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## EDITED BY

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## REVIEWED BY

Jingwei Wu,  
Taizhou University, China  
Gede Adi Wiguna Sudiarta,  
Udayana University, Indonesia

## \*CORRESPONDENCE

Yanping Xu,  
✉ xuziping0635@163.com

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# Evaluation of mesophilic and thermophilic anaerobic co-digestion of agricultural residues for biogas production and antibiotic degradation

Xiaoli Zhu<sup>1,2</sup>, Guowei Chen<sup>1</sup>, Jia Liu<sup>1</sup>, Abdullah Gera<sup>1</sup> and Yanping Xu<sup>1,3\*</sup>

<sup>1</sup>Weifang Institute of Technology, Weifang, China, <sup>2</sup>Weifang Commercial College Shandong Province, Weifang, China, <sup>3</sup>Dezhou Weili Engineering Environmental Protection Technology Co., Ltd, Dezhou, China

Temperature plays a crucial role in determining the operational performance of the anaerobic digestion process. However, a systematic comparison between mesophilic and thermophilic regimes for the anaerobic co-digestion of diverse agricultural residues in terms of biogas production and antibiotic degradation is still lacking. Herein, anaerobic co-digestion of cow manure, chicken manure, corn stover, and wheat straw was investigated under mesophilic (37 °C) and thermophilic (55 °C) conditions using biochemical methane potential testing. The tests were conducted in 500 mL serum bottles with a 350 mL working volume, using acclimated biogas slurry as the inoculum at a substrate-to-inoculum ratio of 3:1 on a volatile solid basis. Methane production, physicochemical parameters, concentrations of three quinolone antibiotics and microbial community were analyzed. The results showed that during the initial 10-day period, daily methane production was consistently higher in the mesophilic digester than in the thermophilic digester. Total methane production over 45 days was approximately twice as high in the mesophilic system. Ammonia, chemical oxygen demand, and volatile fatty acid concentrations were significantly higher under thermophilic conditions, indicating that elevated temperature accelerated the hydrolysis and acidogenesis stages of the anaerobic digestion process. Hydrogenotrophic methanogens (*Methanobrevibacter*) dominated in both the thermophilic and mesophilic systems, but acetoclastic methanogenesis (*Methanosarcina*) was more active during the anaerobic digestion process under mesophilic conditions, which likely contributed to its higher overall methane yield. Furthermore, the removal rates of enrofloxacin and ofloxacin in the thermophilic group exceeded those in the mesophilic group by approximately 8%. Overall, anaerobic co-digestion of these four agricultural wastes at 37 °C was more favorable to the methane production, whereas digestion at 55 °C enhanced the degradation of organic substances, including the three quinolone antibiotics. This study highlights the critical role of temperature in shaping microbial

communities and provides valuable guidance for temperature selection in the co-digestion of diverse agricultural residues.

#### KEYWORDS

agricultural wastes, anaerobism, microbial community, semi-dry fermentation, temperature

## 1 Introduction

Anaerobic digestion (AD), which integrates environmental engineering and microbial technology, is a cost-efficient approach for treating agricultural organic waste, while simultaneously producing renewable energy (biogas) and generating high-value-added products such as organic fertilizer. This process plays a key role in achieving carbon-emission reduction goals (An et al., 2024). Consequently, it is a relatively mature technique that has garnered significant research and commercial interest. However, several key challenges hinder its broader application, including the complexity of feedstock materials, regional and seasonal temperature variations, and uncertainty regarding the composition of key microbial taxa.

The expansion of agricultural production, driven by the rising demand for food coupled with population growth, ultimately causes the massive generation of agricultural waste. Notably, fiber-based agricultural waste alone reaches 1.4 billion tons annually (Xu et al., 2023). Improper management of recyclable agricultural by-products can result in significant resource waste and environmental damage, including water, soil, and air pollution, as well as potential risks to human health. Typical examples include uncontrolled discharge of livestock manure and the release of strong, unpleasant odors. AD facilitates the transformation of organic matter in agricultural waste. Through the processes of hydrolysis, acidification, acetogenesis, and methanogenesis, anaerobic fermentation converts agricultural waste into methane and digestate (Wu et al., 2022). The methane produced has applications in space heating, electricity generation, and as bio-natural gas. Additionally, the solid and liquid digestate (biogas slurry and biogas residues) serve as valuable resources for use as fertilizer, soil conditioner, or animal bedding (Akor et al., 2021). The co-digestion of multiple agricultural wastes, such as animal manure and crop residues, not only optimizes nutrient balance in AD digesters but also facilitates waste management in the same geographic region and regional development. Furthermore, it is more conducive to achieving stable year-round operation and steady biogas production, significantly reducing greenhouse gas emissions, thereby yielding substantial environmental benefits.

Among various physical and chemical indices, temperature is one of the most important parameters that exerts a critical influence on AD performance, such as modulating microbial community structure, interspecies interactions and metabolic activities. A robust and stable microbial community is essential for the sustained operation of AD plants. Throughout the AD process, diverse bacterial communities work together via the formation of an anaerobic food chain: the products generated by one group become the substrate for another. Close cooperation and a balanced state among these communities are prerequisites for effective system function (Sudiartha et al., 2024). According to Nie et al. (2021), methanogens exhibit optimal activity within

the mesophilic (30 °C–40 °C) and thermophilic (50 °C–60 °C) ranges, making these the dominant regimes for commercial AD systems (Sudiartha et al., 2024). Mesophilic conditions are characterized by greater process stability and reduced energy consumption (Singh et al., 2023). In contrast, the thermophilic processes offer significant advantages, including faster waste stabilization, enhanced biogas production with high methane content, accelerated fermentation processes, effective removal of pathogens in the feedstock and improved sludge dewaterability (Xu et al., 2023; Morais et al., 2025). However, maintaining thermophilic conditions, especially in cold climates like northern China in winter, requires substantial auxiliary heating. This high energy demand increases operational costs, compromises system sustainability, and ultimately undermines the economic viability of full-scale projects (Morais et al., 2025). Temperature also regulates reaction kinetics and alters the thermodynamic equilibrium of key reactions, thereby modifying biochemical pathways and exerting a profound influence on the efficiency and stability of syntrophic interactions. (Nie et al., 2021; Sudiartha et al., 2024). Therefore, the optimal fermentation temperatures depend strongly on characteristics of the feedstock and the specific operational conditions. Although extensive research has investigated the influence of temperature on AD, most studies have focused on wet digestion systems using one or two types of substrates (Chen et al., 2021; Liu et al., 2022; Li et al., 2024). Consequently, the precise effects of temperature on the semi-dry co-digestion of multiple agricultural residues remain inadequately understood. In particular, how temperature regulates microbial community succession as a central mechanism controlling biogas production requires further elucidation.

Antibiotics are increasingly recognized as one of the most frequently detected pollutants in wastewater (Vistanty et al., 2025), particularly from livestock farms. Driven by accelerated intensive breeding practices, antibiotic usage in livestock and poultry production has surged, consequently elevating the levels of antibiotic residues in animal manure (Ali et al., 2025). AD has proven effective in mitigating antibiotic contamination (Vistanty et al., 2025). A critical yet often overlooked aspect of using biogas slurry as an organic amendment is its possible contamination with antibiotic residues. Repeated application also risks the accumulation of these compounds in agricultural soils, creating a potential pathway for their entry into food crops and posing a threat to food safety (Wang et al., 2022). Therefore, the effectiveness of AD in removing antibiotics, a process highly dependent on operational parameters, has attracted growing attention (Ali et al., 2025). Additionally, quinolones are generally categorized as recalcitrant antibiotics due to their persistence in anaerobic environments (Sun et al., 2023). However, the removal efficiency of quinolone antibiotics during semi-dry co-digestion of multiple agricultural wastes under different temperature regimes remains unclear.

