Can salt-tolerant sludge mitigate the salt inhibition to acidogenic fermentation of food waste? Insight into volatile fatty acid production microbial community

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Abstract

For treatment of saline wastewater, the feasible approach to mitigate the salt inhibition is using the acclimated salt-tolerant sludge. The aim of this work was to verify if the use of the acclimated sludge (AS) also could alleviate salinity stress on acidogenic fermentation of food waste (FW) under saline environment. The responses of volatile fatty acid (VFA) production and the microbial community to salt stress were investigated. Results showed that VFA production was reduced by high salinity (30 g/L and 70 g/L NaCl) compared with the control (0 g/L NaCl), especially for groups inoculated with the AS, whereas inoculating with the non-acclimated sludge (non-AS) caused less reduction. The impact of salinity was seen on VFA production with accumulation of more propionic acid and acetic acid along with traces of butyric acid. Significant shift on microbial community composition occurred upon biomass exposure to salt. The microbial communities of the non-AS and AS groups at the same NaCl concentrations converged over time. The non-AS groups contained a more proportion of the phyla *Bacteroidetes*, *Atribacteria* and *Chloroflexi* at high salt levels. These findings demonstrate that the non-AS was more conducive to VFA production due to the presence of higher proportions of hydrolytic and fermenting bacteria.

Statement of Novelty

Although anaerobic digestion (AD) would be the most cost-effective and sustainable technology, the salinity is considered to be inhibitory to anaerobic biological treatment processes. The recent applications of salt-tolerant cultures for the treatment of saline wastewaters suggest that biological treatment is promising. Previous studies also reported that acidogenic fermentation as the first step of AD process is inhibited under saline conditions. However, no study to date has focused on acidogenic fermentation for volatile fatty acid production from food waste using salt-tolerant sludge. Therefore, there is a need for improved understanding of high salt stress to resource recovery from organic wastes. This understanding can help in the design of an operating strategy to alleviate the inhibition of waste treatment by salinity.

Introduction

Recovering energy and nutrients from food waste (FW) not only constitutes substantial economic opportunity but is also an essential requirement for the sustainable development of human society. Considering the negative environmental impacts of landfilling, incineration, or composting of FW, anaerobic digestion (AD) has been proposed as a relatively cost-effective technology for renewable energy production and waste treatment of this high-moisture and energy-rich material [1-3].

Salt (e.g., NaCl), used as a type of food flavoring, is accumulated in FW in large amounts when the food is processed. The general mass fraction of NaCl in FW in China ranges between 2% and 5%. Na⁺ is an essential element for the cell synthesis, growth, and metabolism involved in anaerobic digestion system. However, high concentrations of salt can result in cell plasmolysis and cell death due to a dramatic increase in osmotic pressure. As a result, the organic compounds in saline wastewaters often are poorly

biodegraded and seriously affected the efficiency of utilization of this valuable resource [4, 5]. Experimental results have shown that a low level of NaCl (5 g/L) improves hydrolysis and acidification, but inhibits methanogenesis, whereas a high level of NaCl (15 g/L) seriously inhibits acidification and methanogenesis processes [6]. Low concentrations of NaCl (5-9 g/L) were also found to increase the production of polyhydroxyalkanoate (PHA), while higher concentrations (13-20 g/L) inhibited cell viability and decreased PHA content [7]. Short-chain fatty acids, also known as volatile fatty acids (VFAs) are important intermediates in the anaerobic digestion and production of PHA. VFAs have attracted a great deal of interests due to their wide range of potential applications, including the removal of biological nitrogen removal, synthesis of bioplastics, and bioenergy production [8]. The presence of salinity also affects the production of VFA. Zhao et al. [9] showed that VFA production increased from 367.6 to 638.5 mg chemical oxygen demand (COD) g⁻¹ volatile suspended solid (VSS) with increasing concentration of NaCl from 0 to 8 g/L. However, further increases in NaCl resulted in severe inhibition of VFA production. Our previous study investigating the using of FW to produce VFAs under different NaCl concentrations found that a maximum VFA production of 0.542 g/g dry weight of FW occurred under a NaCl concentration of 10 g/L NaCl, whereas that under an NaCl concentration of 70 g/L was about 23% lower [10]. In addition, the time required to reach maximum VFA production increased with increasing NaCl concentration.

