Characterization of microbial evolution in high-solids methanogenic co-digestion of canned coffee processing wastewater and waste activated sludge by an anaerobic membrane bioreactor

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A R T I C L E   I N F O

Article history:
Received 13 March 2019
Received in revised form 18 May 2019
Accepted 4 June 2019
Available online 6 June 2019

Keywords:
Co-digestion
Canned coffee processing wastewater
Waste activated sludge
AnMBRs
Microdynamics

A B S T R A C T

The effects of the microbial community and dynamics on the efficiency of a methanogenic co-digestion system that treats canned coffee processing wastewater and waste activated sludge by means of an anaerobic membrane bioreactor, were investigated and analyzed. The co-digestion system had a chemical oxygen demand (COD) removal efficiency >90%, and a COD to methane ratio >85%. Phyla Synergistetes, Firmicutes, Proteobacteria and Ca. OP9 were dominant bacteria throughout the investigation, and the main contributors to the hydrolysis and fermentation processes. The degradation paths and functional microbes indicated that genera Caldicoprobacter and Clostridium were the main contributors in the hydrolysis process, while genus Anaerobaculum dominated the acidogenesis and acetogenesis at the most efficient hydraulic retention time (HRT) of 10 d (HRT10). The dominant methanogenesis varied from genus Methanosarcina (71.1%, HRT10) to Methanothermobacter (56.4%, HRT3), indicating a transition from acetic methanogenesis to hydrogen-dependent methanogenesis. Furthermore, a microbial analysis indicated that Acinetobacter was the main contributor to caffeine degradation in this system. This also appears to be the first time that Acinetobacter is reported to be capable of degrading caffeine in the anaerobic condition.

1. Introduction

As the main waste in coffee processing, canned coffee processing wastewater (CCPW) requires proper treatment to improve the balance between the required energy and the recovery of energy during coffee production, due to the large amount of wastewater generated (Battista et al., 2016; Dadi et al., 2018; Dinsdale et al., 1996, 1997). Anaerobic digestion (AD) is considered as an effective technology for chemical energy recovery in waste (Beyene et al., 2018; Siddique and Wahid, 2018), and have been demonstrated to be suitable for CCPW treatment due to the rich organic materials (such as carbohydrates, lipids and proteins) with good biodegradability (Dinsdale et al., 1997; Qiao et al., 2013). Thus, CCPW is a potential net supplier of renewable energy by means of the AD process, which provides dual environmental benefits through improved wastewater treatment and sustainable bio-energy generation.

According to previous studies, co-digestion with other substrates has been proven superior to mono-substrate digestion due to balanced nutrients, sufficient alkalinity, and etc. (Razaviarani and Buchan, 2014; Zhu et al., 2011, 2011). While many operational and physicochemical parameters, such as feeding characteristics, organic loading, temperature, pH and among others, are greatly impacting the performance and stability of co-digestion systems (Hardegen et al., 2018; Siddique and Wahid, 2018; Syaichurrozi et al., 2018; Zhao et al., 2018). Moreover, as a synbiotic biological process, anaerobic co-digestion also relies on the activities and community structure of microbes. In particular, for substrates that...
Abbreviation list

| (ACE) | Abundance-based coverage estimator |
| (ALK) | Alkalinity |
| (AD) | Anaerobic digestion |
| (AnMBR) | Anaerobic membrane bioreactor |
| (BALK) | Bicarbonate alkalinity |
| (CCPW) | Canned coffee processing wastewater |
| (COD) | Chemical oxygen demand |
| (CSTR) | Continuous stirred tank reactor |
| (F/M) | Food-to-microorganisms |
| (HRT) | Hydraulic retention time |
| (MLVSS) | Mixed liquor volatile suspended solids |
| (N) | Normalized |
| (ORL) | Organic loading rate |
| (OTUs) | Operational taxonomic units |
| (TALK) | Total alkalinity |
| (TS) | Total solids |
| (VFAs) | Volatile fatty acids |
| (VS) | Volatile solids |
| (WAS) | Waste activated sludge |
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contain specific organic materials, such as cellulose, caffeine and lignin, the community structure or specific microbes are significant for a stable performance of this co-digestion system (Hasina et al., 2013; Zou et al., 2018). Based on this, a deeper comprehensive understanding and resolution of the link between microbial community dynamics and co-digestion performance would therefore be valuable for improving control of the reactor performance.

