# Ferric chloride further simplified the horizontal gene transfer network of antibiotic resistance genes in anaerobic digestion

Junya Zhang[1,2,3], Tiedong Lu[4], Yuan Xin[5], Yuansong Wei[1,3]*

[1] State Key Joint Laboratory of Environmental Simulation and Pollution Control, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[2] Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Leipzig 04318, Germany

[3] University of Chinese Academy of Sciences, Beijing 100049, China

[4] Agricultural Resource and Environment Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, 530007, China

[5] College of Life Science and Technology, Guangxi University, Nanning 530005, Guangxi, China

***Correspondence:**

Tel.: +86-10-62849690; Fax: +86-10-62849690;

E-mail address: jyzhang@rcees.ac.cn (Junya Zhang); yswei@rcees.ac.cn (Yuansong Wei).

# Ferric chloride further simplified the horizontal gene transfer network of antibiotic resistance genes in anaerobic digestion

Junya Zhang[1,2,3], Tiedong Lu[4], Yuan Xin[5], Yuansong Wei[1,3]*

[1] State Key Joint Laboratory of Environmental Simulation and Pollution Control, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[2] Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Leipzig 04318, Germany

[3] University of Chinese Academy of Sciences, Beijing 100049, China

[4] Agricultural Resource and Environment Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, 530007, China

[5] College of Life Science and Technology, Guangxi University, Nanning 530005, Guangxi, China

***Correspondence:**

Tel.: +86-10-62849690; Fax: +86-10-62849690;

E-mail address: jyzhang@rcees.ac.cn (Junya Zhang); yswei@rcees.ac.cn (Yuansong Wei).

**Abstract**

The role of ferric chloride (FC) on the reduction of antibiotic resistance genes (ARGs) in anaerobic digestion (AD) system was investigated from the perspective of vertical (VGT) and horizontal gene transfer (HGT) network through the high-throughput qPCR (HT-qPCR). Although FC showed limited impacts on methane production in AD of swine manure, the tetracycline and MLSB resistance genes were specifically reduced at the end, where *tetQ* of antibiotic target protection and *ermF* of antibiotic target alteration contributed the most to the reduction. Both VGT and HGT network were divided into three modules, and the complexity of HGT network was largely reduced along with AD, where the HGT connection was reduced from 683 (Module III) to 172 (Module I), and FC addition could further reduce the relative abundance of ARG hosts in Module I. The contribution of VGT and HGT to the changes of ARGs in AD was further deciphered, and although the VGT reflected by the changes of microbial community contributed the most to the dynamics of ARGs (68.0%), the HGT contribution could further be reduced by the FC addition. This study provided a new perspective on the fate of ARGs response to the FC addition in the AD system.

**Keywords**: Ferric chloride; Antibiotic resistance genes; Vertical gene transfer; Horizontal gene transfer; Anaerobic digestion.

## 1. Introduction

Antibiotic resistance has arisen to a worldwide highly concerning level, and antibiotic resistance genes (ARGs), as a culprit of antibiotic resistance, constitute a substantial health threat (Abu Al-Halawa et al., 2019). It is reported that infections associated with ARGs are predicted to be a major cause of death by 2050, where the annual death toll is projected to increase from 700,000 to up to 10 million worldwide (O'NEILL, 2016). Due to extensive medical application and livestock use, antibiotic resistance has increased steadily in the environment (Hernando-Amado et al., 2019; Larsson and Flach, 2021; Pruden et al., 2012). Livestock industry is one of the hardest hit areas, where livestock manure serves as an important reservoir of ARGs (Zhu et al., 2013). Swine manure ranked the first in China concerning the amounts produced in the livestock industry, and it is also considered as one of the most important reservoir of ARGs in the environment (Larson, 2015; Zhang et al., 2015). The ARGs in the swine manure could potentially horizontally transfer to human pathogens and contribute to the spread of antibiotic-resistant bacteria in the clinic settlements (Larsson and Flach, 2021). Figuring out the means to prevent the ARGs expansion from the livestock manure is essential for the fight against antibiotic resistance.

Anaerobic digestion (AD) is considered and treated as one of the most important technologies for the livestock manure treatment, because it can not only produce methane for bioenergy but also remove pollutants for resource utilization (Hu et al., 2017). Increasing the methane production and enhancing the pollutant removal are always the two major aims for the investigation of AD (Burch et al., 2017; Zarei-Baygi et al., 2019). Ferric chloride ($FeCl_3$, FC), as a commonly used chemical coagulants in wastewater treatment plants, has been widely demonstrated to be able to improve the

methane production, although the AD of residual sludge is the primary research object (Cheng et al., 2021; Zhan et al., 2021; Zhang et al., 2022; Zhuang et al., 2021). The potential mechanisms are indicated to be associated with the improvement of solubilization, hydrolysis, and acidification via dissimilatory iron reduction (DIR) process, enhancement of key enzyme activities via its role as trace element, reduction of $H_2S$ inhibition etc (Zhang et al., 2022). Nonetheless, the information on how the key functional genes response to FC during the AD of livestock manure is still limited.

There are contrary results on how the FC impacted the fate of ARGs in the environment. As an important coagulant, FC could also effectively remove ARGs from the wastewater treatment plant effluent with 0.5-log to 3.1-log reduction (Grehs et al., 2019; Liu et al., 2021), and the role of FC on the effective reduction of ARGs during the chicken manure composting was also elucidated (Guo et al., 2020). While FC not only increased the abundance of almost all tested ARGs except *tetH*, *aac(6')-Lb-Cr* and $bla_{\mathrm{TEM}}$, but also significantly increased the abundance of *intI1* indicating a higher frequency of HGT and proliferation of ARGs during AD of chemically enhanced primary treatment sludge (Jang et al., 2017). Previous studies generally covered limited target ARGs, and technologies targeting much more ARGs like the high throughput qPCR (HT-qPCR) should be adopted to comprehensively evaluate the role of FC on the fate of ARGs. Besides, the impacts of FC on the fate of ARGs in the AD of swine manure have never been clarified.

There are amounts of ARGs in typical swine manure covering various antibiotic resistance types and mechanisms (Zhang et al., 2019a). The vertical gene transfer (VGT) and horizontal gene transfer (HGT) are the two major molecular mechanisms for the environmental spread of ARGs. As for VGT, ARGs are grown up along with the proliferation of their bacterial hosts, while ARGs can be

disseminated among bacteria of the same or different species through the HGT like transformation, conjugation and transduction, leading to the expansion of ARG-carrying hosts (Jiang et al., 2017; Larsson and Flach, 2021; Wang and Chen, 2020; Wei et al., 2021). The role of VGT on the fate of ARGs could be reflected by the dynamical changes of microbial community, and its importance on the fate of ARGs has been widely demonstrated in various environment not least to the AD system (Luo et al., 2017; B. Zhang et al., 2021; Zhang et al., 2016; Zhao et al., 2021). Nonetheless, it is hard to clarify the role of HGT on the fate of ARGs, because even HGT event happened only once between the donator and receptor, it could be largely amplified through the following VGT of the receptor (Martínez et al., 2014). While there existed complex HGT network for the ARGs in the environment, which could be constructed through the ARG they shared (Zhou et al., 2021). It was indicated that one recent HGT could be identified between two distantly related genomes (less than 97% of 16S rRNA sequence similarity) through the shared region of DNA of at least 500 bp with 99% or greater similarity (Brito1 et al., 2016; Smillie et al., 2011). Then, the HGT network of ARGs could be constructed based on the information of ARG hosts. It was assumed that if the HGT network was simplified, it could facilitate the reduction of HGT and proliferation of ARGs. How the FC impacted the HGT network in the AD of swine manure deserved further investigation, which could decipher the mechanisms from a new perspective.

Thus, batch experiments concerning the AD of swine manure were established to figure out the role of FC on the methane production and fate of ARGs. How the FC impacted the methane production was deciphered from the perspective of key functional genes; HT-qPCR covering 251 ARGs was used to comprehensively clarify the changes of ARGs response to the FC; Microbial

97 community dynamics after the FC addition was followed, and the VGT along with HGT network was
98 constructed to find out the potential mechanisms, which could provide some basis for the future
99 control of ARGs spread in the swine manure.

