Distribution of Antibiotic-resistant Bacteria in Aerobic Composting of Swine Manure With Different Antibiotics

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Abstract

Background: Livestock manure is an important reservoir of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). The community structure and bacterial diversity are usually studied using high-throughput sequencing that cannot provide direct evidence for ARB changes. Thus, little is known about the distribution of ARB, especially in the presence of different antibiotics in composting process. In this study, the fate of ARB was investigated in aerobic composting of swine manure, using chlortetracycline, sulfamethoxazole, lincomycin, ciprofloxacin as typical antibiotics. The abundance and species of ARB were analyzed systematically to evaluate their ecological risk at different stages of composting.

Results: The absolute abundance of total ARB decreased while the relative abundance showed an increasing trend on Day 2. The relative abundance of lincomycin-resistant bacteria was higher than other ARBs during the whole composting process. The absolute abundance of four ARBs was 9.42×10^6 - 2.51×10^2 CFU/g (lincomycin- > chlortetracycline- > sulfamethoxazole- > ciprofloxacin- > multiple antibiotic-resistant bacteria), and they were not completely inactivated at the end of composting. Antibiotics led to a partial proliferation of ARBs including *Corynebacterium_1*, *Sporosarcina*, *Solibacillus*, and *Acinetobacter*. Especially, *Corynebacterium_1*, a pathogenic bacterium, was observed in the treatments of chlortetracycline and lincomycin.

Conclusion: Among the antibiotics studied, lincomycin showed the highest ecological risk, due to it expanded the range of lincomycin-resistant bacteria at the phyla level (Firmicutes, Actinobacteria, and Proteobacteria). The principal co-ordinates analysis indicated that the bacterial community structure was primarily associated with the composting stages rather than the antibiotic types. Possible potential hosts and degrading bacteria for ARGs were indicated based on the network analysis. The decrease of culturable Proteobacteria and the increase of culturable Firmicutes (*Solibacillus*, *Bacillus*) partially explained the high removal rate of various ARGs in this study. These results provided important information for the control of antibiotic resistance in composting.

Background

Antibiotics have been misused widely in animal husbandry, and their residual concentrations were reported in the $\mu\text{g/kg}$ ~ mg/kg level in livestock manure [1-3]. Additionally, inadequate assimilation in the animal body has led to increase residual antibiotics in the manure, which could promote selection for antibiotic resistant-bacteria (ARB) and antibiotic resistance genes (ARGs) and making them a major reservoir of antibiotic resistance [4, 5]. In recent decades, several techniques have been adopted to determine the variations of in microbial structure. Traditional microbiological methods are essential and indispensable as a means to culture, and have continuously been used to successfully isolate various microorganisms [6]. The survival ability of bacteria in the presence of antibiotics is the only way to evaluate whether they are antibiotic-resistant or not, with the minimum inhibitory concentration quantified by agar dilution or broth microdilution assays [7]. *Salmonella* and *Campylobacter*, pathogenic bacteria,

have been detected at 10^5 colony-forming units (CFU/mL) and most of which were antibiotic resistance in the cesspools of pig farms [8]. Numerous antibiotic-resistant human pathogens including *Escherichia coli*, *Alcaligenes faecalis*, and *Enterococcus* have been found in chicken manure [9]. Extensive use of antibiotics in animal husbandry significantly increased ARB and ARGs in livestock settings [10]. This would lead to deleterious implications on human health and environmental quality [5]. Consequently, it is particularly important to reduce the spread and diffusion of ARB and ARGs.

