

Effects of adding osmoprotectant on anaerobic digestion of kitchen waste with high level of salinity

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Anaerobic digestion of kitchen waste can be inhibited by a high concentration of Na⁺ in the substrate. The aim of this study was to determine the extent to which the effect of salinity during KW anaerobic digestion could be reduced by adding osmoprotectant. The results show that when Na⁺ concentrations were increased from 0 to 20 g/L, the yield of cumulative methane production decreased from 623.97 to 0 mL/g volatile solids (VS). Adding 2.0 and 2.5 g/L glycine betaine (GB) to reactors during anaerobic digestion of KW with concentrations of 5 and 10 g Na⁺/L were determined to be the optimal dosages to alleviate Na⁺ inhibition; this resulted in 29.07% and 63.49% improvements in methane yield respectively. The maximum soluble chemical oxygen demand reductions that resulted from adding 2.0 and 2.5 g GB/L to kitchen waste anaerobic digestion with 5 and 10 g Na⁺/L were 90.00% and 82.92%, respectively. Adding GB was helpful to both the production and degradation of acetic and butyric acids, which enhanced the rate of conversion of raw material to methane. Archaeal community changes between the medium and high salt concentration groups were revealed by high-throughput sequencing and by adding GB. The most abundant archaeal phyla in all samples were Euryarchaeota and Crenarchaeota. The hydrogenotrophic methanogens showed more salt tolerance than acetoclastic methanogens. The hydrogenotrophic pathway was predominant for methanogenesis of high-level Na⁺ inhibition anaerobic digestion.

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[Key words: Anaerobic digestion; Archaeal community; Glycine betaine; Osmoprotectant; Saline kitchen waste]

The annual discharge of kitchen waste (KW) in China was estimated in 2011 to be more than 60 million tons (1). This discharge rate is expected to grow by 10% annually due to accelerated urbanization (2). Flawed KW management systems result in many food safety issues, and KW disposal methods are attracting extensive social attention in China (3). Among various possible treatment routes, including landfill and incineration plants, anaerobic digestion (AD) of KW into biogas is an effective way to stabilize KW while generating bioenergy. KW, which comprises a variety of complex components (lipids, proteins, carbohydrates, and inorganic salt) and a high moisture content (75%–90%), has mainly good biodegradability performance (80%–90%) during the process of AD. Nevertheless, the process of AD of KW with a high salt content often suffers from system instability. A moderate Na⁺ concentration (100–200 mg/L) is believed to be essential for the growth of methanogens (4), but high concentrations could cause the failure of the AD process. Salt is the most common food flavoring in China and is therefore inevitably accumulated in KW at high levels. A study has reported that the level of salinity in KW was high in China (5).

Currently, KW treatment is dominated by anaerobic fermentation, aerobic composting, and feedstuff, of which the anaerobic fermentation process accounts for more than 50% and will be used

more and more in the future. Much biogas slurry will be produced from KW AD projects. The recirculation of biogas slurry not only reduces its excretion and treatment but also supplements the nutrients and microorganisms in the system and will become one of the main treatment methods. However, due to the high salt content of KW, this recirculation will cause salt accumulation and inhibit the fermentation system.

More specifically, high salt content in the AD system could cause microbial cell plasmolysis and inhibit cell growth due to the rapid increase in osmotic pressure (6). Protoplasmic separation occurs when osmotic pressure increases, and the dehydration of microbial cells causes dramatic restraint and even failure of the AD process. Su et al. (7) reported that during AD, the level of salinity reached 0.5 mol/L in waste-activated sludge, which caused an observable inhibition of methanogenic activity. Studies have also shown that low salinity facilitates the hydrolysis and acidification processes in AD but inhibits methanogenesis. On the other hand, high salinity concentration seriously inhibits both acidification and methanation processes (3,8,9). Due to Chinese eating habits and the constant need of the anaerobic system for feedstock, the accumulation of Na⁺ in the process of KW AD is continuous and inevitable. It is important to avoid the inhibition caused by excess salinity during the process of AD.

Glycine betaine (GB) is an osmoprotectant that protects cell membranes and induces subtle variations in the dynamic properties of proteins (10,11). GB can also improve salt tolerance even in low concentrations. Its mechanism is used by most living cells.

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External osmotic pressure is equilibrated by osmotically active and compatible solutes. In addition, GB can be either synthesized in cells or accumulated from exogenous additions. However, more microbes excel in absorbing exogenous compatible solutes than synthesize them by themselves. Therefore, the addition of osmoprotectants is considered to be an advanced and proper strategy to reduce saline inhibition in highly saline anaerobic digesters containing KW. In one study, GB was used to alleviate the adverse effects of highly saline wastewater during the process of AD (12). Moreover, it has been reported that due to the presence of GB in grass and leaves, adding them to an anaerobic digester can alleviate brackish toxicity (13). However, GB itself has seldom been applied in KWAD to overcome saline inhibition. Therefore, it is necessary to determine the effect of adding GB to an anaerobic reactor containing highly saline KW.

The level of salinity is a crucial factor during microbial metabolism that can affect the structure of the microbial community (14). Compared with hydrolytic bacteria, methanogens have a higher sensitivity to environmental factors like saline concentration (15). Methanogens are the first to be affected by unstable AD system conditions. When the level of salinity increases, acetoclastic methanogens shift to hydrogenotrophic methanogens (16).

In this study, the effects of different Na^+ concentrations were investigated in a pilot test. To determine the effect of adding osmoprotectants to anaerobic reactors with high-level salinity KW, we added five different concentrations of ectoine, GB, and proline to 5 and 10 Na^+ /L solutions of KW AD. We also evaluated the effectiveness of osmoprotectants on various salinity levels and decomposition pathways of volatile fatty acids (VFAs). Finally, 16S rRNA gene sequence analyses were done to investigate changes in AD after the addition of GB.

MATERIALS AND METHODS

Substrate and inoculum A sample of KW was obtained from the canteen of the Beijing University of Chemical Technology. After the removal of debris such as plastic bags, paper cups, and large bones, the collected KW comprised mainly rice, noodles, meat, oil, and vegetables. The sample was then shredded to a particle size of 5 mm using an electric blender (SS3300, Waste King, North Olmsted, OH, USA). The blended KW was stored at -20°C in a clean and tightly closed plastic bottle. The frozen KW was thawed in a 4°C refrigerator for 24 h before being added to the reactor.