An approach to overcome these challenges posed salinity is to adapt the anaerobic sludge to high saline environment [11]. However, most related previous studies have focused on the treatment of saline wastewater. The various studies on the treatment of saline wastewater through activated sludge have reported different performances due to the differences in processes used and wastewater types [12], with some studies determining that treatment efficiency increased in saline wastewater [13]. Pierra et al. [14] used sediment with a salinity of 67.4 g/L as inoculum within the treatment of wastewater through activated sludge. Their study achieved the highest yield of hydrogen under the highest NaCl concentration of 75 g/L, suggesting a natural adaptation of the sediment inoculum to salt. In addition, many studies have reported on the ability of halophilic microorganisms to continue growth and metabolism under hypersaline conditions [15, 16]. However, no study to date have focused on VFA production from biomass using acclimated anaerobic sludge. Therefore, there is a need for improved understanding of the response of the microbial community to the high salt stress. This understanding can help in the design of an operating strategy to alleviate the inhibition of waste treatment by salinity.

The sequencing batch reactor (SBR) process is often preferred over the continuous flow process (CFP) within waste treatment due to lower energy consumption and enhancement in the selective pressures for biological oxygen demand (BOD), nutrient removal, and control of filamentous bacteria [17]. Batch processes are extensively used to produce specialty chemicals, in biotechnology, and to produce pharmaceutical and agricultural products. Therefore, the present study aimed to evaluate the effect of inoculum on acidogenic fermentation operated in batch mode under highly saline conditions. Duplicate batch reactors were operated at two different high NaCl concentrations of 30 g/L and 70 g/L to observe

the impacts of non-acclimated sludge (non-AS) and acclimated sludge (AS) as inoculum on the product spectrum, the type of acidogenic fermentation, and the respective microbial community.

Experimental

Substrates and Inoculum

FW, containing rice, noodles, vegetables, meat, and tofu, was compounded based on the characteristics of similar FW previously collected from a canteen at Zhejiang Gongshang University (Hangzhou, China). The ratio, source and pretreatment of the substrate were consistent with previous study [10]. Two kinds of anaerobic granular sludge were used as inoculum. The non-acclimated anaerobic sludge (non-AS) from an up-flow anaerobic sludge bed (UASB) reactor at the Snow Beer Brewery in Hangzhou, China. To promote an active bacterial population, the sludge was incubated at ambient temperature with a nutrient solution before inoculation. The acclimated anaerobic sludge (AS) was taken from a lab-scale anaerobic reactor which was used to treat saline wastewater with a NaCl concentration of 30 g/L running for 156 days. Table S1 shows the main characteristics of FW and the two kinds of anaerobic sludge used in this study.

Batch Fermentation Tests

Laboratory-scale batch tests were conducted in brown 1000-mL wide-mouthed bottles with a working volume of 500 mL, capped with a rubber stopper. The reactor material used in the present study consisted of a mixture of 28 g of FW and 7 g of anaerobic sludge (dry weight). The reactors were inoculated with the non-AS or the AS, dosed with different quantities of NaCl to obtain material NaCl concentrations of 30 or 70 g/L. A reactor with non-AS and with no additional NaCl for acidogenic fermentation was used as the control (non-AS_0). Table 1 shows more details on the experimental design. The experimental temperature was maintained at 30±2°C and pH was maintained at 6.0 by the addition of 4.5 M H₂SO₄ or NaOH during the experiment, based on our previous study [18]. Redox potential (ORP) ranged from -100 to -200 mV [19]. All fermentation tests were conducted in duplicate.

Table 1. Experimental groups

Analytical Methods

Sample contents of sugar, lipids, soluble protein, total suspended solids (TSS), volatile suspended solids (VSS), total organic carbon (TOC) , lactate, and VFAs (C2-C5) contents of the samples were determined using methods previously described [10]. Aliquots of the fermentation broth were removed from each reactor at specified times during the fermentation process. These samples were centrifuged at 10,000 rpm for 5 min. The supernatant was then passed through a filtration membrane with a pore size of 0.45 µm, following which the solubility indices (besides for TOC, lactate, and VFA) were measured. The

Groups	NaCl concentration (g/L)	non-acclimated sludge (non-AS)	Acclimated sludge (AS)
non-AS_0 (Control)	0	+	/
non-AS_30	30	+	/
non-AS_70	70	+	/
AS_30	30	/	+
AS_70	70	/	+

solubility indices of TOC, lactate, and VFA were measured after filtering the supernatant through a filtration membrane with a pore size of 0.22 μm .

