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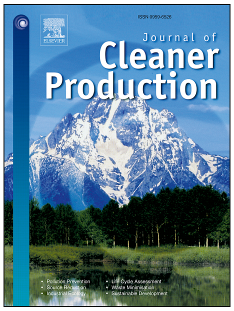

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Achieve clean and efficient biomethane production by matching between digestate recirculation and straw-to-manure feeding ratios

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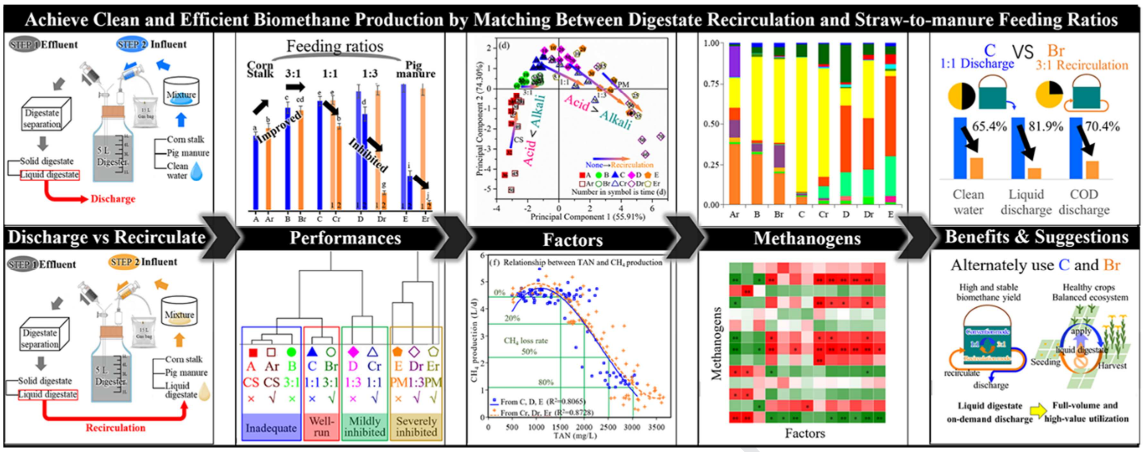

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There are 7671 words, including whole text file, tables, and figure captions.

Abstract

Liquid digestate recirculation can fundamentally solve the problem of voluminous liquid digestate discharge for biogas projects. However, the uncertainty of the effect of recirculation under different feedstock restricts the application of recirculation technology. In this study, the comprehensive effects of straw to manure feeding ratios and liquid digestate recirculation on anaerobic digestion performance and microbial community structure were investigated. Recirculation was beneficial for biomethane production and digestion stability in digesters of corn stalk and straw/manure 3:1 by alleviating the loss of ammonia and alkalinity, while it aggravated the accumulation of ammonia and organic acids and reduced methane yield under 1:1, 1:3 and pig manure. The feeding ratios and recirculation formed a combined effect on ammonia concentration in the fermentation system, and when the total ammonia concentration was above 1,500 mg/L, acids accumulation and decline in methane production would occur whether with recirculation or not. The microbial community structure varied corresponding to the fermentation state. In the well-run digesters, which were 3:1 with recirculation and 1:1 without recirculation, the dominant methanogens were Methanosaeta, while in the inhibited digesters, Methanosarcina and Methanosphaera dominated the methanogen community. Based on producing 1 GJ energy, the strategy of 3:1 with recirculation reduced the discharge of liquid digestate by 81.9 % and the use of clean water by 65.4 % compared to that of 1:1 without recirculation. An integrated

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approach of adjusting feedstock formula and selectively employing liquid digestate recirculation was proposed to produce liquid digestate on-demand with downstream application requirement, which will make biogas industry eco-friendly and cost-effective.

Keywords

Anaerobic co-digestion; Digestate recirculation; Straw-to-manure ratio; Corn stalk; Pig manure.

1 Introduction

Anaerobic digestion is the most commonly used route to deal with much agricultural waste, providing useable and renewable energy in the form of biogas (Frigon and Guiot, 2010; Ward et al., 2008). Biogas production from agricultural waste will help offset fossil fuel demand, reduce pollution from agricultural waste and fossil fuel consumption (Patrizio et al., 2015), and also upgrade matter cycling in agricultural sectors to produce high-quality food and other products (Alburquerque et al., 2012).

Digestate, also known as effluent or residues, is the byproduct of anaerobic digestion, and the disposal of which is the basis for the continuous operation of biogas projects (Monlau et al., 2015). With the increase and expansion of biogas projects, the amount of digestate simultaneous increase. The eco-friendly and low-cost disposal of excessive digestate, especially the liquid part, has become an urgent problem hindering the promotion of anaerobic digestion technology (Gong et al., 2013; Hu et al., 2014). Irrigation is a common practice of liquid digestate (Tambone et al., 2017). However, in

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China, due to the lack of transportation facilities, the liquid digestate can only be poured into the surrounding farmland. More importantly, the positive effect of applying liquid digestate in farmland needs to be based on the demand for crop fertigation, which is seasonal and intermittent. The continuous discharge tends to cause the soil to exceed its bearing capacity and cause serious pollution (Rehl and Muller, 2011). In some European countries, liquid digestate needs to be stored for more than 180 days before irrigation, which occupies a huge space and also emits pollutants (Xia and Murphy, 2016). Seeking alternative approaches to reuse liquid digestate is a global requirement for the economy and sustainability of most biogas projects.

An alternative approach to reduce the volume of effluent is to recirculate liquid digestate back into digesters (Jarvis et al., 1995; Nie et al., 2015; Zuo et al., 2015). Other than discharge reduction, liquid digestate recirculation can also reduce the overall water consumption of biogas projects, which cuts costs and also saves water resources. And the overall energy consumption can also be reduced through recirculation due to the recovery of retained heat in warm liquid digestate (Wang et al., 2018). However, whilst recirculation can improve fermentation efficiency (Peng et al., 2016) and system stability (Gottardo et al., 2017) by recycling nutrients, buffers and microorganisms (Li et al., 2018; Ratanatamskul and Saleart, 2016), it can also result in the accumulation of fermentation inhibitors (Estevez et al., 2014) and non-degradable substances (Ni et al., 2017) leading to inefficient digestion and even process failure (Wu et al., 2016; Zamanzadeh et al., 2016). Previous studies have focused on the effect of liquid digestate

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recirculation under particular one feedstock and/or process. However, the effect of liquid digestate recirculation under different feedstock formula has not been compared. And how liquid digestate recirculation affects the co-digesting system and microbial community structure of crop straw and livestock manure has not been reported. Considering the diversity of feedstock characteristics and many co-digestion situations, studies on the interaction between feedstock ratios and the effect of recirculation will help give full play to beneficial parts of recirculation impacts and avoid the adverse effect.

The aim of this study is to investigate the effect of liquid digestate recirculation under different ratios of crop straw and livestock manure on semi-continuous anaerobic digestion. Corn stalk (CS) and pig manure (PM) were taken as representatives of crop straw and livestock manure since they make up the larger proportion of their types. Systematic data (gas production and fermentation characteristics) was obtained and discussed from ten lab-scaled digesters semi-continuously fed by five feedstock ratios with or without liquid digestate recirculation. Bacterial and Archaeal community structure was investigated by high-throughput sequencing. Emission and consumption per unit energy output were compared between well-performed digesters without and with recirculation. The appropriate feedstock ratios and the suggestion for feedstock adjustment were provided to take advantage of recirculation. The benefits of liquid digestate recirculation were quantified. Results of this study indicated that, by appropriately adjusting the feedstock and fermentation parameters, liquid digestate

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recirculation could be successfully applied to most biogas plants playing the role of reducing waste and recycling matter, water, and energy, which will make biomethane production cleaner and more efficient.

2 Material and methods

Source, characteristic, and preparation of feedstock and inoculum were detailed presented in 2.1. Design, operation and sampling of anaerobic digestion were introduced in 2.2. Methods were classified by analyzing gas, digestate, and microorganisms, and were listed in 2.3. Tools used in data analysis and graphical presentation were summarized in 2.4

2.1 Feedstock and inoculum

Corn stalk (CS) was collected from the Shangzhuang Experimental Station (Beijing, China) of the China Agricultural University, milled with a high-speed pulverizer (FW100, Taisite, Tianjin, China), and stored at room temperature. Pig manure (PM) was collected from a pig farm (Hebei, China) of the China Agricultural University. PM was stored at room temperature in sealed barrels and mixed evenly before daily feeding of the digesters. The inoculum was collected from a 65-L constantly-run continuous stirred tank reactor in Lab of West Campus, Center of Biomass engineering, China Agricultural University. The CSTR was fed by rice straw and dairy manure and run at 37 □ with an average daily methane yield of 90 L/d for over two months. About 5 L effluent of the CSTR was collected every day and stored in a 50-L sealed barrel at room temperature until the barrel was full. Then, the collected effluent was stored anaerobically at 37 □

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for two months, and was thoroughly stirred once a day to eliminate the interference of residual substrates in methane yield and to form homogenous inoculum. The compositions of CS, PM, and inoculum are presented in Table 1.