100 **2. Materials and methods**

101 **2.1 Batch experiments set-up**

102 Automatic Methane Potential Test System II (Bioprocess Control, Sweden) was used to set up
103 the batch experiments. The fresh swine manure (total solids (TS), 29.6%; volatile solids (VS),
104 79.9%) and inoculum sludge (TS, 5.4%; VS, 57.8%) were together collected from the same large-
105 scale swine farm in Beijing, China, where an AD reactor was normally operated. The ratio (swine
106 manure: inoculum sludge) was set as 3:1 (TS) with the final TS of about 8% in each reactor
107 (working volume, 0.4L). Five treatments in triplicate were set up, and certain volume of $FeCl_3$
108 solution (100 g/L) was added in each reactor to reach the final concentrations of iron element at 0
109 mmol (CK), 5 mmol (FC5), 10 mmol (FC10), 25 mmol (FC25) and 40 mmol (FC40), respectively.
110 The mesophilic digestion (37±0.1 °C) were operated in a water bath along with methane production
111 being automatically determined after $CO_2$ was removed using 3M NaOH. The treatment of FC40
112 failed, because the serious sludge bulking happened due to the high amounts of FC. Sampling was
113 conducted through the changes of the daily methane production on days of 0, 6, 15, 22 and 39. The
114 analysis of physicochemical parameters (pH, TCOD, SCOD (chemical oxygen demand), proteins,
115 polysaccharides, VFAs, $NH_4^+$-N, $PO_4^{3-}$ and $SO_4^{2-}$) were conducted as previously described (Zhang
116 et al., 2019c).

117 **2.2 DNA extraction and microbial community analysis**

DNA extraction (0.2 mL of each sample) was done in triplicate through the FastDNA Spin Kit for Soil (MP Biomedicals, USA). The extracted DNA quality control and concentration were determined by gel electrophoresis and NanoDrop ND-1000 (NanoDrop, USA), respectively. The DNA samples from the same treatment were mixed as the representative DNA sample for further analysis.

The primers 515F/806R and the nested PCR primers (Arch340F/Arch1000R and Arch349F/Arch806R) were used for the bacterial and archaeal community analysis, respectively. The samples were sent to Sangon Co., Ltd., (Shanghai, China) for small-fragment library construction and pair-end sequencing using the Illumina Miseq sequencing. The bioinformatics analysis including quality control, pair-end reads merging, chimers filter and normalization was conducted as previously reported (J. Zhang et al., 2021). The clean reads were then submitted to the NCBI Sequence Read Archive (SRA) with the project number of PRJNA802036.

**2.3 Traditional qPCR and High-throughput qPCR (HT-qPCR)**

The functional genes associate with methane production including *cel5* and *cel48* (glycoside hydrolase genes) in cellulose-degrading bacteria, *hydA* (Fe-hydrogenase gene) in the fermentative bacteria, *ACAS* (acetyl-coA synthetase gene) specifically associated with acetoclastic methanogenesis, *dsrA* (dissimilatory sulfite reductase gene) in the sulfate-reducing bacteria, *mcrA* (methyl coenzyme-M reductase gene) participating in non-specific methanogenesis and 16s rRNA were quantified through the traditional qPCR as indicated previously (Zhang et al., 2019b).

The HT-qPCR array adopted 296 primer sets, where 251 ARGs, 28 mobile genetic elements (MGEs), 10 heavy metal resistance genes (MRGs), 6 human pathogens and 16s rRNA were covered

(Table S1). The HT-qPCR was conducted on a Wafergen SmartChip Real-time PCR system (Wafergen Biosystems, USA) in triplicate for each sample as indicated in our previous studies (Cooray et al., 2021; J. Zhang et al., 2021). The amplification efficiencies between 1.7 and 2.3 were retained, while threshold cycle ($C_T$) above 31 and nano-wells with multiple melting peaks were discarded. The relative abundance was calculated as follows:

$$\text{Gene copies} = 10^{((31 - C_T)/(10/3))} \quad (1)$$

$$\text{Relative abundance} = Target\ gene\ copies/16s\ rRNA\ gene\ copies \quad (2)$$

**2.4 Establishment of the VGT and HGT network**

The VGT indicated that the proliferation of ARGs-carrying hosts contributed to the ARGs spread, and the connection between ARGs and potential hosts constituted the VGT network, while HGT network showed the connection between microbes that HGT event ever happened. It was assumed that one recent HGT could be identified between two distantly related genomes (less than 97% of 16S rRNA sequence similarity) through the shared region of DNA of at least 500 bp with 99% or greater similarity (Zhou et al., 2021). Thus, firstly, microbial community was analyzed at the OTU level, which was clustered at the cutoff of 97% of 16S rRNA sequence similarity. Secondly, the potential hosts of the targeted ARGs were identified at the OTU level, where the spearman correlation cutoff was set as $p < 0.01$ and $R > 0.8$. It should be noted that the correlation between ARGs and bacteria is the most widely used approach to figure out the potential hosts of ARGs, although the approach was indirect and putative (Rice et al., 2020). Lastly, if different OTUs shared the same ARG, we considered the HGT happened between these OTUs. The HGT network was further constructed to show the genus (nodes) connected by at least one observed HGT event

(edges).

The VGT and HGT contribution to the fate of specific ARG was further determined. It was hypothesized that for one host, the most abundant ARG was determined; for one ARG, the most abundant host was determined; then, if the host and the ARG were matched in both directions, the host was considered as the preliminary host of the ARG. The VGT contribution was considered as the proportion of the preliminary host divided by all the potential hosts, and the rest was attributed to the HGT contribution (Wei et al., 2021).

### 2.5 Data analysis

Principal component analysis (PCA), nonmetric multidimensional scaling (NMDS), redundancy analysis (RDA) and Procrustes analysis were performed using Canoco 5.0. The Lefse analysis showing the biomarkers was conducted using the galaxy module under the default parameter (Segata et al., 2011). Network analysis based on the spearman correlation ($p < 0.01$) between ARGs and MGEs along with environmental variables was constructed through the Gephi platform (Bastian et al., 2009).

## 3. Results and Discussion

### 3.1 Balance of inhibition and improvement caused by FC in AD.

The effects of FC on the methane production are not dose-dependent. FC5 increased the accumulated methane production by 7.7%, but FC10 reduced it by 15.4%, while the FC25 restored it to the CK level (Figure 1A). FC40 directly led to the AD failure because of the serious sludge bulking caused by the chemical reaction. The dose effects of improvement-reduction-restoration caused by FC have also been demonstrated in AD of other substrate (Qin et al., 2019; Zhan et al., 2021). The

daily methane production was inhibited after FC addition before day 5, and the inhibition showed the dose dependent (Figure 1B). This could be reflected by the VFAs accumulation on D6, especially the butyrate accumulation for FC10 and FC25 (Figure 1C). Then, methane production was largely improved on days 6-11, and the maximum daily methane production was dose dependent, where FC25 reached the 27.4±0.6 mL $CH_4$/gVS. The butyrate accumulation caused by the FC was removed, and all the treatments entered into the propionate inhibition on days 12-16 as reflected by the VFAs composition on D15. The removal rate of propionate inhibition was also dose dependent, and FC25 removed the inhibition much quicker than other treatments reflected by the daily methane production on days 16-30 and VFAs composition on D22. The removal of propionate inhibition was 10 days ahead for FC 25 and 6 days ahead for FC5 and FC10 compared to CK reflected by the maximum daily methane production, although the propionate inhibition was removed finally in all the treatments as shown in VFAs composition on D39. In summary, FC showed two perspectives in AD of swine manure: the cause of butyrate accumulation at the early stage and quick removal of propionate inhibition at the end. The methane production improvement of FC5 happened on days 6-11, while the restoration of FC25 happened on days 15-30.