Bacterial Community Analysis

Samples were collected from all reactors on day 0, day1, and every other day thereafter. The methods used to characterize the bacterial community was consistent with the previous study [10]. In brief, samples were collected from all reactors at specific times during the fermentation process. Genomic DNA was extracted using a DR4011 kit (Bioteke, Beijing, China) according to the instructions of the manufacturer. The methods used to determine the quality (A260/A280) and quantity (A260) of the extracted genomic DNA, to amplify the extracted DNA, and to evaluate the bacterial community have been described in our previous study [10]. Samples were processed through MiSeq high-throughput sequencing (Illumina, San Diego, CA, USA), following with the obtained sequences were aligned and grouped into operational taxonomic units (OTUs) with 97% similarity. Sequences were then phylogenetically assigned to taxonomic classifications and allocated to phylum, class, and genus levels. Hierarchical cluster analysis was performed using R version 3.1.3 (www.r-project.org).

Results And Discussion

Solubilization and Utilization of Substrates

The anaerobic digestion process typically consists of three steps: (1) hydrolysis; (2) acidogenesis; and (3) methanogenesis. The degradation of complex polymers in FW such as lignocellulosic materials, lipids, and proteins to smaller molecules requires the most time during the AD process [20]. Soluble chemical oxygen demand (SCOD) is an important intermediate in the metabolic pathway of AD due to its influence on the yield of VFAs through linking hydrolysis and acidogenesis. As shown in Fig.1A, although there was a difference in SCOD on day 0 of fermentation, that of the non-AS groups significantly exceeded those of the AS groups by day 7. SCOD in the non-AS groups increased rapidly on day 1, almost reaching the maximum value. The maximum value of SCOD was obtained in the AS groups until day 9. These results indicated that non-AS groups produced more soluble organic matter compared to the AS groups at the

hydrolysis stage. It was assumed that many hydrolyzing bacteria are not NaCl-tolerant and are eliminated during the acclimation process.

Soluble substrates such as sugars and proteins are intermediates within a dynamic process in which they are simultaneously and continuously dissolved from FW and consumed to produce other products such as VFAs. As shown in Fig.1B, soluble sugars in the non-AS groups were significantly higher than those in the AS groups by a factor of 2.4-3.4 during the early stages of fermentation. Therefore, it can be speculated that the non-AS groups contained greater quantities of soluble organic matter compared to the AS groups and that organic matter dissolved more rapidly in the non-AS groups. As shown in Fig.1E, the experimental data were consistent with the first-order kinetics equation for the reduction of soluble sugar during fermentation ($R^2 > 0.88$).

The concentration of soluble sugar at any fermentation time can be calculated by: (1)

where C_0 is the initial concentration of soluble sugar, t is the fermentation time, C_t is the soluble sugar concentration at t time, and k_1 is the reaction rate constant. As shown in Fig.1E, the salt concentration had a considerable inverse relationship with the degradation rate of soluble sugar. The rank of the reactor treatments in terms of the rate of soluble sugar degradation was non-AS_0 > non-AS_30 > AS_30 > AS_70 > non-AS_70. At a lower salt concentration, the rates of soluble sugar degradation of the non-AS groups exceeded that of the AS groups. This result can likely be attributed to larger abundances of hydrolytic and fermenting bacteria in the non-AS groups (section 3.3.1). The use of the non-AS as an inoculum resulted in the production of greater quantities of soluble sugar. At a high NaCl concentration of 70 g/L, there was a slightly higher soluble sugar degradation rate in AS_70 compared to that in non-AS_70, which was likely due to the former being better adapted to a high salt environment.

As is shown in Fig.1C, soluble proteins increased with increasing concentration of NaCl. Interestingly, the contents of soluble protein in the non-AS groups during early fermentation exceeded those in the AS groups. However, these differences reduced as the fermentation process progressed, with finally the AS groups contained more soluble protein compared to the non-AS groups. It is likely that NaCl resulted in high extracellular osmotic pressure, thereby triggering the rupturing of non-AS cells and resulting in an increase in soluble protein during the early fermentation stage. The non-AS groups gradually adapted to the high NaCl concentrations by a later fermentation period, resulting in an acceleration of the rate of protein degradation. On the other hand, high NaCl concentrations inhibited the degradation of soluble protein, thereby resulting in high concentrations of soluble protein being maintained.

