Effects of chlortetracycline and copper on tetracyclines and copper resistance genes and microbial community during swine manure anaerobic digestion

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HIGHLIGHTS

- The predominant ARGs could be effectively reduced through mesophilic anaerobic digestion.
- Microbial community evolution was the main driver to the change of ARGs.
- Addition of CTC, Cu and CTC + Cu affected microbial community change and hindered removal of ARGs.
- *Treponema* might indicate the end of anaerobic digestion and compete with ARGs host bacteria.
- CTC + Cu seriously affected *Treponema* and ARGs during anaerobic digestion.

GRAPHICAL ABSTRACT

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ABSTRACT

As antibiotic and heavy metals are over used in the livestock industry, animal manure is a reservoir of antibiotic resistance genes (ARGs). Anaerobic digestion has been reported to have the potential to reduce ARGs. However, few studies investigated whether reduction of ARGs would be affected by different external pressures including antibiotics and heavy metals during anaerobic digestion. The purpose of this study was thus to investigate effects of both chlortetracycline (CTC) and Cu on reduction of ARGs, heavy metal resistance genes (HMRGs) and mobile genetic elements (MGEs) during the swine manure

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anaerobic digestion. The results showed that the predominant ARGs (*tetO*, *tetW*, *tetX*, *tetL*) could be effectively reduced (approximately 1.00 log copies/g TS) through mesophilic anaerobic digestion. Microbial community evolution was the main driver. It was interesting that *Treponema* might indicate the termination of anaerobic digestion and compete with ARGs host bacteria. Addition of CTC, Cu and CTC + Cu affected microbial community change and hindered removal of ARGs, especially, CTC + Cu seriously affected *Treponema* and ARGs during anaerobic digestion.

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1. Introduction

The increasing emergence and spread of antibiotic resistance has been considered to be one of the most serious public health issues and attracted more and more attention in the recent years. It has been estimated that antibiotic resistance is responsible for more than 25,000, 23,000, and 38,000 deaths every year in the European, the United States, and Thailand, respectively (Björn Berglund, 2015). Due to overuse of antibiotics as feed additives to control and prevent diseases, and to promote growth in concentrated livestock and poultry industry (Kemper, 2008; Koike et al., 2007; Sarmah et al., 2006), antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) could be induced under the selective pressure of antibiotics in animal intestinal tract or outer environment, resulting in animal manure being a reservoir of ARB and ARGs (Heuer et al., 2011; Mackie et al., 2006). One important cause of the proliferation of ARGs is the horizontal gene transfer (HGT) with mobile genetic elements (MGEs) as vectors, which have specific structures and ability to capture genes by a site-specific recombination system. Integrins are a kind of MGEs linked with multidrug resistance and facilitate ARGs dissemination (Molla et al., 2007). Some bacteria can capture ARGs housed on the MGEs via HGT (Martinez, 2008) and Martinez et al. (2014) ranked the ARGs which could transfer by MGEs harbored in human bacteria pathogens as the highest risk level.

Tetracyclines (TCs) are broad-spectrum antibiotics, which are extensively used in the livestock industry (Kumar et al., 2005; Sarmah et al., 2006). Tetracycline resistance genes have been reported ubiquitous occurrence in the environments surrounding a pig farm (Wu et al., 2010). Currently, more than 40 classes of tetracycline resistance genes (*tet*) have been described and studied, including three resistance mechanisms (efflux pump proteins, ribosomal protection proteins (RPPs), and inactivating enzymes) (Roberts, 2005). Tetracycline resistance has already emerged in many commensal and pathogenic bacteria because of *tet* genes acquisition (Chopra and Roberts, 2001). Additionally, copper is usually cooperated with antibiotics to enhance their effects in the intensive livestock production (Nicholson et al., 1999). There is growing concern that heavy metals, such as copper, function as a selective agent in the proliferation of ARGs through co-selection (Baker-Austin et al., 2006). The co-occurrence of antibiotic and metal resistance in bacteria has been widely observed due to the cross- or co-resistance phenomena (Pal et al., 2015). Cross-resistance occurs when the same mechanism reduces the susceptibility to metals and antibiotics simultaneously, and co-resistance occurs when separate resistance genes are situated on the same genetic element (Pal et al., 2015).

The previous studies investigated that *tet* genes (such as *tetX*, *tetO*, *tetW*) were the predominant genes in the swine manure (Wang et al., 2016) and the wastewater of swine farm (Sui et al., 2016). Anaerobic digestion has been frequently reported to have the potential to reduce ARGs, e.g., LaPara and Diehl (2010) reported that five *tet* (*tetA*, *tetL*, *tetO*, *tetW*, *tetX*,) and *intI1* genes decreased during the anaerobic digestion, and removal efficiencies of *tet* and *intI1* genes increasing as a function of temperature; additionally,

hydraulic retention time might have an influence on reduction of ARGs. Wu et al. (2016) reported that the acidogenic phase of thermophilic digestion was mainly responsible for reducing the ARGs, such as *tetA*, *tetG*, *tetX*, *sul1*, *ermB*, *dfrA1*, *dfrA2* and *intI1*, while the subsequent methanogenic phase caused a rebound in their quantity; thermophilic digestion could effectively decrease the ARGs and mesophilic one could barely reduce the ARGs. In the study of Sui et al. (2016), mesophilic anaerobic digestion obviously reduced the ARGs and the reduction efficiency was improved by stable operational temperature and longer solid retention time (SRT). However, few studies investigated whether reduction efficiencies of ARGs, as well as HMRGs and MGEs would be affected by different external pressures (including antibiotics and heavy metals) during anaerobic digestion.

