Bioresource Technology 244 (2017) 824–835

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Depth-resolved microbial community analyses in the anaerobic co-digester of dewatered sewage sludge with food waste

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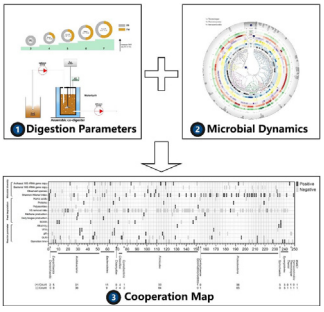
HIGHLIGHTS

- Impacts of high-strength food waste addition was comprehensively evaluated.
- Multivariate analysis and visualization toolkits presented microbial cooperation.
- Microbial dynamics showed possibility to indicate digestion deterioration.
- A wide spread of rare species related to crucial steps in digestion process was observed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 May 2017
Received in revised form 8 July 2017
Accepted 10 July 2017
Available online 12 July 2017

Keywords:
Anaerobic digestion
Dewatered sewage sludge
Food waste
Methane yield
Microbial community
Multivariate analysis

ABSTRACT

This study evaluated the impacts of FW addition on co-digestion in terms of microbial community. Anaerobic co-digestion (AcoD) reactors were conducted at gradually increased addition of food waste (FW) from 0 to 4 kg-VS m^{-3} d^{-1} for 220 days. Although no markable acidification was found at an OLR of 4 kg-VS m^{-3} d^{-1}, the unhealthy operation was observed in aspect of an inhibited methane yield (185 mL g^{-1} VS_{added}), which was restricted by 40% when compared with its peak value. Deterioration of digestion process was timely indicated by the dramatic decrease of archaeal population and microbial biodiversity. Furthermore, the cooperation network showed a considerable number of rare species (<1%) were strongly correlated with methane production, which were frequently overlooked due to the limits of detecting resolution or analysis methods before. Advances in the analysis of sensitive microbial community enable us to detect the early disturbances in AcoD reactors.

1. Introduction

Waste sludge treatment creates a global challenge due to the soaring outputs, landfill shortages, public concerns, legal regulations, and rising costs along with the fast development of wastewater treatment plants (WWTPs). Anaerobic digestion (AD) is a mature technology at municipal WWTPs to treat waste sludge, which provides benefits of sludge volume reduction and renewable energy production (Nguyen et al., 2015; Sawatdeenarunat et al., 2016). Nowadays, many related anaerobic co-digestion (AcoD) process with other organic solid waste have been developed to increase the efficiency of biomethane recovery. (Gou et al., 2014; Luostarinen et al., 2009; Wang et al., 2013; Xu et al., 2015; Ziels et al., 2016). For example, the high organic content and moisture

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http://dx.doi.org/10.1016/j.biortech.2017.07.056
0960-8524/

levels make food waste (FW) an ideal substrate for AcoD (Sawatdeenarunat et al., 2016).

However, previous studies also observed process failures at excessive FW addition (Luostarinen et al., 2009; Wang et al., 2013; Xu et al., 2015; Yang et al., 2016), because co-digestion with such excessive fat-rich or organic-rich waste can result in the accumulation of abundant volatile fatty acids (VFAs) (Ahring et al., 1995; Nagao et al., 2012). The accumulation of VFAs could result in a catastrophic failure of AcoD process (Nguyen et al., 2015). Eventually, the methane production was stopped (Kleyböcker et al., 2012). For AcoD process evaluation or monitoring, the reported threshold value of FW addition is inconsistent (Gou et al., 2014; Luostarinen et al., 2009; Silvestre et al., 2011), which limits to predict the optimal digestion efficiency under the specified conditions. Many digestion parameters (e.g. pH, VFAs concentration, hydrogen partial pressure or methane yield) have been used as critical or direct indicators to evaluate system upsets (Dong et al., 2011; Nguyen et al., 2015). However, such interpretations from individual parameters are not sensitive to indicate the real status of AcoD reactors (Dong et al., 2011; Foley, 2011). AcoD system may under threat before common individual parameters indicating, due to the lack of comprehensive evaluation underlying the digestion processes. Therefore, it is difficult to give a reliable evaluation between "efficiency" and "stability". In many cases, AcoD reactors are subjected to operate at suboptimal organic loading rates (OLRs) to prevent operation instability (Kleyböcker et al., 2012).