Proteins in FW are first degraded to amino acids and then to ammonium, VFAs, and other products. The level of ammonia nitrogen is generally used to assess the level of protein degradation. As shown in Fig.1D, the changes in ammonia nitrogen concentration indicated that under the NaCl concentrations of 30 g/L and 70 g/L, the ammonia nitrogen concentrations in the AS groups significantly exceeded those in the non-AS groups by a factor of 1.6-1.8. This result could be attributed to two possible factors: (1) under higher NaCl concentrations (30 g/L and 70 g/L), the AS increased the degradation of proteins; (2) the

metabolism of amino acids in the AS groups at high salt levels (30 g/L and 70 g/L) shifts after acclimation from mainly decarboxylation to mainly deamination, thereby inducing the release of ammonia nitrogen. Table S2 presents the durations of ammonia nitrogen production in different reactors. The ammonia nitrogen release rate was as follows: AS_30 > AS_70 > non-AS_30 > non-AS_70 > non-AS_0. As reported in our previous result, the release of ammonia nitrogen increased linearly with fermentation time [18], regardless of whether AS or non-AS was used. However, the rates of ammonia nitrogen release in the AS groups exceeded those in the non-AS groups, indicating that salt-tolerant sludge promoted the release of ammonia nitrogen.

Product Spectrum

Figure 2A shows that VFA accumulation in the control group (non-AS_0) increased rapidly from day 3 to day 12 and reached a maximum of 25.1 g/L on day 12. The time required to reach maximum VFA production in all groups was delayed under NaCl concentrations of 30 g/L and 70 g/L. The maximum VFA production for non-AS_30 and AS_30 groups occurred on day 15, whereas a shorter duration was required to reach the maximum VFA production in the AS_70 group compared to that in non-AS_70 group. VFA production generally decreased with increasing salt concentration, with the rank of the reactor treatments according to VFA production being non-AS_0 (25.1 g/L) > non-AS_30 (24.4 g/L) > non-AS_70 (22.6 g/L) > AS_30 (21.0 g/L) > AS_70 (20.5 g/L). Interestingly, the VFA produced by the non-AS groups exceeded that of the AS groups. This result could be attributed to the fact that although AS was better adapted to high NaCl concentrations, the abundance of acid-producing fermenters was not significantly increased (see Section 3.3.2).

The products of fermentation were different under different NaCl concentrations (Fig.2). The control group (non-AS 0) mainly produced acetic acid up to a maximum concentration of 14.9 g/L, equating to 70.1% of the total VFAs produced (Fig.2B). The maximum acetic acid produced by the non-AS group of 13.4 g/L was significantly higher than that of the AS group of 10.8 g/L under a NaCl concentration of 30 g/L. However, the differences in acetic acid production between the AS and non-AS groups decreased with increasing NaCl concentration up to 70 g/L, with an acetic acid concentration in both groups of 8.98 g/L. In comparison, higher quantities of propionic acid and lactic acid were produced at higher salt concentrations (Fig.2C and E), consistent with the results of our previous results [10]. Propionic acids produced in AS_30, non-AS_30, AS_70, and non-AS_70 groups accounted for 51.5%, 48.8%, 60.7%, and 62.2% of total VFA, respectively. Therefore, salt concentration had a greater impact on acidogenic fermentation compared with that of inoculum, regardless of whether the sludge was salt-tolerant or not. Fig.2D shows the change in butyric acid for all groups under different NaCl concentrations. The FW in the reactors under high NaCl concentrations showed low production of butyric acid compared with that of the control. However, Sarkar et al. [21] reported the different results and found VFA production with accumulation of more butyric acid (3.04 g/L) and acetic acid (1.17 g/L) along with traces of valeric acid at 40 g/L NaCl. As shown in Fig.2E, lactic acid production increased and the residence time of lactic acid in the reactors increased with increasing NaCl concentration. During the following days, the concentration of lactate fell below detection limits and propionic acid increased, indicating that propionic acid was

produced by lactate fermentation. These results suggested that the type of acidogenic fermentation of FW changed to propionic acid production as the NaCl concentration increased.