Caffeine is a widely found component in coffee, and is harmful to the ecological system due to its recalcitrant property (Ashihara and Crozier, 1999; Paulo, 2002). Though some studies have been conducted in recent years, the efficient degradation of caffeine still remains a significant challenge, especially in the anaerobic condition, and with the functional microbe counts in this process being very low.

As a typical waste in waste water treatment, waste activated sludge (WAS) is characterized with a low carbon-to-nitrogen ratio of 5.3, so it is capable of adjusting the carbon-to-nitrogen ratio to be appropriate for the anaerobic digestion when co-digestion with CCPW (Chen et al., 2019). The objectives of this study are to investigate the reactor performance linking the microbial community dynamics of thermophilic co-digestion of CCPW and WAS at series organic loading in a submerged anaerobic membrane bioreactor (AnMBR) by changing the hydraulic retention time (HRT), comprehensively comparing the microbial composition and dynamics under different organic loadings, and linking these microbial findings with their respective performances. In addition, microbes related to the degradation of caffeine in this system are also expected to be identified. The work reported here is expected to improve our knowledge about the microbial responding mechanisms and the optimization of operational conditions in a co-digestion system to control this system better for high-efficiency performance.

2. Materials and methods

2.1. Reactor configuration and operation

An AnMBR with a total volume of 10 L (effective volume 6 L) was used, the schematic diagram of which was presented in a previous study (Chen et al., 2017a). A flat sheet membrane made of chlorinated polyethylene with a total area of 0.116 m² was used in this reactor, and the pore size was 0.2 μm (Kubota Membrane Cartridge, Japan). For fouling control, gas sparging was employed and a gas pump (APN-085 LV-1, Iwaki, Japan) was used to circulate the biogas with a flow rate of 5 L/min. The temperature of the reactor was maintained at 55 °C by means of a water bath. Influent and effluent in this system was achieved with peristaltic pumps (Model 7518–10, Cole-Parmer, USA), and the on and off switching of the peristaltic pumps were controlled by several timers. For the biogas production, a wet tip gas meter (W-NK-0.5B, Shinagawa, Japan) was used to record the collected biogas volume before it was released.

Seed sludge for inoculation was taken from a food waste treatment plant. In order to maintain a proper carbon-to-nitrogen ratio, the co-substrate was prepared using CCPW and WAS (with a moisture content about 75%) with a wet weight ratio of 97.2% and 2.8%, respectively, which resulted in the total chemical oxygen demand (COD), soluble COD and total solids (TS) of 42.4 ± 9.9 g/L of 28.7 ± 10.3 g/L and 41.8 ± 5.6 g/L, respectively. The average concentration of proteins, carbohydrates and lipids in the co-substrate were 8.9 ± 1.7 g/L, 12.6 ± 5.1 g/L and 3.9 ± 1.9 g/L, respectively. In addition, caffeine in influent averaged at a concentration of 478 ± 100 mg/L. All the raw materials used in the substrate were supplied by the Tokyo Gas Co. Ltd. For the experiment, six HRTs were set at 36, 15, 10, 7.5, 5 and 3 d, and the corresponding organic loading rates (ORL) were 0.63, 1.52, 4.27, 5.69, 9.18 and 15.3 g COD/L/d, respectively.

2.2. Batch tests for methane potential

In order to explore the methane potential of the substrate, biochemical methane potential (BMP) tests were implemented under serious food-to-microorganisms (F/M) ratios according to the method of Li et al. (2018). The detailed setting and process of the batch test is as follows: 55, 50, 40, 30 and 20 mL sludge taken from the AnMBR was inoculated into serious 120 mL serum bottles, then the co-digestion substrate with volumes of 5, 10, 20, 30, 40 mL were added into the serum bottles, respectively, this resulting in inoculated F/M ratios of 0.11, 0.24, 0.59, 1.18 and 2.36 g COD/g VSS. Headspace of the bottles were purged with nitrogen gas for 2 min, then the serum bottles were incubated in a shaking bath at 115 rpm at 55 °C. After each bottle reached the set temperature in the water bath, the headspace was vented using a syringe to release the pressure caused by the thermal expansion. Each sample was conducted in two replicates to ensure its reliability. Biogas production and composition (percentage of CH4, CO2, H2 and N2) were measured regularly, the cumulative methane production (CMP) was calculated after every measurement and normalized (N) to the value at standard state. When the CMP remained stable, the BMP tests were terminated. The obtained results were simulated by the modified Gompertz equation.

\[
P = P_0 \cdot \exp \left\{ - \exp \left[ \frac{R_{\text{max}} \cdot e}{P_0} \cdot (t_0 - t) + 1 \right] \right\}
\]

Where, \( P \) is cumulative methane production (CMP) (mL), \( P_0 \) is methane production potential (mL), \( R_{\text{max}} \) is the maximum methane production rate (mL/h), and \( t_0 \) is the lag time (h).

