



# Bacterial survival strategies in sludge alkaline fermentation for volatile fatty acids production: Study on the physiological properties, temporal evolution and spatial distribution of bacterial community

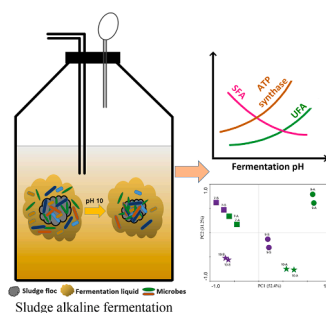
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## HIGHLIGHTS

- Bacterial survival strategies in sludge alkaline fermentation were investigated.
- Increase in ATP synthase activity could benefit bacterial survival at high pH.
- High pH resulted in increased relative abundance of unsaturated fatty acids.
- Microbes mainly lived inside sludge flocs, especially *Firmicutes*.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

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## ABSTRACT

This study investigated the dynamics of ATP synthase activity, phospholipid fatty acid (PLFA) profile, and temporal evolution and spatial distribution of bacterial community to analyze bacterial survival strategies in sludge alkaline fermentation (SAF) for volatile fatty acids (VFAs) production. The results revealed a significant increase in ATP synthase activity at pH 9 and 10 ( $p < 0.05$ ), which could contribute to proton entry into cells and benefit bacterial survival. PLFA analysis indicated that the unsaturated fatty acids content increased with the increase of pH. *Firmicutes* were the dominant microorganisms in the running stage of the pH 10 reactor (35.81–62.34%) and might have been the key microbes that influenced VFAs production. Further analysis of the spatial distribution of microbial community suggested that *Firmicutes* mainly lived inside flocs during SAF. These findings provide an understanding for bacterial survival strategies in SAF, which could help to develop methods to further improve VFAs yield.

## 1. Introduction

Owing to economic and environmental concerns, the management of

excess sludge (ES) is an important issue in wastewater treatment plants (Li et al., 2019; Yang et al., 2015). Anaerobic digestion of ES for volatile fatty acids (VFAs) generation is an effective strategy for ES treatment

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(Jin et al., 2016; Zhang et al., 2009). VFAs have a wide range of applications, such as in the production of biopolymers or renewable energy, and as additional carbon sources for biological nutrient removal process (Huang et al., 2020; Li et al., 2011; Liu et al., 2018). The production of VFAs from sludge anaerobic fermentation involves hydrolysis, acidogenesis and methanogenesis (Wang et al., 2019). Hydrolysis, which provides substrate for VFAs generation, is the rate-limiting step. VFAs are produced by fermentative bacteria in the acidogenesis step, whereas they are consumed in the methanogenesis step. Therefore, improving the performance of sludge hydrolysis and acidogenesis and inhibiting methanogenesis are of great practical significance for the maximization of VFAs yield.

Previous studies have revealed that alkaline fermentation of ES can significantly enhance VFAs yield as hydrolysis can be greatly improved and methanogenesis is greatly inhibited at high pH conditions (especially at pH 10) (Rao et al., 2018; Wu et al., 2010; Yuan et al., 2016). Enhanced VFAs production in sludge alkaline fermentation (SAF) depends on the cooperation of chemical and biological effects (Chen et al., 2017; Yuan et al., 2006). Alkaline pH can promote the dissociation of acidic groups (such as carboxyl) in extracellular polymeric substances (EPS) and the repulsion between the negatively charged EPS, resulting in substantial substrate for VFAs generation (Gonzalez et al., 2018). Acidification of hydrolytic products to VFAs is mainly due to biological effects. Yuan et al. (2006) found that total VFAs concentration in an unautoclaved sludge test (1010.8 mg COD/L) was much higher than that in an autoclaved sludge test (45.9 mg COD/L). Notably, fermentative bacteria can function well in the pH range of 4.0–8.5 (Appels et al., 2008). The activity of bacteria can be adversely affected when pH is beyond this range. Some studies have found that, although an increase in pH can improve VFAs production, the ability of fermentative bacteria to transform hydrolytic products into VFAs was inhibited, thereby hindering the maximization of VFAs yield (Lin and Li, 2018; Ma et al., 2016a). Chen et al. (2017) found that the ratio of VFAs to soluble chemical oxygen demand (SCOD) decreased and low molecular weight proteins accumulated with the increase of pH. A highly probable cause was that acidogenesis was inhibited at high pH. Ma et al. (2016a) found that at pH 7, the acidogenic rate and efficiency of pretreated sludge were higher than those at pH 10. Despite the adverse effects of high pH (9–10) conditions, diverse microbial communities were present and substantial amount of VFAs could be generated in SAF (Li et al., 2014; Wang et al., 2021; Chen et al., 2017), indicating that microbes may develop survival strategies under alkaline pH conditions and thereby maintain the ability to produce VFAs. Therefore, understanding the survival strategies of fermentative bacteria can provide novel insights into the mechanism of enhanced VFAs yield and helps identify potential methods for further improving VFAs production in SAF process. However, our knowledge of bacterial survival strategies in SAF process is limited.

Microorganisms can adjust physiological properties to promote proton capture and retention, and thereby contribute to the stability of cytoplasmic pH and survive under alkaline conditions (Krulwich et al., 2011; Sun et al., 2018). Under such conditions, microbes can upregulate the activity of ATP synthase, and thereby enhance the influx of  $H^+$  into the cell during ATP synthesis (Padan et al., 2005), which could contribute to bacterial survival. Additionally, changes in cell surface layers can also benefit bacterial survival under extreme pH conditions. The cell membrane can regulate the lipid composition (mainly by adjusting the phospholipid fatty acid (PLFA) profile) to maintain fluidity and the ability to transport nutrients and active solute (Ma et al., 2016b), thereby contributing to the optimal growth of microbes under stress. Rousk et al. (2010) found that unsaturated PLFA content increased with the increase in pH of soil. Fang et al. (2007) pointed out that, depending on the environmental conditions, microbial response may result in specific PLFA profiles. Therefore, in SAF process, microbes may regulate these physiological properties to adapt to high pH, and maintain growth. Detailed information on the shifts in ATP synthase activity and PLFA profiles is helpful for obtaining microbial survival

strategies and may provide novel insights into the mechanism of enhanced VFAs production in SAF. However, little is known about the physiological properties of microbes in SAF.