Thus the purpose of this study was to investigate effects of both chlortetracycline (CTC) and Cu, which are extensively used in swine productions, on ARGs, HMRGs and MGEs during the swine manure anaerobic digestion through batch experiments in order to better control the risk from ARGs, HMRGs and MGEs in animal manure spreading to the environment.

2. Materials and methods

2.1. Anaerobic digestion setup

Swine manure was collected from a concentrated pig farm located in eastern of Beijing, China. Inoculum sludge was collected from egg-shaped anaerobic digesters of Xiaohongmen municipal wastewater treatment plant (WWTP) in Beijing, China, treating municipal wastewater with the designed capacity of 600,000 m³/d, and sludge after setting down for ca. 3–5 days was used for inoculum. The characteristics of raw materials were listed in Table 1.

Anaerobic digestion tests were carried out by an Automatic Methane Potential Test System II (Bioprocess Control, Sweden) including a series of serum bottles (total volume: 2 L; working volume: 1.7 L) and plastic caps equipped with agitators and rubber stoppers. Swine manure slurry and inoculums were transferred to each reactor at ratio of 1:1 (v/v), while CTC and Cu were spiked in the reactors in the form of solution in order to determine their influence on ARGs, respectively. Four anaerobic digesters were set up including reactor A (control) without spiked CTC and Cu, B (spiked CTC at 500 mg/kg DW), C (spiked Cu at 5,000 mg/kg DW) and D (spiked CTC at 500 mg/kg DW and Cu at 5,000 mg/kg DW). Spiked concentrations of CTC and Cu were according to their concentration residues in the swine manure (Table 1).

After sealing each reactor, the headspaces were flushed with nitrogen gas for 2 min to remove traces of oxygen. Then the reactors were incubated in a water bath at 37 ± 1 °C. The termination of anaerobic digestion was judged by no obvious biogas production from reactors (daily biogas production < 2 mL). Biogas production yields could be automatically measured by the AMPTS II with displacement of water. Meanwhile, biogas was sampled on day 1, 2, 3, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 34, 36, 38, 41, 49, 52 and 57 for biogas composition analysis, respectively. The mixed slurries

Table 1
Characteristics of raw materials in the swine manure anaerobic digestion.

Characteristics	Swine slurry	Inoculum	Mixture
pH	6.72 ± 0.14	7.48 ± 0.24	7.17 ± 0.25
Moisture (%)	95.14 ± 1.25	98.82 ± 0.44	96.68 ± 0.53
TS ^a (%)	4.86 ± 0.34	1.18 ± 0.08	3.32 ± 0.84
VS ^b (%)	3.95 ± 0.87	0.68 ± 0.07	2.50 ± 0.24
EC (mS/cm)	6.65 ± 1.34	3.46 ± 0.91	4.47 ± 0.61
Cu (mg/kg DW)	538.75 ± 29.93	–	–
Chlortetracycline (mg/kg DW)	146.07 ± 7.32	–	–
Tetracycline (mg/kg DW)	12.31 ± 0.66	–	–
Oxytetracycline (mg/kg DW)	1.11 ± 0.05	–	–

^a Total solids.

^b Volatile solids.

in each reactor were sampled on day 1, 6, 13, 20, 27, 34, 49 and 57, respectively, for DNA extraction and analysis of pH, soluble chemical oxygen demand (SCOD), volatile fatty acids (VFAs) (Support Information).

The accumulative volume of biogas produced (P_{biogas} (t)) over the time course during the batch tests was fitted with the modified Gompertz equation (Zhang et al., 2014):

$$P_{\text{biogas}}(t) = P \cdot \exp \left[- \exp \left(\frac{R_m \cdot e}{P} (\lambda - t) + 1 \right) \right] \quad (1)$$

where P is the biogas potential (mL), R_m is the maximum biogas production rate (mL/day), λ is the lag phase time (day), and e is the exp (1) = 2.7183.

2.2. Genomic DNA extraction

Anaerobic digestion slurry was sampled 5 mL from each digester on 1st, 6th, 13th, 20th, 27th, 34th, 49th, (57th for C digester) in triplicate by syringe, respectively, and centrifuged at 4,000 rpm for 10 min. The solid evenly 0.2 g was transfer to a 2 mL sterilized tubes for total DNA extraction with QIAamp DNA Stool Mini Kit (QIAGEN; Solon, OH) according to manufacturer's instructions. The concentration and quality of the extracted DNA was determined by a NanoDrop 1000 spectrophotometer analysis (NanoDrop, USA) and 1.5% (w/v) agarose gel electrophoresis. DNA extractions were stored at -20°C for the further analysis.

2.3. Quantitative real time PCR

Quantitative real time PCR (qPCR) was used to quantify the absolute abundance of eight tetracycline resistance genes (efflux pump: *tetA*, *tetB*, *tetC*, *tetL*; ribosomal protection protein: *tetO*, *tetM*, *tetW*; enzymatic modification: *tetX*), the three copper resistance genes (*pcoA*, *pcoC* and *pcoD*), the integrase gene of class 1 integrons (*int1*) and the 16S rRNA gene. Primers are described in the Supporting Information (Table S1).