2.3. Physicochemical analysis

The COD and alkalinity were determined according to the standard methods (APHA, 2005). The pH was monitored using a portable pH meter (Horiba, Kyoto, Japan). As for specific organics, the proteins, carbohydrates and lipids in the influent and effluent
were also assayed according to a previous study (Chen et al., 2019).

The composition of biogas generated from the reactor was analyzed by a gas chromatograph (Shimadzu, GC-8A, Japan). For volatile fatty acids (VFAs) analysis, 0.1 mol/L hydrochloric solution and filtrate were added in a gas chromatograph vial with a ratio 1:1 (v/v), then samples were assayed with a gas chromatograph (Agilent-6890, Agilent Technologies, USA).

2.4. Microbial population analysis

When the AnMBR could achieved its steady-state operation at HRT36 (62nd day), HRT15 (83rd day), HRT5 (115th day) and HRT3 (142nd day), 5 mL mixed liquor samples were collected. For DNA extraction, sludge samples were centrifuged at 10000 rpm for 10min, then washed with phosphate buffer solution three times via resuspension and centrifugation. DNA extraction was performed using the PowerSoil® DNA Isolation Kit (MO BIO, USA) as per the instructions. After DNA extraction, the polymerase chain reaction targeting 16s RNA genes was performed for bacteria and archaea. The primers and details about PCR amplification refer to a previous study (Chen et al., 2017b).

3. Results and discussion

3.1. Co-digestion performance

3.1.1. Co-digestion performance in AnMBR

The AnMBR performance of the co-digestion system under different operating OLR is shown in Fig. 1. The concentration of each component in the influent was kept stable throughout the experiment, and the co-digestion system achieved a high COD removal efficiency of the order of 90% at all HRTs with an averaged total COD lower than 4.5 g/L in effluent, which is considered to be efficient organic removal. According to a previous study, the stoichiometric relation for methanogenesis degradation of organic materials can be expressed as in Eq. (2), if the bacteria metabolism (i.e., the synthesis of cell mass and energy for growth and maintenance) is considered (Rittmann and McCarty, 2001).

\[
\begin{align*}
C_nH_{2}O_8N_c + (2n + c - b - \frac{9f_d}{20} - \frac{df_e}{4})H_2O - \frac{df_e}{8}CH_4 \\
+ (n - c - \frac{df_e}{8})CO_2 + \frac{df_e}{20}C_3H_7O_2N + (c - \frac{df_e}{20})NH_4^+ \\
+ (c - \frac{df_e}{20})HCO_3^-
\end{align*}
\]

where \( d = (4n + a - 2b + 3c) \), \( f_d \) represents the fraction of waste organic matter synthesized or converted to cells, the values of which are 0.28, 0.08 and 0.06 for carbohydrates, proteins and lipids, respectively. \( f_e \) represents the portion converted into energy, with \( f_d + f_e = 1 \).

When the representative molecular composition of proteins \((C_6H_{13}O_7N_2)\), carbohydrates \((C_6H_{10}O_5)\) and lipids \((C_{16}H_{22}O_2)\) (Kytheriotou et al., 2014; Rittmann and McCarty, 2001) are taken into consideration, the stoichiometric equations of proteins, carbohydrates and lipids for anaerobic microbial digestion can be expressed as Eqs. (3)–(5), respectively. Although proteins, carbohydrates and lipids all contribute to the generation of methane, the main contributors are proteins and carbohydrates. The proportions of methane produced from carbohydrates, proteins and lipids in the co-digestion system, which accounts for the bio-generated methane, were 46.3%, 64.2%, 103.7%, 88.5%, 91.8% and 86.6%, respectively, corresponding to HRT36 to HRT3 (Fig. 2b).