Microorganisms are essential for composting, and the compositions of these microbial communities are altered during this process. For example, lincomycin (LIN) residues promoted the abundance of Firmicutes, Actinobacteria, Proteobacteria, Chloroflexi, and Bacteroidetes in composting [2]. Previous studies found that the proportion of Chloroflexi was significantly higher than other phyla, and the composting process also resulted in increases of Bacteroidetes abundance. The increase paralleled the antibiotic concentration, indicating that Bacteroidetes were tolerant to high levels of tetracycline [1, 11, 12]. Community structure and bacterial diversity have been reported using high-throughput sequencing. However, there is a lack of research on ARB community structure. Besides, this type of metagenomic sequencing data cannot provide direct evidence for ARB changes [7]. Even culturable bacteria also accounted for a low proportion of the total bacteria; the traditional pure culture method is the only way to understand ARB at present. Therefore, it is very meaningful that the development of antibiotic resistance of specific bacteria during composting should be monitored dynamically and analyzed comprehensively in combination with the traditional pure cultures.

Aerobic composting can effectively remove ARB from livestock manure. Previous studies concluded that cultured bacteria resistant to tetracycline and erythromycin decreased by 7 and 4 logs, respectively; substantial degradation of ampicillin and tetracycline-resistant *Escherichia coli* as well as a decrease in the abundance of *erm* and *tet* genes abundance, was also observed during composting of cattle manure [13, 14]. ARBs are the source and hosts for ARGs [4]. Most of the previous studies only analyzed the abundance of ARB and did not focus on the evolution of community structure. Besides, it is even little known that the influence of antibiotic types on the abundance and community structure of ARB, as well as the potential relationship of ARB and ARGs. In this study, chlortetracycline (CTC), sulfamethoxazole (SMX), CIP, and LIN were selected according to their types and residual concentrations in our previous study [12]. These four antibiotics are frequently used in veterinary and human treatments worldwide, and have been detected in different environmental matrices [15]. The research attempted to achieve the following objectives: (1) how different types of antibiotics affect the abundance of ARB; (2) whether composting stages impart any role in the ARB community structure; (3) to determine the correlations between ARB and ARGs to mitigate the public health risk. This study provides a better understanding on the fate of ARB, ARGs and antibiotic resistance in aerobic composting.

Materials And Methods

Composting and sampling procedures

The detailed experimental design was the same as in our previous article [12]. Briefly, the treatment without added antibiotic was used as the control (CK). Composting was performed in rectangular foam boxes (55×45×45 cm) with 2×2 cm diameter holes spaced 15 cm apart in all 4 walls to allow facilitate ventilation. The basic physical and chemical properties of the composting materials are shown in Table S1. Composting materials were composed of 14.28 kg pig manure: straw: coconut shell powder (6.67:4.94:2.67) with the C:N ratio of 30:1. Moisture content was set at 65 %. The residual concentrations of various antibiotics varied, but 50 mg/kg was selected as a standardized antibiotic concentration to ensure study comparability.

CTC, SMX, CIP and LIN obtained from Beijing DHLH Biotechnology Co. Ltd. China. CTC and LIN were mixed by methanol, respectively; SMX and CIP were dissolved in hydrochloric acid, respectively. Treatments were named as T0 (CK), T1 (CTC), T2 (SMX), T3 (LIN), T4 (CIP) and T5 (the four mixed antibiotics). All treatments were replicated for three times. The boxes were set in a row and ventilated for the duration of the 35-day experiment. Samples were collected on the 2nd, 7th, 14th, 21st, 28th and 35th days, and at the top, middle and bottom of the reactor, then combined and mixed. The fresh samples were divided into two parts: one was stored at 4 °C for bacterial culture, and the other was stored in a -80 °C refrigerator for the determination of ARGs.

Determination of the total culturable heterotrophic and antibiotic-resistant bacteria

Samples (5.00 g) were added to a conical flask with 45 mL sterile water and then shaken at 200 rpm and 25 °C for 30 min. The total culturable heterotrophic bacteria (TCB) were determined by beef extract-peptone agar medium (Beijing Solarbio Science & Technology Co. Ltd. China). In brief, the concentration of CTC, SMX and LIN and CIP in the different mediums was 16, 76, 16, and 4 µg/mL according to the Clinical and Laboratory Standards Institute guidelines to determine the abundance of ARB [16]. The plates were incubated for 24 h at 35 °C. TCB and ARB levels were calculated from CFU on non-selective and selective mediums. Plates containing all four antibiotics were used to detect multiple antibiotic-resistant bacteria. ARBs were denoted as chlortetracycline-resistant bacteria (CRB), sulfamethoxazole-resistant bacteria (SRB), lincomycin-resistant bacteria (LRB), ciprofloxacin-resistant bacteria (CIRB), multiple antibiotic-resistant bacteria (MRB), respectively.