TABLE 1 Fermentation substrates and inoculum characteristics.

Item	Total solid/%	Volatile solid (dry basis)/%	Total organic carbon content/%	Total nitrogen content/%
Wheat straw	89.8	92.0	44.5	0.446
Corn stalk	91.4	89.9	44.3	0.601
Cow manure	15.7	85.7	40.5	1.57
Chicken manure	39.1	77.8	38.1	4.22
Biogas digestate	5.50	64.9		

This study systematically compared the performance of semi-dry AD of a mixture of corn stalk, wheat straw, cow and chicken manure under mesophilic and thermophilic conditions. The aim was to assess the impact of temperature on the anaerobic co-digestion of diverse agricultural waste, evaluate system performance and stability by monitoring methane yield and key physicochemical Indicators, analyze the corresponding changes in the microbial community, identify the optimal temperature conditions for AD and disposal of agricultural by-products, and provide operational insights for minimizing the environmental risk of antibiotic residues in biogas slurry.

## 2 Materials and methods

### 2.1 Materials

Chicken manure and cow manure were collected from local livestock farmers in Weifang, Shandong Province. The corn stover and wheat straw were also harvested from local cultivation areas and air-dried. The dried materials were ground using a grinder (FW135, Beijing Ever Bright Medical Treatment Instrument Co., Ltd., Beijing, China) and sifted using a 40-mesh sieve. The biogas slurry, used as inoculum, was obtained from an operational 6,000 m<sup>3</sup> anaerobic digester (Shandong Luxi Dasheng Environmental Protection Technology Co., Ltd., Weifang) that primarily treats cow, chicken, and duck manure. Before use, the biogas slurry was pre-incubated under anaerobic conditions for 2 weeks at 37 °C ± 0.2 °C for the mesophilic trial and 55 °C ± 0.2 °C for the thermophilic trial, respectively. The characteristics of all materials are presented in Table 1.

### 2.2 Anaerobic fermentation equipment experiment design

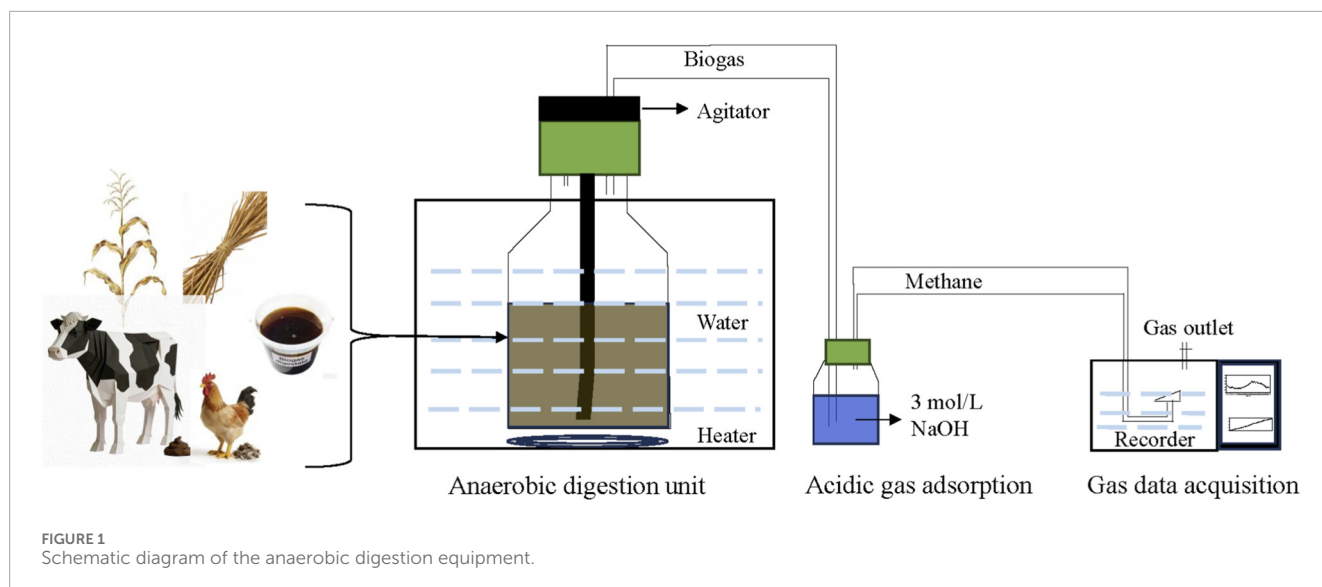
The experiment was conducted using MultiTalent 203 (Nova Skantek Instruments Co., Ltd., Beijing, China) to automatically record the methane yield. The fully automatic methane potential tester consists of an AD unit, an acidic gas adsorption module, a gas flow rate and a data acquisition module (Figure 1). The AD unit includes an electric-heated thermostatic water bath and 500 mL sealed glass bottles equipped with separate mechanical stirrers. The

acidic gas adsorption module comprises 100 mL glass bottles, each containing 80 mL of 3 mol/L sodium hydroxide solution.

The experiment comprised two treatments: a mesophilic group (MT, 37 °C ± 0.2 °C) and a thermophilic group (HT, 55 °C ± 0.2 °C) with three replicates per treatment. Each bottle was loaded with 350 g (wet weight) of the substrate-inoculum mixture, resulting in a working volume of approximately 350 mL. Based on experience in engineering practice, the substrate was formulated on a dry matter basis as a mixture of chicken manure, cow manure, wheat straw and corn stalks in a 1.6:1:1:1 ratio, with a total carbon-to-nitrogen ratio (C/N) of 20.2. The total solid (TS) content was adjusted to 12% (semi-dry) using biogas slurry. The feedstock contained naturally occurring quinolone antibiotics, with initial concentrations of ciprofloxacin (24.6 µg/kg), enrofloxacin (379 µg/kg) and ofloxacin (467 µg/kg). After sealing, the bottles were incubated in a thermostatic water bath for a 45-day AD period. The mixture was agitated intermittently at 80 rpm for 30 s, followed by a 3-min rest period.

### 2.3 Sampling and measurements

Samples were collected on day 10 and day 45, with three replicates being tested. The pH value was measured using PHS-3E (INASE Scientific Instrument Co., Ltd., Shanghai, China) instantly after halting the AD process by rapid cooling in an ice box. Aliquots of fermentation liquid from each bottle were collected and pooled with the samples from the same group, rapidly frozen in liquid nitrogen, and stored at -80 °C until microbial community analysis. The fermentation liquid supernatant was obtained by centrifugation (20,000 × g, 15 min, 4 °C) and subsequently analyzed for ammonia nitrogen, chemical oxygen demand (COD), and volatile fatty acid (VFA). TS were determined by drying 20 mL of fermentation liquid at 105 °C for 48 h in a laboratory oven (WGL-45B, Tianjin Taisite Instrument Co., Ltd., China). Subsequently, volatile solids (VS) were determined by combusting the dried residues in a chamber furnace (SX-4-10, Tianjin Taisite Instrument Co., Ltd., China) at 550 °C for 5 h. The residual fermentation liquid of each group was pooled by group and submitted to Anqiu Agricultural Product Quality and Safety Management Service Center (Weifang, China) for the analysis of three antibiotics (ciprofloxacin, enrofloxacin and ofloxacin). The analysis was performed using high-performance liquid chromatography-triple



quadrupole mass spectrometry, following the standardized protocol outlined in the Chinese National Standard HJ 1399-2024 (2024). Elemental analysis was performed on a Vario MAX cube system (Elementar Analysensysteme GmbH, Germany) to quantify total carbon and nitrogen content. The VFA concentrations were analyzed following the method of Sun et al. (2009). Ammonia nitrogen concentrations were quantified via Nessler's reagent spectrophotometry (HJ 535-2009, 2009, China), and COD was assessed using the fast digestion-spectrophotometric method (HJ/T 399-2007, 2007, China).