\[
\begin{align*}
C_6H_2O_5 + 1.554H_2O + 0.336NH_4^+ + 0.336HCO_3^- \rightarrow 5.16CH_4 & + 5.16CO_2 + 0.336C_3H_7O_2N \\
C_{16}H_{30}O_8N_4 \rightarrow 10.35CH_4 & + 11.1CO_2 + 0.36C_3H_7O_2N \\
& + 3.64NH_4^+ + 3.64HCO_3^- + 0.736H_2O \\
C_{18}H_{32}O_2 + 8.535H_2O & + 0.2766NH_4^+ + 0.2766HCO_3^- \rightarrow 10.81CH_4 \\
& + 15.31CO_2 + 0.276C_3H_7O_2N
\end{align*}
\]

With the shortening of the HRT, the proportion of CH4 in the biogas gradually decreased from 66.4% to 42.5%. The proportion of CH4 reduced to less than 60% when the OLR was over 5.69 g COD/L/d (corresponding to HRT < 7.5 d). This indicates that a higher OLR will result in lower biogas quantities, and the balance of the co-digestion system tend to be destroyed when HRT < 7.5 d. The proportions of CO2 confirmed that a CO2 proportion higher than 40% in biogas resulted when HRT < 7.5 d, especially when HRT was 5 and 3 d (Fig. 1c). Data of pH shown a step decrease with the shorting of HRT, which means the co-digestion system was not balanced. Similar conclusion can also be drawn from the data and results for the VFAs of the system (see Fig. S1 in the supplementary materials).

Interestingly, the total alkalinity in the reactor showed a gradual increase from HRT10, which is not in agreement with the variation of the pH. In contrast, the bicarbonate alkalinity remained mostly stable throughout the experiment, meaning that a stable effective alkalinity in the reactor, and the increase of total alkalinity (TALK) may be due to hydrolysis of sulfide. Comparison about the co-digestion performance between this and previous studies is given in Table 1. It demonstrates that this study achieved an efficient co-digestion performance at a high OLR of 9.18 g COD/L-reactor/d, the achieved OLR in this study is much higher than that in other studies. Though Xiao et al. (2017) also achieved a high OLR equals to that in this study, the COD removal is only 68.8%, and the methane yield is only 0.192 NL CH4/g COD removed, much lower than the COD removal (>90%) and methane yield (0.215 NL CH4/g COD removed) in this study. Those indicated that this study achieved an efficient co-digestion by AnMBR for COD removal and energy recovery than previous studies.

3.1.2. Methane potential in batch experiment

Methane potential test was implemented to investigate the biochemical methane potential under different F/M ratios, the results are shown in Fig. 2. The cumulative methane production showed a stepwise increase when F/M ratio increased from 0.11 to 0.59 gCOD/gMLVSS, and then it dramatically decreased with F/M ratio increased to 2.36. The inhibition effect occurred when the F/M ratio was over 0.59 gCOD/gMLVSS, suggested that the sludge in the AnMBR was able to bear an F/M ratio lower than 0.59 gCOD/gMLVSS. Simulation results by Gompertz equation showed that methane production occurred under all F/M ratios with a short lag time, and the maximum methanogenic production rate \( (R_{\text{max}}) \) occurred as 15.5 mL/h when the F/M ratio was 0.24 gCOD/gMLVSS, indicated that \( R_{\text{max}} \) was achieved at an equivalent HRT of 3.0 d (Table 2). COD converting to CH4 rate showed that a high conver-
3.2. Variation of microbial community structure

3.2.1. Community richness and diversity

Nearly 2 million sequences were obtained from the five different samples. Operational taxonomic units (OTUs) were clustered at 97% of the sequences, which means a dissimilarity level of 0.03. In the bacterial community, the OTU numbers, the abundance-based coverage estimator (ACE) and the Chao richness estimator all decreased significantly from HRT10 to HRT5, where they reached the lowest values for all the HRTs (Table 3), indicating that the species richness was greatly reduced and the diversity was greatly increased as a result of a significant increase in Shannon’s diversity index, similar trend was also reported in a previous study (Jang et al., 2014). This may be because of the decrease in the OLR and substrate components from the seed sludge to the initial stage of the study. When the HRT decreased from 5 to 3 d, the OTU numbers, the ACE richness estimator and the Chao richness estimator all showed a significant increase, which means that the species richness was greatly increased, and a significant decrease of diversity was shown according to the Shannon diversity index. Noticeably, the Shannon index showed that the diversity suddenly increased at HRT5 within the bacteria community, this probably due to the large proliferation of certain non-dominating species at the HRTs longer than HRT5. In contrast to the bacterial community, the archaeal community showed irregular changes during all the HRTs. The Shannon diversity remained at a high level, which means that no dominant methanogens were formed in the overall process, and that in these co-digestion processes the different archaeal species cooperate with each other.

