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Sludge anaerobic digestion with high concentrations of tetracyclines and sulfonamides: Dynamics of microbial communities and change of antibiotic resistance genes

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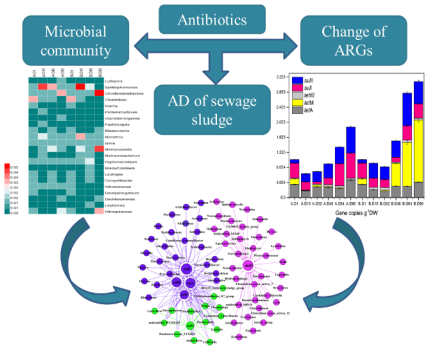
GRAPHICAL ABSTRACT



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ABSTRACT

This study established two mesophilic anaerobic digesters to ascertain the microbial dynamics and variation characteristics of antibiotic resistance genes (ARGs) during sludge anaerobic digestion (AD) with high concentration of antibiotics. System parameters, microbial community, ARGs (*tet*A, *tet*M, *tet*W, *sul*I, *sul*II) and integrase gene of class 1 (*intI*1) were analyzed. General performance of AD showed methane production was inhibited by 17.1% under the pressure of antibiotics. Microbial 16S rRNA high-throughput sequencing results showed the richness of microbial community decreased, but a higher diversity was found with antibiotics added. Furthermore, microbial community structure at genus level was significantly changed. Real-time quantitative PCR of several target genes demonstrated that the adjunction of high concentration of antibiotics exerted a significant induction influence on ARGs, however, the abundance of *intI*1 decreased observably. Correlation analysis showed *intI*1 only played a small role in ARGs' transfer during AD, change of potential hosts was the key factor instead.

1. Introduction

Since the penicillin was found by Fleming in 1928, antibiotics has been a new powerful weapon against pathogens for human beings, which opened a new era of infection against pathogens including bacteria, mycoplasma, chlamydia, spirochetes, rickettsia and so on.

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However, there is an indisputable fact that the global abuse of antibiotics has led to the widespread spread of antibiotic resistance genes (ARGs) which can pose a serious threat to the ecological environment and human health (Guo et al., 2018; Sun et al., 2018). Particularly, wastewater treatment plants (WWTPs) have been pertinent reservoirs for antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Compared with the effluent of sewage plant, sludge is considered to be the main source that is responsible for the transmission of ARGs in environment (Zhang et al., 2016). This is because the hydraulic retention time (HRT) for typical municipal wastewater treatment plants is usually too short to achieve the biodegradation process of most antibiotics (such as sulfonamides), which leads to the residue of most antibiotics in sludge that can absorb a large amount of antibiotics, ARGs and resistant bacteria during the treatment (Yang et al., 2011). Tetracyclines and sulfonamides are two broad-spectrum antibiotics, which are frequently used in the livestock industry (Ezzariai et al., 2018; Liu et al., 2017). It has been reported that the concentration of sulfonamides in activated sludge has reached μg/kg level (Xu et al., 2007). The abundance of ARGs in the digested sludge can be as high as 10^6–10^{10} copies/g (Ma et al., 2011). Obviously, the sewage sludge has a very high risk of resistance transmission. In China, it is estimated that the production of municipal sludge will reach 60–90 million tons by 2020 (Yang et al., 2015). If there is no proper method to deal with these sludge, it will inevitably lead to the rapid migration and spread of antibiotic resistance to the environment (Peng et al., 2015). Thus, developing a technology that can effectively control the propagation of ARGs in the sludge treatment and disposal process is of great importance.

Anaerobic digestion (AD) is an effective way to dispose the sewage sludge. (Xu et al., 2018). Meanwhile, the application prospects of it will be even broader under the premise of energy shortage all over the world for its low cost and high harmlessness (Zhao et al., 2017). However, currently, the primary goal of anaerobic digestion is based on the efficient removal of organic matters (such as COD, volatile organic compounds) and the increase of methane production. The removal of trace contaminants in sludge is very limited (Zhang et al., 2016). Among those trace pollutants that have been frequently reported, the reduction of antibiotics and ARGs in the anaerobic digestion process has been a hot topic. Although some studies found that the residual antibiotics in sludge could be effectively removed through AD, the removal rates on ARGs were quite different, and even some opposite conclusions were drawn. For example, it was found that most ARGs could be reduced in the process of anaerobic digestion except *sul*II and *tet*M (Polesel et al., 2016; Zhang et al., 2018). Meanwhile, there was another study found that mesophilic anaerobic digestion could remove part of ARGs including *sul*I and *sul*II, while some ARGs increased, such as *tet*W and so on (Ma et al., 2011). Thus, there has not been an agreement on how to effectively reduce ARGs in the sludge treatment process until now. In order to correctly evaluate the effect of anaerobic digestion on ARGs, it is necessary to learn the correlation between different anaerobic digestion conditions and the evolution of ARGs.

With a complicated mechanism, ARGs proliferated mainly through vertical gene transfer (by the reproduction of bacterial hosts) and horizontal gene transfer (via mobile gene elements). Some studies have found that ARGs and microbial bacteria have a good relevance through correlation analysis (Liu et al., 2018; Zhang et al., 2017). Microorganisms in different nutrient environments can affect the spread of ARGs to some extent. Therefore, it is believed that changes in community structure play an important role in the evolution of ARGs in sludge, especially the succession of ARGs hosts (Song et al., 2017). In other words, it is of great importance to track the dynamic changes of key microbial flora in AD system and further investigate the succession of the potential hosts. The complex microbial community in sludge creates favorable condition for the horizontal gene transfer. Therefore, as one of the most important ways for the spread of ARGs in the environment, the interspecific propagation process dominated by horizontal gene transfer with mobile genetic elements (MGEs) may have a certain influence on the evolution of ARGs in anaerobic digestion system. Most researches in this area were based on the correlation analysis between ARGs and MGEs (Guo et al., 2017). MGEs, including the integrase gene of class 1 integron (*intI*1), *ISCR1* and *Tn916/1545* have been found to have significant impacts on the distribution of ARGs (Tong et al., 2017). Moreover, it has also been found that the *sul*I was present in a conserved region of the *intI*1 sequence, which could be used as a microbial vector for its transfer in the environment (Nigro et al., 2013). Therefore, anaerobic digestion not only needs to reduce the abundance of ARGs, but also should pay attention to the MGEs closely related to ARGs, controlling them from the perspective of preventing the transmission of ARGs.