Genomic DNA was extracted from the fermentation liquid samples using the E. Z.N.A.<sup>®</sup> Soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). Following extraction, DNA quality was evaluated by measuring purity and concentration with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States), and assessing integrity through 1% agarose gel electrophoresis. Next-generation sequencing of the 16S rRNA gene was performed by Shanghai Biozeron Biotechnology Co., Ltd. (Shanghai, China). For microbial community analysis, the V3-V4 hypervariable regions of the bacterial 16S rRNA were amplified using primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), while archaeal communities were targeted using primers Arch519F (5'-CAGCCGCCGCGTAA-3') and Arch915R (5'-GTGCTCCCCCGCCAATTCCT-3'). Sequencing was carried out using NovaSeq PE250 (Illumina, Kapa Biosciences, Woburn, MA, United States) following library construction with the NEXTflex Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, United States).

## 2.4 Statistical analysis

Data was analyzed using SPSS Statistics 22 software (IBM, Armonk, NY, United States). Measurements of ammonia nitrogen content, pH value, COD, TS, VS, VFA and cumulative methane yield on day 45 (n = 3) were subjected to an independent-samples T-test. Significant differences were considered at  $P \leq 0.05$ . GraphPad Prism

6 (GraphPad Software, San Diego, CA, United States) was used to create the graphs.

## 3 Results and discussion

### 3.1 Methane production

The efficiency of methanogenesis in anaerobic digestion is governed by both the extent of substrate decomposition and the conversion of intermediate compounds into methane (Ali et al., 2025). As shown in Figure 2, the mesophilic (MT) digester's daily methane fluctuated from 1.05 to 3.09 mL/g-VS during the first 17 days, after which it increased gradually to a first peak of 7.34 mL/g-VS on day 26. Conversely, the thermophilic (HT) digester achieved its first peak on day 14, but at a lower yield of 3.43 mL/g-VS. These trends suggest a rapid process initiation under mesophilic conditions, compared to a brief lag phase under thermophilic conditions. The second peak in the MT group occurred on day 36 (3.89 mL/g-VS) and then declined. Although a similar pattern was observed in the HT group, its daily methane production remained consistently lower than that of the MT group. These results indicate that the mesophilic AD of the four agricultural residues was more favorable for methane production than the thermophilic condition, a finding further supported by the cumulative methane production data (Figure 2). Ultimately, the cumulative gas production of the mesophilic group was significantly higher than that of the thermophilic group ( $p = 0.010$ ), reaching approximately twice the total volume over the 45-day operational period. This result aligns with the findings of Yu et al. (2025), who reported that the methanogenic potential of corn stover was lower under thermophilic conditions (52 °C) than under mesophilic conditions (37 °C). However, it contrasts with a study by Hupfauf et al. (2018) on the anaerobic co-digestion of corn straw and cow manure, which observed a higher methane yield at 55 °C than at 37 °C. This discrepancy, in which the optimal temperature varies across studies, collectively underscores the importance

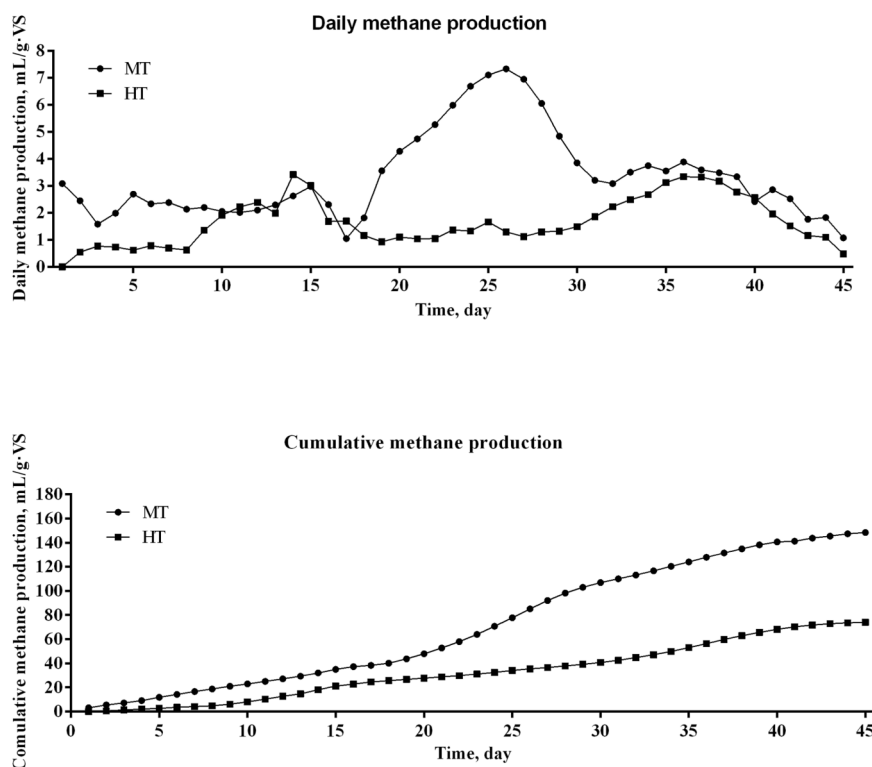


FIGURE 2 Daily methane production and cumulative methane production of semi-dry anaerobic digestion of a mixture of agricultural residues under mesophilic and thermophilic conditions.

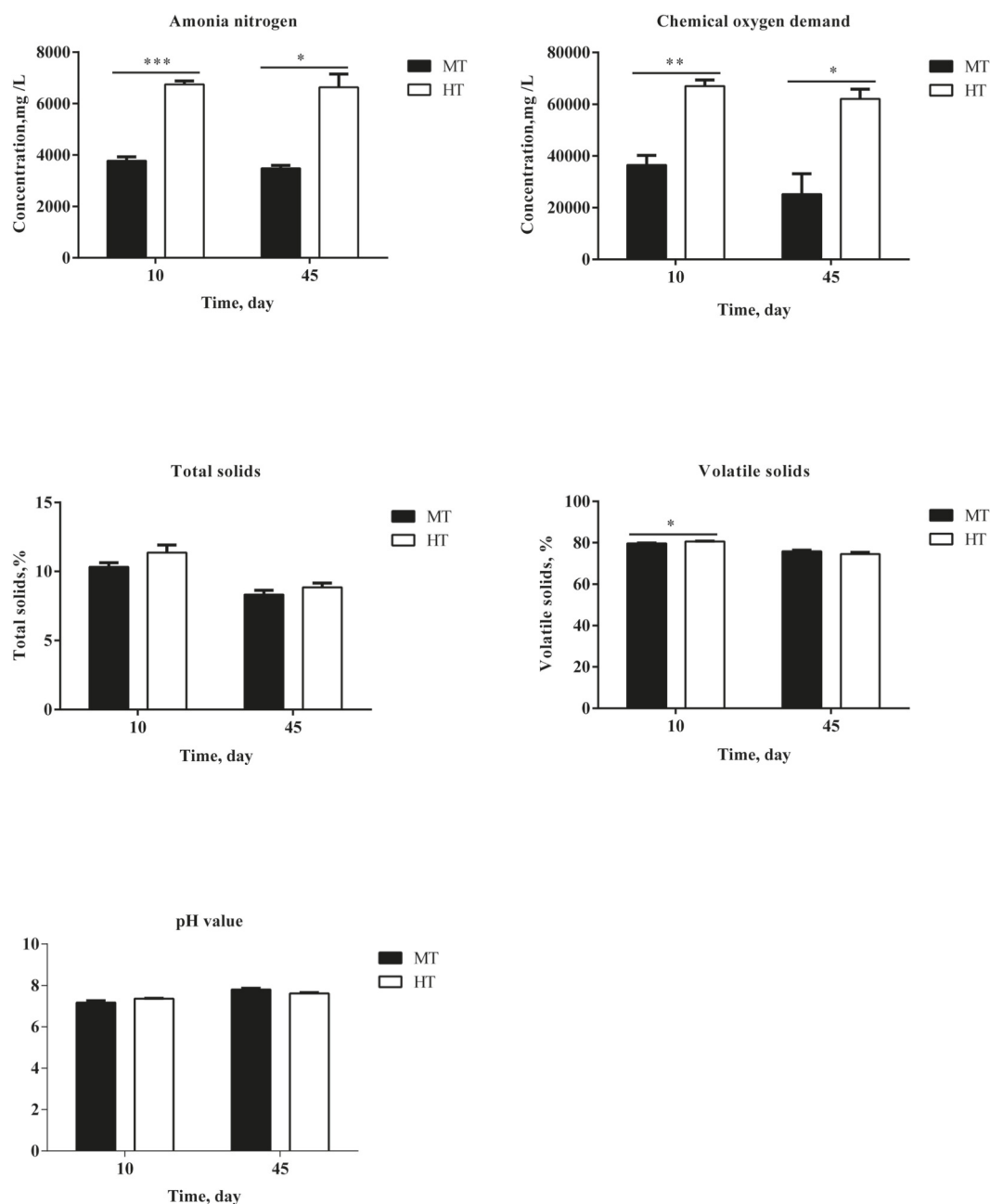
of selecting an appropriate temperature regime for process optimization.