The assembly of microbial community can greatly change with fluctuations in the fermentation pH. Alkaline pH can result in an effective increase in fermentative bacteria in SAF reactors, which can benefit VFAs production (Wu et al., 2020). When environmental parameters change, the microbial community will gradually vary over time until it reaches a stable state (Peces et al., 2018; Vanwonterghem et al., 2014). Hence, investigating the temporal evolution of microbial community helps to understand the alkaline adaptability of bacteria in SAF. Previous studies have found that living in flocs can provide protection for microbes against alkaline pH. Charles et al. (2017) reported that, under alkaline conditions, microorganisms tended to form sustainable aggregations and bacterial cells were mainly located at the centers of the aggregations. Sung et al. (2007) found that an increase in the pH enhanced bacterial attachment to the substrate during rumen fermentation of rice straw; however, different bacterial species exhibited different attachment capacities. In SAF process, some aggregates were distributed among dispersed flocs (Li et al., 2014). Protease and amylase were mainly distributed in tightly-bound EPS and pellets during SAF (Yu et al., 2008). A reasonable inference is that microorganisms may mainly survive in sludge flocs, which benefit their survival in SAF. Therefore, it is desirable to know whether the bacterial community mainly live inside sludge flocs (the spatial distribution of microbial community) during SAF. Investigations on the temporal evolution and spatial distribution of bacterial community may help to clarify the bacterial survival mechanisms in SAF.

This study analyzed the bacterial survival strategies in ES alkaline fermentation for VFAs production. The ATP synthase activity, ATP content and PLFA composition of the microorganisms were evaluated. The dynamics of the temporal evolution and spatial distribution of the bacterial community in SAF were also studied. In addition, the influence of key fermentative bacteria on the VFAs production in SAF was quantitatively evaluated using grey relational analysis (GRA). And multiple fluorescence staining method and confocal laser scanning microscopy (CLSM) analysis was used to identify the distribution of EPS and cells in sludge flocs. The results provide an insight of bacterial survival strategies in SAF, which may advance the knowledge on the mechanism of enhanced VFA production by SAF and lay the foundation for further improving VFAs yield.

## 2. Materials and methods

### 2.1. Characteristics of excess sludge and reactors operation

ES was taken from a municipal wastewater treatment plant in Nanjing, China. The collected ES was filtered through a sieve with a pore diameter of 1.6 mm, and was then stored in a refrigerator at 4 °C (maximum 2 weeks). The sludge discharged from a lab-scale mesophilic anaerobic digestion reactor was used as the seeding sludge (detailed information about the reactor can be found in Ma et al. (2019a)). The characteristics of ES and seeding sludge are presented in Table 1. To better understand the survival strategies of microorganisms in SAF process, five reactors (pH of uncontrolled, 7, 8, 9 and 10) with effective and total volume of 400 and 800 mL, were used in this study. The ratio of

**Table 1**  
Characteristics of excess sludge and seeding sludge.

Parameter	Excess sludge	Seeding sludge
TS (g/L)	19.36 ± 0.68	17.94 ± 0.41
VS (g/L)	11.91 ± 0.37	9.23 ± 0.22
pH	6.7 ± 0.1	7.1 ± 0.1
SCOD (mg/L)	124.7 ± 14.34	398.3 ± 31.25
TCOD (g/L)	17.98 ± 1.02	13.55 ± 1.14

volatile solids (VS) in seeding sludge and ES was 1:1. 2 M sodium hydroxide was used to regulate the pH of the reactors thrice a day, which was measured using a pH meter (FE20, Mettler Toledo, USA). The fermentation reactors were placed in a shaking cultivating chamber, with mixing intensity of 100 rpm and temperature of 37 °C. Solids retention time of the reactors was 8 days (Li et al., 2014), and 50 mL of fermented sludge was replaced with an equal amount of ES every day.

## 2.2. Analytical methods

Total chemical oxygen demand (TCOD), SCOD (APHA 5220), total solids (TS) and VS (APHA 2540) were detected by the Standard Methods (APHA, 2005). CH<sub>4</sub>, H<sub>2</sub> and VFAs yield were analyzed by 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) according to the method of Wang et al. (2019). COD of VFAs, CH<sub>4</sub> and H<sub>2</sub> was calculated based on: 1.07 g COD/g acetic acid, 1.51 g COD/g propionic acid, 1.81 g COD/g butyric acid, 2.04 g COD/g valeric acid, 1 g COD/382 mL CH<sub>4</sub> and 1 g COD/1528 mL H<sub>2</sub>. Each reactor was run for more than 10 SRTs prior to monitoring ( $\geq 3$  samples) to ensure steady state (Ma et al., 2019b) and then the samples were collected every 10 days for 30 days. For VFAs analysis, the digested sludge was centrifuged at 4000 g for 10 min, and then 0.45  $\mu$ m fiber filter was used to filter the supernatant. The acidification percentage was calculated as follows: acidification percentage = (COD<sub>VFAs</sub> + COD<sub>CH<sub>4</sub></sub> + COD<sub>H<sub>2</sub></sub>)/(SCOD + COD<sub>CH<sub>4</sub></sub> + COD<sub>H<sub>2</sub></sub>) (Chen et al., 2017). To detect the ATP synthase activity and ATP content, the TS of the collected digestate was diluted approximately to 3000 mg/L, and then six diluted samples were collected and used for subsequent analysis. ATP synthase activity was measured using a commercial ELISA (Enzyme-Linked Immunosorbent Assay) kit (Shanghai Hengyuan Biotechnology Co., Ltd, China), and Bac Titer-Glo™ (Promega, USA) was used to detect the ATP content. Experimental operations were conducted according to the manufacturer's protocols. PLFA analysis was conducted according to the method described in Niu et al. (2012).

