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Effect of reactor operation modes on mitigating antibiotic resistance genes (ARGs) and methane production from hydrothermally-pretreated pig manure

Chunchun Guo [a,1], Shupeng Lin[a,1], Tao Lyu[b], Yanfang Ma[a], Renjie Dong[a], Shan Liu[a,c,*]

[a] *College of Engineering, China Agricultural University, Beijing 100083, P. R. China*

[b] *School of Water, Energy and Environment, Cranfield University, College Road, Cranfield, Bedfordshire MK43 0AL, United Kingdom*

[c] *Yantai Research Institute, China Agricultural University, Yantai, 264670, P. R. China*

* Corresponding author. liu610shan@cau.edu.cn (S. Liu).

[1] Chunchun Guo and Shupeng Lin contributed equally to this work.

Abstract

Numerous efforts have been made to enhance the performance of anaerobic digestion (AD) for accelerating renewable energy generation, however, it remains unclear whether the intensified measures could enhance the proliferation and transmissions of antibiotic resistance genes (ARGs) in the system. This study assessed the impact of an innovative pig manure AD process, which includes hydrothermal pretreatment (HTP) and a two-stage configuration with separated acidogenic and methanogenic phases, on biomethane (CH_4) production and ARGs dynamics. Results showed that HTP significantly increase CH_4 production from 0.65 and 0.75 L/L/d in conventional single-stage AD to 0.82 and 0.91 L/L/d in two-stage AD. This improvement correlated with a rise in the relative abundance of *Methanosarcina*, a key methanogenesis microorganism. In the two-stage AD, the methanogenic stage offered an ideal environment for methanogens growth, resulting in substantially faster and higher CH_4 production by about 10% compared to single-stage AD. Overall, the combined use of HTP and the two-stage AD configuration enhanced CH_4 production by 40% compared to traditional single-stage AD. The abundance and diversity of ARGs were significantly reduced in the acidogenic reactors after HTP. However, the ARGs levels increased by about two times in the following methanogenesis stage and reached similar or higher levels than in single stage AD. The *erm(F)*, *erm(G)*, *ant(6)-Ia*, *tet(W)*, *mef(A)* and *erm(B)* were the six main ARGs with significant differences in relative abundances in various treatments. The two-stage AD mode could better remove *sul2*, but it also had a

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rebound which elevated the risk of ARGs to the environment and human health. Network analysis identified pH and TVFAs as critical factors driving microbial communities and ARG proliferation in the new AD process. With the results, this study offers valuable insights into the trade-offs between AD performance enhancement and ARG-related risks, pinpointing essential areas for future research and practical improvements.

Keywords: Antimicrobial resistance (AMR); livestock manure; metagenomics investigation; sustainable biowaste treatment; two-stage anaerobic digestion

1. Introduction

Anaerobic digestion (AD) has emerged as an important technology for sustainable management of various organic wastes, sourcing from sewage treatment sludge, agricultural crop residues, and municipal biomass waste (Gurmessa et al., 2020). The generation of renewable energy in the form of biogas or even (bio)hydrogen has contributed to the substitution of conventional fossil fuels and has supported global net-zero emission targets (Ni et al., 2017; Zhao et al., 2023). In addition to the bioenergy recovery, anaerobic digestate can also serve as a nutrient-rich biofertilizer with numerous readily available nutrients (Kizito et al., 2017; Li et al., 2021). Large-scale AD applications have been implemented worldwide, however, continued efforts are essential to improve AD performance and reduce risks associated with the operation and resource re-utilisation (García-Depraect et al., 2022).

Mesophilic AD (30-40 °C) is more common in large-scale applications, compared to thermophilic AD (50-60 °C), as it enables greater microbial diversity and community stability, system resilience, and less energy consumption (Flores-Orozco et al., 2022). Nevertheless, appropriate pretreatment and operational adaptations are important to overcome challenges related to relatively slower biogas yields and organic biodegradation rates. Hydrothermal pretreatment (HTP) has proven to be a promising approach for accelerating the rate-limiting hydrolysis process in AD by decomposing complex substances into smaller molecules (Zhang et al., 2021). Previous

investigations have primarily focused on HTP for lignocellulosic biomass, e.g., corn straw and rice straw (Liu et al., 2022), but whether it is necessary and worthwhile to have HTP for animal manure-based AD have not been sufficiently studied. Additionally, two-stage AD configurations, involving separate acidogenic and methanogenic reactors, have been developed to optimise grow conditions for methane-producing archaea in the methanogenic stage and benefit CH_4 production (García-Depraect et al., 2022). However, the extent to which the integration of HTP and two-stage AD flowsheets could improve the AD performances and the associated mechanisms are not yet clear.

Along with the wide acceptance of using AD for bioresource recover, the risks associated with both chemical and microbiological micropollutants in AD digestate residues have garnered significant attention (Zubair et al., 2023). The extensive use of antibiotics in livestock breading has resulted in 50%–95% of these compounds being present in manure (Zhi et al., 2021). The biological treatment of manure, either through aerobic or anaerobic processes, could trigger the generation and proliferation of antimicrobial resistance (AMR), including both antibiotic resistance genes (ARGs) and antibiotic resistance bacteria (ARB) (Bai et al., 2022; Zubair et al., 2023). HTP has been demonstrated to decrease the abundance of ARGs during the AD of municipal waste-activated sludge (Sun et al., 2022). Similarly, many other efforts are being made to reduce ARGs in livestock manure during AD, with most studies focusing on traditional single-stage AD systems rather than updated two-stage AD systems. Therefore, understanding whether the optimised AD flowsheet designed specifically to benefit bioenergy recovery from livestock manure could affect the fate of ARGs is crucial for providing evidence-based practices.

The AD process, including both CH_4 production and the proliferation of ARGs, involves complex microbial-mediated processes driven by environmental and operational conditions (Yadav et al., 2022). The prior HTP of livestock feedstock could potentially enhance the breakdown of organic structures, including the antibiotics (Paranhos et al., 2023). Moreover, acidogenic bacteria have been shown to harbour more ARGs

compared to methanogenic archaea in single-stage AD (Flores-Orozco et al., 2022). In a two-stage AD system, acidogenesis and methanogenesis are separated into two compartments, reducing their interactions. The overall microbial community structure and characteristics, which may significantly affect the fate of ARGs, are hypothesized to be significantly altered.

Giving those aforementioned gaps in research, this study examined the potential trade-offs of enhanced mesophilic AD of pig manure, through HTP and two-stage AD flowsheets, and the mitigation of ARGs. The productions of biogas and CH_4 were quantified in the optimised flowsheet compared to traditional single-stage AD and systems without HTP. Metagenomic analysis was conducted to investigate how HTP and AD configurations affect ARG fate and microbial community dynamics. Moreover, network analysis was conducted to explore key factors affecting AGRs and microbial communities. With these results, this study aims to provide new insights for upgrading AD technology for bioresources recovery while minimizing environmental risks from ARGs.

2. Materials and Methods

2.1. Substrate and inoculum

In the study, raw pig manure (PM) was collected in Changping Base of Animal Husbandry Research Institute, Chinese Academy of Agricultural Sciences. No antibiotics were applied in the pig breeding process, and the typical nine types of antibiotics (i.e., tetracycline, chlortetracycline, oxytetracycline, sulfadiazine, sulfamethoxazole, sulfamethoxazole, ofloxacin, norfloxacin, and ciprofloxacin) were not detected in the raw PM before the experiment. Therefore, sulfadiazine (SDZ), as the target antibiotic, was added to the PM in this study with an initial concentration of 75 mg/L. The initial feedstock of pig manure had a total solid (TS) of 21.6±0.1% and a volatile solid (VS) of 17.0±0.1%. The ammonium nitrogen (NH_4^+-N) content was 2.5 ± 0.1 g/L, and the soluble chemical oxygen demand (SCOD) was 64.1 ± 0.4 g/L. The inoculum sludge used in the AD reactors was collected from a stable mesophilic AD

reactor for wastewater treatment at Beijing Drainage Group. The TS and VS in the sludge were 6.3±0.2% and 3.2±0.1%, respectively. The sludge was acclimated at 37 °C for 1 week before being added to the different AD reactors to enrich the microbes.

2.2. Experimental setup and operations

Six continuously stirred tank reactors (CSTRs) were used in this study, each equipped with motor-driven stainless-steel shafts and paddles (Fig. 1). Four different processes were conducted in parallel. 1) A single-stage mesophilic AD system using PM as the feedstock with a reactor labelled R0. 2) A two-stage mesophilic AD system with PM as the feedstock, consisting of a hydrolysis acidification reactor labelled Ra and a methanogenesis reactor labelled Rm. 3) A single-stage mesophilic AD system using PM after HTP as the feedstock with a reactor labelled R0h. 4) A two-stage mesophilic AD system followed by HTP, consisting of a hydrolysis acidification reactor labelled Rah and a methanogenesis reactor labelled Rmh. The HTP was operated at 180 °C for 30 min in a stainless-steel reactor with heat-conducting oil as the medium.