There are two explanations about the production of ARGs in the environment. One is defined as “intrinsic resistance” by Davies (Davies, 1996), which means the genetic materials that exist in microbial cells. Genes with high resistance to β-lactams, tetracyclines, and glycopeptide antibiotics found in ancient DNA 30,000 years ago proved this suppose (D’Costa et al., 2011). In addition, exogenous pollutants such as antibiotics can also induce DNA mutations or intrinsic resistance gene expression in microorganisms, thereby producing resistant genes. It has been found that the abuse of antibiotics induced intestinal microbial resistance genes in animals (Sørum and Sunde, 2001; Sunde and Norström, 2006). Although, there might be certain corrections between the production of ARGs and antibiotics, the effect of antibiotics on the sludge anaerobic digestion process and its microbiological mechanism have not reached an agreement. Thus, the purpose of this study was to investigate the connections among systematic parameters, microbial communities and ARGs as well as *intI*1 in the process of sludge anaerobic digestion under the pressure of antibiotics, which was helpful for better understanding the key factors and mechanism in the evolution of ARGs. Meanwhile, it provided a theoretical basis for applying appropriate methods to regulate the process of sludge anaerobic digestion containing antibiotics.

2. Materials and methods

2.1. Anaerobic digestion setup

Inoculum sludge was collected from a mature mesophilic anaerobic digester according to the previous study (Yang et al., 2016). Therefore, the startup period of anaerobic digestion could be cut down. A mixture of secondary sludge and dewatered sludge collected from Yuelu municipal wastewater treatment plant (WWTP) in Changsha, China was used as daily feed sludge. The characteristics of raw materials were listed in Supporting information. Anaerobic digestion tests were carried out in two cylindrical reactors made of glass with an effective volume of 3 L. A glue plug with two holes was inserted into the reactor. One hole was used for sample injection, and the other was connected to a bottle used for collecting gas. Reactor A was set as control without any antibiotics added, while 6 kinds of antibiotics (Tetracycline, Oxytetracycline, Chlortetracycline, Sulfathiazole, Sulfamethizole and Sulfamethoxazole) with a high concentration of 2 mg/L were added in Reactor B. Since there were generally different kinds of antibiotics existed in actual sewage sludge, the concentration of these antibiotics added in this study referred to some published papers (Gao et al., 2012; Pei et al., 2006). The hydraulic retention time (HRT) of both reactors was 15 days. To maintain a constant working volume, 200 mL of digested/untreated sludge was removed/refilled every day (Jang et al., 2016). After the reactor was completely sealed, nitrogen gas was pumped in to remove oxygen. Then both reactors were incubated in a water bath at 35 ± 1 °C (mesophilic condition).

2.2. Chemical analytical methods

Sludge samples were collected periodically. They were crushed to

about 2 mm with large particles removed. Some of them were stored at −20 °C for the further analysis, others were centrifuged and the supernatant was used for chemical analysis including pH, total alkalinity (TA), volatile fatty acids (VFAs), soluble chemical oxygen demand (SCOD), proteins and polysaccharides. Methods used for measuring pH, Total Solid (TS), VS (Volatile Solid), TA (Total Alkalinity), SCOD, protein and polysaccharides were described according to Gou's study (Gou et al., 2014). Biogas production yield was measured daily. Analysis of the component of VFAs and biogas were determined by a gas chromatograph (GC2010-plus, Shimazu) with a flame ionization detector.

2.3. DNA extraction and storage

Sludge samples collected on 1 d, 15 d, 32 d, 46 d, 64 d and 90 d from the control and antibiotics added groups, respectively, were thawed in a 35 °C water bath at first. Then, after being centrifuged for 2 times, the precipitate was transferred to a ziplock bag and pre-frozen at −20 °C for 48 h. Lastly, it was placed in a vacuum freeze dryer at −80 °C for 48 h when the sludge could be grinded into powder. Samples of 0.5 g was used for Genomic DNA extraction by FastDNA® spin kit (MP bio, Santa Ana, USA) according to the manufacturer's instructions. In this experiment, 1% (w/v) agarose gel electrophoresis was used to test the quality of DNA and subsequent purification. All the extractions were stored at −20 °C for further analysis.

