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# Effects of earthworms on the fate of tetracycline and fluoroquinolone resistance genes of sewage sludge during vermicomposting

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**Abstract**

Diverse antibiotic resistance genes (ARGs) present in sewage sludge are difficult to be eliminated using conventional sludge treatment processes. To date, little remains known on the fate of the ARGs during vermicomposting of sludge. This study aimed to investigate the effect of earthworms on the fate of tetracycline and fluoroquinolone resistance genes, and integrons during vermicomposting of sewage sludge through contrasting two systems of sludge stabilization with and without earthworms. Compared to the control without earthworms, vermicomposting significantly ($p$<0.05) decreased the abundances of tetracycline and fluoroquinolone resistance genes and *int*1, with complete removal for *par*C. Variations in ARGs were associated with environmental factors, horizontal gene transfer, bacterial community composition, and earthworms during vermicomposting. In addition, earthworms strongly affected the possible host bacteria encoding ARGs and *Int1*, abating the pathogenic bacteria in vermicomposting product. These results imply that vermicomposting could effectively reduce tetracycline and fluoroquinolone resistance genes in the sludge.

**Keywords**: Antibiotics; Antibiotic resistance gene; Earthworms; Sewage sludge; Vermicomposting

## Introduction

The excessive use of antibiotics for human and veterinary applications results in widespread occurrences of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) in the natural environment. Waste water treatment plants (WWTP) receive sewages from different sources, making them possible the hotspots for ARB and ARGs (Guo et al., 2017; Rizzo et al., 2013). As the by-product of WWTP, high abundances of diverse ARB and ARGs have also been documented in dewatered sewage sludge (Li et al., 2013; Guo et al., 2017; Karkman et al., 2017). In contrast to the removal efficiency of ARGs in sewage treatment, their removal in sludge treatment seems less efficient (Yang et al., 2014). Moreover, the sewage sludge containing high organic matter and diverse microorganisms enables the ARGs to propagate and disseminate among bacterial species through horizontal gene transfer (HTG, Li et al., 2013; Guo et al., 2017). The HTG can help spreading the ARGs from commensal and free-living species to pathogenic ones through three canonical modes of conjugation, transformation and transduction, making the ARGs difficult to be completely removed from the environment (Von Wintersdorff et al., 2016). Consequently, to control the ARB and ARGs in sludge, several studies have investigated their fates in different sludge treatment systems (Su et al. (2015; Yang et al., 2016; Zhang et al., 2016; Yang et al., 2014; Wu et al., 2016). Aerobic composting and anaerobic digestion are considered potential recycling methods for treating sludge. However, previous studies showed that the abundance and diversity of ARGs were significantly elevated during thermal composting for sludge (Su et al. (2015; Yang et al., 2016; Zhang et al., 2016). In addition, Su et al. (2015) found 156 unique ARGs and mobile genetic elements in composted sewage sludge. Compared to composting, thermophilic anaerobic digestion

at temperatures between 50ºC-55ºC appears to produce better results in terms of reducing the ARGs (Diehl and LaPara, 2010; Wu et al., 2016). However, only a small portion of certain types of ARGs can be limited by anaerobic digestion (Yang et al., 2014; Wu et al., 2016). As a result, there is an ongoing search for a suitable approach to control the ARGs during sludge recycling.

Vermicomposting is a biochemical decomposition process of organic wastes involving the interaction of earthworms and microbes. Compared to the usual compost, vermicomposting product has higher contents of plant-available nutrients and much more diverse agricultural and aquacultural probiotics (Huang et al., 2016; Sharma and Garg, 2018). Thus, vermicompost is deemed as a microbial fertilizer mostly applied to agricultural lands (Sharma and Garg, 2018) and aquacultural operations (Godara et al., 2015a, 2015b). Vermicomposting for recycling sewage sludge has also been successfully demonstrated by several studies (Yasir et al., 2009; Rodríguez-Canché et al., 2010; Xing, et al., 2012; Fu et al., 2015; Fernández-Gómez et al., 2015; Villar et al., 2016). In addition, previous studies also found that human pathogenic bacteria present in the sludge could be significantly reduced after vermicomposting, in contrast to usual composting methods (Rodríguez-Canché et al., 2010; Godara et al., 2015c; Soobhany et al., 2017). However, to the best of our knowledge, only a few attempts have been made to investigate the effects of earthworms on the fate of ARGs during sludge vermicomposting.

It has been reported that the combined actions of earthworms and microorganisms helped in degrading the organic matter component during vermicomposting (Domínguez et al., 2010; Gómez-Brandón et al., 2011; Villar et al., 2016). Simultaneously, earthworms also strongly affect microbial growth and reproduction (Domínguez et al., 2010; Gómez-Brandón et al., 2011; Villar et al., 2016). Accordingly,

the microbial community is directly and indirectly regulated by the gut behavior during digestion of earthworms and their non-trophic behaviors such as burrowing, mucus excretion and castings (Gómez-Brandón et al., 2011; Hoang et al., 2016; Huang et al., 2018). It is well known that the ARGs are harbored in possible microbial hosts (Li et al., 2015). Recent studies have focused on the relationship between earthworms and microbes (Gómez-Brandón et al., 2011; Yasir et al., 2009; Villar et al., 2016; Huang et al., 2018), but little information is available on the relationship between earthworms and possible hosts of ARGs in vermicomposting systems. To effectively eliminate the ARGs in vermicompost, it is of utmost importance to investigate the effects of earthworms on the ARGs and their possible host.

This study then aims to investigate the effects of earthworms on the fate of the ARGs and to further understand the relationship between earthworms and the possible hosts of ARGs. For this, tetracycline and fluoroquinolone resistance genes, the first two main sub-types ARGs in sludge, were monitored during vermicomposting. Moreover, the Int1 of integron gene involved in the horizontal gene transfer of the ARGs in microbes was also monitored in this study.

## 2 Methods

### *2.1 Materials*

The earthworm *Eisenia foetida* was chosen as the model species for this study. Prior to the experiment, earthworms were cultured in a mixture of dewatered sludge and cow dung (1:1 dry basis) for 2 months in the laboratory. The vermicomposting reactor was made of a plastic box with dimensions of 46 cm×17 cm×13 cm. To provide an

aerobic environment, all reactors were drilled on the bottom. Freshly dewatered sewage sludge was collected from the dewatered sludge workshop of the WWTP in Anning Distinct, Lanzhou city. Then, the fresh sludge was immediately pelleted by squeezing it with wire meshes having sizes of 5 mm×5 mm in the laboratory, following the methods of Fu et al. (2015). The properties of fresh sludge are given in the Table 1.