## 2.3. Analysis of temporal evolution and spatial distribution of bacterial community

To analyze the temporal evolution of bacterial community, the seeding sludge and the samples collected on the 1th, 8th, 16th, 32th, 64th, and 96th days were centrifuged at 7600 g (8000 rpm) for 10 min, then mixed with alcohol (50%) and stored at -80 °C for further analysis. VFAs of the samples (the 32th, 64th and 96th days) were also analyzed to conduct GRA analysis.

To identify whether the bacterial community mainly live inside sludge flocs during SAF, the spatial distribution of bacterial community was analyzed (using the samples collected from the pH 7, pH 9 and pH 10 reactors). The microorganisms in the reactors were divided into two parts, referred to as microbes in flocs (inner) and other microbes (other), according to the methods described by Luo et al. (2015). 10 mL sludge mixture was diluted to 50 mL, and then vortex-mixed for 1 min and centrifuged at 700 g for 5 min. The supernatant was transferred to a tube. 50 mL phosphate buffered saline (PBS) (consisting of 8 g/L NaCl, 0.2 g/L KCl, 1.14 g/L Na<sub>2</sub>HPO<sub>4</sub> and 0.24 g/L KH<sub>2</sub>PO<sub>4</sub>) was added to the sediment, and the mixture was vortex-mixed for 30 s and centrifuged at 700 g for 5 min. The supernatant was transferred to a tube. To separate the microbes loosely attached to flocs, this process was repeated thrice, twice with PBS containing 0.1% Tween-80. All the supernatants were mixed. The microbes in the supernatant were regarded as planktonic microbes and labelled as "P", and the microorganisms in the final residue were regarded as microbes in flocs and labelled as "I". Three samples (10 mL sludge mixture) were collected from each reactor (on the 90th and 110th day, respectively) and the combined samples were used for further analysis.

DNA was extracted using the FastDNA™ Spin Kit for soil (MP Bio-medicals, Santa Ana, CA). The bacterial community structure was

analyzed using high-throughput 16S rRNA gene amplicon sequencing. During the analysis of the spatial distribution of bacteria, the total bacteria in the "P" and "I" samples was quantified using real-time PCR assays, for which the universal primers 341F (5'-CCTACGGGAGGCAG-CAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3') were used. qPCR was performed using a 7500 Real Time PCR System (Applied Biosystems, USA) based on SYBR-Green. The experimental protocol was as described by Zheng et al. (2013).

To further analyze the spatial distribution of bacterial cells and EPS at pH 10, an inverted CLSM (Zeiss LSM880 with Airyscan) was employed to probe the internal structure of sludge flocs. The TS of the sample was diluted to about 3000 mg/L, and the flocs were fixed in 4% para-formaldehyde. Then the flocs were stained using FITC for proteins, Syto 63 for total cells and DNA, and concanavalin A for  $\alpha$ -mannopyranosyl and  $\alpha$ -glucopyranosyl sugars according to the method outlined by Chen et al. (2007). The CLSM scanned the sample at xyz mode and images analysis was performed using Zen 2.1 (Zeiss Microscopy).

## 2.4. Statistical analysis

"R" statistical packages were used to draw heat map. Significant correlations and the significant differences (Student's *t* test) were calculated using SPSS 17.0 software. CANOCO 4.5 software was employed to perform principal component analysis (PCA) and redundancy analysis (RDA). GRA was performed according to the procedures of Ma et al. (2019a) and Xu et al. (2011).

## 3. Results and discussion

### 3.1. Acidogenesis in the reactors with different pH

The total VFAs concentration in the fermentation reactors increased with the increase in pH (Fig. 1). An increase in the pH from uncontrolled to 10, significantly increased the concentration of acetic acid from 341.4 to 1717 mg COD/L ( $p < 0.05$ , *t*-test) (Table 2). The concentration of other VFAs also increased as the pH of the fermentation reactors increased from uncontrolled and 7 to 10. These findings are in agreement with previous studies (Yu et al., 2008; Chen et al., 2019), implying that controlling the fermentation pH at 10 can greatly enhance the production of VFAs in sludge anaerobic fermentation. However, the acidification efficiency of the total SCOD decreased with the increase of pH (Fig. 1), which is agreed with the results of Ma et al. (2016a). Some studies have found that easily biodegradable substrates (i.e., low molecular weight proteins and carbohydrate, tyrosine-like and tryptophan-

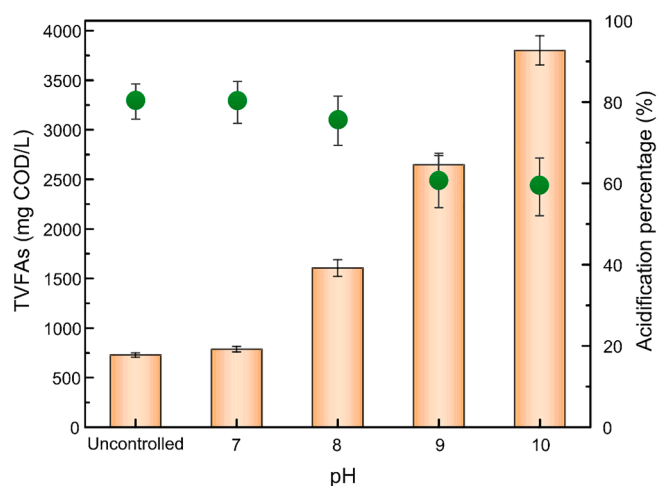


Fig. 1. The total VFAs concentration and the acidification percentage in the reactors with different pH. The columns represent total VFAs concentration and the dots represent the acidification percentage.