For both one-stage CSTRs (R0 and R0h), a working volume of 5-L was prepared. They were operated continuously for 80 days, maintaining a fixed hydraulic retention time (HRT) of 25 days with organic load rate (OLR) of 3.5 gVS/L/d. The two-stage CSTR groups were operated continuously in a 7-L overall working system for 80 days. HRT of the acidogenic reactor in the 2-L working system was 5 days, and the subsequent methanogenic reactor in the 5-L working system had an HRT of 20 days. The OLR of the acidogenic reactor and acidogenic reactor was 3.5 and 17.0 gVS/L/d, respectively. Consequently, the overall HRT in both two-stage ADs was also 25 days.

Throughout the experiment, all six reactors were incubated within a 37 ± 1 °C water bath and stirred automatically at a speed of 180 r/min per day. Manual feeding of the reactors was carried out once daily when effluent was discharged. Reactors R0 and Ra were fed with PM, while reactors R0h and Rah were fed with PM after HTP. The methanogenic reactor was initiated, followed by the acidogenic reactor, each running one HRT in the two-stage AD system. No pH adjustments were completed, and no exogenous chemicals were added during AD process. Biogas was collected using an

airbag to measure the gas volume and gas composition every day. Effluents were collected in the reactors at 2-4-day intervals for the detection of pH, TS, VS, VFAs, and NH_4^+-N.

2.3. Chemical analytical methods

The wet gas meter (LML-1, China) was utilised for testing biogas volume daily. Meanwhile, a gas chromatograph (Shimadzu GC-8A, Japan) was used to analysis the gas composition in biogas gas (N_2, CH_4, CO_2). TS, VS, SCOD, and NH_4^+-N were determined following the procedures outlined by the American Public Health Association (Walter, 1961). Another gas chromatograph (GC-2010 Plus, Japan) was used to determine the concentrations of individual VFAs, such as acetic acid, butyric acid, caproic acid, isobutyric acid, isovaleric acid, propionic acid, and valeric acid, following the methods developed by Jiang et al. (2020). The individual VFA concentrations were then converted into acetic acid equivalent, while the total VFAs content was calculated as the combined concentration of individual VFAs (Lin et al., 2022).

2.4. DNA extraction and metagenomic analyses

Triplicate samples from the original pig manure feeding stock, inoculum sludge, and six AD reactors were collected during the stabilisation period (day 60) to process the metagenomic analysis. The FastDNA Spin Kit for Soil (MP Biomedicals, USA) was used to extract total genomic DNA following specific instructions. The NEXTFLEX Rapid DNA-Seq kit (Bioo Scientific, Austin, TX, USA) was adopted for the construction of paired-end library. Metagenomic analyses were carried out using Illumina Novaseq 2000 (Illumina Inc., San Diego, CA, USA) with NovaSeq Reagent Kits, following specific protocols (www.illumina.com). The obtained sequences underwent quality control, splicing, assembly, amino-acid sequence translation, non-redundant gene set construction, and abundance calculations, all in accordance with established methods (Chi et al., 2022). Finally, data analysis was conducted using the public Majorbio Cloud Platform (www.majorbio.com). Diamond (http://www.diamondsearch.org/index.php,

version 0.8.35) was applied to compare representative sequences in non-redundant gene sets in the NR database with an e-value ≤$1e^{-5}$ for taxonomic annotations. To compare ARG levels within samples and in the database (ARDB, http://ardb.cbcb.umd.edu/) based on amino-acid sequences in non-redundant gene sets (identity > 70%, e-value ≤ $1e^{-5}$) (Buchfink et al., 2015).

2.5. Energy balance assessment

The theoretical energy balance of reactors was estimated from experimental data. The processing capability of the different AD systems was defined as 1 cubic meter per day, with a work volume of 25 m^3 for R0, R0h, Rm and Rmh reactors and 5 m^3 for Ra and Rah reactors. The energy consumption and generation were calculated following the method proposed by Passos and Ferrer (2014). The input energy includes consumption for feedstock heating, pump operation, reactor mixing, and heat losses. The output energy includes the generated CH_4, and the conversion rate was determined as 90% (Passos and Ferrer, 2014). Finally, result was expressed as energy balance for R0, R0h, Rm and Rmh reactors. The energy balance was calculated as the difference between the energy output and energy input. The parameters used for the energy assessment are summarized in Table S1.

2.6. Statistical analysis

SPSS 18.0 was employed for one-way analysis of variance (ANOVA), with $p < 0.05$ being considered to be of statistical significance. Alpha-diversities, including Chao1 index, Shannon estimator, and Shannoneven index were determined using Mothur (v 1.30.1, available at http://mothur.org/). Beta-diversities, which included principal coordinate analysis (PCoA) and redundancy analysis (RDA), were performed with R software (version 3.4.2) using vegan package. The “pheatmap” package (version 1.0.8) was used to generate heat maps. The Pearson’s rank correlation coefficient (r) showed correlations of environmental factors and main microorganisms, ARGs. Besides, corrplot and ggplot2 packages were employed for constructing correlated graph. This study also determined Pearson’s correlation coefficients ($p < 0.05$) between main ARGs

levels and dominant microbial taxa levels for every sample. The $|r| \geq 0.5$ being considered to be of significant correlation.

3. Results and discussion

3.1. Enhanced methane production

3.1.1. The effects of hydrothermal pretreatment (HTP)

During the stable methane (CH_4) production phase (Fig. 2), the volumetric CH_4 production rate of 0.82 L/L/d in the single-stage reactor with HTP (R0h) was significantly higher than that (0.65 L/L/d) in the single-stage reactor without HTP (R0). A similar trend was obtained for the two-stage reactors, where the HTP approach boosted the CH_4 production from 0.75 L/L/d in the methanogenesis reactor of the two-stage mode without HTP (Rm) to 0.91 L/L/d in the methanogenesis reactor of the two-stage reactor with HTP (Rmh). The VS of the feeding stock is the key factor controlling the overall CH_4 yields in AD (García-Depraect et al., 2022). Attributing to the HTP, the value increased from 192.30 and 212.08 L/gVS/d in R0 and Rm reactors to 242.18 and 260.82 L/gVS/d in R0h and Rmh groups, respectively (Fig. 2). In summary, the HTP approach benefits both volumetric CH_4 production rate and CH_4 yield with approximately a 26% improvement in the single-stage AD reactor. The result was slightly lower but in the similar range of the increment (30%) from a previous AD study after HTP under mesophilic conditions (Liu et al., 2023). Nevertheless, the extent of HTP benefits in two-stage reactors was lower and reached a 21% and 19% improvement in volumetric CH_4 production rate and CH_4 yield, respectively. It may be due to the two-stage AD configuration itself, which could accelerate the CH_4 production (see the section below) and overrode part of the effect from HTP.

The NH_4^+-N contents did not exceed 3.5 g/L in all reactors (Table 1), which has been shown to be an appropriate level that would not affect AD performances at moderate temperatures (Jia et al., 2023). The changes in total solid (TS), volatile solid (VS), and volatile fatty acids (VFAs) contents reflected the degradation and digestion efficiency

of organic matters in AD (Shi et al., 2021). The total VFAs content was about 13% higher in Rah of 17.37 g/L compared with Ra of 15.39 g/L (Table 1). It supported that the HTP could destroy the rigid organic structure and benefit hydrolysis enzymes to perform (Liu et al., 2020). Such differences were not determined in the single-stage ADs (R0 and R0h) as the transformations of organics through acidogenic and methanogenic processes were mixed in one reactor and may affect the determinations of the alternatives. Excess VFAs may lead excessive propionate content causing a slight decline in CH_4 proportion of biogas from 68.87% in Rm to 66.12% in Rmh (Wang et al., 2006). However, due to the HTP, the overall biogas/CH_4 production was still stimulated.

3.1.2. The effects of two-stage anaerobic digestion (AD) configuration

The acidogenesis reactors in both two-stage AD (Ra or Rah) reactors achieved pH values of 5.59-5.98 (Table 1), which were much lower than the preferred pH environment for methanogenesis (Chen et al., 2008). It indicated that the two distinct stages were successfully separated, moreover, this was supported by the ignorable CH_4 yield in the acidogenesis reactors. In the traditional single-stage AD mode, methanogens and acidogenic bacteria were inhibited due to the ill-fitting pH value (Chen et al., 2008). However, the two-stage mode separated acidogenic and methanogenic phases, which could provide better microorganisms living conditions (Zhu et al., 2022). The results from the current study demonstrated that the optimized mode not only reduced the system stabilisation period but also the overall CH_4 yield.