2.4. Quantitative real time PCR (qPCR)

Quantitative real time PCR (qPCR) was used to quantify the absolute abundance of 3 tetracycline resistance genes (*tet*A, *tet*M, *tet*W), 2 sulfonamide resistance genes (*sul*I, *sul*II), the integrase gene of class 1 integrons (*intI*1) and microbial 16S rRNA genes for archaea/bacteria. The qPCR reaction was performed on iQ5 real-time PCR thermocycler (BIO-RAD, USA) with Super Real fluorescence premixing (SYBR Green) kit. A 20 μL reaction mixture contained 10 μL of 2 × Power PreMix (Tiangen, China), 0.6 μL of forward/reverse primer (10 μM), 1.0 μL of template DNA, and 8.8 μL sterile ultrapure water. All the above steps were carried out on ice. The procedure for qPCR was set according to the kit, using two-step method with the melting curve analysis which could demonstrate the specificity of the amplifications. The thermocycling steps for qPCR amplification were as follows: 40 cycles of pre-denaturation at 95 °C for 15 min, denaturation at 95 °C for 10 s, annealing/extension for 30 s, and simultaneous scanning of the fluorescent signal. The temperature set for melting curves analysis was between 50 °C and 95 °C with 0.5 °C/30 s per cycle. Primers and annealing temperature for qPCR were shown in Appendix. 16S rRNA genes for archaea/bacteria were selected as the internal reference genes that could minimize the basis caused by different microbial abundance (Ju et al., 2016; Tian et al., 2016). Each gene was quantified in triplicate for each sample using a calibration curve by the purified DNA template that have been amplified by conventional PCR and a negative control (Lü et al., 2013). Concentrations of the standard samples (ng/μL) were determined with the Nanodrop ND-1000 (Nanodrop, USA), and their copy concentrations (copies/μL) were then calculated by this formula: Log copy of genes/μL DNA $= \log \frac{\text{DNA concentration (ng / μL)}}{\text{DNA molecular weight (g/mol)}} \times n \times 6.02 \times 10^{23} \times 10^{-9}$, n represents the dilution multiple of DNA when prepared to qPCR. Serial dilutions of the standard samples from 10×10^{10} to 10×10^{3} were used as calibration standards. Standard curves were constructed in each PCR run and the copied numbers of the genes in each sample were interpolated using these standard curves. All standards, samples, and negative control (sterile water) were quantified in triplicate. The result of qPCR was reliable when the R^2 was higher than 0.99 for standard curves over 8 orders of magnitude and the amplification efficiencies based on slopes were between 90% and 110% (Pei et al., 2016; Zhang et al., 2016).

2.5. High-throughput sequencing of 16S rRNA genes

DNA extractions of sludge samples collected from digesters on day 1, 15, 46, 90 were used to conduct 16S rRNA sequencing. The primers for PCR reactions targeting the V3-V4 regions were 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) (Sundberg et al., 2013; Ziels et al., 2016). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equidensity ratios. Then, mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The sequencing library was generated using the TruSeq® DNA PCR-Free kit according to the operating manual. The library quality was evaluated by the Qubit@ 2.0 fluorometer and the Agilent Bio 2100 system. Effective tags merged using FLASH (V1.2.7) and filtered by QIIME (V1.7.0) were clustered into OTUs (Operational Taxonomic Units) with 97% consistency (Caporaso et al., 2010). For each representative sequence, the GreenGene database based on the RDP classifier algorithm was used to annotate the classification information (DeSantis et al., 2006). Alpha diversity indices including Observed-species, Chao1, Shannon, Simpson, ACE and Good-coverage were calculated based on OTUs classification (Li et al., 2013).

2.6. Correlation analysis

Correlation between microbial communities and digestive system parameters was assessed by Redundancy Analysis (RDA) with the Canoco 5.0 (Microcomputer Power, USA). Relationship between class I integron gene (*intI*1) and ARGs was analyzed using SPSS 21.0 statistical software package (IBM, USA) based on the Pearson correlation coefficient, and a p value < 0.05 was considered statistically. Network analysis of ARGs, *intI*1 and potential hosts was performed using Gephi (V0.9.1). OTUs at phylum level were selected and only the correlation index that was higher than 0.8 ($p < 0.05$) would be considered as a potential host for a target gene.

3. Results and discussion

3.1. General performance of anaerobic digestion

The two digestors were operated for 90d at mesophilic condition.

Table 1
Summary of digestion parameters for each reactor.

Item	Reactor A (the control group)						Reactor B (the antibiotics added group)					
Time (day)	1	15	32	46	64	90	1	15	32	46	64	90
pH	$7.42_{(0.12)}$	$7.45_{(0.07)}$	$7.48_{(0.06)}$	$7.35_{(0.03)}$	$7.33_{(0.05)}$	$7.39_{(0.07)}$	$7.35_{(0.09)}$	$7.47_{(0.04)}$	$7.29_{(0.11)}$	$7.34_{(0.05)}$	$7.36_{(0.10)}$	$7.38_{(0.08)}$
SCOD (mg L^{-1})	$1006_{(136)}$	$1120_{(158)}$	$1360_{(220)}$	$1377_{(316)}$	$1640_{(266)}$	$1736_{(218)}$	$936_{(94)}$	$1020_{(121)}$	$1480_{(117)}$	$1628_{(233)}$	$1862_{(274)}$	$2125_{(255)}$
VFAs (mg L^{-1})	$226_{(17)}$	$263_{(13)}$	$774_{(38)}$	$864_{(43)}$	$467_{(27)}$	$517_{(25)}$	$211_{(12)}$	$270_{(16)}$	$619_{(42)}$	$1258_{(73)}$	$947_{(57)}$	$694_{(44)}$
TA (mg L^{-1})	$1907_{(103)}$	$2250_{(112)}$	$2551_{(128)}$	$2796_{(140)}$	$3453_{(200)}$	$3610_{(180)}$	$1970_{(101)}$	$2134_{(122)}$	$2568_{(159)}$	$2913_{(175)}$	$3822_{(233)}$	$3500_{(224)}$
Pn (mg L^{-1})	$72.4_{(3.4)}$	$87.1_{(2.8)}$	$85.3_{(2.6)}$	$120.1_{(4.1)}$	$128.4_{(3.8)}$	$121.9_{(3.3)}$	$81.4_{(2.9)}$	$90.0_{(3.2)}$	$92.4_{(3.6)}$	$125.3_{(3.5)}$	$139.0_{(4.7)}$	$125.2_{(2.8)}$
Ps (mg L^{-1})	$77.1_{(2.6)}$	$82.3_{(3.1)}$	$101.0_{(4.9)}$	$132.2_{(4.6)}$	$109.2_{(3.2)}$	$80.5_{(2.5)}$	$79.4_{(2.4)}$	$78.4_{(3.2)}$	$106.0_{(4.2)}$	$92.9_{(2.8)}$	$91.0_{(2.2)}$	$72.1_{(3.0)}$

VFAs: volatile fatty acids; TA: total alkalinity; SCOD: soluble COD; Pn: proteins; Ps: polysaccharides.