*2.2 Experimental set up*

Around 4 kg fresh pelleted sludge was placed into the vermicomposting reactor. Then, 100 young *E. fetida* with a mean individual weight of 0.3 g and individual length of 3 cm - 5 cm were randomly selected from the culture bins and inoculated into the vermicomposting reactor. In parallel, the reactors containing the same sludge but without earthworms were used as the control treatment. Both vermicomposting and control treatments were designed with three replicates. All reactors were covered with a shade cloth and kept at room temperature (18 ºC- 26 ºC). To maintain water moisture, the tap water was sprinkled once every 3 days. To make the environment aerobic, all reactors were turned over every week. After being thoroughly mixed, an approximate 100 g fresh sample was collected from each reactor at 20 days intervals. In this study, each sample was collected in duplicate. After 80 days of experiment, earthworms and their cocoons were picked up and counted by hands, respectively. The collected samples were divided into two sub-samples for the other analysis. One was stored into -20 ºC for enzyme and DNA related analysis while the other half was dried under room condition and used for measuring chemical properties.

*2.3 Chemical properties analysis*

151

152 The dry samples were ground and sieved through 80 mesh for the next chemical
153 analysis. All chemical analyses were based on the the Chinese standard of determination
154 method for municipal sludge in waste water treatment plant (CJ/T 221-2005), with some
155 modifications suggested by Huang et al. (2017). The mixed sample and deionized water
156 (dry sample/water = 1/50, w/v) was used in measuring pH and electrical conductivity
157 using a pH meter (PHS-3C, LEICI, China) and electrical conductivity (DJS-1, LEICI,
158 China) at 20 ºC, respectively. Total carbon and nitrogen were measured by an elemental
159 analyzer (Yanaco CHN CORDER MT-6, Japan). The mixture of dry sample and Milli-Q
160 water (dry sample/water = 1/200, w/v) filtered through a 0.45 μm membrane was
161 divided into triplicates. One portion was used to determine nitrate, ammonia and
162 phosphate by ion chromatography (SHIMADZU, Japan). The other portion was used in
163 determining dissolved organic carbon (DOC) by TOC analyzer (SHIMADZU, Japan).
164 The rest of the mixture was utilized for three-dimensional fluorescence excitation
165 emission matrix spectroscopy (3D-EEM) using a fluorescence spectrophotometer
166 (RF-5300PC, SHIMADZU, Japan). Following the methods of Fernández-Gómez et al.
167 (2015), the fluorescence-based humification index of $A_{435-480}/A_{300-345}$ was calculated
168 using the fluorescence emission spectrum at an excitation wavelength of 254 nm. This
169 was determined by dividing the area from 435 nm to 480 nm by the area from 300 nm
170 to 345 nm. Dehydrogenase activity was determined by triphenyl tetrazolium chloride
171 method using chromatometry at the 485 nm by spectrophotometer.

172

173 *2.4 DNA extraction and absolute quantification of 16S rDNA gene*

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175 Total genomic DNA was extracted with the DNA Isolation kit DNeasy®

PowerSoil® Kit (QIAGEN, Germany) according to the manufacturer's instructions. The extracted DNA was stored under -20 ºC before use.

The universal primers 341F and 518R (given in Supplementary information) were used to quantify the 16S rDNA gene copies in the Thermal Cycler Dice Real Time System (TP800, TaKaRa, Japan). The standard curve was established using the 16S rDNA of *Escherichia coli*. The SYBR® Premix Ex Taq™ (TaKaRa, Japan) was used as the fluorescent dye for the quantitative PCR reaction. The qPCR program was composed of an initial denaturation at 95 ºC for 5 min, 35 cycles of 95 ºC, 15 s, 57 ºC, 30 s and 72 ºC, 30 s, followed by a melting curve from 60 ºC to 95 ºC. The data was automatically collected at the last step of each cycle. The control without DNA was also set up for comparison. All templates were amplified three times.

*2.5 High-throughput quantitative PCR*

The primers for tetracycline resistance genes (*tet*C, *tet*G, *tet*M, *tet*O, *tet*W and *tet*X), fluoroquinolone resistance genes (*gry*A, *par*C and *qnr*S) and class 1 integron gene (*int*1) as well as the 16S rDNA gene were used for the relative quantitative PCR. Additional details on the primers are given in Supplementary information. The relative quantification reaction was conducted with a high through-put quantitative real time PCR system (Applied Biosystems, ViiA$^{TM}$7, USA) at Wcgene Biotechnology Co. Ltd, (Shanghai, China). The FastStart Universal SYBR Green Master (ROX) (Roche, USA) was selected as the fluorescent dye. The PCR reaction procedure was initialized at 95 ºC for 10 min and followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 30 s. The program incorporated an automatic heating and melting curve analysis. Each reaction was quantified in triplicate. A comparative CT method was used to calculate the fold change

(FC value) of each ARG, as described by Su et al. (2015).

*2.6 PCR and high through-put sequencing of 16S rDNA*

The V3-V4 region of the 16S rDNA gene was amplified using the primers 341F and 806R (given in Supplementary information) conjugated with barcode base pairs. All PCR reactions were carried out with the Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The resulting PCR products were detected using 2% agarose gels and then purified by GeneJET Gel Extraction Kit (Thermo Scientific, USA). The Ion Plus Fragment Library Kit 48 rxns Kit (Thermofisher, USA) was utilized in establishing the sequencing library and its quality was assessed using the Qubit®2.0 Fluorometer (Thermo Scientific, USA). Subsequently, the library was sequenced on a Life Ion S5$^{TM}$ platform at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

The raw reads were first trimmed to obtain high-quality clean tags based on Cutadapt (V1.9.1, http://cutadapt.readthedocs.io/en/stable/) quality control pipeline. Then, the reads were compared to the reference database "Gold database" (http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html). After removing the chimeric sequences, the high quality tagged sequences were obtained. Clustering of the OTUs (Operational Taxonomic Units) were set at >97% similarity using Uparse package (Uparse v7.0.1001, http://drive5.com/uparse/). A representative sequence for each OTU was classified taxonomically by comparing with the SILVA SSU rRNA database (http://www.arb-silva.de/) via Mothur (Schloss et al., 2009).

