Antibiotic resistance genes variation pattern and microbial community succession during swine manure composting under different aeration strategies

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Abstract

This study adopted 3 common composting aeration methods, including forced aeration (with 3 ventilation rate of 0.0/0.1/50.25 $L \cdot kg^{-1}DM \cdot min^{-1}$ respectively), turn windrow and static pile; with the aim to clarify the actual effects of aeration methods on the removal of antibiotic resistance genes (ARGs) in swine manure composting. Representative ARGs abundances and microbial communities were determined via qPCR and 16S rRNA gene high-throughput sequencing technology. Results showed that most of ARGs (including *tetW/tetO/tetH*, *qnrS*, *ermB* and bla_{TEM}) in swine manure could be significantly reduced to low levels after composting for 77 days, but sulfonamide resistance genes (*sulI/sulII*) and integrase gene (*intI*) were highly enriched, closely correlated with the increase of genera belonged to *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* and the decrease of genera affiliated with *Firmicutes*. Temporal variations of ARGs were consistent under different aeration treatments. Further study should pay more attention to the recalcitrant ARGs in compost.

Keywords: Compost; Swine manure; Antibiotic resistance gene; Microbial community; Aeration methods.

1. Introduction

The world is undergoing the antibiotic crisis and entering a “post-antibiotic” era. The bloom of antibiotic resistance is threatening human health [1, 2]. China, as one of the largest antibiotic production and consumption country, the situation is even more serious due to the widespread overuse of antibiotics in clinic and agriculture [3]. As the vital initiator of the antibiotic crisis, the antibiotic resistance genes (ARGs) have been recently identified as emerging environmental contaminants [4], subsequently arousing a burst of attentions in related research fields.

In agriculture, the fate and dissemination pathways of ARGs have been largely investigated [5-7]. Animal manure has been reported as one of the significant reservoirs of ARGs due to the abuse of veterinary antibiotics as disease treatment or prevention and growth promotion agent. Among the multifarious types of livestock breed, swine manure was especially obtained much more researches in terms of ARGs occurrence state. Compared with other manures, swine manure harbored relatively more diverse ARGs with higher abundances and mobility that was positively correlated with antibiotics and heavy metal [8, 9], which was deserved further studies on its ARGs removal.

Composting process has been declared to reduce the diversity and quantity of ARGs in swine manure to certain extent [10]. Numerous studies focused on enhancement of ARGs attenuation via composting with versatile methods, e.g., adding biochar [11], surfactants [12], natural zeolite [13], superabsorbent polymers [14], co-composting with Chinese medicinal herbal residues [15], vermicomposting using housefly larvae [16] and continuous thermophilic composting [17]. However, controversy about the actual effect of composting to ARGs were still existed. For instance, it was found that efflux pump tetracycline ARGs, sulfonamide ARGs and integrase gene *intI* generally increased significantly after manure composting for 32 days with 3.5 days of thermophilic periods [18]. Another study also indicated that composting process enriched ARGs instead of elimination using sewage sludge as raw materials [19]. Hence, it should be necessary to further explore the composting method itself in terms of monitoring the fate of ARGs.

Mounting evidences verified that microbial community structure was the core determinants of ARGs variation pattern during composting [12, 15-16]. Considering that the environmental factors correlated with microbial growth and metabolism were distinct under different composting methods, this study adopted 5 composting methods (including static pile , turn windrow and forced aeration with 3 different ventilation rate of 0.05/0.1/0.25 $L \cdot kg^{-1}DM \cdot min^{-1}$) and mainly focused on the temporal variation of ARGs and also the succession of microbial community structures, with the aim to answer questions below: 1)

Whether ARGs were eliminated or enriched after composting? 2) Which composting method was best in ARGs removal? 3) What was the relationship between ARGs pattern and microbial community structure?

2. Materials and methods

2.1. Raw materials and composting instrument

Swine manure and cornstalk were collected as mentioned previously [20]. Rotting boxes (1.08×0.8×1.4 m) were used to simulate central parts of composting piles in practice. The detail information was shown in Fig. 1.

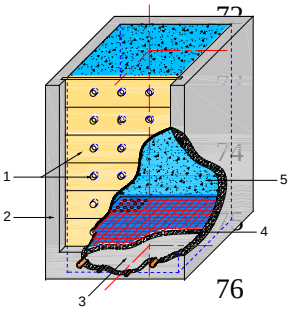


Fig. 1 Schematic diagram of rotting box for composting.