Analysis of Microbial Community

Microbial community of inoculums

Then we analyzed the differences in microbial community structure between the AS and non-AS groups. Figure 3 shows the differences in bacterial composition resulting from the non-AS and the AS as an inoculum.

Among the 15 phyla, nine showed extremely significant differences (Fig.3A). *Proteobacteria* (39.7%), *Firmicutes* (27.5%), *Bacteroidetes* (12.6%), and *Chloroflexi* (6.85%) dominated in non-AS groups, whereas *Proteobacteria* (40.3%), *Synergistetes* (14.1%), *Bacteroidetes* (13.2%), *Nitrospirae* (12.5%), and *Firmicutes* (8.54%) dominated in the AS. Significant difference in the abundances of *Firmicutes*, *Chloroflexi*, *Synergistetes*, *Nitrospirae* and *Bacteroidetes* was observed between the non-AS and the AS. *Proteobacteria*, *Firmicutes* and *Bacteroidetes* are obligate or facultative bacteria characterized by high hydrolytic capacities during anaerobic digestion and an ability to produce VFAs from organic compounds [22]. *Firmicutes* was found at relatively high and low abundances in the non-AS and AS, respectively. This result indicated that high NaCl concentration strongly inhibited the *Firmicutes* phylum [23, 24]. Most species in phylum *Chloroflexi* are filamentous bacteria capable of degrading macromolecular organics [25]. The relatively high abundances of *Firmicutes* and *Chloroflexi* in the non-AS groups during the early stage of fermentation might result in hydrolysis rates far exceeding those of the AS groups for fermentation experiments. In contrast, the AS had a relatively higher abundance of *Synergistetes* (14.1%) than that in the non-AS (3.90%), indicating that enriched *Synergistetes* played an important role in the anaerobic fermentation of FW under high salt stress, consistent with the report of Zhang et al. [24]. Interestingly, the relative abundance of *Nitrospirae* increased in the AS. Wan et al. [26] similarly found that phylum *Nitrospirae* was dominant in the hydrogen reactors of thermophilic alkaline fermentation.

Characterization of the microbial communities at the genus level showed that *Thioclava* (24.0%), *Nitrospira* (12.5%), *Blvii28_wastewater-sludge_group* (6.64%) and *norank_f_Synergistaceae* (6.48%) were dominant in the AS (Fig.3B). *Thioclava* (*Proteobacteria* phylum) always isolated from surface seawater [27], marine sediments [28] and sea sand [29]. *Nitrospira* (*Nitrospirae* phylum) populations were detected in hypo- and sub-saline (1.3-12.8 g salt/L) environments and highly alkaline (pH 8.9-10.3) lakes, and were also considerably diverse, representing *Nitrospira* lineages I, II and IV [30]. *Blvii28_wastewater-sludge_group* (*Bacteroidetes* phylum) found in the mesophilic anaerobic reactors treating sewage sludge [31] and saline industrial wastewater [23] were reported as mesophilic acetogens, producing acetate and hydrogen. *norank_f_Synergistaceae* (*Synergistetes* phylum) can improve the hydrolysis acidification process and the acetotrophic pathway [32, 33]. In the non-AS, *unclassified_f_Enterobacteriaceae* (17.5%), *Clostridium_sensu_stricto_1* (13.9%), *Tolumonas* (11.8%), *norank_c_Bacteroidetes_vadinHA17* (6.50%) and *norank_f_Anaerolineaceae* (5.57%) were dominant bacterial groups. Wang et al. [33] reported *unclassified_f_Enterobacteriaceae* (*Proteobacteria* phylum) and *Clostridium_sensu_stricto_1* (*Firmicutes*

phylum) to be the most abundant genus identified during acidogenesis of anaerobic digestion under acidic conditions. *norank_c_Bacteroidetes_vadinHA17* (*Bacteroidetes* phylum) is classified as hydrolytic/fermentative bacteria and plays an important role in the degradation of complex organics [34]. *norank_f_Anaerolineaceae* (*Chloroflexi* phylum) can decompose organic acids into acetate with the production of H₂ in co-culture with H₂-consuming methanogens [32].