226 *2.7 Statistical analysis*

227

228 Significant differences among the chemical parameters and ARGs with time
229 between vermicomposting and control treatments were analyzed by repeated measures
230 ANOVA (n=6) at 95% confidence level using STATISTIC 10.0 software. The relative
231 abundances of ARGs and bacterial community were presented as heatmap diagrams
232 generated using Heml 1.0 software. The alpha and beta diversities of bacterial
233 communities were calculated using weighted UniFrac distance between samples within
234 QIIME (Version 1.9.1, http://qiime.org/index.html/) (Li et al., 2015). LDA Effect Size
235 (LEfSe) analysis was used to differentiate the abundant and biologically relevant
236 features, ranked by effect size after undergoing linear discriminant analysis (LDA).
237 Redundancy analysis (RDA) was performed with the selected environmental variables
238 and the ARGs using CANOCO 4.5 software. A correlation matrix among ARGs and
239 bacterial community was constructed statistically, and only those with Spearman's
240 correlation coefficient ($R^2$) of > 0.9 at $P < 0.01$ were retained (Junker and Schreiber,
241 2008). Cytoscape 3.6.0 software was used to visualize the network graphs using the
242 attribute circular layout (Li et al., 2015).

243

244 **3. Results and Discussions**

245

246 *3.1 Effects of earthworms on sludge stabilization*

247

248 The stability and maturity of sludge should be assessed using a set of
249 complementary parameters while considering the complexity of sludge samples,
250 (Fernández-Gómez et al., 2015). Consequently, the content and characteristic of

dissolved organic carbon (DOC) as well as microbial activity and abundance were combined to evaluate sludge stabilization in the present study. As shown in Fig. 1a, DOC contents significantly decreased in both vermicomposts and control treatments but with significant differences ($F=8871.5$, $p<0.001$). Specifically, a more rapid decrease in DOC content was observed in the treatment with earthworms compared to those without earthworms. The DOC contents in the final vermicomposting products and control were at 3.78 mg/kg and 15.61 mg/kg, respectively. Accordingly, a threshold of DOC lower than 4.0 mg/kg in the manure vermicompost suggests that a manured vermicomposting product was harvested in this study (Xing et al., 2012). Fig. 1b displays the change of humification index based on $A_{435-480}/A_{300-345}$ during the experiment derives from the 3D-EEM results. Compared to the control, vermicomposting produced significantly higher values (HST test, $p<0.001$) fro humification index from the 60th day of the experiment, which indicates that the activity of earthworms boosted the humification process in the last 20 days. The humification index of vermicomposting product was associated with the gradual increment in humic-like and fulvic-like substances in the vermicompost, as shown in Supplementary information. Similarly, Xing et al. (2012) reported that earthworms could influence the conversion of aromatic-like and protein-like substances into humic-like and fulvic-like substances during vermicomposting.

As depicted in Fig. 1c, a gradually decreasing trend of dehydrogenase activity was observed in both treatments, with the vermicomposting product having significantly ($F=379.3$, $p<0.05$) lower value. For the bacterial 16S rDNA gene abundance, it increased continuously in the control treatment, which was significantly higher ($F=5541.6$, $p<0.001$) than those in the vermicomposting treatment. Whereas, vermicomposting treatment displayed an increase on the first 20 days followed by a

sharp decrease on the 40$^{th}$ day, and finally stabilized towards the end. This break point at the beginning of vermicomposting could be probably due to the fact that the addition of earthworms carrying a certain amount of mucus stimulated microbial activity and bacterial numbers within the initial experiment (Huang and Xia, 2018). In contrast, vermicomposting product with lower microbial activity and abundance but higher humification index implies that the sludge was stabilized and matured by earthworms.

*3.2 Effects of earthworms on bacterial community during sludge stabilization*

After sequencing and quality filtering, an average of 847 high quality OUTs were obtained in each sample. Compared to the control, vermicomposting exhibited a relatively higher Shannon and Chao 1 indices during the experiment (shown in Supplementary information), indicating that the inoculation of earthworms could enhance the diversity and evenness of the bacterial community in the sludge. In addition, the compositions of bacterial community showed distinct variations in both treatments (Fig. 2a). Proteobacteria, Bacteroidetes, Actinobacteria, Saccharibacteria, and Frimicutes were the five most dominant phyla in the initial substrate, accounting for over 90% of the bacterial 16S rRNA gene abundance. Being the most abundant phylum in the sludge, Saccharibacteria decreased dramatically from the 20$^{th}$ day, and almost disappeared in the final products of both treatments. In contrast, Proteobacteria, Bacteroidetes, and Frimicutes became abundant from the 20$^{th}$ day onwards in the reactors. Moreover, from the 40$^{th}$ day, a remarkable difference in bacterial community compositions between vermicomposting and control was observed. At the end of the incubation, the vermicomposting product was already dominated by Proteobacteria (31.3%), Bacteroidetes (27.1%), and Actinobacteria (21.1%). In contrast, the

Proteobacteria (56%), Bacteroidetes (16.3%), and Frimicutes (15.4%) were found to dominate in the end products without earthworms. Yasir et al. (2009) also reported that the largest group were the Proteobacteria (47.9%), followed by Bacteroidetes (31.2%) and Actinobacteria (6.4%) in the vermicompost obtained from cow dung and sludge.

Further analysis using LDA effect size revealed significant differences ($p$<0.05) between vermicomposting and control treatments, as displayed in Fig. 2b. Results showed that the Actinobacteria, specifically the *Micrococcales*, significantly increased in the vermicomposting system. The Actinobacteria are capable of decomposing refractory organic matter and producing antibiotics, which are considered estimators of composting product (Xiao et al., 2011). The higher abundance of the Actinobacteria should be linked with the gut digestion of earthworms and their castings (Knapp et al., 2009; Yasir et al., 2009). Pathma and Sakthivel (2013) reported that the abundance of the *Micrococcale*s could reach to 12% in the vermicomposting product. In addition, the Rhizobials also became enriched as part of the agricultural probiotics in the final vermicomposting product, suggesting that the earthworms promoted the potential of vermicompost from sludge as microbial fertilizer. On the other hand, the control treatment had higher abundances of the Bacteroidales and Pseudomonadales, which are often considered strong degraders of organic matter (Gao et al., 2016). The anaerobic environment and non-stable product without earthworms could be responsible for the enrichment of the degrading-bacteria.