The anaerobic seed sludge used in this study was collected from a continuously operated stable anaerobic digester at the Nanma Biogas Station (Shunyi, Beijing, China), which was fed with cow manure and crop straw at $35 \pm 2^\circ\text{C}$. Before we applied it, the anaerobic seed sludge was inoculated for 7 d to concentrate the activity of the sludge.

The characteristics of the KW and the anaerobic seed sludge used in this research are summarized in Table 1.

Batch anaerobic digestion Batch AD was carried out in 250-mL serum bottles, each with a working volume of 150 mL. The inoculum and substrates were adequately mixed before being added to the digesters. All AD reactors were flushed with pure nitrogen for 60 s to provide an anaerobic condition. Each anaerobic bottle was tightly closed with a butyl rubber stopper and sealed with parafilm. The bottles were then incubated by a thermostatically controlled water bath at $35 \pm 2^\circ\text{C}$ and manually shaken twice per day.

TABLE 1. Characteristics of kitchen waste and anaerobic sludge.

Parameter	Crushed kitchen waste	Anaerobic sludge
Total solid (TS) (%)	23.87 (± 1.02)	8.94 (± 0.91)
Volatile solid (VS) (%)	19.4 (± 0.74)	4.73 (± 0.53)
Total carbon (TC) (%)	48.3 (± 1.83)	19.09 (± 1.02)
Total nitrogen (TN) (%)	2.56 (± 0.32)	2.14 (± 0.35)
C/N	18.9	8.9
Protein (%)	15.32 (± 0.94)	—
Crude fat (%)	23.04 (± 2.17)	2.92 (± 0.15)
Na^+ (g/L)	5.3143	0.0577
Cl^- (g/L)	8.93	0.0577

The organic loading of the KW was 10 g volatile solids (VS)/L, and a food-to-microorganism ratio (F/M, VS) of 1.0 was added to all digesters. During the period of AD, the methane content was measured daily to determine the performance of the process. After this, a drainage method was used to measure the volume of methane. The biogas production was monitored daily by a water displacement method.

Study on anaerobic digestion performance at a high Na^+ concentration To investigate the effects of Na^+ concentrations on AD of KW, five batch reactors with various Na^+ additions were used. First, the concentration of Na^+ in the KW and sludge was measured. Then NaCl was added before AD to adjust the Na^+ concentrations to 0, 5, 10, 15, and 20 g/L. The AD biogas production and methane contents at various NaCl concentrations were then compared with the control.

Effect of glycine betaine on anaerobic digestion of kitchen waste The osmoprotectant, including ectoine, GB, and proline, was separately added into the AD reactor to amend 5 and 10 g/L solutions of Na^+ . Five concentrations of osmoprotectants (0.5, 1.0, 1.5, 2.0, and 2.5 g/L) were set up to examine the effects of osmoprotectants on the AD of KW, and two digesters without osmoprotectants were set as controls for two different Na^+ concentrations at the same digestion condition. Each sample was triplicated with the same condition.

Analytical methods The qualities of all reagents used in this study were analytical grade. Total solids, VS, and soluble chemical oxygen demand (sCOD) were measured according to American Public Health Association standard methods (17). A Thermo Dionex ICS-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the Na^+ concentration. Each test was done in triplicate with the same conditions.

Daily biogas production was measured using the water displacement method, and its composition was analyzed by an SP-2100 gas chromatograph (BeiFen, Beijing, China) with a thermal-conductivity detector and a 2.0-mm stainless column (TDX-01). The concentrations of individual VFAs were analyzed by a gas chromatograph (GC2014, Shimadzu, Kyoto, Japan) with a flame-ionization detector. Detailed protocols for the determination of biogas and VFAs were followed in accordance with De La Rubia et al. (18).

DNA extraction and polymerase chain reaction amplification DNA samples were extracted from the anaerobic reactors on day 5 and day 25 of the digestion period. Then the genomic DNA was isolated from each sample using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA). Genomic DNA coding for the bacterial 16S rRNA V4 region was targeted by a polymerase chain reaction (PCR) forward bar-coded primer 519F (5'-CAGYMGCCRCGGKAAHACC-3') and reverse primer 806R (5'-GGACTACHVGGGTWCTAAT-3'). The PCR reactions were run using a GeneAmp 9700 system (Thermo Fisher Scientific), and we did a preliminary denaturation step at 95°C for approximately 5 min, cycled 30 times at 95°C for 30 s, annealed at 58°C for 30 s, an extension at 72°C for 25 s, and an extension for 7 min at 72°C . Purified PCR products were sent to Sinobiocore Inc. (Beijing, China) for Illumina Miseq PE300 sequencing. The reaction was done using a Real-time PCR 7500 (Thermo Fisher Scientific).

High-throughput 16S rRNA gene sequencing and analysis Each sample matched to bacteria sequencing represented the basic type in the particular barcode of each sample. The barcode and primers were eliminated in subsequent operations. Sequence data were analyzed using a combination of software UPPARSE (19), QIIME (20), and R version 3.4.4. (www.r-project.org/). The low-quality raw 16S rRNA reads were filtered out and trimmed by Trimmomatic (21) with default parameters, and pair-end reads were merged by PandaSeq (22). To characterize the microbial diversity, operational taxonomic units (OTUs) were clustered with a 97% similarity

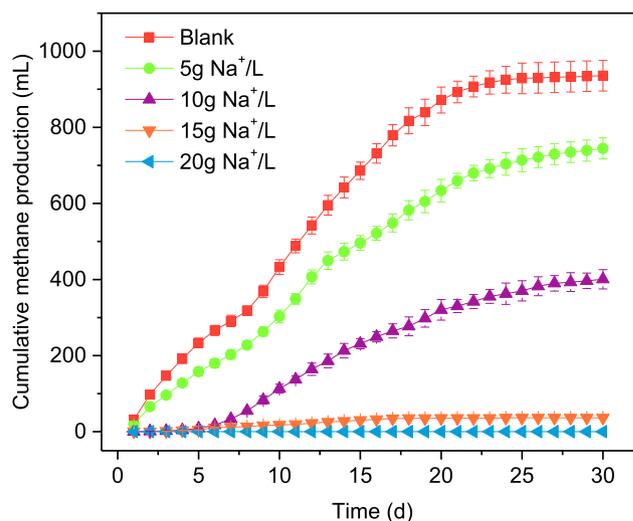


FIG. 1. Profile of cumulative methane production from kitchen waste containing different concentration of Na^+ .