**Table 2**

Volatile fatty acids (mg COD/L) concentration in the fermentation reactors.

VFAs	pH uncontrolled	pH 7	pH 8	pH 9	pH 10	SD <sup>a</sup>
Acetate	341.4	376.1	562.4	935.3	1717	547.2
Propionate	175.3	212.9	405.7	661.8	650.6	209.3
Butyrate	24.1	37.5	93.6	203.6	361.0	126.7
Iso-butyrate	49.1	57.4	165.0	338.1	365.7	134.6
Valerate	38.2	50.6	54.5	141.8	91.3	36.2
Iso-valerate	98.8	72.1	273.2	411.4	587.2	193.5

<sup>a</sup> SD represent the standard error of the six VFA concentration among the five reactors.

like substances) were accumulated in SAF at pH 10 (Chen et al., 2017; Ma et al., 2019b). A longer SRT or operating time was not able to decrease the substrates, and the inhibited acidogenesis by high pH is highly likely to account for this phenomenon (Chen et al., 2017; Ma et al., 2016a). Although the acidogenesis of total SCOD can be inhibited, large amounts of VFAs can also be produced during SAF process. It is known that, VFAs are produced by large densities of fermentative bacteria. The activity of fermentative bacteria can be adversely impacted when pH is higher than 8.5. Meanwhile, microorganisms develop several survival strategies to mitigate the pH stress caused by alkaline conditions, which can help to maintain their growth and function. Therefore, it is desirable to know how the microorganisms adapt to alkaline conditions and thereby maintain the ability for VFAs production in SAF reactors.

### 3.2. Variations in bacterial physiological properties in the fermentation reactors

#### 3.2.1. ATP synthase activity and ATP content

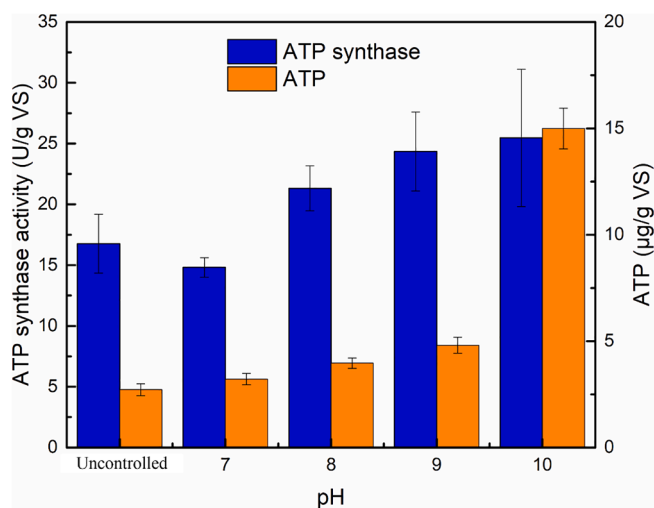
The ATP synthase activity of the microorganisms in the fermentation reactors is shown in Fig. 2. The activity of ATP synthase significantly increased from  $16.77 \pm 2.41$  and  $14.82 \pm 0.8$  U/g VS to  $25.46 \pm 5.65$  U/g VS as the increase of pH from uncontrolled and 7 to 10 ( $p < 0.05$ ). Compared with that in the pH uncontrolled reactor, the activity of ATP synthase increased by 45.14% and 51.88% (in average) in the pH 9 and 10 reactors, respectively. Under alkaline conditions, microbes can elevate ATP synthase activity, enhancing the capture and retention of  $H^+$  in cells during ATP synthesis (Padan et al., 2005). Such an increase in ATP synthase activity can enhance the conversion of ADP to ATP. Thus, proton entry into cells during ATP generation can be improved, which can benefit microbial adaptation to high pH during SAF. Krulwich et al. (2011) reported that proton capture can be enhanced by increasing

expression of ATP synthase in *Escherichia coli* under alkaline conditions. To further illustrate that the activity of ATP synthase improved at alkaline conditions, the concentration of total ATP in the cells among the fermentation reactors was investigated. The concentration of ATP increased with the increase in pH (Fig. 2). The ATP concentration in the pH 10 reactor was  $14.99 \pm 0.95$   $\mu$ g/g VS, which was 5.5 times higher than that in the pH uncontrolled reactor. This result is agreed with the observed increase in ATP synthase activity during SAF. However, the ATP content showed a sharp increase at pH 10. Hydrolysis is the rate-limiting step of sludge anaerobic fermentation, and sludge hydrolysis can be greatly enhanced at pH 10. It is possible that the effective hydrolysis induced at pH 10 can result in large amounts of substrate that are easily available to the microbes, which can also contribute to the increase of ATP content. The improved ATP content also implied that the retention of  $H^+$  in cells during ATP synthesis was enhanced at pH 10. Therefore, the increased ATP synthase activity and ATP content may favor bacterial adaptation to high pH during SAF.