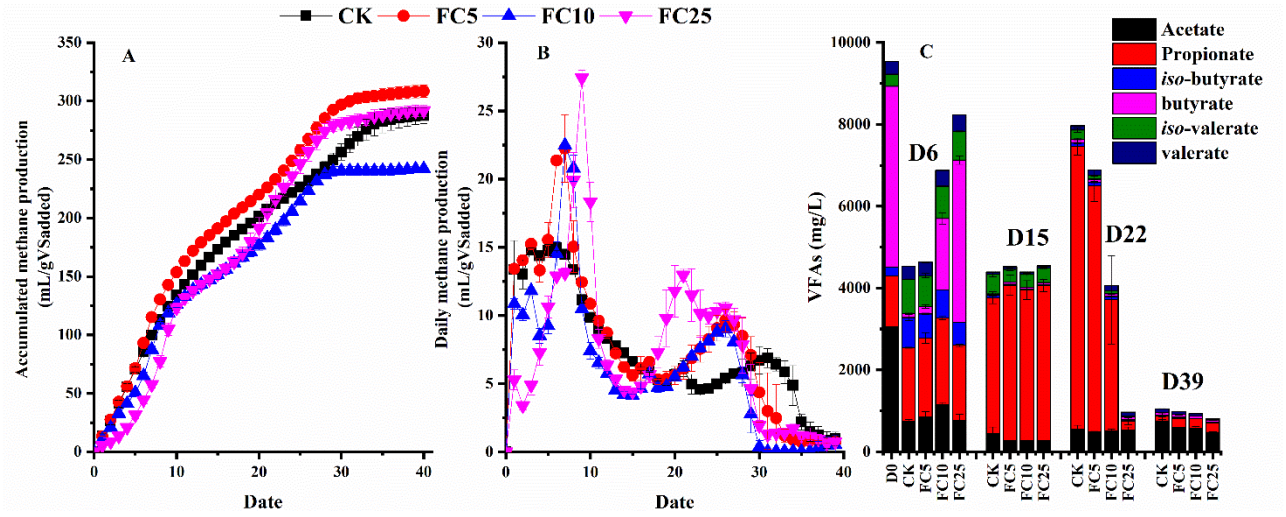


**Figure 1.** Dynamics of accumulated methane production (A), daily methane production (B) and VFAs (C) in anaerobic digestion response to ferric chloride.

The changes of TCOD and SCOD on D6 indicated the inhibition caused by FC at the early stage, while the concentration of SCOD on D22 showed the improvement effects by FC (Figure S1). The changes of polysaccharides indicated that FC inhibited the degradation of polysaccharides on D6, but the degradation was whereafter enhanced along with the AD. The degradation of proteins was improved response to the FC addition in all the treatments. Higher concentration of FC25 facilitated the $PO_4^{3-}$ removal due to the formation of vivianite (Prot et al., 2020), but there existed the phenomenon of re-release of $PO_4^{3-}$ at FC5 and FC10 at the end of the AD. FC facilitated the removal of $SO_4^{2-}$ at the early stage of D6, but showed limited effects whereafter. The pH was largely reduced by FC at the early stage, which was in accordance with the VFAs concentration. The reduction of pH was associated with $Fe^{3+}$ hydrolysis reactions (Guerrero et al., 2021). Nonetheless, the pH was comparable with CK along with AD, as $H^+$ was flushed out in the form of $CH_4$. When no more $CH_4$ was produced, the pH was soon decreased due to the reaction of FC. The changes of free ammonia showed the similar trend with pH, but lower free ammonia (FA) on D6 did not facilitate the methane production, which indicated that the FA inhibition in AD of swine manure was not the key factor when FC was involved. Interestingly, the addition of FC solution into the AD system did not increase but even reduce the concentration of soluble iron at the early stage. This could be associated with the coagulation effects of FC, and only the concentration of soluble iron in the FC25 increased after D22. This indicated that part of the flocs caused by higher concentration of FC could have been degraded by the microbes, and the iron elements could be released into the soluble phase.

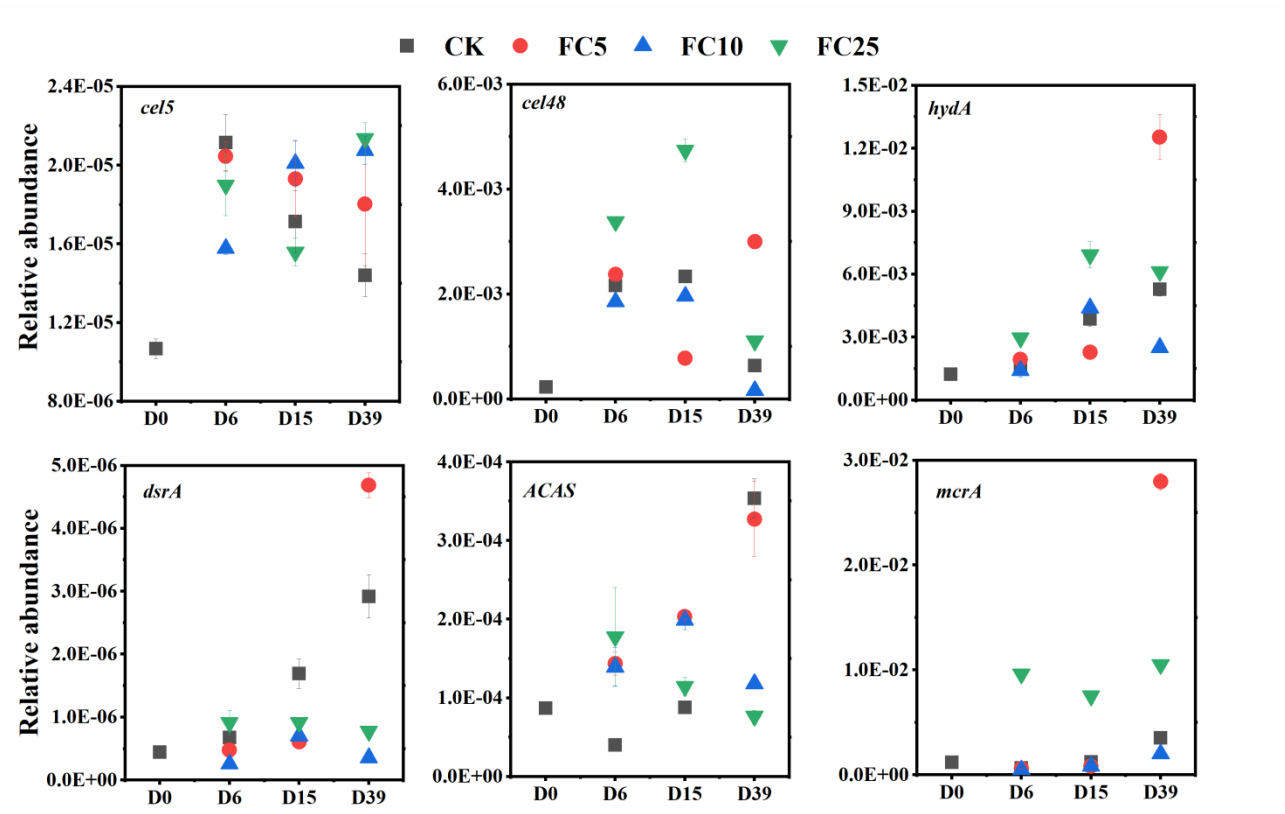



**Figure 2.** Changes of key functional genes associated with methane production in anaerobic digestion response to ferric chloride.

The glycoside hydrolases of families 5 (*cel5*) was inhibited on D6, and the inhibition for FC25 continued to D15, but FC addition significantly increased the *cel5* at the end. While FC25 increased the relative abundance of *cel48* and *hydA* throughout the AD process, and the *hydA* was closely associated with the $H_2$ production. The higher concentration of FC facilitated the inhibition of sulfate reduction bacteria as reflected by the *dsrA*. The changes of *ACAS* could reflect the acetoclastic methanogenesis, and FC could increase the relative abundance of *ACAS* on D6 and D15. While only FC25 significantly increased the relative abundance of *mcrA* throughout the AD process. These indicated that the inhibition of methane production at early stage might be associated with the inhibition of hydrogenic methanogenesis. Nonetheless, FC addition significantly decreased the

relative abundance of *ACAS* at the end, but the relative abundance of *mcrA* was still much higher for FC25. These indicated that the enhancement of methane production at the later stage, especially for FC25 and FC5, might also be associated with the enhancement of hydrogenic methanogenesis.

### 3.2 FC can enhance the reduction of the relative abundance of ARGs in AD.

FC addition could enhance the reduction of the relative abundance of ARGs at the end of AD, where the enhancement was dose dependent, and the maximum reduction was realized at FC25 by 33.9% (Figure 3). Tetracycline, aminoglycoside, MLSB and streptothricin resistance genes were dominant throughout the AD process, which accounted for 40.0%, 27.7%, 24.6% and 5.7%, respectively (Figure S2). While from the perspective of resistance mechanisms, ARGs associated with antibiotic inactivation, target protection, target alteration and efflux were abundant accounting for 39.1%, 31.9%, 15.5% and 12.9%, respectively.