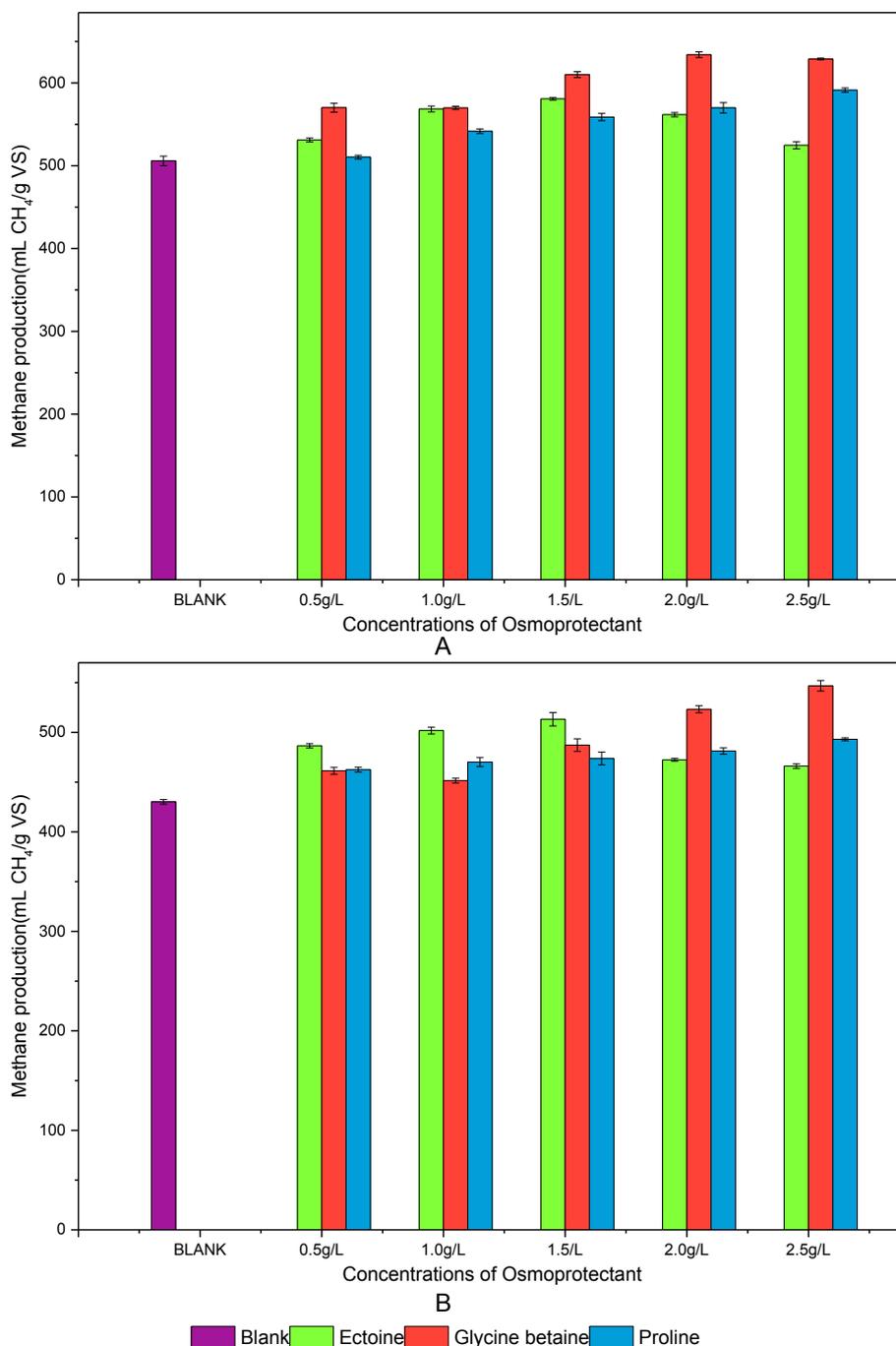


FIG. 2. Effect of different osmoprotectants addition on methane production from kitchen waste between two kinds of dosages of Na⁺: (A) 5 g/L and (B) 10 g/L.

cutoff using UPARSE. The classification of the sequences of each sample was determined individually using the RDP classifier. Furthermore, the relevant RDP pipeline modules were used to determine the diversity and richness indices (23).

Mothur was used to calculate various diversities (richness, evenness, coverage) and to describe the rank abundance curves. Also used for each sample were the Shannon diversity index, the Chao 1 richness estimator, and the abundance coverage-based estimator (ACE). Statistical significance between any two groups was determined with a Wilcoxon rank-sum test, and $\alpha = 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Inhibition of anaerobic digestion of kitchen waste by NaCl The production of methane during the process of AD of KW at various Na⁺ concentrations is displayed in Fig. 1. It shows

that methane production decreased with increasing Na⁺ concentrations. At Na⁺ concentrations of 0, 5, and 10 g/L, the yields of methane reached 935.69, 744.52, and 400.84 mL, respectively. However, at 15 and 20 g/L of Na⁺, methane production was only 36.44 and 0 mL, respectively. This shows that the inhibiting effect of Na⁺ became acute on AD as the concentrations of Na⁺ increased.

Similar methane production trends were obtained between 0 and 5 g/L of Na⁺, and both concentrations showed high methane production on the primary stage after a lag phase of 1 day. However, it showed a clear increase on the 6th day at a concentration of 10 g of Na⁺/L. Zhang et al. (23) found a 7-d lag phase in AD of KW with 35 g/L of NaCl. It induced a 20.4% inhibition of methanogenic activity at an Na⁺ concentration of 5 g/L, whereas it caused a 57.2%