To identify the key drivers of methane yield, correlations with key physicochemical parameters and microbial taxa were evaluated (Supplementary Figure S1). Methanogens play a pivotal role in biogas production, and the diversity of their methanogenic pathways significantly influences yield variations (Sudiartha et al., 2024). In line with this, our analysis revealed a positive correlation between cumulative methane yield and the relative abundance of *Methanoculleus*, a hydrogenotrophic archaeon. Its prevalence was higher under mesophilic conditions (2.76% of total archaea) than under thermophilic conditions (1.48%) on day 45, consistent with the higher methane yield observed at mesophilic temperatures. This suggests that *Methanoculleus* was an important contributor to methane production in the present system, where hydrogenotrophic methanogenesis dominated, which is consistent with the reported positive association between its abundance and enhanced methane production (Sudiartha et al., 2024).

### 3.1.1 Physicochemical indicators

In engineering practice, physicochemical parameters are widely employed as indicators to assess the operational status of AD systems. In this study, no significant differences in pH or TS were observed between the two groups ( $p > 0.05$ , Figure 3). The pH values remained within the optimal range of 6.5–8.0 for microbial activities during AD (Suanu et al., 2018), indicating that both AD systems operated stably. Ammonia nitrogen levels were

significantly higher in the thermophilic (HT) group compared to the mesophilic (MT) group by day 10 of fermentation ( $p < 0.001$ ), and differences remained significant through day 45 ( $p = 0.018$ ), aligning with the results of Li et al. (2024). In locally operated biogas plants that primarily use livestock and poultry manure as feedstock and operate stably, an ammonia nitrogen concentration between 3,000 and 4,000 mg/L is considered normal. Therefore, the mesophilic group was more closely aligned with practical conditions and less likely to experience ammonia inhibition. In contrast, the thermophilic group exhibited ammonia nitrogen concentrations of over 6,000 mg/L. This may be due, on the one hand, to the accelerated degradation of protein-rich substrates at elevated temperatures and, on the other hand, to the potential onset of ammonia inhibition, which impairs substrate utilization efficiency (Zhang et al., 2011; He et al., 2025). Free ammonia nitrogen, which is a fraction of total ammonia nitrogen, is more toxic to methanogens as it can penetrate cell membranes and disrupt intracellular enzyme systems. Given that ammonia nitrogen concentration increases substantially with temperature (Liu et al., 2022), the methanogenic activity in our HT group was likely subject to inhibition. Another explanation for the reduced methanogenesis under ammonia nitrogen stress is that ammonia-tolerant taxa, regardless of their functional roles in AD (e.g., hydrolytic bacteria, methanogens), must allocate additional carbon, nitrogen, and energy resources to counteract ammonia toxicity. This metabolic reallocation compromises central carbon metabolism, ultimately reducing metabolic methane yields (Finn et al., 2023).



**FIGURE 3** Effects of fermentation temperature on ammonia nitrogen content, pH value, chemical oxygen demand, TS and VS in anaerobic fermentation of agricultural residues.

Moisture content (Figure 3) is a key factor for microbial activity, as it supports the motility and growth of microorganisms, promotes the dissolution and transport of nutrients, and alleviates mass transfer limitations associated with heterogeneous or particulate substrates (Suanu et al., 2018). In the present study, the higher VS content in the HT group on day 10 ( $p = 0.011$ ) indicates a lower initial organic matter degradation efficiency compared to the mesophilic group. However, this difference disappeared by day 45. These findings suggest that temperature exerts distinct regulatory effects on the anaerobic microbiota, and independently

modulates the initial start-up rate and the long-term organic matter degradation potential of the system.

Overall, the HT group consistently exhibited elevated VFA levels at both time points (Figure 4). Higher levels of acetic acid, butyric acid, isobutyric acid, isovaleric acid and total VFAs were observed in the HT treatment compared with the MT treatment ( $p < 0.05$ ). This pattern reflects a more intensive substrate decomposition in the thermophilic group during the early stage of AD, suggesting a higher potential for subsequent methane production—this was further supported by the increased methane yield observed from

day 10 to day 14. Moreover, the presence of unconsumed VFAs in the HT group on day 45 indicates the potential for continued fermentation and subsequent biogas generation. The present study observed that the propionate concentration in the MT group was significantly higher than in the HT group by day 45 ( $p < 0.001$ ). The syntrophic oxidation of propionate is crucial for methanogenesis, as approximately 30% of the electrons derived from complex substrate breakdown pass through propionate during AD. This process requires acetogenic bacteria to form syntrophic partnerships with hydrogenotrophic archaea, which consume  $H_2$  to maintain a low partial pressure, thereby enabling thermodynamically favourable propionate degradation (Sudiartha et al., 2024). Therefore, the propionate accumulation observed in the MT group by day 45 likely served as a substrate reservoir for continued methanogenesis. The elevated abundances of *Fermentimonas* (a propionate-producing genus) and the hydrogenotrophic methanogen *Methanobrevibacter* during this period support the scenario that propionate was further converted to acetate and  $H_2$ , with the latter being efficiently consumed for methane production. A positive correlation was observed between propionate content and the relative abundance of Bacteroidota (Supplementary Figure S1). The relative abundance of Bacteroidota increased on day 45 in the mesophilic group, and the active metabolic activity of bacteria in this phylum might lead to the accumulation of propionate at this time point. Previous studies have reported that ammonia toxicity is primarily caused by the free ammonia fraction, which can promote VFA accumulation (Liu et al., 2022). The interaction between free ammonia and VFAs may establish a dynamic equilibrium, resulting in a stable yet suboptimal inhibited steady state characterized by lower methane yield. This mechanism may help explain the performance observed under thermophilic AD conditions.

COD quantifies the dissolved, oxidizable organic matter in digestate and is a critical parameter for assessing its residual organic load (Abid et al., 2021). Its levels in the HT group were significantly elevated, measuring 1.84 and 2.46 times higher than in the mesophilic (MT) group on day 10 and 45, respectively ( $p < 0.05$ ). This increase can be attributed not only to the generally accelerated degradation rate under thermophilic conditions but more specifically to the substantial accumulation of VFAs, key components of COD.