### *3.3 Effects of earthworms on the ARGs and Int1*

Six tetracycline resistance genes (*tet*C, *tet*G, *tet*M, *tet*O, *tet*W, *tet*X), three fluoroquinolone resistance genes (*gry*A, *par*C, *qnr*S), and class 1 integron (*Int1*) were

monitored during sludge stabilization, as shown in Fig. 3. For tetracycline resistance, the *tet*X was detected as the dominant gene, followed by *tet*G and *tet*M in all reactors (Fig. 3a). Compared to the initial sludge, the tetracycline resistance genes in control treatments increased by 1-27 fold during the experiment, showing the largest increment in *tet*X. This finding indicates that the potential host bacteria with ARGs propagated in the reactor. However, except for *tet*X and *tet*G, other tetracycline resistance genes showed a declining trend after vermicomposting. In contrast to the control product, the abundances of the *tet*C, *tet*G, *tet*M, *tet*O, *tet*W and *tet*X in the end vermicompost were reduced by 83.1%, 39.6%, 99%, 80.2%, 94.1% and 60.9%, respectively. Such result indicates that the inoculation of earthworms could attenuate the abundance of tetracycline resistance genes. In addition, the effects of earthworms on all of tetracycline resistance genes were exceedingly significant ($p<0.001$) during vermicomposting, as described in Fig. 3b. As for fluoroquinolone resistance genes, there is a similar trend to tetracycline resistance genes, displaying lower content of fluoroquinolone resistance genes in vermicomposting treatment (Fig. 3a). Compared to the end-product of the control, the abundances of *gry*A, *par*C, *qnr*S in the vermicompost decreased by 57.2%, 100% and 90%, respectively. Moreover, the *Int*1 content in both reactors increased in the first stages of the incubation decreased towards the end (Fig. 3a). Compared to the control, the abundance of *Int*1 was 68.1% lower in the final vermicomposting product, suggesting that vermicomposting abated the potential risk of the ARGs dissemination.

The increased abundances of ARGs in the control system is similar to previous studies in sludge composting (Su et al. 2015; Wei et al., 2014). Su et al. (2015) reported that the total ARGs and tetracycline resistance genes detected significantly increased during sludge composting process. Although high temperature can slightly reduce the proportion of ARGs in the thermophilic stage, the abundance of *tet*X still increased in

the maturation phase (Wei et al., 2014; Zhang et al., 2016). However, high removal efficiency for ARGs was observed in the composting system treating animal wastes (Zhang et al. 2017; Qian et al., 2018). This difference could be explained by the environmental pressures that anaerobic gut microorganisms dominating in animal wastes could not survive in aerobic and high temperature conditions during composting (Su et al., 2015). In the present study, earthworms significantly lowered the abundances of the ARGs and *Int*1 during vermicomposting (Fig. 3b), notably for the complete removal of the *par*C. This finding could be mainly associated with earthworms being able to regulate the bacterial community and environmental factors in vermicomposting system. Similarly, over 80% of ARGs abundance was significantly reduced after short-term gut digestion process in a larvae (Wang et al., 2017). Further, antibiotics decomposition was accelerated by earthworms associated with symbiotic microorganisms (Cao et al., 2018), diminishing the selective pressure of antibiotic microbes (Zhang et al., 2013; Qian et al., 2016), thus resulting in the reduction of ARGs. However, the final vermicompost still containing a certain amount of ARGs is consistent with the vermicompost produced from swine manure by larvae (Wang et al., 2017).

*3.4 Relationships among environmental factors, ARGs and bacterial community in vermicomposting*

The relationship between environmental parameters and ARGs was evaluated by RDA analysis (Fig. 4). Results showed that the selected variables could account for 74.3% of the total variations in the first two axes. The *tet*G, *tet*M, *tet*X, *tet*W, *tet*O, *qnr*S and *Int*1 correlated positively with pH, $NH_4^+$, phosphate, total carbon and total nitrogen

($p$<0.05) in the control treatment after 40 days. Meanwhile, *Int*1 exhibited significant ($p$<0.05) positive relationship with *tet*G, *tet*M, *tet*X, *tet*W, *tet*O, *qnr*S, indicating that these ARGs could rapidly be disseminated through horizontal gene transfer in the control treatment from the 40$^{th}$ day. Qian et al. (2016) highlighted that *Int*1 played an important role in the variation of ARGs during composting of manure wastes. Interestingly, vermicomposting displayed a significantly negative relationship ($p<0.05$) with the ARGs profile during vermicomposting. Accordingly, the frequency of horizontal gene transfer was affected by pH, temperature and cell density (Johnsen and Kroer, 2006). Thus, an affinity of ARGs and *Int*1 should be linked with a higher microbial number in the control (Fig. 1). Moreover, the high nutrient enriched in control could be another contributor for the distribution of ARGs (Zhao et al., 2017). The above results suggest that the environmental factors strongly affected the variation of ARGs, especially in control system.

Network analysis could provide new insights into ARGs and their possible hosts in complex environmental scenarios if the ARGs and the co-existing bacterial taxa had significantly positive correlations (Li et al., 2015; Qian et al., 2018). As shown in Fig. 5, there are two different network modules in control and vermicomposting systems. A total of 12 bacterial orders emerged as possible hosts for the co-occurring ARGs in vermicomposting system. The dominant possible hosts encoding ARGs were affiliated with Proteobacteria and Antinobacteria in vermicomposting. In addition, the control showed 11 possible host bacterial orders carrying ARGs, with the largest group in Proteobacteria, followed by the Bacteroidetes, and Frimicutes. In this study, the possible host bacteria group was consistent with the dominant bacteria in each treatment system, which indicates that the bacterial community plays an important role in varying the ARGs during vermicomposting process. Such result is coherent to previous studies of

sludge composting system (Su et al., 2015; Zhang et al., 2016). However, the members of Bacteroidetes that dominated in vermicomposting did not carry any ARGs in this study, suggesting that the earthworms also exerted a strong effect on the possible bacterial host during vermicomposting. The forward gut organs and anaerobic environment of the gut could directly shift bacterial community (Drake and Horn, 2007; Gómez-Brandón et al., 2011). Mucus excretion and aerobic burrowing of earthworms also modified microbial community diversity (Huang et al., 2018; Hoang et al., 2016). Similar finding was reported by Wang et al., (2017), where they suggested that ARG attenuation during vermicomposting with larvae was significantly correlated with changes in microbial community succession, especially reduction in Clostridiales and Bacteroidales.