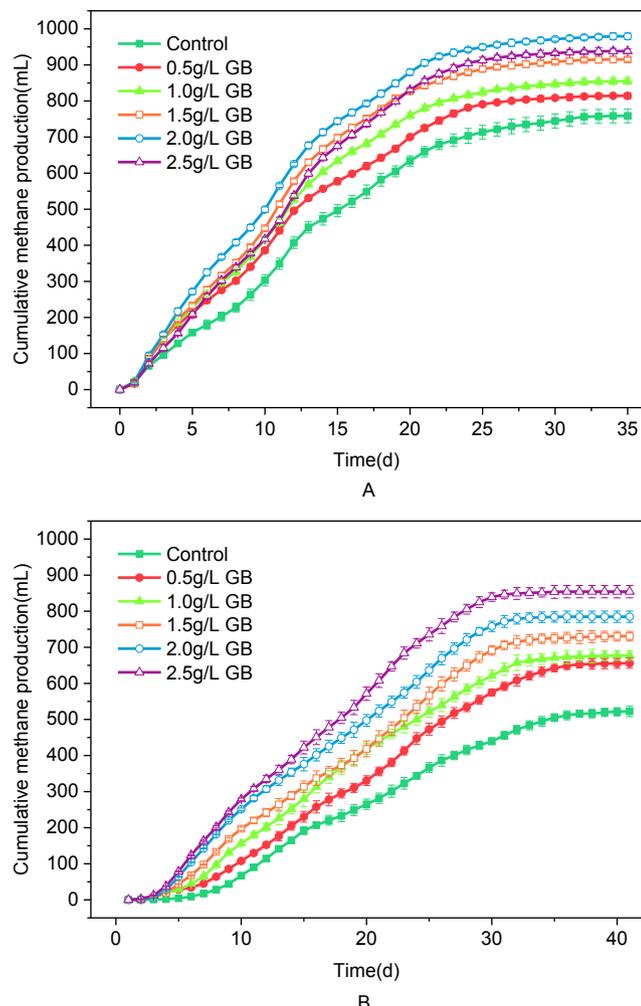


FIG. 3. Profile of cumulative methane production of adding GB into anaerobic digestion of kitchen waste containing 5 g/L (A) and 10 g/L Na⁺ (B).

inhibition of methanogenic activity at a Na⁺ concentration of 10 g/L. The KW at 20 g Na⁺/L without osmoprotectants showed low methanogenic activity. Those results are in agreement with previous studies, where sodium concentrations ranging from 10 to 35 g/L caused a 49.8% and an 89.2% inhibition of methane production (23). Oh et al. (24) showed that the treatment process for highly saline wastewater (10.2 g Na⁺/L) was difficult to scale up due to the unstable process.

Effect of osmoprotectant on anaerobic digestion of kitchen waste with high NaCl Because there were critical inhibitions from Na⁺ in AD of KW, we added three kinds of osmoprotectants, namely ectoine, GB, and proline, to the medium-level (Na⁺ 5 g/L) and high-level (Na⁺ 10 g/L) salt-containing KW to investigate whether the inhibitory effects of Na⁺ could be overcome. Fig. 2 shows the effect of the osmoprotectant addition on daily biogas production from AD with two different salt doses. In the whole process of AD, methane yields of all groups improved after adding osmoprotectants to saline-containing KW AD. In the case of medium salinity, the average methane yields after adding ectoine and proline were 553.40 and 554.45 mL CH₄/g VS, respectively, whereas the sample with GB showed a 602.65 mL CH₄/g VS average methane yield. As for 10 g/L of Na⁺, the group with 2.0 and 2.5 g/L GB added showed the greatest production of methane, which reached 523.19 and 546.83 mL CH₄/g VS, respectively. The groups with 0.5, 1.0, and 1.5 g/L ectoine added

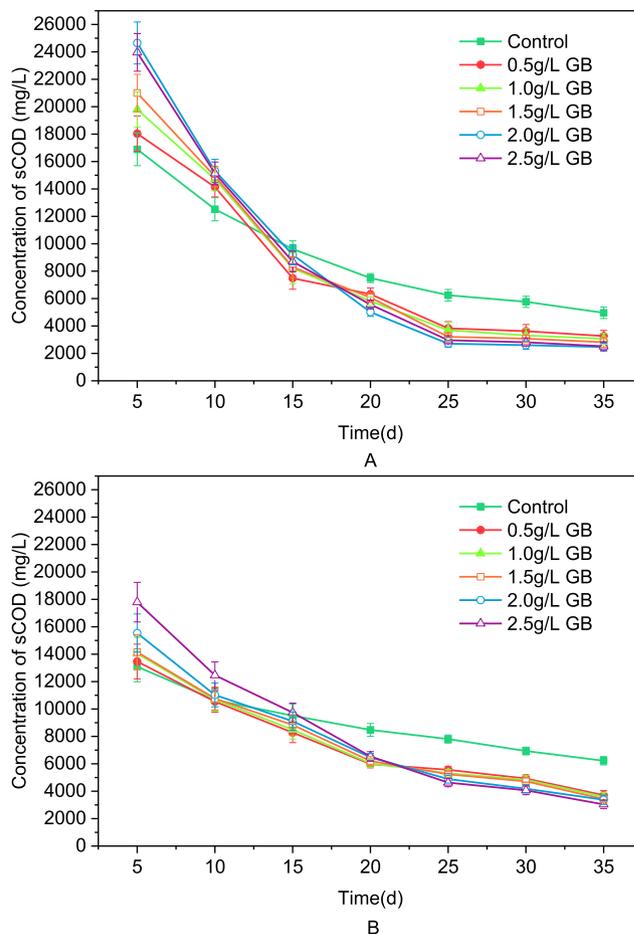


FIG. 4. Profiles of anaerobic digestion of sCOD with different glycine betaine addition on 5 g/L (A) and 10 g/L Na⁺ (B).

showed better performance than the same concentration of added GB, but the production of biogas was still lower than the 2.0 and 2.5 g/L GB groups. On the other hand, the GB was more cost-effective than the ectoine: the GB was 2.66 CNY/g, and the ectoine was 237.6 CNY/g. Compared with other osmoprotectants, the GB showed better performance and cost-effectiveness on alleviating the inhibitory effects of the Na⁺. GB was selected as the optimal osmoprotectant to reduce the inhibition of salinity, and we investigated the underlying mechanism in a subsequent experiment.

At 5 g/L of Na⁺, the net methane production of the KW for GB amounts of 0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/L were 505.80, 570.17, 569.87, 610.14, 652.85, and 625.54 mL CH₄/g VS, respectively. The methane production of the reactor with high salinity showed an observable difference in the trend of production. Obviously, 2.0 g/L exogenous GB was determined to be the best amount to add to the reactor, and it increased methane production by 63.49%. We assumed that a small quantity of osmoprotectants initially existed in the KW, which enabled the KW to resist slight salinity. The most effective concentration of GB for AD of KW was 2.0 g/L at 5.0 g Na⁺/L and 2.5 g/L at 10.0 g Na⁺/L.

Fig. 3A shows the daily methane yield of KW AD with GB additions of 5 and 10 g Na⁺/L. The cumulative methane production of the control group was 758.70 mL. A 28.94% greater incremental production of cumulative methane than that of the control group was obtained when the concentration of GB was 2.0 g/L. Notably, the methane yield of the 2.5 g GB/L addition showed decreasing trends due to the adverse effects of superfluous addition.