#### 3.2.2. Profiles of phospholipid fatty acid

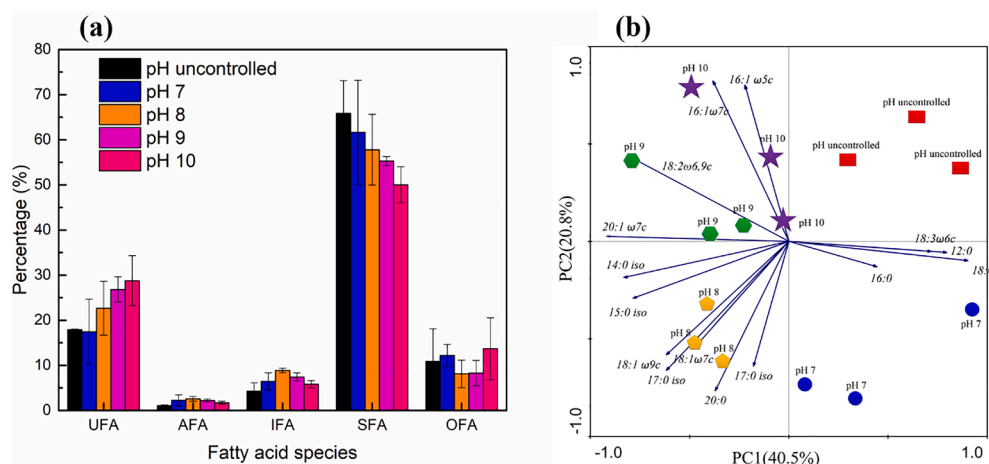
The PLFA compositions of the microbes in the sludge fermentation reactors are shown in Fig. 3a. According to the structural features, total PLFA was divided into five categories: unsaturated fatty acid (UFA), anteiso-branched fatty acid (AFA), iso-branched fatty acid (IFA), saturated fatty acid (SFA) and other fatty acid (OFA) (Ma et al., 2016b). The PLFA of the microbes in the fermentation reactors mainly consisted of SFA and UFA. When the fermentation pH was increased from uncontrolled to 10, the abundance of UFA significantly increased from  $17.91 \pm 0.12\%$  to  $28.78 \pm 5.54\%$  ( $p < 0.05$ ), whereas SFA decreased from  $65.87 \pm 7.24\%$  to  $50.02 \pm 3.98\%$  ( $p < 0.05$ ). Rousk et al. (2010) found that the PLFA composition of microbes clearly changed along the soil pH gradient, and an increase in pH led to increase in the relative concentrations of some UFA (such as C16:1 $\omega$ 5c, C16:1 $\omega$ 7c and C18:1 $\omega$ 7c). The increase in the contents of C14:0 and C16:0 and decrease in the content of C18:0 may be a strategy for *Listeria* to adapt to acidic conditions (Cotter and Hill, 2003). In this study, the abundance of 16:1 $\omega$ 5c and 16:1 $\omega$ 7c (3.49% and 12.46%, on average) was the highest at pH 10, whereas the abundance of C16:0 and C18:0 decreased with an increase of pH. Shifts in the PLFA composition can be beneficial to maintain the fluidity of the cell membrane and bacterial survival or growth under changes in the environmental conditions (Fang et al., 2007). UFA have a lower phase transition temperature than SFA (Niu et al., 2012; Fang et al., 2007), thus the increase of UFA and the decrease of SFA can disrupt the close packing of phospholipid acyl chains, contributing to maintenance of good mobility of the cell membrane during SAF. Moreover, an increase in the relative abundance of UFA and decrease of SFA has been reported as an adaptive mechanism employed by microbes under stressful conditions, such as low temperature (Niu et al., 2012) and fullerene (Fang et al., 2007). The increase in fermentation pH has been shown to improve the relative abundance of UFA, which may contribute to bacterial survival in SAF reactors (Rousk et al., 2010; Tan et al., 2020) and thereby favor VFAs production. In future study, it could suggest to enhance VFAs production in SAF by improving the ratio of UFA of fermentative bacteria. Previous studies have found that weak magnetic field and electric field can improve the UFA content of microorganisms (Niu et al., 2013; Wick et al., 2010).

To further analyze the changes of PLFA composition among the reactors, PCA was employed to assess the overall differences (Fig. 3b). PC1 and PC2 explained 40.5% and 20.8% of total data variability, respectively. The PLFA samples collected from reactors with the same pH were clustered together, and samples derived from different pH reactors can be distinguished. C16:1 $\omega$ 5c and C16:1 $\omega$ 7c clustered around the pH 10 samples, indicating that these fatty acids were related to high pH. This phenomenon indicated that different pH resulted in clear differences in the PLFA composition of the microbes during sludge anaerobic fermentation.