DNA extraction and quantitative polymerase chain reaction

DNA extraction from samples was performed with a TIANamp Stool DNA Kit (Tiangen Biotech, Beijing, China) according to instructions provided by the manufacturer. DNA quality was checked on 1% agarose gel. DNA concentration was determined by Nano Drop one (Thermo, USA). DNA samples stored at -20 °C until use. Quantitative polymerase chain reaction (qPCR) was performed from the extracts to quantify tetracycline, sulfonamide, macrolide, quinolone resistance genes, two integrons, one multiple resistance gene. The detailed protocols for qPCR are described in supplementary materials.

Pretreatment of TCB and ARB, and sequence analysis

Sequencing analysis was performed on representative samples (Day 0, 7, 21, and 35). The pretreatment method referred to a previous study with some modifications [9]. All bacteria colonies in plate were combined and washed with ddH₂O. Bacteria solution was mixed and centrifuged for 5 minutes (10000 rpm) and then the supernatant was dumped. The 16S rRNA sequence analysis was used to identify the taxonomic status of a bacterial species. The procedure used was as follows:

High throughput sequencing was conducted using the Illumina Miseq PE300 platform (Illumina, San Diego, CA, USA) by Shanghai Majorbio Bio-pharm Technology. The primers 338F and 806R were used to target the V3-V4 region of 16S rRNA gene. The raw data was spliced and filtered for quality control and aggregated using Usearch software (<http://www.drive5.com/usearch/>), and non-repeat sequences were identified and single sequences were removed to get rid of repetition. Clustering of operational taxonomic units (OTU) was conducted into minimum taxons using a similarity level of 97 % to generate and a Biological Observation Matrix (BIOM) table. The Bayesian algorithm using the RDP (Ribosomal Database Project) classifier (<https://omictools.com/>) was applied for the taxonomic analysis of representative OTU using Qiime (<http://qiime.org/>). This was accomplished by first extracting and added from each OTU as the representative sequence, compared with the RDB (Relational Database), and OTU was associated with bacterial species to form an OTU abundance table.

Statistical analysis

Basic statistical calculations were performed using Origin 9.1 (Origin Lab, San Diego, CA, USA). Significant differences were calculated using one-way analysis of variance (ANOVA) with IBM SPSS 23.0 Statistics (IBM, Chicago, ILL, USA). Heatmaps and principal co-ordinates analysis (PCoA) were constructed using Vegan, PCoA of R 3.5.2 (<https://www.r-project.org/>), respectively. The networks were built using Networkx software (<http://networkx.github.io/>) according to the relative contents of each genus after classification.

Results & Discussion

Variation in abundance of TCB and ARB at different stages of composting

Compared with Control (T₀), the absolute abundance of TCB decreased in T₁-T₅, ranging from 1.48×10^{14} to 3.04×10^{11} CFU/g (Fig. 1). The addition of antibiotics significantly affected on the bacterial community in the raw material. In T₅, the absolute abundance of TCB was significantly lower than other treatments. It was mainly because of the high concentration of antibiotics, which strongly inhibited on microorganisms [17]. In the first 7 days of composting, the absolute abundance of TCB decreased significantly. There were two main reasons: (1) the composting temperature was above 60 °C, inhibiting the activity of some bacteria [4, 12]; (2) most antibiotics were degraded rapidly in the first 13 days [18], which also indicated that antibiotics were most effective in killing or inhibiting bacteria in the early stage. At the end of composting, the absolute abundance of TCB decreased to 6.96×10^7 CFU/g in T₀; while, it was similar in the presence of CTC, SMX, and LIN (T₁-T₃). In contrast, TCB was reduced to 9.8×10^5

CFU/g in T4 and 1.06×10^6 CFU/g in T5 (Fig. 1). Sulfadiazine and CTC were completely within 3 days and 21 days, respectively, while 1.17 mg/kg of CIP was extracted after 56 days of composting [19]. The influence of CIP on bacterial community was more substantial than other antibiotics at the same concentration in composting. The most obvious influence was also observed on composting's physical and chemical properties in the treatment with CIP [12].