Compared to single-stage AD, where CH_4 production initiated on the 12^{th} day and stabilised after 20 days (Fig. 2a), the two-stage AD systems reduced the stabilisation period to 12 days with the initial CH_4 production on the 2^{th} day (Fig. 2b). At the same time, the TS, VS and VFAs production and consumption by methanogens in the two-stage mode were nearly twice as much as the single-stage mode (Table 1). Although the two-stage operation did not alter the biogas yield, an 8%-10% increment of CH_4 content in the biogas was observed in the two-stage AD. Consequently, the volumetric CH_4 production rate and overall CH_4 yield of the two-stage AD elevated by 8%-16% compared to the one-stage mode and reached the peak in the Rmh reactor (Fig. 2).

3.1.3. The synergistic effect of HTP and two-stage AD

Compared to the traditional single-stage mode, the combination of HTP and the two-stage AD flowsheet triggered a significant enhancement of CH_4 production by 40%, from 0.65 to 0.91 L/L/d. The overall results demonstrated that the optimized flowsheet promoted livestock manure's biodegradation as well as increased CH_4 production without affecting the stability of reactors. Moreover, the energy balance of the proposed flowsheet was evaluated to compare with the traditional single-stage AD towards guiding future implementation (Table S2). These values indicate that the extra energy requirements for the 150 °C HTP two-stage process would be covered by the energy produced from the enhanced CH_4 production, and in addition, there would be a significant energy surplus (529220 kJ/d). Thus, HTP + two-stage mode flowsheet for the AD of pig manure resulted not only in increased organic matter destruction efficiency, but also enhanced CH_4 production that surpasses the extra energy consumption.

3.2. The response of ARGs development under the HTP and two-stage AD operations

Although no antibiotics were applied in the pig breeding process and the typical antibiotics were not detected in the raw PM, the trace level of compounds (ng/L) may still lead significant ARG accumulation through horizontal and vertical gene transfer process (Chakraborty et al., 2023). Therefore, the metagenomics analysis was conducted to evaluate the dynamics of overall ARGs in the system. As significant sulfadiazine was added in the feedstock during the study, further quantitative analysis of sulfadiazine ARGs were carried out to illustrate the mechanisms.

3.2.1. Metagenomics analysis of overall ARGs development

The Chao1 and Shannon index values reflect the richness and diversity of ARGs, respectively. In the single-stage AD system, the abundance and diversity of ARGs with HTP (R0h) were slightly lower than those in the traditional reactor (R0) (Table 2). This aligns with the previous study reporting that HTP effectively reduce ARGs growth in AD (Han et al. 2023). Due to HTP, a significant decrease in the abundance and diversity

of ARGs by 24%-53% was observed in the acidogenesis reactor (Rah) compared to the Ra reactor. Besides the shared 15 ARGs species in all reactors (Fig. 3a), the two-stage systems with HTP showed much fewer specific ARGs (4 species in Rah) compared to the system without HTP (20 species in Ra). Although HTP treatment could promote ARGs removal in acidogenesis stage, the Chao1 values of ARGs elevated again by about two times in the subsequent methanogenesis stage (Rmh). However, the ARGs diversity in terms of Shannon index (Table 2) and ARGs species (Fig. 3a) were not increased along with the abundance.

These results were also supported by the principal coordinate analysis (Fig. 3b), which showed clearly different clusters between reactors without HTP (R0 and Ra) and the reactors with HTP (R0h and Rah). What's more, the methanogenic reactors of two-stage AD (Rm, Rmh) had similar ARGs composition with R0, while ARGs in Ra, Rah were quite different. It may be caused by different treatment modes making changes in the host microbial community (Han et al., 2023).

The *erm(F)*, *erm(B)*, *erm(G)*, *tet(W)*, *ant(6)-Ia*, and *mef(A)*, were the six main ARGs with the highest relative abundances and showed more distinct distributions in different reactors (Fig. 3c and d, $p < 0.05$). The *erm(F)* tends to accumulate in AD due to its recalcitrance under anaerobic conditions (Sui et al., 2016; Wu et al., 2018). For the single-stage AD, compared to the R0 reactor, the relative abundance of *erm(B)* and *erm(G)* in the R0h reactor decreased by 29.50%-49.53% while *erm(F)* increased significantly by 53.75% ($p < 0.05$). For the acidogenesis of the two-stage AD, the relative abundances of *erm(G)* and *erm(F)* in the Rah reactor were increased about 6 to 7 times compared to the Ra reactor ($p<0.01$). The sum of their relative abundances was more than 66% in the Rah reactor after HTP. To the contrary, the relative abundances of *tet(W)* declined by 67.31% from 21.35%-6.98%. The decreased abundance also applied to *ant(6)-Ia*, with a 76.93% reduction in the Rah reactor ($p < 0.01$). The *mef(A)* presents in a variety of Gram-positive and Gram-negative bacteria with the main action mechanism of drug efflux (Suzuki et al., 2022), also declined by 53.48% after HTP in both single and two-stage AD.

In summary, HTP could reduce the proliferation and accumulation of ARGs in the single-stage AD system and the acidogenic stage of the two-stage AD system. In the following methanogenesis stage, the abundance of ARGs, rather than diversity, significantly regrew, posing potential risks.

3.2.2. Quantitative analysis of sulfonamide resistance genes dynamics

It was agreed that SDZ is recalcitrant under traditional mesophilic AD processes, and HTP could benefit the subsequent AD biodegradation of SDZ (Chen et al., 2022). The current study took a step further to evaluate whether the associated ARGs can be simultaneously reduced in the optimized AD configuration. The sulfonamide genes exhibit sulfonamide resistance by encoding dihydropteroate synthease (Adekanmbi et al., 2020), and four sulfonamide ARGs, including *sul1-4*, were identified in all systems (Fig. 4).

The *sul2* disseminates in the environment quickly because it is often situated on mobile genetic elements, such as plasmids (Haenelt et al., 2023), which showed significantly different abundance in the studied systems (p = 0.008335, Fig. 4b). In the single-stage mode, the relative abundance of *sul2* in the reactor (R0h) after HTP was more than four times higher than that in the R0 reactor. It was consistent with Zou et al. (2023) finding that intracellular *sul2* increased in AD system after 160 ℃ HTP treatment. In the two-stage mode with HTP, the relative abundance of *sul2* decreased significantly by more than 90% in the acidogenic stage, with a large increase in the following methanogenic stage (p < 0.05). Such changes were probably associated with diverse high temperature resistance of corresponding resistant bacterial hosts (Awad et al., 2021). Overall, after HTP, the *sul2* abundance in the two-stage AD mode treatment was 66.74% lower than the single-stage mode.

The *sul1*, *sul3*, and *sul4* showed relative abundances of less than 1% in the Rah reactor, possibly due to low pH values (Fig. 4a, c, and d). As suggested by Lin et al. (2020), lowering manure pH value decreased some sulfonamide genes and bacteria with sulfonamide resistance while inhibiting horizontal gene transfer. They rebounded in the methanogenic stage of the two-stage AD mode. Wu et al. (2016) reported that the

sulfonamide genes also increased again in the thermophilic methanogenic reactors, with the highest increase of 1.72 log units. It indicated sulfonamide genes may transfer and proliferate in the methanogenesis. The acidogenic stage exhibited the highest efficiency in reducing sulfonamide genes after HTP. However, the *sul1-4* genes proliferation in the hydrothermal-pretreated methanogenic stage was a cause for concern. The fluctuations in sulfonamide genes abundance could be mainly attributed to the different composition of possible ARGs-containing microbes.

3.3. Microbial community composition in different AD modes

3.3.1. The archaeal community

The archaeal community had critical effects on biogas generation performance in the AD system (Zhi et al., 2021). The phylum *Euryarchaeota* which commonly contained methanogens was the predominant phylum with relative abundance up to 99% in all AD systems (Fig. 5a). *Methanobrevibacter* and *Methanosarcina* were the most dominant genera whose relative abundances had significant different in reactors (p = 0.0054, Fig. S1a). In PM as well as the acidogenesis stage (Ra and Rah), the genus *Methanobrevibacter* accounted for more than 95% of the relative abundance (Fig. 5b). It was reported that *Methanobrevibacter*, abundant in animal intestines and rumens, could use substrates such as hydrogen and carbon dioxide to produce CH_4 and exhibit optimal activity at the pH value of 6.6–7.8 (Kim et al., 2022; Liu and Whitman, 2008). However, the condition of the acidogenic reactors was ill-fitting for *Methanobrevibacter* working which caused no CH_4 production. In contrast, *Methanosarcina*, a versatile methanogen, was dominant in the single-stage mode (R0, R0h) and methanogenesis stage (Rm, Rmh) reactors (94.37%-99.67%). *Methanosarcina* had a strong adaptability which could flexibly change between hydrogenotrophic and acetoclastic methanogenesis in these reactors (Capson-Tojo et al., 2018). Compared to the reactors without HTP (R0, Rm), *Methanosarcina* was slightly elevated by 1%-5% in the R0h and Rmh reactors. These trends in the archaeal community were consistent with the changes in CH_4 production observed earlier which could indicate that HTP could promote CH_4 production by increasing correlative

methanogenic microbial activity.