Details were as follows:

1. Wooden boards with sampling holes;

2. Concrete side wall;

3. Concrete floor and aeration and leachate cavum;

4. Bottom board with aeration holes;

5. Compost materials.

2.2. Experimental design and sampling

In total, 5 different composting methods (including static pile, turn windrow and forced aeration with 3 ventilation rate of 0.05/0.1/0.25 $L \cdot kg^{-1}DM \cdot min^{-1}$) were conducted for each rotting box. The static pile was under no artificial interference, neither forced ventilation nor turning occurred during the whole process. The turning windrow and forced aeration treatments were all turned weekly. All piles were composted for 77 days.

Sampling was carried out on 3 time points of day 21, day 49 and day 77 before turning. 9 samples of each composting system were randomly collected through the sampling holes on the front wooden boards, 3 of which were thoroughly mixed as one sample. Bulk cornstalk residues were picked out to avoid the disturbance introduced by plant. Then the left fresh composting samples were stored in -80 °C for further molecular experiment.

2.3. DNA extraction

In total, 16 groups of samples (including the original swine manure and compost samples at day 21, 49 and 77 of the five treatments) in triplicate were ready to extract DNA. Before the extraction, samples were pre-washed three times by phosphate buffer solution (pH 8.0) and 1.7% polyvinylpyrrolidone K30 to remove impurity like humic acids. Then DNA was extracted from 0.2 g of each sample via the Fast-DNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. DNA concentrations and purity were determined via the NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific, MA, USA).

2.4. Quantitative PCR (qPCR)

In this study, 8 ARGs including resistence genes of tetracycline (*tetW/tetO/tetH*), sulphonamide (*sulI/sulII*), fluoroquinolone (*qnrS*), macrolide (*ermB*), beta-lactam (bla_{TEM}) and one integrase gene (*intI*) were determined by qPCR. Besides, the total bacteria was quantified through the general 16S rRNA gene. The detail information of the primers targeted the above genes were shown in Table 1.

Table 1 Detail information of primers and qPCR condition used in this study

Target Gene	Prime	Sequence(5'-3')	Annealing temperature (℃)	Product length (bp)	qPCR efficiency (%)
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	60	146	87.4
	R1194	ACGTCRTCCMCACCTTCCTC			
sulI	sul(I)-FX	CGCACCGGAAACATCGCTGCAC	55	163	95.1
	sul(I)-RX	TGAAGTTCCGCCGCAAGGCTCG			
sulII	sul(II)-FX	TCCGGTGGAGGCCGGTATCTGG	58	190	94.6
	sul(II)-RX	CGGGAATGCCATCTGCCTTGAG			
tetW	tet(W)-FX	GAGAGCCTGCTATATGCCAGC	60	168	100.2
	tet(W)-FV	GGGCGTATCCACAATGTTAAC			
tetO	tetO-F	ACGGARAGTTTATTGTATACC	57	171	84.1
	tetO-R	TGGCGTATCTATAATGTTGAC			
tetH	tetH-F	CAGTGAAAATTCACTGGCAAC	60	185	105.7

	tetH-R	ATCCAAAGTGTGGTTGAGAAT			
qnrS	qnrSrtF11	GACGTGCTAACTTGCGTGAT	62	240	91.4
	qnrSrtR11	TGGCATTGTTGGAAACTTG			
ermB	erm(B)-91f	GATACCGTTTACGAAATTGG	58	364	90.0
	erm(B)-454r	GAATCGAGACTTGAGTGTGC			
bla $_{TEM}$	RTblaTEMFX	GCKGCCAACTTACTTCTGACAACG	60	247	93.8
	RTblaTEMFR	CTTTATCCGCCTCCATCCAGTCTA			
intI	intI-F	GGCTTCGTGATGCCTGCTT	55	145	80.1
	intI-R	CATTCCTGGCCGTGGTTCT			

