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Metagenomic analysis reveals the effects of long-term antibiotic pressure on sludge anaerobic digestion and antimicrobial resistance risk

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ABSTRACT

Continuous stirred-tank digesters with tetracyclines and sulfonamides were operated to investigate the impacts of antibiotic pressure on sludge anaerobic digestion. The versatile methanogen *Methanosarcinales* and strictly hydrogenotrophic methanogen *Methanobacteriales* increased and decreased by 21.1% and 10.9% under antibiotic pressure, respectively. KEGG analysis revealed that hydrogenotrophic and acetoclastic methanogenesis pathways were all affected. The decrease in abundance of function genes involved in lipid metabolism, carbohydrate metabolism, and fatty acid degradation, would lead to a reduction in methane production by 25%. Network analysis indicated positive associations among tetracycline residuals, abundance of resistance genes (ARGs), and specific member of potential hosts. Over 1000 ARG subtypes were widely detected in sludge, including macrolide (28%), tetracycline (24%), fluoroquinolone (20%), and peptide (20%) resistance genes. AD process exposed to long-term antibiotic would increase the diversity and abundance of ARG, enhance the association of ARG with specific microbes, and select bacteria able to perform chemotaxis mechanism.

1. Introduction

Antimicrobial resistance (AMR) is the ability of microorganisms to become increasingly resistant to antimicrobial agents (e.g. antibiotics, antivirals, antifungals and antiprotozoals) to which they were previously susceptible (EU, 2017; Qiao et al., 2018). The abuse and misuse of antibiotics, such as in human medicine and the industrial production of livestock, leads to their continuous discharge into the environment and propagates the global growth of antibiotic resistant bacteria (ARB) and corresponding antibiotic resistance genes (ARG) (O'Neill, 2014; Qiao et al., 2018). The large-scale co-occurrence patterns of antibiotic compounds, ARB, and ARG has been widely reported in both natural and engineered ecosystems, like soil, surface water, groundwater, livestock farm, wastewater treatment plants (WWTPs), landfill (Feng et al., 2016; Guo et al., 2016; Yang et al., 2013; Zhu et al., 2017). AMR has become a major health concern because it is estimated that the mortality from drug-resistance infections would increase up to 10 million by 2050 if no appropriate action is taken (Brown and Wright, 2016; O'Neill, 2014).

As the gut of cities, WWTPs are recognized as a major source of AMR because they receive a large amount of domestic sewage containing various biological contaminants (Feng et al., 2016; Yang et al., 2014). WWTPs are not specifically designed to remove the pharmaceutically active compounds. Besides, due to the strong adsorption capacity of sludge biomass, many antibiotics and ARG are retained in the sludge during the wastewater treatment process (Li et al., 2015). When using anaerobic digestion (AD) technology to treat such AMR-concentrated sludge, the dense and diverse range of microorganisms in digesters may provide an ideal setting for the acquisition of antibiotic resistance. In-depth investigation of the emerging prevalence and dissemination of AMR contaminants in digesters is critical (WHO, 2017).

Many studies have investigated the diversity, distribution, co-occurrence, fate, and removal of various antibiotics and the corresponding ARG and ARB during the AD process (Diehl and Lapara, 2010; Feng et al., 2016; Li et al., 2015). Literatures reported that only 27% of the total ARG were removed via sludge digestion, suggesting that effluents may cause serious problems by spreading antibiotic resistance when released into the downstream environment (Forsberg and Dantas, 2012;

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Yang et al., 2014). Reports of human bacterial pathogens from sludge that carry various ARG via the horizontal gene transfer (HGT) are also on the rise, like *Pseudomonades*, *Acinetobacter*, *Enterobacteriaceae*. Correlation between antibiotic concentrations and the evolution of ARG and ARB is often unclear. Moreover, the specific mechanisms leading to different microorganisms to acquire resistance and how long-term antibiotic pressure affect survival strategies during the AD process remain unknown. One of the key ways to address this challenge is the measurement, collection, and determination of various genotypes from sludge samples. Through metagenomic sequencing, a large amount of biological data is available to obtain the important information regarding ARG genotypes including intrinsic resistance, dedicated ARG, mutation of antimicrobial targets, associated elements, potential hosts, and the mechanisms of resistance (Guo et al., 2017; Li et al., 2015; McArthur and Wright, 2015; Zhu et al., 2017).

Several studies have shown the adverse impacts of antibiotics on the stability and process efficiency of laboratory or field-scale digesters (Beneragama et al., 2013; Hu et al., 2018; Yi et al., 2016). For example, the presence of sulfonamide was reported to be toxic to sludge biomass (Hu et al., 2018). Alvarez et al. observed a significant inhibition of methane production under a combination of chlortetracycline and oxytetracycline at concentrations of 50–100 mg L^{-1} (Álvarez et al., 2010). Sludge digestion is known as a very complex engineered ecosystem in terms of microbial richness, biodiversity and evenness. The introduction of antibiotics may also exert a persistent selection pressure on microbial communities, which could influence anaerobic digester function. Antibiotics do not specifically target genes in methanogenic archaea, and archaea do not harbor ARG like bacteria do. Archaea are only affected to the extent that the bacteria responsible for providing their food are affected. However, the degree to which microbial metabolic networks (e.g., hydrolysis, acidogenesis, acetogenesis, and methanogenesis) are affected by the concentrated antibiotics in digesters remains poorly understood. The different hydrolytic and acetogenic bacteria and methanogenic archaea involved in the AD process form an extremely complex and specialized microbiome. Therefore, both the composition of microorganisms and their metabolic potential must be clarified and correlated with environmental variables (Ma et al., 2017; Ye et al., 2018).

Several recent studies have characterized AD-related microbiomes based on the 16S rRNA encoding genes across different antibiotic conditions. A consensus regarding the core composition and its possible function is emerging, e.g., the phylum *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Bacteroidetes*, *Euryarchaeota*. (Liu et al., 2018; Xu et al., 2018b, 2017). These studies have typically determined microbial functions through inferences based on correlation analysis including both statistical methods and taxonomic assignments. To our knowledge, there is little direct evidence derived from gene pools within the AD system. This is further complicated by the difficulty of sampling, DNA sequencing, and gene annotation, as well as operational conditions. A metagenomic approach can directly characterize the taxonomic and functional gene profiles providing stronger evidence of genetic potential and correlating bioinformatics with performance data for AD reactors (Feng et al., 2016).

This study applied a metagenomic approach to reveal the commonalities and differences among microbial community, functional genes, and ARG from two AD reactors with or without long-term antibiotic pressure. The main goals of the study were to (1) clarify the impacts of long-term antibiotic pressure on AD-related metabolic pathway networks, and (2) assess whether the propagation of AMR would increase under antibiotic pressure, by determining the broad correlations of potential ARG genotypes and microbial community structure. This would enable the determination of the most important drivers shaping AMR and support the global surveillance of resistome risk within AD ecosystems.

2. Materials and methods

2.1. Characterization of substrates and feedstock

Anaerobic inoculum (seed sludge) was obtained from a mature mesophilic anaerobic digester in this lab (Xu et al., 2017). Feed sludge was a mixture of dewatered sludge and secondary sludge. Sludge collected from Yuelu WWTPs, Changsha. The pH of seed and feed sludge were 7.21 and 6.82; the total solids (TS) were 38 and 480 g L^{-1}; the volatile solids (VS) were 9 and 140 g L^{-1}, respectively. Detail characteristics can be found in previous report (Xu et al., 2018a).