The populations of bacteria resistant to each antibiotic were not significantly different from T0, with the abundance ranging from 21.6 to 1.44×10^{12} CFU/g (Fig. 1). In the first 2 days, the absolute abundance of ARB decreased gradually, while the relative abundance increased significantly. For example, CRB increased from 5.90 % to 12.85 % in T1; SRB increased from 5.66 % to 12.14 % in T2 (Fig. S1). The higher occurrence of ARB after composting was most likely due to mutations of local bacteria in the manure [20], horizontal gene transfer [20], as well as selective pressure of the antibiotics [21]. In this study, mutation or horizontal transfer of CRB and SRB might be more likely to occur under the selection pressure of CTC or SMX. Therefore, the relative abundance of CRB and SRB increased within 2 days of composting. The absolute abundance of ARB was reduced by > 6 orders of magnitude during the 35-d composting. A previous study indicated that ARB was correlated with antibiotic concentrations [22]; the antibiotics degraded gradually over time, so ARB abundance was decreased. In T4 and T5, the absolute abundance of MRB was $0.24\text{--}1.75 \times 10^4$ CFU/g (Fig. 1). However, MRB was not found after Day 21. *Escherichia-Shigella* and *Enterococci* were the main MRB that existed extensively in livestock manure [4, 23]. They were sensitive to temperature, inactivated in 1.7 to 8.4 d at 37 °C and only 2-3 d at 55 °C [23]. This might be partially explaining the absence of MRB at the end of composting. The absolute abundance of four ARBs was 9.42×10^6 - 2.51×10^2 CFU/g, the order of ARB abundances was LRB > CRB > SRB > CIRB > MRB in the end (Fig. 1).

Variation of TCB community structure

Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were the primary phyla of TCB (Fig. 2). The abundance of Proteobacteria was >65 % in all treatments and of which differences were insignificant, indicating that Proteobacteria was insignificantly affected by the presence of antibiotics in the initial stage. The major genera were *Escherichia-Shigella* (Fig. 3). The abundance of Proteobacteria decreased, and the most apparent reduction was in T4, where they were reduced from 80 % to 0.056 % due to the abundance of *Escherichia-Shigella* decreased on Day 7 (Fig. 3). *Escherichia-Shigella* was probably particularly sensitive to temperature as indicated above [23]. In addition, CIP had an inhibitory effect on almost all bacteria in the composting [24]. The combination of high temperature and CIP might be the reduction of culturable Proteobacteria. On Day 7 and 21, *Alcaligenes*, *Pseudomonas*, *Comamonas* and *Acinetobacter* were main genera in T1, indicating a relative insensitivity or strong tolerance to CTC as compared with other antibiotics. On Days 21, *Comamonas* displayed a notable decrease to 1 %, indicating that *Comamonas* were sensitive to temperature and their decline significantly correlated with CTC levels.

The abundance of Bacteroidetes was 20.36 %-0.65 % in T0-T5, indicating that antibiotics impacted on Bacteroidetes in the initial stage. *Sphingobacterium* and *Flavobacterium* were dominant genera. On Day 7, Bacteroidetes increased to 36.59 %, 29.24%, and 34.76 % in T0, T2, and T5, respectively, while decreased in other treatments (Fig. 2). Bacteroidetes has been shown to increase dramatically in cow manure and rice straw composts during the thermophilic phase due to the presence of thermophilic species [25]. This indicated that CTC, LIN and CIP inhibited the growth and reproduction of Bacteroidetes or even killed them. On Day 21, Bacteroidetes increased to 29.80 % and 25.72 % due to the high abundance of *Sphingobacterium* in T4 and T5. In the end, Bacteroidetes decreased except for T2. This result was similar to Proteobacteria which was relatively heat-sensitive. The abundance of Bacteroidetes was increased, accounted for 62.02 % in T2 due to *Parapedobacter* and *Niabella* rising to 36.61 % and 21.51 %, respectively. In contrast, these genera were inhibited by SMX in the initial stage. They eventually regained growth or developed antibiotic resistance as the SMX was degraded and the temperature was reduced [3].