Degradation of organic substrate in AD process is complex due to the participation of diverse microbial population supporting a series of synergistic biochemical reactions (Nguyen et al., 2015). Methane production from feedstocks includes four major steps: (i) hydrolysis of complex polymers; (ii) fermentation of simple soluble products; (iii) anaerobic oxidation of VFAs and (iv) methane production from acetate and hydrogen (Nguyen et al., 2015). An efficient and stable digestion process depends on a synergistic effort of two groups of microbial communities for metabolizing diverse organic substrates, namely *Bacteria* and methanogenic *Archaea* (Jang et al., 2016; Nguyen et al., 2015; Vanwonterghem et al., 2014). It is reported that methanogens are the most sensitive to environmental conditions among the many microorganisms detected in AD systems (Rivière et al., 2009). For this reason, previous efforts mainly evaluated the community structure of methanogens and found several members (e.g. *Methanosarcina* and *Methanosaeta*) are the largest fraction in AD reactors (Jang et al., 2015b; Traversi et al., 2011; Yang et al., 2016). In addition to these important groups, which have been well covered in many previous studies, are there some specific members with low abundance but strongly correlated with digestion functions? In fact, microbial richness and diversity are key factors in maintaining the functional stability of different AD conditions, such as pH, substrate or FW concentration (Wittebolle et al., 2009). When a digestion community is highly diverse, it will not strongly rely on the dominant members. The functional stability will be less distracted by environmental fluctuations (Wittebolle et al., 2009). But the complexity of methane production process makes it difficult to identify key members capable of carrying out specific metabolic functions, not to mention the rare species. Therefore, this work proposed a possible indicator using multivariate statistical methods that correlating microbial community dynamics with performance parameters to comprehensively evaluate the impacts of FW addition on AcoD.

One of the key issues in this study is to identify the primary members that rapidly respond to AcoD operation, in terms of richness and diversity. Previous studies collectively suggested that FW co-digestion are affected by the rapid adaptation of diverse microbial population to change stressful conditions (Briones & Raskin, 2003; Silvestre et al., 2011; Yang et al., 2016). But in-depth knowledge of the microbial adaption and their response to AcoD function is incomplete and likely biased, because the involvement of diverse microbial communities participating various pathways to produce methane (Briones & Raskin, 2003; Traversi et al., 2011). Thus, the impacts of biodiversity on digestion functions are of great interest (Wittebolle et al., 2009). Besides, variation of AcoD conditions complicates the identification of functional species. For instance, two *Archaea* genera of *Methanosarcina* and *Methanosaeta* are the most detected in AD systems (Rivière et al., 2009). But in a subsequent study based on 16S rDNA gene amplicon sequences in AD system, Nelson et al. found that more than 50% of sequences can't be affiliated to any established genus (Nelson et al., 2011), which implies that microbial diversity is far more extensive than had ever been imagined. Over the last decade, a wide application of molecular microbiology techniques (e.g. real-time quantitative PCR, high-throughput sequencing) has advanced our understanding of complex microbial world in AD, accompanying with the unprecedented size of high-dimensional species datasets (Vanwonterghem et al., 2014). These methods can provide sufficient data to cover the overall taxonomic composition, but also create increasingly challenges at data visualization and multiple analysis to explain a large phylogenetic and function diversity in AD (Vanwonterghem et al., 2014; Wong, 2012). The increasing ability to collect numerous sequence data using modern microbiology techniques is arguably outpacing the rate that we can process and analyze (Krzywinski et al., 2009). It is of equal importance for reactor states evaluation and extracting useful "hidden" information from the large datasets of microbial population. Such data should be possibly used to discover key members, develop novel pathways and effectively manage current AD process (i.e. methane production and organic matter removal).

This work evaluated the correlation between FW co-digestion parameters and microbial population under FW threshold. Particularly, advanced molecular techniques (PCR-DGGE, real-time quantitative PCR, and Illumina Hiseq sequencing) were applied to investigate microbial community changes as well as digestion performance for 220 days. Multiple bioinformatics analysis (i.e. Circos toolkit, Pearson correlation, and redundancy analysis) were combined to provide interpretable indicators that relating microbial diversity to functional complexity. Results will allow to discover a fundamental understanding on possible metabolic pathway occurring in AD process.