The qPCR analysis was conducted using an ABI7300 apparatus (ABI, USA). Each qPCR run consisted of 2 min initial denaturation at 95°C , followed by 40 cycles of denaturation at 95°C for 10 s, and anneal and extended at 60°C for 40 s. A 20 μL reaction mixture contained 10 μL of $2 \times$ Power SybrGreen qPCR Master Mix (Applied Biosystems, USA), 0.5 μL of forward and reverse primers (10 μM), 2 μL of template DNA and 7 μL of ddH_2O . Samples were analyzed in triplicate independent qPCR runs, with a standard curve and negative control included in each run. The quantity of target DNA in unknown samples was calculated based on a standard curve generated using known quantities of template DNA. Standards for qPCR were prepared by PCR amplification of genes from positive controls, followed by ligation into pMD-18T cloning vector following manufacturer's instructions (Takara), and transformation into JM109 Escherichia coli (Takara). Plasmids were purified using

SK1191 UNIQ-10 kit (Sangon Biotech, Shanghai, Co., Ltd. CN). 10-fold serial dilutions (10^{-1} – 10^{-8}) of plasmid DNA were prepared and run on the thermal cycler to generate standard curves ($R^2 > 0.99$). Following qPCR, melting curves were generated automatically to verify that nonspecific amplification did not occur. DNA copy concentrations (copies/ μL) were calculated by the following formula.

DNA copy concentration (copies μL^{-1})

$$= \frac{\text{DNA mass concentration (ng } \mu\text{L}^{-1})}{\text{DNA molecular weight (g mol}^{-1})} \times 6.02 \times 10^{23} \times 10^{-9}$$

Gene quantities are presented on a per gram anaerobic digestion materials total solid (TS) basis because the total quantity of genes are of greatest importance (absolute abundance).

2.4. High-throughput sequencing and bioinformatics analysis

DNA extractions of fresh swine manure and samples on days 1, 13, 27, 49 (day 57 only for C test) of the swine manure anaerobic digestion, respectively, were used to determine the microbial community analysis using PCR primers 515F (GTGCCAGCMGCCGCGG-TAA) and 806R (GGACTACHVGGGTWTCTAAT) targeting the bacteria 16S V4 region according to the protocol described in Caporaso et al. (Caporaso et al., 2010). PCR amplicons were further purified with a DNA purification kit (BioFlux, Japan), and concentrations were determined using spectrometry NanoDrop-1000 (Thermo Scientific, USA). Amplicons from different samples were then mixed to achieve equal mass concentrations in the final mixture, which were sent out to Novogene Co., Ltd. in Beijing for small-fragment library construction and pair-end sequencing using Illumina MiSeq sequencing system (Illumina, USA).

Sequencing reads from the original DNA fragments were merged using FLASH and then filtered by QIIME quality filters (Caporaso et al., 2010). PCR chimeras were filtered out using UCHIME (Edgar et al., 2011). And then the reads are "clean reads". The average length of all the clean reads was 253 bp, and the minimum number of selected sequences in the 18 samples was 15, 218 for bacterial community analysis. To fairly compare the 18 samples at the same sequencing depth, normalization of the sequence number was conducted by extracting the first 15, 218 sequences in each sample for bacterial community analysis. And all the normalized sequences were uploaded to MG-RAST (<http://metagenomics.anl.gov/>, No. project was 10976). Taxonomic classification of the sequences of each sample was carried out individually using the RDP Classifier. A bootstrap cutoff of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels (Zhang et al., 2016).

2.5. Data analysis

Statistical calculations and data analysis were performed using the SPSS 16.0 statistical software package (IBM, USA) and Origin 9.0 (Origin Lab, USA). A heat map of the top 24 families in each sample was built using HemI (<http://hemi.biocuckoo.org/>). According to the relative content of each family based on the classification, a principal component analysis (PCA) was performed using Canoco 5.0 (Microcomputer Power, USA).

3. Results and discussion

3.1. Performance of anaerobic digestion

The durations of the four anaerobic digestion tests were totally different (Table 2). Although the control one lasted for 37 days, the biogas yield had already arrived at 95% of the total yield on 21th

Table 2

Estimated values of parameters in the Modified Gompertz Equation for swine manure anaerobic digestion.

Anaerobic digestion tests	Total reaction time (days)	The lag phase time λ (day)	The biogas potential P (mL)	The maximum biogas production rate R_m (mL/day)	R^2	Biogas yield comparing with control (%)
A	37	0.3	9445.53	1506.56	0.99	0
B	55	0.1	11974.30	978.06	0.98	26.77
C	55	2.5	12587.55	771.41	0.99	33.26
D	32	0	6540.34	1137.69	0.99	−30.75

A: Control test; B: CTC spiked test; C: Cu spiked test; D: CTC+Cu spiked test.

day. Actually, the whole anaerobic digestion could divide into two stages: increment stage and plateau stage, approximately 95% of the total biogas was produced at the increment stage. The biogas production was fitted using the modified Gompertz equation (Table 2). The maximum biogas production rate (R_m) somehow reflects the reaction rate of anaerobic digestion, including organic matter degradation and methane production rate.

The lag phase time (λ) in the Cu spiked test (C) was much higher ($\lambda = 2.5$) than that in the others (A = 0.3; B = 0.1; D = 0) tests, and the maximum biogas production rate (R_m) in C test was much lower (771.41 mL/day) than that in the others (Table 2). These results implied that Cu at this level had a significant influence on the biogas production rate of anaerobic digestion. However, the biogas potential in the C test was the highest (12587.55 mL) among the four tests, which was 33% more than that in the control. This fact might indicate that although Cu addition inhibited the biogas production rate, it increased the biogas potential. On the contrary, the biogas potential in the D test was the lowest (6540.34 mL),

which was 30% less than the control one, but its maximum biogas production rate was much higher (1137.69 mL/day) than that in the B (978.06 mL/day) or C (771.41 mL/day) test, lower than that in the A (1506.56 mL/day) test. This fact indicated that Cu and CTC addition together decreased the total biogas yield, and inhibited R_m , but R_m in the D was much higher than that in the B or C test. These results demonstrated that adding CTC, Cu separately would seriously slow down the reaction rate but increase the biogas potential; adding CTC + Cu together would decrease the biogas potential and the reaction rate, to some extent, impede the anaerobic digestion.