2.2 Experimental design

To simulate different feedstock conditions, the following five recipes were selected as follows: only CS, 3:1, 1:1, 1:3, and only PM, the proportions of which (CS:PM) were based on their volatile solid (VS) contents. In each case, experiments were carried out to compare performance with and without recirculation of the liquid digestate. The experimental design is summarized in Table 2. Treatments without recirculation were labeled A to E; treatments with recirculation were labeled Ar to Er. Digestion was carried out in ten digesters, each with a total volume of 6.2 L, and a working volume of 5.0 L. Digestions were carried out under mesophilic conditions (37 ℃), and were fed once a day. The organic loading rate (OLR) was 4.0 g-VS/(L·d) and both the hydraulic retention time (HRT) and the solid retention times (SRT) were 20 d. Digestions were initiated by the addition of 5.0 L of inoculum and then discharged 250 mL digestate and injected 250 mL feedstock every day. For digesters with recirculation, the discharged 250 mL digestate was liquid-solid separated by centrifuging 5 min at 3,000 rpm (CR3i, Thermo Electron Co., Waltham, MA, USA), after which, 150 mL of the liquid digestate was recirculated to digester by replacing 150 mL water in preparation of feedstock. The biogas generated by each digester was collected in a 15-L gas bag. The compositions and volumes of biogas produced by each digester were measured daily. Approximately

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20 mL of effluent from each digester was sampled every other day and frozen at -35 □ prior to further analysis.

2.3 Analytical methods

Biogas: volumes were monitored using the water displacement method and then converted to a volume at standard conditions (0°C, 101.325 kPa). Methane content was analyzed using a biogas analyzer (GA5000, Geotech, Britain).

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Digestates: total solid (TS) and volatile solids (VS) contents, alkalinity, total carbon, and total nitrogen were analyzed using standard methods (APHA, 2017). The pH was determined using a pH meter (SX610, Sanxi, China). Cellulose and hemicellulose contents were analyzed using a fiber analyzer system (Ankom 2000, Ankom Technology Corp., Fairport, NY, USA). Concentrations of lactic acid and volatile fatty acids (VFAs) including formic, acetic, propionic and butyric acids were analyzed using a high performance liquid chromatography (HPLC) system equipped with an ion-exchange column (Aminex HPX-87H; 30 0 mm x7.8 mm, BioRad Laboratories, Hercules, CA, USA) and a diode array detector (SPD-M20A, Shimadzu, Kyoto, Japan). Samples were centrifuged at 12,000 rpm for 30 min (CR3i, Thermo Electron Co., Waltham, MA, USA), and the supernatants were filtered through a 0.22 μm filter (Nylon66, Jinteng Experimental Equipment Co., Ltd., Tianjin, China). Dilute sulfuric acid (5 mmol/L) was applied as the eluent (0.6 mL/min) at 35°C (Cai et al., 2018). The concentration of total ammonia nitrogen (TAN) was measured by a flow injection analyzer (AA3, SEAL, Germany). The concentration of the free ammonia nitrogen (FAN) was calculated based

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on equation Eq. (1) (Hansen et al., 1998).

$$[FAN] = [TAN]\times\left(1+\frac{10^{-\mathrm{pH}}}{10^{-\left(0.09018+\frac{2729.92}{T(k)}\right)}}\right)^{-1} \qquad \text{Eq. (1)}$$

Where [TAN] is the concentration of total ammonia (mg/L), [FAN] is the concentration of free ammonia (mg/L), and T(K) is the temperature (kelvin).

Microbial analysis: total microbial DNA was extracted from 3 mL of effluent from digesters at the 60th day using a bead beating plus column purification (RBB + C) as previously reported by Zhao et al. (2017). High-throughput (Illumina Miseq) sequencing was conducted by Majorbio Co., Ltd. (Shanghai, China). PCR and Illumina Miseq sequencing were conducted as described by Zhao et al. (2017). The primer sets 338F and 806R (5’-ACTCC TACGGGAGGCAGCA-3′and 5’-GGACTACHVGGGTWTCTAAT-3’) were used for bacteria, and primer sets 524F-10-extF and Arch958-modR (5’-TGYCAGCCGCCGCGGTAA-3’ and 5’-YCCGGCGTTGAVTCCAATT-3’) were used for archaea. The reads were assigned operational taxonomic units (OTUs) at 97 % sequence similarity. Community structure was analyzed at the phylum and genus levels using the Silva database (Release 115 http://www.arb-silva.de) (Zhao et al., 2017).

2.4 Statistical analysis

Statistical significance was evaluated by using univariate analysis of variances at a P-value of less than 0.05 in IBM SPSS Statistics 25. Cluster analysis and principal component analysis (PCA) were conducted using all measured parameters, including methane production, pH, alkalinity, lactic acid, and volatile fatty acids. Origin 9.0 and

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Canoco for Windows 4.5 was used for data analysis and graphical presentation.

3 Results and discussion

The responses of digester performance to liquid digestate recirculation under varying straw/manure ratios were investigated. The interaction between feeding ratios and recirculation was analyzed and discussed. Microbial communities in digesters of different strategies, including feeding ratio and whether with recirculation, were observed, and the relationship among operation strategies, fermentation parameters, and microbial community was discussed. Finally, the benefits of proper use of recirculation were quantified, and practical suggestions were draw to guide the appropriate application of recirculation by summarizing the optimized strategies.

3.1 Effects of feeding ratios and recirculation on digester performance

The effect of digestate recirculation under varying feeding ratios on digester performance were discussed by promotion in section 3.1.1 and inhibition in section 3.1.2. Performance enhancement by liquid digestate recirculation was observed in digesters fed with only CS and CS:PM 3:1, and performance inhibition was observed in digesters fed with CS:PM 1:1, 1:3, and only PM. The interaction of feeding ratios and digestate recirculation was discussed in section 3.1.3.

3.1.1 Improved effects of recirculation under CS and CS:PM 3:1

The stable period of methane production, which was day 6-20 for A and day 6-35 for Ar, was prolonged 15 d with the recycled utilization of liquid digestate (Fig. 1a). The methane production significantly ($P<0.05$) increased from 3.08±0.16 L/d for A to 3.38±

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0.20 L/d for Ar with an increased efficiency of 10 %, showing the effect of liquid digestate recirculation on methane production enhancement when fed with only CS.. The pH, alkalinity, and TAN of stable periods of both A and Ar decreased as digestion proceeded, indicating that the buffer substances and nitrogen nutrients discharged along with digestate were more than those added along with feedstock and produced by digestion. The decline of pH, alkalinity, and TAN in Ar was slower than that of A, because soluble matter in liquid digestate recycled back to digesters, which made a decreased dilution of buffer substances and nitrogen nutrients. As a result, the stability of digesters was improved by recirculation through reutilization of the buffer substances and nitrogen nutrients, so the longer period of high process performance was achieved in Ar than A. Consistent conclusions were draw in Li et al. (2018) and Peng et al. (2016), where liquid digestate recirculation achieved methane production enhancement of 11 % and 21 % along with stability improvement. When the pH decreased to under 6.8, alkalinity decreased to under 2.0 g/L, and TAN decreased to under 50 mg/L (day 24 for A and day 40 for Ar), total acids concentration sharply increased (Fig. 2a&b), showing an imbalanced state of digesters. It is obvious that the supplementation of buffering capacity by liquid digestate recirculation did not fill the loss of buffer substances through discharge when fed by only CS, so Ar still failed at the end. Before the concentration of acids increased, there was no significant difference ($P>0.05$) in the concentration of total acids between A and Ar, all of which were 0.24±0.01 g/L. Since hydrolysis of crop straw is the key rate-limiting step in anaerobic digestion (Yu et al.,

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2016), and acids (mainly substrates for methanogens) was in a deficiency in Ar, it was inferred that the acids returned by recirculation was directly converted into methane and formed the increase of methane production in Ar.