NMDS analysis of fermentation process

The differences in microbial community composition were evaluated by comparing the AS with the non-AS groups using a nonmetric multidimensional scaling (NMDS) analysis based on unweighted Unifrac full tree similarity distance (Fig.4). As shown in Fig.4, samples from the reactors with the same inoculum generally clustered more closely and were separated from each other. This revealed a strong difference in microbial community compositions among different groups due to different inoculum and salts. The same inoculated sludge also showed a certain regularity with increasing of NaCl concentration.

Changes in microbial diversity

Table 2 summarizes the results of microbial alpha diversity analysis. The Chao and Ace indices represent microbial richness, whereas the Shannon and Simpson indices represent microbial diversity. Compared with the inoculum, microbial diversity and richness decreased under high NaCl conditions. Microbial diversity and richness decreased first in the control, following which they increased up until the levels of the inoculum. This result demonstrated that microbes in the control were selected through acclimation to new conditions. In addition, the non-AS_30 and non-AS_70 groups showed decreased microbial richness. Although microbial diversity also decreased in both groups, there was an increasing trend in microbial diversity from day 6 to day 15. These results showed although high salinity decreased microbial richness and diversity, the microorganisms showed a capacity to adapt to the saline environment. Microbial diversity and richness increased with time in the AS_30 group, while increased microbial diversity and decreased microbial richness were observed in the AS_70 group. Under the same NaCl concentrations, compared with AS groups, non-AS groups had higher microbial richness but comparable microbial diversity, which is in alignment with the VFA production.

Table 2 Qualified reads, OTU counts, and alpha diversity estimates of microbial populations.

	Samples ^a	Reads	OTU	Shannon	Simpson	Ace	Chao	Coverage
Inoculum	non-AS	47139	486	3.71	0.07	526	524	0.999
Operation	non-AS 6_0	50915	284	1.21	0.60	497	420	0.998
	non-AS 15_0	44437	391	2.83	0.19	500	496	0.998
	non-AS 21_0	45158	449	3.67	0.06	520	522	0.998
	non-AS 6_30	47075	443	2.29	0.36	555	551	0.998
	non-AS 15_30	47037	434	3.50	0.09	516	523	0.998
	non-AS 21_30	37174	343	2.95	0.13	460	466	0.997
	non-AS 6_70	38019	418	2.56	0.20	522	527	0.997
	non-AS 15_70	39759	387	2.82	0.15	476	475	0.998
	non-AS 21_70	37086	367	2.43	0.23	458	466	0.997
Inoculum	AS	48626	418	3.52	0.09	478	490	0.998
Operation	AS 6_30	50738	313	2.09	0.37	390	392	0.998
	AS 15_30	46227	324	2.97	0.13	395	401	0.998
	AS 21_30	42844	331	2.99	0.12	415	415	0.998
	AS 6_70	47094	435	2.86	0.19	517	524	0.998
	AS 15_70	44171	360	2.77	0.20	516	442	0.998
	AS 21_70	41537	373	3.34	0.10	429	442	0.998

^a non-AS and AS represents the seed sludges of non-acclimated anaerobic sludge and acclimated sludge, respectively. The number 6, 15 and 21 represent the sampling day. The number 0, 30 and 70 represent the NaCl concentrations of 0 g/L, 30 g/L and 70 g/L, respectively.

Microbial composition and difference analysis

At the phylum level, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Atribacteria*, *Synergistetes* and *Chloroflexi* were the most abundant phyla in five groups of non-AS_0, non-AS_30, non-AS_70, AS_30 and AS_70, and together they make up more than 95% of the total, as shown in Fig.5A. However, *Firmicutes* (43.4%) and *Bacteroidetes* (27.7%) dominated the control group (non-AS 0). Reactors containing 30 g/L NaCl showed relatively higher abundances of *Firmicutes* (50.8-68.8%) compared to the reactors containing 70 g/L NaCl. But greater abundances of *Proteobacteria* (36.7-60.7%) were observed in the reactors containing 70 g/L NaCl, regardless of the inoculum used. Similar microbial communities were observed in reactors under the same salt concentrations. The abundances of *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Atribacteria* and *Nitrospirae* were significantly different in five groups (Fig.5B). It was worth

noting that the abundance of *Nitrospirae* was remarkably higher in the AS_70 group, compared with the other four groups ($p < 0.002$). The non-AS groups contained a larger proportion of the phyla *Bacteroidetes*, *Atribacteria*, and *Chloroflexi*, which are able to degrade macromolecular organics to VFAs, especially at high salt levels (Fig.S2). This result is consistent with the fact that a little higher VFA production was observed in the non-AS groups.