3.3.2. The bacterial community

Firmicutes and *Bacteroidetes* accounted for main bacterial phyla in reactors (Fig. 5c). *Firmicutes* was significantly dominant in the single-stage system reactors (R0 and R0h) and the methanogenic reactors (Rm and Rmh) with the range of 46.08%–64.00% in relative abundance. Meanwhile, in the acidogenic reactors (Ra and Rah), *Bacteroidetes* had a significant advantage, with a relative abundance of 44.40%–80.94%. They played important roles in rate-limiting step during hydrolysis and acidogenesis. It was reported that *Firmicutes* was important for degrading cellulosic materials to sugars, while *Bacteroidetes* was involved in converting monomeric sugars to acid intermediates that were subsequently converted in acetate for CH_4 production (Arelli et al., 2022). Compared to reactors without HTP (R0, Ra, Rm), the relative abundance of *Firmicutes* had decreased by 24%-126% while *Bacteroidetes* elevated by 21%-62% within all reactors after HTP (R0h, Rah, Rmh). The most significant alterations of *Firmicutes* and *Bacteroidetes* abundances were found in the Ra and Rah reactors, because HTP could promote the sugar production and transformation which occurred during the acidogenic stage (Liu et al., 2020). The action of HTP changed the substrate of microorganisms and then altered their relative abundances.

The microbial composition at the genus level was significantly different ($p < 0.01$, Fig. S1b). Bacteria in the acidogenic reactors of the two-stage system (Ra and Rah) varied from those in other reactors (R0, R0h, Rm, and Rmh). In the Ra and Rah reactors, *Sodaliphilus*, which belonged to *Bacteroidetes*, was prominent at a range of 34.20%–41.21% in relative abundance (Fig. 5d). However, data on the functional studies of *Sodaliphilus* were lacking as it is a newly isolated strain from PM. It was only confirmed that the growth of *Sodaliphilus* was slow under strict anaerobic conditions forming long filament cells (Holman et al., 2022). After HTP, the relative abundance of *Prevotella* which was known to convert various sugars into carboxylates with its moderate saccharolytic potential increased by 27 times in acidogenic reactors (Sakamoto and Ohkuma, 2012). Reportedly, *Prevotella* was significantly positively

related to VFAs concentration while negatively related to the pH value (Liang et al., 2021). Therefore, such elevation of VFAs content and reduction of the pH value in reactors brought by HTP, as previously explained, may have caused the increase in *Prevotella* abundance to promote the yield of AD. *Lactobacillus*, which was related to production of acetic, caproic and butyric acids, disappeared after pretreatment in the Rah reactor. The thermophilic temperature (>70 °C) usually had an inhibitory role which was the major factor leading to reduced *Lactobacillus* abundance after HTP (Shi et al., 2020).

Microbial composition within reactors of the single-stage system (R0 and R0h) and the methanogenic phase of the two-stage system (Rm and Rmh) exhibited some similarities. *Petrimonas* and *Acetivibrio*, which did not appear in the acidogenic phase, were predominant. They were typical acetogenic bacteria that play an important role in inducing butyrate biodegradation into acetate to produce CH_4 (Yi et al., 2020). The relative abundance of *Acetivibrio* did not show a significant change, ranging from 9.46% to 12.81% in the single-stage mode and methanogenesis stage, regardless of HTP involvement. This finding proved that *Acetivibrio* was relatively stable under different conditions caused by pretreatment. For *Petrimonas*, HTP had a significant effect on the single-stage mode with a large increase in relative abundance by 142% from the R0h reactor to the R0 reactor. However, in methanogenic reactors (Rm and Rmh), the relative abundance of *Petrimonas* increased only slightly by HTP. It indicated that HTP mainly acted on the bacterial community in the acidogenic stage. And the separation of reactors could not only provide good growth conditions for the acid-producing bacteria but ensure the stability of the community structure in the methanogenic stage. In summary, the HTP + two-stage mode flowsheet could simultaneously promote the growth of related bacteria and guarantee stable systems operation to increase the production of AD systems.

3.4 Relations of ARGs, environmental factors, with microbial communities

3.4.1 Relations of environmental factors with microbial communities and ARGs

The redundancy analysis and network analysis were conducted to demonstrate the correlations between environmental factors, such as pH, TVFAs, VS, and NH_4^+-N, microbial communities, and ARGs. The results showed that the pH value and TVFAs content exhibited significant correlations with the microbial community, more so than VS and NH_4^+-N content (Fig. 6a). The pH value was found to be negatively associated with the TVFAs content and had positive correlation with NH_4^+-N content. The pH value was positively correlated with *Paenibacillus* and *Acetivibrio* ($|r| > 0.6$, $p < 0.01$) and negatively correlated with *Sodaliphilus* and *Prevotella* ($|r| > 0.5$, $p < 0.05$) (Fig. S2a). *Petrimonas* and *Acetivibrio* had a negative correlation ($|r| > 0.7$, $p < 0.001$) with the TVFAs content. However, *Prevotella* showed significant positively with the TVFAs content ($|r| > 0.9$, $p < 0.001$), consistent with the research of Liang et al. (2021). As mentioned above, the relative abundance of *Prevotella* increased sharply in Rah reactor, causing the accumulation of TVFAs and a decrease in the pH value.

Environmental factors also showed correlations with ARGs during the AD process (Fig. 6b). The TVFAs content and pH value were significantly correlated with ARGs with a negative correlation with each other. The *erm(F)* and *tet(W)* had a positive correlation with the pH value ($|r| > 0.6$, $p < 0.01$). The *erm(B)* and *ant(6)-Ia* were negatively correlated with the TVFAs content ($|r| > 0.6$, $p < 0.01$), while the *lnu(C)* had a positive correlation with TVFAs ($|r| > 0.6$, $p < 0.01$).

The four sulfonamide genes demonstrated a positive correlation with the pH value, especially *sul2*. Chen (2019) reported that pH-limited *intI*1 abundance as well as pH-limited plasmid and extracellular DNA delivery capacities of sulfonamide suppressed horizontal genes transfer in sludge bacteria. But an increased pH level possibly induces SOS response of DNA within certain microbes, which thus elevates specific ARGs abundances (Miller et al., 2014). Based on Song et al. (2017), *sul1* and *sul2* had positive

correlations with pH in AD by adopting three diverse PM proportions in comparison with wheat straw. In this present study, compared with the R0h reactor (pH=7.49), *sul2* and *sul4* abundances within Rah reactor declined by 98% and 100% (pH =5.59), respectively (Figs. 4b and 4d). Thus, in the acidogenic phase, the abundance of sulfonamide genes could be reduced. Acidified manure was found to contain decreased *sul1* and *sul2* abundances at relative and absolute levels compared with those under the neutral pH level through the adjustment of original pH from neutral level (7.4) to acidic level (5.4–4.8) (Lin et al., 2020).

According to the result of two RDA results, changes in the environmental factors via HTP can elevate abundance of *Prevotella* which may cause an increase in CH_4 production. The abundance of ARGs can be reduced by decreasing the pH value, especially sulfonamide resistance genes.

3.4.2 Network analysis between microbial communities and ARGs

Generally, network analysis which identifies possible ARGs hosts at the genus level can be applied in expressing ARGs co-existing with microbes (Guo et al., 2020; Zhi et al., 2021). Nine potential bacterial genera were closely related to the ARGs in the top 20 abundance and sulfonamide genes (Fig. 6c). Specifically, *Lactobacillus* had significant positive correlations with four ARGs, including *tet (40)*, *aad9*, *tet (W)*, and *lnuC* ($|r| > 0.8$ and $p < 0.05$). *Acetivibrio* had great positive correlations with three ARGs, including *msrD*, *sat4*, and *ant (6)-Ia* ($|r| > 0.8$ and $p < 0.05$), while *Megasphaera* had great negative correlations with three ARGs, including *tet (M)*, *sul3*, and *ant (6)-Ia* ($|r| > 0.8$ and $p < 0.05$). *Petrimonas* demonstrated great positive correlations with three ARGs, including *tet (M)*, *aadS*, and *sat4* ($|r| > 0.8$ and $p < 0.05$). The *erm(G)* gene and *Bacteroides* and *Prevotella* genera exhibited significantly positive correlations ($|r| > 0.8$ and $p < 0.05$). *Lactobacillus* abundance relatively decreased within the reactors in two-stage system that underwent HTP (R0h, Rah, and Rmh) than in the corresponding reactors without HTP (R0, Ra, and Rm) (Fig. 5d). It suggested that HTP may reduce the risk of propagation of *tet(40)*, *aad9*, *tet(W)*, and *lnuC* ARGs in AD reactors.

