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Influence of zeolite and superphosphate as additives on antibiotic resistance genes and bacterial communities during factory-scale chicken manure composting

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ABSTRACT

Factory-scale chicken manure composting added with zeolite (F), superphosphate (G), or zeolite and ferrous sulfate (FL) simultaneously, were evaluate for their effects on the behaviors of antibiotic resistance genes (ARGs) and bacterial communities. After composting, ARGs in manure decreased by 67.3% in the control, whereas the reductions were 86.5%, 68.6% and 72.2% in F, G and FL, respectively. ARGs encoding ribosomal protection proteins (*tetO*, *tetB(P)*, and *tetM*) were reduced to a greater extent than *tetG*, *tetL*, *sul1* and *sul2*. Bacteria pathogens were also effectively removed by composting. Network analysis showed that *Firmicutes* were the important potential host bacteria for ARGs. The bacterial communities and environmental factors, as well as the *intI* gene, contributed significantly to the variation of ARGs. The ARGs and integrons were reduced more when zeolite was added than when superphosphate was added; thus, it may be useful for reducing the risks of ARGs in chicken manure.

1. Introduction

The increasing emergence and spread of antibiotic resistant bacteria (ARB) and resistance genes (ARGs) are of great concern worldwide, threatening the efficacy of modern medicines and posing risks to human health (Levy and Marshall, 2004; Su et al., 2015). Antibiotics are used widely in intensive animal husbandry to prevent diseases and promote growth; thus, animal manure is a significant source of antibiotics (Zhang et al., 2016a) and ARGs (Zhang et al., 2016b), which can enter the soil following direct land application (Fang et al., 2015) and can further spread to food and groundwater (Heuer et al., 2011; Brichta-Harhay et al., 2011). It has been found that livestock manure contains numerous types and amounts of ARGs (Zhu et al., 2013) and that they can be readily disseminated among bacteria in the environment via bacterial reproduction and horizontal gene transfer (HGT) (Binh et al., 2008). Therefore, it is important to implement appropriate management practices that minimize the risk of disseminating ARGs and pathogens from manure.

As an established and well-developed technology for the stabilization of organic matter and reduction of pathogens and odors, composting is widely applied in animal manure treatment and reclamation (Wu et al., 2011). Composting was demonstrated to significantly reduce the levels of antibiotics (Selvam et al., 2012a; Selvam et al., 2012b; Wu et al., 2011; Wang et al., 2012) and ARGs (Selvam et al., 2012a; Zhang et al., 2016b; Wang et al., 2016). Selvam et al. (2012b) reported that composting could effectively remove chlortetracycline and sulfadiazine that were spiked, even at high concentrations, and that resistance genes for tetracycline and sulfonamide were undetectable after 42 days of composting. Wang et al. (2016) also found composting could effectively reduce the abundance of ARGs in swine manure. Therefore, composting is a promising method for the management of manure to decrease the abundance of ARGs.

One the other hand, Qian et al. (2016) found that composting did not remove most ARGs and that the compost remained a reservoir of ARGs. Conventional aerobic composting was found to not effectively control the proliferation and diffusion of ARGs and mobile genetic elements (MGEs) (Youngquist et al., 2016; Zhu et al., 2013; Wang et al., 2015). Therefore, the development of more efficient techniques to improve the removal of ARGs in composting is vital. High temperatures (55 vs 35 °C) has been shown to be important in reducing ARGs more efficiently from digested sludge (Tian et al., 2016), and Liao et al. (2018) found hyperthermophilic composting (temperatures reach up to 90 °C during the fermentation process) could significantly enhance the removal of ARGs and MGEs from sewage sludge. Therefore, elevated

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thermophilic composting temperatures might increase the removal rate of ARGs during chicken manure composting.

Additives, such as mushroom biochar and different surfactants (rhamnolipid and tween 80), were also found able to increase the removal rate of ARGs during chicken manure composting (Cui et al., 2016; Zhang et al., 2016b), while rice straw biochar addition yielded opposite results (Cui et al., 2016). As commonly used additives, mineral substances such as zeolite and calcium superphosphate were demonstrated to have a positive effect on the conservation of nitrogen in compost (Chan et al., 2016; Jiang et al., 2014). Zeolite has also been used as bulking agents to increase sludge porosity (Villasenor et al., 2011), while the additive ferrous sulfate can remove the phosphorous in manure and modify zeolite with a reduced pH level. Zhang et al. (2016c) reported that the total ARGs copies reduced by 1.5% when natural zeolite was added during sludge composting. However, no study has yet investigated and compared the impact of these additives on the abundance and variation of ARGs and bacteria communities during full-scale chicken manure composting.

Thus, the present study tested the effects of added zeolite (F), calcium superphosphate (G), or zeolite and ferrous sulfate (FL) simultaneously, on the removal rate of ARGs during chicken manure composting, under the condition of elevated thermophilic composting temperature up to 70–80 °C without exogenous heating, which is 10–15 °C higher compared to conventional composting. The fate of seven ARGs (*tetG, tetL, tetM, tetO, tetB(P), sul1*, and *sul2*) during full-scale chicken manure composting was evaluated. Bacterial communities strongly affected the dynamic of ARGs during sludge composting (Su et al., 2015) and integrase genes can capture ARGs and facilitate their transfer in the environment (Sun et al., 2017). Therefore, the relationships among integrase gene (*intI1* and *intI2*), the bacterial community structure, bacterial pathogens and ARGs were explored to understand the variation of ARGs during composting. The main objectives of this study were to investigate (1) the effects of aerobic composting on ARGs abundances and bacterial communities with higher thermophilic composting temperature (approximately 70–80 °C); (2) the effects of zeolite and calcium superphosphate on the levels of ARGs and the integrase gene during composting with an elevated thermophilic composting temperature; and (3) the degree to which different variables, including environmental factors and the bacterial community, affect the behaviors of ARGs.