2. Material and methods

2.1. Collection and preparation of feedstock

FW used in this study was collected daily from the dining hall of Hunan University, Changsha, China. FW was crushed to particle size of ca. 2 mm and stored at 4 °C before utilization. Seed sludge and dewatered sewage sludge (DS) used for co-digestion were collected from Yuelu WWTP, Changsha. Main characteristics of seed sludge, DS and FW are summarized in Table 1.

2.2. Anaerobic co-digestion test

Experiments were conducted at three parallel continuous anaerobic co-digesters with a 15-day hydraulic retention time (HRT) for 220 days (Jang et al., 2016). Initially, the seed sludge was condensed to ca. 15 g-VS L^{-1} then transferred to each bottle with 1 L working volume. After sealing, nitrogen gas was flushed for 5 min to create an anaerobic condition. Then bottles were incubated and stirred in a water bath for mesophilic digestion (35 ± 0.5 °C). Reactors were mixed at 60 rpm (1 min on and

Table 1
Main characteristics of seed sludge, dewatered sewage sludge and food waste.

Item	Seed sludge	DS	FW
pH	7.72 ± 0.1	7.53 ± 0.1	4.16 ± 0.1
sCOD (g L^{-1})	0.8 ± 0.5	16.1 ± 5	172.4 ± 25
TS (g L^{-1} substrate)	33.7 ± 0.5	414.2 ± 5	763.2 ± 5
VS (g L^{-1} substrate)	10.2 ± 0.5	133.4 ± 5	811.4 ± 5
VS/TS (%)	31.4 ± 0.2	32.0 ± 0.2	94.8 ± 0.2
C/N (w/w)	4.8 ± 1.5	8.6 ± 1.1	21.3 ± 7.9
TN (g L^{-1})	ND	ND	2.2 ± 0.5
NH_4^+-N (g L^{-1})	ND	ND	0.7 ± 0.1
Polysaccharides (g L^{-1} glucose)	ND	13.0 ± 1.7	97.3 ± 1.1
Proteins (g L^{-1})	ND	11.7 ± 1.2	58.8 ± 2.4
Saturated fat (g L^{-1})	ND	ND	240.3 ± 5.6
Monounsaturated fat (g L^{-1})	ND	ND	253.1 ± 9.8
Polyunsaturated fat (g L^{-1})	ND	ND	50.6 ± 3.3
Trans fat (g L^{-1})	ND	ND	94.9 ± 6.5
TVFAs (mg L^{-1})	ND	ND	2477.5 ± 83

DS = dewatered sewage sludge; FW = food waste; TN = total nitrogen; TVFAs = total VFAs.
ND = not detect.

10 min off). Operation of AcoD can be divided into 5 stages (Table 2). To achieve a stable start-up, co-digesters were fed with DS only and operated at first 60 days (stage 0) at 3 kg VS m^{-3} d^{-1}. Subsequently, organic loading rates (OLRs) gradually increased incrementally from 3 to 4–7 kg VS m^{-3} d^{-1} in the following 4 stages (40 days/stage) with FW addition. VS load of FW in feedstock increased in a stepwise manner over 57% at the end of experiment. Feedstock was automatically fed into reactors 1 time each day and withdrew same volume of digested samples. Digested samples were collected for further analysis.

2.3. DNA extraction and quantification

Digested samples were collected on 1, 30, 60, 80, 100, 120, 140, 160, 180, 200, and 220 day from each of the triplicates reactors. 500 mg centrifuged sludge samples were prepared for total genomic DNA extraction using Fast DNA® Spin Kit for Soil (MP Biomedical, USA) according to manufacturer's instructions. Concentration of extracted genomic DNA were determined by Micro-Ultraviolet spectrophotometer (NanoDrop Inc., USA). Triplicates genomic DNA were mixed for microbial analysis.