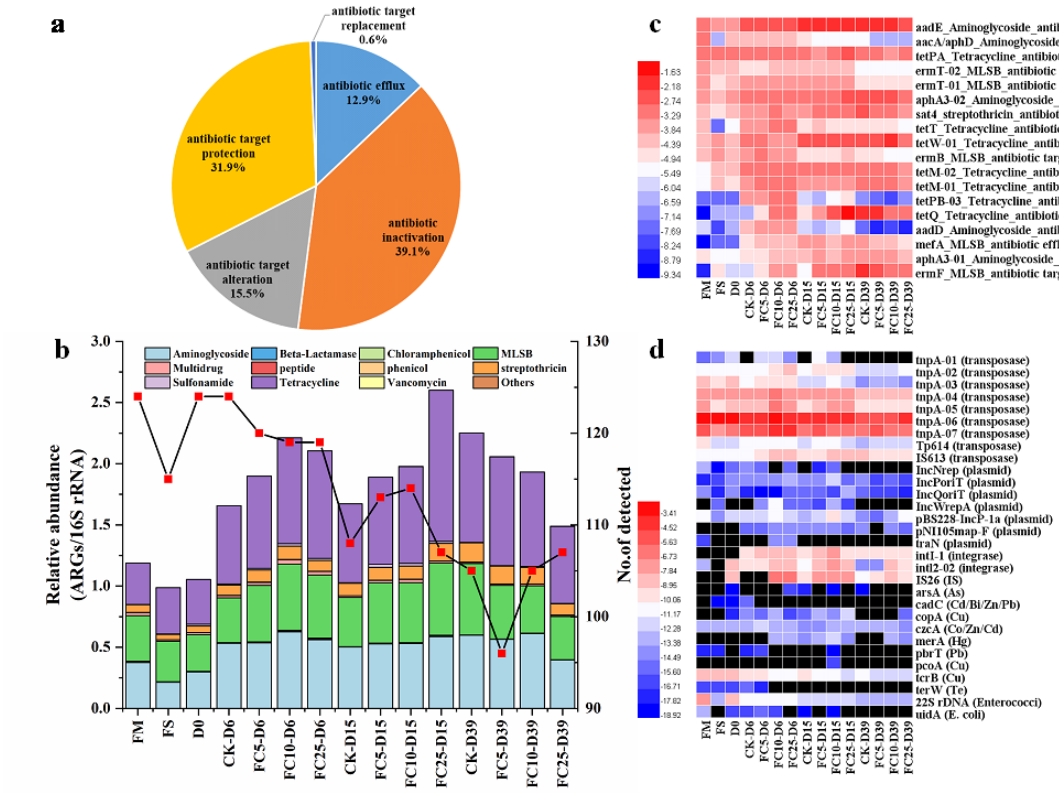


**Figure 3**. Distribution of ARGs under resistance mechanism (a); Changes of ARGs at types level (b), subtype level (c) and MGEs along with MRGs and VFs (d) response to ferric chloride in anaerobic digestion.

The impacts of FC on the fate of ARGs varied a lot from different stages of AD. FC increased the relative abundance of ARGs on D6 and D15, although FC could reduce the relative abundance of ARGs at the end (D39). Interestingly, the dynamic effects of FC mainly happened on the tetracycline and MLSB along with streptothricin resistance genes, and aminoglycoside resistance genes were limitedly affected. The changes of tetracycline and MLSB resistance genes contributed 68.5% to the dynamics of the ARGs response to the FC. As for the resistance mechanisms, FC significantly impacted the antibiotic target protection and alteration along with inactivation significantly, but limited effects on the antibiotic efflux (Figure S3). The *ermF* belonging to MLSB of the antibiotic target alteration, *tetQ* belong to tetracycline resistance genes of antibiotic target protection and *sat4* belonging to streptothricin resistance genes of antibiotic inactivation were the key ARG subtypes impacted by the FC throughout the AD process. The *ermF*, *tetQ* and *sat4* contributed to the variance of ARGs by 16.0%, 9.9% and 6.8%, respectively, throughout the AD process at subtype level. The abundant MGE was transposase, and *tcrB* was the dominant MRG, while only 2 of 6 human pathogens were detected with the *Enterococci* prevalence in the AD process. Although AD process could reduce the MGEs, MRGs and human pathogens, the impacts of FC on these elements were limited.

### 3.3 Changes of microbial community response to FC in AD.

FC showed no significant impacts on the diversity indexes in this study as shown in Table S2.

263 The phylum of Firmicutes accounted for 86.1%±4.5% in all the samples (Figure S4), and the abundant
264 genus was *Clostridium sensu stricto* (39.8%±7.4%). According to the NMDS analysis based on the
265 genus level, the AD process was separated into three stages along with time (Figure 4A). One-way
266 ANOSIM based on Bray-Curtis indicated that FC did not significantly impacted the changes of
267 microbial community during the AD process ($p > 0.05$).

268 The *Clostridium* sensu stricto was abundant throughout the AD process, which was associated
269 with its high function diversity. The Clostridium could not only take part in complex organics
270 decomposition at the early stage of AD, but also constitute a syntrophic metabolism with
271 methanogens (J. Zhang et al., 2021). Thus, its relative abundance was not significantly changed
272 along with time, and the FC addition also showed limited effects on its dynamics. The biomarkers
273 indicated by the Lefse analysis at the early stage (D6) were *Anaerococcus*, *Peptostreptococcus*,
274 *Peptoniphilus*, and *Anaerosphaera* etc. The biomarkers at the early stage could be divided into two
275 types, one type was closely associated with the swine manure. They entered into the AD system
276 through the swine manure, but they cannot adapt to the AD system, and then the relative abundance
277 was reduced along with time. The *Anaerococcus* belonged to the first type, its relative abundance
278 was 25.8% in the swine manure, but only 0.03% in the inoculum sludge. The relative abundance
279 decreased along with time from 7.5% on D0 to 2.4% (average value) on D6, and 0.7% at the end of
280 AD. The other type was associated with the AD process, and their abundance could be enriched a
281 lot on D6. The *Peptostreptococcus*, *Peptoniphilus* and *Anaerosphaera* could be considered as this
282 type. The relative abundance of *Peptostreptococcus* was below 0.1% in both swine manure and
283 inoculum sludge, while its relative abundance could be increased to 6.1% on D6, so did the

*Anaerosphaera*, whose relative abundance was increased from 0.02% to 2.7%. The *Peptostreptococcus* could produce acetate and propionate but it could not produce butyrate, and the major fermentation products of *Peptoniphilus* and *Anaerosphaera* were butyrate and acetate (Lagier et al., 2018). These butyrate-producing microbes on D6 could have contributed to the butyrate accumulation caused by the FC addition. On D15, the propionate accumulation happened, the daily methane production was low, and the system was under inhibition. The biomarkers at this stage were *Clostridum III* and *Alkaliflexus*, and it was reported that *Alkaliflexus* could help the degradation of cellulose, and propionate was the major fermentation product in AD, which well explained its biomarker role on D15 under the propionate accumulation. The FC addition also did not significantly impact their relative abundance. The abundant biomarkers on D39 were *unclassified_Ruminococcaceae* and *Syntrophomonas*, and as the key syntrophic bacteria, *Syntrophomonas* can not only participate in the degradation of long-chain fatty acids, but also metabolize syntrophy with methanogens (Sousa et al., 2007). Besides, it could produce methane not only with hydrogenotrophic methanogens using the $H_2$ and $CO_2$ but also with acetoclastic methanogens through DIET. This was in accordance with the removal of propionate inhibition. FC addition decreased the relative abundance of *unclassified_Ruminococcaceae* and *Syntrophomonas*, although the removal of propionate inhibition was improved response to the FC addition.

The *Methanosphaera* and *Methanosarcina* were the two abundant archaea microbes in AD of swine manure (Figure 4D), and they mainly came from swine manure and inoculum sludge, respectively. The *Methanosphaera* was also considered as the biomarker on D6, which specially used $H_2$ to reduce methanol to methane (Garcia, 1990). Its dominance at the early stage of AD was

associated with the higher release of $H_2$ and methanol. Along with AD, the *Methanosarcina* became dominant, and *Methanosarcina* could use the $H_2$ and $CO_2$, acetate or methyl compounds. At the end, *Methanosphaera* was largely reduced, and *Methanosarcina* became dominant. It seemed that FC could reduce the relative abundance of *Methanosarcina*, which showed the similar trend with *Syntrophomonas*. The significant correlation between *Syntrophomonas* and *Methanosarcina* ($p <$ 0.01) indicated the important role of syntrophy with methanogens at the later stage of AD.