2. Materials and methods

2.1. Description of raw materials and experimental setup

Fresh chicken manure was collected at a selected farm in July 2014 in Huizhou of Guangdong province, China. Composting materials comprised a mixture of fresh chicken manure and rice bran at a ratio of 4:1. The physicochemical properties of the raw materials are described in Table 1. Chicken manure composting was carried out in four windrow tests, including four experimental tests of every 7t raw materials (on wet weight) with the addition of (1) 350 kg natural zeolite (F), (2) 350 kg calcium superphosphate (G), (3) 350 kg natural zeolite and 210 kg ferrous sulfate (FL), and (4) a control test (CK) without additive. The tests were carried out from July 14 to September 2, 2014, in a windrow composting plant of Haina bioorganic fertilizer Co Ltd. located in Huidong County, Huizhou, Guangdong. The shape of the windrow pile was a three-prism type, at a height of 1.1–1.2 m and a width of 2.0–2.2 m at the bottom of the pile. The full-scale windrow composting was turned by a turner (BACKHUS 15.30, Germany) every two days (48 h) during the thermophilic stage of 15 days. Then, the piles sat quietly for 31 days (mature stage). The pile temperature, at a depth of 30 cm, was recorded from 5 points in each pile every day.

Table 1
Physical and chemical properties of the raw materials used for composting.

	Total carbon (g/kg)	Total nitrogen (g/kg)	pH	Moisture (%)
Chicken manure	371.6	26.8	7.56	80.0
Rice bran	413.0	9.8	7.29	5.08

2.2. Sample collection and DNA extraction

The surface material was removed from each pile, and samples were collected by inserting a hollow PVC circular tube ($\phi = 2$ cm) at six points in each composting pile, and then mixing completely to obtain a representative sample. Samples were collected on days 1, 5, 15, 28, and 39. Each sample was divided into two parts: the first was stored at 4 °C for later analysis, and the second was stored at −80 °C before DNA extraction.

Total community DNA was extracted from 0.5 g manure using the FastDNA® SPIN Kit for feces (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. The extracted DNA was dissolved in 80 μL of TES buffer, quantified by microspectrophotometry (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE), and stored at −20 °C until use. The DNA samples were diluted to 10× and 50× to avoid inhibitors to the PCR reaction.

2.3. Quantification of ARGs, intI gene and 16S-rRNA

The presence of tetracycline resistance genes (TRGs) (*tetG*, *tetL*, *tetM*, *tetO*, *tetB(P)*), sulfonamide resistance genes (*sul1* and *sul2*), and *intI1*, *intI2* were quantified by Real-Time quantitative PCR (qPCR) analysis with a C1000™ Thermal Cycler equipped with the CFX96™ Real-Time system (Bio-Rad, USA). In addition, the 16S rRNA gene abundance, which has been used previously to assess the overall bacterial abundance, was quantified by using primer set 519F/907R so that the ARGs abundance could be standardized against the bacterial populations.

Plasmids carrying each targeted ARGs were extracted and purified using the E.Z.N.A.™ Plasmid Mini Kit (OMEGA, USA). Plasmid concentrations were determined by NanoDrop, and the abundance of ARGs per microliter of plasmid solution was calculated according to Zhang et al. (2009). A 10-fold dilution series of plasmid DNA was made to generate a six-point calibration curve (Ct versus log of initial ARGs copy) for qPCR. Assays were set up using the SYBR *Premix Ex Taq™ Kit* (TaKaRa). The 20 μL reaction mixture contained 10 μL of SYBR® *Premix Ex Taq™*, 0.5 μM of the primers described previously (Peng et al., 2017), and 1.0 μL of template containing approximately 2–9 ng of DNA. Blanks were always run with water as the template instead of soil DNA extract. Specific target gene amplification was confirmed by agarose gel electrophoresis and melt curve analysis. qPCR was performed in triplicate and amplification efficiencies of 95–110% were obtained with R^2 values of 0.990–1.000. Based on the calibration curves, the Ct value of a test sample was used to calculate the abundance of ARGs. The extracted DNA was checked by qPCR using serially diluted samples to minimize PCR inhibition.

2.4. Illumina sequencing and analyses of bacterial pathogens

The 16S rRNA gene sequencing was performed at Novegene (Beijing, China) using the Illumina HiSeq platform. The 16S V4 region was amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′). The bar-coded PCR products from all samples were normalized in equimolar amounts, purified and then sequenced using the HiSeq 2500 (PE 250) following the manufacturer's protocols. The raw pair-end reads were assembled using FLASH. Raw reads were quality filtered using QIIME (available at http://www.qiime.org) (Caporaso et al., 2010), and the high quality sequences generated were processed and analyzed. Operational

taxonomic units (OTUs) was defined at the 97% similarity level using Uparse. A representative sequence of each OTU was assigned to a taxonomic level in the SILVA SSUrRNA database. Pathogenic bacteria were detected according to the methods described by Peng et al. (2017).

2.5. Statistical analysis

Statistical analyses were performed with the software package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± standard deviation (SD). Mean separation was assessed by ANOVA. Differences were considered significant at $P < 0.05$. Correlation tests were performed using spearman correlation coefficient. Network maps based on the spearman correlation analysis between ARGs and *intI*, the bacterial communities and bacterial pathogens were drawn using the Gephi platform.

3. Results and discussion

3.1. Changes of pile temperature and pH

Temperature and pH are the main indicators of composting. As shown in Fig. 1, the pile temperature in F and CK increased more rapidly than the other treatments, reaching 74 °C and 75 °C, respectively, on the 6th day, while FL need 10 days, and G need 13 days. The addition of G and FL delayed the arrival of high temperatures, indicating that G and FL might inhibit the thermophilic microbial activity, which plays a leading role in the heating stage. This result was partly consistent with the results observed by Lee et al. (2009). The maximum pile temperatures (79.5 °C) observed in this study were higher than the findings of previous studies, in which the highest temperature was 76 °C(Li, et al., 2017), 71 °C (Zhang et al., 2016b) or 44 °C (Cui et al., 2016). The stage with temperatures above 70 °C (at least 21 days) was also far longer than those observed in these previous studies.