In vermicomposting system, the members of Antinobacteria (Corynebacteriales, PeM15, Actinomycetales, Solirubrobacterales and Micromonosporales) encoded several ARGs such as *tet*C, *tet*G, *tet*X and *gry*A, which was related to the excretion of their antibiotics. The Antinobacteria contain resistance genes as a self-protecting mechanism towards antibiotics (Thaker et al., 2013). Additionally, the *Int*1 was present in Lactobacillales and Rhodocyclales, and positively correlated with the *qnr*S. The *Int*1 was not fully removed by vermicomposting, which could be due to the contributions of the gut microbiota of earthworms, since the members of Lactobacillales and Rhodocyclales inhabited and predominated in the gut of earthworms (Wüst et al., 2017).

In control system, the gene-type *tet*X was strongly harbored in Flavobacteriales. Also, Pseudomonadales encoded four ARGs including *tet*M, *tet*C, *tet*G and *gry*A. The *Acinetobacter* and *Pseudomonas* genera, as members of Pseudomonadales, have been affirmed as the persistent ARGs in manure-treated soils (Leclercq et al., 2016). Moreover, Zhang et al. (2016) also found that the Pseudomonadales, Bacillales, and

Bacteroidales were significantly correlated with some tetracycline resistance genes during sludge composting. The *Int*1 also showed significant correlation ($p$<0.01) with *tet*G and *tet*M in the control system. Previous studies have reported that the *Int*1 was commonly associated with multiple drug resistant genes (Chen et al., 2015). Compared to vermicomposting system, the *Int*1 harbored in different bacterial hosts and encoding different ARGs suggest that the addition of earthworms could lead to dissimilar dissemination of ARGs.

It is worthy to note that Flavobacteriales, Campylobacterales and Spirochaetales enriched human pathogenic bacterial species, which also took along diverse ARGs (*tet*X, *tet*G and *tet*O) in control system as compared to vermicomposting system. The ability of earthworms to effectively decrease pathogenic bacterial abundance has been documented by several studies (Rodríguez-Canché et al., 2010; Soobhany et al., 2017). This could be mainly due to the excretion of fibrinolytic enzymes and antibacterial substances from earthworms, which have negative effects against the pathogenic bacteria (Li et al., 2011). Additionally, considering that the complex relationships among earthworms, environmental factors, microbial communities and ARGs are present in a vermicomposting system, it is still difficult to exactly know the main contributor to the abundance and diversity of ARGs in this study. Hence, investigations on the underlying mechanisms regarding how earthworms affect ARGs are still required to be explored further.

## 4. Conclusions

Compared to the control without earthworms, the abundances of tetracycline and fluoroquinolone resistance genes, and class 1 integron were reduced by

vermicomposting, with 100% removal for *par*C. The variations of ARGs were influenced by environmental factors, bacterial community abundance and horizontal gene transfer, notable for the control system. The members of Proteobacteria and Antinobacteria were the potential hosts in vermicomposting. The inoculation of earthworms strongly affected the possible host bacteria encoding ARGs and *Int1*, decreasing the pathogenic bacteria in vermicomposting product. This study suggests that vermicomposting could be effectively used to lower tetracycline and fluoroquinolone resistance genes of the sludge.

E-supplementary data for this work can be found in e-version of this paper online.

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629 **Captions of tables and figures**

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631 **Table 1** Chemical properties of the initial sludge used

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633 **Fig. 1** Changes of DOC, humification index, dehydrogenase activity and 16S rDNA
634 gene abundance of vermicomposting and control treatments during sludge stabilization
635 process.

636 **Fig. 2** Bacterial community of weighted UniFrac distances (a) and of LDA score
637 diagram (b) in vermicomposting and control treatments during sludge stabilization
638 process.

639 **Fig. 3** Abundances of ARGs (a) of vermicomposting and control treatments and their
640 significant differences (b) during sludge stabilization period.

641 **Fig. 4** Redundancy analysis of the relationship between environmental factors and
642 antibiotic resistance genes.

643 **Fig. 5** Network analysis of co-occurring ARGs and possible host bacteria (top 50 order)
644 based on Pearson's correlation coefficients ($P < 0.01$, $R^2 > 0.90$) in control (a) and
645 vermicomposting (b) systems.

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**Table 1** Chemical properties of the initial sludge used.

| Parameters | Initial sludge |
|---|---|
| pH | 6.80 ± 0.03 |
| Water content (%) | 78.4 ± 0.30 |
| Electrical conductivity (mS/m) | 1.71 ± 0.01 |
| Organic matter (%) | 66.0 ± 0.70 |
| Total nitrogen (g/kg) | 60.97 ± 0.03 |
| Total carbon (g/kg) | 261.56 ± 0.21 |
| Ammonium (mg/kg) | 947.27 ± 87.86 |
| Nitrate (mg/kg) | -- |
| Phosphate (mg/kg) | 604.13 ± 115.63 |