Before qPCR detection, DNA standards for the absolute quantification were prepared from purified plasmid DNA containing the targeted gene ranging from 1.0×10^{2} to 1.0×10^{8} copies·ul^{-1}. The standard plasmid was constructed as follows. Firstly, targeted gene was amplified using the above extracted DNA as template. Then PCR products were purified and ligated into the pMD19-T vector (TaKaRa) according to the manufacturer's instructions. The recombinant plasmids were then transformed into *Escherichia coli* JM109 competent cells (TaKaRa) and the selected positive clones were sequenced using ABI 3730 XL sequencing platform (Majorbio, Shanghai, China). After phylogenetic identification of the targeted gene, the plasmids were re-extracted from positive clones. The initial copy numbers of plasmid DNA were calculated as follows: 6.02×10^{23} × (plasmid DNA concentration, ng/μl) $\times10^{-9}$/ (plasmid DNA length, bp) ×660. Then gradient diluted from 1.0×10^{8} to 1.0×10^{2} copies·ul^{-1}, ready for the next qPCR.

qPCR were carried out in 7500 real-time PCR system (Applied Biosystems) using primer pairs listed in Table 1. Specifically, it was performed in a total volume of 25 μl, containing 12.5 μl of 2×Power SYBRs Green PCR Master mix kit (Applied Biosystems), 0.5 μl of each primer (10 μM), 0.5 μl of bovine serum albumin (10 mg/ml) and 2 μl of DNA (20:1 dilution). Each measurement was performed in three replicates.

2.5. 16S rRNA gene high-throughput sequencing

The 16S rRNA gene sequencing was performed at Majorbio Technology (Shanghai, China) using the Illumina Miseq platform. Microbial community structures were determined using primers 338F (5'-barcode-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeted the V3-V4 region of 16S rRNA gene [21]. The raw paired-end reads were merged by Flash [22].

Then the obtained data were analyzed using QIIME pipeline [23]. The representative sequences of each OTU were picked using uclust method. The taxonomy was annotated with the RDP classifier.

2.6. Data analysis

The redundancy analysis (RDA) of ARGs and its relationship with physicochemical factors (temperature, pH, moisture and O_2) were performed using CANOCO5 (Microcomputer Power, Ithaca, NY). The heatmap of variation pattern of representative ARGs was based on the ratio of absolute quantity of each ARG with the total bacterial copy numbers. Both the heatmaps of ARGs and microbial community structure at genus level were performed via RStudio (RStudio Inc., Boston, Massachusetts). The network analysis was performed using Cytoscape 3.4.0. Only correlations with coefficient (ρ-value) above 0.6 and a significance level (P-value) below 0.05 were displayed in the networks.

3. Results and discussion

3.1. *Variation pattern of representative ARGs during composting under different aeration strategies*

In this study, all of the 8 representative ARGs (including *bla*$_{TEM}$, *ermB*, *qnrS*, *tetH/tetO/tetW* and *sulI/sulII*) and integrase gene *intI* were detected in the original swine manure, with the absolute quantities ranged from 1.2×10^{8} to 9.84×10^{10} copies per gram of dry compost (Fig. 2), which was at the same level of ARGs content in previous studies [14, 17].

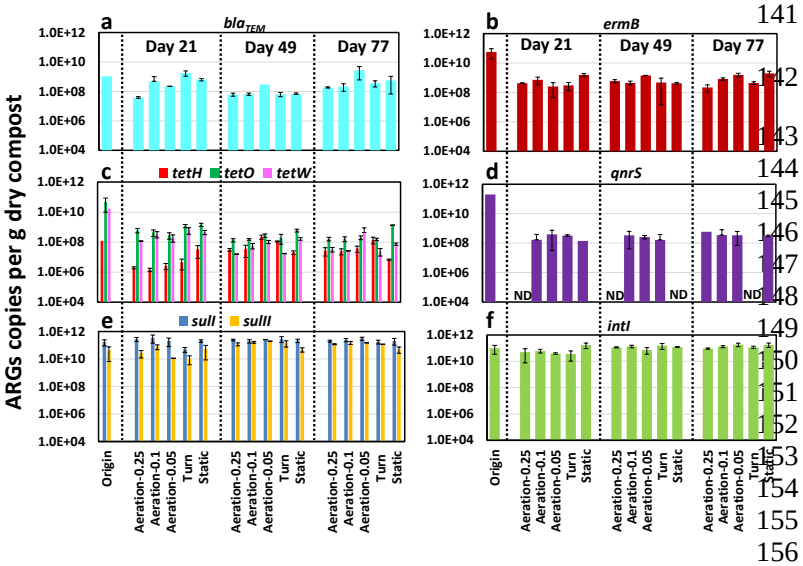
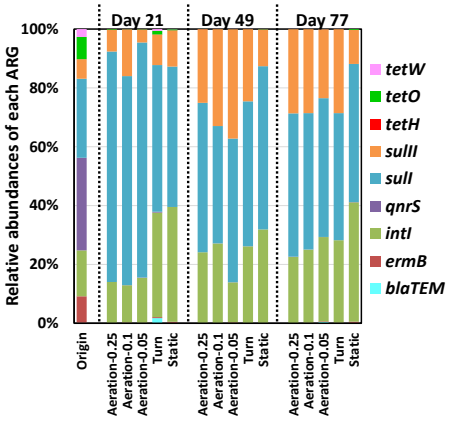