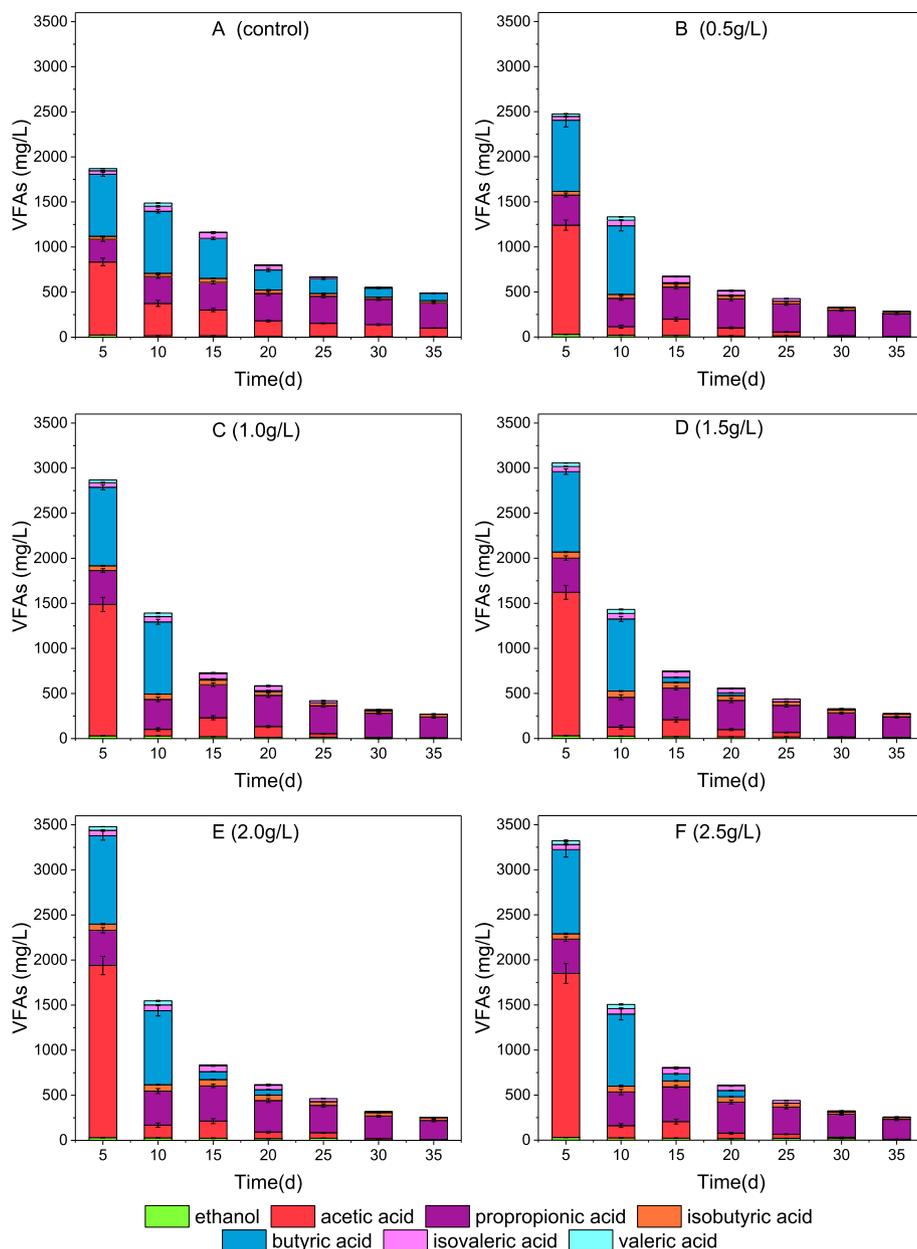


FIG. 5. Volatile fatty acids of control (A) and adding 0.5 (B), 1.0 (C), 1.5 (D), 2.0 (E) and 2.5 g/L glycine betaine (F) into the anaerobic digestion of kitchen wastes containing 5 g/L Na^+ .

As the concentration of exogenous GB increased, the inhibition of the high salinity to the AD of KW decreased. The maximum cumulative methane production was 563.5 mL/g VS when 2.5 g GB/L was added; a 61.76% increase over the control (Fig. 3B). However, the yields of methane were lower than those in the medium salinity with the same volume of GB added. This phenomenon might be explained by the stress of the salt being alleviated by the addition of exogenous GB, which promoted cell metabolism in the circumstance of high salinity (10,25).

Consequently, adding exogenous GB could shorten the lag phase of an anaerobic reactor with a 10 g/L Na^+ concentration. It was apparent that the methanogenesis of KW with the addition of both 2.0 and 2.5 g/L GB was substantially promoted after a lag phase of 3 d. In contrast, the lag phase of other groups was much longer, especially the control (6 d). Gebauer (25) also found that adding GB shortened the lag phase in both glucose and KW AD with 35 g of NaCl/L . Some early studies also reported similar results where

adding GB can shorten the lag phase and the fermentation progress as well (9,16).

A high concentration of Na^+ could cause hyperosmotic stress, which leads to loss of the intracellular water of the methanogens and decreases the dehydrogenase activity in the microorganisms (23). One explanation is that GB can protect microorganisms, is conducive to the stabilization of methanogen enzymes, and improves the biological activities on AD with salt stress (11,26).

Effect of exogenous glycine betaine on the sCOD Most macromolecular organic matter like proteins and carbohydrates are initially insoluble in water; however, these substances compose most of the KW. They decompose into soluble molecules before the process of fermentation. Day 5 illustrated various decompositions of the insoluble organic matter in various GB additions (Fig. 4). At a 5 g Na^+/L concentration, the sCOD concentration reached its peak value (23,962 mg/L) in the reactor with 2.0 g GB/L, which was