As in digesters fed with CS:PM of 3:1 without (B) and with (Br) liquid digestate recirculation, gas production continued at a steady and high level for the duration of experiment (Fig. 1b), and no significant difference ($P>0.05$) between B and Br was observed on methane production and proportion. It is consistent with results obtained by Hu et al. (2014), who investigated anaerobic digestion of CS added with urea (C/N=25) with and without liquid digestate recirculation and no difference in methane production was found between treatments. However, the pH, alkalinity, total acids concentration (Fig. 2c&d) and ammonia concentration (Fig. 3b) increased from 7.34±0.02 for A to 7.52±0.02 for Ar, from 3.50±0.54 g/L to 5.94±0.76 g/L, from 0.98±0.30 g/L to 1.55±0.56 g/L and from 378±70 mg/L to 760±177 mg/L, with an increasing rate of 2 %, 70 %, 58 % and 101 %, showing the throttling effect of liquid digestate recirculation on alkalinity, acids and ammonia, the same with A and Ar. Among the above parameters, only the alkalinity of B showed a significant decrease trend ($P<0.05$). According to the formula of its trend line [$y=-0.025x+4.376$, $R^2=0.7256$, where y is alkalinity (g/L), and x is time (d)], the alkalinity of digester B will decline to 2.0 g/L by the 95th day of digestion, at which time, the stability of digester B would collapse just as in digesters A and Ar. Whereas, risk of fermentation failure cannot be predicted in digester Br based on available data at present, showing a good effect of recirculation on the stability of

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digesters fed with CS:PM 3:1.

In summary, the improved effect of liquid digestate recirculation occurs in the fermentation system characterized by a lack of VFA, buffering capacity, and specific nutrients. Usually, this kind of fermentation system is caused by feedstock that is relatively difficult to degrade and imbalance in nutrients, such as crop straw. And the reason for the improvement is to alleviate the loss of insufficient substances through recirculating liquid digestate to make full use of the limited substances and render high performance.

3.1.2 Inhibited effects of recirculation under 1:1, 1:3, and PM

The dynamics of daily methane production and proportion of the digesters fed by CS:PM 1:1 (C and Cr), 1:3 (D and Dr), and only PM (E and Er) without and with liquid digestate recirculation are shown in Fig. 1c-e. Among these six treatments, only digester C (1:1 without recirculation) performed stable and efficient after start-up till the end. No significant change trend ($P>0.05$) was found in methane production, VFAs, alkalinity, and ammonia concentration, indicating the equilibrium between hydrolysis, acidification, and methanogenesis in digester C. However, the balanced state of fermentation was disturbed by liquid digestate recirculation shown as VFAs, alkalinity, and TAN cumulated in Cr over time. Imbalanced digestion also occurred in Cr, D, Dr, E, and Er. The methane production of these digesters went through four periods: start-up period, stable period 1, decline period, and stable period 2. For digesters Cr, D, Dr, E and Er, the methane production in stable period 2 was 24 %, 19 %, 85 %, 73 %, and 93 % lower

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than that in stable period 1, indicating these digesters were in an inhibited state. The methane production in stable period 2 in digesters with liquid digestate recirculation was only 77 %, 18 % and 23 % of the methane production in stable period 2 in digesters without recirculation under CD: PM 1:1, 1:3 and only PM, which reflected the aggravated inhibition with liquid digestate recirculation.

Unlike methane production, the dynamics of VFAs, alkalinity, pH (Fig. 2f-j), and ammonia concentration (Fig. 3c-e) in digesters Cr, D, Dr, E, and Er couldn’t be divided into periods, but continuously changed over time. The reason to this asynchrony is that inhibitors need to accumulate to exceed limits before they exert their inhibitory effect. In anaerobic digestion, ammonia is an essential nutrient for microbial growth and a good buffer for digester stabilization when it is in an appropriate dose. But when ammonia accumulated to a relatively high dose, it becomes an inhibitor to restrict methanogenesis ((Rajagopal et al., 2013; Wang et al., 2016). In this study, whether with liquid digestate recirculation or not, when the TAN above 1,500 mg/L, and FAN above 50 mg/L, the corresponding methane production ended the stable peried 1 and entered to the decline period. The duration of stable period 1 in digesters with liquid digestate recirculation was shorter than that without recirculation under the same feedstock condition, indicating inhibitors were enriched by liquid digestate recirculation.

It is accepted that ammonia strongly inhibited methanogenesis but minimally affected hydrolysis and acidification, as a result to which, VFAs accumulation will occur (Rajagopal et al., 2013; Wang et al., 2016). The concentration of acetic acid, propionic

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acid, and butyric acid increased along with the increase of TAN. The increased VFAs usually results in a decrease in pH and further limits methanogenesis activity, just like the final rancidity of A and Ar. However, in Cr, D, Dr, and E, VFAs, alkalinity and ammonia neutralized each other so that the pH did not decrease. The interactions between VFA, alkalinity, and ammonia led to an inhibited steady-state (Li et al., 2015), where the pH was in the suitable range recommended by literature for the anaerobic digestion (Chen et al., 2008), but methane production remained relatively stable in an inefficiency state, shown as the stable period 2 in these inhibited digesters. These results were in agreement with those of former investigations conducted by Huang et al. (2015) and Wu et al. (2016). It is noteworthy that butyric acid, which is an important chemical raw material and organic intermediate (Fu et al., 2017), counted over 55 % of the cumulated VFAs in digesters Cr, D, Dr, E, and Er. The role of accelerated VFA accumulation through liquid digestate recirculation might be performed with further research to benefit the biological production of butyric acid.

In summary, the recirculation of liquid digestate in digesters, which is in equilibrium and steady-state, will break the balance and cause accumulation and then come to the inhibition. Identically, the recirculation of liquid digestate in digesters, which is in excessive and accumulation state, will accelerate accumulation and lead to aggravated inhibition. As in this study and previous studies, the main inhibitor turned out to be the accumulation of ammonia when performing recirculation in digesting animal manure. Other inhibitors, such as TS accumulation (Estevez et al., 2014) and

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increased viscosity (Ni et al., 2017), were also found to be the main cause of inhibition in a few types of research.

3.1.3 Interaction of feeding ratios and liquid digestate recirculation

Cross comparison by cluster analysis and principal component analysis (PCA) was conducted on gas production and proportion as well as the internal digestion parameters of all the digesters.

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Cluster analysis divided the digestion strategies into four categories (Fig. 4a). Digesters A, Ar, and B were clustered together because their nitrogen (TAN<500 mg/L) and buffer (alkalinity<10 g/L) content were low and declining over time. Digesters C and Br were clustered because of the excellent performance in both gas production and system stability. Digesters D and Cr were clustered as the slightly inhibited group, and the digesters E, Dr, and Er were clustered as the seriously inhibited group.

From the perspective of the influent of digesters, the changes in feeding ratios and the use of digestate recirculation are both measures of changing influent composition. The high carbon to nitrogen ratio (C/N ratio) of crop straw could lead to an imbalance in nutrients and a lack of buffering capacity (Neshat et al., 2017; Sawatdeenarunat et al., 2015). And the low C/N ratio of animal manure could cause the inhibition of ammonia accumulation (Rajagopal et al., 2013). So the different straw-to-manure ratios formed a C/N gradient from high to low in influent from digester A to E. If the recirculated liquid digestate was regarded as a co-substrate just like CS and PM, the C/N ratio of influent was changed by exchanging CS-PM-water influent to CS-PM-digestate influent through liquid digestate recirculation. When calculated the C/N ratios of influent from the composition of raw materials and liquid digestate, the C/N ratios of influent in recirculated digesters were 1.3-3.2 lower than that in digesters without recirculation (Table S1), because some carbon in raw materials has dissipated as CH_4 and CO_2 while little nitrogen has gone as NH_3. All recirculation digesters were clustered with non-recirculation digesters which were fed with more CS and less PM, namely Br with A, Cr with B, Dr with C and Er with D, because their similar C/N ratios could service similar nutrition and environment for anaerobic organisms. When the C/N ratio of the raw material is higher than the suitable range, the C/N ratio of the new substrate composed of liquid digestate will be closer to the suitable range, or enter the suitable range, providing more balanced nutrients for microorganisms and obtaining more efficient digestion process. Similarly, when the C/N ratio of raw material is lower than the appropriate range, the recirculated liquid digestate will make the C/N ratio of the new substrate lower and farther away from the appropriate range, resulting in a greater imbalance in digesters. Based on this, liquid digestate recirculation is more suitable in anaerobic digestion of materials with a slightly high C/N ratio (a C/N of 25 in this study), such as lignocellulosic materials.