3.2.2. Variation of microbial community

At the genus level, *Methanosarcina* and *Methanothermobacter* were the only two genera observed, with an abundance of over 0.1% as shown in Fig. 3a, which is consistent with a previous study that the *Methanosarcina* genus tends to be the predominant acetoclastic methanogens when the acetate concentrations are higher than 1 mM (Hori et al., 2006). With the shortening of the HRT, the abundance of the *Methanosarcina* genus gradually decreased, and a rapid decrease was observed when the HRT was shortened from 10 to 3 d, where it finally reached a low relative abundance of 35.5% at HRT3. In contrast, the abundance of the *Methanothermobacter* index, similar trend was also reported in a previous study (Jang et al., 2014). This may be because of the decrease in the OLR and substrate components from the seed sludge to the initial stage of the study. When the HRT decreased from 5 to 3 d, the OTU numbers, the ACE richness estimator and the Chao richness estimator all showed a significant increase, which means that the species richness was greatly increased, and a significant decrease of diversity was shown according to the Shannon diversity index. Noticeably, the Shannon index showed that the diversity suddenly increased at HRT5 within the bacteria community, this probably due to the large proliferation of certain non-dominating species at the HRTs longer than HRT5. In contrast to the bacterial community, the archaeal community showed irregular changes during all the HRTs. The Shannon diversity remained at a high level, which means that no dominant methanogens were formed in the overall process, and that in these co-digestion processes the different archaeal species cooperate with each other.

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genus also showed a decrease from the seed sludge to HRT10, at which it reached the minimum abundance of 23.3% and then suddenly increased to 56.4% at HRT3. This indicates a transition of methanogenesis from HRT36 to HRT3, which may be the main reason for the suddenly depressed COD to methane rate. The transition of methanogenesis observed in this study suggests that syntrophic interactions involving hydrogenotrophic methanogens will increase during unstable operation, as reported by Demirel and Coates (2012).
Scherer (2008), which may be attributed to the increased activity of hydrogenotrophic methanogens during these unstable condition of the reactor (Padmasiri et al., 2007).

Bacteria that were detected with a relative abundance of over 1% belong to nine different phylum level, namely, Synergistetes, Firmicutes, Proteobacteria, Ca. OP9, Bacteroidetes, Chloroflexi, Planctomycetes, Spirochaetae and Actinobacteria (Fig. 3b). This is consistent with the bacteria distribution reported in a previous study (Temesgen et al., 2017). Among them, phyla Synergistetes, Firmicutes, Proteobacteria and Ca. OP9 are the predominant phylogenetic groups due to a high abundance in all operating conditions, which should be attributed to their ability to degrade a wide range of substances such as cellulose, proteins and fatty acids (Dodsworth et al., 2013; Sousa et al., 2009). Notably, the production of the high abundance of these four phyla are different. For Proteobacteria and Firmicutes, their high abundance at HRT36 and HRT10 is due to a high abundance in the seed sludge, with a gradual decrease from the seed sludge to HRT10 and a dramatic increase at HRT3. This indicates that a short HRT will stimulate their growth, while the disappearance of Proteobacteria at HRT3 may be due to its low tolerance to excess organic acids. Though the variations of Ca. OP9 and Synergistetes were different, they both showed sudden decreases at HRT5, and continued to decrease at HRT3, suggesting that a short HRT has an adverse effect on their growth.

In comparison with the aforementioned four phyla, the abundance of the Bacteroidetes, Spirochaetae, Thermotogae and Chloroflexi phyla in the seed sludge was very low. However, a dramatic abundance increase was observed at HRT10 (for phyla Bacteroidetes, Spirochaetae and Thermotogae) and HRT5 (for phylum Chloroflexi), although a slight decrease of Thermotogae and Spirochaetae was observed at HRT3. In contrast, the phylum Planctomycetes showed a high abundance in the seed sludge and at HRT36, and its abundance was lower than 0.5% at HRT10 to HRT3. This means that phylum Planctomycetes is prone to occur at long HRTs (36 d), which is again consistent with a previous study that indicated that the presence of Planctomycetes was related to long HRTs and short OLRs (Krakat et al., 2011).