3.2. Effects of CTC, Cu and CTC+Cu on reduction of ARGs, HMRGs and MGE

3.2.1. Reductions of ARGs, HMRGs, MEG in anaerobic digestion

The absolute abundance of all the ARGs (including eight *tet* genes), HMRGs (including three *pco* genes) and MGE (including

Table 3

ARGs reduction of A, B, C and D tests by the swine manure anaerobic digestion.

ARGs, HMRGs, MGEs	Genes reduction values (log copies/g TS)			
	Control	CTC	Cu	CTC+Cu
<i>tetA</i>	-0.3157	-0.3834	-0.1426	-0.9761
<i>tetC</i>	-0.1864	0.0868	0.0394	0.0764
<i>tetO</i>	1.4520	0.8032	1.1240	0.7881
<i>tetM</i>	0.0344	-0.2887	0.9498	0.1134
<i>tetX</i>	1.5175	1.3794	1.3063	1.4507
<i>tetL</i>	0.8008	1.1248	0.9890	0.4608
<i>tetW</i>	0.7790	0.3937	0.7945	0.3244
<i>tetB</i>	0.8663	0.2981	0.2960	0.3764
<i>pcoA</i>	0.8349	0.7140	0.6525	0.5938
<i>pcoC</i>	1.3736	1.0866	1.3020	0.8457
<i>pcoD</i>	0.8758	0.4380	0.5435	0.5005
<i>Int1</i>	-0.0834	0.1274	-0.2828	-0.8859
16S rRNA	0.4932	0.2652	0.3808	0.2676

This table is red-yellow-green color gradation table, the red color means higher value and the green color means lower value.

one *int1* genes) in the fresh swine manure were higher (approximately 0 to 3 log copies/ g TS) than those in the inoculum from WWTP (Fig. S2). It was obvious that the dominant genes between the fresh swine manure and the inoculum from WWTP were totally different (Fig. S2 b, c); e.g., relative abundance of *tet* genes in the fresh swine manure (6.20%) was much higher than that in the inoculum (0.96%); *tetW*, *tetX*, *tetO*, *tetL* dominated evidently in the fresh swine manure. These results illustrated that tetracycline resistance existed abundantly in the swine manure. Relative abundance of *int1* gene was dominant in the inoculum, which was much higher (0.93%) than that in the fresh swine manure (0.12%). Additionally, *tetA*, *tetC* showed relative high levels in the inoculum.

On the whole, most of the *tet*, *pco* and *int1* genes showed decreasing trends among the four anaerobic digestion, e.g., especially the dominant ARGs in the swine manure, such as *tetW*, *tetX*, *tetO*, *tetL*, were almost reduced by one order of magnitude (Table 3). The reduction of *tetX* was the largest among all the ARGs, which was decreased by 1.52, 1.38, 1.31, 1.45 log copies/g TS in the control, CTC, Cu and CTC + Cu spiked tests, respectively. The three *pco* genes decreased obviously and were reduced by approximately one order of magnitude as well at the end of the anaerobic digestion (Table 3). However, *tetA* and *int1* showed obviously increasing trends during the anaerobic digestion; and *tetC* and *tetM* were barely reduced, even increased in the control (*tetC*) and the CTC (*tetM*) tests. However, in the Cu test, *tetM* was almost reduced by one order of magnitude, this indicate that Cu might affect *tetM* evolution. It should pay more attention to *tetA*, *tetC*, *tetM* and *int1*, which were hard to be reduced by the anaerobic digestion process. These results were opposite to results of the research (LaPara and Diehl, 2010), which demonstrated that *tetA* and *int1* could be

effectively removed from the municipal wastewater solids and decreased by more than one order of magnitude at the end of the 37 °C mesophilic anaerobic digestion. Sui et al. (2016) investigated the distribution of ARGs in swine wastewater anaerobic digestion and the results showed that *tetX* was effectively removed but *int1* was barely reduced after the mesophilic anaerobic digestion. These reduction differences of *int1* and ARGs might be caused by different substrates (e.g., swine manure, sewage sludge), inoculums, or different operating conditions, and further investigation is needed.

Pearson correlations analysis among the ARGs, HMRGs, MGE and 16S rRNA genes during the swine manure anaerobic digestion were shown in Table 4, *tetO*, *tetX*, *tetL*, *tetW* and *pcoA*, *pcoC*, *pcoD* presented a strong positive correlation. This might be attributable to co-resistance between antibiotics and heavy metals (Baker-Austin et al., 2006), e.g. the predominant ARGs showed a significant positive correlation with HMRGs (Wang et al., 2016). In addition, *tetA*, *tetC* and *int1* presented a strong positive correlation. These results were in accordance with the PCA analysis (Fig. 4), which *tetO*, *tetX*, *tetL*, *tetW*, *pcoA*, *pcoC*, *pcoD* clustered as a one group and *tetA*, *tetC*, *int1* clustered as another group; *tetM* separated from the others.