At the genus level, the microbial compositions of the five groups of non-AS_0, non-AS_30, non-AS_70, AS_30 and AS_70 were significantly different. The *Bacteroidetes* (*Bacteroidetes* phylum), *Veillonella* (*Firmicutes* phylum), *Streptococcus* (*Firmicutes* phylum), *Mangrovibacter* (*Proteobacteria* phylum) and *Candidatus_Caldatribacterium* (*Atribacter* phylum) were the most abundant in five groups of non-AS_0, non-AS_30, non-AS_70, AS_30 and AS_70 (Fig.6A).

Beneficial bacteria of *Bacteroidetes* and *Veillonella*, capable of producing VFAs, were remarkably increased in the non-AS_0 group, compared with other groups, as shown in Fig.6B. The abundances of *Streptococcus*, *Proteus* (*Proteobacteria* phylum) and *Lactococcus* (*Firmicutes* phylum) observably increased both in groups of the non-AS_30 and AS_30, while the non-AS_30 group had higher abundance of *Proteus* than the AS_30 group (Fig.6B). The abundance of *Mangrovibacter* in the AS_70 group was extremely significantly higher than that in other groups ($p < 0.0005$). In the non-AS_70 group, the abundances of *Enterococcus* (*Firmicutes* phylum), *Clostridiisalibacter* (*Firmicutes* phylum) and *Weissella* (*Firmicutes* phylum) obviously increased.

Effect of NaCl concentration on microbial composition difference

As shown in Fig.6B, the beneficial bacteria of *Bacteroidetes* and *Veillonella* significantly increased in the control group. The main by-products of anaerobic respiration by *Bacteroidetes* include acetic acid, iso valeric acid, and succinic acid. *Veillonella* spp., which is well known for its ability to ferment lactate, mainly appeared from day 6 to day 15 during the fermentation (Fig.S1), consistent with the degradation of lactate in the control reactors.

Under NaCl concentration of 30 g/L, the phylum *Firmicutes* mainly contained the genus *Streptococcus* (Fig.6B), which was observed in the non-AS_30 and AS_30 groups (Fig.S3), particularly from day 0 to day 9, following which their abundance clearly decreased with time (Fig.S1). The genus *Streptococcus* encompasses Gram-positive, catalase-negative, facultatively aerobic and homofermentative cocci which produce L(+)-lactic acid as major end product of glucose fermentation [35]. The genus *Proteus* appeared mainly from day 9 to day 21, particularly in the non-AS_30 group (Fig.S1). *Proteus* spp. decompose organic substances and oxidatively deaminate amino acids, hydrolyze urea and exhibit proteolytic activity [36]. Beneficial bacteria of *Lactococcus*, which are homofermentative and are used to produce L (+) lactic acid from glucose, had a higher relative abundance from day 6 to day 9 in both the non-AS_30 and AS_30 groups (Fig.S1). Correspondingly, abundant production of lactic acid was observed from day 3 to day 9 (Fig.2). Further, the microbial composition difference between AS_30 and non-AS_30 groups was analyzed (Fig.S2). The relative abundances of *Candidatus_Caldatribacterium* and *norank_f_Synergistaceae* were significantly higher in the non-AS_30 group. *Candidatus_Caldatribacterium*

played a vital role in the anaerobic fermentation of carbohydrates to VFAs [37, 38].

norank_f_Synergistaceae can improve the hydrolysis acidification process and the acetotrophic pathway [32]. This result supported the fact that more VFAs were produced in the non-AS_30 group.