Actinobacteria was relatively low at 0.63 %-6.46 % in all treatments. Actinobacteria increased to 61.09 % and 25.67 % due to the presence of *Glutamicibacter* in T1 and T2 (Fig. 2, Fig. 3). Therefore, CTC and SMX inhibited the growth and reproduction of Actinobacteria, but *Glutamicibacter* gradually recovered or either developed or was intrinsically resistant. In the end, only in T0 and T3, the abundance of Actinobacteria increased to 28 % and 10.13 %, respectively (Fig. 2). In the absence of antibiotics, *Corynebacterium_1* was the dominant genera at 28 % and demonstrated the resilience of this genus to composting, and has developed LIN resistance. These results were also consistent with a previous composting study which indicated *Corynebacterium_1* was a major genus [26].

The abundance of Firmicutes was 3.36 %-22.72 % in all treatments (Fig. 2). The primary genera were *Bacillus*, *Lysinibacillus*, *Sporosarcina*, *Enterococcus*, and *Staphylococcus* (Fig. 3) and most were endospore formers and resistant to high temperatures [27]. *Bacillus* was isolated at the highest frequency in composting benefiting from their thermotolerance, and greatly impacted on degradation of waste [28, 29]. The abundance of Firmicutes reached 99.94 %; the dominant genera were *Lysinibacillus* and *Solibacillus* in T4 (Fig. 2, Fig. 3). Both of these also developed CIP resistance. On Day 35, Firmicute had a complete comparative advantage (except for T2), included *Lysinibacillus*, *Bacillus*, *Solibacillus* and *Sporosarcina*. The abundance of Firmicutes was relatively low because of the high proportion of *Bacteroidetes* in T2. These results indicated that the influence of antibiotics on microbial community structure was a continuous process despite the degradation of antibiotics.

The community structure of ARB

Proteobacteria took a relatively high ratio in the initial stage, while Firmicutes became the dominant phylum with the extension of composting (Fig. 4). MRB was found only in the early stages of composting. On Day 7, Proteobacteria (99.93 %) was the dominant MRB; *Enterobacter* was the main genus (Fig. 4, Fig. 5). *Enterobacter* is an opportunistic pathogen that can cause extra-intestinal infections in the urinary, respiratory tracts, and wounds, which possess resistance to different antibiotics [9, 22]. The

optimum growth temperature of *Enterobacter* was 25°C which exhibited enzyme production and good growth at a temperature range of 15-35°C, pH 5-10 [30]. In this study, the first 20 days of composting were above 50 °C [12] Therefore, MRB was not detected at the end of composting.

The dominant CRB genera were *Escherichia-Shigella*, *Enterobacter*, *Psychrobacter*, *Sphingobacterium*, and *Alcaligenes* in the initial stage. Compared with Control (T0), the abundance of *Escherichia-Shigella* and *Pseudomonas* increased, indicating that the existence of antibiotic enhanced bacterial resistance to CTC. As the composting continued, Firmicutes gradually replaced Proteobacteria becoming the primary CRB phylum. The dominant CRB genera were *Sporosarcina*, *Lysinibacillus*, *Bacillus*, *Solibacillus*. *Corynebacterium_1* accounted for 10.64 % in T1. It is a facultative anaerobe and a pathogen. Its CTC resistance was consistent with previous studies [31]. Composting generally had a heterogeneous population composed of animal and human pathogens that could cause disease in livestock [32, 33]. These findings suggested that CTC increased the risk of human pathogenic bacteria circulating in the environment and posed a threat to human health.