2.4. Real-time quantitative PCR (RT-qPCR) analysis

Total 16S rDNA gene copies of *Bacteria* and *Archaea* were detected by RT-qPCR. The targeting region of bacterial 16S rDNA was amplified using 341F and 518R (Deng et al., 2015). *Archaea* were specially amplified by nested PCR approach. Initially, a primer set consisting of Arch 46F and Arch 1017R were employed for a first round of amplification. The obtained PCR product was used as a template in the second PCR round using the primers Arch 334F and Arch 522R (Roling et al., 2006). Raw PCR products were gel-purified using the GENECLEAN Turbo Kit (MP bio, USA) and detected by 1.5% (w/w) agarose gel. A two-step RT-qPCR amplification protocol with SYBR-Green I fluorophore was followed by Deng (Deng et al., 2015). RT-qPCR was operated in a Cycler iQ5 thermocycler (Bio-Rad, USA). Negative control, standard curves and melting curve analysis were followed by Lu (Lunhui et al., 2014).

2.5. PCR-DGGE

Regarding for DGGE, the targeting region of bacterial 16S rDNA was amplified using ahead primers but with a GC clamp. DGGE was performed using the DCode system (Bio-Rad, Hercules, CA, USA) as described by Deng et al. (2015). Gels containing 8% (wt/vol) polyacrylamide were used with a denaturing gradient of 30–60%. Gels were performed for 14 h at 85 V in a 1X TAE buffer at 60 °C, then stained with GeneGreeen solution (TianGen, China) for 30 min and visualize with the Gel Doc XR System (Bio-Rad, USA). DGGE bands profiles were analyzed by Quantity One (v4.62, Bio-Rad, USA).

2.6. Illumina high-throughput sequencing (HTS) and statistical analysis

For Illumina Hiseq 2500 sequencing, the barcoded-primers 341F and 806R targeting microbial 16S rDNA fragment were selected (Yang et al., 2016). Filtration of sequencing data was performed as previously described by Zheng et al. (2014). Complete sequences data were submitted to NCBI with the accession number SUB2118508. Final OTUs were taxonomically annotated using QIIME (v. 1.9.0, http://bio.cug.edu.cn/qiime/). Statistical analysis, including relative abundance, Good's coverage, rarefaction curves, alpha diversity of microbial community were calculated using Mothur (www.Mothur.org/) (Zheng et al., 2014). Correlation map was built with Pearson indexes ($r > 0.8$ and P-value <0.05) for phylotypes using microbial abundance data in SPSS (v. 19) (De Vrieze et al., 2016). A phylogenetic tree was constructed in MEGA (v. 5.0) using neighbor-joining method. Heatmap of the top genus in each sample based on the relative abundance was visualized using the HemI (http://hemi.biocuckoo.org/). Integrative circular graph represented the taxonomy, abundance and phylogenetic trees was constructed by Circos (http://circos.ca/). Also, redundancy analysis (RDA) of microbial communities, operational parameters and digestion samples were calculated using Canoco (v. 4.5).

2.7. Analytical techniques

Digester system was monitored for biogas daily production, total solids (TS), volatile solids (VS), soluble COD (SCOD), pH, total volatile fatty acids (TVFAs), and total alkalinity (TA). Biogas production and composition (mainly methane, carbon dioxide) were analyzed weekly by a gas chromatograph (Shimadzu GC 2010,

Table 2
Continuous operation mode of AcoD reactor of dewatered sewage sludge and food waste.

	Stage 0	Stage 1	Stage2	Stage 3	Stage 4
Days	1–60	61–100	101–140	141–180	181–220
DS (mL d^{-1})*	22.6	22.6	22.6	22.6	22.6
FW (mL d^{-1})*	0	1.2	2.5	3.7	4.9
Dilute to (mL d^{-1})	66.6	66.6	66.6	66.6	66.6
DS (kg VS m^{-3} d^{-1})	3	3	3	3	3
FW (kg VS m^{-3} d^{-1})	0	1	2	3	4
Total OLRs	3	4	5	6	7
FW-OLRs (%)	0	25	40	50	57

DS = dewatered sewage sludge; FW = food waste; OLRs = organic loading rates.
* Approximate.

Japan) with a thermal conductivity detector (TCD) (Xu et al., 2015). TS, VS, SCOD, pH, TA were measured according to the Standard Method (Yang et al., 2016). TVFAs (mainly acetate, propionate, butyrate, *iso*-butyrate, valerate, *iso*-valerate, capronate, and *iso*-capronate) were measured by a gas chromatograph (Agilent 7890A, USA) with a flame ionization detector (FID) (Yang et al., 2016). Centrifuged pellets were used to extract and measure concentrations of proteins, polysaccharides, and humic acids by heating-extraction method, as described by Xu et al. (2015). The fat content of FW was measured following procedures outlined in AOAC International method (International, 1995). All above analyses were performed in triplicates and the data were expressed as mean value ± standard deviations.