Table 1 summarized the operating parameters and performances. During the experiment, the pH fluctuated between 7.29 ± 0.11 and 7.48 ± 0.06 in two systems. Besides, VFAs in reactor A increased to the maximum value for about 864 ± 43 mg/L on 46th day. However, the VFAs yield of reactor B reached to 1258 ± 73 mg/L at the same time. Furthermore, as shown in Supporting information, the averaged methane production during the process of anaerobic digestion was lower in reactor B of 349 ± 11 mL/gVS/d compared with that of 421 ± 14 mL/gVS/d in reactor A. In addition, the data for biogas composition on the 70th day for two reactors were similar, with methane counting for 68% in Reactor A and 69% in Reactor B, respectively. It could be speculated that the accumulation of VFAs in Reactor B negatively affected the activities of methanogens which have been proved to be sensitive to the environment. Therefore, the methanogenesis process of anaerobic digestion was inhibited with the addition of antibiotics. This observation was also consistent with previous studies that had reported reduction in methane production during anaerobic digestion of sewage sludge under the presence of different kinds of antibiotics with high concentrations. For example, it was found that methane production was reduced by 62% at oxytetracycline and chlortetracycline concentrations of 100 mg/L (Álvarez et al., 2010).

3.2. Changes in microbial abundance and structure

According to the Alpha diversity indices, the abundance and diversity of the microbial community in two digesters were different from each other during anaerobic digestion. As shown in Table 2, during the first 15 days of the experiment, the number of OTUs in the antibiotics added group was larger than that of the control. As the number of OTUs in the control increased gradually, the disparity between two systems became smaller. This was consistent with the results shown by Chao1 index and ACE index, indicating that the addition of high concentrations of antibiotics reduced the microbial community richness of the anaerobic digestion system. On the other hand, Shannon index of the control was always higher than that of the antibiotics added group which demonstrated that the microbial community distribution in the control was more uniform. Furthermore, Simpson index of the antibiotics added group remained smaller than that of the control, which indicated that the addition of antibiotics increased the diversity of the sludge digestive system. Principal component analysis (PCA) showed in Appendix also indicated that the microbial community structure changed over the course of sludge anaerobic digestion with the addition of antibiotics. A possible explanation for this difference could be that antibiotics promoted the rapid adaptation of certain microorganisms to the environment and inhibited the growth of other types of microorganisms at the same time, thus showing a decrease in systemic microbial richness. The increased diversity of microbial community in anaerobic system could be a result of resisting the impact of toxic and harmful substances. However, this kind of resistance was limited considering the observation of AD performance.

To evaluate the effect of antibiotics on microbial community structure, the composite of the microbial community at genus level were analyzed. Details of each sample could be visualized in Fig. 1.

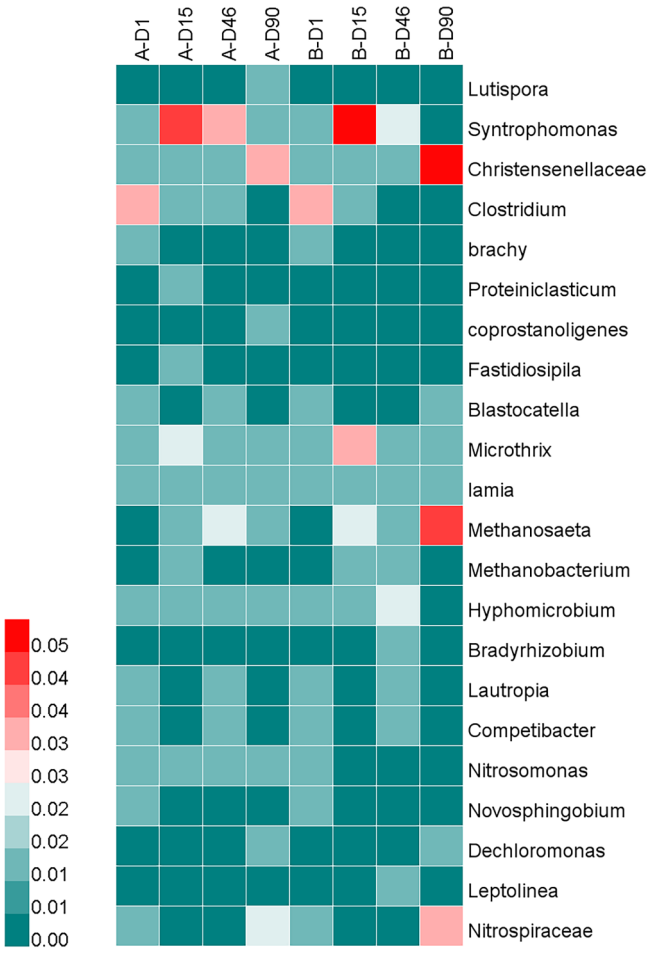

Fig. 1. Heatmap of changes in genus during the anaerobic digestion in two reactors. The relative abundance of genus with more than 1% was selected for discussion. The color intensity shows the relative abundance of each genus as the color key indicates at the bottom left.

Since there were many microorganisms (50% of each sample) that could not be identified at genus level, and the abundance of classified microbial community were very low, the relative abundance of flora with more than 1% was selected for discussion. As shown in Fig. 1, the addition of antibiotics exerted significant influence on the microbial community structure at genus level. Both *Syntrophomonas* and *Christensenellaceae* were dominant bacteria for two systems while the relative abundance of *Nitrospiraceae*, *Hyphomicrobium*, *microthrix* and *Methanosaeta* were much higher in the antibiotics added group. At the beginning of the experiment, *Clostridium* took an absolute advantage in AD system and its abundance has been decreasing over time, from the initial of 3.1% to 0.7% in the end. The abundance of the genus *Lautropia* and *Competibatcer* changed closely, both of which experienced a decrease form 1.2% at the beginning to the final abundance of 0.8%.