Fig. 2 Variation patterns in terms of absolute quantities of each ARG during the whole composting process among five different managements. Fig. 2a-f was corresponding to *bla*$_{TEM}$ (a), *ermB* (b), *tetW/O/H* (c), *qnrS* (d), *sulI/II* (e) and *intI* (f), respectively. Bars represent standard deviations (n = 3). Note: origin, original swine manure; the number followed "Aeration" indicated the ventilation rate; Turn, turn windrow management; Static, static pile management.

The relative abundance of *qnrS* was the most dominant (31%) in swine manure, followed by *sulI* (27%), *intI* (16%), *ermB* (9%), *sulII* (7%) and *tetO/tetW* (8% / 3%), while that



of bla_{TEM} and *tetH* were less than 0.5% of the total ARGs (Fig. 3). Even though the detection of such high content of *qnrS* was uncommon in other studies, it was indeed exhibited that antibiotics of fluoroquinolone, sulfonamide, macrolide and tetracycline routinely feeding pig with therapeutic or subtherapeutic concentration could enrich diverse and abundant ARGs in manure, posing potential risk for direct application of manure into soil.

Fig. 3 The diversity patterns of the representative ARGs during the whole composting process among 5 different aeration methods. Data were shown in average. Note: origin, original swine manure; the number followed "Aeration" indicated the ventilation rate; Turn, turn windrow management; Static, static pile management.

Similar with prior study [10], the situation turned to be better monitored after composting. The diversity of ARGs in manure significantly decreased via composting, with the only overwhelming dominance of sulfonamide resistance genes *sulI/sulII* and integrase gene *intI* (Fig. 3), fluctuating around ~10^{11} copies per gram of dry compost (Fig. 2e&f). However, inconsistent with previous study reporting the undetectable of most of ARGs at the end of composting, the seemingly disappearing ARGs were still above the detection limitation during the whole composting process. Specifically, the original dominant *qnrS*, *ermB* and *tetO/W* nearly dropped two orders of magnitude from ~10^{10} to ~10^{8} copies per gram of dry compost, while the less abundant bla_{TEM} and *tetH* were even detected with variable low levels of ~10^{6} to ~10^{8} copies per gram of dry compost (Fig. 2a-d). Considering that ARGs could exist for long term, either confined as genetic materials within a cell of the microbe or aggregated with humus in the form of naked nucleotide acid fragments exempt from degradation by extracellular enzymes, it was rational to deduce that composting could not completely remove ARGs from manures [24]. Nevertheless, compared with spreading manure to crops directly, composting was still an efficient measurements in terms of controlling ARGs dissemination, since the decline of most of ARGs instead of elimination could be available to weaken the transmission possibility of ARGs in the environment [25, 26].

It had been reported that high temperature was key factor to control ARGs through killing or inhibiting microbes during the thermophilic phase of composting [17]. The thermophilic period with