At the beginning of the composting process, the pH value of CK was 7.12. The pH values of CK and F markedly increased to maximum values of 9.03 and 9.43 on days 11 and 21 respectively, while pH of G and FL reached a maximum value of 7.97 and 7.75 on day 9 (Fig. 1). The pH of F and CK increased immediately in the early stage before stabilizing at approximately 9.0, which is consistent with Zhang et al. (2016b). This was likely due to the high content of organic matter in chicken manure, the rapid degradation of this matter and subsequent decomposition of organic acids, which released ammonium and volatile ammonia, thus increases pH values. While the pH values in G and FL were not changed a lot and maintained stability near 7.6 and 7.3 as a result of the neutralization effects of calcium superphosphate and ferrous sulfate.

3.2. Variation in ARGs during composting

As shown in Fig. 2, *sul1* was observed to have the highest relative abundance. The total relative abundance of ARGs in all four treatments was reduced along with the composting process (Fig. 2). In terms of each ARG, the trend of decreasing relative abundance was observed in all treatments during the chicken manure composting period. Therefore, these additives did not substantially change the trends of these ARGs during chicken manure composting, but F and G did hinder the recovery of the total absolute abundance of these ARGs at day 28 (Fig. 2A).

Some studies reported that composting can reduce the abundance of ARB and ARGs in manure and biosolids, but there is also evidence that the composting process can increase the levels of ARGs (Youngquist, et al. 2016). In this study, the maximum removal rates of *tetM, tetO*, and *tetB(P)* at the end of the composting experiment were 99.9%, 99.6%, and 98.5%, respectively (Table 2), while for *sul1*, *sul2*, *tetG*, and *tetL*, they were 82.1%, 92.8%, 69.2% and 87.6%, respectively (Table 2). No matter whether the chicken manure compost was added with additives, TRGs encoding ribosomal protection proteins (RPPs) (*tetO*, *tetB(P)* and *tetM*) were reduced to a greater extent than *tetG*, *tetL*, *sul1* and *sul2*. Yu et al. (2005) also found that in swine manure, TRGs encoding RPPs were reduced about 4-log after composting, while *tetG* was not changed. Wang et al. (2015) also reported those TRGs encoding efflux pump (EFPs) and enzymatic inactivation proteins and sulfonamide resistance genes increased when the composting was complete, while the TRGs encoding RPPs remained relatively stable throughout the composting process. Generally, RPPs TRGs and a few TRGs encoding EFPs were mainly harbored by Gram + bacteria, while EFP TRGs dominated in Gram- bacteria (Liu et al., 2012; Chopra and Roberts, 2001), Gram + bacteria may be more easily removed than Gram- bacteria, which was considered to be the one important factor affecting ARGs variation.

At the end of the experiment, *sul1* was the main ARG and *intI1* was the main integrase gene in the compost, the removal rates of *sul1* and *intI1* in CK were 52.6% and 28.5% in relative abundance, indicated that these two genes were difficult to remove by composting. Addition of F increased the removal rate of *sul1* and *intI1* to 82.1% and 63%. Class I integrons are typically associated with *sul1* and often found in Gram- bacteria (Heuer and Smalla, 2007), *sul1* was found to be extremely tolerant to high temperatures or it can use thermophilic bacteria as their hosts (Liao et al., 2018), hence, some complementary strategies are needed to remove this stubborn ARG.

After the maturation phase (day 39), the relative abundance of these ARGs were all significantly reduced in each treatment compared with their original abundance in chicken manure before composting (Table 2). F decreased ARGs the most (86.5%) in relative abundance,

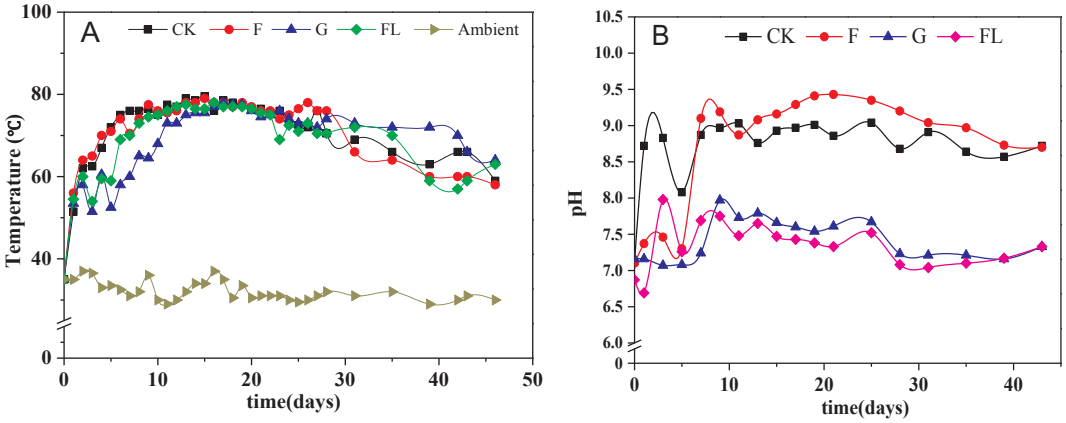

Fig. 1. Changes in temperature (A) and pH (B) during chicken manure composting. CK–raw materials (fresh chicken manure and rice bran); F–raw materials + natural zeolite; G –raw materials + calcium superphosphate; FL– raw materials + natural zeolite + ferrous sulfate.