## 3.2 Antibiotic degradation

The extensive use of antibiotics to enhance feed efficiency, prevent infections, and treat diseases is a ubiquitous feature of intensive animal production systems (Chen et al., 2025). They are excreted in urine and feces, leading to high concentrations in manure, with a substantial fraction remaining in their original form or as transformed metabolites (Zubair et al., 2023). Figure 5 illustrates the degradation rates of three antibiotics during anaerobic fermentation under different temperature conditions. For enrofloxacin and ofloxacin, the degradation efficiency of AD under high-temperature treatment exceeded that of mesophilic treatment by 8.05% and 7.92%, respectively. After 45 days of anaerobic fermentation, ciprofloxacin was completely degraded in both the MT and HT groups, likely due to the relatively low initial concentration ( $<25 \mu\text{g/kg}$ ). These antibiotics belong

to quinolones, a class of synthetic antimicrobial agents with strong bactericidal activity and broad-spectrum antibacterial properties (Wu et al., 2024). Anaerobic fermentation markedly reduced their concentrations, supporting the standardized use of the resulting organic fertilizer and minimizing potential adverse impacts on soil and plants. Overall, thermophilic AD achieved greater removal of quinolone antibiotics, consistent with Zahedi et al. (2022), who found thermophilic conditions superior for removing various veterinary pharmaceuticals. This alignment suggests a potentially common, thermally enhanced degradation mechanism (Zubair et al., 2023). Zhang et al. (2022) stated that key enzymes involved in quinolone biodegradation in AD systems, such as hydrolases (deaminases and peptidases), ligases, transferases, and lyases, are positively associated with the phylum Bacillota. Their activity is sensitive to the accumulation of propionic acid. Consistent with this finding, our results demonstrated that the mesophilic group exhibited both a lower relative abundance of Bacillota and a significantly higher concentration of propionic acid compared to the thermophilic reactor at day 45. This combination of an unfavourable microbial community structure and inhibitory metabolic conditions likely suppressed the relevant enzymatic activity, thereby explaining the lower enrofloxacin and ofloxacin degradation efficiency observed under mesophilic conditions.

## 3.3 Microbial community

Studies have shown that anaerobic co-digestion systems of straw with livestock and poultry manure optimize the microbial community composition and structure (Huang et al., 2024). This is critical because the acid-forming and methane-forming microorganisms in these systems have distinct physiologies and growth requirements. Since different bacterial species synergistically produce a diverse array of enzymes, the composition and diversity of the bacterial community in AD systems significantly influence the extent of substrate degradation. Furthermore, the composition of methanogenic archaea strongly affects methane production, as acidolysis produces a variety of substrates that necessitate different methanogenic pathways for efficient methanogenesis (Niya et al., 2024).

In the present study, pretreatment and sequencing generated 727 bacterial operational taxonomic units (OTUs) and 612 archaeal OTUs across all samples. Among the alpha diversity indexes, the bacterial Chao1 index of the MT group was 1.93 and 2.52 times higher than that of the HT group on days 10 and 45, respectively (Figure 6). They exhibited a similar temporal pattern in bacterial richness, with higher species numbers in the early stage and lower richness in the late stage. It is widely recognized that greater microbial diversity in AD systems is associated with enhanced biogas production (Kong et al., 2018). Although the archaeal Chao1 indices were higher in the HT group, the greater bacterial diversity under mesophilic conditions likely played a more pivotal role. Diverse bacterial communities supply a broader spectrum of metabolic intermediates (e.g., hydrogen, formate, acetate) that are essential substrates for various methanogenic archaea (Finn et al., 2023). Therefore, the MT environment was more conducive to maintaining the complex syntrophic networks necessary for efficient methanogenesis,

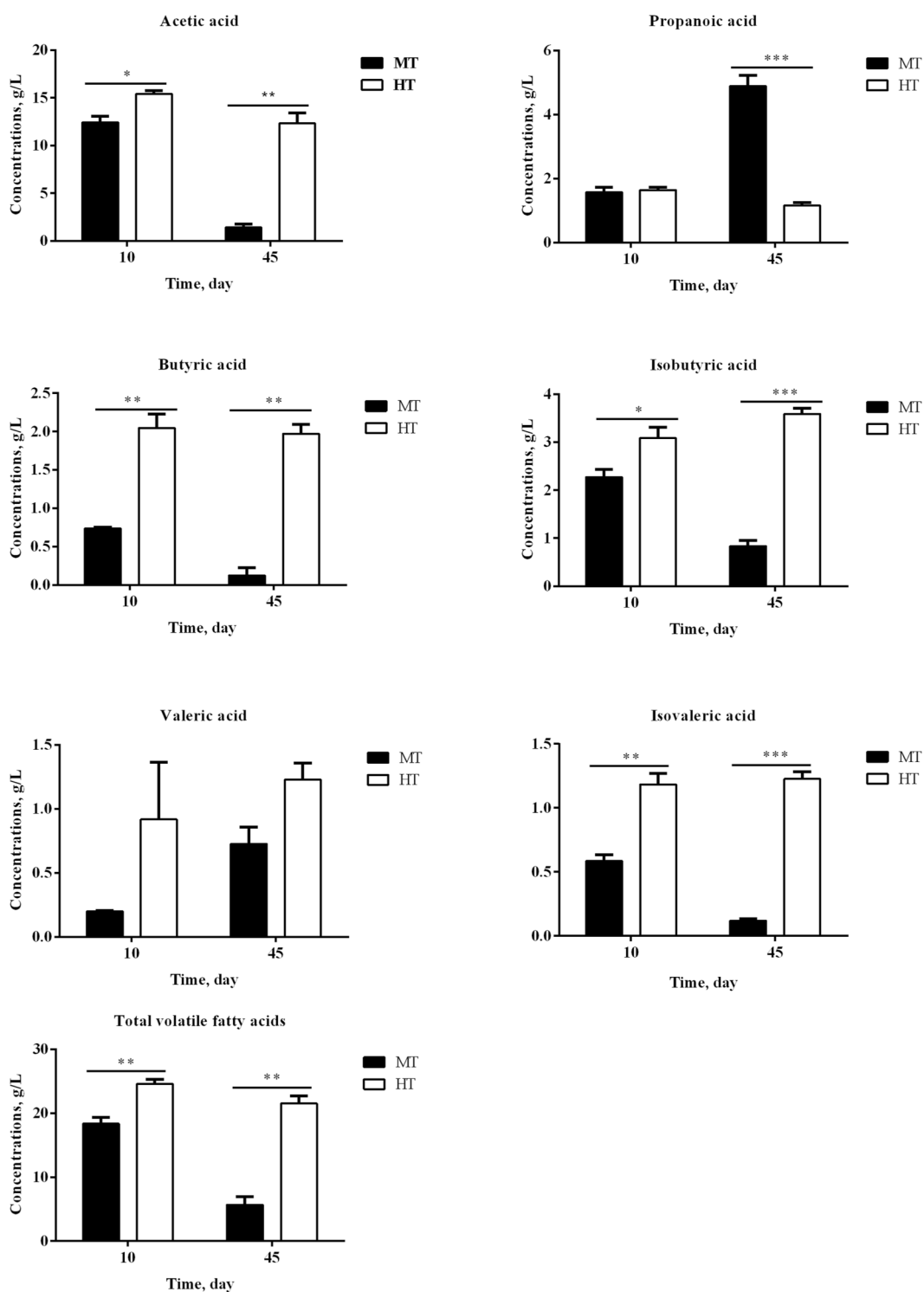
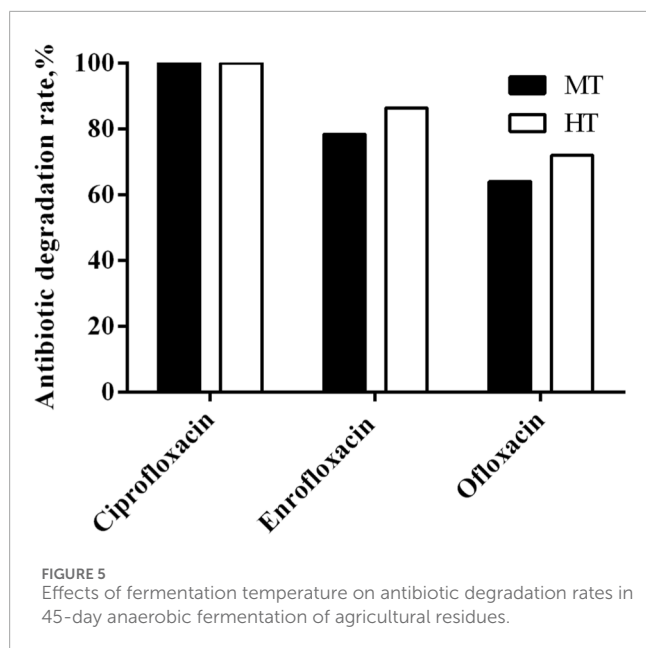


FIGURE 4 Effects of fermentation temperature on volatile fatty acids production in anaerobic fermentation of agricultural residues.

which may explain the higher methane production observed in the MT group.