3. Results and discussion

3.1. FW co-digestion performance

AcoD performance was monitored for 220 days to compare difference between sludge mono-digestion (day 1–60) and FW co-digestion (day 61–220) (Table 2). At stage 1, the VS load of feedstock increased to 4 VS m^{-3} d^{-1}. With a continuous addition of FW, VS load of feedstock at stage 4 reached 2.3 times to the stage 0. Consequently, increased FW load in feedstock was major reason for the AcoD process variation, as revealed by the change of VS concentration and methane production (Fig. 1-c, d). To better understand the digestion status, pH, TVFAs, and TA concentration were also monitored at each stage to compare reactor stability (Fig. 1-a, b and Table 3). Notably, pH was slightly reduced at stage 1 (ca. 6.95) then recovered to ca. 7.20 after day 120, without any artificial assistance. The pH range of 6.5–7.5 is suggested as a suitable condition for substrates degradation and methane production in a single-stage reactor (Xu et al., 2015). Unlike the pH, TVFAs, and TA concentrations fluctuated apparently with FW addition. TVFAs concentration remained below 300 mg L^{-1} at stage 0 then reached a peak value at stage 2. TVFAs/TA ratio is calculated to assess an acidification-risk during digestion process. This ratio <0.4 is suitable for methanogenesis without inhibition by acidification (Vanwonterghem et al., 2014). The TVFAs/TA ratio remained below 0.4 at stage 0. When FW was added at stage 1 and stage 2, this ratio exceeded the threshold value (>0.4). From these perspectives, results implied that FW addition disturbed reactor stability. After that, the TVFAs/TA ratio dropped and no more than exceeded 0.4 throughout stage 3 and 4, which means reactor has sufficient buffering capacity at the prevailing TVFAs conversion rates.

On the contrary, methane production and related metabolic products (e.g. polysaccharides, proteins, and humic acid substances) showed an unexpected decreasing trend (Fig. 1-d and Table 3). Various organic substrates are decomposed into simple intermediates for subsequent methane production. During the mono-digestion stage, daily methane production fluctuated slightly and stabilized at 300 ± 50 mL g^{-1} VS_{added}. With an increased addition of FW, the averaged methane production increased by 20 to 43 ± 1.4% in the co-digestion stage 1–3 (360, 430 and 350 ± 5 mL g^{-1} VS_{added}, respectively), implying that FW co-digestion offers potential advantages over sludge mono-digestion. Also, the release of microbial metabolic by-products (polysaccharides, proteins, and humic acid substances) were stimulated by the addition of FW from day 80 to day 160. This finding is in accordance with many researches that higher microbial activity and methane productivity can be achieved under FW co-digestion (Wang et al., 2013; Ziels et al., 2016). FW has been reported as a desirable co-digestion substrate due to the potential methane productivity (mL per gVS_{added}), which can reach as higher as 300% than sludge (Luostarinen et al., 2009).

Notably, when FW-OLR continued increasing to 4 kg VS m^{-3} d^{-1} (stage 4), methane yield dropped to 185 ± 5 mL g^{-1} VS_{added}, which