Horizontal gene transfer, which could be carried out by MGE such as integron1 (*int1*), is a very important pathway for ARGs' proliferation, especially under certain selective pressure (Agersø and Sandvang, 2005). Integron is considered as the most common MGEs, which is reported to coexist with *tet* genes (such as *tetA*, *tetC*) in conjugative plasmids by which they can be easily co-transferred into recipients (Agersø and Sandvang, 2005; Liu et al., 2012). Although no *tet* genes have been detected in the *int1*, they have been found on ISCR2 and ISCR3 (insertion sequence common region), respectively, the latter is linked to *int1* (Toleman et al.,

Table 4

Pearson Correlations among ARGs, HMRGs, MGE, 16S rRNA genes during the swine manure anaerobic digestion (R^2).

	<i>tetA</i>	<i>tetC</i>	<i>tetO</i>	<i>tetM</i>	<i>tetX</i>	<i>tetL</i>	<i>tetW</i>	<i>tetB</i>	<i>pcoA</i>	<i>pcoC</i>	<i>pcoD</i>	<i>int1</i>	16S
<i>tetA</i>	1.0000												
<i>tetC</i>	0.4219	1.0000											
<i>tetO</i>	-0.2837	0.0921	1.0000										
<i>tetM</i>	0.0099	0.2843	0.1412	1.0000									
<i>tetX</i>	-0.0835	0.1418	0.7777**	-0.1300	1.0000								
<i>tetL</i>	-0.1186	0.1638	0.7581**	0.1415	0.8378**	1.0000							
<i>tetW</i>	-0.2273	0.1426	0.9316**	0.3299	0.7156**	0.8216**	1.0000						
<i>tetB</i>	0.0695	0.4413	0.5866*	0.1460	0.3788	0.2961	0.4322	1.0000					
<i>pcoA</i>	-0.3061	-0.0455	0.7878**	0.0261	0.7107**	0.8584**	0.7930**	0.2987	1.0000				
<i>pcoC</i>	-0.2961	-0.1641	0.8045**	-0.0465	0.8080**	0.8325**	0.7640**	0.2263	0.9075**	1.0000			
<i>pcoD</i>	-0.3387	-0.1063	0.7961**	0.1419	0.6642*	0.8045**	0.8184**	0.2826	0.9233**	0.8768**	1.0000		
<i>int1</i>	0.8006**	0.6168*	-0.2206	0.1044	-0.1709	-0.1248	-0.1974	0.3326	-0.2489	-0.3636	-0.2937	1.0000	
16S	0.1887	0.5778*	0.6051*	0.4366	0.4912	0.6132*	0.6098*	0.6841*	0.5427*	0.3966	0.4710	0.3660	1.0000

Correlation is significant at the 0.01 level ($p < 0.01$); *: Correlation is significant at the 0.05 level ($p < 0.05$).

This table is red-yellow-green color gradation table, the red color means higher value and the green color means lower value.

2006). Liu et al. (2012) indicated that *int11* might be involved in *tetA* and *tetC* proliferation in antibiotics production wastewater treatment system. In this study, *int11* might also play an important role on the transfer and proliferation of *tetA* and *tetC*, which exhibited significant positive correlations with *int11* ($R^2 = 0.80, 0.61, p < 0.05$) during the swine manure anaerobic digestion.

3.2.2. Effects of CTC, Cu and CTC+Cu on ARGs, HMRGs and MGE

Addition of CTC, Cu or CTC + Cu could impact on the reductions of ARGs, HMRGs and MGE to some extent, as well as the reductions of 16S r RNA genes, e.g., the influences of CTC, Cu or CTC + Cu were almost negative on the removal efficiencies. The average reductions values of all the genes (including *tet*, *pco* and *int11*) were

Fig. 1. Absolute abundance of ARGs, HMRGs and MGE change during the swine manure anaerobic digestion of A, B, C and D tests (a: histogram; b: heatmap).

0.66, 0.48, 0.63, 0.31 log copies/g TS in the control, CTC, Cu and CTC + Cu, respectively. Evidently, the selective pressure on the Cu spiked test was much less than the CTC or CTC + Cu spiked tests, and the CTC + Cu exerted a relative large pressure on the removal efficiencies (Table 3), especially on *tetA* and *intI1* genes, which increased dramatically during the last few days of the anaerobic digestion. Actually, in the CTC + Cu test, all of the *tet*, *pco* and *intI1* genes showed decreasing trends until 34th day when the biogas production had already stopped (32th day). After that, *tetA* and *intI1*, which were the predominant genes in the inoculum from WWTP, increased dramatically during the last several days.

Several researchers reported that the fate and behavior of ARGs were related to microbial community evolution (Wang et al., 2016; Zhang et al., 2016). It was assumed that anaerobic digestion had the potential to remove *tet*, *pco* and *intI1* genes due to the microbial community change. In the process of anaerobic digestion, the microbial community, especially functional microorganisms, evolved little by little, and took up the ecological niche (Mei et al., 2016). Once anaerobic digestion had stopped, the functional microorganisms did not occupied the ecological niche, meanwhile other microorganisms would take it over, in which might include several host bacteria of ARGs and MEG. This might explain that the dominant genes in the inoculum, such as *tetA* and *intI1*, increased and occupied a large proportion on the 49th day of the anaerobic digestion in the CTC + Cu test (Figs. 1, S3). Additionally, as mentioned above, *intI1* played an important role on the proliferation of *tetA* under CTC + Cu pressure.