Based on the bacterial Shannon index, the species evenness in the thermophilic group was higher than in the mesophilic group on day 10. However, by day 45, evenness in the HT group had

decreased to a level below that in the MT group, which exhibited an increase over time. This pattern suggests that high temperature imposed a strong environmental filter on the bacterial communities, selecting for a subset of thermotolerant species. On the 10th day of AD, the archaeal evenness of the HT group showed slightly higher



evenness than the MT group, but by day 45, the two groups exhibited nearly identical evenness. On day 10, the HT group harboured a community with high evenness and transient diversity. This was consistent with the reported decoupling between methanogenesis and biodiversity in AD (Finn et al., 2023). As fermentation progressed, however, sustained thermophilic conditions likely led to the gradual loss of less tolerant species, resulting in a community dominated by a few competitive, thermotolerant taxa. Additionally, the Simpson index values over 0.859 across all samples, confirming that several dominant species constituted a large proportion of the microbial community. Given that efficient methanogenesis relies on the participation of multiple microbial species, and higher microbial abundance and diversity can facilitate more robust methane conversion (Huang et al., 2024).

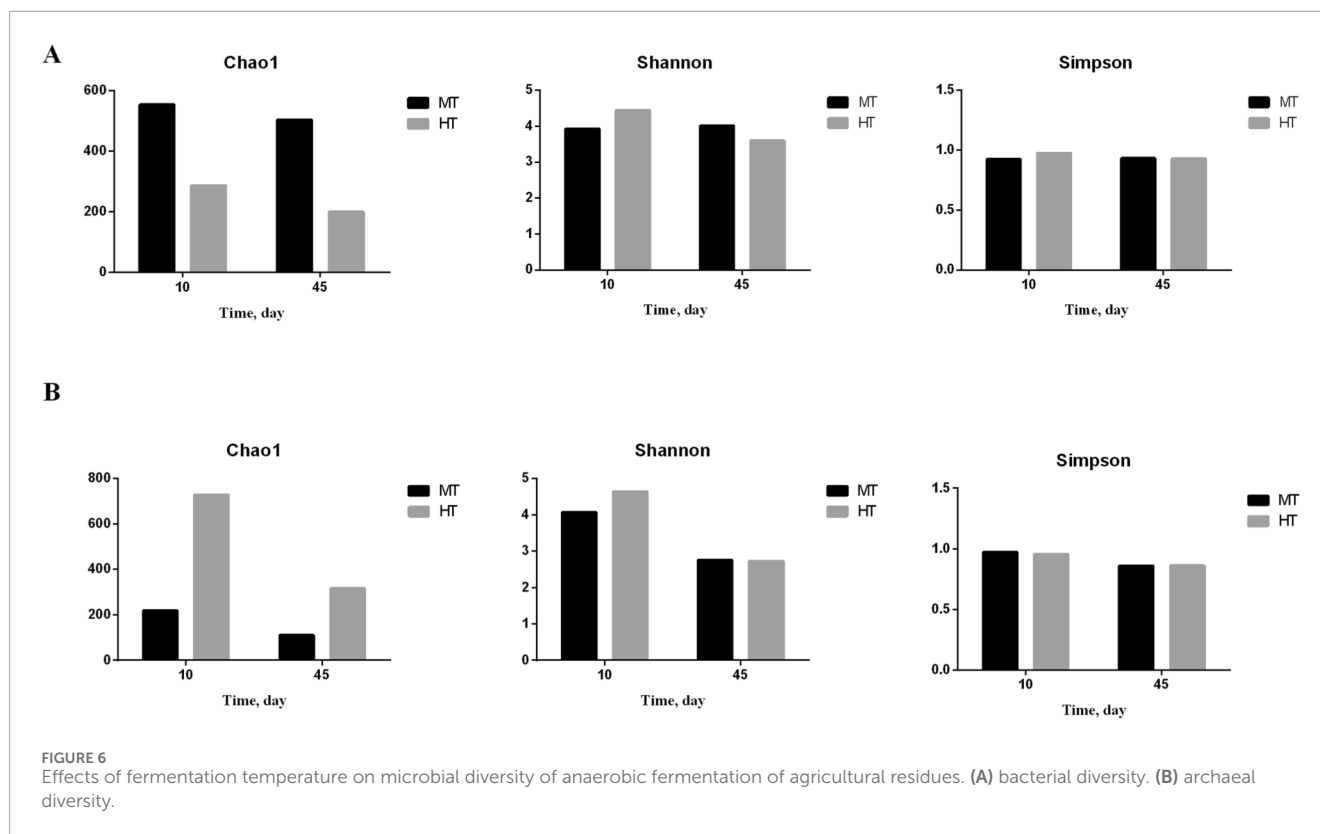
The analysis of diversity indices revealed distinct thermal responses between bacterial and archaeal communities. High temperature acted as an environmental filter, reducing bacterial richness while promoting archaeal diversity. This selective pressure ultimately shaped a functionally streamlined yet less resilient thermophilic community, dominated by specialist taxa adapted to the dual stresses of heat and potential ammonia. In contrast, the mesophilic system fostered a functionally robust and diverse community, characterized by a higher proportion of generalist taxa and complex interaction networks (Supplementary Figure S2), thereby explaining its superior and more stable methane production. To explore the specific differences in bacterial and archaeal communities at different taxonomic levels induced by temperature, the following sections provide a detailed analysis of community composition at both the phylum and genus levels for each domain.

### 3.3.1 Bacterial community

The dominant bacterial communities, comprising the top 10 phyla and genera, are present in Figure 7 (the remainder are classified as “Others”). At the phylum level, the

mesophilic community was dominated by Bacillota, Bacteroidota, Pseudomonadota, and Actinomycetota. A distinct shift occurred under thermophilic conditions, where the top five phyla were Bacillota, Halanaerobiaeota, Actinomycetota, Bacteroidota and Pseudomonadota. In both cases, these dominant phyla collectively represented over 98% of the community, highlighting their strong adaptive capacity to the microenvironment and utilize organic substances and sustain rapid growth (An et al., 2024). Bacillota and Bacteroidota comprise most of the hydrolytic and acidogenic taxa, which efficiently degrade macromolecular organic matter, including protein, lipid and cellulose, and thereby establishing the foundation for the methanogenic stage (Huang et al., 2024). Bacteroidota was more dominant in the mesophilic group, consistent with findings from anaerobic digestion of poultry and cattle manures (Zahedi et al., 2022). By day 45, the relative abundance of Bacillota increased under elevated temperature, in agreement with Pap et al. (2015). Interestingly, Halanaerobiaeota, a phylum of halophilic and halotolerant anaerobic bacterium, was detected exclusively in the HT group, where its relative abundance rose from 2.61% on day 10–11.5% on day 45. This pattern suggests that thermophilic fermentation may promote the formation of locally hypersaline microenvironments, likely due to water evaporation and consequent salt concentration, that favour its growth. In contrast, the salt concentration in the mesophilic system presumably remained below the threshold required to activate its salt-in mechanism, reducing its competitiveness relative to other microbial taxa.