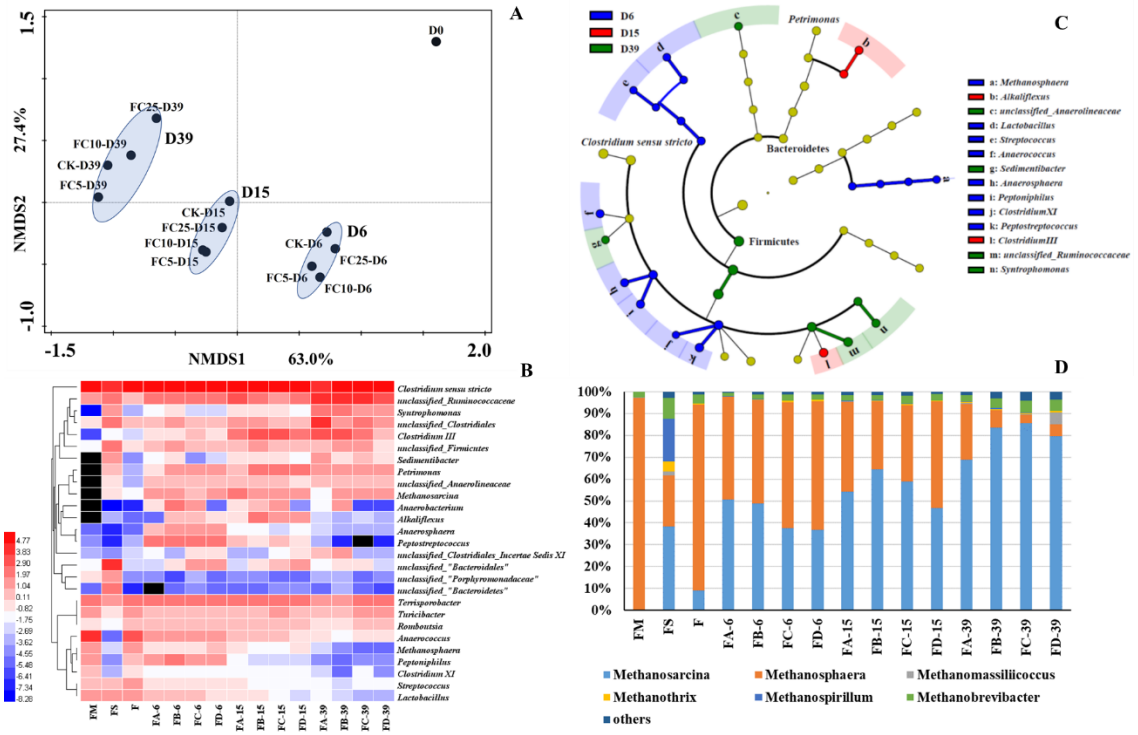
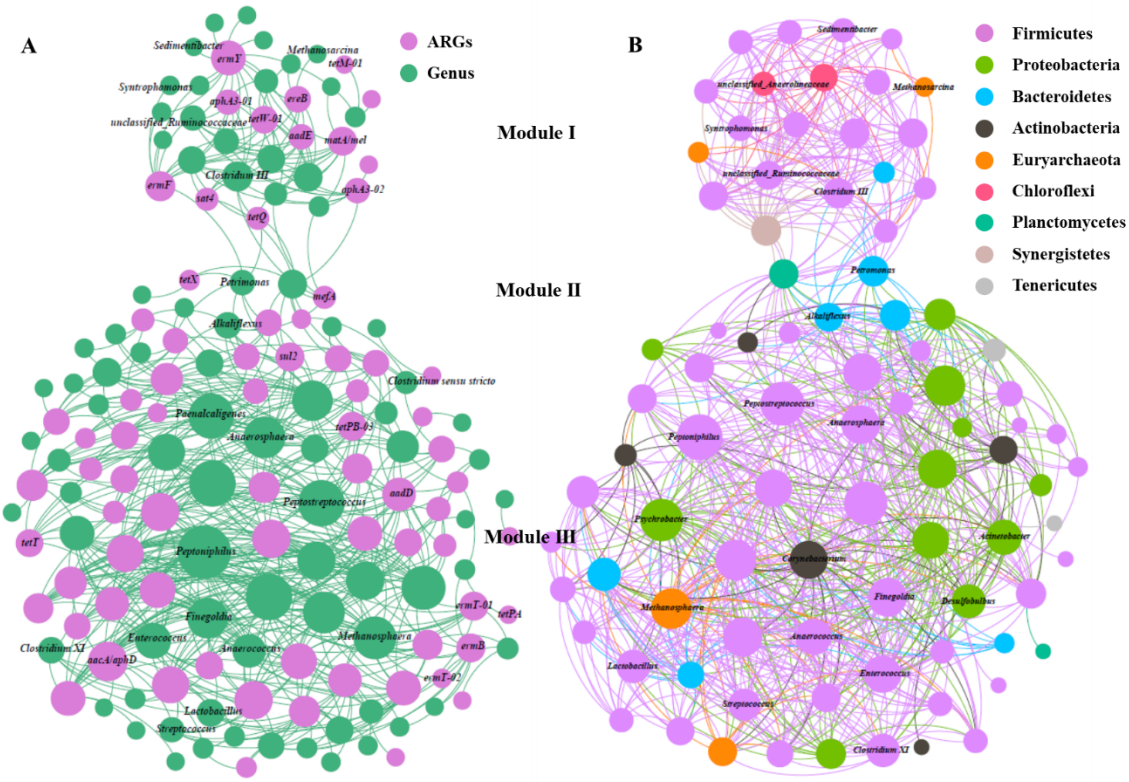



**Figure 4**. Nonmetric multidimensional scaling (NMDS) analysis (A); heatmap showing the top 10 genus (B); lefse analysis showing the biomarkers on D5, D15 and D39 (C); changes of archaeal community in anaerobic digestion response to ferric chloride (D).

### 3.4 Vertical gene transfer determined the changes of ARGs response to FC

The spread of ARGs through the VGT could be reflected by the changes of microbial community. The results of Procrustes analysis and mantel test showed that there existed significant

positive correlation ($p < 0.01$) between changes of ARGs and dynamics of microbial community (Figure S5). The VGT reflected by microbial community contributed 68.0% to the changes of ARGs in AD of swine manure. Although FC can help the reduction of specific ARGs at the end, it did not significantly impact the fate of ARGs throughout the process ($p > 0.05$). Meanwhile, no significant correlation between MGEs and ARGs was figured out at type level, and so did the physic-chemical parameters with tetracycline and MLSB resistance genes that were reduced at the end by FC addition. Nonetheless, the important role of MGEs, physic-chemical parameters, MRGs and VFs on the changes of ARGs could be reflected at subtype level (Figure S6). There existed significant positive correlation between MGEs and ARG subtypes extensively ($p < 0.01$), and *tnpA* belonging to the transposase played more important role on the HGT of ARGs compared with other MGEs, which showed significant correlation with 48 ARG subtypes. The 22S rDNA representing the pathogen of *Enterococci* was found to be closely associated with 25 ARG subtypes including *ermT-01*, *tetT* etc., and the *copA* and *tcrB* also showed the co-occurrence with 13 kinds of ARG subtypes. These indicated that although the VGT caused by the changes of microbial community played the critical role on the dynamics of ARGs, other factors like MGEs, VFs, MRGs and physic-chemical parameters should also not be overlooked.




334

335 **Figure 5**. The vertical gene transfer (VGT) network (A) showing the potential hosts of ARGs and
336 horizontal gene transfer (HGT) network (B) showing the genus (nodes) connected by at least one
337 observed HGT event (edges). The genus was colored according to the taxonomy at phylum level.