A total of three tetracyclines including tetracycline (TC), oxytetracycline (OTC), chlortetracycline CTC) and three sulfonamides including sulfathiazole (STZ), sulphamethoxazole (SMX), and sulfamethizole (SML) antibiotics was prepared to make a stock solution in the phosphate buffer solution at the concentration of 2 g L^{-1}, respectively. The stock solutions were stored without light (4℃). The selection of antibiotic classes was due to their extensive usages in the world (Li et al., 2015; Lu et al., 2016). All the antibiotics (purity > 98%) were purchased from Sigma-Aldrich (Shanghai, China).

2.2. Setup of anaerobic digestion

Anaerobic digestion experiment was performed in two group triplicated continuous stirred-tank reactors (CSTR) with a working volume of 3 L. One group received dewatered sludge with an organic loading rate of 3 g VS L^{-1} d^{-1} as the control reactor (Ct). Another received the same volume of sludge plus a mixture of antibiotics as the antimicrobial pressure reactor (Anti). Considering the environmentally relevant concentrations of antibiotics in dewatered sludge (~mg kg^{-1} dw) (Qiao et al., 2018), this study added each antibiotic with ca. 2 mg L^{-1} every day to create a long-term antibiotic pressure in Anti reactor. Each of the two reactors were initially inoculated with 2 L seed and 1 L feed sludge, then operated at mesophilic condition (in 35℃ incubator) for 130 days. Each day, 200 mL sludge was removed and replaced with equal volume of feed sludge to maintain the hydraulic retention time at 15 days.

2.3. Chemical analysis

Methane production was monitored daily using a gas chromatograph with a thermal conductivity detector (Shimadzu GC-2010). Centrifuged (10 min for 7000 rpm) and filtered (via 0.45 μm) samples were used for the analysis of pH, TS, VS, total alkalinity (TA), volatile fatty acids (VFA), chemical oxygen demand (COD), which were detected via the standard methods to indicate the performance of two reactors. Detail descriptions of above measurements can be found in previous reports (Xu et al., 2017).

To detect the concentration of antibiotics, all the samples were filtered to pre-extract antibiotic from both liquid and solid phase using the solvent extractor. The detail extraction conditions, including pressure, flow rate, heating period and validity were described previously (Gao et al., 2012; Hu et al., 2018). The total antibiotics (sum of solid phase and liquid phase) were measured using high-performance liquid chromatography/triple quadrupole mass spectrometry (Applied Biosystems, USA) with an electrospray ionization (Gao et al., 2012; Zhang and Li, 2018). All the measurements were performed in triplicates.

2.4. DNA extraction and metagenomic sequencing

Genomic DNA was extracted from triplicate 0.5 g samples of well-mixed digested sludge from the Ct and Anti reactors on 1 d, 30 d, 50 d and 130 d, namely “Ct 1–4” and “Anti 1–4”. The FastDNA SPIN Kit (MP bio, USA) was used to extract genomic DNA within 24 h. Extraction details can be found in previous reports (Xu et al., 2018b). DNA quality was evaluated by gels electrophoresis and Nanodrop UV/VIS

spectrophotometer. The meta-library was prepared as the Illumina manufacture's guideline (www.mrdnalab.com, Shallowater, USA). Metagenomic sequencing was performed at Illumina HiSeq-PE150 platform. Raw data have been deposited at JGI databank with the sequencing project ID Gp0272271–Gp0272281 and analysis project ID Ga0236237–Ga0236247.

2.5. Bioinformatic analysis

IDBA-UD software was used to assemble the metagenomic sequencing data (Peng et al., 2012). The structural and functional annotation of metagenomic datasets with nucleotide sequences was conducted in the DOE-JGI Metagenome Annotation Pipeline (MAP, v.4) (Huntemann et al., 2016), according to the standard operating procedure by three stages: (i) Sequence data pre-processing: this step was implemented for all the metagenomic datasets to reduce noise, including file quality control, trimming, low complexity filtering, dereplication. (ii) Structural annotation: scaffolds that have stretches of ≥50 Ns were separated into contigs to predict gene. After annotation, scaffolding information was retained and contigs were re-assembled into scaffolds. Then, protein coding genes were conducted using a combination of Hidden Markov Models and *ab initio* gene callers. (iii) Functional annotation (including the Clusters of Orthologous Groups of proteins-COGs and KEGG Orthology-KO terms) and phylogenetic lineage prediction for scaffolds/contigs. COGs related genes were annotated by comparing protein sequences to COG PSSMs from the Conserved Domain Database (CDD) database using RPS-BLAST (2.2.31) (with an e-value of 0.1) (Aron et al., 2007). KO terms related genes were annotated by comparing metagenome proteins against an isolate genome reference database based on the USEARCH (6.0.294) results (with default maxhits and an e-value) (Edgar, 2010). One top USEARCH hit per gene was also retained for the Phylogenetic Distribution tool in Integrated Microbial Genomes (IMG) and assignment of phylogenetic lineage to scaffolds and contigs. Phylogenetic lineage was assigned as the last common ancestor of USEARCH hits of the genes on the scaffold/contig provided that at least 30% of the genes have USEARCH hits. Details of structural, functional and phylogenetic annotation can be found in the Appendix. Moreover, the annotation of ARG ontology, associated phenotypes, AMR gene family and resistance mechanism was performed with Resistance Gene Identifier (RGI) against the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017). The ARG-annotation was conducted with online tool and default criteria (https://card.mcmaster.ca/analyze/rgi). A resistance gene was annotated when the read hit ≥90% amino acid identity over > 90% of the length of the target sequence. ARG were grouped according to their resistance mechanism (Li et al., 2015). The abundance of a gene was weighted by counting the number of reads that align to the gene normalizing by the gene length and the total number of reads aligned to any contig (Karlsson et al., 2012).

2.6. Statistical analysis and biological data visualization

Principal component analysis (PCA) among gene contents was performed in Canoco (v4.5). The differences in the taxonomic and functional profiles of two groups ("Ct": Ct 1–4 and "Anti": Anti 1–4) were calculated in the Statistical Analyses of Metagenomic Profiles (STAMP) software, including two-sided Welch's *t*-test and Fisher's test with a *P*-value < 0.05 (Parks et al., 2014). Enrichment analysis of COG and KEGG pathway were conducted with the OmicShare tools (http://www.omicshare.com/tools). Analysis of KEGG module, mapper and pathway were conducted with online tools (http://www.genome.jp/keeg/). Pearson's correlation was calculated to indicate the association between the ARG-ARG and ARG-microbe pairwise. The composition of detected microbes was visualized on the Krona platform (http://krona.sourceforge.net). To detect the potential hosts of ARG from complex microbiomes, the co-occurrence network of ARG and genus was constructed using similarity correlation-based statistical matrix (Feng et al., 2016). Strong pairwise-correlations (r > 0.8, *P*-value < 0.05) were selected to eliminate the poorly correlated ARG or microbe's links. Profiles of network topology were visualized by Cytoscape (v3.6.1). Above input data were normalized using log-transformation in SPSS (v19.0) when necessary.