The results of PCA closely complemented those of cluster analysis (Fig. 4b). The four performance groups shown by cluster analysis (inadequate, well-run, mildly inhibited and severely inhibited), could be clearly positioned across the arched distribution. The proportion of CS gradually decreases and PM increases along the arch

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from left to right. The points that are positioned at the left-hand end of the arch reflected a shortage of buffering matter, while the points lying at the right-hand end of the arch reflected inhibition by ammonia and accumulation of organic acids, indicating that the lignocellulosic materials tends to the digestion performance type of inadequate, while livestock manure tends to the type of excessive. The factor loading plot for the PCA is presented in Fig. 4c. Alkalinity, TAN and FAN contribute to the positive direction of principle component 1 (PC1) and principle component 2 (PC2). All acids contribute to the positive direction of PC1 and negative direction of PC2. If a point moves close to the positive direction of PC1 it means both acid and alkali increase, and if a point moves close to the negative direction of PC1it means a decrease in acid and alkali. Liquid digestate recirculation moved the distribution of all feedstock mixtures to the right, among which only CS (A and Ar) was moved right and upwards while 1:1 (C and Cr), 1:3 (D and Dr) and only PM (E and Er) were moved right and downwards (Fig. 4d). The results indicated that the effect of liquid digestate recirculation on anaerobic digestion under all tested feedstock conditions is to increase alkalinity, ammonia and organic acids concentrations. For digesters in inadequate state such as digesters fed with CS or CS:PM 3:1, the increasing of alkali and acid by recirculation relieved or eliminated the shortage. While for digesters in balance or excessive state such as digesters fed with CS:PM 1:1, 1:3 and PM alone, the increasing of alkali and acid by recirculation triggered or aggravated the accumulation of ammonia and VFA, causing subsequent adverse fermentation performance.

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In summary, feeding ratios and digestate recirculation both are measures to change influent. Different feeding ratios from CS to PM provided different C/N ratio from high to low for the digesters, and formed different levels of metabolites from inadequate to accumulated. On the basis of feeding ratios, liquid digestate recirculation played a role in reducing the C/N ratios of influent, and recycling parts of metabolites, and benefited digesters with high C/N and inadequate metabolites, inhibited digesters with low C/N and accumulated metabolites.

3.2 Effects of feeding ratios and recirculation on microbial community

Anaerobic digestion is conducted by a community of microbes. To further understand the microbial responses to feeding ratios and liquid digestate recirculation, microbial analysis by Illumina MiSeq sequencing were carried out. The relationship between fermentation parameters and microbial community was analyzed by redundancy analysis (RDA). The detailed microbial community structure of methanogens and the correlations between factors and methanogens was shown and discussed.

3.2.1 Summary of the observed sequence

The summary of microbial analysis including richness and diversity indexes were in Table S2. Corresponding to the fermentation performance of digesters, inhibited digesters (Cr, D, Dr and E) showed lower richness and diversity in bacterial community, which was indicated by less OTUs and species, lower Chao and Shannon indices, and higher Simpson indices in digesters Cr, D, Dr and E than in digesters Ar, B, Br and C. In

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terms of archaea, this difference between uninhibited and inhibited digesters was not obvious, but the diversity of archaea in digester C, which produces the highest methane yield, was lower than that of other digesters, indicating the methane metabolism of high efficient digesters was relatively pure and simple.

3.2.2 Redundancy analysis

The relationship between fermentation parameters and microbial community was analyzed by redundancy analysis (RDA) (Fig. 5). When the digester points were projected perpendicularly onto the arrow of CS and PM, the projections of digesters with liquid digestate recirculation located closer to the positive end of PM and further to the negative end of CS than digesters fed by the same feedstock ratios but without liquid digestate. The microbial community structure changed to that of more PM and less CS under the influence of liquid digestate recirculation, which was corresponding to the fact summarized from the fermentation performance data. Among all the fermentation parameters, the arrows of TAN and propionic acid were the longest in Fig 5a, indicating the major effects of TAN and propionic acid to bacterial community changes. While, the arrows of TAN and Alkalinity were the longest in Fig 5b, indicating their major impacts on archaeal community dynamics. The different straw/manure ratios in feedstock caused the varying amount of flow-in nitrogen and then resulted in difference in TAN. The discharge or recirculation of liquid digestate caused the change in the amount of flow-out TAN. Then, under the major effect of different TAN, the microbial community evolved into different structure and formed different digester performance types.

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Bacteria species from phylum of Firmicutes, Cloacomonetes, Tenericutes and Proteobacteria; Methanogens from genus of Methanoculleus, Methanosarcina, Methanobrevibacter, Methanosphaera and Methanobacterium survived the high TAN environment, and formed the methanogenic metabolism under severely inhibition of ammonia.

3.2.3 Microbial community structure

The community structures of archaea at genus level were shown in Fig. 6a. Combined with digester performance, the dominant methanogen in digester Ar were Methanocorpusculum (19.3 %) and Methanosaeta (18.7 %), and the unique archaeal community of digester Ar corresponded to its inadequate and out-run state. The dominant methanogen was Methanosaeta in digesters B (51.8 %), Br (51.9 %), C (83.4 %) and Cr (67.3 %), and these digesters were all in steady methane production. However, digesters B, Br and C was in steady-state from beginning, but digester Cr was in steady-state after mildly inhibition. Corresponding to the slightly inhibition in digester Cr, Methanobacterium (12.4 %), Methanosphaera (7.2 %) and Methanosarcina (5.9 %) in digester Cr were enriched. In digesters D, Dr and E, which were also in the fermentation state of inhibitory, Methanosarcina was abundant, accounted 41.2 % for D, 32.1 % for Dr and 49.7 % for E. In addition, Methanosphaera, Methanobrevibacter, Methanoculleus were more abundant in digesters D, Dr and E than in other digesters. An interesting phenomenon was that the relative abundance of Methanosaeta were 35.4 % and dominant in archaea in the severely inhibited digester Dr with the TAN of 3,000

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mg/L. However, it has been widely reported that Methanosaeta are sensitive to high ammonia concentrations (Wilson et al., 2012; Zamanzadeh et al., 2016). In the study by Zamanzadeh et al. (2016) on anaerobic digestion of food waste, the digester with liquid digestate recirculation also had a higher relative abundance (91 %) of Methanosaeta than that of digesters without recirculation (65 %) under mesophilic condition. It was speculated that the toxicity of ammonia to Methanosaeta was relieved in digesters with liquid digestate recirculation.

The relationship between fermentation parameters and methanogens was shown in Fig. 6b by correlation analysis. Among all the parameters, the percentage of CS in feedstock showed the significant correlation ($P<0.05$) with methanogens from 6 genera, indicating the change in feeding ratios was one of the main factors that led to change in methanogen community structure. Also, TAN was one of the main factors who showed significant correlation ($P<0.05$) with methanogens from 5 genera. Methanogens from genus of Methanoculleus, Methanosarcina, Methanobrevibacter, and Methanosphaera showed significant positive correlation ($P<0.05$) with concentrations of acids and ammonia, which was consistent with results from RDA, and showing the tolerance of these methanogens under inhibition of acids and ammonia accumulation.

The changes in bacteria community structure were not as dramatic as methanogens. (Fig. S1). Firmicutes and Bacteroidetes accounted for over 60 % of the total bacteria and were dominant phylums in all digesters, and the relative abundance of Firmicutes gradually increased along with the proportion of CS decreased in feedstock. TAN was

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the main factor in changing bacteria community structure because TAN showed significant correlation with 12 bacterial phyla among the top 14 phyla.

In summary, the microbial community structure varied corresponding to the fermentation state. The dominant methanogens in the well-run digesters were Methanosaeta, while Methanosarcina and Methanosphaera dominated in the inhibited digesters. TAN was the main factor in microcial dynamics. And liquid digestate recirculation might relieve the toxicity of ammonia to Methanosaeta in inhibited digester.