Table 2
Alpha-diversity indexes of microbial community at different digestion stage.

Sample	Shannon	Chao1	Simpson	ACE	OTUs	Sequence
A-D1	$7.739_{(0.1)}$	$1694_{(115.6)}$	$0.987_{(0.001)}$	$1737_{(70.3)}$	1700 (25.1)	45,008 (5054)
A-D15	$7.994_{(0.2)}$	$1837_{(311.7)}$	$0.990_{(0.002)}$	1875 (324.9)	1638 (20.5)	39,302 (8392)
A-D46	$7.918_{(0.1)}$	$1737_{(43.8)}$	$0.988_{(0.001)}$	$1838_{(56.4)}$	1800 (56.4)	43,204 (2574)
A-D90	$8.328_{(0.2)}$	$1902_{(85.7)}$	$0.992_{(0.002)}$	$1961_{(116.3)}$	1943 (115.2)	40,320 (2284)
B-D1	$7.734_{(0.1)}$	$1699_{(110.6)}$	$0.983_{(0.001)}$	$1749_{(75.3)}$	1718 (21.8)	45,022 (4951)
B-D15	$7.885_{(0.1)}$	$1817_{(146.3)}$	$0.988_{(0.002)}$	$1826_{(67.1)}$	1738 (15.5)	48,391 (3629)
B-D46	$7.650_{(0.1)}$	$1542_{(31.8)}$	$0.985_{(0.003)}$	$1627_{(38.3)}$	1616 (37.1)	43,966 (1389)
B-D90	$7.885_{(0.1)}$	$1730_{(85.0)}$	$0.987_{(0.001)}$	$1795_{(81.3)}$	1763 (49.1)	44,453 (4063)

Additionally, *Hyphomicrobium*, *Nitrosomonas* as well as *Methanosaeta* showed relatively high abundance in the antibiotics added group during the later period of AD. As a kind of denitrifying bacteria that can grow with methanol methylamine, the genus *Hyphomicrobium* plays an important role in the degradation of organic matter and the removal of nitrogen (Wei et al., 2017). Changes in the relative abundance of *Hyphomicrobium* and *Methanosaeta* in the two systems could be the result of the accumulation of VFAs in reactor B. Since in previous studies, *Methanosaeta* has been proved to be a kind of strict aceticlastic methanogen (Lü et al., 2016).

3.3. Correlation between system parameters of AD and microbial dynamics

The correlation between the microbial community and the reactors' performance was investigated by performing the Redundancy Analysis (RDA). The Pearson correlation coefficient between the 224 microbial floras at genus level and the digestive system parameters pH, volatile fatty acids (VFAs), polysaccharides (Ps), proteins (Pn), as well as biogas were calculated. 6 strong-related strains ($p < 0.05$, $r > 0.8$) were subjected to RDA analysis together with the top 10 species in relative abundance in the sludge samples. As shown in Fig. 2, both ordination axes of the RDA triplet combined to explain 89.3% of the microbial community variations, indicating that these system parameters were major factors shaping the microbial community dynamics. The microorganisms were mainly distributed in two regions, one of which was associated with Ps and pH and the others were related to biogas, VFAs and Pn. Interestingly, *Lutispora* and *Methanosaeta* showed strong relationship with proteins, VFAs and biogas. Specifically, *Lutispora* belonging to the phylum *Firmicutes* has been found to be responsible for the degradation of proteins in anaerobic fermentation systems (Jang et al., 2015). Besides, as important methanogens in the environment, both *Methanosaeta* and *Methanobacterium* showed positively relationship with biogas production, suggesting that such microorganisms could be involved in the methanogenesis process in the anaerobic digestion system. The results of RDA also demonstrated that some kinds of microorganisms such as *Lutispora* and *Proteiniclasticum* with relatively lower abundance in anaerobic digesters could also have important impacts on digestive efficiency.

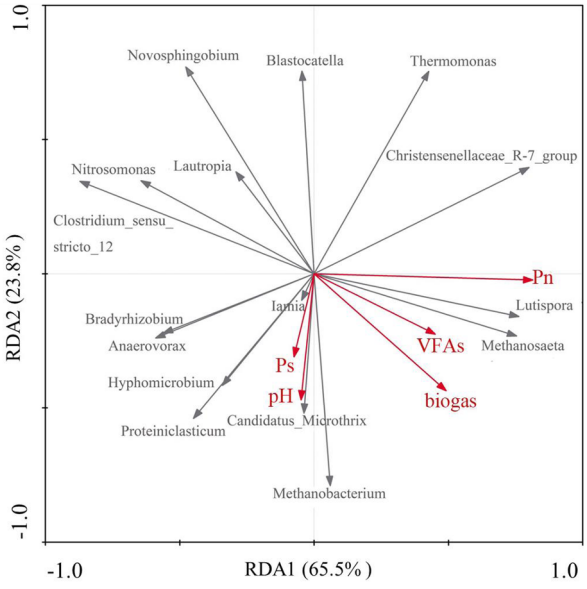

Fig. 2. RDA analysis revealing the relationship between the microbial community and the reactors' performance, including pH, Ps, Pn, VFAs and biogas in anaerobic digestion. 6 strong-related strains ($p < 0.05$, $r > 0.8$) were selected together with the top 10 species in relative abundance in the sludge samples.