To increase the understanding of bacterial community structures and dynamics, the bacteria community at genus level was also considered (Fig. 4). It is clear that the abundance of genera Anaerobaculum, Soehngenia, Caldicoprobacter, Enterococcus and an unclassified genus in phylum Ca. OP9 all reached a maximum at HRT10. Genus Anaerobaculum is a thermophilic anaerobe that grows at 40–65 °C, and was found to be active in the fermentation of organic acids and carbohydrates into acetate, hydrogen and CO₂, and therefore may be able to interact syntrophically with hydrodptic methanogens (Temesgen et al., 2017).

Genus Soehngenia has the ability to produce hydrogen in the anaerobic condition (Zhang et al., 2017) and can grow on various carbon sources (Parshina et al., 2003). Enterococcus is a facultative anaerobe present in the intestinal tract of various animals, which metabolizes a diversity of fermentable substrates (Dang et al., 2016; Massé et al., 2011). For genera 060F05—B-SD-P93 and Peptostreptococaceae genus Incertae Sedis, the abundance increased when the HRT decreased from 36 to 5 d and then suddenly dropped at HRT3. In particular, the abundance of Peptostreptococaceae genus Incertae Sedis decreased from 10.0% to almost zero when the HRT decreased from 5 to 3 d. Genus 060F05—B-SD-P93 can produce large quantity of extracellular polymeric substances, and hence enhance the formation of stable cellular aggregates and facilitate interspecies hydrogen transfer (He et al., 2017). Peptostreptococaceae genus Incertae Sedis were the fermentative acidogenic bacteria (Jin et al., 2016). The changes of these two genera therefore indicate that they were stimulated at middle HRTs (10 and 5 d) but were inhibited at low HRT (3 d).

The metabolism of genus Proteiniphilum in the decomposition of complex carbohydrates yields carbon dioxide, hydrogen, and all kinds of fatty acids as end products of the fermentation process. The high abundance of this genus in the seed sludge, and at HRT36 and HRT3, indicates that it was a significant genus in the degradation of organics, while the variation tendency is difficult to explain where an extremely low abundance at HRT10 and HRT5 was observed. Notably, genus Acinetobacter, a strictly aerobic species (Steven and David, 2014), was detected. It was reported as is high tolerant to adverse environmental conditions, such as those that contain toxic substances, and is even capable of degrading caffeine (Yamaokayano and Mazzafera, 1998). As reported in our previous study, the average caffeine removal efficiency reached 87.5 ± 5.3% in this system (Chen et al., 2018), which may be attributed to the presence of genus Acinetobacter.

3.2.3. Correlation between environmental parameters and microbial dynamics

The relationship between the relative abundance of microorganisms (bacteria at the phylum level and archaea at the genus level) and environmental variables were investigated by statistical and dynamic analysis (Canoco, version 5.0). The bacterial and archaeal abundances shown in Fig. 5 represent more than 70% and 91% of the total sequence abundance of bacteria and archaea in all the detected data, respectively. Both coordinate axes of the canonical correspondence analysis triplot combine to explain over 84% of the microbes, indicating that these environmental variables were major factors shaping the microbial community dynamics.
As shown in Fig. 5, environmental variations are significantly divided into two groups, while the pH shows an apparent divergence from the other environmental variations. Interestingly, the negative correlation between the pH and TALK and bicarbonate alkalinity (BALK) are difficult to explain. Environmental variables have relatively little effect on the archaea (Methanothermobacter and Methanosarcina) because the OLR in the seed sludge is higher than the OLR in HRT10, and results in a decrease of the Methanothermobacter from the seed sludge to HRT10. Their positions indicate that Methanothermobacter tends to be prevalent at low pH values and Methanosarcina prefers a high pH. The positions of phyla Planctomycetes and Actinobacteria are far from the origin, but near to the HRT36 sample, indicating that these phyla were abundant at HRT36, yet sensitive to the environmental changes and with a positive correlation with the pH values. This is consistent with the results obtained for these bacteria at long HRTs and high pH values.