Proteobacteria and Bacteroidetes were the main SRB phyla in the initial stage (Fig. 4). The dominant genera included *Escherichia-Shigella*, *Enterobacter*, *Alcaligenes*, and *Sphingobacterium* (Fig. 5). The abundance of *Escherichia-Shigella* increased in T2, indicating that SMX induced bacterial resistance, which was consistent with a previous study [34]. *Escherichia-Shigella* has been frequently reported in livestock and poultry feces and was a common fecal-resistant microorganism [9, 35]. On Day 7, *Sporosarcina* and *Bacillus* dominated at 23.79 % and 54.49 %, respectively (Fig. 5). *Bacillus* had a strong ability to adapt to harmful factors environment, and it might be associated with SMX removal [36]. These might be explaining the high abundance of *Bacillus* in the early stage. In the end, the dominant genera for SRB included *Sporosarcina* and *Solibacillus* in T2.

Proteobacteria and Bacteroidetes were also the main phyla of LRB in the early composting stage (Fig. 4). *Escherichia-Shigella*, *Pseudomonas*, and *Empedobacter* were the primary LRB in T3. *Escherichia-Shigella* and *Pseudomonas* have been found to be resistant to various antibiotics [23]. Compared with T0, the abundance of Firmicutes increased, the dominant LRB were *Psychrobacillus*, *Comamonas* and *Flavobacterium* on Day 7, indicating that these genera showed strong tolerance to LIN. At the end of composting, Firmicutes, Actinobacteria and Proteobacteria dominated in the phyla of LRB with a ratio of 43.44 %, 36.22 % and 19.36 %, respectively (Fig. 4). In addition, *Lysinibacillus*, *Acinetobacter* and *Corynebacterium_1* were increased (Fig. 5). It was reported that *Corynebacterium_1*, a pathogenic bacterium, was a major genus in animal manure and organic fertilizer [32, 33]. It was also found to be resistant to CTC. As a result, the risk of *Corynebacterium_1* should be considered in composting. Antibiotic residues in manure exerted a selective pressure on the bacterial community and induced the emergence of diverse ARGs even at low concentrations [37]. LIN might enhance horizontal transfer of ARGs, leading to the increase of LRB at phylum and genera, and indicating that the ecological risk of LIN was higher than other antibiotics.

CIP resistance was dramatically changed in T4 (Fig. 4, Fig. 5). The dominant CIRB was Proteobacteria in the first 21 days of composting; Firmicutes (98.87 %) became the dominant phylum in the end in T0. On Day 7, Firmicutes increased, the dominant genera were *Escherichia-Shigella* and *Solibacillus* (Fig. 4, Fig. 5). These genera were common ARB in manure. Previous researchers obtained similar results, who found the primary ARB was *Enterobacter*, *Alcaligenes*, *Escherichia-Shigella*, *Acinetobacter*, *Enterococcus*, and *Bacillus* in livestock and poultry manure [22]. In the end, Firmicutes was the absolute dominant phylum at 91.57 %, *Sporosarcina* was the dominant genus (Fig. 4, Fig. 5). A study monitored the bacterial community spiked with CIP at two different levels (2, 20 mg/kg) during swine manure composting; found that *Sporosarcina* were selectively enriched in antibiotic treatments [19]. These results indicated the sensitivity of *Sporosarcina* to CIP, and the potential selection of specific bacteria.

Previous studies have found that ARBs from chicken feces were *Alcaligenes*, *Lampropedia*, *Escherichia*, *Enterococcus*, *Corynebacterium*, *Lactobacillus*, *Citrobacter*, *Bacillus* and *Pseudomonas* [22]. *Sporosarcina*, *Bacillus*, *Enterobacter*, *Lysinibacillus*, *Enterococcus*, and *Escherichia-Shigella* were resistant to all tested antibiotics in this study. The changes in the environment by the composting reduced the bacteria diversity, and favored the growth of tolerant bacteria [19]. This might be the reason for the difference between the results of ARB and those of previous studies. The widespread use of antibiotics has resulted in ARB development, which often co-carried aminoglycoside, quinolone, chloramphenicol, and sulfonamide resistance [38]. Bacteria developed antibiotic resistance due to the presence of ARGs; horizontal gene transfer was a primary reason driving alterations in antibiotic resistance. When ARB from feces entered the soil, they carried ARGs that were capable of horizontal transfer to indigenous bacteria and even to some human pathogens such as *Salmonella*, *Campylobacter*, and *Shigella* [37, 39]. In this study, ARB still existed at the end of composting. Therefore, ecological risks of manure must be evaluated.