Microbes are ARGs carriers, which have a vital effect on transmitting ARGs. Different correlations were found among four sulfonamide ARGs (*sul1-4*) and microorganisms. Correlation analysis showed that *sul1* had a negative correlation with *Bacteroides* and *Prevotella*, whereas *sul2*, *sul3*, and *sul4* had a positive correlation with *Petrimonas* ($|r| > 0.6$, Fig. 6c). This suggests that the higher abundance of *Petrimonas* in R0h and Rmh reactors may be the reason for the enrichment of *sul2*, *sul3*, and *sul4* (Fig. 4 and 5d). *sul3* and *sul4* were significantly negatively related to *Sodaliphilus* and *Megasphaera* (Fig. 6c). Such decreased *sul3* and *sul4* abundances in acidogenic phase within Ra and Rah (Fig. 4c and 4d) may be due to the enrichment of potential hosts such as *Sodaliphilus* and *Megasphaera* (Fig. 5d).

The nine different genera, including *Lactobacillus*, *Acetivibrio*, *Petrimonas*, *Prevotella*, *Clostridium*, *Clostridioides*, *Sodaliphilus, Megasphaera*, and *Bacteroides,* belong to *Firmicutes* and *Bacteroidetes*, implying that these two phyla had important effects on transmitting ARGs. Guo et al. (2020) found *Firmicutes* to be one of the five potential host phyla containing most possible ARGs hosts at the genus level (7/14) in high-solid anaerobic PM co-digestion. As suggested by Song et al. (2017), the phylum *Firmicutes* could be a host for certain ARGs, such as *tet(G)*, *tet(X)*, *erm(X)*, *erm(B)*, *sul1*, *sul2*, together with *dfrA7*. Although the link between the detected ARGs and the pathogenic microorganisms could not be determined precisely, the high abundance of *Petrimonas, Prevotella*, *Lactobacillus*, and *Acetivibrio* genera in PM and the relatively high enrichment of ARGs possibly increased antibiotic resistance. In this study, acidogenic phase in the two-stage system was found to be efficient for reducing antibiotic-resistant bacteria and ARGs by HTP of PM. *Lactobacillus*, *Acetivibrio*, and *Petrimonas* did not appear in the acidogenic phase after HTP, and they may be the potential hosts of *tet (40)*, *aad9*, *tet (W)*, *lnuC*, *msrD*, *sat4*, *ant (6)-Ia*, *sul2*, *sul3*, and *sul4*. *Firmicutes* and *Bacteroidetes* made the highest contributions to possible ARGs host at phylum level, which are possibly impacted by AD modes and HTP.

4. Conclusion

This study investigated the role of HTP in CH_4 production and the fate of ARGs during single-and two-stage AD modes of pig manure. HTP was found to increase volumetric CH_4 production rate in both AD modes, with the two-stage mode exhibiting better promotion than the single-stage system. Critical factors, like pH, NH_4^+-N and TVFAs, were altered by stage separation and HTP, which then facilitated the growth of key microorganisms correlated with CH_4 production. HTP and AD management were effective in reducing ARGs' richness and abundances. However, HTP binding enabled stronger removal of ARGs during the acidogenic phase, while ARGs abundances increased without an increase in species during the methanogenic phase. Firmicutes and Bacteroidetes were identified as potential host phyla for ARGs and maybe influenced by AD modes and HTP. These findings provide insights into strategies for optimizing AD performance and reducing the spread of ARGs in the environment. The rebound of ARGs in response to HTP in the methanogenic phase of AD process needs further investigation.

Data availability

Data will be made available on request.

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CRediT author statement

Chunchun Guo: Conceptualization, Investigation, Data curation, Writing - original draft. Shupeng Lin: Conceptualization, Data curation, Formal analysis, Software, Writing - original draft. Tao Lyu: Conceptualization, supervision, Writing - Review & Editing.

Yanfang Ma: Data curation, Investigation. Renjie Dong: Conceptualization, Methodology. Shan Liu: Supervision, Conceptualization, Writing - review & editing, Funding acquisition.

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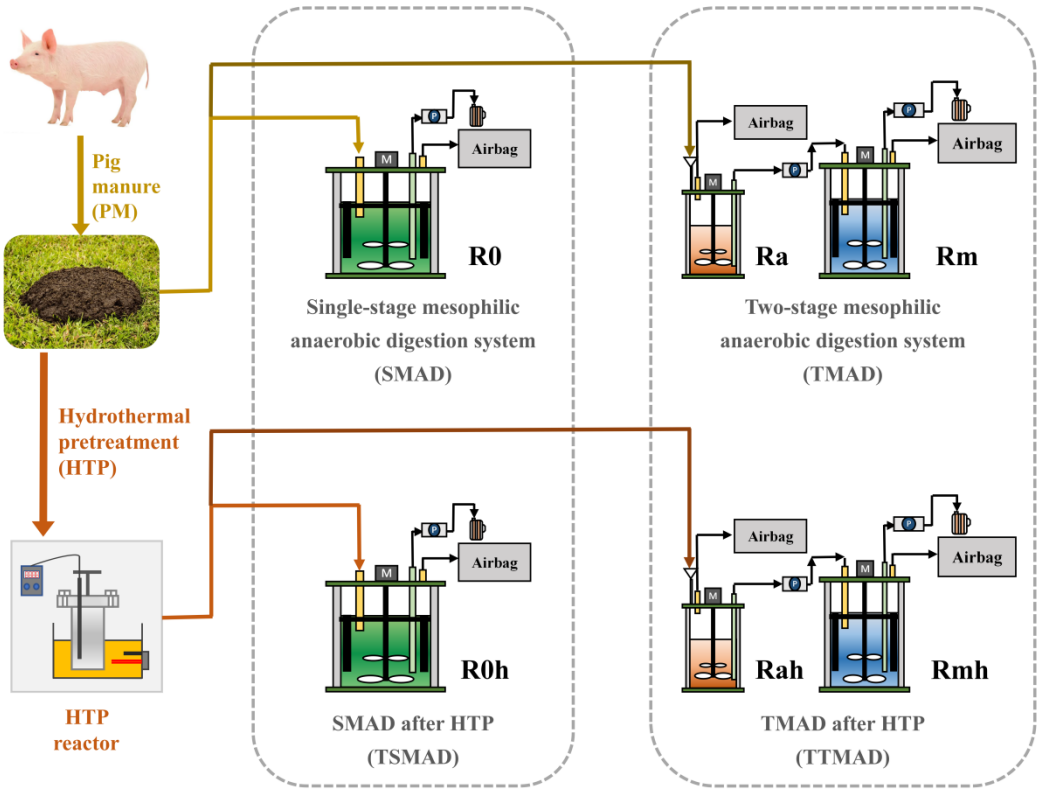


Fig. 1. Schematic of the different AD systems used in the test.