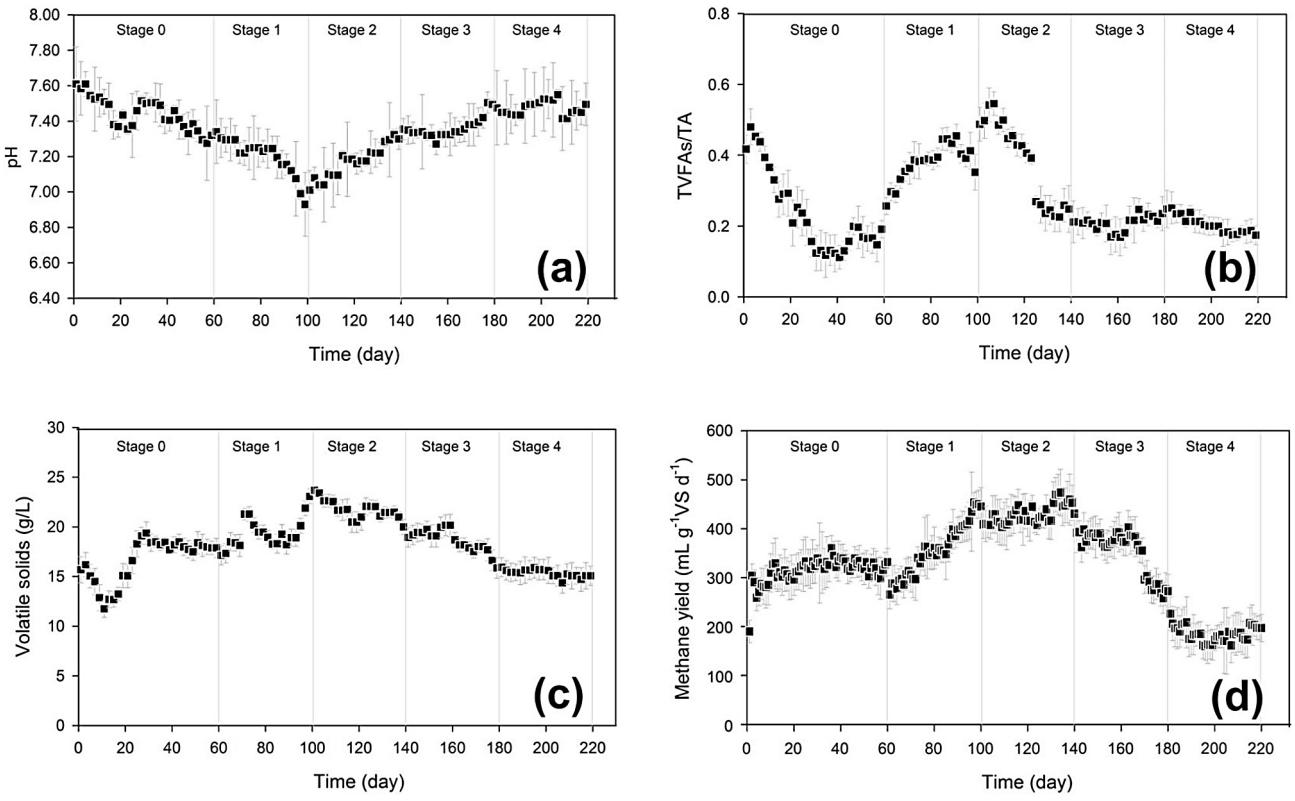

Fig. 1. Operation of mesophilic anaerobic codigesters of dewatered sewage sludge and gradually increased food waste for 220 days: (a) pH; (b) total VFAs/total alkalinity ratio; (c) volatile solids content; (d) methane yield.

Table 3
Summary of digestion parameters(stand deviation) at each stage.

Parameters	D1	D30	D60	D80	D100	D120	D140	D160	D180	D200	D220
TS (g L^{-1} substrate)	42.9	38.9	39.7	39.6	43.8	37.1	36.3	34.0	30.2	26.6	26.8
TVFAs (mg L^{-1})	670.0$_{(7.4)}$	293.8$_{(8.5)}$	423.0$_{(12.3)}$	516.5$_{(16.0)}$	659.1$_{(34.3)}$	568.0$_{(6.8)}$	340.0$_{(4.1)}$	297.0$_{(3.6)}$	476.9$_{(24.8)}$	392.6$_{(11.0)}$	334.4$_{(19.1)}$
TA (mg $CaCO_3$ L^{-1})	1607.1$_{(109.3)}$	2376.2$_{(161.6)}$	1649.7$_{(19.8)}$	1337.6$_{(16.1)}$	1352.2$_{(39.2)}$	1400.0$_{(43.4)}$	1608.9$_{(109.4)}$	1768.7$_{(21.2)}$	1924.9$_{(55.8)}$	1964.3$_{(133.6)}$	1920.1$_{(23.0)}$
Polysaccharides (mg L^{-1})	90.3$_{(3.1)}$	109.3$_{(5.1)}$	96.4$_{(5.0)}$	97.5$_{(7.6)}$	135.5 $_{(4.2)}$	152.3$_{(4.0)}$	158.4$_{(7.6)}$	128.4$_{(5.3)}$	71.6$_{(2.5)}$	63.3$_{(1.8)}$	61.5$_{(1.0)}$
Proteins (mg L^{-1})	102.6$_{(8.0)}$	123.0$_{(3.8)}$	101.4$_{(2.6)}$	69.5$_{(1.8)}$	210.8$_{(10.1)}$	206.8$_{(8.5)}$	236.1$_{(11.1)}$	117.5$_{(6.1)}$	69.9$_{(5.4)}$	77.2$_{(4.0)}$	71.7$_{(5.6)}$
Humic acid substances (mg L^{-1})	269.1$_{(8.3)}$	260.0$_{(6.8)}$	256.6$_{(12.3)}$	226.4$_{(9.3)}$	316.1$_{(14.9)}$	330.8$_{(15.5)}$	324.9$_{(16.9)}$	263.9$_{(20.6)}$	191.1$_{(5.9)}$	178.1$_{(4.6)}$	199.1$_{(9.6)}$