**Fig. 2.** ATP synthase activity and ATP content in the sludge fermentation reactors with different pH.



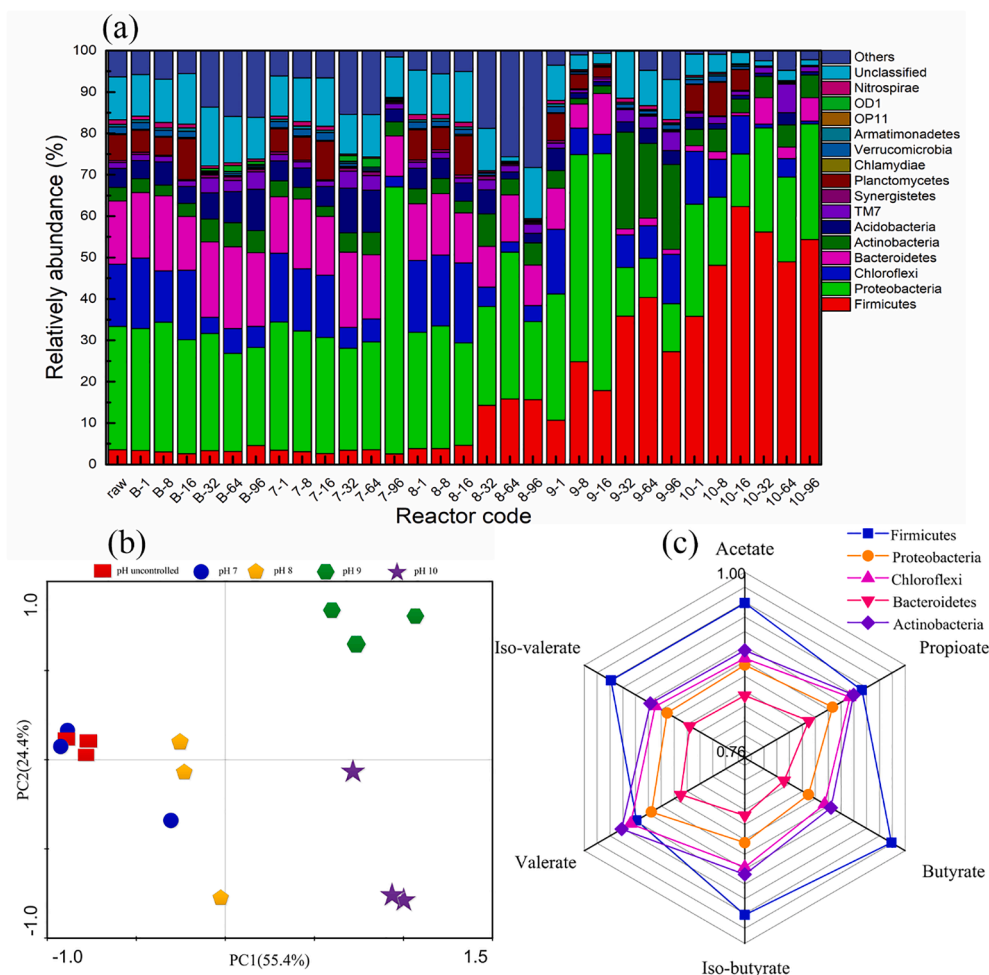


**Fig. 3.** Phospholipid fatty acid (PLFA) composition of the microbes in the reactors with different pH: (a) Relative abundance of UFA, AFA, IFA, SFA and OFA; (b) PCA of PLFA profiles. UFA, AFA, IFA, SFA and OFA denote unsaturated, anteiso-branched, iso-branched, saturated and other fatty acids, respectively.

### 3.3. Temporal evolution of bacterial community in the sludge fermentation reactors

The microbial community structure of the seeding sludge and sludge samples collected from the fermentation reactors at different times are

shown in Fig. 4a. The microbial community in the reactors primarily consisted of *Firmicutes*, *Proteobacteria*, *TM 7*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and *Acidobacteria* (Fig. 4a), which is consistent with the results of previous studies (Chen et al., 2017; Ping et al., 2020). These microbes play important roles in hydrolysis and acidogenesis during



**Fig. 4.** The bacterial community of the sludge fermentation reactors at different time points and grey relational grades ( $\gamma$ ) between microorganisms and VFAs. (a) bacterial community structure at phylum level; (b) principal component analysis (PCA); and (c) the  $\gamma$  between the key microorganisms and VFAs. B, 7, 8, 9 and 10 represent the pH of uncontrolled, 7, 8, 9 and 10 reactors, respectively.

sludge anaerobic fermentation (Lu et al., 2019; Ma et al., 2019a). The results of PCA based on the samples collected on the 32th, 64th and 96th days are shown in Fig. 4b. They suggest that changes in pH resulted in obviously different microbial communities. The bacterial community at pH uncontrolled and 7 varied slightly with the operation of the reactors, which could be attributed to the similar pH in the two reactors and the reactor for seeding sludge collection. In the pH 8 reactor, the relative abundance of *Firmicutes* gradually increased to 14.31%–15.85%. On the first day, the relative abundance of *Firmicutes* in the pH 9 and 10 reactor increased from 3.53% (seeding sludge) to 10.66% and 35.81%, respectively. In the pH 9 reactor, *Firmicutes* continuously increased to 27.24%–40.38%, whereas *Proteobacteria* initially increased and then decreased to 9.46–11.76%. The relative abundance of *Actinobacteria* was the highest at pH 9 (18.04–23.35%). *Firmicutes* were the dominant microorganisms in the entire running stage of the pH 10 reactor, and their relative abundance (48.96–62.34%) was the highest at pH 10. The higher relative abundance of *Firmicutes* at higher pH is consistent with the findings of Zhou et al. (2020). *Firmicutes* have a thick cell wall, which could benefit their survival in extreme conditions (such as alkalization and acidification) (Zhou et al., 2020). Many species of *Firmicutes* possess the ability to utilize a broad spectrum of substrates and diverse pathways to produce fermentable components (Tracy et al., 2012), which may be helpful for organic acids generation in various environments. The production of organic acids is beneficial to bacterial survival under alkaline conditions (Padan et al., 2005). Furthermore, Chen et al. (2017) also found that *Firmicutes*, *Proteobacteria* and *Actinobacteria* were the dominant microbes involved in SAF, and that the abundance of *Firmicutes* was the highest (60%) in the reactor with the maximum VFAs yield. At the genus level, the relative abundances of *Guggenheimella*, *Tissierella* and *Clostridium XI* increased over time in the pH 10 reactor, and the content of those microbes in this reactor was higher than that in the other reactors. These microbes are well known to degrade organic matter and produce VFAs during sludge anaerobic fermentation (Jin et al., 2016). Their thick cell walls and the ability for acids production may benefit the

accumulation of *Firmicutes*, which can further contribute to the generation of VFAs during SAF.