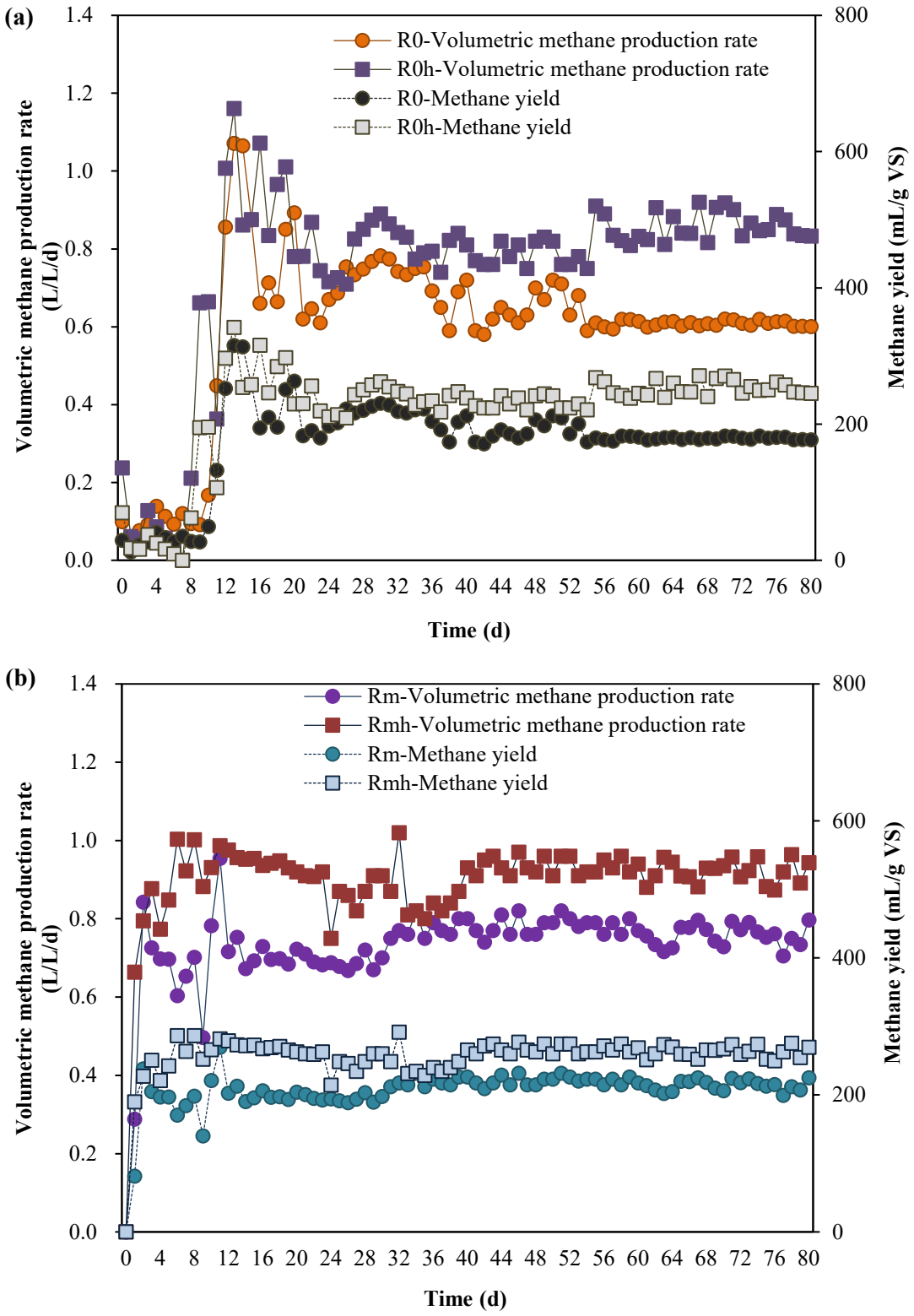


Fig. 2. Volumetric methane production rate and methane yield of single-stage mode (a) and methanogenesis reactor of two-stage mode (b).

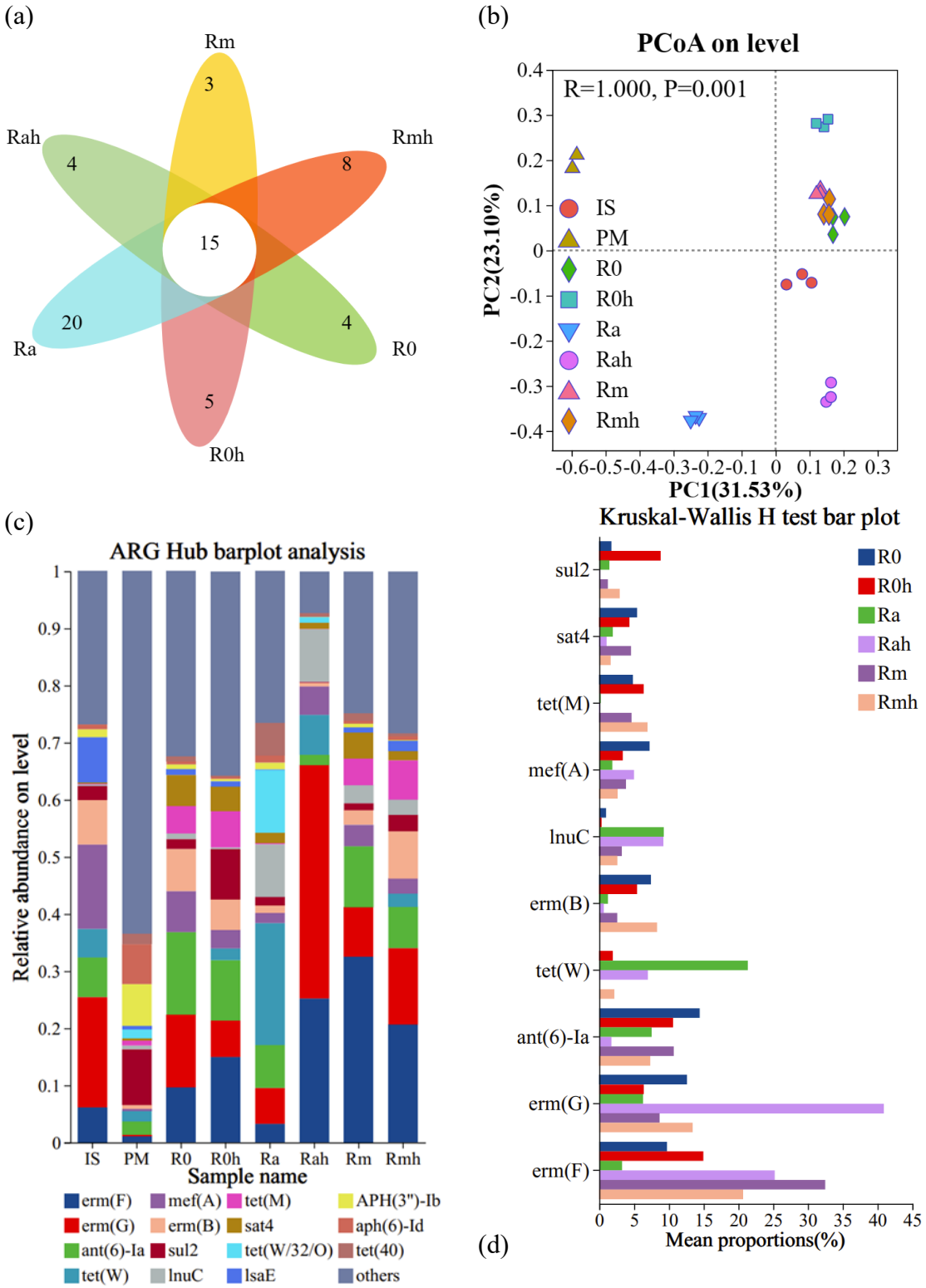


Fig. 3. Changes in ARGs among different treatment groups. Venn maps of ARG types (a); principal coordinate analysis (PCoA) of ARG distances among different groups(b); relative abundance of ARGs (genus level; others < 0.05) (c); multigroup comparison based on Kruskal–Wallis test during different AD modes (d).

(a) *sul1* (b) *sul2*

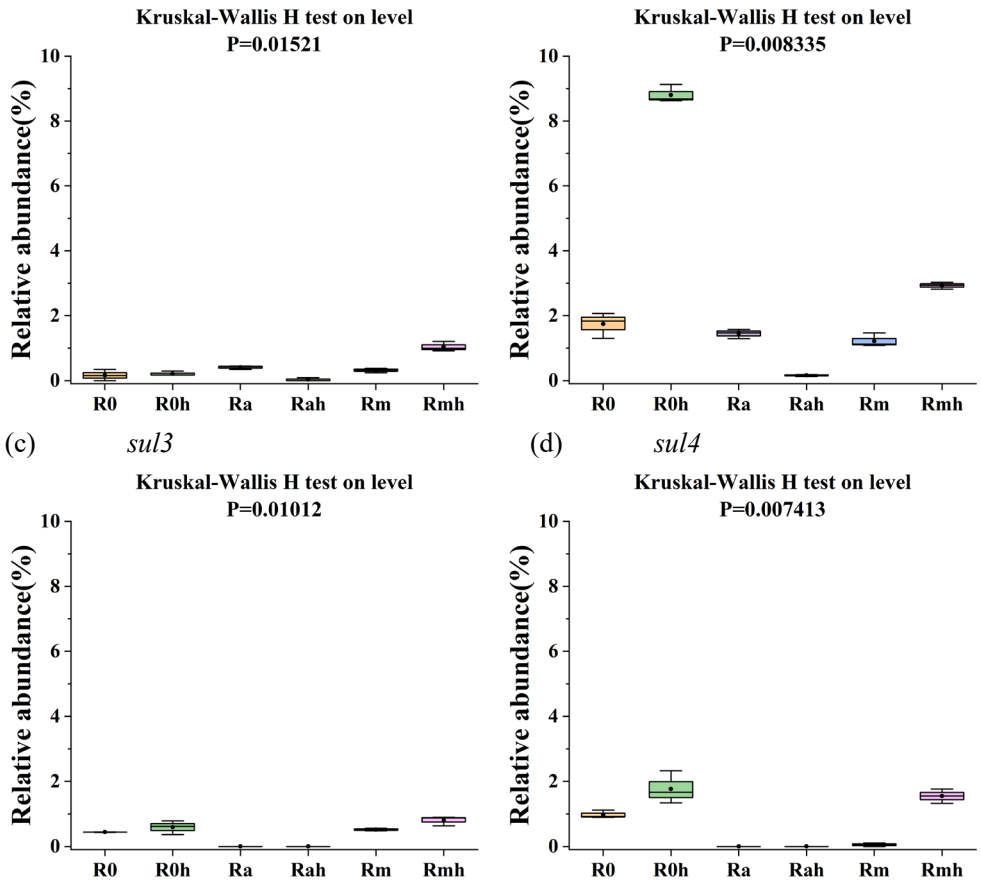


Fig. 4. Relative abundance of four sulfonamide ARGs in different groups.