3. Results and discussion

3.1. Antibiotic pressure affects the AD process

3.1.1. General AD performance

Tetracyclines and sulfonamides are widely detected in both sewage and sludge from WWTPs because they are two important classes of antibiotic to treat human diseases. This study operated two anaerobic digesters with (-Anti) or without (-Ct) antibiotics addition. Detail performance of two digesters are summarized in Appendix. Generally, during the whole process of operation, pH in Ct and Anti reactors ranged from 7.2 to 7.7 without manual intervention. Total VFA in Ct stabilized at 696 ± 132 mg L^{-1}, while Anti increased by 45% with the value of 1009 ± 168 mg L^{-1}. Averaged methane production in Ct is 453 ± 18 mL g^{-1}VS d^{-1}, while Anti was inhibited by 25% with the value of 342 ± 16 mL g^{-1}VS d^{-1}. In the Ct reactor, the mean concentrations of three tetracyclines (TC, OTC, and CTC) ranged from 37 ± 5 to 614 ± 77 μg kg^{-1} dw. Whereas those of three sulfonamides (STZ, SMX, and SML) ranged from 7 ± 2 to 79 ± 16 μg kg^{-1} dw. Under long-term addition of antibiotics in the Anti reactor, the mean concentrations of three tetracyclines and three sulfonamides ranged from 613 ± 78 to 870 ± 56 μg kg^{-1} dw and from 119 ± 14 to 315 ± 32 μg kg^{-1} dw, respectively. The AD process had a higher removal efficiency for sulfonamides (63–81%) than tetracyclines (15–42%) because sulfonamides are readily biodegradable antibiotics. The higher concentrations of tetracycline residues indicated that they were mainly absorbed by sludge rather than biodegraded (Zhang and Li, 2018).

3.1.2. Global composition of the microbial community structure

Metagenomic sequencing yielded approximately 10 Gb of raw data per sample. The number of predicted genes ranged from 1,026,234 to 1,177,130. To identify the phylogenetic diversity of the samples from the Ct and Anti reactors, global profiles of microbiomes were constructed using the Krona platform. Most sequenced reads belonged to bacteria (95–98%), whereas, archaea, eukaryotes and viruses accounted for only 2–5%, 0.1–0.2%, and 0.04–0.06%, respectively. Details are provided in Appendix.

The bacterial communities in the two reactors had a similar composition from the phylum to species levels. For example, the major species had a similar distribution pattern between the Ct and Anti groups, while the archaeal community were separated by antibiotics (Fig. 1-a). The dominant microbiomes comprised the phyla *Proteobacteria* (38 ± 2%), *Chloroflexi* (18 ± 2%), *Bacteroidetes* (13 ± 1%), *Firmicutes* (7 ± 1%), and *Actinobacteria* (6 ± 1%) (Fig. 1-b), which agreed with the results of a previous investigation based on 21 full-scale anaerobic facilities (Sundberg et al., 2013). Different hydrolytic, acidogenic and fermentative genera belonged to *Bacteroidetes* and *Firmicutes* (Venkiteshwaran et al., 2017).

The phylogenetic composition of methanogens archaea was highly variable between the two reactors. For example, a large proportion of methanogens belonged to the order *Methanosarcinales*, which increased from 39.4 to 47.7% (enriched by 21.1%) in the Anti reactor, while *Methanobacteriales* decreased from 21.6 to 19.5% (depleted by 10.9%, Fig. 1-c). At the genus level, the relative abundances of *Methanothrix*, *Methanothermus*, *Methanobacterium*, *Methanococcoides*, *Methanosarcina*, and *Methanomethylovorans* also displayed significant variations (Fig. 1-d). These results indicate that methanogens, rather than bacterial populations, were strongly affected by long-term antibiotic pressure. A

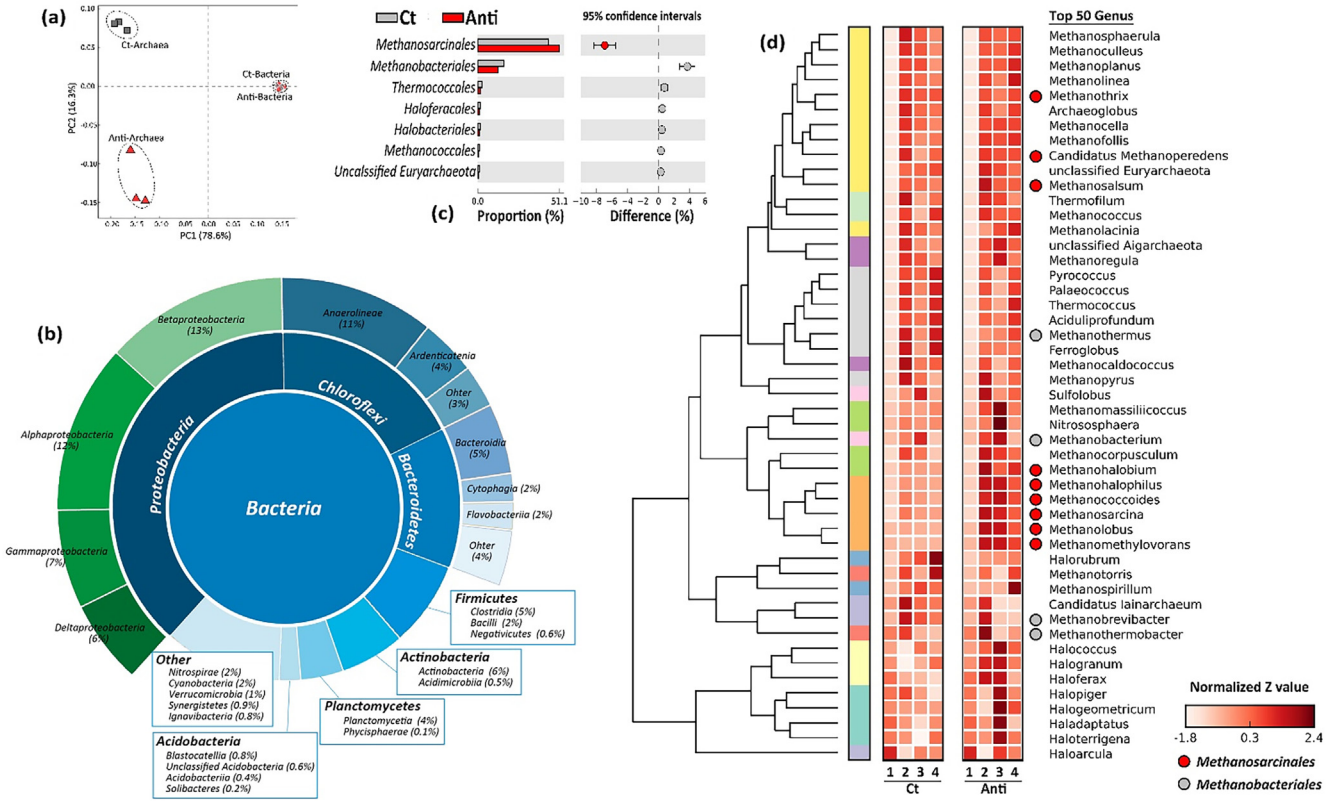

Fig. 1. Profiles of microbial community from Ct and Anti reactors. (a) Distribution of archaeal and bacterial community composition (specie level) by PCA analysis. (b) Similar bacteria compositions (class level) in two reactors. (c) Variation analysis of archaeal community (order level). Bar chart indicates the mean proportion (%) of each order by Welch's-test (two sided). (d) Heatmap reveals the dynamics of the top 50 genera (row) during the AD process (columns). The relative abundance of each genus is normalized by z-value and transferred to color intensity. The sort of rows is clustered using average neighbor method (UPGMA) at the threshold of 0.75. Grey/red dots represent each genus that belonged to the order *Methanobacteriales/Methanosarcinales*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

similar survey reported that the presence of sulfonamide antibiotics strongly inhibited the methanogenesis process, but did not affect acidogenesis or acetogenesis (Hu et al., 2018).