TVFAs = total volatile fatty acids; TA = total alkalinity.

was restricted by 40 ± 1.3%. It is reported that high loading of FW can disturb AD stability and even deteriorate methane production. This study tested the threshold of FW addition. According to the AcoD performance, although no markable acidification was observed when FW increased to 4 kg VS m^{-3} d^{-1} (total OLRs reached 7 kg VS m^{-3} d^{-1}), but methane production dropped significantly at such high OLRs condition. It is suggested that an optimum mixing ratio of DS at 3 kg VS m^{-3} d^{-1} and FW at 3 kg VS m^{-3} d^{-1} is highly recommended to maximize the methane yield without any penalty. Decline of methane yield and microbial activity is usually interpreted as the methanogenesis inhibition or organic overloading. In this case, VFAs could not be utilized by methanogens immediately and result in reactor acidification, even process failure (Dong et al., 2011). This research supplied a unique sample to evaluate the impacts of FW addition. Because co-digestion with FW at stage 1 and 2 promoted methane production and increased TVFAs/TA ratio. While co-digestion at stage 4 inhibited the methane yield, but the operation process remained stable (no VFAs accumulation or pH drop). Using common monitoring indicator alone, such as pH, VFAs concentration, biogas production or OLRs will not always reliably and clearly reflect a process disturbance. pH is very easy to monitor and important for biological reactor. However, it has been suggested that pH alone is not sensitive to indicate sudden change in a digestion system with high buffering capacity (Angelidaki & Ahring, 1994). Nakakubo et al. found that it was more sensitive to indicate imbalanced reactors by *iso*-butyric, butyric, and *iso*-valeric acids individually, instead of total VFAs concentration (Nakakubo et al., 2008). It is also pointed out that biogas composition (e.g. CH_4, CO_2, and H_2) could not be served as a universal indicator for early warning against disturbance because methane yield will not decrease following inhibition (Bruni et al., 2013). Charles et al. proposed that using the methane production rate and OLRs to indicate an early warning of reactor imbalance (Charles et al., 2011). However, previous studies also reported the successful operation of anaerobic digesters at relatively high OLRs (7–15 kg VS m^{-3} d^{-1}) without acidification, but the methane yield was low (140–314 mL g^{-1} VS_{added}) (Bolzonella et al., 2003; Nagao et al., 2012). To address this issue, an improved evaluation combined with microbial population aspects jointly is required to determine reactor status.

3.2. PCR-DGGE and RT-qPCR revealed the change of microbial community structure

FW addition also strongly affected the microbial community in AD process, in terms of structure and 16S rDNA gene copy concentration. As showed in PCR-DGGE fingerprints profile of *Bacteria* (Fig. 2-a), several bands were observed in AD process all the time, but their brightness changed, as detected from band intensity. Besides, band b disappeared gradually then many unique bands emerged with FW co-digestion from day 60 (e.g. band c, d, and e). The changing of bands pattern in microbial composition could be clustered into two major shifts. The first one was observed at FW co-digestion start-up stage (from day 60 to day 100) and the second one was observed at FW overloaded stage (from day 180 to day 220). At these two timing, methane productivity and reactor stability also changed significantly, as revealed in Fig. 1. It was suggested that specific functional or FW-favorites populations (e.g. the representative bands c-e) were disturbed in AcoD systems by FW addition, then resulted in the change of digestion performance. Such consistency implied the potential of microbial biodiversity to sensitively indicate the impact of FW on AD process.

