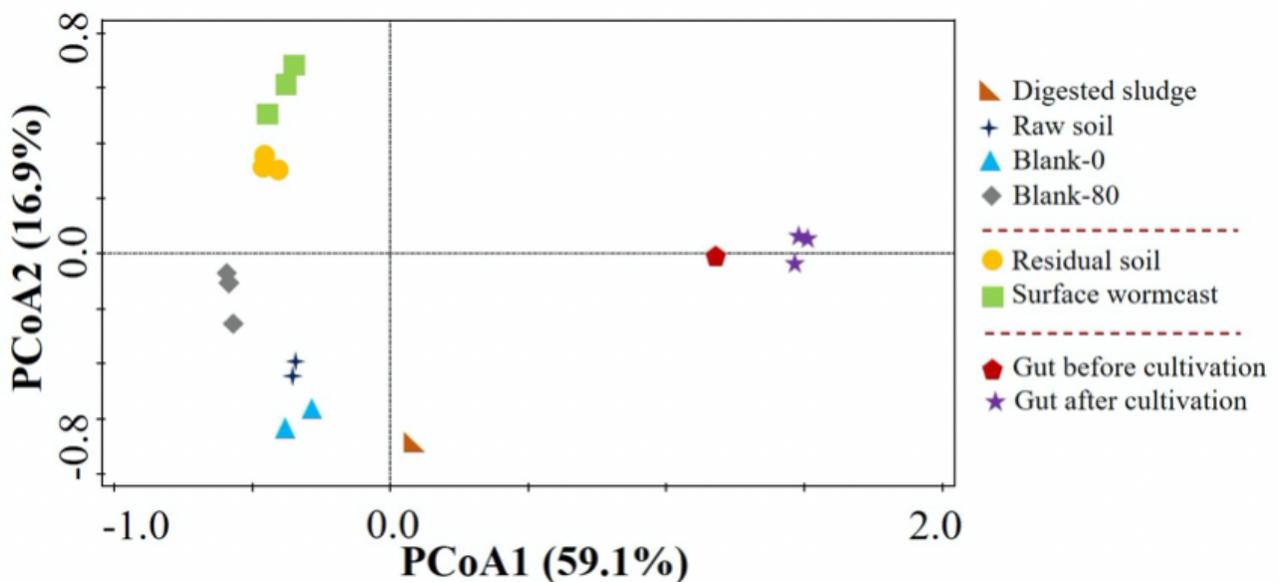


Figure 4

Differences in microbial communities between soil and gut samples. PCoA analysis showing the microbial community pattern in soil and earthworm gut samples before and after 80-day cultivation. "Blank-0" and "Blank-80" represented the sludge-amended soil (without earthworms) before and after 80-day cultivation, respectively.