3.1.3. Clusters of gene functional profile

To estimate the conservative functional profile of each sample, the predicted genes were annotated using contigs through analysis of COGs. The abundance of differentially identified COGs was used to construct a purely functional matrix. PCA result indicated that the COGs could be separated into two clusters by antibiotics (Fig. 2-a). Specifically, COG0492, COG1185, COG0285, and COG0820 were enriched in the Ct group, while COG0145, COG5276, COG0599, and COG0575 were enriched in the Anti group. Each could be further assigned to different functional categories to predict differences in metabolic function (Fig. 2-b and c). In the Anti group, the abundance of genes involved in posttranslational modification, protein turnover, and chaperones decreased by 4.3%, while amino acid transport and metabolism increased by 14.8%. Similarly, functional profiles from the Ct and Anti reactors based on KEGG annotation also showed distinct patterns under antibiotic pressure. Only 88.5% of the KO entries were shared between the two reactors. The reduction of differentially identified genes in the Anti reactor mainly encoded nucleotide metabolism, metabolism of cofactors and vitamins, lipid metabolism, carbohydrate metabolism, and energy metabolism (see Appendix).

3.1.4. Metabolic pathway analysis in KEGG

Functional differences at the KEGG pathway level were mapped to reveal how antibiotic pressure affects microbial survival strategies. The details of the assigned genes are summarized in Table 1. Using KEGG functional modules, genes involved in fatty acid degradation, RNA degradation and pyrimidine metabolism were significantly decreased in the Anti group ($P < 0.05$). Genes encoding fatty acid degradation (paaF, echA, fadA, fadI and atoB) mainly participated in the metabolism of coenzyme A (CoA), including the trans-Hexadec-2-enoyl-CoA, (S)-3-Hydroxyhexadecanoyl-CoA, trans-Tetradec-2-enoyl-CoA, 3-Oxohexadecanoyl-CoA, and Acetoacetyl-CoA, Acetyl-CoA, which verified the lower VFA degradation capacity as mentioned above. Since CoA is critical in the synthesis and oxidation of fatty acids in cells (Shi and Tu, 2015).

Simultaneously, in the Anti group, genes associated with sulfur metabolism were increased likely because a large quantity of sulfonamides were introduced, which were easily biodegraded (Zhang and Li, 2018). The abundant sulfur metabolism-coding genes in the Anti reactor indicated that such antibiotics partially promoted the reduction of organic sulfur compounds. These findings might enable a better understanding of sulfonamide biodegradation in the AD process.

3.1.5. Influence mechanism

During the AD process, macromolecules (e.g. carbohydrates, proteins or lipids) are first hydrolyzed and then further degraded by bacteria to generate VFA (e.g. acetic, propionic or butyric acids) in addition to H_2 and CO_2. Methanogenic archaea are then responsible for the conversion of acetate or H_2/CO_2 into methane via the acetoclastic or hydrogenotrophic pathways (Ma et al., 2018; Ye et al., 2017). Generally, antibiotics do not specifically target archaea that harbor ARG, but they could affect the bacterial functions that provide food for archaea. For example, methanogens are affected when hydrolysis, acetogenesis, and acidogenesis are altered. If digesters accumulate a lot of

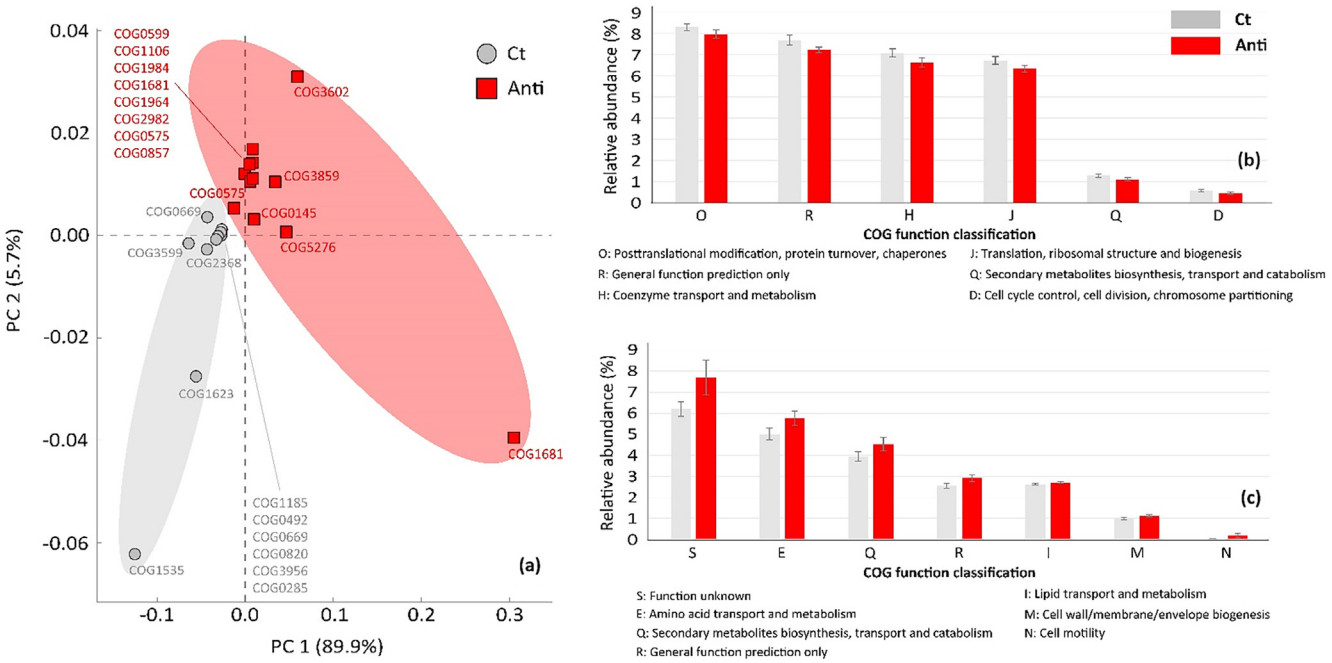

Fig. 2. Profiles of the Clusters of Orthologous Groups of proteins (COGs) in Ct and Anti reactor. (a) PCA shows two distinct clusters of COGs in Ct and Anti. These differentially identified genes can be assigned to different COGs functional categories and enriched in Ct (b) or Anti (c) reactor, respectively.

VFA, methane production will decrease because methanogens are generally sensitive to excessive acidification (Bai et al., 2019; Xu et al., 2018b). Studies have reported that methane production from AD facilities is adversely impacted by high concentrations of antibiotics (ranging from 8 to 100 mg L^{-1}), such as tetracycline, sulfamethazine, thiamphenicol, chlortetracycline, and oxytetracycline. (Álvarez et al., 2010; Beneragama et al., 2013; Hu et al., 2018; Yi et al., 2016). Antibiotics can cause the accumulation of acetogenic substrates, which was also confirmed in this study by the higher VFA concentration and lower methane production in the Anti reactor (Section 3.1). Results indicate that high levels of antibiotic residues in sludge may seriously inhibit the operation of sludge anaerobic treatment.