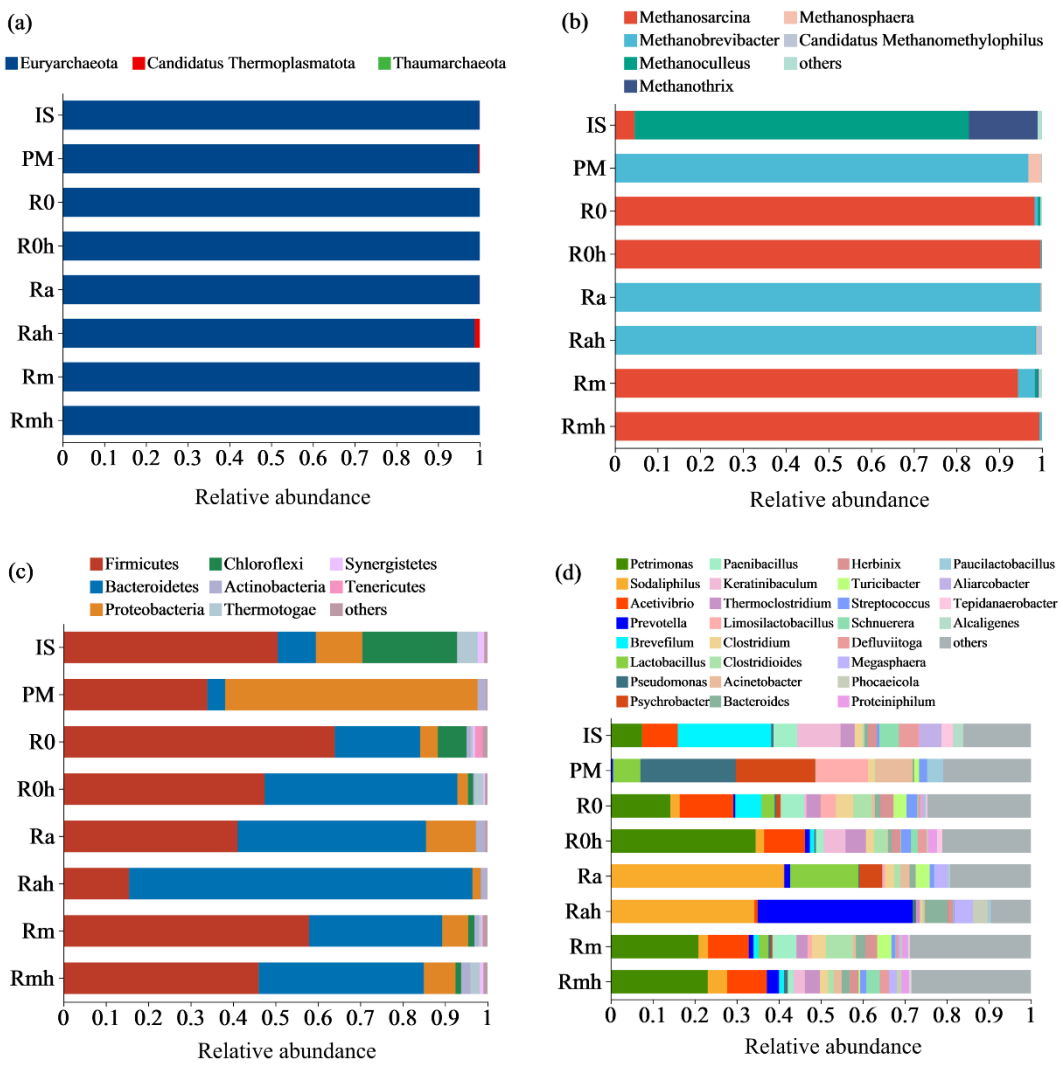
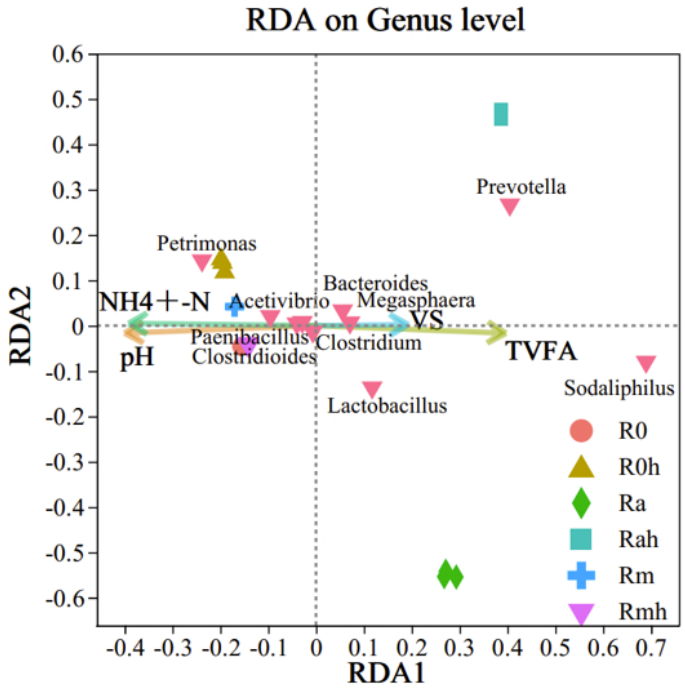
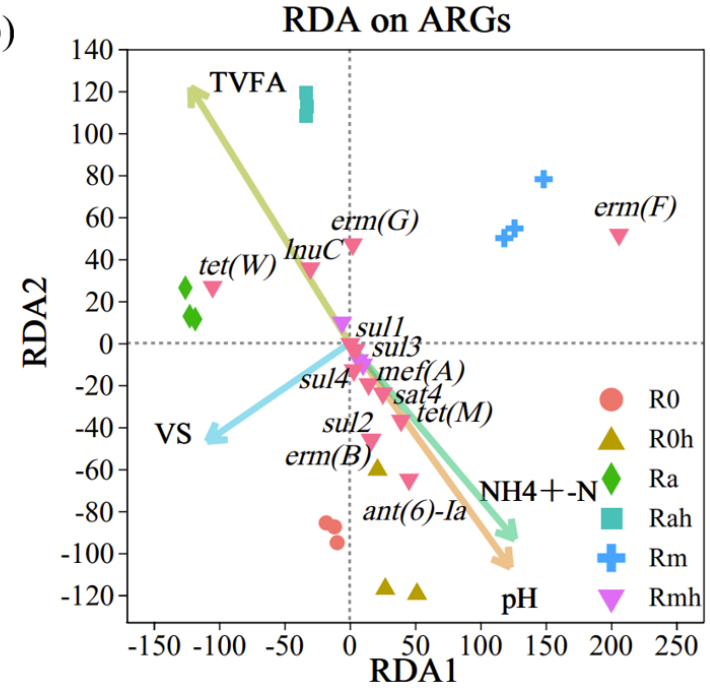


Fig. 5. Bar plots for archaeal and bacterial communities at the phylum level (a and c) and genus level (b and d).

(a)


RDA on Genus level
RDA2
RDA1
Prevotella
Petrimonas
Bacteroides
Megasphaera
Acetivibrio
NH4+-N
VS
TVFA
pH
Paenibacillus
Clostridioides
Clostridium
Lactobacillus
Sodaliphilus
R0
R0h
Ra
Rah
Rm
Rmh

(b)


RDA on ARGs
RDA2
RDA1
TVFA
erm(G)
lnuC
tet(W)
erm(F)
sul1
sul3
sul4
mef(A)
sat4
tet(M)
sul2
erm(B)
ant(6)-Ia
VS
NH4+-N
pH
R0
R0h
Ra
Rah
Rm
Rmh