3.3 Benefits analysis

In order to quantify the benefits of liquid digestate recirculation, the consumption and emission data under the same energy output were calculated based on the data in this study from the best process without recirculation (digester C, marked as convention mode) and the best process with recirculation (digester Br, marked as recirculation mode) (Table 3). The discharge of liquid digestate of recirculation mode was reduced by 81.9 % compared to that of convention mode, and soluble chemical oxygen demand (sCOD) emission was reduced by 70.4 %. The water consumption (water used in diluting feedstock into desired influent TS) of recirculation mode was cut down by 65.4 % of convention mode. The warm liquid digestate, instead of water at ambient temperature mixed with raw materials, could reduce the heat needed to raise the influent to the fermentation temperature due to heat recovery. According to the equation expressed by Wang et al. (2018), the theoretical heat required to raise the influent to fermentation

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temperature in recirculation mode was 56.3 % lower than that in convention mode, reducing the influent heat load from 9.7 % to 4.2 % of total energy production (energy of total produced biomethane). The reduced water consumption and influent heat load in recirculation mode, on the one hand, marked the contribution of recirculation in saving resource and energy; on the other hand, indicated the benefits of cost-saving. Under the buying water price of 2-7 CNY and the selling biomethane price of 2-4 CNY, the saved cost in the recirculation mode of water and heat was 7.1-15.7 % of total revenue from sales of biomethane.

When scaled up to daily biomethane production of 10,000 m^3 and compared to convention mode, the recirculation mode could reduce the discharge amount of liquid digestate of 335.7 t/d, reduce sCOD emission of 1.22 t/d, save water of 280 t/d and theoretically recover heat from liquid digestate of 19.0 GJ/d, showing the advantages of recirculation mode in reducing pollutant, saving resource and energy, and reducing cost. However, a more detailed calculation is needed under a certain situation and include the cost of handling raw material and solid digestate which have huge regional differences.

As a potential benefit, the production time and amount of liquid digestate might be controlled by changing proportions of agricultural waste in feedstock and selectively employing liquid digestate recirculation while ensured efficient production of renewable methane. A new operation strategy of alternate using CS:PM 3:1 with recirculation (Br) and CS:PM 1:1 without recirculation (C) could be performed in the same one digester, and run C when liquid digestate was needed while run Br when not needed. The

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integrated approach will achieve on-demand production of liquid digestate and facilitate downstream utilization of liquid digestate for seed germination promotion (Aihemaiti et al., 2019), pest control and growth regulation (Li et al., 2016) in the seasonal agricultural process, which will achieve high value utilization of liquid digestate and promote sustainable development of agriculture.

3.4 Production recommendation

Liquid digestate recirculation provides a possibility of not discharging continuously produced, large amounts of liquid digestate in scaled biomethane projects, which attracts the attention of practitioners and researchers. However, because of the difference of raw materials and processes, the liquid digestate obtained is also very different, which makes the recirculation of liquid digestate perform different effects under different situation. The possible negative effects of recirculation restrict its popularization and application. The interaction between feedstock formula and liquid digestate recirculation on anaerobic digestion was investigated in this study. Results indicated that recirculation was a process to reduce material loss from digesters. For this reason, the improved effect of recirculation occurred in digesters that were lack of nutrients and buffers, while the inhibited effect occurred in digesters that were abundance of nutrients and buffers. Since the inhibitory effect of liquid digestate recirculation is caused by inhibitor accumulation, the inhibition is not immediately apparent with the beginning of recirculation. In the process of liquid digestate recirculation, it is recommended to grasp the changing trend of possible inhibitors to keep high efficient and to adjust in time.

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As under the same operation condition of this study, digesters fed by more-straw feedstock performed into inadequate state and improved by liquid digestate recirculation while digesters fed by more-manure feedstock performed into excessive state and inhibited by liquid digestate recirculation. In addition, both the fermentation parameters and the microbial analysis indicated that digesters with liquid digestate recirculation were similar to those without liquid digestate recirculation but fed by more manure and less straw. That is, if the liquid digestate recirculation will be applied to a well-run digester, it is necessary to adjust the feedstock formula, appropriately increase the proportion of straw and reduce the proportion of manure, to match the liquid digestate recirculation so as to avoid inhibitor accumulation and achieve desired performance. And the appropriate proportion under conditions of this study was 25 %. Conversely, some external conditions may cause the raw materials to change, such as the manure in the area is not enough to be used as co-digestion material, or the straw is eager to be disposed of because of the seasonal harvest. If a well-run digester had to change feedstock formula to fed by less manure or more straw, the liquid digestate recirculation is recommended to be introduced to make full use of buffers and nutrients to match the change in feedstock formula.

This study focused on the response of digestion performance to liquid digestate recirculation under different feedstock conditions with uniform process conditions, rather than optimize the recirculation process of different feedstock conditions. So, the final failure or decline of methane production occurred in digesters fed by only CS,

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CS:PM 1:3, and only PM. However, it is worthwhile to further study the optimization of liquid digestate recirculation under these feedstock conditions and other types of raw material in the future, which is conducive to reducing the discharge of liquid digestate from the source and making full use of water, heat, and substances in liquid digestate. Li et al. (2018) and Peng et al. (2016) also studied the effect of liquid digestate recirculation on digesting CS, pretreatment methods (NaOH), buffering agents ($NaHCO_3$), and longer HRT (40-45 d) were used, which could make straw easier to degrade, provide better conditions, and more time for microorganisms to degrade. As a result, no process failure occurred, and long-term, stable, and efficient fermentation was achieved. An integrated approach including pretreatment, pH control, liquid digestate recirculation, etc. may be a potential scheme to solve the problem of mono-digestion of crop straw in areas where manure and other co-digestion materials are scarce.

In studies on liquid digestate recirculation in manure digesters, appropriate interventions were introduced to reduce the negative impact of liquid digestate recirculation on digesting animal manure and provide the possibility of ensuring both high efficient digestion and the long-term operation of recirculation. In a study by Nie et al. (2015) on mono-digesting chicken manure with liquid digestate recirculation, 72 % of ammonia in liquid digestate was removed through decompression steam stripping before recirculation, and long-term stable digestion was achieved. Besides, air stripping was used by Wu et al. (2016) to mitigate ammonia in liquid digestate before recirculation in anaerobic digestion of chicken manure. Results showed the ammonia

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concentration in digester was declined after ammonia stripping of liquid digestate, and methane yield was back to the same level as the control digester without recirculation. However, either vacuum stripping or air stripping requires additional equipment and operation costs, which limits these technologies to be promoted. Further research is needed to reduce costs and simplify operation.

Some studies have been optimized and explored the more detailed process optimization, including recirculation ratio (partially recirculation) (Ratanatamskul and Saleart, 2016; Zuo et al., 2015), pre-mixing time of liquid digestate with raw material before recirculation (Elsayed et al., 2019; Liu et al., 2019), etc., to achieve high efficient digestion with liquid digestate recirculation. In this way, the liquid digestate recirculation will have the potential to be applied under a wider variety of raw material conditions and operating processes. And the advantage of controllable discharge of liquid digestate, waste reduction, and water conservation of liquid digestate recirculation will benefit more regions and people.

4 Conclusions

Ten lab-scaled semi-continuous anaerobic digesters, fed by corn stalk, pig manure, and their 3:1, 1:1, 1:3 mixtures, with or without liquid digestate recirculation, performed as four states of inadequate, well-run, mildly inhibited and severely inhibited. Methane production was promoted by recirculation when fed with corn stalk and 3:1, while inhibited under 1:1, 1:3 and pig manure. Microbial community varied to their performance states. The regimes of 3:1 with recirculation and 1:1 without recirculation

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outperformed the other digesters, and their dominant methanogens were Methanoseata. Recirculation mode showed 81.9 % liquid digestate reduction, 70.4 % COD reduction, and 65.4 % water saving to that of convention mode, which achieving efficient and cleaner biomethane production.

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Table 1.

Characteristics of corn stalk (CS), pig manure (PM) and inoculum.

Parameter	Unit	Corn stalk	Pig manure	Inoculum
Moisture content	% Wet weight	8.58±0.33	68.20±0.57	93.14±0.42
Total solids	% Wet weight	91.42±0.31	31.80±0.41	6.86±0.34
Volatile solids	% Total Solids	98.55±0.18	78.00±0.23	58.93±0.19
Hemicellulose	% Total Solids	30.47±0.01	14.56±0.04	11.56±0.02
Cellulose	% Total Solids	34.08±0.19	9.28±0.44	13.76±0.03
Total carbon	% Total Solids	61.81±0.62	47.02±1.08	40.39±0.07
Total nitrogen	% Total Solids	1.22±0.00	4.78±0.00	1.86±0.00
C/N ratio		50.7±0.7	9.8±1.13	21.8±0.00
pH		Not determined	7.7±0.0	7.5±0.0

Average±standard deviation (n=3)

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Table 2.