The impacts of antibiotics on a diverse range of microbial interactions, functional genes, and metabolic pathways were also identified from a metagenomic perspective. For example, decreased methane production might be associated with the low-abundance methanogens, such as *Methanosarcinales* and *Methanobacteriales*, which only contribute 0.5–2% of the total microbial community (Section 3.2). The generalist *Methanosarcinales* can use both acetate and H_2/CO_2. *Methanobacteriales* are only known to perform hydrogenotrophic methanogensis (Xu et al., 2018b). Due to their higher growth rate and lower affinity for acetate, *Methanosarcinales* generally outcompete other strict acetoclastic methanogens (e.g., *Methanosaeta*) in digesters with high acetate concentration (Venkiteshwaran et al., 2017). The enrichment of *Methanosarcinales* in the Anti reactor (by 21.1%) also demonstrated an association between antibiotic pressure and VFA accumulation (by 45%).

Using the differentially identified genes, the methane metabolic pathway was re-constructed to highlight the impacts of antibiotic pressure on the methane generation process (Fig. 3). Methanogenesis is the final step in the generation of methane and determines the methane yield of AD reactors (Xu et al., 2018b). All methanogenesis pathways (KEGG module number, M00567, M00357, M00356, and M00563) were affected by antibiotics. Among them, the hydrogenotrophic (M00567, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) and acetoclastic (M00357, $CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$) methanogenesis pathways significantly changed in samples ($P < 0.05$). Together with the shifts in phylogenetic composition and methanogenesis pathways, this is the first metagenomic-based evidence that microbes can produce energy from fermentation substrates via different routes under antibiotic pressure, further lowering methane production in the Anti reactors.

It should be noted that the bacterial compositions (i.e., the dominant phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*) in the Ct and Anti reactors were remarkably similar throughout the AD process (Fig. 1-a and b). However, the abundances of according to COG and KEGG entries clearly varied between the two reactors, implying that metagenomic indicators from the gene markers were more robust than the taxonomies. In addition, microbes in the AD system experienced

Table 1
Changes of KEGG pathway in Anti reactor.

KEGG pathway	Involved genes	Definition	Under antibiotic pressure in Anti
Fatty acid degradation	*paaF, echA, fadA, fadI, atoB*	Enoyl-CoA hydratase, Acetyl-CoA acyltransferase, Acetyl-CoA C-acetyltransferase	Decreased
RNA degradation	*pnp*	Polyribonucleotide nucleotidyltransferase	Decreased
Pyrimidine metabolism	*pnp, trxB*	Polyribonucleotide nucleotidyltransferase, Thioredoxin reductase (NADPH)	Decreased
Purine metabolism	*rdgB*	XTP/dITP diphosphohydrolase	Decreased
Sulfur metabolism	*tauD, cysH*	Taurine dioxygenase, Phosphoadenosine phosphosulfate reductase	Increased
Phosphonate and phosphinate metabolism	*phnA*	Phosphonoacetate hydrolase	Increased
Nonribosomal peptide structures	*tycC, srfAA*	Tyrocidine synthetase III, Surfactin family lipopeptide synthetase A	Increased

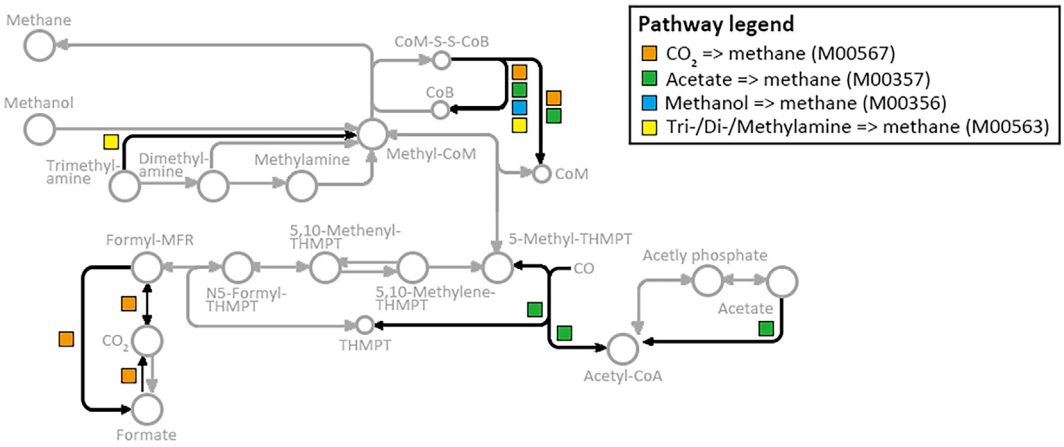

Fig. 3. Variations of identified genes involved in methanogenesis pathways from Anti reactor using the re-construction of KEGG modules. Circles represent the metabolic product. Grey lines represent the potential pathways from KEGG modules. Black lines represent the detected pathways, which are influenced by the long-term antibiotics pressure in this study. Colorful squares represent different methanogenesis pathways.

selective pressure from various competitors in the AD process, which led to some specific groups being abundant and many more being rare (known as the "long-tail" effect, Fig. 1-c). The findings suggested that the abundant groups could not represent the entire functional complexity within AD microbes. Further metagenomics studies are needed to clarify the diversity of functions and unveil the genomic potential of low-abundance microbes.

3.2. Antibiotic pressure increase the risk of AMR

3.2.1. Dynamics of critical pathogens

Sewage sludge is recognized as a hotspot for ARG-exchange events via bioaccumulation or for boosting the growth of multi-drug resistant bacteria (Bondarczuk et al., 2016; Feng et al., 2016). It has been documented that antibiotics adsorbed to sludge are more stable than those found in wastewater (Bondarczuk et al., 2016). Findings further suggest that AMR could be propagated when sludge is exposed to the long-term effects of antibiotics through the AD process. The high concentrations of antibiotics and microorganism density in sludge enable microbes to acquire drug resistance (Yi et al., 2016). When searching the priority pathogens list for ARB according to the latest World Health Organization report (WHO, 2017), twelve potential pathogens belonging to three priorities (critical, high and medium) were detectable (Table 2). *Pseudomonas aeruginosa* and *Enterobacteriaceae* accounted for 1–2% of *Gammaproteobacteria*. 6 critical potential pathogens were enriched by 4.8–34.9% under long-term antibiotic pressure, including *Acinetobacter baumannii*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Only five potential pathogens could be effectively reduced by 2.5–25.1% within the AD process. Specifically, *Acinetobacter* were critical members that shaped the ARG distribution in sludge samples (Yang et al., 2014). These findings increase the urgency for the development of effective sludge treatments.

3.2.2. Abundance and variation of ARG

Most studies have used quantitative PCR (qPCR) and microarray-based techniques (e.g., GeoChip) to investigate ARG profiles in complex samples (Yi et al., 2016; Zhou et al., 2015). However, few ARG (mainly tetracycline and sulfonamide resistant ARG) could be detected via such PCR-based approaches, due to the limited primer design among the highly diverse ARG (Gao et al., 2012). This study found a diverse range of genotypes of environmental ARG through the wide-spectrum detection using a metagenomics approach. Metagenome sequences were searched against CARD to determine the ARG profiles. More than 1000 known subtypes can potentially confer resistance to 25 classes of antibiotics (out of 28) and were widely detected in samples (see Appendix).