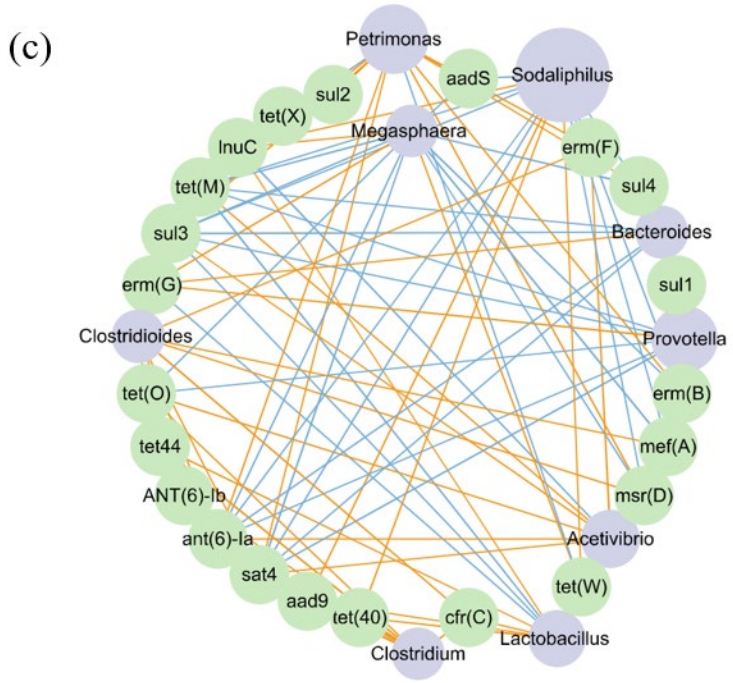


Fig. 6. Redundancy analysis (RDA) of the relationship among environmental factors and microbial community during AD (a); RDA of the relationship among environmental factors and ARGs during AD (b); network analysis of co-occurrence patterns between ARGs and microbial community (c). Connection represents significant correlation ($|R| > 0.5$; yellow: positive, blue: negative)

Supplementary Material

Effect of reactor operation modes on mitigating antibiotic resistance genes (ARGs) and methane production from hydrothermally-pretreated pig manure

Chunchun Guo[a,1], Shupeng Lin[a,1], Tao Lyu[b], Yanfang Ma[a], Renjie Dong[a], Shan Liu[a,c,*]

[*] Corresponding author. liu610shan@cau.edu.cn (S. Liu).

[1] Chunchun Guo and Shupeng Lin contributed equally to this work.

[a] College of Engineering, China Agricultural University, Beijing 100083, P. R. China

[b] School of Water, Energy and Environment, Cranfield University, College Road, Cranfield, Bedfordshire MK43 0AL, United Kingdom

[c] Yantai Research Institute, China Agricultural University, Yantai, 264670, P. R. China

Table of Contents

Table S1 - Energy assessment parameters 32
Table S2 - Energy assessment of pig manure anaerobic digestion with and without HTP 33
Figure S1 - Multigroup comparison based on Kruskal–Wallis test for archaeal and bacterial communities at the genus level 34
Figure S2 - Heatmap analysis of the relationship among environmental factors and microbial community and the relationship among environmental factors and ARGs during AD 35

Table S1 Energy assessment parameters

Parameter	Unit	Value	Reference
Density of water (ρ)	kg /m^3	1000	Fabiana et al. (2014)
Specific heat of water (γ)	kJ/kg °C	4.18	Fabiana et al. (2014)
Ambient temperature (T_a)	°C	20	this study
Anaerobic digestion temperature (T_d)	°C	37	this study
Pretreatment temperature (T_p)	°C	150	this study
Flow rate (Q)	m^3/d	1	this study
Heat transfer coefficient (k)	W/ m^2·°C	1	Fabiana et al. (2014)
Heat recovery by heat exchanger (ϕ)	%	85	Fabiana et al. (2014)
Surface area of the reactor wall (A)	m^2	25.12; 9.04	calculated
Useful volume (V)	m^3	25; 5	calculated
Electricity consumption for pumping (ϑ)	kg /m^3	1800	Fabiana et al. (2014)
Electricity consumption rate for mixing (ω)	kg /m^3·d	300	Fabiana et al. (2014)
Lower heating value of methane (ξ)	kg /m^3	35800	Fabiana et al. (2014)
Methane production rate(P_{CH4})	m^3_{CH4} /m^3·d	Fig.2a	this study
Energy conversion efficiency (η)	%	90	Fabiana et al. (2014)

Table S2 Energy assessment of pig manure anaerobic digestion with and without HTP

Parameter	Single-stage mode		Two-stage mode	
	R0	R0h	Rm	Rmh
$E_{i,heat}$ (kJ/d)	107956	178807	121234	192085
$E_{i,electricity}$ (kJ/d)	9300	9300	11700	11700
E_0 (kJ/d)	523575	660510	604125	733005
ΔE (kJ/d)	415619	472403	471191	529220

*R0, the single-stage reactor without hydrothermal pretreatment (HTP); R0h, the single-stage reactor with HTP; Rm, the methanogenesis reactor of the two-stage mode without HTP; Rmh, the methanogenesis reactor of the two-stage reactor with HTP.

*$E_{i,heat}$: input heat (kJ/d); $E_{i,electricity}$ (kJ/d): input electricity (kJ/d); E_0:output energy (kJ/d); ΔE: energy balance(kJ/d).

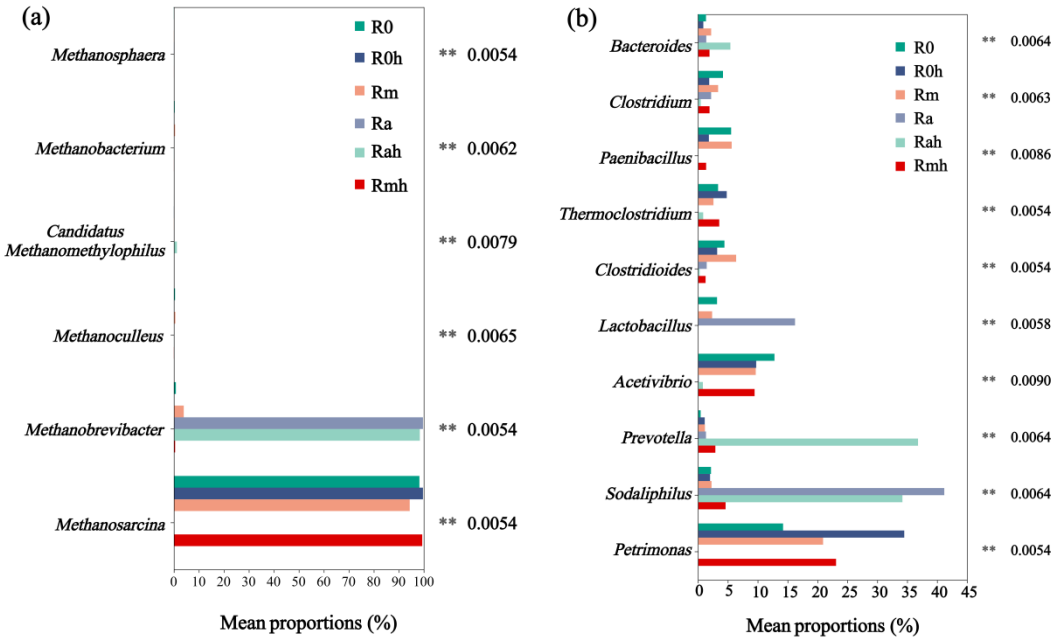


Fig. S1. Multigroup comparison based on Kruskal–Wallis test for archaeal (a) and bacterial (b) communities at the genus level, * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$.

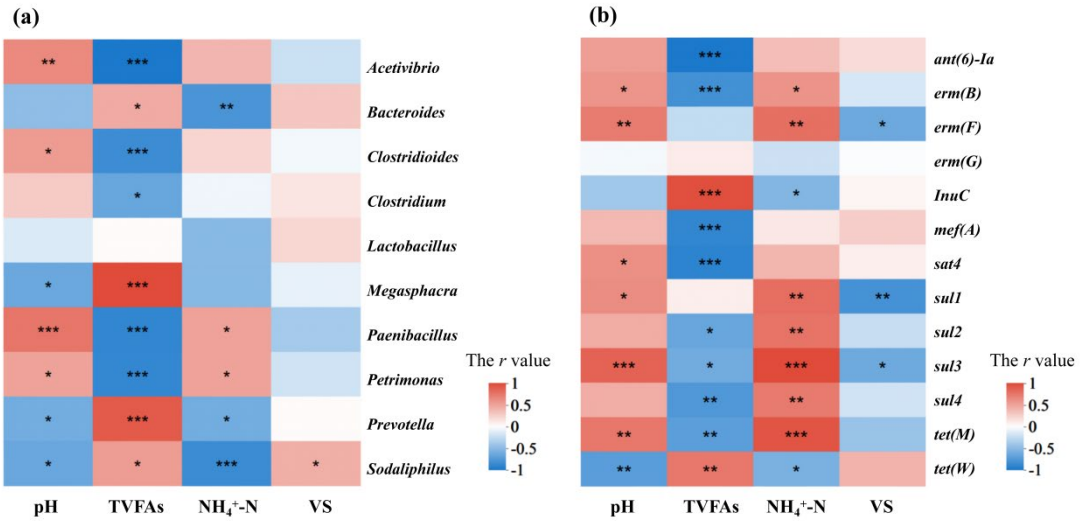


Fig. S2. Heatmap analysis of the relationship among environmental factors and microbial community (a) and the relationship among environmental factors and ARGs during AD (b), * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $p \leq 0.001$.



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