Experimental design in this study.

Setup	Feedstock (or CS:PM[a])	C/N Ratio[b]	Liquid digestate	Name[c] in the study
1	Only CS	50.7	Discharge	A
2	Only CS	50.7	Recirculation	Ar
3	3:1	25.2	Discharge	B
4	3:1	25.2	Recirculation	Br
5	1:1	16.7	Discharge	C
6	1:1	16.7	Recirculation	Cr
7	1:3	12.4	Discharge	D
8	1:3	12.4	Recirculation	Dr
9	Only PM	9.8	Discharge	E
10	Only PM	9.8	Recirculation	Er

a. The CS:PM is the ratio of corn stalk to pig manure in feedstock, and is based on volatile solid (VS) content.

b. Calculated from proportion and composition of raw material

c. The letter “r” represents recirculation.

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Table 3.

Comparison of emission and consumption per unit energy output between convention mode (based on digester C) and recirculation mode (based on digester Br).

	Unit	Convention mode	Recirculation mode	Difference [a]
Energy output	GJ	1	1	0.0 %
Biomethane [b]	m^3	27.9	27.9	0.0 %
Revenue of selling gas [c]	CNY	57.5-115.0	57.5-115.0	0.0 %
Influent				
Corn stalk	kg [d]	68.8	112.4	-63.3 %
Pig manure	kg	249.9	136.8	45.3 %
Clean water	m^3	1.23	0.43	**65.4 %**
Liquid digestate	m^3	0.00	1.01	--
Effluent				
Solid digestate [e]	kg	387.5	470.9	-21.5 %
Liquid digestate	m^3	1.16	1.22	-5.2 %
-Recirculation	m^3	0.00	1.01	--
-Discharge	m^3	1.16	0.21	**81.9 %**
-sCOD discharge	kg	5.00	1.48	**70.4 %**
Heat required [f]	GJ	0.097	0.042	56.3 %
Methane required	m^3	2.70	1.18	56.3 %
Clean water cost [g]	CNY	2.5-8.6	0.9-3.0	65.4 %
Heat cost [h]	CNY	5.4-10.8	2.4-4.7	56.3 %
Sum of water and heat cost	CNY	8.0-19.7	3.3-7.8	58.0-61.9 %
Proportion of cost in revenue	%	**12.2-25.4**	**5.1-9.7**	58.0-61.9 %

a. Difference was calculated as (x-y)/x*100 %,where x is the value of convention mode and y is value of recirculation mode.

b. 1 GJ energy is converted from 27.9 m^3 biomethane, the heat value of which is 35.9 MJ/m^3.

c. Gas sold is bio-natural gas with a methane proportion of 97 %, and the price is 2-4 CNY according to practice in China.

d. The values, which are united by kg, are based on wet weight.

e. The moisture content of solid digestate is 78.8 % after solid-liquid separation.

f. Heat required to raise the influent to fermentation temperature (35 □). The ambient temperature is assumed as 20 □.

g. The commercial water price is assumed 2-7 CNY according to practice in China.

h. Heat cost is calculated as the reduced income of selling biomethane by the combustion of biomethane to obtain these heat.

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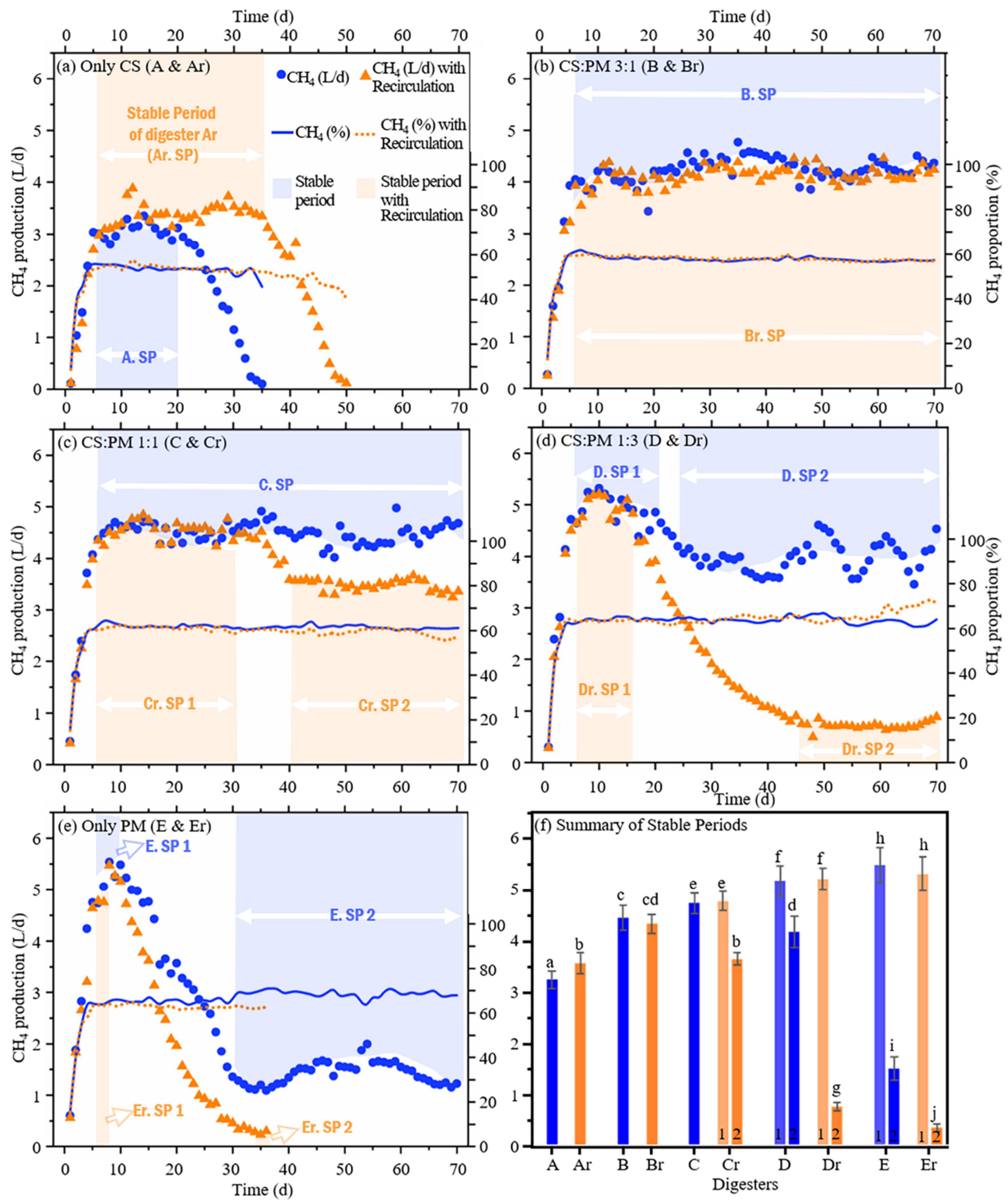

Fig. 1. Methane production (scatters) and proportion (lines) without (blue) or with (orange) liquid digestate recirculation (a-e). And bar chart (f) shows the average methane production of stable periods (periods that covered by shadows in a-e).