Table 2
Detection of 12 critical antibiotic-resistant pathogens in this study.[a]

Pathogens ID	Enriched rate under antibiotics pressure	Removed rate after AD
Level 1-Critical		
Acinetobacter baumannii	13.0%	N.A.
Pseudomonas aeruginosa	N.A.	8.4%
Enterobacteriaceae	N.A.	2.5%
Level 2-High		
Enterococcus faecium	N.A.	N.A.
Staphylococcus aureus	34.9%	5.7%
Helicobacter pylori	6.5%	13.8%
Campylobacter	N.A.	N.A.
Salmonellae spp.	4.8%	N.A.
Neisseria gonorrhoeae	N.A.	N.A.
Level 3-Medium		
Streptococcus pneumoniae	32.1%	N.A.
Haemophilus influenzae	12.6%	25.1%
Shigella spp.	N.A.	N.A.

N.A.: Not enriched or removed.
[a] According to the "Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics" by World Health Organization (2017).

The most frequently detected ARG were macrolide (28%), tetracycline (24%), fluoroquinolone (20%), and peptide antibiotic resistance (20%). Sulfonamide ARG only accounted for a small proportion of the total in both reactors (2%) (Fig. 4-a). A Venn diagram showed that 870 subtypes of ARG were simultaneously observed between the two reactors. There were 129 new ARG subtypes that emerged in the Anti reactor, indicating that antibiotic pressure increased the diversity of ARG (Fig. 4-b). WWTPs receive sewage from hospitals or households where many antibiotics are applied and contain a large amount of human feces. A previous survey found that tetracycline and macrolide ARG were the major components found in the human gut (Hu et al., 2013). This result suggests a strong association in ARG abundance between human gut microbiota (source) and sludge (terminal).

Levels of many ARG types/subtypes were also significantly higher in the Anti than the Ct reactor ($P < 0.05$) when exposed to long-term antibiotic pressure (Fig. 4-c, d, e and f), such as the genes conferring resistance to macrolide (*clbB*), macrolide/tetracycline (*muxA*), tetracycline (*tetX*), and aminocoumarin (*mdtC*). Studies have suggested that high concentrations of tetracycline and sulfonamide antibiotics could increase the development and transmission of several corresponding ARG, such as *tetA*, *tetW*, and *sul1* (Feng et al., 2016; Guo et al., 2017; Li et al., 2015). The findings of this study provide evidence that several

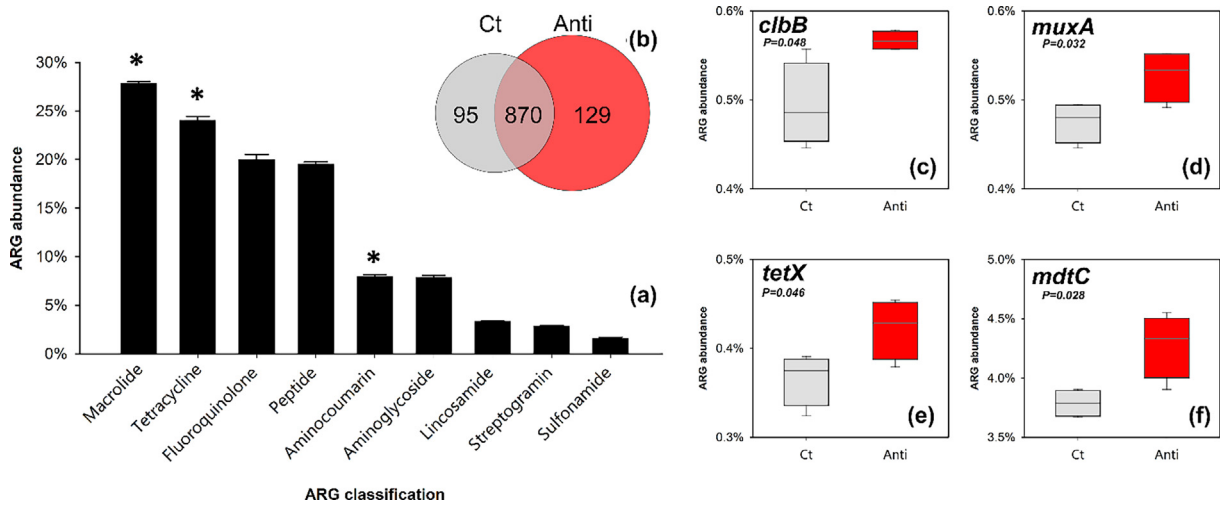

Fig. 4. Profiles of ARG classes. (a) Bar chart shows the relative abundance of dominant ARGs in two reactors. (b) Venn analysis reveals the number of overlapping and distinct ARG subtypes. (c)–(f) shows the enriched ARGs under antibiotics pressure.

genes encoding tetracycline resistance (e.g. *muxA* and *tetX*) tend to be directly enriched under long-term antibiotic pressure. It is speculated that bacteria residing in sludge may be intrinsically resistant to these antibiotics and confer antibiotic resistance through clinically relevant mechanisms (Bondarczuk et al., 2016).

In addition, this study revealed the co-occurrence of ARG among different antibiotic categories. For example, the abundances of some macrolide and aminocoumarin resistance genes (e.g. *clbB* and *mdtC*) increased simultaneously. This result indicated that antibiotic pressure (tetracycline and sulfonamide) not only led to an increase in specific ARG associated with these antibiotic categories (tetracycline or sulfonamide), but also simultaneously enriched the ARG with other antibiotic categories (e.g., macrolide or aminocoumarin resistance) (Fig. 4). The development of cross-resistance, whereby resistance to one stressor results in resistance to another stressor (Zhu et al., 2017), may also stimulated by tetracycline or sulfonamide antibiotics. This was likely because the same mobile genetic elements (MGEs, including integrons, transposons, plasmids, and combinations of them) carried multiple ARG related to different antibiotic residue categories. For instance, *tetW* gene has been identified in *Streptomycetes* and was determined to be potentially transferred into other microbes with conjugative transposons (Gao et al., 2012). The *sul1* gene was reported to be associated with sulfonamide resistance bacteria. But clinical treatment with sulfonamides for methicillin resistant infections was used (O'Neill, 2014). Co-occurrence patterns of different ARG classes from this study was also supported by other studies of WWTP effluent, primary sludge, and landfill leachate (Bondarczuk et al., 2016; Feng et al., 2016).

3.2.3. Association between ARG and potential hosts

The ARG profiles identified in sludge samples prompted us to detect the main factors affecting their distributions and predict the dissemination of the resistome in the future. Microbial community structure was a critical determinant of ARG in sludge. Microorganisms are not only important sources of antibiotics but are also hosts to many ARG (Forsberg et al., 2014). Microorganisms can excrete specialized antibiotics or their derivatives under natural conditions, which may result in changes to ARG (Forsberg and Dantas, 2012). In this study, not only the diverse range of ARG, but also the potential hosts could be detected simultaneously via the metagenomic approach. To highlight possible co-occurrence patterns within microbial consortiums and ARG under antibiotic pressure, this study screened the differentially enriched strains and subsequently associated them with the resistance genes of 11 and 124 subtypes that conferred to resistance to sulfonamides and tetracyclines, respectively (Fig. 5). Pearson's correlation analysis revealed associations in diversity between ARG and their potential hosts. The tetracycline resistance genes had a more intensive interaction with hosts. A total of 135 edges and 92 nodes were detected in the tetracycline resistance network, while only 43 edges and 40 nodes were determined in the sulfonamide resistance network. Considering the higher tetracyclines residuals and higher relative abundance of tetracycline resistance genes, there was a possible association among antibiotic concentrations, ARG abundances, and microbial interaction.