338 The hosts of ARGs were identified through the network analysis based on spearman correlation
339 ($p < 0.01$, $R > 0.8$), and 84 kinds of genus were found out to be the potential hosts of ARGs. The
340 VGT network was then established and could be divided into three modules (Figure 5A). The
341 Module I and Module III were connected through the Module II which only contained *Petrimonas*
342 and *unclassified_Planctomycetaceae*. The changes of the relative abundance of the hosts could well
343 explain the dynamics of ARGs. In Module I, the relative abundance of the hosts increased after AD,
344 while the hosts in Module III were generally decreased. For instance, *Syntrophomonas*,
345 *Methanosarcina* and *Sedimentibacter* were abundant hosts in Module I, they were generally
346 enriched on D39. The abundant ARGs including *aadE*, *tetW-01*, *aphA3-02*, *ermF*, *sat4* and *tetQ*

were located in Module I. FC specially impacted the ARGs in Module I. For instance, the FC could reduce the relative abundance of *Syntrophomonas* as indicated previously on D39, which well explained the final enhanced reduction of *ermF*. Most of the ARGs in Module III were reduced after AD, which could also be reflected by their hosts. For instance, the *Anaerococcus* and *Methanosphaera* were derived from swine manure as indicated by the microbial community, and the relative abundance of them were largely reduced after AD. The *Enterococcus* as the hosts of various ARGs also confirmed the results reflected through the 22s rDNA. The VGT network could well explain the fate of ARGs at subtype level.

**3.5 Horizontal gene transfer network was largely simplified in AD.**

The HGT event happened between 84 different genus (nodes) covering 8 phylum, and there were 858 HGT edges between them (Figure 5B). Among them, genus belonging to Firmicutes accounted for the 60.9% following by Proteobacteria (12.6%) and Bacteroidetes (8.1%). The HGT network was also divided into three modules, and Module II containing the *Petromonas* connecting the Module I and Module III. It can be seen that the Module III was much more complex than Module I. There were 21 nodes and 172 edges in Module I, but there were 61 nodes with 683 edges in Module III. The relative abundance of the genus constituting the HGT network accounted for 39.8%±7.4% in all the samples (Figure 6A). Although the relative abundance of genus in the HGT network increased along with AD, HGT network was largely simplified pushing the changes of HGT network from Module III to Module I. The relative abundance of nodes in Module III was largely reduced along with AD from 26.9% on D0 to 7.0% on D39, while the relative abundance of nodes in Module I was increasing from 2.8% to 36.7%, where the HGT network was largely simplified. FC addition could further

reduce the relative abundance of nodes in Module I, which indicated that FC could further simplify and reduce the HGT frequency.

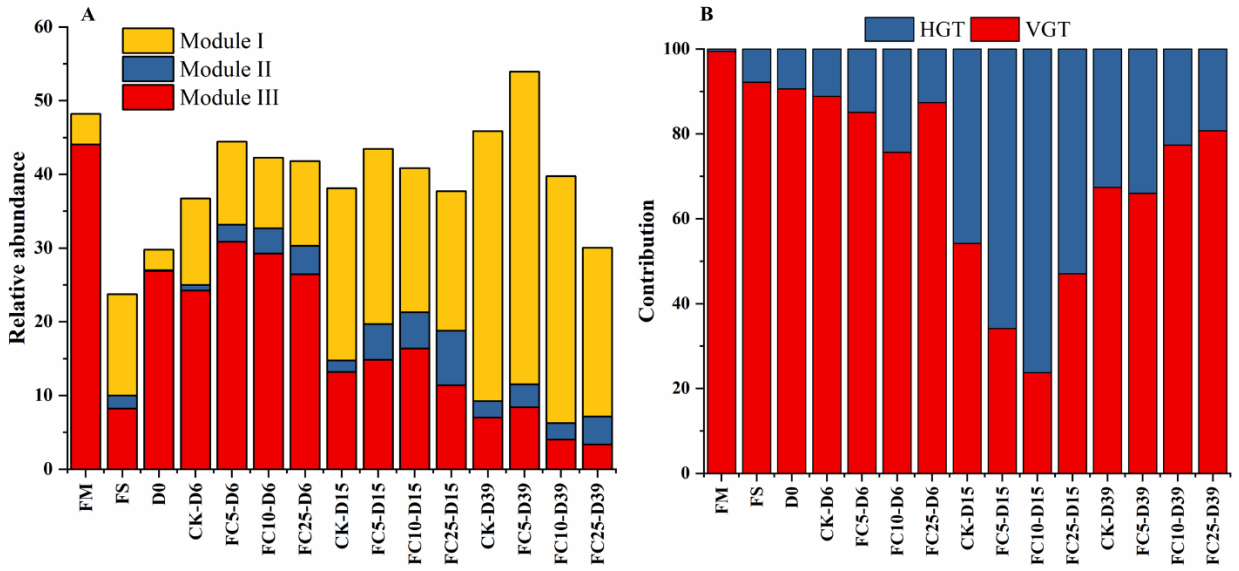



**Figure 6.** Changes of relative abundance of genus in different modules in the HGT network (A) and dynamical changes of VGT and HGT contribution to the fate of the abundant *aadE* (B) response to the ferric chloride in AD of swine manure;

### 3.6 Comparison of VGT and HGT on the fate of ARGs in anaerobic digestion

Although FC showed limited effects on the improvement of methane production in AD of swine manure, it could specifically reduce the tetracycline and MLSB resistance genes of *tetQ* and *ermF*, respectively. Statistical analysis indicated that VGT reflected by the changes of microbial community contributed the most to the dynamics of ARGs (68.0%, $p < 0.01$). The VGT network showed the potential hosts of ARGs, it was found that many hosts of ARGs were important functional microbes in the AD process. The abundant *Clostridium sensu stricto* was found to be carrying 5 ARG subtypes and was also responsible for the degradation of complex organics. The biomarkers on D6 including *Peptostreptococcus*, *Peptoniphilus* and *Anaerosphaera* contributing to the VFAs production were

383 also the critical ARG-carrying hosts in Module III. The *Syntrophomonas* as the most important
384 syntrophy along with *Methanosarcina* were also found to be important hosts of ARGs in Module I,
385 while they were the most important microbes responsible for the methane production. From the
386 perspective of ARGs control, we can reduce the ARGs in AD just by killing these ARGs hosts, but
387 the methane production would be largely impacted. It was not wise to discuss ARGs reduction letting
388 out the methane production in AD system, where the changes of microbial community were
389 responsible for the methane production. Thus, it would not be a big issue that VGT determined the
390 fate of ARGs. However, the HGT event that happened in AD would cause some risks. Compared to
391 VGT, HGT is of greater concern due to the high potential to create antibiotic-resistant "superbugs"
392 in various anthropogenic environments like clinics, urban surface water, and wastewater treatment
393 plants (Montassier et al., 2021; A. Zhang et al., 2021; Zhu et al., 2017). Thus, it would be a good
394 choice to control the ARGs spread focusing on the HGT network not VGT network in the AD system.
395 In Module III, the HGT network is more complex, and the potential human pathogens like
396 *Enterococcus*, *Streptococcus* and *Acinetobacter* were among them. The HGT network in Module III
397 could bring unknown risks from the perspective of ARGs spread. In Module I, the complexity of
398 HGT network was largely reduced. AD process could simplify the HGT network from Module III to
399 Module I, which indicated that the HGT events could be largely reduced in AD. This was also in
400 accordance with the changes of MGEs in AD. FC addition did not significantly change the dynamics
401 of microbial community, while FC largely reduced the relative abundance of genus in Module I,
402 which could further simplify and reduce the HGT events. The significantly impacted ARGs by FC
403 including *ermF*, *sat4* and *tetQ* also located in Module I.

Separating the HGT and VGT would further help clarify the changes of ARGs response to FC in AD of swine manure. We took the abundant ARG of *aadE* for example. The most abundant host of *aadE* was *unclassified_Ruminococcaceae*, and the most abundant ARG it carried was *aadE*. Based on this, the contribution of VGT and HGT on *aadE* was clearly determined (Figure 6B). The average contribution of VGT on *aadE* was 71.3%, which was comparable with the results of Procrustes analysis (68.0%) between ARGs and microbial community. The HGT contribution increased along with AD from 0.1% to 45.9%, and reached to the maximum value on D15. Nonetheless, the HGT contribution decreased at the end of the AD. During the active methane production phase on D6 and D15, the interaction between organisms was active and the degradation of organics provided enough energy for the HGT (Lopatkin et al., 2016; Nielsen and Townsend, 2004), the HGT contribution to the fate of *aadE* would increase accordingly. At the end of AD, the degradation of organics was not active, and the interaction between microbes was largely reduced, which unfavored the HGT. These could be reflected by the changes of the daily methane production. FC addition, especially FC5 and FC10, increased the HGT contribution on D6 and D15, but FC10 and FC 25 could further reduce the HGT contribution at the end of AD.