3.3. Relationship among organic matters transformation, bacterial communities and ARGs, HMGRs, MEG

3.3.1. Organic matter transformation and ARGs, HMGRs, MEG

Redundancy analysis (RDA) was used for analyzing the relationship between organic matter transformation (including COD, VFAs, biogas production flow rate, methane percentage) and ARGs (Fig. 4), and between bacterial communities and ARGs (Fig. 5), respectively, in order to further study driver of ARGs changes during the swine manure anaerobic digestion. All of the organic matter transformation characteristics were positively correlated with the first axis (explaining 56.34% of total variance). TCOD, SCOD, VFAs and biogas production flow rate showed a significant positive correlation with the predominant ARGs (*tetW*, *tetL*, *tetO*, *tetX*, *tetB*) and HMGRs (*pcoA*, *pcoC*, *pcoD*) which clustered together (Fig. 4). TCOD, SCOD, VFAs and biogas production flow rate represented organic matters degradation in the anaerobic digestion, which all of them were controlled by the functional bacteria. Therefore, it was reasonable that these four parameters directly or indirectly affected the variations of resistance genes.

3.3.2. Microbial communities and ARGs, HMGRs, MEG

Evolution of microbial community exhibited temporal variations during the swine manure anaerobic digestion among the four tests by comparing the composition and structure of bacterial 16S rRNA genes (Figs. 2 and S7). Bacteroidetes (39.09%), Proteobacteria (21.71%) and Firmicutes (27.81%) were the most abundant bacteria

Fig. 2. Bacterial community evolution during the swine manure anaerobic digestion of A, B, C and D tests (genus).

in the fresh swine, which accounted for approximately 90%. All of them took on a decreasing trend throughout the anaerobic digestion among the four tests, which accounted for approximately 45% in the end. Spirochaetes and chloroflexi, which accounted for 1.74% and 0.24% in the fresh swine manure, increased evidently and reached to around 27% and 6% in the end of anaerobic digestion.

Pearson correlations between the most abundant microorganisms at phylum level and ARGs, HMRGs, MEG were shown in the Table S2. The predominant ARGs in the fresh swine manure such as *tetO*, *tetX*, *tetL*, *tetW* exhibited a significant strong positive correlation with Bacteroidetes and Firmicutes, which were the most abundant bacteria in the fresh swine manure as well and might contribute to the reduction of the predominant ARGs during the anaerobic digestion. Additionally, Tenericutes showed a significant positive correlation with *tetX* and *tetL*; Proteobacteria and Verrucomicrobia showed a significant positive correlation with *tetM*; Euryarchaeota showed a significant positive correlation with *tetA* and *int11*, which increased dramatically in the final few days of CTC + Cu test (Fig. S8) and might contribute to the evidently increment of *tetA* and *int11* in the end of CTC + Cu test. In other words, CTC and Cu spiked together might have a great influence on Euryarchaeota variation during anaerobic digestion. The Euryarchaeota are a phylum of the Archaea, which survive extreme concentrations of salt and other extremely environment. This kind of microorganism is often found in intestines (Woese et al., 1990), which explain their high levels in the fresh swine manure (Fig. S8).

However, Spirochaetes and Chloroflexi showed a significant negative correlation with most of the predominant ARGs during the swine manure anaerobic digestion (Table S2), which

speculated that they might have a competitive relationship with the host bacteria of the predominant ARGs.

For detailed analysis of microbial community structure, the 18 most abundant genus (abundance above 1% in any sample) were selected and compared among the four tests (Fig. 2). The composition of microbial community binned at the genus level showed remarkable difference during the swine manure anaerobic digestion. *Prevotella* was the most abundant bacteria in fresh swine manure (20.60%) and in the beginning of anaerobic digestion (A1: 22.22%; B1: 21.48%; C1: 27.61%; D1: 10.35%). Then they decreased evidently and accounted for less than 1% (A49: 0.05%; B49: 0.13%; C57: 0.04%; D49: 0.19%) at the end of tests. *Prevotella* showed a significant positive correlation with the predominant ARGs (*tetO*, *tetL*, *tetW*) and HMRGs (*pcoA*, *pcoC*, *pcoD*) (Table 5, Fig. 5), which might contribute to the reductions of these resistance genes during the anaerobic digestion. When CTC and Cu were spiked together, they really inhibited *Prevotella* in the beginning, which the abundance (D1: 10.35%) was around a half of the others (A1:22.22%). The corresponding resistance genes, including *tetO*, *tetL*, *tetW* and *pcoA*, *pcoC*, *pcoD* in the D test were evidently less than these in the other tests on the first day (Fig. 1). *Bacteroides*, *Veillonella* showed a positive correlation with *tetO*, *tetX*, *tetL*, *tetW* and *pcoA*, *pcoC*, *pcoD*, which all of them decreased among the four tests. Previous studies reported that tetracycline resistance was related to *Veillonella* (Olsvik et al., 1995), in this study, it might be the host bacteria of the predominant ARGs and HMRGs.

It was interesting that *Treponema* showed an obviously increasing trend and was the most abundant bacteria in the end of the anaerobic digestion, which accounted for 22.15%, 16.09%, 23.76%, 10.56% in the control, CTC, Cu and CTC + Cu tests

Fig. 3. Potential pathogens change during the swine manure anaerobic digestion of A, B, C and D tests (genus).

Fig. 4. Redundancy analysis assessing the relationship between environmental factors (red arrows) and ARGs, HMRGs and MGE (deep blue arrows) during the swine manure anaerobic digestion of A, B, C and D tests. The percentage of variation explained by each axis is showed, and the relationship was determined significant ($p < 0.001$) based on 499 permutations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(A49, B49, C57, D49), respectively. The result from Pearson correlation showed that it presented a significant negative correlation with the predominant ARGs (*tetO*, *tetX*, *tetL*, *tetW*) and HMRGs (*pcoA*, *pcoC*, *pcoD*) (Table 5, Fig. 5). It was assumed that *Treponema* might have a competitive relationship with the host bacteria of the predominant ARGs. During anaerobic digestion, *Treponema* took over the ecological niche from the dominant bacteria such as *Prevotella* and *Bacteroides* in the fresh swine manure and might result in the corresponding resistance genes decreasing.