All ARG–host linkages could be separated into two distinct groups according to the abundance patterns of their potential hosts: (i) ARG prevalent with a higher relative abundance in the Anti reactor including eight subtypes conferring tetracycline resistance (e.g., *emrK*, *smeE*, *tetX*, *mexL*, *muxA*, *evgA*, *tap*, *marA*) and one subtype conferring sulfonamide resistance (*sul3*); and (ii) ARG that decreased in the Anti reactor and increased in the Ct reactor (e.g., *tetB*, *tet32*, *adel*, *mexA*, *mexB*, *cpxR*).

When searching the nodes of potential host, it is found that tetracycline resistance genes were strongly connected with *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, and *Gammaproteobacteria* (alpha–beta order). Sulfonamide resistance genes were mainly connected with *Actinobacteria* and *Gammaproteobacteria*. In a soil survey, *Actinobacteria* and *Proteobacteria* were the most abundant potential hosts of multi-drug resistance genes (D'Costa et al., 2006; Forsberg et al., 2014), which was also confirmed by previous results (Fig. 5). Notably, the metagenomic data suggested that many hosts with resistance to sulfonamides were significantly enriched in the Anti reactor, although no sulfonamide ARG were identified as being directly enriched (Fig. 4).

Furthermore, many hosts belonging to human bacterial pathogens that could be enriched by antibiotic pressure including *Pseudomonas*, *Enterobacter*, *Streptomyces*, and *Mycobacterium*. This study also detected possible multi-drug resistance in native environmental groups, such as *Ruminococcus*, which is a typical cellulose digester under anaerobic conditions and potentially carries three ARG subtypes (*adel*, *otrC*, *opmE*). The presence of these environmental ARB may be explained by (1) possible HGT between environmental bacteria and pathogenic bacteria and (2) antibiotic selection pressures that propagate environmental ARB (Guo et al., 2017). This study provides evidence of the co-occurrence of potential pathogens and environmental bacteria under antibiotic pressure. Previous studies have indicated that environmental factors (e.g., the antibiotic concentrations, hydraulic retention time, operation temperature, sludge concentration, heavy metals, and MGE) may impact the microbial community by selecting for ARB and further enhance the HGT of ARG (Guo et al., 2017). Many environmental ARG have identical sequence identities to those in human pathogens from feces, soil, or sediment samples (Forsberg and Dantas, 2012). Studies

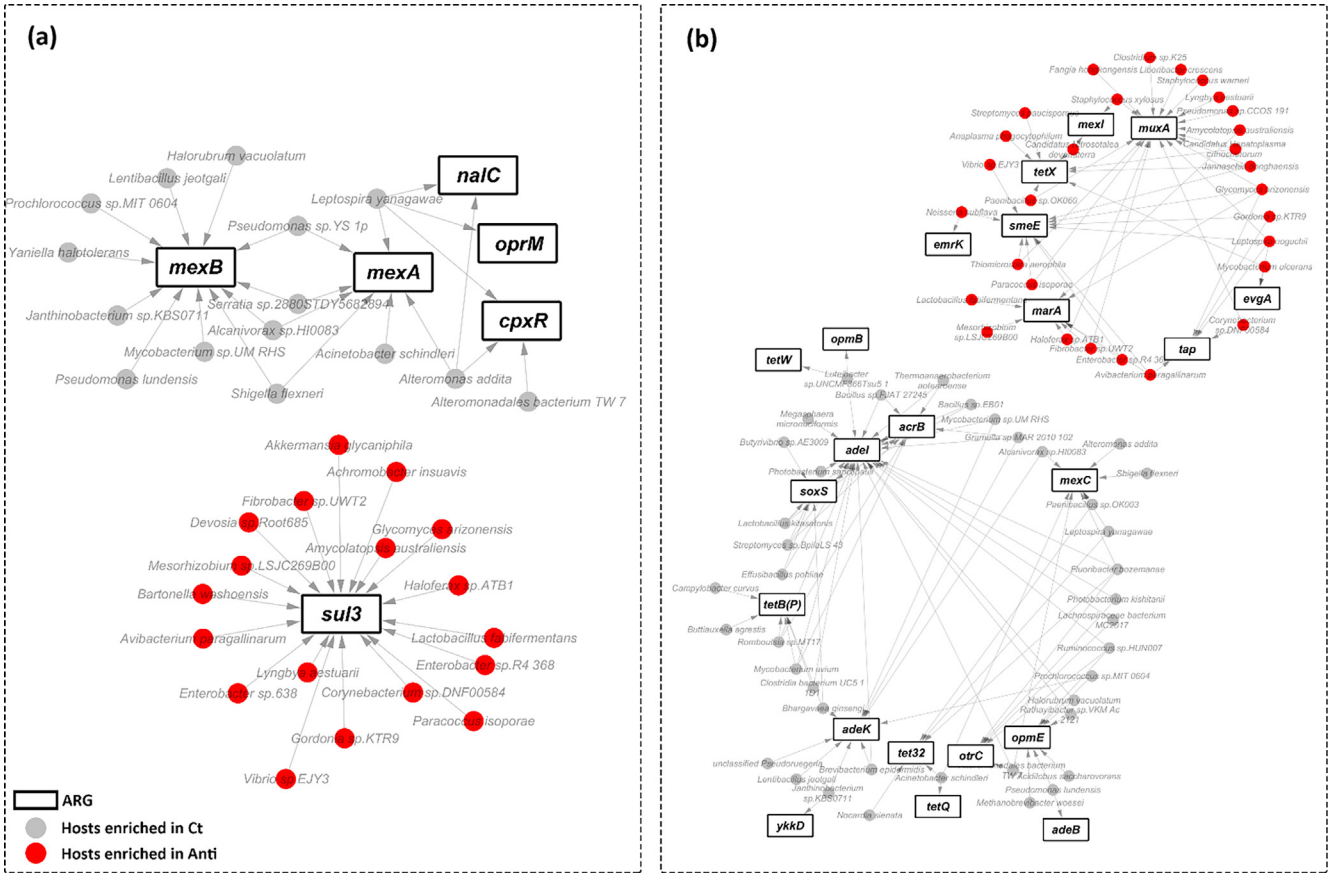

Fig. 5. Co-occurrence networks within (a) sulfonamide ARGs and (b) tetracycline ARGs and their potential hosts. The arrow indicates a strong association ($r > 0.8$, $P < 0.05$). Squares and circles represented ARG classes and hosts, respectively. For interpretation of the high-resolution figure, the reader is referred to the web version of this article.

suggest potential gene flow within clinical and environmental resistomes (Forsberg and Dantas, 2012; Forsberg et al., 2014). Knowledge of non-pathogenic environmental microbes within AD ecosystems, which could carry or spread ARG, is limited (Guo et al., 2017). Therefore, more studies are needed to evaluate the underlying risks of such non-pathogenic environmental microbes, especially under high antibiotic pressure. The density of microbes in sludge may provide an ideal setting for the HGT of ARG among different groups via MGEs (Guo et al., 2017; Yang et al., 2014). As a result, more multidrug-resistant bacteria have emerged and there are now superbugs that do not respond to any drugs (O'Neill, 2014). The potential threat of propagation of multidrug-resistant bacteria may be a major concern for the operation of AD processes.