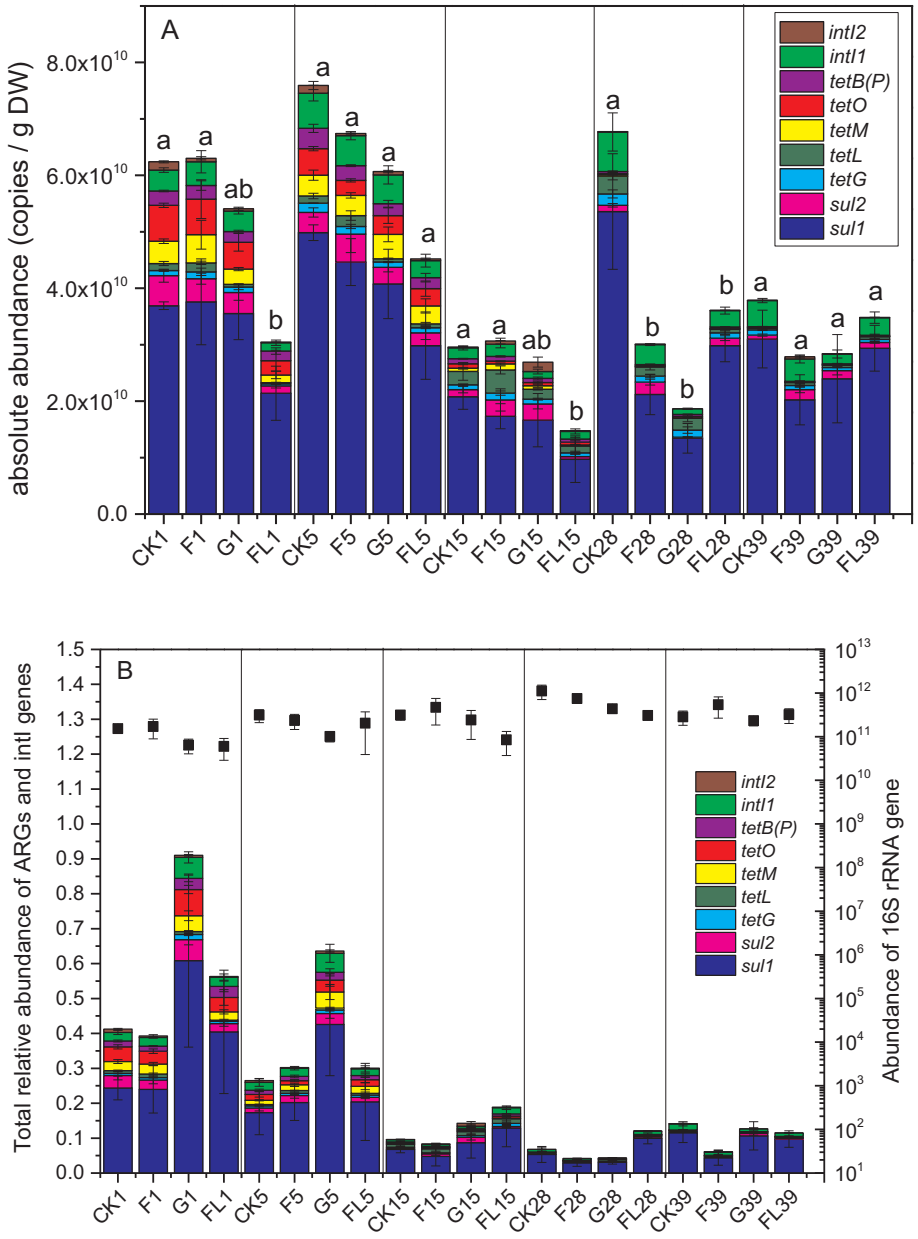

Fig. 2. Changes in the absolute abundance (A) and relative abundance (B) of ARGs in different additives treated chicken manure. Bars denote standard errors.

Table 2
The remove rate the absolute abundance and the relative abundance of each ARG or total ARGs after the chicken manure were composted with addition of different additives (day 39) when compared with their initial content in raw material.

	Treatments	sulfonamide resistance genes		TRGs encode efflux pumps (EFPs)		TRGs encode ribosomal protection proteins (RPPs)			Integrase gene		
		sul1	*sul2*	*tetG*	*tetL*	*tetM*	*tetO*	*tetB(P)*	*intI1*	*intI2*	total ARGs
absolute abundance change	CK	16.0%	86.7%	0.4%	75.9%	98.6%	98.7%	94.4%	−24.8%	96.8%	42.0%
	F	45.1%	66.4%	17.3%	58.1%	98.7%	99.0%	96.0%	−6.4%	73.7%	58.9%
	G	35.0%	73.1%	38.9%	65.2%	99.7%	98.9%	94.6%	53.1%	98.9%	53.5%
	FL	20.4%	80.6%	40.6%	59.6%	99.7%	98.9%	94.1%	15.7%	96.8%	44.7%
relative abundance change	CK	52.6%	92.8%	42.6%	85.3%	99.2%	99.3%	96.9%	28.5%	98.1%	67.3%
	F	82.1%	89.4%	67.0%	87.6%	99.5%	99.6%	98.5%	63.0%	91.7%	86.5%
	G	56.2%	81.2%	58.2%	75.8%	99.8%	99.3%	96.4%	68.6%	99.2%	68.6%
	FL	60.1%	90.5%	69.2%	77.3%	99.9%	99.4%	96.8%	59.9%	98.3%	72.2%