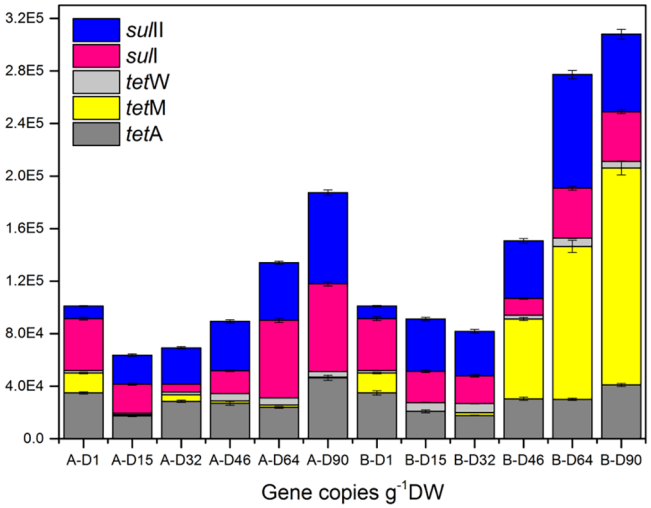

Fig. 3. Absolute gene copies of ARGs of different stages in anaerobic digesters.

3.4. Evolution of ARGs in anaerobic digestion

Standard curves and amplification efficiency of target genes by qPCR were summarized in Supporting information. Fig. 3 showed the absolute copy numbers of total ARGs. Results indicated that the addition of antibiotics certainly affected the evolution of total ARGs. At the beginning of the anaerobic digestion, the total gene copies of ARGs for the control group experienced a reduction of 37.1% within 15 days, while the valley gene copies of total ARGs for the antibiotics added group were delayed until Day 32 with a slighter decrease of 19.1%. Then, both the total gene copies of ARGs in two systems remained increasing until the last day. The relatively lower reduction of total gene copies during this period in the antibiotics added group largely attributed to the increase of *sul*II from Day 1 to Day 15. In terms of the absolute gene copies, AD showed some enrichment of ARGs in the control (1.85 times) and the antibiotics addition group (3.05 times). This result indicated that sludge anaerobic sludge digestion at mesophilic condition could not effectively remove most ARGs. Conversely, the absolute gene copies of ARGs increased after this disposal method, which was corroborated with previous studies (Diehl and LaPara, 2010). Additionally, it also demonstrated that the addition of antibiotics in sludge could contribute positively to the accumulation of total ARGs in the process of anaerobic digestion.

As shown in Fig. 4, the relative abundance of ARGs was normalized by 16S rRNA genes. Changes in the prevalence of most ARGs showed a similar pattern during sludge anaerobic digestion. All the selective ARGs in two reactors showed an increase at different levels except the *tet*M gene for the control. By the end of the experiment, the relative abundances of *tet*A, *tet*W, *sul*I, and *sul*II for the control and the antibiotics added group increased by 1.001 logs/1.041 logs, 1.001 logs/1.006 logs, 1.008 logs/1.033 logs, and 1.029 logs/1.087 logs, respectively. However, the change of *tet*M gene in two systems was totally different from each other. It decreased by 0.996 logs for the control, while the largest rise (1.192 logs) was observed in reactor B. It was clear that sludge anaerobic digestion reduced the abundance of *tet*M in the control, while others were enriched, including *tet*A, *tet*W, *sul*I and *sul*II in both systems. Meanwhile, the addition of antibiotics strengthened this kind of enrichment to some extent. According to previous studies, the resistance mechanism of the *tet*M gene and the *tet*W gene is similar, both of which are a kind of coding gene for ribosomal protected proteins (Warsa et al., 1996). However, the different performance of them in this study probably due to the result that the *tet*M was reported to have a large number of hosts, including Gram-positive bacteria, Gram-negative bacteria, aerobic bacteria and anaerobic bacteria (Salyers et al., 1995). Interestingly, the relative expression levels of three