temperature above 55 °C extended to almost 6 weeks in this study (data not shown) and even persisted along the whole process in the static pile treatment, especially longer than prior studies with high temperature less than 3 weeks [10, 18]. Here, ARGs contents were determined at 3 time points of the 5 treatments, corresponding to mid-stage of thermophilic phase (day 21), early stage of maturing phase (day 49) and end state of experiment (day 77). Unexpectedly, the scenario of ARGs variation pattern seemed complicated, neither exhibiting regular temporal dynamic nor clustering with composting aeration methods (Fig. 4). Compared with original status, most of the representative ARGs including *qnrS*, *ermB*, *tetO/W/H* and bla_{TEM} decreased and kept at low levels among the 3 time points of different treatments, while *sulI/sulII* and *intI* were highly abundant all along the experiment (Fig. 3&4). It had been found that continuous thermophilic composting with 40 days could effectively reducing the abundances of most of ARGs [17], which reoccurred in our study with extraordinarily long-term of high temperature. However, the decrease of ARGs were not strengthened in static pile with longer thermophilic phase of 77 days. Moreover, no obvious clue correlated aeration rate and aerobic status with the decline of ARGs. The removal patterns of each ARG in different aeration methods were consistent, hence it was tricky to screen out the best methods in terms of ARGs controlling.

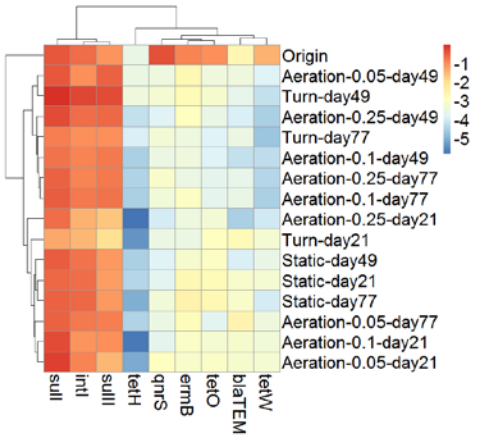


Fig. 4 The heatmap of ARGs variation pattern during the whole composting process among five different managements. Data were analyzed based on the ratio of absolute quantities of ARGs and 16S rRNA gene. Note: origin, original swine manure; the number followed "Aeration" indicated the ventilation rate; Turn, turn windrow management; Static, static pile management.

Even though 6/9 genes detected in this study exhibited removal trend via composting, it was noteworthy that sulphonamide resistance genes *sulI/sulII* and integrase gene *intI* were enriched instead of elimination via composting. With regard to the frequent detection of *sulI/sulII* and *intI* in high abundance from manure and compost samples, further study should be carried out to focus on the removal of these

recalcitrant genes and relevant evaluation of the risk it might be imposed to the accepted environment like soil and water.

3.2. *Microbial community succession under different aeration methods*

After normalization, 46788 sequences of each sample were randomly selected to further analyze the microbial community structure. In total, 1373 OTUs were obtained from the 11 samples with 0.97 similarity level. The good's coverages were all above 99% (Table 2), indicating the sequencing depth of this study was reliable to reflecting the microbial community structure. The richness and diversity indexes were shown in table 2. Compared with original raw material, the microbial richness conveyed via ACE and Chao1 was decreased after composting, especially in the mature ending point. However, the diversity did not change a lot among different aeration methods except the turn windrow treatment at day 77 with a slight reduction.

Table 2 Microbial richness and diversity indexes

Samples	ACE	Chao1	Shannon	Simpson	Coverage
Origin	868	887	4.38	0.04	99%

Aeration-0.25-day21	855	853	4.50	0.04	99%
Aeration-0.1-day21	674	701	4.57	0.02	99%
Aeration-0.05-day21	754	779	4.53	0.03	99%
Turn -day21	753	754	4.35	0.03	99%
Static-day21	718	695	4.56	0.02	99%
Aeration-0.25-day77	604	600	4.08	0.05	100%
Aeration-0.1-day77	605	610	4.57	0.02	99%
Aeration-0.05-day77	611	606	4.51	0.03	99%
Turn-day77	539	536	3.88	0.07	100%
Static-day77	676	656	4.37	0.03	99%

The microbial community composition at phylum level kept steady and consistent across the whole process and among different aeration modes, with the main dominant members containing *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*, accounting for 89-97% of the total sequences (Fig. 5a), which had also been frequently detected with high abundances in previous studies [14-17]. Moreover, the microbial community structures were undergone similar temporal successions among different aeration treatments. Specifically, compared with the original swine manure, the relative abundance of *Firmicutes* was increased at high temperature stage (day 21) but decreased at the end of the experiment (day 77), owing to the fact that most of members belonged to *Firmicutes* were thermophile. *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* were especially promoted at maturity stage in all treatments, which might be correlated with the enrichment of *sulI/sulII* and *intI*. The dominance of *Actinobacteria* at day 77 marked the maturation status of composting [27]. However, *Actinobacteria* was also well-known for its capability to produce antibiotics and harbor diverse ARGs [28]. Hence, it should be cautious to evaluate the compost quality with the abundance of *Actinobacteria*.