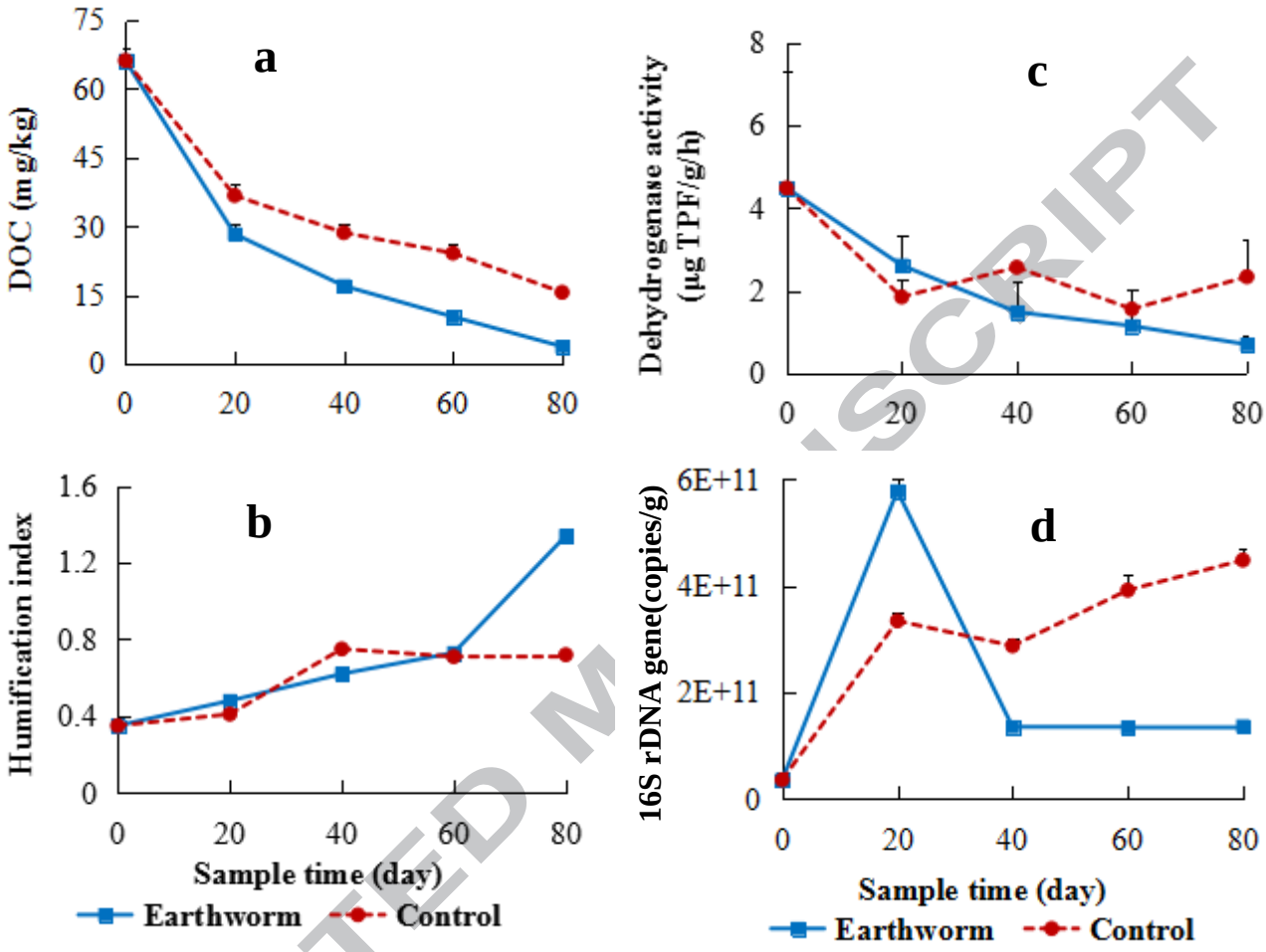
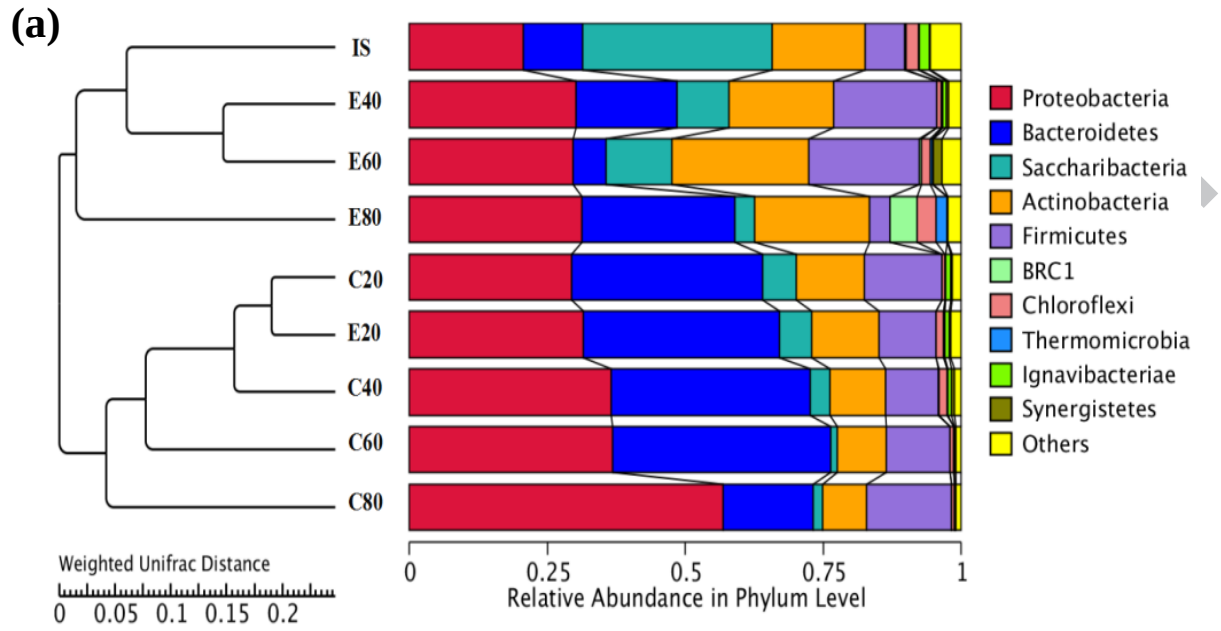



**Fig. 1** Changes in DOC, humification index, dehydrogenase activity and bacterial 16S rDNA gene abundance in the vermicomposting and control treatments during sludge stabilization process. Data are presented as mean ± standard deviation (n=6). Repeated measures ANOVA was used to test for significant differences between vermicomposting and control treatments with experimental time.