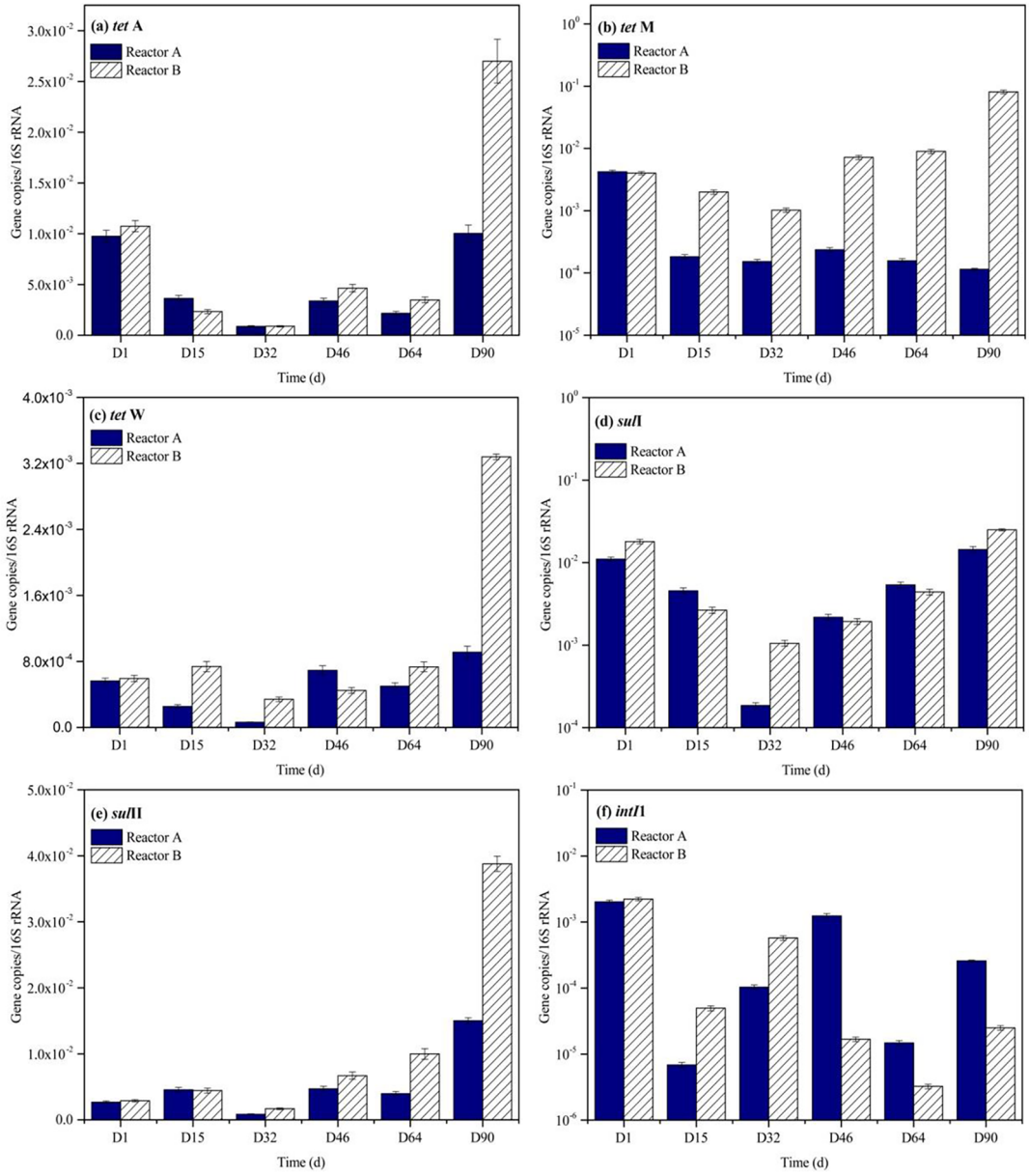

Fig. 4. Relative abundance of tetracycline resistance genes (*tet*A, *tet*M, *tet*W) and sulfonamides resistance genes (*sul*I, *sul*II) as well as the class I integrons (*intI*1) during the process of anaerobic digestion in two reactors. The quantity of genes detected in this study was normalized by the 16S rRNA gene copies, an addition of bacterial and archaeal, to minimize the basis caused by different microbial abundance.

tetracycline resistance genes (*tet*A, *tet*M and *tet*W) and two sulfonamide genes (*sul*I and *sul*II) detected in the antibiotics added group suddenly increased rapidly during the final stage of anaerobic digestion. The increase rate of *sul*I was relatively smaller than others, indicating *sul*I was less sensitive to the pressure caused by antibiotics. However, this was in contrast with a previous study (Ma et al., 2011). The discrepancy might be caused by the different feedstock used in two studies (dewatered sludge/a mixture of primary and secondary sludge) as well as the operating conditions, which could lead to a totally different microbial community structure during the process of anaerobic digestion.

3.5. The role of intI1 in the change of ARGs

The generation and evolution of ARGs in the environment are associated with a combination of factors, such as antibiotics and heavy metals that can affect the growth and metabolism of resistant bacteria and induce the vertical transfer of ARGs. There could also be certain connections between different kinds of ARGs. On the other hand, integrons, usually located on mobile gene elements (MGEs) such as transposons and plasmids, are considered to be important indicators for horizontal gene transfer. The integrase gene of class 1 integron (*intI*1) detected in this study is one of the most common type of MGEs (Chen et al., 2015). Miller found that its abundance could be reduced by one to two orders of magnitude during anaerobic digestion (Miller et al., 2013). This conclusion was also verified in this experiment. The relative abundance of *intI* significantly reduced in both reactors, with 1.001 logs and 1.005 logs for the control and the antibiotics added group, respectively. Meanwhile, there was slightly increase in the abundance of *intI*1 for the control from Day 15 to Day 46, which were consistent with the change of some ARGs including *tet*W and *sul*II, indicating these ARGs were likely to be amplified by horizontal gene transfer (HGT) at this stage. However, at the end of the experiment, the abundances of all the detected ARGs were increased except the *tet*M in the control, indicating that the vertical gene transfer caused by the increase in

Table 3
Pearson Correlations between ARGs and *intI*1 during the sludge anaerobic digestion.

		*tet*A	*tet*M	*tet*W	*sul*I	*sul*II	*intI*1
*tet*A	r						
	p						
*tet*M	r	0.920*					
	p	0.001					
*tet*W	r	0.921*	0.970*				
	p	0.001	0.002				
*sul*I	r	0.960*	0.792	0.832*			
	p	0.001	0.019	0.010			
*sul*II	r	0.902*	0.934*	0.965*	0.836*		
	p	0.002	0.001	0.001	0.010		
*intI*1	r	−0.051	−0.283	−0.284	0.008	−0.435	
	p	0.905	0.497	0.496	0.984	0.281	

Correlation is significant at the 0.01 level ($p < 0.01$)*.

microbial community which has adapted to the environment exerted more effects on the evolution of the ARGs in this study.

In order to further investigate the connection of *intI*1 and ARGs in AD system, Pearson correlation coefficient between three kinds of tetracycline resistance genes, *tet*A, *tet*M and *tet*W, two kinds of sulfonamide resistance genes, *sul*I and *sul*II, as well as *intI*1, was calculated (Table 3). No significant correlation between the five ARGs detected in this experiment and the *intI*1 was observed, indicating that the occurrence of horizontal shift in integron-resistant genes did not play an important role in the evolution of ARGs, compared to the vertical gene transfer process, which was consistent with the study conducted by Su (Su et al., 2015). However, some studies have found significant positive correlations between the *tet*A and *intI*1 as well as the *sul*I and *intI*1 (Zhang et al., 2009). The relative abundance of ARGs was mainly influenced by two factors: change in the proportion of probable host microorganisms and the horizontal transfer of genes between microorganisms (Resende et al., 2014; Tian et al., 2016). The reason why there were different conclusions drawn by researchers may be that the resistance mechanism of ARGs in different environment could be different. In this study, different kinds of antibiotics were added in one reactor, which might greatly change the structure of microbial community. Potential hosts of ARGs could be beneficially affected, therefore enhancing the process of horizontal gene transfer between microorganisms.