It seemed that CTC + Cu had a great influence on the *Treponema*, which varied differently comparing to the other groups. In the control, CTC and Cu spiked tests, *Treponema* increased gradually along with anaerobic digestion, and reached to the maximum values in the end; however, in the CTC + Cu test, it increased and reached to the maximum (28.78%) on the 27th day, and then decreased

to a relatively low value (10.56%) in the end. Poirier et al. (2016) demonstrate that *Treponema* is a kind of potential syntrophic microorganisms with methanogens, which fluctuates along with the concentration of total ammonia nitrogen (TAN) in anaerobic digestion. Therefore, to some extent, the abundance of *Treponema* signified the process of anaerobic digestion, which the maximum abundance implied the termination of anaerobic digestion. In this case, CTC + Cu group reached to the termination of anaerobic digestion on the 27th day, while the biogas production had almost stopped (1.7 mL/day). It was hypothesis that *Treponema* competed with some of the ARGs host bacteria, in the final few days of CTC + Cu test, abundance of *Treponema* decreased dramatically from 28.78% to 10.56%, oppositely, several *tet* genes and MEG increased, including *tetA*, *tetO*, *tetX*, *tetL*, *tetW*, *int11*, especially *tetA* and *int11* ascended dramatically. However, further study is needed to reveal the real reason.

Fig. 5. Redundancy analysis assessing the relationship between bacterial communities (red arrows) and ARGs, HMRGs and MGE (deep blue arrows) during the swine manure anaerobic digestion of A, B, C and D tests. The percentage of variation explained by each axis is showed, and the relationship was determined significant ($p < 0.001$) based on 499 permutations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

16 potential pathogenic bacteria (from 30 potential pathogens based on <http://www.mgc.ac.cn/cgi-bin/VFs/jsif/main.cgi>) were detected and showed in the Fig. 3, and more detailed information about the Pearson correlations between the most abundant bacteria, the potential pathogens at genus level and ARGs, HMRGs, MEG were shown in the Table 5, red characters represented the potential pathogens. The total potential pathogens detected in this study took on a decreasing trend among the four tests, which was 5.56% in the fresh swine manure, and decreased to 2.89%, 3.85%, 5.31% and 2.40% in the control, CTC, Cu and CTC + Cu tests, respectively. This implied that anaerobic digestion could somehow eliminate the potential pathogens.

Acinetobacter was the most abundant genus in the beginning (2%–5%) and decreased most obviously in the course of anaerobic digestion. *TetC* and *tetX* showed a positive correlation with

Acinetobacter, *tetX* declined down largely, *tetC* fluctuated and was barely reduced in the end of the tests. Previous studies reported that *tetA* has been distributed in *Acinetobacter*, *Escherichia*, *Pseudomonas* (Chopra and Roberts, 2001), however, it did not exhibit any positive correlation with these bacteria in this study. Actually, *tetA* and *int1* did not show any significant correlation with the most abundant bacteria and the potential pathogens listed in the Table 5.

Escherichia accounted for approximately 1.2% in the beginning and exhibited an evidently decreasing trend among the four tests as well (A49: 0.32%; B49: 0.39%; C57: 0.33%; D49: 0.30%). *TetL* and *tetX* showed a positive correlation with *Escherichia*, which both of them decreased largely. It was speculation that *Escherichia* contributed to the reduction of *tetL* and *tetX*. *Clostridium* was barely removed and kept a relatively stable level (around 2%) during the

Table 5Pearson Correlations among ARGs, HMRGs, MGE and host genera (R^2).