## 4. Conclusions

This study clarified the role of FC on the methane production and reduction of ARGs in AD of swine manure using the HT-qPCR. The VGT and HGT network were both established based on the spearman correlation analysis. Although VGT reflected by the microbial community contributed the most to the dynamics of ARGs, the complexity of HGT could be largely reduced along with AD, and FC further simplified the HGT network. This study provided a new perspective on the fate of ARGs

response to FC addition in AD of swine manure. However, how the expression of ARGs response to FC in AD system need further investigation, and the technology like epic-PCR should be used to directly link the ARGs with the host. Considering the coagulation effects of FC, the fate of extracellular and intracellular ARGs should also be investigated separately in the future.

**Acknowledgement**

This work was supported by National Natural Science Foundation of China (51808540) and Guangxi Key Research and Development Program (AB21196036). Dr. Junya Zhang is also grateful for the financial support from Alexander von Humboldt Foundation.

**References**

Abu Al-Halawa, D., Sarama, R., Abdeen, Z., Qasrawi, R., 2019. Knowledge, attitudes, and practices relating to antibiotic resistance among pharmacists: a cross-sectional study in the West Bank, Palestine. Lancet 393, S7. https://doi.org/10.1016/S0140-6736(19)30593-8

Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: An open source software for exploring and manipulating networks. Proc. Third Int. ICWSM Conf. 2, 361–362.

Brito1, I.L., Yilmaz, S., Huang, K., Xu, L., Jupiter, S.D., Jenkins, A.P., Naisilisili, W., Tamminen, M., Smillie, C.S., Wortman, J.R., Birren, B.W., Xavier, R.J., Blainey, P.C., Singh, A.K., Gevers, D., Alm, E.J., 2016. Mobile genes in the human microbiome are structured from global to individual scales. Nature. https://doi.org/10.1038/nature18927

Burch, T.R., Sadowsky, M.J., LaPara, T.M., 2017. Effect of Different Treatment Technologies on the Fate of Antibiotic Resistance Genes and Class 1 Integrons when Residual Municipal

446 Wastewater Solids are Applied to Soil. Environ. Sci. Technol. 51, 14225–14232.
447 https://doi.org/10.1021/acs.est.7b04760
448 Cheng, Y., Wang, X., Wu, J., Chen, Y., Shen, N., Wang, G., Liu, X., 2021. Improvement of
449 Methane Production and Sludge Dewaterability by FeCl 3 -Assisted Anaerobic Digestion of
450 Aluminum Waste-Activated Sludge . ACS ES&T Water.
451 https://doi.org/10.1021/acsestwater.1c00223
452 Cooray, T., Zhang, J., Zhong, H., Zheng, L., Wei, Y., Weragoda, S.K., Jinadasa, K.B.S.N.,
453 Weerasooriya, R., 2021. Profiles of antibiotic resistome and microbial community in
454 groundwater of CKDu prevalence zones in Sri Lanka. J. Hazard. Mater. 403, 123816.
455 https://doi.org/10.1016/j.jhazmat.2020.123816
456 Garcia, J., 1990. Taxonomy and ecology of methanogens. FEMS Microbiol. Lett. 87, 297–308.
457 https://doi.org/10.1016/0378-1097(90)90470-B
458 Grehs, B.W.N., Lopes, A.R., Moreira, N.F.F., Fernandes, T., Linton, M.A.O., Silva, A.M.T.,
459 Manaia, C.M., Carissimi, E., Nunes, O.C., 2019. Removal of microorganisms and antibiotic
460 resistance genes from treated urban wastewater: A comparison between aluminium sulphate
461 and tannin coagulants. Water Res. 166, 115056. https://doi.org/10.1016/j.watres.2019.115056
462 Guerrero, A., Duan, H., Meng, J., Zhao, J., Song, Y., Yu, W., Hu, Z., Xu, K., Cheng, X., Hu, S.,
463 Yuan, Z., 2021. An integrated strategy to enhance performance of anaerobic digestion of waste
464 activated sludge. Water Res. 195, 116977. https://doi.org/10.1016/j.watres.2021.116977
465 Guo, H., Gu, J., Wang, X., Nasir, M., Yu, J., Lei, L., Wang, Q., 2020. Elucidating the effect of
466 microbial inoculum and ferric chloride as additives on the removal of antibiotic resistance

467 genes from chicken manure during aerobic composting. Bioresour. Technol. 309, 122802.
468 https://doi.org/10.1016/j.biortech.2020.122802
469 Hernando-Amado, S., Coque, T.M., Baquero, F., Martínez, J.L., 2019. Defining and combating
470 antibiotic resistance from One Health and Global Health perspectives. Nat. Microbiol. 4,
471 1432–1442. https://doi.org/10.1038/s41564-019-0503-9
472 Hu, Y., Cheng, H., Tao, S., 2017. Environmental and human health challenges of industrial
473 livestock and poultry farming in China and their mitigation. Environ. Int. 107, 111–130.
474 https://doi.org/10.1016/j.envint.2017.07.003
475 Jang, H.M., Shin, J., Choi, S., Shin, S.G., Park, K.Y., Cho, J., Kim, Y.M., 2017. Fate of antibiotic
476 resistance genes in mesophilic and thermophilic anaerobic digestion of chemically enhanced
477 primary treatment (CEPT) sludge. Bioresour. Technol. 244, 433–444.
478 https://doi.org/10.1016/j.biortech.2017.07.153
479 Jiang, X., Ellabaan, M.M.H., Charusanti, P., Munck, C., Blin, K., Tong, Y., Weber, T., Sommer,
480 M.O.A., Lee, S.Y., 2017. Dissemination of antibiotic resistance genes from antibiotic
481 producers to pathogens. Nat. Commun. 8, 1–7. https://doi.org/10.1038/ncomms15784
482 Lagier, J.C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., Levasseur, A., Rolain,
483 J.M., Fournier, P.E., Raoult, D., 2018. Culturing the human microbiota and culturomics. Nat.
484 Rev. Microbiol. 1–11. https://doi.org/10.1038/s41579-018-0041-0
485 Larson, C., 2015. China's lakes of pig manure spawn antibiotic resistance. Science (80-. ). 347,
486 704–704. https://doi.org/10.1126/science.347.6223.704
487 Larsson, D.G.J., Flach, C.F., 2021. Antibiotic resistance in the environment. Nat. Rev. Microbiol.

488 0123456789. https://doi.org/10.1038/s41579-021-00649-x
489 Liu, X., Wu, Y., Xu, Q., Du, M., Wang, D., Yang, Q., 2021. Mechanistic insights into the effect of
490 poly ferric sulfate on anaerobic digestion of waste activated sludge. Water Res. 189, 116645.
491 https://doi.org/10.1016/j.watres.2020.116645
492 Lopatkin, A.J., Huang, S., Smith, R.P., Srimani, J.K., Sysoeva, T.A., Bewick, S., Karig, D.K., You,
493 L., 2016. Antibiotics as a selective driver for conjugation dynamics. Nat. Microbiol. 1–8.
494 https://doi.org/10.1038/nmicrobiol.2016.44
495 Luo, G., Li, B., Li, L., Zhang, T., Angelidaki, I., 2017. Antibiotic Resistance Genes and
496 Correlations with Microbial Community and Metal Resistance Genes in Full-Scale Biogas
497 Reactors As Revealed by Metagenomic Analysis. Environ. Sci. Technol. 51, 4069–4080.
498 https://doi.org/10.1021/acs.est.6b05100
499 Martínez, J.L., Coque, T.M., Baquero, F., 2014. What is a resistance gene? Ranking risk in
500 resistomes. Nat. Rev. Microbiol. 1–8. https://doi.org/10.1038/nrmicro3399
501 Montassier, E., Valdés-Mas, R., Batard, E., Zmora, N., Dori-Bachash, M., Suez, J., Elinav, E.,
502 2021. Probiotics impact the antibiotic resistance gene reservoir along the human GI tract in a
503 person-specific and antibiotic-dependent manner. Nat. Microbiol. 6, 1043–1054.
504 https://doi.org/10.1038/s41564-021-00920-0
505 Nielsen, K.M., Townsend, J.P., 2004. Monitoring and modeling horizontal gene transfer. Nat.
506 Biotechnol. 22, 1110–1114. https://doi.org/10.1038/nbt1006
507 O'NEILL, J., 2016. Tackling drug-resistant infections globally: final report and recommendations.,
508 The review on antimicrobial resistance. https://doi.org/10.4103/2045-080x.186181