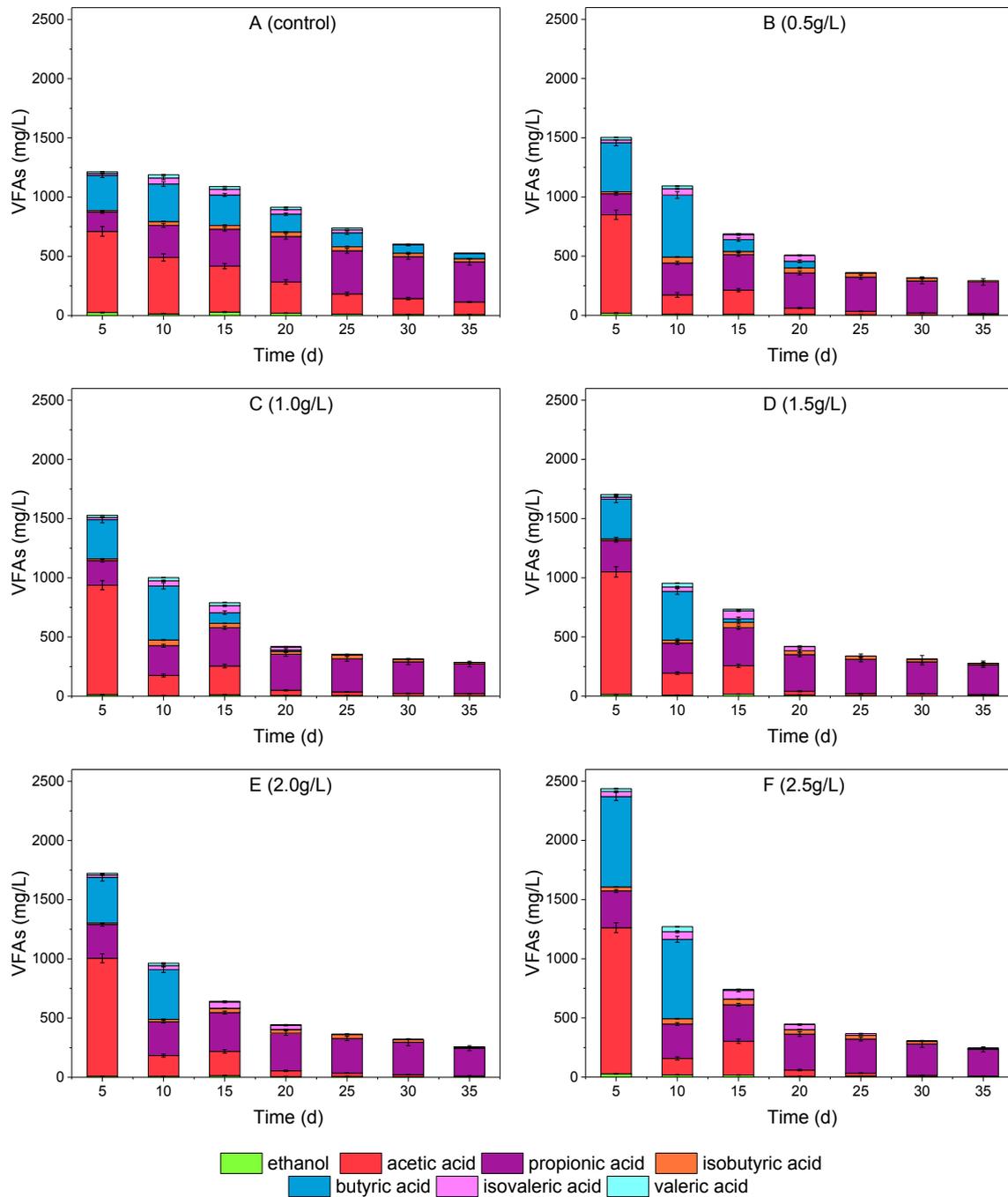


FIG. 6. Volatile fatty acids of control (A) and adding 0.5 (B), 1.0 (C), 1.5 (D), 2.0 (E) and 2.5 g/L glycine betaine (F) into the anaerobic digestion of kitchen wastes containing 10 g/L Na^+ .

41.87% higher than that of the control. Adding GB could have improved the decomposition of the macromolecular organic matter, which was inhibited by substrate salinity. As a result, the fermentation of the soluble organic matter could have been improved. The maximum sCOD reduction (22,186 mg/L) was obtained with 2.0 g/L GB, whereas only 11,928 mg/L reduction was achieved with the control (Fig. 4A). The maximum sCOD reduction rate reached 90.00%, with only a 70.63% reduction rate on the control. Fig. 4B shows a clear decrement of initial sCOD concentration on all the 10 g Na^+ /L groups compared with 5 g Na^+ /L, which was in the range of 13,087–17,795 mg/L. This can be explained by hyperhaline retarding the decomposition of the macromolecular organic matter. Also, adding 0.5–1.5 g/L GB had negligible effects on the decomposition of the macromolecular

organic matter. The decomposition rate of the raw material with an addition of 0.5–2.5 g/L GB ranged from 72.46% to 82.92%, whereas the decomposition rate of the control was 52.43%. As a result, the decomposition of both the macromolecular material and the degradation of the sCOD were elevated by adding exogenous GB to the KW AD, inhibiting the salinity.

Effect of exogenous glycine betaine on VFA concentration To obtain favorable conditions for microbial metabolism and system stability of the digester, the degradation of VFAs should reach a level sufficient for the reactor to maintain an optimal pH level. As shown in Figs. 5 and 6, maximum values of VFAs during AD were reached in 5 d and then decreased for all groups. At 5 g/L Na^+ (Fig. 5), the maximum accumulation of VFAs

TABLE 2. Results of 16S rRNA OTU reads, presenting the coverage, the alpha diversity indices.

Sample	Sampling date	Na ⁺ concentration	GB concentration	OUT reads	Ace	Chao	Shannon index	Simpson index	Coverage
M5	5	5	0	305	310.4981	309.7895	3.59023	0.067999	0.999867
M25	25	5	0	251	275.8475	281.5652	3.303278	0.102139	0.998664
MA5	5	5	2.0	266	286.6677	293.0667	2.565223	0.211455	0.999696
MA25	25	5	2.0	289	307.1081	308.8947	3.244927	0.085782	0.999855
H5	5	10	0	257	268.8149	264.3077	2.807759	0.146223	0.999711
H25	25	10	0	253	276.4921	286.4615	3.228628	0.082946	0.99977
HA1	5	10	2.5	297	315.4098	312.9545	3.851017	0.041999	0.999765
HA2	25	10	2.5	258	283.5074	284.4	3.574922	0.055736	0.999522

(3479.618 mg/L) was obtained in the presence of a 2.0 g/L exogenous GB addition, which was 85.99% higher than the control (1870.865 mg/L). The observed VFA degradations for 0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/L GB were 73.80%, 88.54%, 90.55%, 91.06%, 92.63%, and 92.30%, respectively. The statistics were concurrent with cumulative methane production. This shows that the optimal hydrolysis and methanation occurred with the addition of 2.0 g/L exogenous GB.