Under NaCl concentration of 70 g/L, the genus *Mangrovibacter* belonging to the phylum *Proteobacteria* dominated (Fig.6B), which was detected in both the non-AS_70 and AS_70 groups (Fig.S4). Moreover, the abundance of *Mangrovibacter* in the AS_70 group (45.7%) was much higher than that in non-AS_70 group (22.3%). Members of genus *Mangrovibacter* are facultatively anaerobic and nitrogen-fixing bacteria which are slightly halophilic. The optimal NaCl concentration and temperature for growth of *Mangrovibacter* were 1% and 30°C, respectively [39]. Li et al. [40] found that *Mangrovibacter* was the dominant bacteria in halotolerant aerobic granular sludge for treating saline wastewater with a salinity of 3%. In addition, the abundances of *Enterococcus*, *Clostridiisalibacter* and *Weissella* were significantly higher in the groups with 70 g/L NaCl (Fig.6B). Růžicková et al. [41] reported that *Enterococcus* is a large genus of lactic acid bacteria and can adapt up to 6.5% NaCl. *Clostridiisalibacter* is a Gram-positive moderately halophilic strictly anaerobic and motile bacterial genus with an optimum at 50 g/L NaCl [42]. *Weissella* are obligately heterofermentative bacteria that produce CO₂ from carbohydrate metabolism, with lactic acid and acetic acid being the other major end products of sugar metabolism [43]. Several genera also were identified accounting for most of the differences in microbial community between the non-AS_70 group and AS_70 group, including *Mangrovibacter*, *norank_c_Bacteroidetes_vadinHA17*, *Thioclava* and *Nitrospira* (Fig.S4). *norank_c_Bacteroidetes_vadinHA17* was more dominant in the non-AS_70 group. Greater abundances of halophilic bacteria were found in the AS_70 group, including the genera of *Mangrovibacter*, *Thioclava* and *Nitrospira*, and less hydrolytic/acidogenic bacteria could cause low VFA production.

Therefore, salt inhibition seems not to be dependent on the inoculum. A selection of *Streptococcus* and *Mangrovibacter* as a result of gradual increase of NaCl from 30 g/L to 70 g/L was observed in both the AS groups and non-AS groups. So NaCl concentration had a greater impact on the acidogenic fermentation process, and if grown under identical saline conditions sludges had similar microbial populations.

Conclusions

The non-AS was more conducive to the hydrolysis and acidogenesis process of FW compared to the AS. The degradation of organic matter was inhibited in all groups under high NaCl concentrations. Although the AS can shorten the time required to reach maximum VFA production, VFA production could not be increased. Microbial diversity and richness decreased under high NaCl conditions as compared with that in the inoculum. However, the microbial community also presented an ability to adapt to the saline environment. The microbial communities showed clear differences in NaCl concentrations. The non-AS contained a greater abundance of microbial species such as in the phyla *Bacteroidetes*, *Atribacteria* and *Chloroflexi*. The species in these phyla can degrade macromolecular organics and produce enzymes to promote the hydrolysis of FW. Therefore, more VFA produced in the non-AS groups, while more salt-

tolerant bacteria were found in the AS groups. Nevertheless, the NaCl concentration had a greater impact on the process of acidogenic fermentation.

Declarations

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Conflict of Interest The authors declare that we have no conflict of interest.

Availability of data and material The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions Material preparation, data collection and analysis were performed by Xiaozheng He. Data analysis, writing-review & editing and supervision were performed by Jun Yin. Conceptualization, writing-review & editing and project administration were performed by Ting Chen. The first draft of the manuscript was written by Xiaozheng He and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Changes in concentrations of (A) soluble chemical oxygen demand (SCOD); (B) soluble sugar; (C) soluble protein, (D) ammonia nitrogen concentration at different NaCl concentrations during acidogenic fermentation and (E) degradation model of soluble sugar.

Figure 2

Changes in concentrations of (A) Volatile fatty acids (VFAs); (B) Acetic acid; (C) Propionic acid; (D) Butyric acid and (E) Lactic acid; concentration at different NaCl concentrations during acidogenic fermentation.

Figure 3

Analysis of the differences in the bacterial communities between inoculums of non-AS and AS at the (a) phylum level and (b) genus level (*p < 0.05, **p<0.01, ***p<0.001)

Figure 4

A nonmetric multidimensional scaling (NMDS) ordination based on microbial community composition.

Figure 5

Community abundance on phylum level. (A) Microbial community bar plot with the relative abundance higher than 5%; (B) Kruskal–Wallis H test bar plot. The asterisk represents significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 6

Community abundance on genus level. (A) Microbial community bar plot with the relative abundance higher than 5%; (B) Kruskal–Wallis H test bar plot. The asterisk represents significance (*p < 0.05)

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