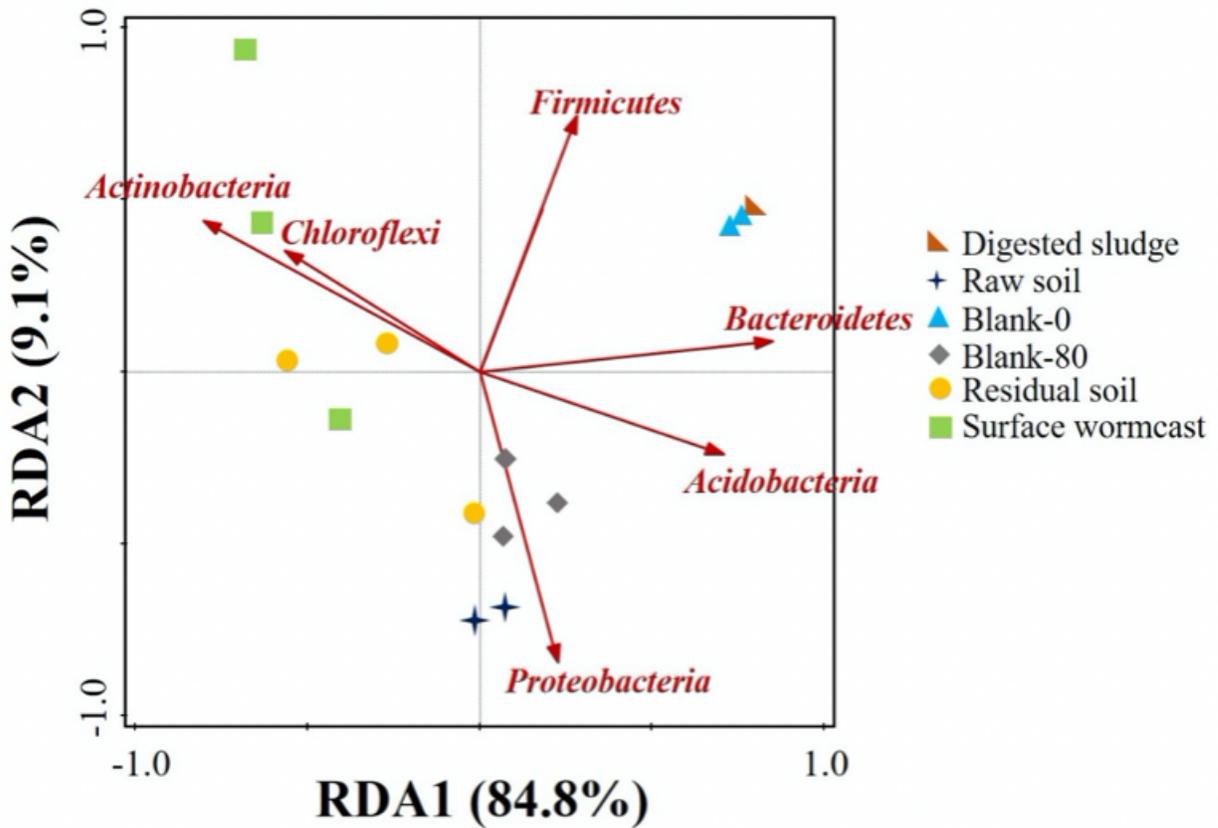


Figure 5

Redundancy analysis (RDA) of the quantitative correlation between microbial taxa and ARGs (based on relative abundance). "Blank-0" and "Blank-80" represented the sludge-amended soil (without earthworms) before and after 80-day cultivation, respectively.

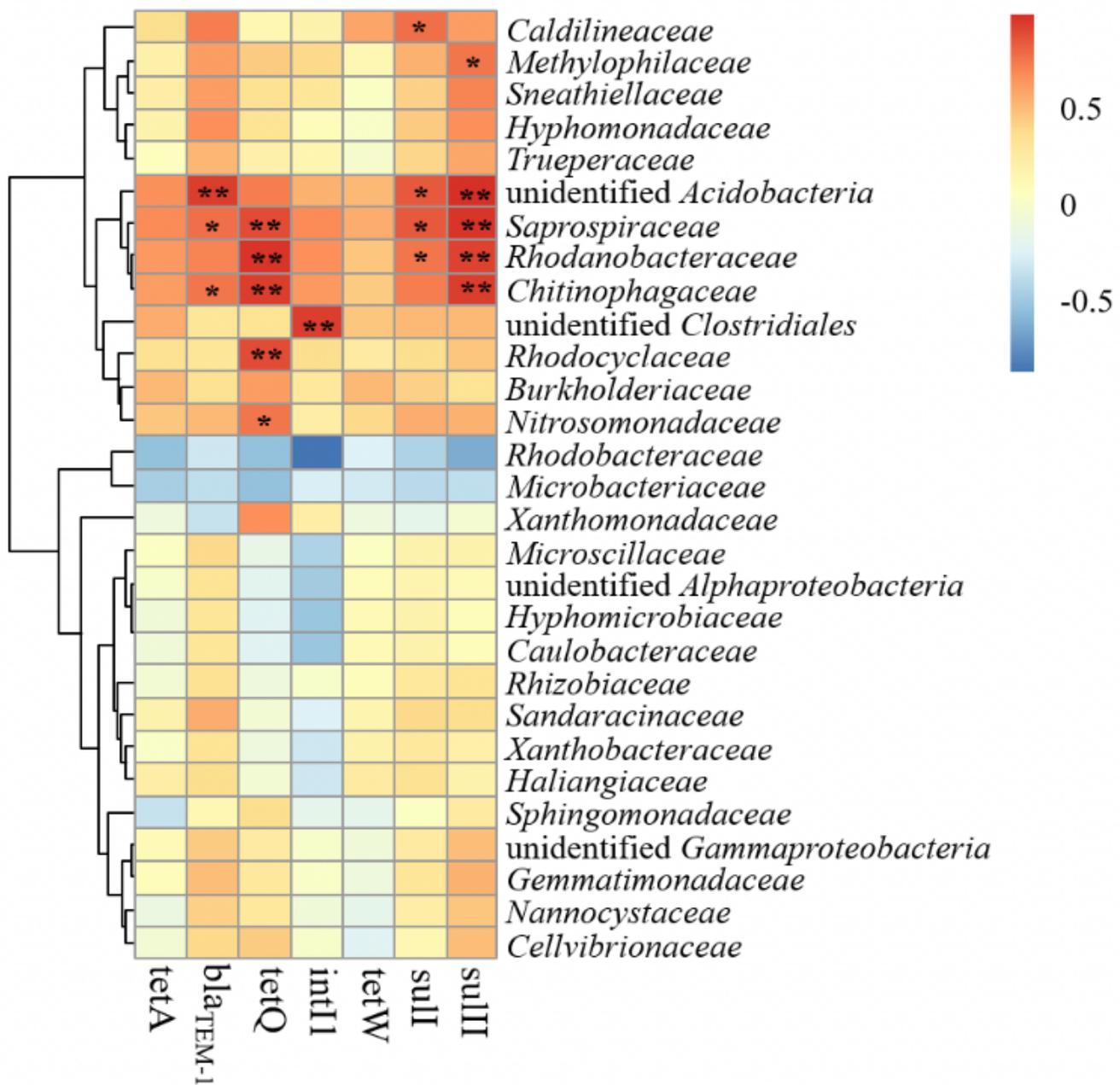


Figure 6

Relationships between ARGs and microbial community at family level. Heatmap describing the quantitative correlation of the bacterial family and ARGs in soil and earthworm gut samples. The scale bar shows the Spearman index (R) that red represents a positive correlation and blue represents a negative correlation, Significant difference was indicated at *P < 0.05 or **P < 0.01 levels.

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