Temporal succession of microbial community was prominent at genus level. According to the heatmap shown in Fig. 5b, the core microbial community was significantly distinct among and clustered with the composting stage, but not the aeration modes. Specifically, cluster 1 including *Ruminofilibacter* (8%, *Bacteroidetes*), *Ignatzschineria* (3%, *Proteobacteria*) and *Streptococcus / Lactobacillus / Sporosarcina* (4% / 2% / 2%, *Firmicutes*) were mainly emerged in original swine manure, but declined to

lower abundances with less than 0.5% after composting (Fig. 5b). The succession of these bacteria might contribute to the decrease of diversity pattern of ARGs via composting (Fig. 3). During the composting process, the core microbial community shifted dramatically from cluster 2 of thermophilic phase to cluster 3 of maturity state. In cluster 2, *Bacillus*, *Clostridium sensu stricto 1*, *Oceanobacillus*, *Paucisalibacillus*, *Sinibacillus*, *Terrisporobacter*, *Turicibacter*, uncultured *Thermoactinomycetaceae* and OPB54 with no rank accounted for the increase of *Firmicutes* at high temperature stage, mostly due to their property of thermo-tolerance. While distinct genus affiliated with *Actinobacteria* were abundant in day 21 and day 77 respectively, with frequent detection of *Corynebacterium*, *Dietzia*, *Georgenia* and *Saccharomonospora* in cluster 2 but *Actinomadura*, *Longispora*, *Thermobifida* and uncultured *Acidimicrobiales* in cluster 3. Such shifts were also reported in prior researches, which was attributed to temperature selection [29,30].

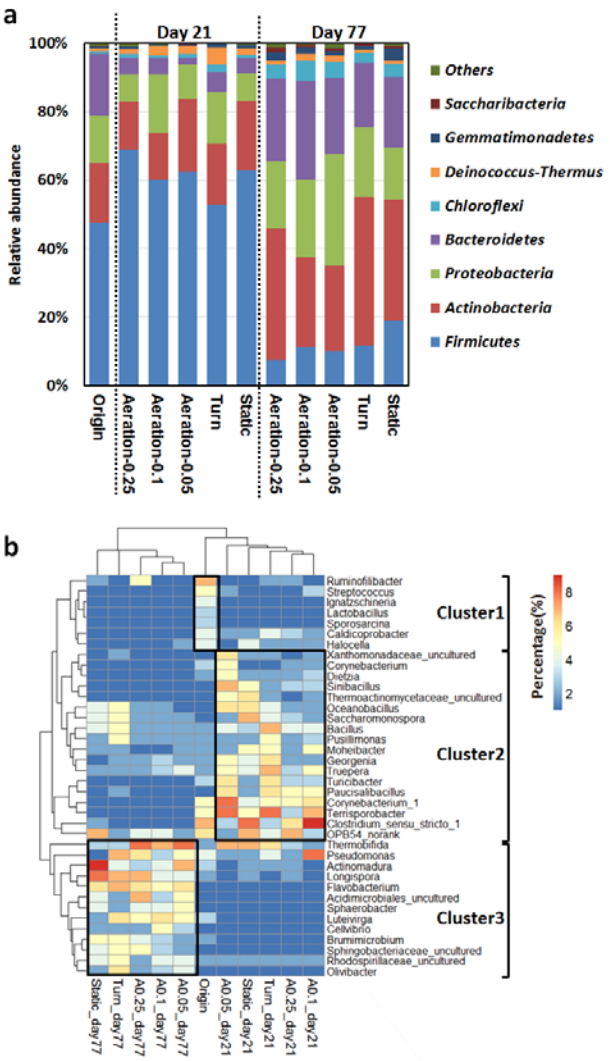


Fig. 5 Microbial community structure at phylum level (a) and heatmap of the top 38 genera with the relative abundance above 0.5% (b). Note: origin, original swine manure; the number followed "Aeration" indicated the ventilation rate; Turn, turn windrow management; Static, static pile management.