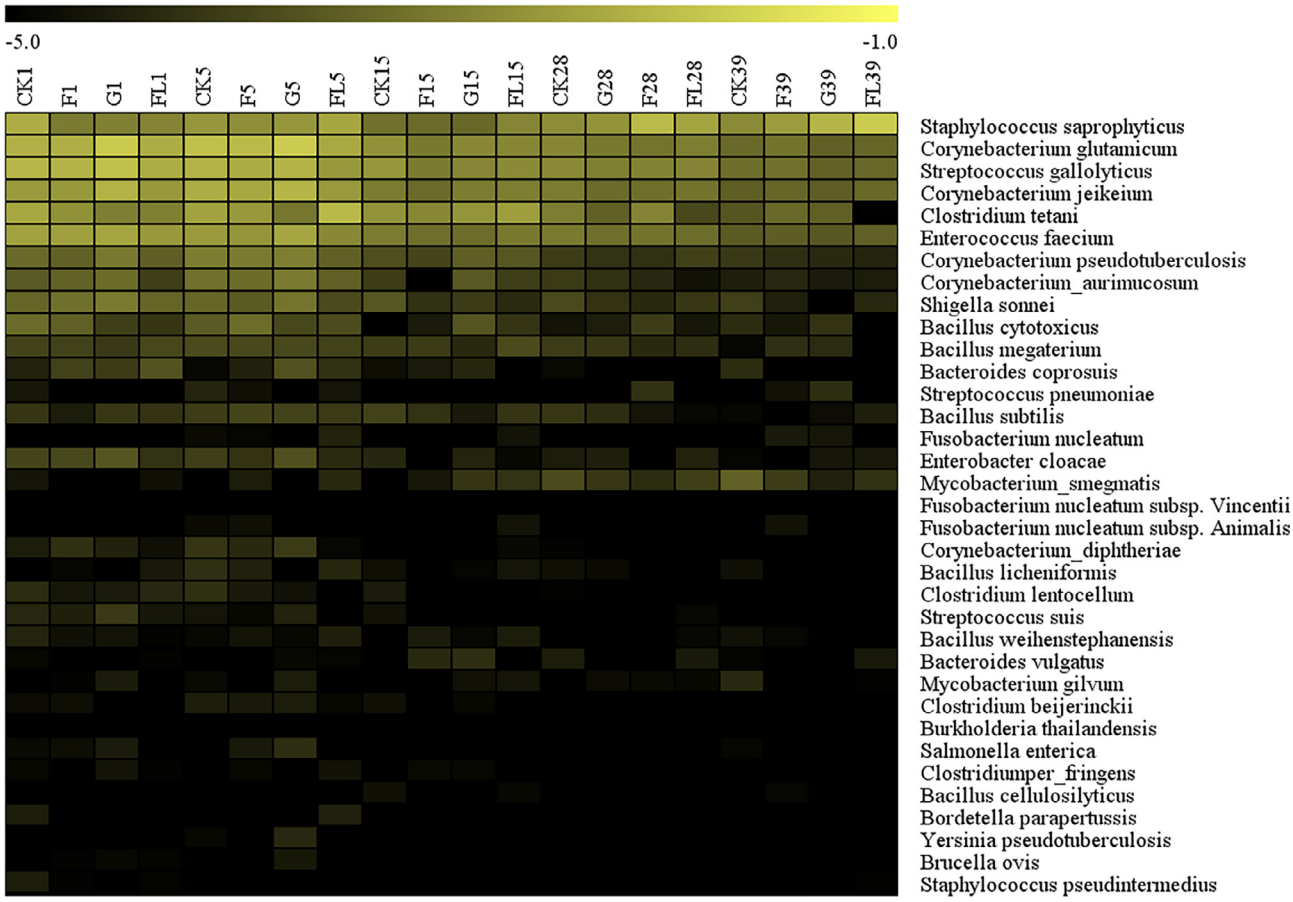

Fig. 3. The relative abundance of bacterial pathogen in chicken manure compost with different treatment. The color intensity in each panel shows the average relative abundance (log scaled) of each species in four samples.

followed by FL (72.2%), addition of F increased the removal rate of composting by 19.2%. This indicated that composting could effectively reduce the abundance of these ARGs in chicken manure, addition or combined addition of zeolite and ferrous sulfate promoted the effects of compost on ARGs. Additives, such as bamboo charcoal, investigated in previous studies, were found to be able to reduce some ARGs by 21.6–99.5%, while increasing *sul1* by 7.5–17.7 times (Li et al., 2017). Mushroom biochar reduced ARGs in chicken manure compost, while rice straw biochar increased abundance of ARGs (Cui et al., 2016). Both mushroom biochar and rice straw biochar additions had a negative influence on the average removal value of ARGs in duck manure compost (Cui et al., 2017). Zhang et al. (2016c) found that total ARGs copies were reduced 1.5% by natural zeolite in sludge composting, while in this study, 58.9% of the total ARGs copies were removed by natural zeolite.

3.3. Occurrence and abundance of bacterial pathogens

ARGs are a concern because they may transfer into bacterial pathogens and make antibiotics ineffective (Sun et al., 2017); therefore, changes in bacterial pathogens were also analyzed. Twelve pathogenic bacteria were dominant at the beginning of the experiment, but six of the pathogens were decreased by composting (Fig. 3). The removal rate of pathogenic bacteria in CK reached 70% on day 15, and 86.8% on day 39 (Table 3), indicating that composting is an effective method for the removal of pathogenic bacteria from chicken manure. Previous studies have shown that more than 94% of human pathogenic bacteria could be removed by anaerobic digestion of pig manure (Sun et al., 2017). On day 15, addition of F increased the removal rate of the pathogenic bacteria compared with CK (Table 3), this result may be caused by the higher temperature and the higher pH in F compared to CK, because most pathogenic bacteria cannot survive continuous high temperature, continuous thermophilic composting could remove 96.9% of human pathogenic bacteria (Qian et al., 2016). In addition, alkaline environment (pH > 8.5) may also be detrimental to the survival of the pathogenic bacteria.

Table 3
The remove rate of the pathogenic bacteria after the chicken manure were composted with addition of different additives when compared with the initial content of pathogenic bacteria in raw material.