509 Prot, T., Wijdeveld, W., Eshun, L.E., Dugulan, A.I., Goubitz, K., Korving, L., Van Loosdrecht,
510 M.C.M., 2020. Full-scale increased iron dosage to stimulate the formation of vivianite and its
511 recovery from digested sewage sludge. Water Res. 182, 115911.
512 https://doi.org/10.1016/j.watres.2020.115911
513 Pruden, A., Arabi, M., Storteboom, H.N., 2012. Correlation between upstream human activities and
514 riverine antibiotic resistance genes. Environ. Sci. Technol. 46, 11541–11549.
515 https://doi.org/10.1021/es302657r
516 Qin, Y., Chen, L., Wang, T., Ren, J., Cao, Y., Zhou, S., 2019. Impacts of ferric chloride, ferrous
517 chloride and solid retention time on the methane-producing and physicochemical
518 characterization in high-solids sludge anaerobic digestion. Renew. Energy 139, 1290–1298.
519 https://doi.org/https://doi.org/10.1016/j.renene.2019.02.139
520 Rice, E.W., Wang, P., Smith, A.L., Stadler, L.B., 2020. Determining Hosts of Antibiotic Resistance
521 Genes : A Review of Methodological Advances. Environ. Sci. Technol. Lett.
522 https://doi.org/10.1021/acs.estlett.0c00202
523 Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C.,
524 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.
525 https://doi.org/10.1186/gb-2011-12-6-r60
526 Smillie, C.S., Smith, M.B., Friedman, J., Cordero, O.X., David, L. a., Alm, E.J., 2011. Ecology
527 drives a global network of gene exchange connecting the human microbiome. Nature 480,
528 241–244. https://doi.org/10.1038/nature10571
529 Sousa, D.Z., Smidt, H., Alves, M.M., Stams, A.J.M., 2007. Syntrophomonas zehnderi sp. nov., an

anaerobe that degrades long-chain fatty acids in co-culture with Methanobacterium formicicum. Int. J. Syst. Evol. Microbiol. 57, 609–615. https://doi.org/10.1099/ijs.0.64734-0

Wang, J., Chen, X., 2020. Removal of antibiotic resistance genes (ARGs) in various wastewater treatment processes: An overview. Crit. Rev. Environ. Sci. Technol. 0, 1–60. https://doi.org/10.1080/10643389.2020.1835124

Wei, Z., Feng, K., Wang, Z., Zhang, Y., Yang, M., Zhu, Y.G., Virta, M.P.J., Deng, Y., 2021. High-Throughput Single-Cell Technology Reveals the Contribution of Horizontal Gene Transfer to Typical Antibiotic Resistance Gene Dissemination in Wastewater Treatment Plants. Environ. Sci. Technol. 55, 11824–11834. https://doi.org/10.1021/acs.est.1c01250

Zarei-Baygi, A., Harb, M., Wang, P., Stadler, L.B., Smith, A.L., 2019. Evaluating Antibiotic Resistance Gene Correlations with Antibiotic Exposure Conditions in Anaerobic Membrane Bioreactors. Environ. Sci. Technol. 53, 3599–3609. https://doi.org/10.1021/acs.est.9b00798

Zhan, W., Tian, Y., Zhang, J., Zuo, W., Li, L., Jin, Y., Lei, Y., Xie, A., Zhang, X., 2021. Mechanistic insights into the roles of ferric chloride on methane production in anaerobic digestion of waste activated sludge. J. Clean. Prod. 296, 126527. https://doi.org/10.1016/j.jclepro.2021.126527

Zhang, A., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L., Loosdrecht, M.C.M. Van, Topp, E., Gillings, M.R., Hanage, W.P., Tiedje, J.M., Moniz, K., Alm, E.J., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. Nat. Commun. 12, 4765. https://doi.org/10.1038/s41467-021-25096-3

Zhang, B., Qin, S., Guan, X., Jiang, K., Jiang, M., Liu, F., 2021. Distribution of Antibiotic

551 Resistance Genes in Karst River and Its Ecological Risk. Water Res. 203, 117507.
552 https://doi.org/10.1016/j.watres.2021.117507
553 Zhang, J., Lu, T., Chai, Y., Sui, Q., Shen, P., Wei, Y., 2019a. Which animal type contributes the
554 most to the emission of antibiotic resistance genes in large-scale swine farms in China? Sci.
555 Total Environ. 658, 152–159. https://doi.org/10.1016/j.scitotenv.2018.12.175
556 Zhang, J., Lu, T., Wang, Z., Wang, Y., Zhong, H., Shen, P., Wei, Y., 2019b. Effects of magnetite on
557 anaerobic digestion of swine manure: attention to methane production and fate of antibiotic
558 resistance genes. Bioresour. Technol. 291, 121847.
559 https://doi.org/10.1016/j.biortech.2019.121847
560 Zhang, J., Lu, T., Zhong, H., Shen, P., Wei, Y., 2021. Zero valent iron improved methane
561 production and specifically reduced aminoglycoside and tetracycline resistance genes in
562 anaerobic digestion. Waste Manag. 136, 122–131.
563 https://doi.org/10.1016/j.wasman.2021.10.010
564 Zhang, J., Sui, Q., Tong, J., Buhe, C., Wang, R., Chen, M., Wei, Y., 2016. Sludge Bio-drying:
565 Effective to Reduce both Antibiotic Resistance Genes and Mobile Genetic Elements. Water
566 Res. 106, 62–70. https://doi.org/10.1017/CBO9781107415324.004
567 Zhang, J., Wang, Z., Lu, T., Liu, J., Wang, Y., Shen, P., Wei, Y., 2019c. Response and mechanisms
568 of the performance and fate of antibiotic resistance genes to nano-magnetite during anaerobic
569 digestion of swine manure. J. Hazard. Mater. 366, 192–201.
570 https://doi.org/10.1016/j.jhazmat.2018.11.106
571 Zhang, Q.Q., Ying, G.G., Pan, C.G., Liu, Y.S., Zhao, J.L., 2015. Comprehensive evaluation of

antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance. Environ. Sci. Technol. 49, 6772–6782. https://doi.org/10.1021/acs.est.5b00729

Zhang, Z., Li, X., Liu, H., Zamyadi, A., Guo, W., Wen, H., Gao, L., Nghiem, L.D., Wang, Q., 2022. Advancements in detection and removal of antibiotic resistance genes in sludge digestion: A state-of-art review. Bioresour. Technol. 344, 126197. https://doi.org/10.1016/j.biortech.2021.126197

Zhao, Q., Guo, W., Luo, H., Xing, C., Wang, H., Liu, B., Si, Q., Ren, N., 2021. Deciphering the transfers of antibiotic resistance genes under antibiotic exposure conditions: Driven by functional modules and bacterial community. Water Res. 205, 117672. https://doi.org/10.1016/j.watres.2021.117672

Zhou, H., Beltrán, J.F., Brito, I.L., 2021. Functions predict horizontal gene transfer and the emergence of antibiotic resistance. Sci. Adv. 7, eabj5056.

Zhu, Y.-G., Johnson, T.A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. U. S. A. 110, 3435–3440. https://doi.org/10.1073/pnas.1222743110

Zhu, Y., Zhao, Y., Li, B., Huang, C., Zhang, S., Yu, S., Chen, Y., Zhang, T., Gillings, M.R., Su, J., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. 16270. https://doi.org/10.1038/nmicrobiol.2016.270

Zhuang, H., Amy Tan, G.-Y., Jing, H., Lee, P.-H., Lee, D.-J., Leu, S.-Y., 2021. Enhanced primary treatment for net energy production from sewage – The genetic clarification of substrate-

593 acetate-methane pathway in anaerobic digestion. Chem. Eng. J. 133416.
594 https://doi.org/10.1016/j.cej.2021.133416
595