To further identify the key microorganisms that influence VFAs production during SAF, the GRA method was employed (Fig. 4c). A larger  $\gamma$  value represents a higher influential degree (Xu et al., 2011). *Firmicutes* showed the highest  $\gamma$  with acetate (0.959), propionate (0.932), butyrate (0.979), *iso*-butyrate (0.962) and *iso*-valerate (0.958), whereas *Actinobacteria* exhibited the highest  $\gamma$  with valerate (0.942). In addition to valerate, the  $\gamma$  generally decreased as follows: *Firmicutes* > *Actinobacteria* > *Chloroflexi* > *Proteobacteria*. The concentration of valerate was the lowest among the six VFAs in SAF reactors, especially at pH 10. Furthermore, RDA was used to analyze the correlation between the microbial community and VFAs concentration (Fig. 5). *Firmicutes* showed a significantly positive correlation with the six VFAs ( $p < 0.05$ ). However, *Bacteroidetes*, *Synergistetes*, *Acidobacteria* ( $p < 0.05$ ), and *Proteobacteria* ( $p > 0.05$ ) negatively correlated with the six VFAs. The abundance of these microbes decreased while the VFAs yield increased with the increase of fermentative pH, which could account for the negative correlation between these microbes and VFAs. Zhou et al. (2020) found that the abundance of *Bacteroidetes* and *Proteobacteria* decreased, and *Firmicutes* increased with the increase of pH during sludge fermentation. Thus, it suggested that *Firmicutes*, not other microbes, might have been the key functional microorganisms and their accumulation benefited the VFAs production during SAF in this study.

### 3.4. Dynamics of spatial distribution of bacterial community at different pH

The pH 7, 9 and 10 reactors were selected (two parallel samples) to analyze the dynamics of spatial distribution of bacterial community induced by pH (Fig. 6a). Microbial community of the I (microbes in flocs) and P (planktonic microbes) samples in the pH 7 reactor was similar (Fig. 6). *Bacteroidetes* were more abundant in P samples, whereas *Proteobacteria* were more abundant in the I samples. When the pH

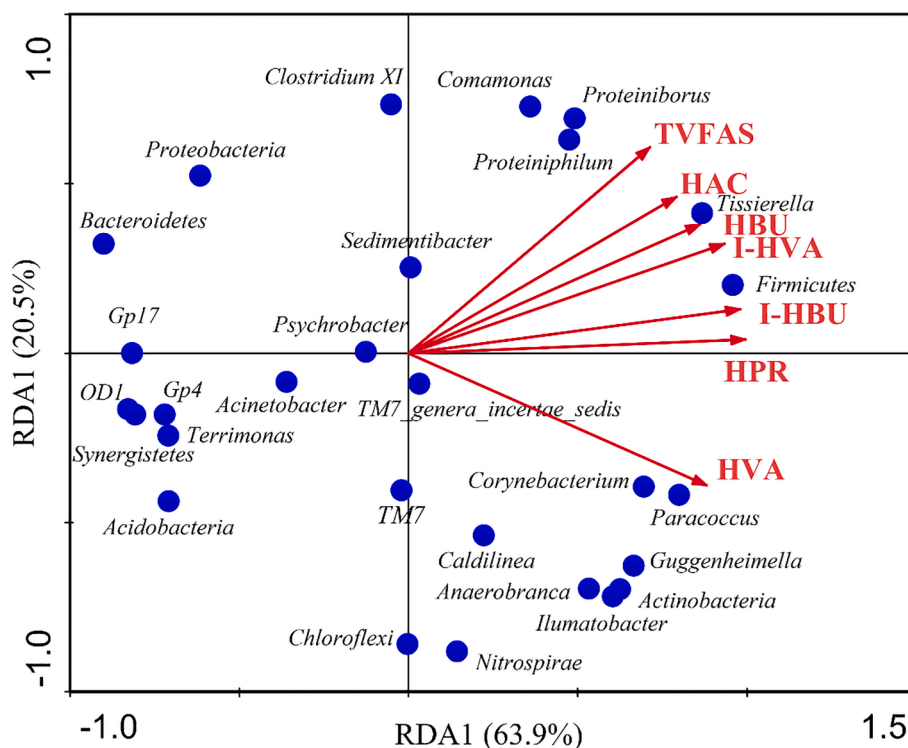
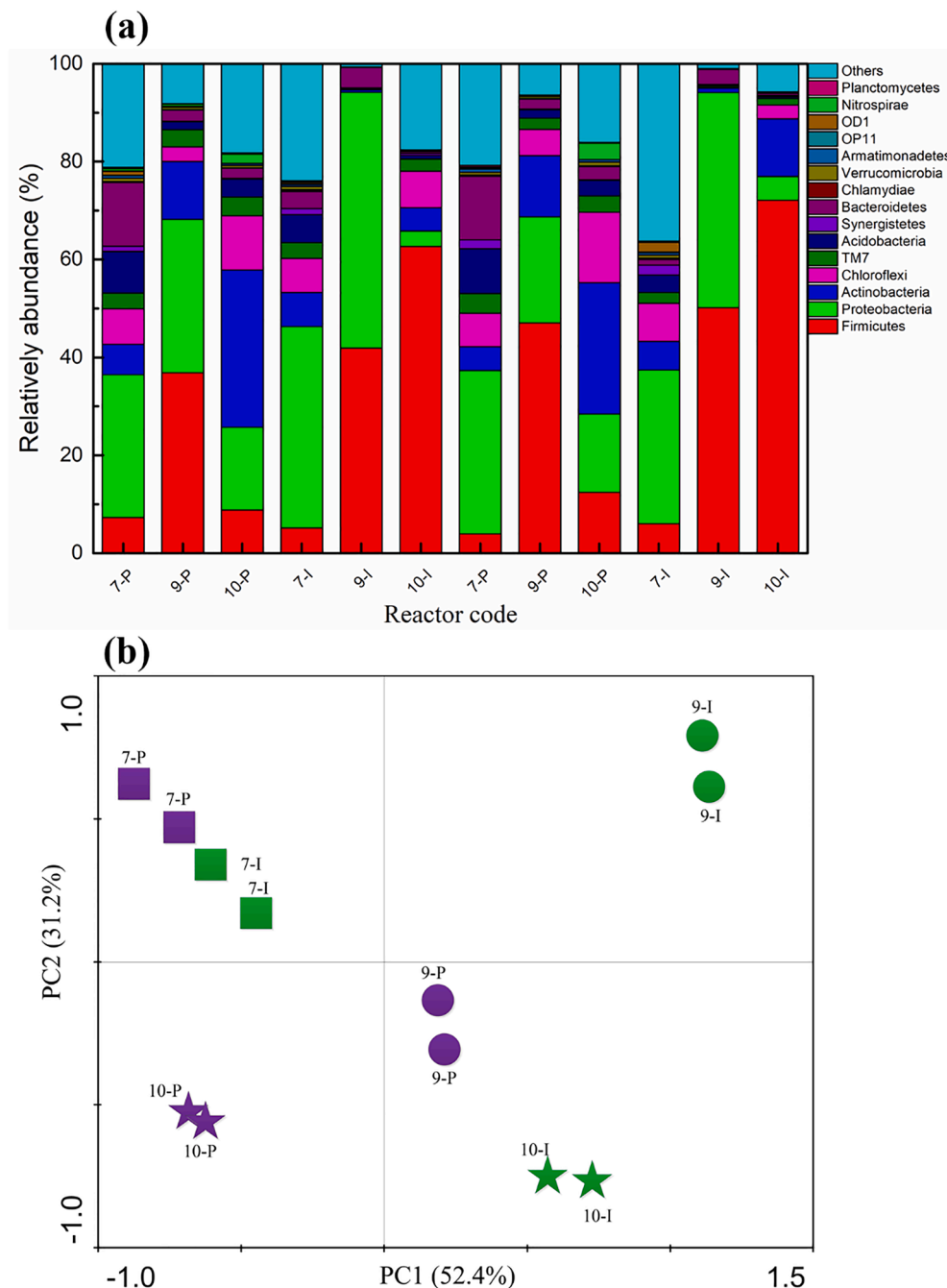