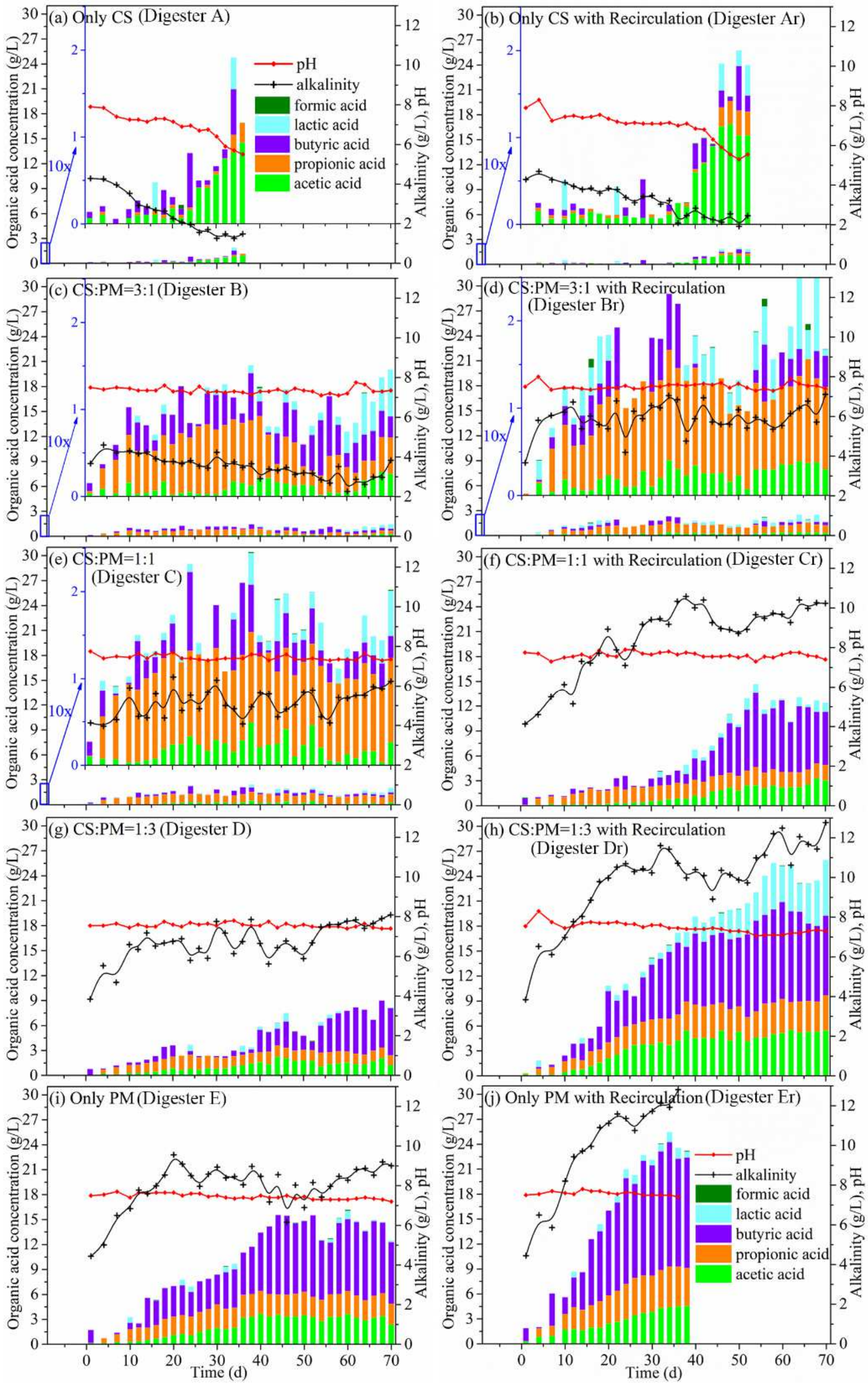

Fig. 2. Changes of pH (red symbol-line, dot), alkalinity (black symbol-line, cross) and organic acids (acetic, propionic, butyric, lactic, formic which are represented as five colored and stacked columns) concentrations.

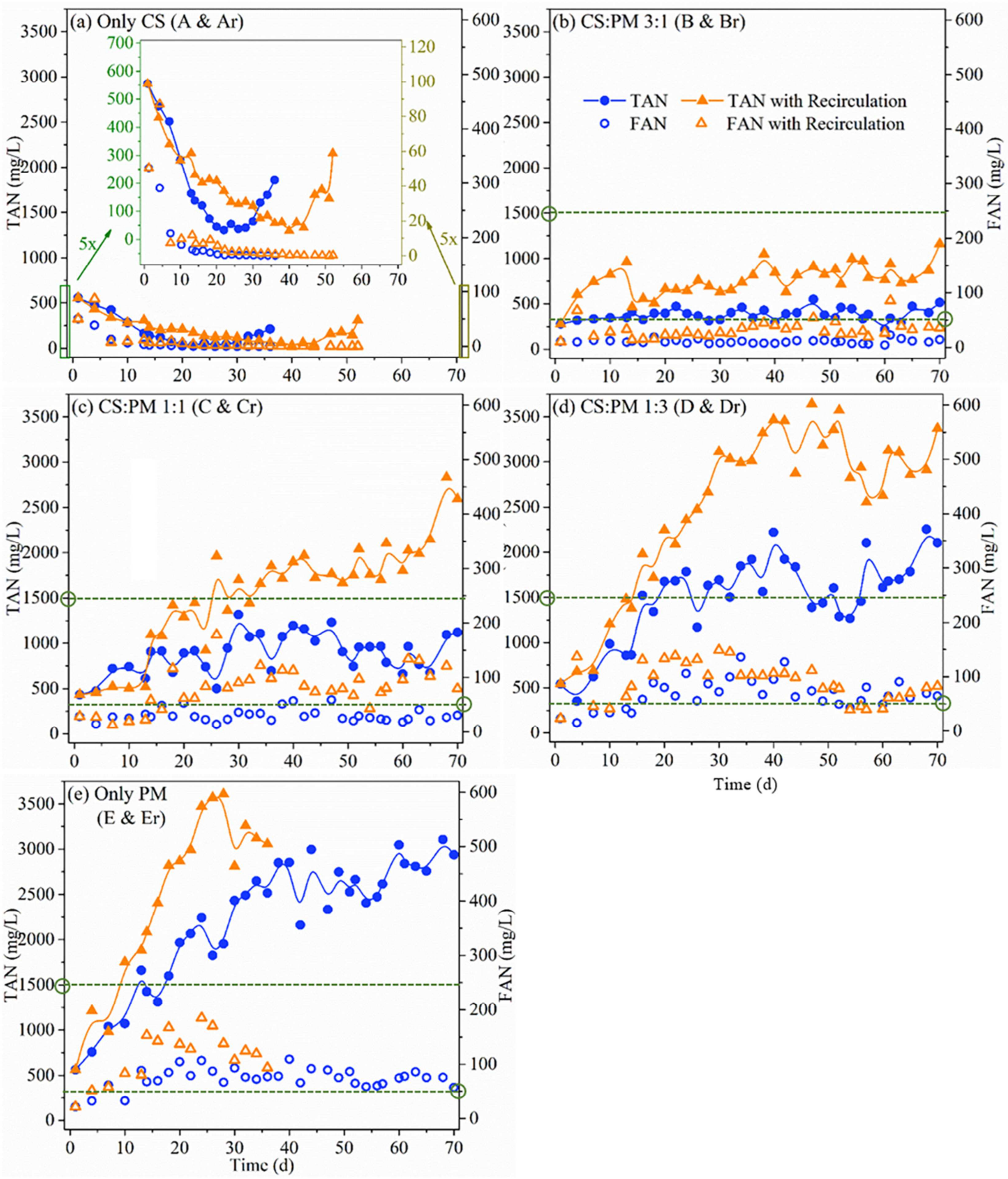

Fig. 3. Total ammonia (TAN), free ammonia (FAN) concentrations (symbol-lines) without (blue circle) or with (orange triangle) liquid digestate recirculation.