The positions of phyla Ca. OP9, Proteobacteria, as well as Synergistetes, indicate that they all have a positive correlation with the pH values. Actinobacteria was reported efficiently degrading complex organic materials to organic acids (Jang et al., 2015), while Planctomycetes can convert various monosaccharides into acetic acid and hydrogen gas (Zheng et al., 2015). The short distance between these two species appears to suggest a symbiosis relation between them. The positions of phyla Spirochaetae and Bacteroidetes mean that they also tend to be significantly affected by environmental variables, such as TALK, TS and OLR, and these bacteria tend to be present at short HRT (high OLR). Phylum Bacteroidetes is highly relevant for the degradation of complex organic, while Thermotogae produced H2 via fermenting a variety of organic compounds (van Ooteghem et al., 2002). Therefore,
Spirochaetae will produce various substrates for Thermotogae, so they may be symbiotic. Phylum Spirochaetae showed close affinity with TVFA, which fits the behavior that Spirochaetae is a common and dominant acidogenic bacterium in the AD process (Li et al., 2017).

The cluster analysis in Fig. 6 shows that the archaeal community structure was significantly affected by the HRT. The archaeal community structure at HRT36 and HRT10 are similar to that in the seed sludge, while it shows a significant difference when the HRT is shortened to 5 and 3 d. Contrary to archaea, the bacterial community structure shows a step change from the seed sludge to HRT3, which means that the bacteria may be directly influenced by changes in the HRT and that the variation of the bacterial community promotes the changes of archaea.

### 3.3. Functional microbes in the methanogenic process

As mentioned before, methane generated from carbohydrates, proteins and lipids accounts for over 86% of the total methane when HRT shorter than 10 d. Understanding the functional microbes in each degradation process is of great value for optimizing and controlling an efficient co-digestion system. At HRT10, in which an optimized COD conversion efficiency was achieved, the main degradation of the process can be described as the upper part of Fig. 7.

Genera Caldicoprobacter and Clostridium are the main bacteria for the hydrolysis of proteins and lipids, respectively, similar function of these bacteria were also reported in previous studies (Ning et al., 2018). Meanwhile, the hydrolysis of carbohydrates under this condition should be attributed to the phylum Ca. OP9. In the acidogenesis process, the amino acids and saccharides from the hydrolysis of proteins and carbohydrates, respectively, were further fermented to acetic acid and other fatty acids by genus Anaerobaculum, this step was also reported by Li (Li et al., 2019). The process of amino acids and saccharides to lactic was not listed because the bacteria (genus Lactobacillus) abundance correlating to it was very low (<0.5%). Genus Anaerobaculum also was the main bacteria in the lipid acidogenesis process, while genus Clostridium sp. was another important supplement. In the acetogenesis process, genera Anaerobaculum and Tepidanaerobacter degraded fatty acids to acetic acid and hydrogen. The acetic acid was converted to methane by the genus Methanosarcine, which is the main path in methanogenesis. The hydrotropic methanogenesis driven by genera Methanosarcine and Methanothermobacter is the secondary path.

In addition to the above, related syntrophic acetate-oxidizing and driven by genera Tepidanaerobacter and Thermacetogenium, was also believed to have occurred. When the HRT was shortened to 3 d, microbial species and their abundance all showed significant changes. Genus Proteiniphilum became the main species in the hydrolysis process of proteins and carbohydrates, while the predominant species in acidogenesis and acetogenesis were replaced by genera Clostridium and Syntrophomonas. Furthermore, hydrotropic methanogenesis driven by genera Methanothermobacter and Methanosarcine evolved into the predominant process.

### 4. Conclusions

The effect of microbes on the efficiency of a co-digestion system for the high-solids methanation digestion of CCPW and WAS, under thermophilic conditions, was analyzed. Phyla Synergistetes, Firmicutes, Proteobacteria and Ca. OP9 were dominant bacteria and responsible for hydrolysis and fermentation. Synergistetes specifically showed a close relationship with the performance of the co-digestion system. Genera Caldicoprobacter and Clostridium, and
phylum Ca. OP9 are the main bacteria for the hydrolysis of proteins, lipids and carbohydrates, respectively, and a resulting efficient co-digestion performance. The evolution of dominant methanogens from the genus Methanosarcina to Methanothermobacter indicates the dynamic character of the process as the OLR increases. In addition, the genus Acinetobacter was identified as being responsible for the efficient caffeine degradation in this system. Achievements in this study will contribute to the development of promising technologies for efficient co-digestion of CCWP and WAS in industrial applications.

Declarations of interest

None.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (No. SQ2017YFGH001891), Shaanxi Provincial Key Program for Science and Technology Development (No. 2018KWZ-06), and Shaanxi Provincial Program for Innovative Research Team (No. 2019TD-025).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2019.06.045.

References