Fig. 5. Redundancy analysis of the correlation between bacterial community and VFAs. The blue circles represent the microorganisms. TVFAS, HAC, HPR, HBU, I-HBU, HVA and I-HVA represent total VFAs, acetate, propionate, butyrate, *iso*-butyrate, valerate and *iso*-valerate, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** The spatial distribution of bacterial community in the sludge fermentation reactors at pH of 7, 9 and 10: (a) bacterial community at phylum level; (b) PCA analysis of the bacterial community. “I” represent microbes living in flocs; “P” represent planktonic microbes; 7, 9 and 10 represent the pH of 7, 9 and 10 reactors, respectively.

increased to 9 and 10, the microbial community of the P and I samples exhibited obvious differences (Fig. 6b). The abundance of *Firmicutes* was similar in the P and I samples, whereas *Proteobacteria* were more abundant in the I samples than in the P samples at pH 9. At pH 10, *Firmicutes* were the dominant microbes and their abundance was 62.67–72.10% in the I samples, whereas it was only 8.79–12.42% in the P samples. Vanwonterghem et al. (2014) found that *Clostridiales* (belonging to *Firmicutes*) were predominantly attached to substrate particles during cellulose anaerobic digestion. In this study, *Clostridiales* accounted for 75.08–79.58% of *Firmicutes*. Furthermore, the numbers of total bacteria in the P and I samples decreased with the increase of pH. Zheng et al. (2013) also reported that operation at pH 10 decreased the total bacteria in SAF. The numbers of total bacteria in the I samples were

higher than those in the P samples, which is consistent with the stand-point described by Mason and Stuckey (2016). Moreover, the ratio of total bacteria in I and P samples increased from 6.40 to 8.69 and 8.85 (on average) when pH increased from 7 to 9 and 10, respectively. This phenomenon suggested that the microbes living in flocs were less inhibited than the other microbes during SAF. Previous studies have reported that, due to the acid production by microbes living inside biofilms or granules, the pH stress experienced by bacteria can be reduced (Charles et al., 2017; Mason and Stuckey, 2016). Therefore, promoting the formation of flocs or similar structure could be beneficial to bacterial survival in SAF. Adding carriers with internal pores (such as biochar) can provide habitat for microbes (Luo et al., 2015), which may benefit to the survival of fermentative bacteria and VFAs production in

## SAF.

The results of CLSM analysis revealed that the outer layer of the flocs was mainly composed of proteins, and  $\alpha$ -mannopyranosyl and  $\alpha$ -glucopyranosyl sugars. The general shapes and sizes of flocs in panel (a), representing cellular nucleic acids (cells), are smaller than other panels. Meanwhile, the edges of the flocs are mainly red and green, respectively. These phenomena suggested that the bacterial cells were primarily located at the inner layer of flocs. This phenomenon is consistent with the finding observed by Charles et al. (2017), which also found bacterial cells were concentrated in the center of the floc and surrounded by EPS in anaerobic digestion of cellulose at alkaline conditions. Taken together, these results indicated that the microbes mainly lived inside flocs in SAF, especially the key functional microorganisms (*Firmicutes*), which could benefit the survival of the microbes and further favor VFAs production.

#### 4. Conclusions

The results showed that the activity of ATP synthase and UFA content increased with the increase of pH. *Firmicutes* were the dominant microorganisms in the entire running stage of the pH 10 reactor and might have been the key microorganisms that influenced VFAs production. *Firmicutes* mainly lived inside flocs during SAF. These shifts in the physiological properties and the temporal and spatial distribution of bacterial community benefited bacterial survival during SAF. Not only the widely reported improvement in hydrolysis but also the survival strategies developed by bacteria against alkaline pH could contribute to VFAs production.

#### CRediT authorship contribution statement

**Sijia Ma:** Methodology, Data curation, Validation, Writing - review & editing. **Dongli Yang:** Methodology. **Ke Xu:** Data curation, Supervision. **Kan Li:** . **Hongqiang Ren:** Conceptualization, Supervision, Resources, Funding acquisition, Project administration, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2021.125701>.

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