3.3. *Correlation of representative ARGs and microbial community structure*

Numerous studies had highlighted the role of microbial community on ARGs pattern in manure and compost samples [13,15-16]. In this study, diverse microbes were significantly negative or positive correlated with most of the detected ARGs except the less abundant *tetH* gene ($P<0.05$). It was noticeable that different genera under the same phylum correlated with distinct ARGs. As shown in Fig. 6, 2 of 12 genera belonged to *Bacteroidetes* (*Ruminofilibacter* and *Petrimonas*) were potential carriers of *qnrS*, *ermB*, *tetO* and *tetW*, while the other genera were mainly positive correlated with *sulII* and *intI*, among which *intI* were co-occurred with *sulII* via the connection of *Flavobacterium* and unclassified *Flavobacteriaceae*. Moreover, the decrease of different genera of *Firmicutes* could lead to the decline of *qnrS*/*ermB*/*tetO*/*tetW* and the enrichment of *sulII*, respectively. In terms of *sulI*, there was only one negative correlation established with *Hahella* affiliated with *Proteobacteria*. Such weak relationship with microbial community was probably due to the redundant existence of *sulI* highly widespread in diverse microbes, rendering it to persist for long term undisturbed by the variation of microbial community [26]. Generally, the enrichment of s*ulI/sulII/intI* at the end of the composting was correlated with the increase of genera belonged to *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* and the decrease of genera affiliated with *Firmicutes*. Given that the microbial succession exhibited similar trends among different treatments, it was well explained the consistent variation patterns of ARGs under different aeration strategies.

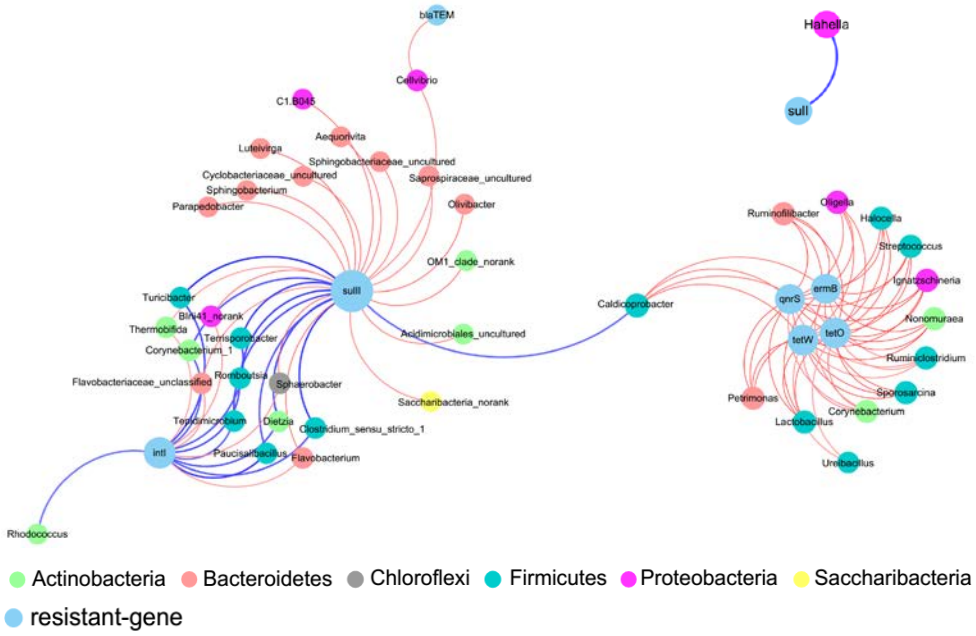


Fig. 6 Network analysis based on the co-occurrence of representative ARGs and their potential host bacteria. A connection represents a significant positive correlation ($P < 0.05$) according to Spearman's rank analysis. The blue and red lines were designated with negative and positive correlations, respectively.

4. Conclusion

Combined with the above results, the questions listed in the introduction could be answered as follows. Firstly, composting was an efficient tool in terms of reducing most of ARGs in original swine manure, but sulfonamide resistance gene (*sulI/sulII*) and integrase gene (*intI)* were enriched instead of removal. Secondly, since the aeration methods showed little influence on the variation patterns of ARGs, especially the intractable stubborn ARGs like *sulI/sulII/intI*, innovative methods should be further developed for the elimination of these genes in compost. Lastly, the variation pattern of ARGs was highly depended on the succession of microbial community.

Acknowledgments

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