Fig. 6 illustrates the effect of adding various concentrations of exogenous GB in the KW during AD with 10 g/L Na⁺. The maximum value of VFA accumulation on AD of KW with 10 g/L Na⁺ was 2437.27 mg/L, which was lower than that of the sample containing 5 g/L Na⁺ due to the more severe inhibition caused by the higher Na⁺ content. The range of VFA degradation was between 80.56% and 90.00% when exogenous GB was added, whereas the VFA degradation of the control was only 56.73%. This shows the similar restorative effects on VFA degradation with an added GB of 5 and 10 g/L Na⁺. We concluded that the most likely reason that GB can promote methane yield on KW AD with salt was that the GB has an effective recovery of the degradation of VFAs.

Acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid are the main types of acids generated during the fermentation of KW (27). Each type of acid shows a different effect during AD because acidogenic fermentation has multifarious metabolic pathways (28). Moreover, in our study the prevailing pathway alterations were affected in the different periods of KW AD by adding GB, which affected the production and distribution of VFAs. Especially from day 5 to day 10, acetic acid was degraded as the main substrate and was rapidly converted into methane and carbon dioxide with the addition of exogenous GB. The cumulative methane production of the control was diminished by the tardy degradation of acetic acid. Simultaneously, the concentrations of the propionic and butyrate acids were changed insignificantly even with supplementary GB. With regard to the

degradation of acetic acid, the rates of degradation were 80.56%–90.00% for groups with added GB, while the control rate was 43.27%. These results show that the addition of GB effectively ameliorated the inhibition of saline to acetoclastic methanogen metabolism. The extent of propionic acid degradations by adding GB was from 24.3% to 45.7%. Nevertheless, the propionic acid was increased by 12.1% without GB addition. This confirmed that propionic acid was accumulated without adding exogenous GB, indicating that the degradation of propionic acid was promoted by adding exogenous GB, thereby improving methane production.

The GB addition enhanced the hydrolysis and acidogenesis of the KW, especially for acetic acid and butyrate acid producers, and played a lesser role in the propionic acid decomposition pathway. These indicated that GB exerted various effects on different anaerobes resisting high osmotic pressure. On one hand, GB was beneficial for both acetic acid and butyrate acid utilizers and acetoclastic methanogens, whereas it had a negligible effect on the propionic acid. This result concurs with a previous study finding that adding 1 mM GB to a KW AD with 35 g/L NaCl could promote acetic acid decomposition, but had no appreciable effect on the propionic acid (12). On the other hand, hydrolysis and acidogenesis were limited by adding extra GB, but apparently no adverse effects were discovered on the methanogenesis.

As to the influence of 10 g Na⁺/L on the methanogenic step, the degradation rate of acetic acid was inhibited. The average degradation rate of acetic acid on adding exogenous GB was 99.90%, but 84.55% for the control. This result shows that salinity concentration is an important factor that can affect the activities of acetotrophic methanogens. Moreover, propionic acid showed recalcitrant characteristic when the propionic acid degradation rate was low on the control and after adding 0.5–1.0 g/L exogenous GB. Even in the best result, the degradation rate of propionic acid with the addition of 2.5 g/L exogenous GB was 27.34%. Additionally, we found that adding GB to KW during AD with 10 g Na⁺/L had positive effects on

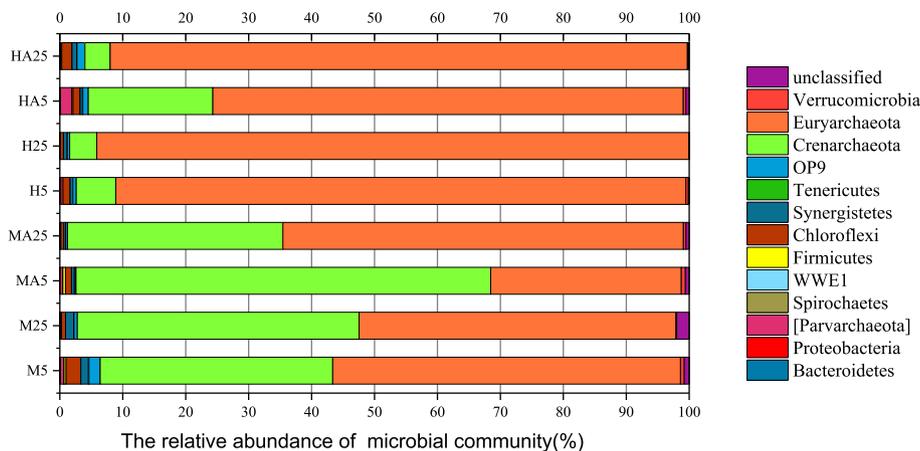


FIG. 7. Taxonomic composition of the microbial community at phyla level in each sample.