Among the top 10 most abundant genera in the MT group, *Alkaliphilus*, *Caldicoprobacter*, *Fastidiosipila*, *Tissierella* and *Anaerocolumna* (Bacillota), as well as *Fermentimonas* and *Proteiniphilum* (Bacteroidota), were all capable of degrading organic substances such as cellulose, sugars and proteins, exhibiting functional redundancy (Finn et al., 2023). Notably, the relative abundance of *Fermentimonas* in the MT group increased 9.50-fold from day 10 to day 45, suggesting potential syntrophic interactions with hydrogenotrophic methanogens (Finn et al., 2023). The dominant genera in the HT group differed substantially from those in the MT group. Aside from *Corynebacterium* (Actinobacteriota) and *Proteiniphilum* under (Bacteroidota), the remaining seven dominant genera belonged to the phylum Bacillota. As fermentation progressed, the combined relative abundance of these 10 genera in the HT group decreased sharply (by 22.0%), mainly because the abundances of UCG-012, *Keratinibaculum*, *Proteiniphilum*, *Jeotgalicoccus*, and *Lachnospiraceae* NK3A20 each fell below 1% on day 45. *Caldicoprobacter*, *Halocella* and *Tepidimicrobium* are thermotolerant, moderately halophilic and strictly anaerobic bacteria capable of fermenting and utilizing cellulose, starch, and protein to produce VFAs and hydrogen. Among them, *Halocella* became increasingly important for substrate hydrolysis during the later stage of thermophilic fermentation, indicating that a considerable amount of recalcitrant substrate remained. In contrast, in the MT group, a large quantity of organic matter remained on day 10, with *Caldicoprobacter* showing relatively high degradation activity. By day 45, its abundance had declined by 24.7%; however, as system pH increased, the abundance of the mesophilic alkaliphilic bacterium *Alkaliphilus* rose by 28.2%, becoming the dominant taxon responsible for degrading complex organic matter.

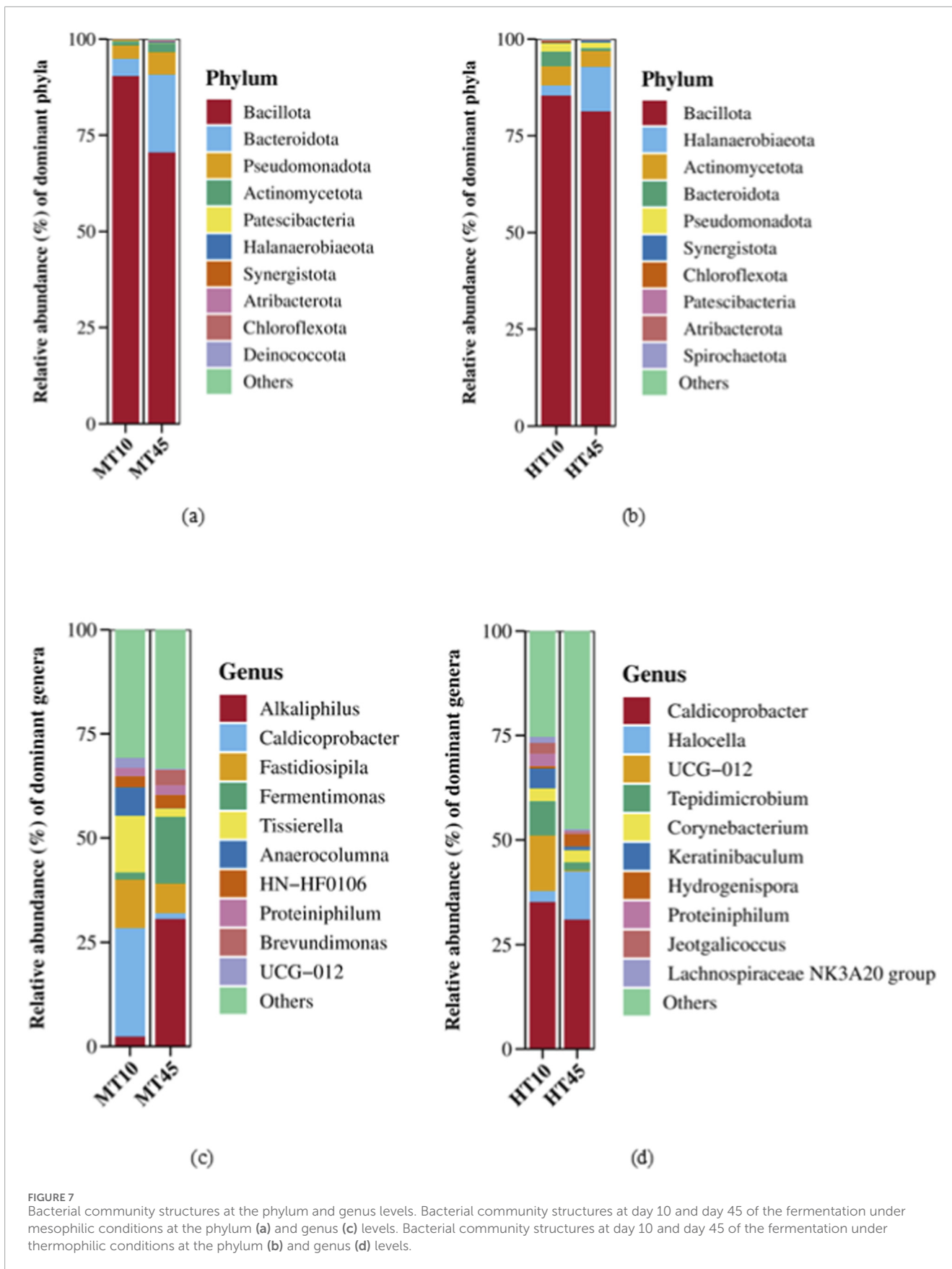


### 3.3.2 Archaea community

During the methanogenesis stage, multiple species of methanogenic archaea taxa are involved in reducing C1–C2 intermediates to methane (Finn et al., 2023). At the phylum level (Figure 8), Methanobacteriota, Thermoplasmatota, Thermoproteota, and Halobacteriota were detected across all samples (Huang et al., 2024), with Methanobacteriota being the most dominant phylum. Thermoplasmatota was abundantly present only in the mesophilic group at day 10, reaching a relative abundance of 31.7%, which likely reflects the activity of specific genera within this phylum, such as Candidatus Methanoplasma and its role as an early adaptive specialist. Members of this phylum are known for versatile metabolism (Zhang et al., 2022), possibly thriving on initial fermentation intermediates. Its decline on day 45 coincides with the rise of Methanobacteriota, indicating a functional succession in the archaeal community. As the system stabilized, specialized hydrogenotrophic methanogens (Methanobacteriota) outcompeted other taxa, optimizing the community for efficient methanogenesis from H<sub>2</sub> or CO<sub>2</sub>. Notably, some Thermoplasmatota are methylotrophic methanogens (Zheng et al., 2022), and their presence suggests a complementary methanogenic pathway under mesophilic conditions that may have contributed to methane production. Thermoproteota exceeded 9% in both the mesophilic and thermophilic groups on day 10, but declined to below 1% by day 45, indicating its role as an early colonizer rather than a keystone lineage for sustained methanogenesis. The elevated abundance of Halobacteriota in the mesophilic group by day 45 was notable. Since this phylum contains methanogens with diverse substrate preferences (hydrogenotrophic, acetoclastic,

and methylotrophic) (Lyu et al., 2025), this observation directly supports the coexistence of multiple methanogenic pathways in the mesophilic system.