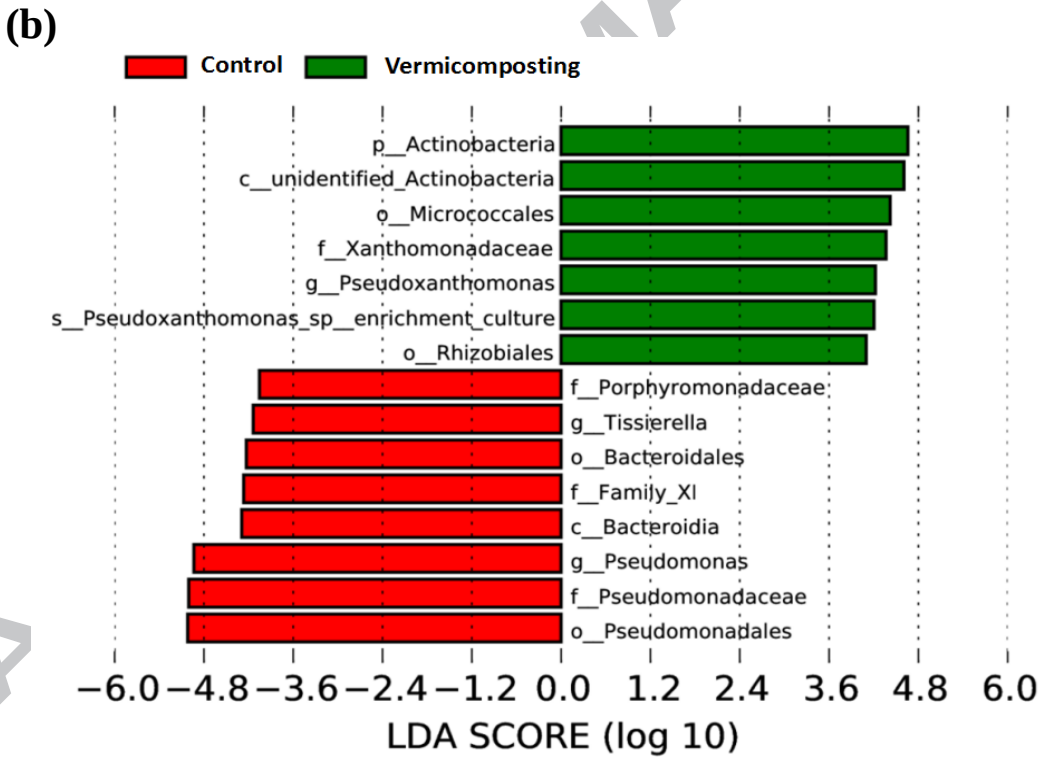
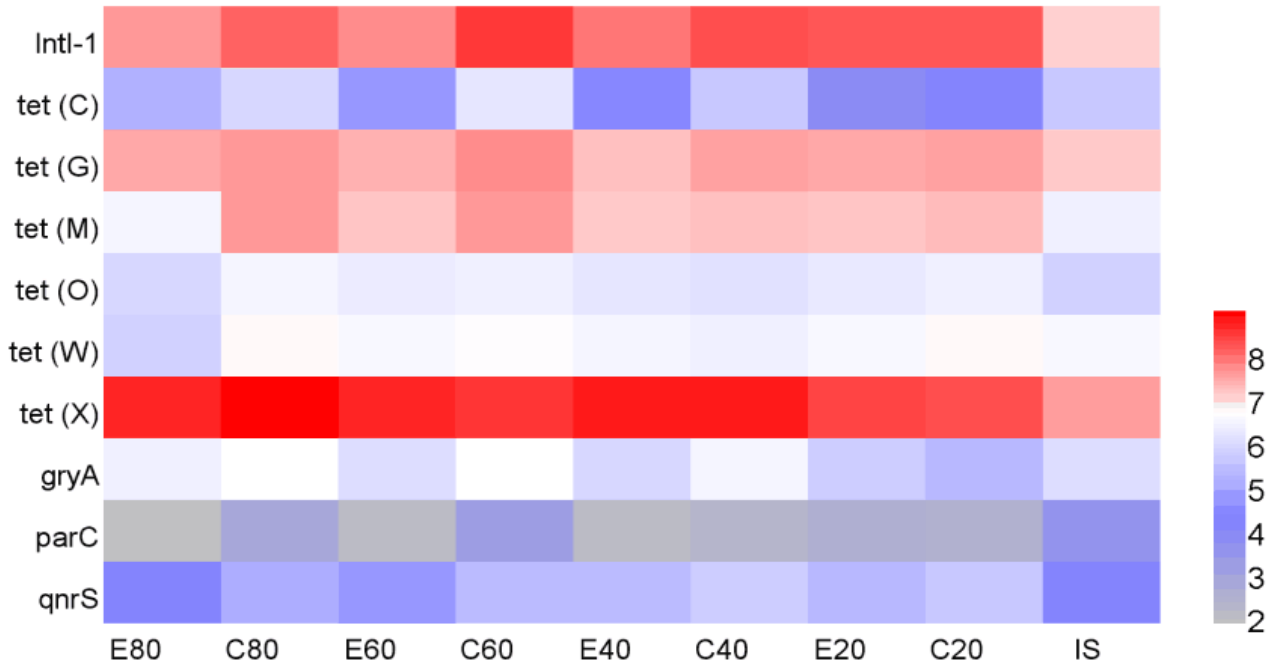




**Fig. 2** Bacterial community clustering based on weighted UniFrac distances (a) and the LDA score diagram (b) of vermicomposting and control treatments during sludge stabilization process. The tree was calculated by weighted UniFrac distance based on relative abundances at the phylum level. LDA score ($Log_{10}$ > 3.0) was adopted as those that differentiate key OTUs between vermicomposting and control samples.

**(a)**




**(b)**

| | *Int*1 | *tet*C | *tet*G | *tet*M | *tet*O | *tet*W | *tet*X | *gry*A | *par*C | *qnr*S |
|---|---|---|---|---|---|---|---|---|---|---|
| Time | *** | *** | *** | *** | *** | *** | *** | *** | * | *** |
| Treatment | *** | *** | *** | *** | *** | *** | *** | ** | * | *** |
| Time×Treatment | *** | *** | *** | *** | *** | *** | *** | *** | ** | *** |

**Fig. 3** Abundances of ARGs (a) in vermicomposting and control treatments and their significant differences (b) during sludge stabilization period. Repeated measures ANOVA was used to test for significant difference between vermicomposting and control treatments with experimental time.