3.2.4. Mechanisms of bacterial resistance

Most known antibiotics are produced by specific species in natural settings. Bacteria usually use these compounds as weapons to compete for valuable resources or as signaling molecules to communicate with other species (D'Costa et al., 2006). In the AD process, many selection pressures (e.g., high antibiotic concentrations derived from sludge and long-term residues of indigenous ARG) may also contribute to the development of AMR. Although this study revealed the taxonomic composition and broad-spectrum of ARG, their correlations were complex. Hence, this work re-constructed the bacterial chemotaxis pathway to assess the possible mechanisms of bacterial resistance (Fig. 6). The metagenomic data revealed that many KOs related to the bacterial chemotaxis pathway (e.g., *CheA*, *CheB*, *CheC*, and *CheD*) were significantly higher (3–133%) in the Anti reactor. These abundant KOs mainly participated in signal recognition and transduction (*MCP* and *CheD*), adaptation (*CheR*, *CheB* and *CheV*), excitation (*CheA*, *CheW* and *CheY*), and signal removal (*CheC* and *CheZ*). Similarly, Li et al. found that *CheA*, *CheR*, and *CheW* were involved in a chlortetracycline-resistant strain (Li et al., 2018). The abundance of genes in the chemotaxis pathway helped clarify the microbial resistance mechanism under long-term antibiotic pressure. Bacterial chemotaxis is usually considered to be the response of cells to environmental parameters enabling them to move toward more favorable conditions.

The chemotaxis mechanism has been identified in many organisms, and the core mechanisms are conserved across all archaea and bacteria (Szurmant and Ordal, 2004). Among them, two core KOs are most important: (i) events at the receptors determine the autophosphorylation of the sensor kinase *CheA*; and (ii) the chemotaxis protein *CheY* regulates the transfer of the phosphoryl to a conserved aspartate, using phosphohistidine as the substrate. The resulting *CheY*-P (dephosphorylation) in the motor can interact with the switch mechanism. This process usually leads to a change in behavior, such as the direction or rotational speed of flagella (Szurmant and Ordal, 2004). Meanwhile, bacteria could use a quorum-sensing mechanism to control gene expression according to the inherent cell density and adjust their behavior pattern of antibiotic resistance. Chemotaxis gives species with an extra pattern-formation mechanism to increase their density, thus reaching a threshold level of quorum sensing molecules within the cell (Wong-Ng et al., 2018).

3.3. Perspectives

The possible linkages among sewage sludge, the fate of ARG and ARB, and the global human and ecological risks introduced during the AD process should be carefully evaluated. Despite the significant impacts of antibiotic pressure on AD-related microbes and ARM risks

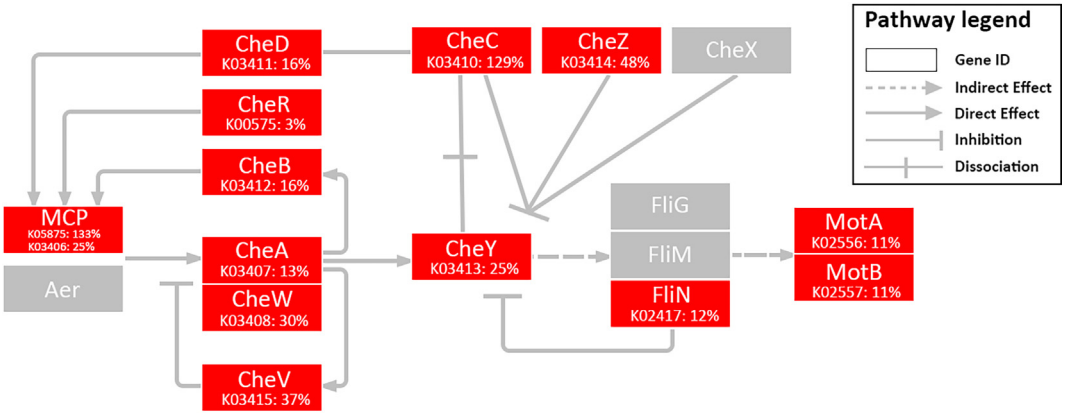

Fig. 6. Re-construction of the bacterial chemotaxis process in Anti reactor. Grey squares indicate the potential regulators from the entire pathway module. Red squares indicate the major regulators, which are significantly increased in Anti reactor ($P < 0.05$). The representative KOs and their enrichment rate (%) are also shown. (CheA: sensor kinase; CheB: protein-glutamate methylesterase/glutaminase; CheR: chemotaxis protein methyltransferase; CheW: purine-binding chemotaxis protein; MCP: methyl-accepting chemotaxis protein; FliN: flagellar motor switch protein; CheC, CheD, CheV, CheY, CheZ, MotA, and MotB: chemotaxis proteins). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

identified in this study, many other drivers are also important, including temperature, pH, substrates, and reactor's configuration. These environmental factors are closely linked to the distribution of the microbial community and functional genes across different digesters. To better study the patterns of AMR derived from AD systems, it is necessary to assess additional operational conditions to better characterize the relationship between microbes and ARG. Additionally, the metagenomic approach demands improvements in data management, analysis, and accessibility. Several resistance gene databases in the AMR field have been proposed, providing details of antimicrobial compounds, as well as many genes that encode regulators of resistance phenotypes, drug targets, and transporters (Jia et al., 2017). However, understanding the taxonomic origins of ARG that underlie this category is a major challenge. There is a need to develop a new framework to annotate antibiotic resistance sequences, which could determine the pathways and origins of ARG by linking them with microbial community composition. More importantly, the identified ARG indicate the potential resistance to antibiotics. However, the potential HGT of ARG in the AD process has not been clearly demonstrated. Therefore, simultaneous characterization of ARG and MGE (e.g., integrons, plasmids, and transposons) in sludge warrants further study, which could provide a full profile of the fate of ARG in AD systems and offer an important proxy for ARG being transferred horizontally to other pathogens or environmental bacteria (Zhu et al., 2017).

It is highlighted that the effluent discharged from AD process contained many ARG that could possibly enter the downstream environment via gene flows. The widely existed ARG may occur at levels of clinical significance if they are harbored by human bacterial pathogens. Therefore, eliminating the spread of ARG and ARB should be a new target for biological treatment processes. Several studies have investigated the role of sludge AD in reducing ARG. However, many studies have reported that traditional AD technology has mixed outcomes in terms of the elimination of ARG and ARB. Some ARG can be reduced, but many ARG have also emerged or increased after AD (Diehl and Lapara, 2010; Guo et al., 2017; Xu et al., 2018a). Findings support that the continuous input of antibiotics from WWTPs is a critical factor that propagate the ARG and ARB from digested sludge.

4. Conclusions

Long-term selective pressure by addition of a mixture of tetracycline and sulfonamides within an anaerobic digester lead to the accumulation of VFA and reduction in methane production. Antibiotics affected archaeal composition, hydrogenotrophic and acetoclastic methanogenesis pathways and lipid/carbohydrate/fatty acid metabolism. Positive association were found among the residual tetracycline concentrations, corresponding ARG abundances, and microbial interactions. A broad profile of ARG were identified from sludge samples. Antibiotic pressure further increased the diversity and abundance of ARG subtypes, increased the microbes able to perform chemotaxis, propagated the potential hosts, and promoted the interactions of ARB and indigenous environmental bacteria.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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