	Day 15	Day 39
CK	70.01%	86.83%
F	83.91%	81.97%
G	74.99%	71.61%
FL	69.25%	42.58%

Pathogenic bacteria assigned to 35 genera were detected during composting (Fig. 3), where *Staphylococcus saprophyticus, Corynebacterium glutamicum, Streptococcus gallolyticus*, *Corynebacterium jeikeium*, *Clostridium tetani*, and *Enterococcus faecium* were dominant, as they accounted for 91.14% of the total pathogenic bacteria (Fig. 3). The relative abundance of these pathogenic bacteria were reduced during composting except for *Staphylococcus saprophyticus* (Fig. 3), which increased in G and FL on day 39 compared with CK. *S. saprophyticus* is a common cause of community-acquired urinary tract infections, the real abundance of this pathogen at the end of the composting should be measured by culture-depended method, because bacterial 16S rRNA genes may persist until the DNA structure is degraded, yet the bacterium may have been rendered nonviable long before the DNA is

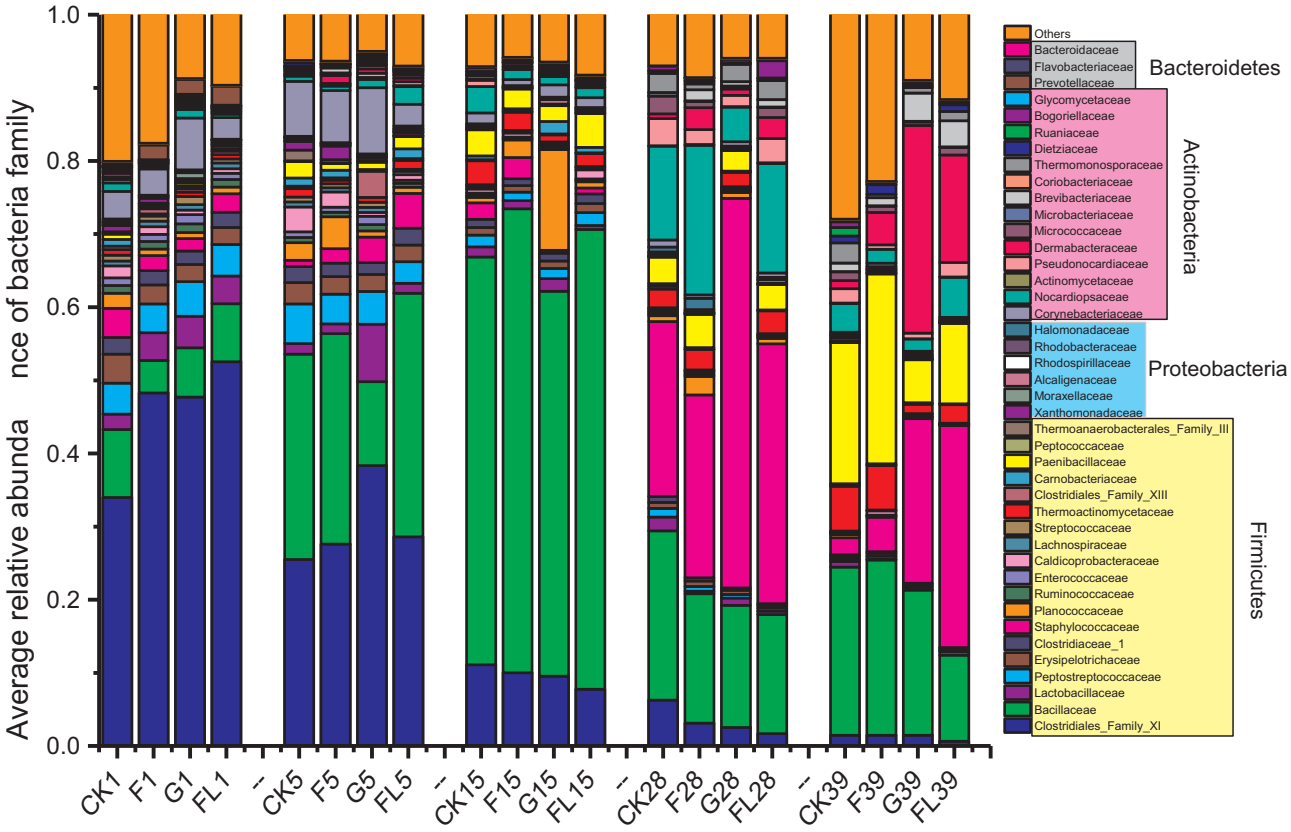

Fig. 4. The relative abundance of 16S rRNA gene sequences classified to each family during chicken manure composting.

completely degraded.

3.4. Changes in the bacterial communities

16S rRNA gene sequencing was performed to reveal bacteria community profiles during chicken manure composting. The 16S rRNA HiSeq sequencing generated a total of 3,640,731 high quality sequences from all samples, where the number of sequences per sample ranged from 32,883 to 61,348. The relative abundances of bacteria, at the family level, displayed distinct variations at different phases of composting (Fig. 4). Most of these bacteria belong to *Fimicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, accounting for 73–96.2% of the total bacterial 16S rRNA gene sequences, which is consistent with previous studies (Zhang et al., 2016c; Cui, et al., 2016; Song et al., 2017). *Firmicutes* are reported to be the key players in the degradation of cellulose (Lebuhn et al., 2014), and were the most abundant phylum (71–90% on days 1, 5, and 15, 53–83% on days 28 and 39) throughout the composting process in all treatments (Fig. 4). This result differs from the results obtained by Cui et al. (2016), in which the relative abundance of *Firmicutes* was lower than 31.5% throughout the chicken manure composting process, and the relative abundance of *Bacteroidetes* and *Proteobacteria* were far greater than what was observed in this study. Thus, the bacterial communities in chicken manure from different sources is quite different.