Genus	<i>tetA</i>	<i>tetC</i>	<i>tetO</i>	<i>tetM</i>	<i>tetX</i>	<i>tetL</i>	<i>tetW</i>	<i>tetB</i>	<i>pcoA</i>	<i>pcoC</i>	<i>pcoD</i>	<i>Int1</i>	16s
<i>Prevotella</i>	-0.200	-0.246	0.828**	0.148	0.397	0.555*	0.825**	-0.017	0.902**	0.975**	0.932**	-0.330	0.395
<i>Bacteroides</i>	-0.192	-0.104	0.788**	0.071	0.560*	0.587*	0.797**	-0.042	0.856**	0.914**	0.864**	-0.302	0.496*
<i>Acinetobacter</i>	-0.120	0.590*	0.238	-0.179	0.613**	0.409	0.298	0.148	0.296	0.296	0.275	-0.005	0.397
<i>Escherichia</i>	-0.130	0.480	0.277	-0.182	0.718**	0.497*	0.347	0.251	0.374	0.358	0.324	0.001	0.416
<i>Pseudomonas</i>	-0.176	0.317	0.120	-0.311	0.386	0.230	0.125	0.418	0.197	0.220	0.224	0.063	0.141
<i>Providencia</i>	0.167	-0.337	-0.568*	0.211	-0.710**	-0.584*	-0.586*	-0.329	-0.586*	-0.572*	-0.534*	0.086	-0.580*
<i>Vibrio</i>	0.037	-0.071	-0.395	-0.244	0.092	0.122	-0.259	0.001	-0.217	-0.368	-0.336	0.124	-0.342
<i>Neisseria</i>	-0.176	0.251	0.006	-0.167	0.366	0.342	0.116	0.035	0.183	0.175	0.127	-0.104	-0.099
<i>Clostridium</i>	-0.065	0.178	-0.267	0.338	-0.645**	-0.534*	-0.348	0.208	-0.556*	-0.606**	-0.500*	0.037	-0.014
<i>Lactobacillus</i>	0.115	-0.348	0.041	-0.556*	0.199	0.252	0.064	-0.018	0.352	0.230	0.354	0.092	-0.115
<i>Streptococcus</i>	-0.082	0.164	0.524*	0.181	0.252	0.169	0.471	0.033	0.325	0.434	0.390	-0.047	0.596*
<i>Bacillus</i>	-0.161	-0.136	-0.390	0.095	-0.529*	-0.410	-0.440	-0.203	-0.350	-0.401	-0.295	-0.110	-0.298
<i>Staphylococcus</i>	-0.009	0.024	0.256	0.095	0.323	0.245	0.292	0.419	0.025	-0.074	-0.079	-0.071	0.036
<i>Veillonella</i>	-0.162	0.146	0.557*	-0.119	0.817**	0.694**	0.626**	0.168	0.685**	0.674**	0.616**	-0.156	0.445
<i>Butyrivibrio</i>	-0.175	-0.160	0.249	-0.146	0.789**	0.574*	0.345	-0.183	0.419	0.304	0.297	-0.328	0.086
<i>Bifidobacterium</i>	-0.184	-0.304	-0.375	-0.023	-0.303	-0.218	-0.354	-0.256	-0.115	-0.223	-0.107	-0.212	-0.385
<i>Mycobacterium</i>	-0.004	0.160	0.021	-0.133	0.342	0.453	0.175	-0.206	0.401	0.263	0.382	-0.013	0.098
<i>Treponema</i>	-0.069	-0.280	-0.571*	-0.181	-0.554*	-0.512*	-0.594*	-0.228	-0.581*	-0.603*	-0.527*	-0.035	-0.580*
<i>Burkholderia</i>	-0.143	0.292	-0.227	0.617**	-0.209	-0.257	-0.215	-0.162	-0.351	-0.298	-0.321	-0.167	-0.155
<i>Campylobacter</i>	-0.136	0.327	0.427	-0.145	0.529*	0.258	0.406	0.261	0.282	0.379	0.300	-0.070	0.367
<i>Helicobacter</i>	-0.019	0.495*	0.149	-0.042	0.679**	0.552*	0.299	0.052	0.225	0.140	0.132	0.018	0.270
<i>Yersinia</i>	-0.014	0.490*	0.110	0.443	0.009	-0.011	0.079	-0.045	0.011	0.056	0.064	0.140	0.660**
<i>Corynebacterium</i>	0.070	-0.301	-0.630**	0.054	-0.741**	-0.651**	-0.681**	-0.345	-0.652**	-0.645**	-0.603*	0.026	-0.636**
<i>Mycoplasma</i>	0.197	-0.360	-0.501*	-0.358	-0.563*	-0.592*	-0.569*	-0.284	-0.472	-0.478	-0.457	0.058	-0.582*

**: Correlation is significant at the 0.01 level ($p < 0.01$); *: Correlation is significant at the 0.05 level ($p < 0.05$).This table is red-yellow-green color gradation table, the red color means higher value and the green color means lower value. Red characters represent the potential pathogens according to (<http://www.mgc.ac.cn/cgi-bin/VFs/jsif/main.cgi>).

anaerobic digestion. *TetX*, *tetL* and *pcoA*, *pcoC*, *pcoD* showed a significant negative correlation with *Clostridium*. Other potential pathogens such as *Corynebacterium* and *Mycoplasma* also presented a significant negative correlation with the predominant ARGs and HMRGs during the swine manure anaerobic digestion, which indicated that they might compete with the host bacteria of ARGs and HMRGs. It was notable that *Corynebacterium* obviously increased during the swine manure anaerobic digestion and accounted for approximately 0.18% in the end. The previous study demonstrated

that *Corynebacterium* had a significant positive correlation with the *tet* and *pco* genes during the swine manure composting (Wang et al., 2016). These opposite results indicated that the relationship between ARGs and bacteria was complicated and different operated conditions or different technologies could impact both of them variations. Certain bacteria might be host of ARGs in one condition, but might not be the host of the same ARGs in the other condition. Therefore, the relationship between the host bacteria and ARGs needs further studies.

Fig. 6. Procrustes analysis of the correlation between ARGs, HMRGs and MGE and microbial communities during the swine manure anaerobic digestion of A, B, C and D tests. The percentage of variation explained by each axis is showed, and the relationship was determined significant ($p < 0.001$) based on 499 permutations.

Procrustes analysis was conducted by rotating the ordination of changes in the microbial communities to match the profiles of resistance genes based on a PCA analysis (Fig. 6), and the results indicated that most of the resistance genes and bacterial communities clustered during the swine manure anaerobic digestion. The explanatory variables accounted for 44.6%, and the correlation between the first two axes was strongly positive ($R = 0.91$ and 0.42 , respectively). Therefore, evolutions in the microbial communities' structures along with the swine manure anaerobic digestion contributed evidently to the resistance gene profiles.

4. Conclusions

The predominant ARGs (*tetO*, *tetW*, *tetX*, *tetL*) and HMRGs (*pcoA*, *pcoC*, *pcoD*) in fresh swine manure could be effectively eliminated (approximately $1.00 \log$ copies/ g TS) through mesophilic anaerobic digestion. Addition of CTC, Cu and CTC + Cu could impact the changes of ARGs, to some extent, especially CTC + Cu spiked could obviously inhibit the removal of ARGs; and affect shaping of bacterial communities during the anaerobic digestion. It is better to control the usage of antibiotics and heavy metals in swine industries as feed additive to better control the pollution of resistance genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2017.03.134>.

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