Analysis of differences in TCB and the relationship between ARB and ARGs

Bacterial genera were reduced by 32 in T4 compared with other treatments (Fig. S2). This indicated that CIP had the greatest impact on bacteria and significantly reduced species richness, which was most likely the result of a wide bactericidal spectrum [24]. PCoA was used to identify any significant factors that influenced experimental results. PC1 and PC2 accounted for 75.92 % of the changes in bacteria levels (Fig. 6). The result showed that the bacterial community of different treatments was not significantly separated, while there was a significant difference at different composting stages. The existence of antibiotics caused only a transient perturbation and the bacterial community structure was restored after 3 weeks of composting [19]. Therefore, these results showed alterations in the bacterial communities by composting were primarily influenced by the composting duration but not antibiotics. A study has shown that the microbial community can be affected by the compost materials and antibiotic concentration [40].

Based on the above results, an interactive network of 30 primary genera was generated to analyze the potential hosts of ARGs at different composting stages (Fig. 7). Only *Psychrobacillus* was positively correlated with *tetX*, indicating that *Psychrobacillus* might be the potential host of *tetX* on Day 7.

Psychrobacillus belongs to Firmicutes. A Previous study reported that Actinobacteria and Firmicutes were prevalent during the thermophilic phase of composting, and the prevalence of ARGs increased in the end of composting [41]. However, *Solibacillus* and *Enterococcus* of Firmicutes, *Corynebacterium_1* of Actinobacteria were negatively correlated with various ARGs (Fig. 7a). In this study, the bacterial genera belonged to ARB. The culture-based methods captured only a small proportion of the whole bacterial community (0.1-10 %); ARB resistance cannot be mediated by the ARGs being analyzed [42]. Previous studies have shown that Bacteroidetes might be important hosts for *tetC*, *tetX*, *sul2*, and *int1* [43]. On Day 7, Proteobacteria was the main phylum of ARB, and the abundance of ARB decreased significantly (Fig. 4). These might be partially explaining the high removal of ARGs (Table S3). As the composting continued, we found that *ermA* was positively correlated with *Corynebacterium_1*, *Bhargavaea* and *unclassified-Bacillales*, which might be the host bacteria or synergistic bacteria for *ermA*. A previous study indicated that the concentration of ARGs was positively correlated with the abundance of corresponding bacteria hosts [44]. However, the removal rate of *ermA* reached levels > 99 % by 35 days (Table S3). We found that *ermA* was correlated negatively with *Solibacillus* (Fig. 7c). *Solibacillus* might be the primary *ermA* degrader as the composting matured. In addition, *Escherichia-Shigella* of Proteobacteria was reported as the carrier of *tetA*, *Pseudomonas* was the shelter of *tetA* and *tetG* [45]. *Bacillus* was negatively correlated with both *tetA* and *ermC* (Fig. 7c). Similar results were found for *tetO*. Overall, the decrease in abundance of culturable Proteobacteria and the increase of partially culturable Firmicutes (*Solibacillus*, *Bacillus*) might be explaining the high removal rate of various ARGs. In the processing of composting, pathogenic bacteria (*Corynebacterium_1*, *Acinetobacter*, and *Enterococcus*) could not be completely removed, and the existence of antibiotics led to a partial proliferation. Pathogenic bacteria were significantly correlated with ARGs, indicating that pathogenic bacteria could become a major host of ARGs [32, 46, 47]. *Enterococcus* was correlated positively with *tetX*. No positive correlations were observed between other pathogenic bacteria and ARGs, which might be based on culturable bacteria in this study. Anyhow, the spread risk of pathogenic bacteria to farmland soil and crops cannot be ignored in the application of swine manure.