The composting process is dominated by aerobic bacteria and fungi that convert raw materials into energy and cell biomass and produce humified organic matter. *Clostridia* are reported to be prevailing in reactor substrates with lignocellulose-rich agricultural residues with *Clostridium* III or *Clostridium* IV members being dominant (Lebuhn et al., 2014). In this study, *Clostridiales* Family XI was the most abundant bacteria at the beginning of composting, and it decreased during the composting process (Fig. 4). In contrast, the relative abundance of *Bacillaceae* was increased during the thermophilic stage and observed to be the main bacteria on day 15. *Staphylococcaceae* are aerobic or facultatively anaerobic, with a few being obligate anaerobes, in this study, *Staphylococcaceae* was prevailing on day 28 when turning the piles was stopped (Fig. 4). At the end of the composting, the bacterial communities of CK and F were similar to each other, with both treatments containing more abundance of *Bacillaceae* and *Paenibacillaceae*, while the main bacterial families in G and FL were *Staphylococcaceae*, *Bacillaceae* and *Dermabacteraceae*. This may be a result of the relatively lower pH of G and FL (7.1–7.3) than that of CK and F (8.5–8.8).

3.5. Relationships among bacterial communities, bacteria pathogens, intI genes and ARGs

The co-occurrence patterns of ARGs, *intI1*, *intI2*, and bacterial families during chicken manure composting were investigated using network analysis (Fig. 5). Microbes are the main carriers of ARGs, so changes in the succession of the bacterial community may lead to variations in ARGs (Udikovic-Kolic et al, 2014). Spearman's correlation coefficient ($P < 0.05$) was significant between the bacterial families and the abundance of ARGs (Fig. 5). Although *Firmicutes* and *Actinobacteria* were the groups probably carrying and disseminating ARGs (Huerta et al., 2013; Cui et al., 2016), the relative abundances of these two phyla were not reduced during the thermophilic stage, yet the relative abundance of ARGs was obviously decreased. Therefore, the decrease in the relative abundance of some special bacteria which are associated with ARGs might explain the removal of ARGs during the composting process. Consistent with previous observations (Song et al., 2017), the bacteria that are positively correlated with these ARGs belong mainly to *Clostridia, Bacilli, Bacteroidia* with some classes belonged to *Actinobacteria* or *Proteobacteria*, and these potential ARG hosts were strongly reduced in these treatments.

Twenty bacterial families belonging to *Firmicutes* were significantly correlated with all ARGs. *Clostridiales* Family XI, *Lactobacillaceae*,

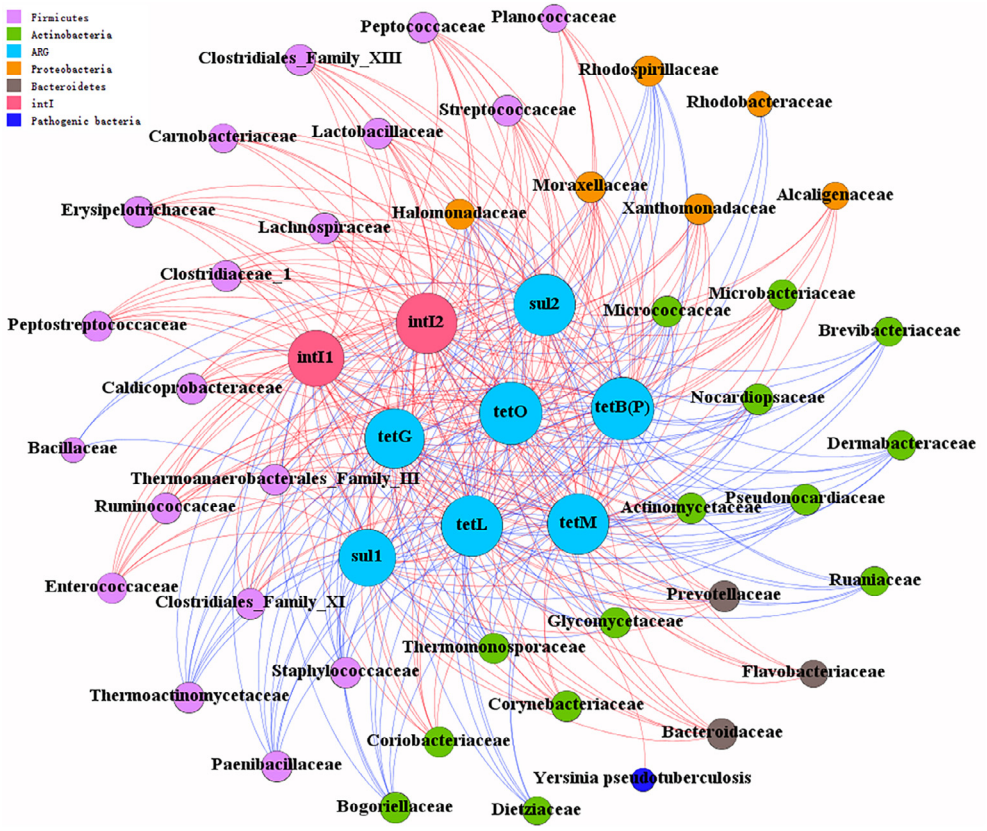

Fig. 5. Network analysis of the co-occurrence patterns between ARGs and microbial taxa. A connection represents a significant (p-value < 0.05) correlation based on spearman correlation analysis (r > 0.3). The size of each node is proportional to the number of connections, i.e., the degree. The rose red and blue lines represent positive and negative correlations, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Erysipelotrichi and *Clostridiaceae* 1 were significantly positively correlated with all ARGs and intI genes, while no ARGs was positively correlated with *Bacillaceae* (Fig. 5). The *tetM*, *tetO* and *tetB(P)* genes were most correlated with *Clostridiales* Family XI (r = 0.92, 0.88 and 0.88); the decrease of this potential host during the composting process (Fig. 4) might explain the reduction of these ARGs to a certain extent. A previous study found that the bacterial classes including *Gammaproteobacteria* and *Clostridia* in sediment samples were significantly selected for by tetracyclines (Xiong, et al., 2015). The results presented here are somewhat coincident with this study, with approximately 56% of the detected bacteria belonged to *Clostridia,* and all of them being significantly positively correlated with the ARGs analyzed in this study.