At the genus level, Methanobrevibacter was the predominant species across treatments. Under mesophilic conditions on day 10, Methanobrevibacter, Candidatus Methanoplasma and Methanobacterium collectively accounted for 85.4% of the methanogenic community. In contrast, Methanobrevibacter alone represented more than 85% of the relative abundance in each of the three remaining groups. Anaerobic methane production typically proceeds through three metabolic routes, whose energetic efficiency generally follows the order: hydrogenotrophic > methylotrophic > acetotrophic (Niya et al., 2024). As hydrogenotrophic methanogens, Methanobrevibacter and Methanobacterium are characterized by reducing CO<sub>2</sub> to CH<sub>4</sub> using H<sub>2</sub> and/or formic acid serving as electron donors (Ayob et al., 2023; Huang et al., 2024). Members of Candidatus Methanoplasma form syntrophic associations with hydrogen-producing bacteria and are obligate methylotrophs that utilize methylated compounds for methane production; a related methylotrophic genus, Methanomassiliicoccus, was primarily observed in the MT group. On day 10, Candidatus Nitrosocosmicus reached a relative abundance of 7.32% in the mesophilic group, while Candidatus Nitrosopumilus accounted for 8.27% in the thermophilic group. As ammonia-oxidizing archaea within Methanobacteriota, both taxa can tolerate high ammonia concentrations and play crucial roles in nitrification and the nitrogen cycle, enabling them to colonize a wide spectrum of extreme environments (Ayob et al., 2023). By day 45, however, their abundances had declined markedly to below



**FIGURE 7** Bacterial community structures at the phylum and genus levels. Bacterial community structures at day 10 and day 45 of the fermentation under mesophilic conditions at the phylum (a) and genus (c) levels. Bacterial community structures at day 10 and day 45 of the fermentation under thermophilic conditions at the phylum (b) and genus (d) levels.

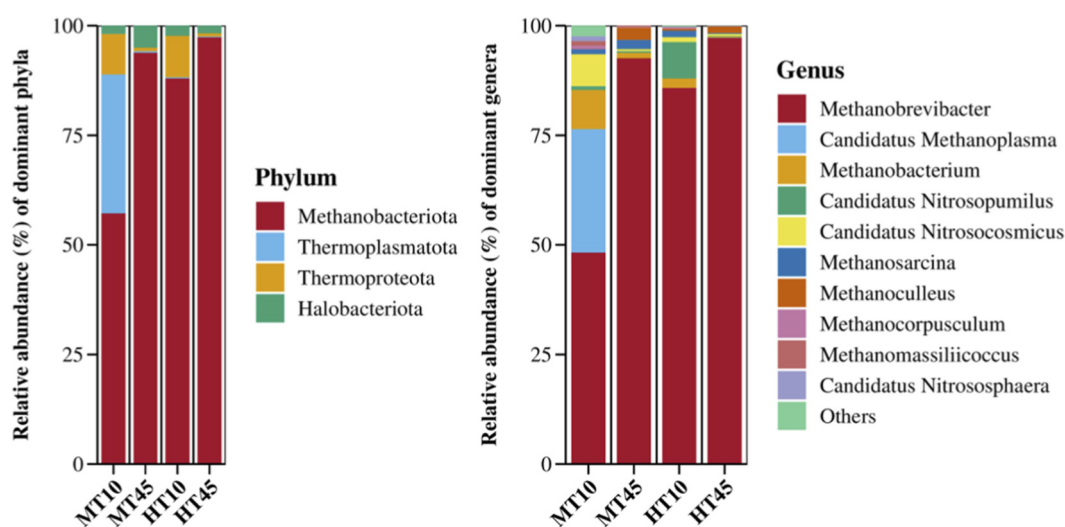


FIGURE 8  
Archaeal community structures at day 10 and day 45 of the fermentation at the phylum and genus levels.

0.5%, which is ultimately beneficial for the stability of the AD system because the nitrite they produced can be toxic to methanogens and anaerobic bacteria. Finn et al. (2023) reported that methanogenesis under ammonia stress is likely supported by interactions among ammonia-tolerant taxa, which are found across all functionally important prokaryotic phyla. Numerous studies have also shown evidence that microbial communities can adapt to total ammonia nitrogen concentrations from 2000 mg/L to 7,000 mg/L (Finn et al., 2023). A consistent observation is that, under ammonia-inhibitory conditions, the dominant methanogenic pathway shifts from acetoclastic to hydrogenotrophic methanogenesis (Finn et al., 2023).

Methanosarcina which exhibits high metabolic flexibility by utilizing multiple methanogenic pathways, served as the primary acetotrophic methanogen in this study. They are capable of oxidizing the carboxyl group of acetate to  $\text{CO}_2$  while reducing the methyl group to methane (Huang et al., 2024). Their abundance increased over time in the mesophilic group but decreased in the thermophilic group, suggesting that acetoclastic methanogenesis was better sustained under mesophilic conditions. Based on the combined analysis of VFAs and ammonia nitrogen levels, it can be inferred that under mesophilic conditions, the methanogenic process was dominated by hydrogenotrophic methanogenesis, with methylotrophic methanogenesis and acetoclastic pathways acting as supplementary routes. In contrast, elevated temperature increased the relative abundance of Methanobacteriota at the phylum level and Methanobrevibacter at the genus level, particularly on day 10. Our findings are consistent with Pap et al. (2015), who reported that increasing temperature from 37 °C to 55 °C enhances hydrogenotrophic rather than acetoclastic methanogenesis. In our HT system, this shift was marked by the reduced abundance of acetoclastic Methanosarcina and the concurrent proliferation of hydrogenotrophic genera like Methanobrevibacter.

## 4 Conclusion

The results of this study demonstrate that stable biogas production is achievable from the anaerobic co-digestion of complex agricultural waste under both mesophilic and thermophilic conditions, but with different features. The thermophilic system exhibited faster organic matter degradation, including enhanced removal of quinolone antibiotics, whereas the mesophilic system achieved a higher overall methane yield, attributable to a more robust and functionally diverse microbial ecosystem. The mesophilic anaerobic digestion involved multiple methanogenic pathways (acetoclastic, hydrogenotrophic, methylotrophic), which granted greater resilience and metabolic flexibility. In contrast, the thermophilic system evolved toward a specialized but susceptible state, dominated by hydrogenotrophic methanogens. Ultimately, ammonia inhibition, high VFA accumulation, and a scarcity of acetoclastic methanogens (e.g., Methanosarcina) collectively resulted in its lower methane production. Therefore, this study confirms that temperature is a decisive engineering parameter that directly shapes microbial community structure and function, thereby determining process performance in the co-digestion of heterogeneous feedstocks. The choice between mesophilic and thermophilic operation is not absolute but should be strategically aligned with specific waste characteristics and operational priorities.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

XZ: Methodology, Conceptualization, Writing – original draft, Funding acquisition. GC: Writing – review and editing, Formal Analysis, Methodology, Investigation. JL: Writing – review and editing, Data curation, Resources. AG: Writing – review and editing. YX: Writing – review and editing, Methodology, Funding acquisition.

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## Conflict of interest

Author YX was employed by Dezhou Weili Engineering Environmental Protection Technology Co., Ltd.

The remaining author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

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### SUPPLEMENTARY FIGURE 1

Heatmap of Pearson correlations between physicochemical characteristics and cumulative methane production, as well as microbial communities based on treatment condition means. NH<sub>3</sub>-N: ammonia nitrogen; COD: chemical oxygen demand; TS: total solids; VS: volatile solids; VFA: total volatile fatty acids; CMP: cumulative methane production. \* Correlation is significant at the 0.05 level; \*\* Correlation is significant at the 0.01 level.

### SUPPLEMENTARY FIGURE 2

Schematic diagram of anaerobic co-digestion of agricultural residues for biogas production under mesophilic and thermophilic conditions. Red arrow: inhibitory effects; green arrow: facilitative effects; black arrow: common pathway.

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