Conclusions

In the first 7 days, the abundance of TCB decreased significantly. At the end of composting, the lowest TCB abundance was found in T4 (9.8×10^5 CFU/g) and T5 (1.06×10^6 CFU/g), respectively. Total ARB decreased while their relative abundance increased on Day 2. CRB and SRB increased from 5.90 % to 12.85 %, 5.66 % to 12.14 %, respectively. LRB had the highest relative abundance in the whole composting process. The absolute abundance of ARB was LRB > CRB > SRB > CIRB > MRB. Antibiotics led to a partial proliferation of ARBs including *Corynebacterium_1*, *Sporosarcina*, *Solibacillus*, and *Acinetobacter*. Especially *Corynebacterium_1*, a pathogenic bacterium, was observed in the treatments of CTC and LIN. In addition, the existence of LIN expanded the range of LRB at the phyla level (Firmicutes, Actinobacteria and Proteobacteria) at the end of composting. It indicated that these bacteria were more likely resistant to LIN, and thus the ecological risk of LIN was higher compared with other antibiotics studied in this work. PCA analysis showed that the composting stages primarily influenced the alteration of bacterial

community structure. The network analysis indicated that the decrease in the abundance of culturable Proteobacteria and the increase of culturable Firmicutes might partially explain the high removal rate of various ARGs in composting process. These results provide valuable reference for the ecological risk control of antibiotic resistance during swine manure composting.

Abbreviations

ARB: Antibiotic-resistant bacteria; ARGs: Antibiotic resistance genes; CFU: Colony-forming units; CTC: Chlortetracycline; SMX: Sulfamethoxazole; CIP: Ciprofloxacin; LIN: Lincomycin; TCB: Total culturable heterotrophic bacteria; CRB: Chlortetracycline-resistant bacteria; SRB: Sulfamethoxazole-resistant bacteria; LRB: Lincomycin-resistant bacteria; CIRB: Ciprofloxacin-resistant bacteria; MRB: Multiple antibiotic-resistant bacteria; QPCR: Quantitative polymerase chain reaction; OUT: Operational taxonomic units; BIOM: Biological Observation Matrix; RDP: Ribosomal Database Project; RDB: Relational Database; PCoA: Principal co-ordinates analysis.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

TS and HL contributed to the experiments, original draft writing and revising. BL, JY, MS, and MY were responsible for the data analysis. LL and YT were contributors in the data visualization. SX and CZ were responsible for manuscript correction and supervision. All authors already read and approved the manuscript.

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Availability of data and materials

The datasets obtained and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no known competing personal relationships or financial interests that could have appeared to affect the work reported in this paper.

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Figures

Figure 1

The absolute abundance of total cultivable heterotrophic bacteria and antibiotic-resistant bacteria. CRB: chlortetracycline-resistant bacteria; SRB: sulfamethoxazole-resistant bacteria; LRB: lincomycin-resistant bacteria; CIRB: ciprofloxacin-resistant bacteria; MRB: multiple antibiotic-resistant bacteria

Figure 2

Community abundance succession of total culturable heterotrophic bacteria in the phylum

Figure 3

Top 30 genera of total culturable heterotrophic bacteria

Figure 4

Community abundance succession of culturable antibiotic-resistant bacteria in the phylum

Figure 5

Top 30 genera of culturable antibiotic-resistant bacteria

Figure 6

The principal co-ordinates analysis (PCA) of total culturable heterotrophic bacteria

Figure 7

Network diagram analysis of ARB and ARGs. (a) Day 7, (b) Day 21, and (c) Day 35. The calculated the Spearman rank correlation coefficient between species reflected the correlation between species. Species with $p < 0.05$ are shown by default. The node sizes represent species abundance of species and different colors represent different species. A red line color represents a positive correlation and green represents a negative correlation. Line thickness is proportional to the correlation coefficient.

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