In addition, *Enterococcaceae* and *Streptococcaceae* were significantly positively related with all of the ARGs and *intI* genes. And *intI1* was positively correlated with *sul1* (r = 0.94), *tetG* (r = 0.83), *tetB(P)* (r = 0.76), *tetM* (r = 0.76), *tetO* (r = 0.74) and *sul2* (r = 0.76), *intI2* was also correlated with all of these ARGs, and they were significantly correlated with various bacteria. These results indicated that *Firmicutes* were the most important potential host bacteria for ARGs, which contains potential human pathogen groups such as *Enterococcaceae* and *Streptococcaceae*. Their roles in disseminating ARGs should not be underestimated, although only one pathogenic bacteria (*Yersinia pseudotuberculosis*) was found significantly positively correlated with only one ARG (*tetL)* (Fig. 5).

3.6. Relationships between the ARG profiles and environmental factors

VPA analysis (Fig. 6) showed that a total of 87.7% of the variance of ARGs could be explained by selected variables including integrase genes, bacterial communities and environmental factors. RDA analysis between the relative abundance of ARGs and environmental factors such as pH, temperature, moisture and the concentrations of nitrogen and total organic carbon (TOC) showed that pH, temperature and the concentration of nitrite nitrogen were negatively correlated with the abundance of ARGs and *intI1* or *intI2* (Fig. 6). Li et al. (2017) found that the ARG profiles during chicken manure composting with added with bamboo charcoal were affected greatly by the temperature, pH and C/N ratio. While Zhang et al (2016b) found that temperature, water-soluble carbon and pH greatly explained the ARG profiles during chicken manure composting treated with different surfactants. Temperature is a critical variable with respect to the destruction and inactivation of ARB and ARGs (Diehl and LaPara, 2010; Zhang et al., 2016b), and played an important role in the reduction of ARGs and the evolution of microbial communities (Wang et al., 2016; Liao et al., 2018). In addition, pH can impose a selective pressure on microbes, and thus affect the ARGs profile during composting. Alkaline fermentation could greatly decrease the quantities of target ARGs in sludge, the quantities of genetic vectors (plasmid DNA, extracellular DNA and phage DNA) were also decreased, which might limit the transfer of ARGs via conjugation, transformation and transduction (Huang et al., 2017). In this study, the thermophilic composting temperature was 70–79.5 °C, composting could effectively reduce the abundance of ARGs, probably because of the high temperature, high pH value and partially aerobic conditions.

The bacterial community has been determined to have a dominant contribution to ARG profiles in soils (Forsberg et al., 2014; Chen et al., 2016), sludge composts (Zhang et al., 2016c; Zhang et al., 2016d), rivers (Czekalski et al., 2014, Xiong et al., 2014), wastewater treatment plants (Yang et al., 2014) and others (Xiong et al., 2015; Udikovic-Kolic et al., 2014). The succession of the bacterial community appears to have a greater influence on the variation of ARGs during composting than the presence of antibiotics (Qian et al., 2016). While in this study, the interaction between environmental factors, bacterial communities and *intI* genes explained most (51.7%) of the variation of the ARGs (Fig. 6). Thus, changes in environmental factors, as well as the bacterial community structure and *intI* genes resulted by the effects of these additives and composting, all contributed significantly to the resistance gene profiles during chicken manure composting.

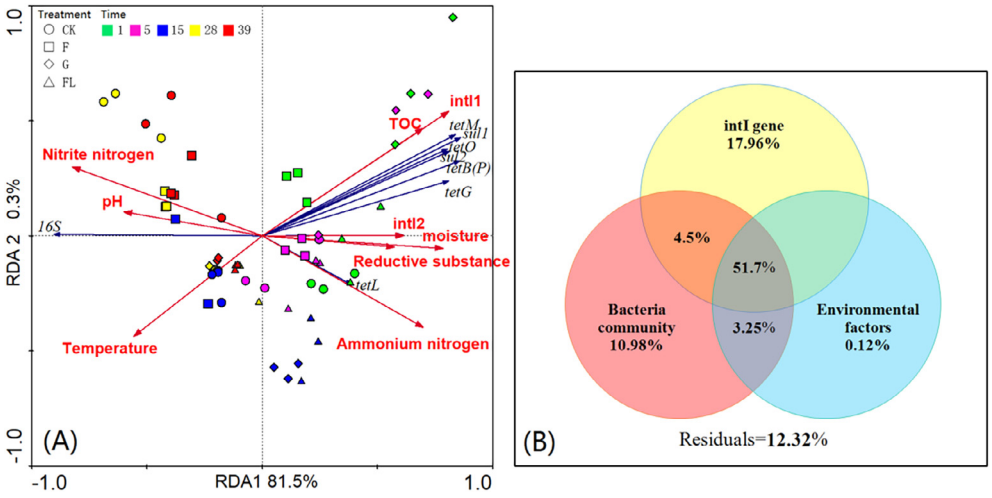

Fig. 6. Redundancy analysis (RDA) differentiating the effects of environmental factors (red arrows except *intI1* and *intI2*) on the shift of overall ARGs (A) and variation partitioning analysis (VPA) differentiating effects of environmental factors, bacterial community and intI genes on the resistome alteration (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

Most ARGs were reduced along with the composting process in chicken manure, and the addition of zeolite and calcium superphosphate promoted the effects of compost on ARGs, hindered ARGs recovery at day 28. Composting also effectively removed the pathogenic bacteria, and the addition of zeolite accelerated the removal of pathogenic bacteria. Furthermore, potential ARG bacterial hosts were strongly reduced. Indicating these additives are promising in the terms of ARGs reduction during chicken manure composting. Environmental changes (mainly temperature, pH and concentration of nitrite nitrogen), integron abundance and bacterial succession were all responsible for the variation in the ARG profiles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.04.107.

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