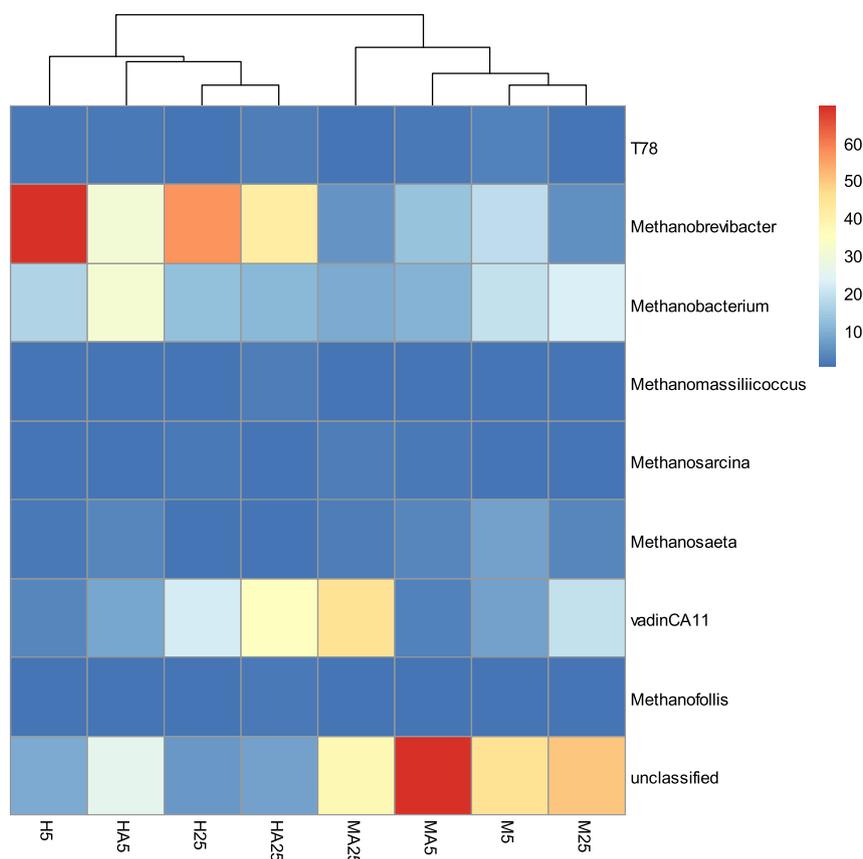


FIG. 8. Heat map of microbial populations in phylum level present at a relative abundance \geq average of 1% in at least one sample. Sample names are listed on the x-axis, and genus names are listed on the y-axis.

butyric acid methanation. Results show that the range of butyric acid on day 5 from the control to the 2.0 g/L exogenous GB addition was 297.78–415.16 mg/L. However, 763.36 mg/L butyric acid was generated by adding 2.5 g/L exogenous GB. In spite of that addition, the acidogenesis pathway of the butyric acid was decreased. This decrease appeared after a GB addition of less than 2.5 g/L, and the generation of butyric acid was abruptly boosted when the concentration of added GB reached 2.5 g/L. This result suggests that the inhibitions caused by high salinity on the hydrolysis and acidification of acetic acid and butyric acid were quite different.

Effect of additional glycine betaine on the archaeal community structure To obtain insights into the relation between the saline concentrations, the volume of GB addition, and microbial activity, we analyzed the microbial communities during AD. For eight of the obtained samples, the total 16S rRNA gene sequencing gave approximately 251–305 OTUs after checking for chimera and singletons. Before the analyses, Good's coverages were calculated to confirm the representativeness of the OTU set. As shown in Table 2, all Good's coverages were equal to or greater than 99%, thereby confirming the representativeness of all samples. The Chao, Shannon diversity, and ACE indices had similar results. This shows that the exogenous GB positively affected the diversity of the archaeal community. Moreover, the higher salinity had no significant effect on the archaeal community.

With regard to the archaeal communities, the category of sequences of each sample at the phylum level is shown in Fig. 7. Clear microbial successions in the phylum level were obtained from four different groups. The most abundant archaeal population phyla were Euryarchaeota and Crenarchaeota. They often co-occurred in the same AD systems and had cooperating synergistic interactions

(29). Euryarchaeota, which include all known methanogens, were more dominant in the 10 g Na⁺/L groups. The abundance of Euryarchaeota in the 10 g Na⁺/L groups was 74.76% (HA5) to 94.09% (H25), but only 30.28% (MA5) to 63.63% (MA25) in the 5 g Na⁺/L groups. We supposed that Crenarchaeota had worse salt tolerance than Euryarchaeota. In spite of the enhancement of Crenarchaeota in the 10 g Na⁺/L on day 5 by added exogenous GB, it was temporarily inhibited at the end due to the overwhelming salinity inhibition.

Further revealing the archaeal shift under high salinity inhibition and GB addition, the distribution of archaea at the genus levels was considerably different among the four groups (Fig. 7). Combining the taxonomic distribution of bacterial diversity at the phylum level (Fig. 8), most of the unclassified were contributed by uncultured Crenarchaeota. The genera *Methanobrevibacter*, *Methanobacterium*, and *VadinCA11* were predominant in the eight groups. *Methanobrevibacter* and *Methanobacterium* are known to exist in most common mesophilic AD systems as hydrogenotrophic methanogens (30–33). However, due to the higher Na⁺ (34), the acetoclastic methanogens, such as *Methanosaeta* and *Methanosarcina*, were rarely detected. With higher Na⁺ concentrations, hydrogenotrophic methanogens demonstrated positive correlations, whereas acetoclastic methanogens were more vulnerable. Especially, the relative abundance of *Methanosarcina* ranged from 0.2% to 1.4%. Simultaneously, the increase in concentration of Na⁺ during AD as a result of the sharp decrement of *Methanosaeta* was obtained in all groups. According to Ventorino et al. (34), acetoclastic methanogens yielded more methane than hydrogenotrophic methanogens (35). The relative abundance of *Methanobrevibacter* ranged from 30.07% (HA5) to 69.24% (H5) in the 10 g Na⁺/L solution and decreased with decreasing Na⁺. Remarkably, the relative

abundance of *Methanobrevibacter* ranged from 4.41% (MA25) to 18.19% (M5) in the 5 g Na⁺/L solution (Fig. 8). This result indicates that *Methanobrevibacter* showed more salt tolerance than other hydrogenotrophic methanogens. This result is opposite to that of Wang et al. (16), whose study suggested that hydrogenotrophic methanogens showed lower resistance to high salinity than acetoclastic methanogens. *VadinCA11* from *RCC Thermoplasmata* were the main genera in MA25 (44.62%). Its energy resource is methylamine, and it has not proved to be a hydrogenotrophic methanogen (36). It is noteworthy that a good salt tolerance of *VadinCA11* was obtained, and the relative abundance of *VadinCA11* increased dramatically from 5.22% (average in all the 5-d groups) to 29.86% (average in all the 25-d groups). We assume that this result occurred because GB was metabolized to methylamine, which is the source of energy of *VadinCA11*. This genus was also detected in other KW AD systems (31).

On the 5 g Na⁺/L level, adding GB could shift the archaeal community from *Methanobrevibacter* to *VadinCA11*. On the contrary, although adding GB to AD with 10 g Na⁺/L could recover the methane production, it had little effect on recovering the archaeal community. This result shows the irreversible inhibition when excess Na⁺ was added in the AD system.

In summary, GB was effective in overcoming the inhibitory effect of salt on AD of KW. Adding GB to the sample with 5 g Na⁺/L was beneficial for both acetic and butyric acid producers and utilizers. GB also played a negligible role in propionic acid fermentation. As to the fate of the archaeal community, acetoclastic methanogens were more able to resist high-level salinity concentrations. These findings are expected to provide deep insight into both the dosage of GB additions to high-salinity KW AD and the changes of VFAs and archaea caused by various dosages of GB additions. In further research, the stability of osmotolerant substances in continuous systems should be focused on.

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