3.6. Network analysis of microbial community and ARGs

It has been proved that network analysis was able to investigate the potential hosts of ARGs in environment (Zhang et al., 2016). As shown in Fig. 5., 73 species were found to be significantly ($p < 0.05$) associated with the ARGs and *intI*1 quantified in this study. The network

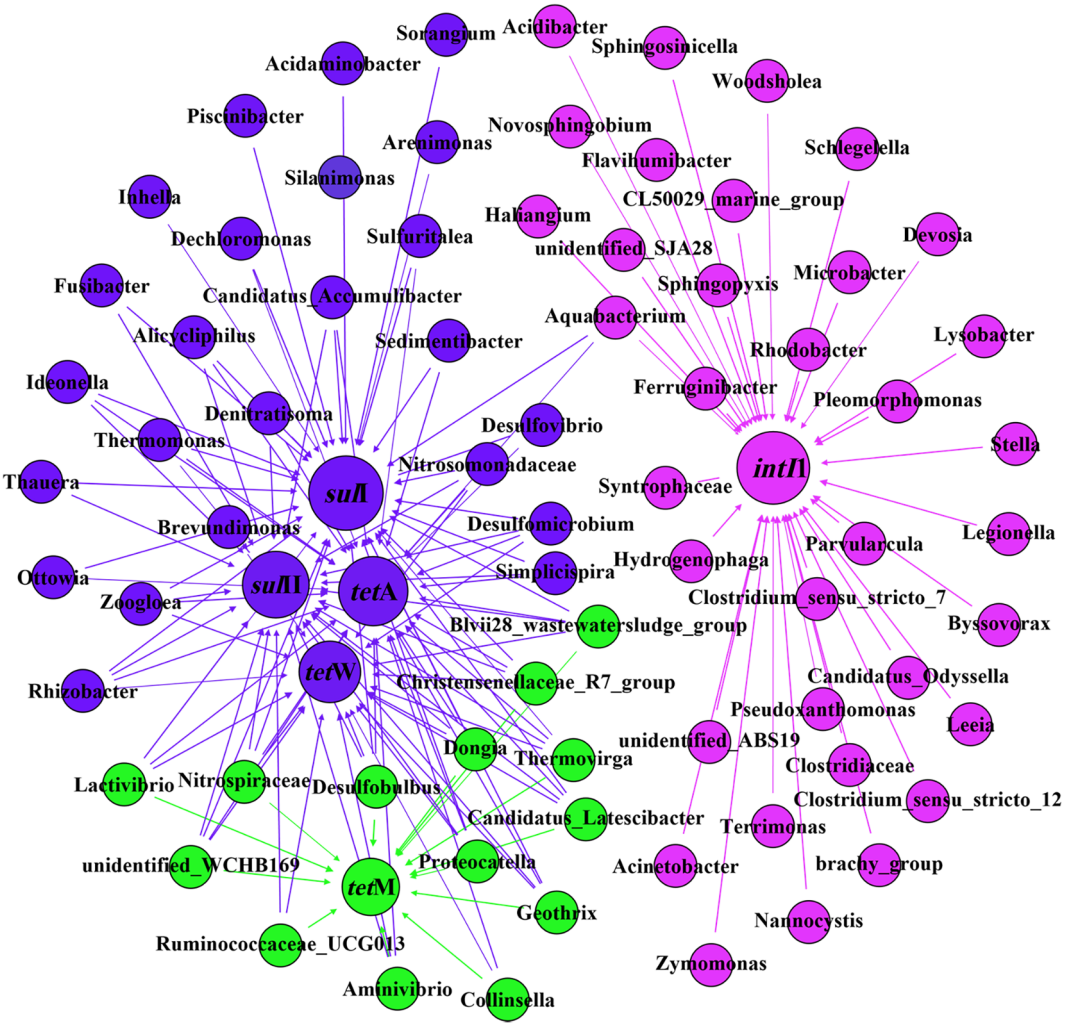

Fig. 5. Network analysis showing the co-occurrence patterns between the microbial communities and ARGs as well as the class I integrons (*intI*1) in anaerobic digestion. The thickness of arrows and lines were based on Pearson's correction coefficient.

was clearly divided into 3 modules: (1) the *intI*1 that decreased in two reactors, (2) the *tet*M that was the only decreased gene in the control group, while it increased in the antibiotics added group, and (3) the remaining 4 ARGs that showed similar patterns during sludge anaerobic digestion. The *sul*I gene that was the most abundant in the control had the most potential hosts (35), followed by the *tet*A gene (29), the *sul*II gene (26), the *tet*W gene (19) while the *tet*M showed the least (14). The number of potential hosts for the *intI*1 was 35. From the perspective of microbial communities, 10 genera were related to 5 ARGs, while they showed no significant connection with *intI*1. Interestingly, changes of these genera in the anaerobic digestion generally synchronized with the evolution of ARGs. Specifically, *Christensenellaceae R-7 group* that belongs to the phylum *Firmicutes* increased from 1.08% to 3.04% and 5.04% for the control and the antibiotics added group, respectively. On the other hand, most potential hosts (over 90%) of *intI*1 decreased a lot, which demonstrated that anaerobic digestion was a critical method to control the horizontal gene transfer possibility among bacteria. Consequently, network analysis further proved the strong impact of bacteria community on the evolution of ARGs.

4. Conclusions

Methane production was inhibited under the pressure of antibiotics due to the accumulation of VFAs. The addition of antibiotics could impact the fate of ARGs and affect shaping of microbial communities in anaerobic digestion. Most of the ARGs detected were induced by antibiotics, ranging from 1.006 logs to 1.087 logs while the *intI*1 showed a significantly decrease with 1.005 logs. *IntI*1 only played a small role in ARGs' evolution and transfer during anaerobic digestion, vertical transfer by the potential hosts of ARGs was the key factor instead.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.12.066.

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