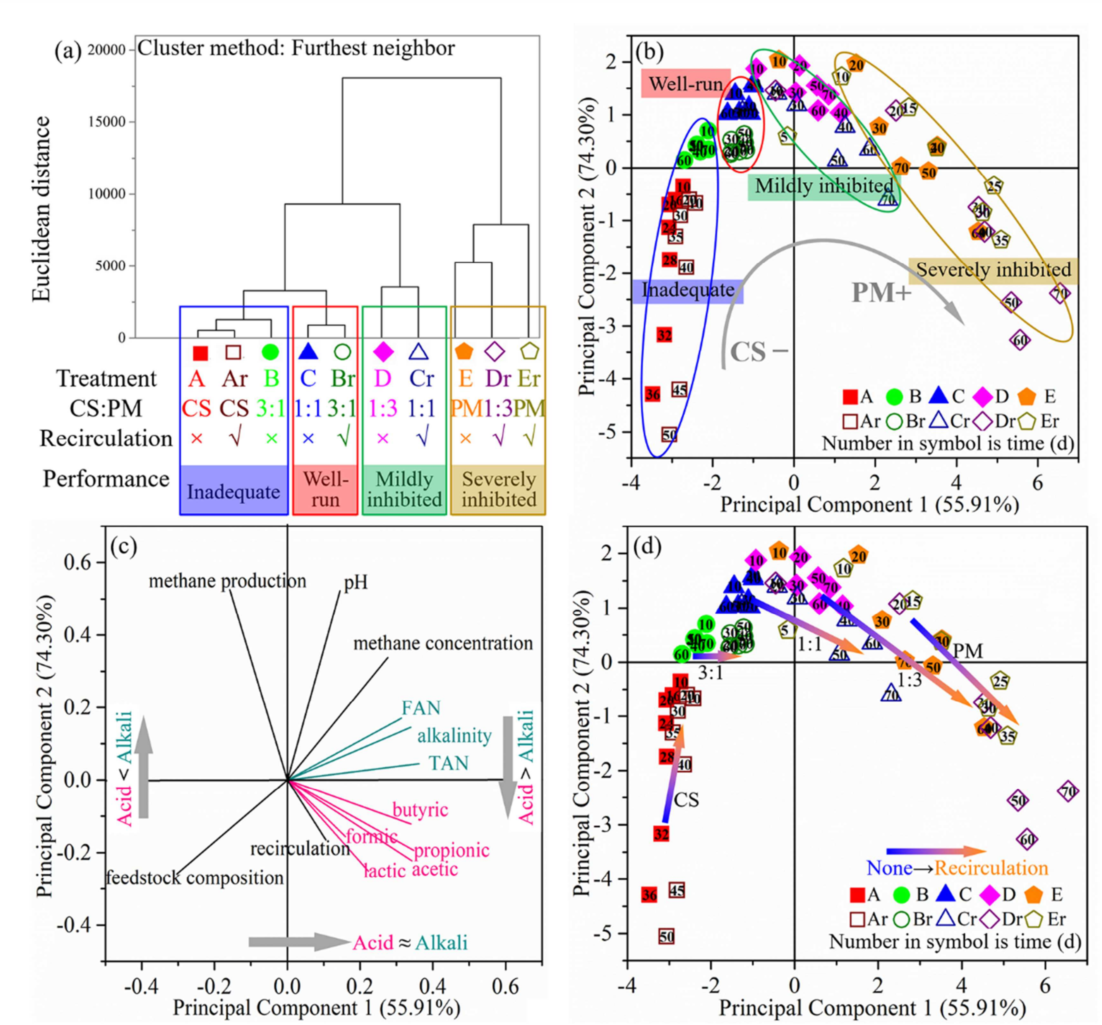

Fig. 4. Cross comparison by [a] cluster analysis where ten digesters are clustered into four performance types of inadequate, well-run, mildly inhibited and severely inhibited; and by [b] principal component analysis (PCA). [c] The factor loading plot for PCA. [d] Position changes in PCA caused by liquid digestate recirculation.

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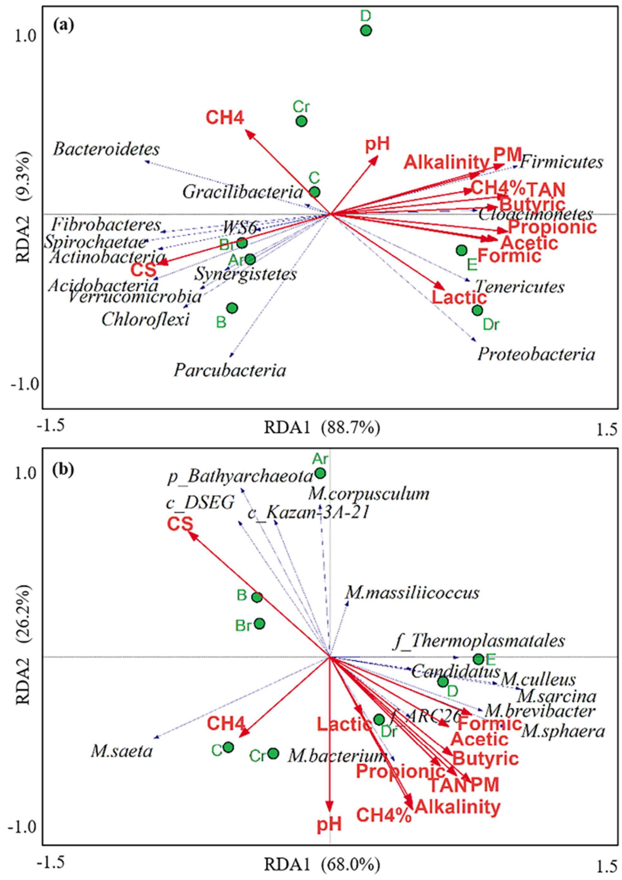
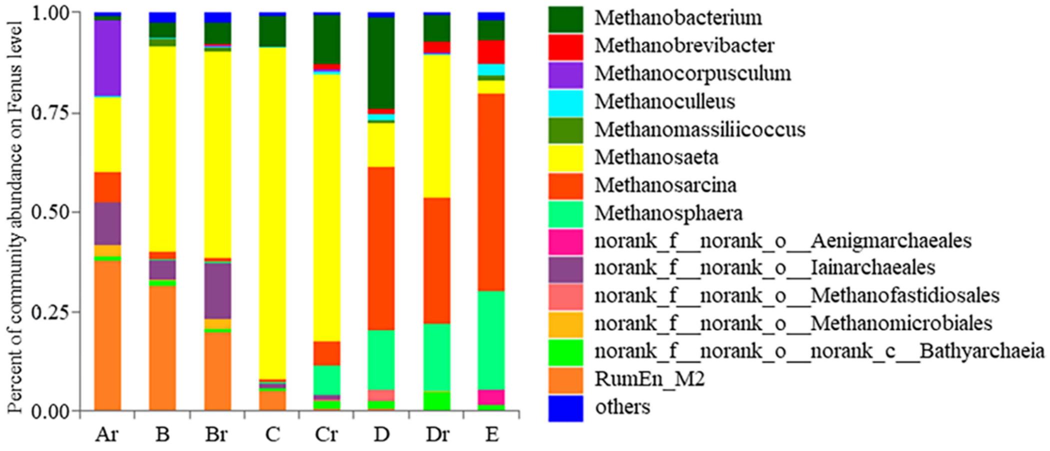


Fig. 5. Redundancy analysis of bacteria on phylum level (a) and archaea on genus level (b) with fermentation parameters.

(a) Community barplot analysis



(b) Pearson Correlation Heatmap

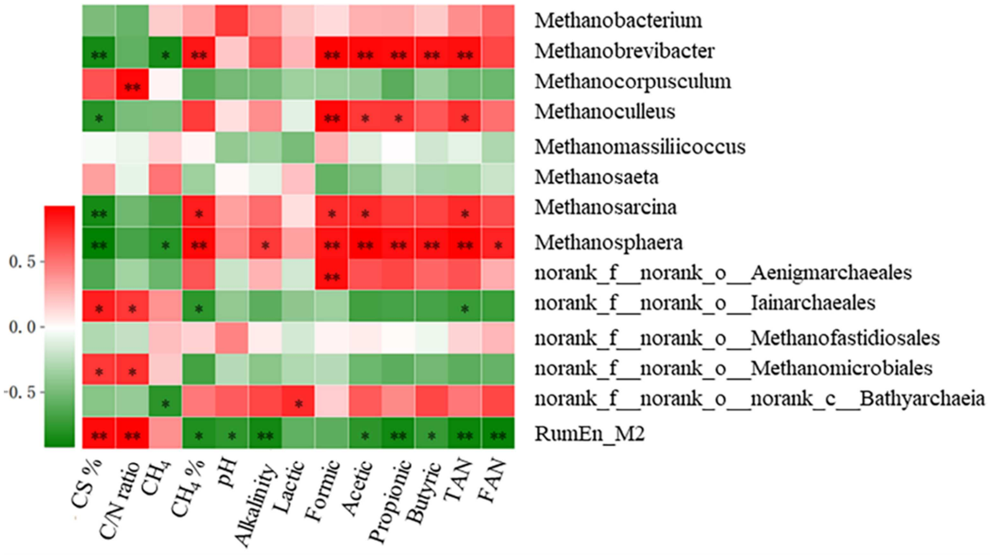


Fig. 6. Community barplot analysis (a) of archaeal community at genus level, and pearson correlation heatmap (b) between archaeal genus and fermentation parameters (* means p<0.05, ** means p<0.01, and ***means p<0.001).

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Highlights

- Cross effects of liquid digestate reuse and feed-formula on performance and microbes.
- Recirculation is beneficial when straw to manure ratio is 3:1 or higher in feedstock.
- Recirculation mode ran well and reduced 81.9% liquid discharge and 65.4% water usage.
- Cooperation of feedstock and recirculation may achieve controllably liquid discharge.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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