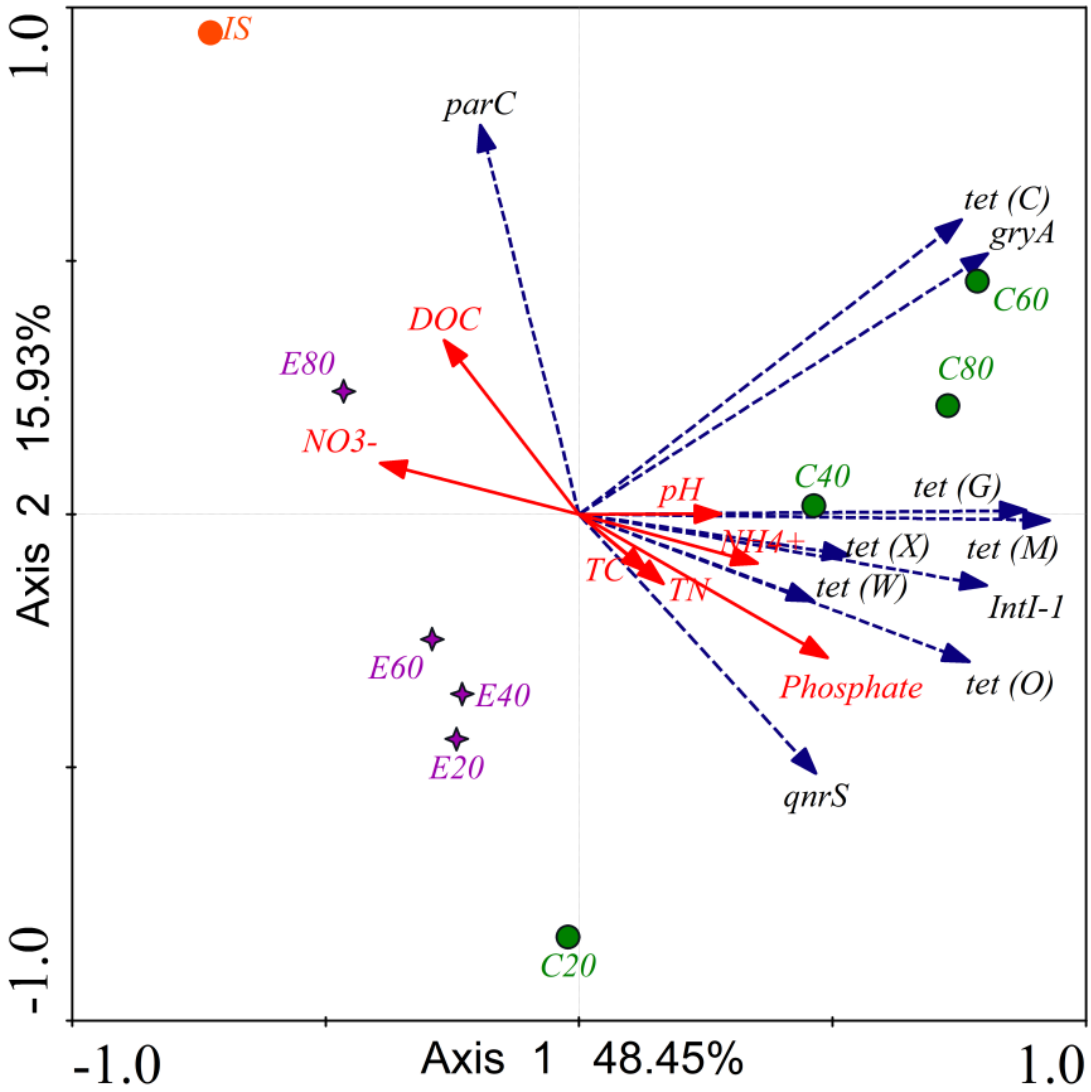



**Fig. 4** Redundancy analysis (RDA) of the relationship between environmental factors (red arrows) and antibiotic resistance genes (blue arrows). IS means initial sludge. 20, 40, 60 and 80 behind E and C represent sampling days of 20, 40, 60 and 80 from earthworms and control reactors, respectively.

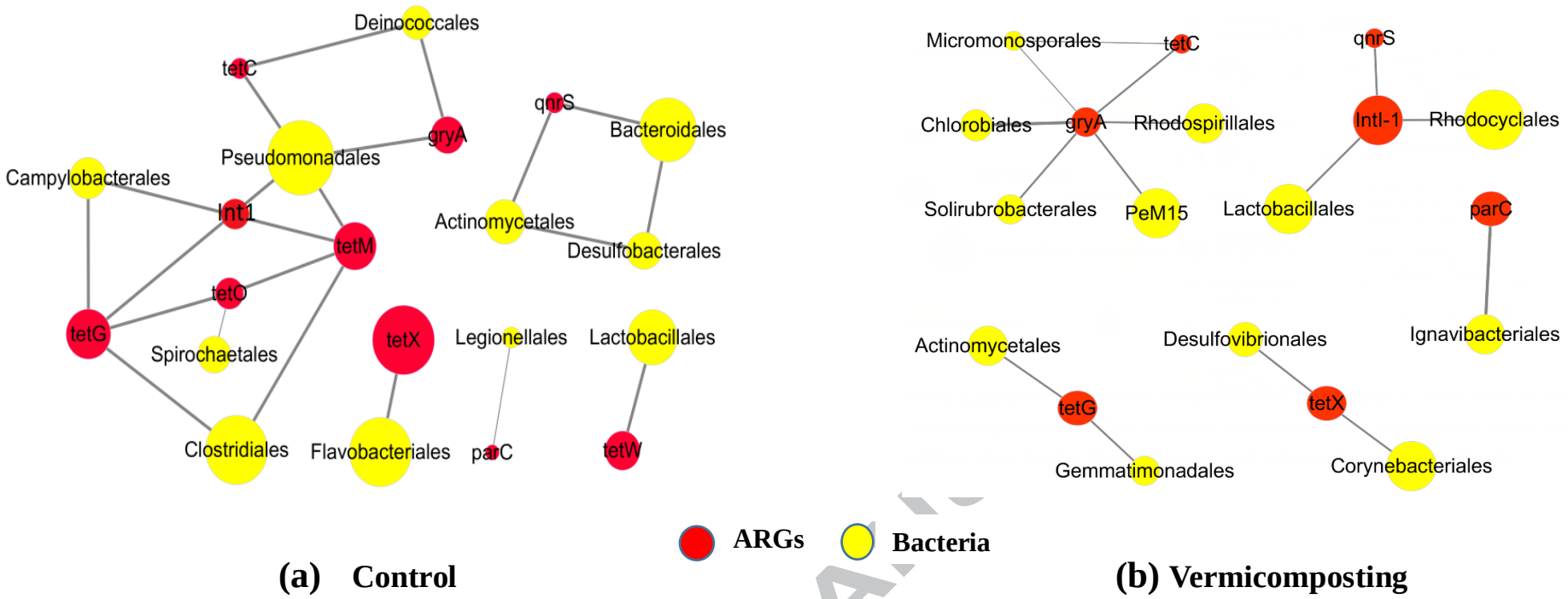



**Fig. 5** Network analysis of co-occurring ARGs (relative abundance) and possible host bacteria (top 50 Orders) based on Pearson's correlation coefficients ($p < 0.01$, $R^2 > 0.90$) in control (a) and vermicomposting (b) systems. The node represents an ARG or bacterium, where the node size is proportional to each abundance. An edge represents a positive significant correlation, where the edge thickness is proportional to Pearson's correlation coefficients.

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760 **Highlights**

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762 1. Fate of selected ARGs and *Int*1 during vermicomposting was studied.

763 2. Earthworms significantly reduced the selected ARGs and *Int*1.

764 3. Proteobacteria and Antinobacteria were potential host of ARGs in vermicomposting.

765 4. Earthworms strongly affected the possible host bacteria encoding ARGs and *Int1*.

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