RT-qPCR method targeting 16S rDNA gene concentration provides a detection of microbial population number (including *Bacteria* and *Archaea*) to response different operation parameters. As showed in Fig. 2-b, the number of bacterial 16S rDNA gene copies

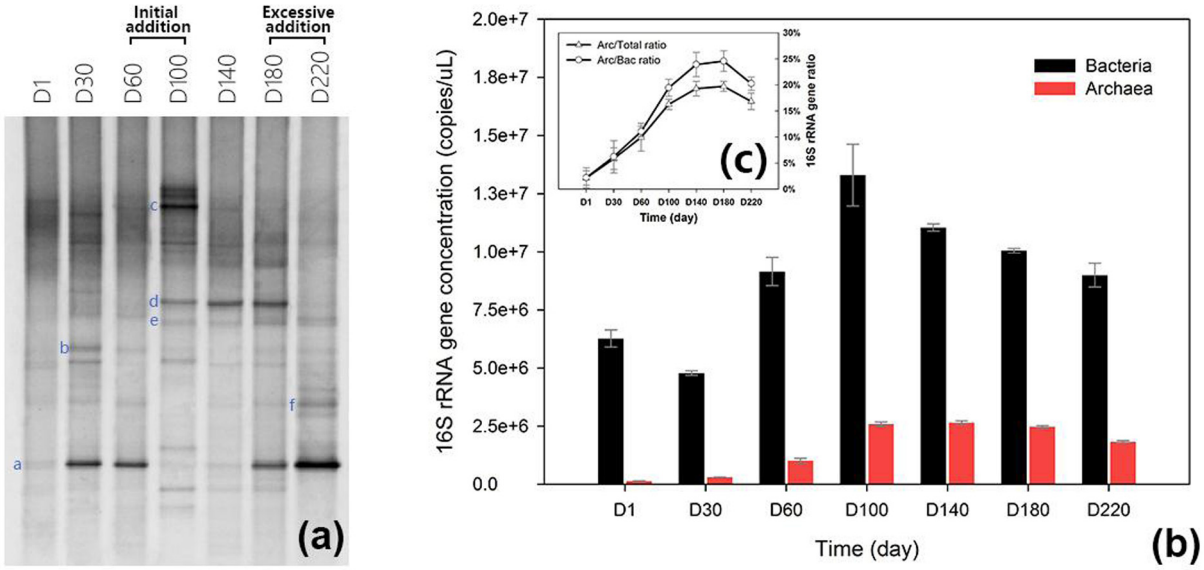

Fig. 2. Dynamics of microbial structure during 220 d digestion process: (a) denaturing gradient gel electrophoresis (DGGE) fingerprints of targeting bacterial 16S rRNA gene fragments. Representative bands were marked by blue; (b) real-time quantification of targeting 16S rRNA gene copy concentration for bacteria and archaea; (c) specific ratios of archaea/bacteria and archaea/total populations. All reactions are performed in triplicates. Error bars indicate the standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in day 1 was $6.27 \pm 0.06 \times 10^6$ copies μL^{-1}; The highest population ($1.33 \pm 0.10 \times 10^7$ copies μL^{-1}) was observed at day 100 (stage 1), but decreased consistently with FW further addition (from day 100 to day 220). Finally reached at $9.00 \pm 0.06 \times 10^7$ copies μL^{-1}. RT-qPCR results also showed that gene copies number for *Archaea* was rare (ca. 3–25% of bacterial gene copies concentration). The relative lower archaeal 16S rDNA gene copies were observed at stage 1. After that, a consistent increase in archaeal took place and remained stable from day 100–220 (ca. $2.48 \pm 0.02 \times 10^6$ - copies μL^{-1}). Compared with day 1, the ratio of archaeal 16S rDNA gene copies in both whole community (Arc/Total) and bacterial community (Arc/Bac) increased 10-times by day 180. After that, two ratios slightly dropped from 20–25% to 15–20%.

Results suggested that FW co-digestion might lead to higher development of both *Bacteria* and *Archaea*. High organic substrates (e.g. FW) are mainly hydrolyzed through a specific anaerobic chain reaction. Secondly, some acetogenic microbe convert intermediate product into acetate and H_2 through β-oxidation process (Schink, 1997; Weng & Jeris, 1976; Ziels et al., 2016). Finally, acetate and H_2 are used by methanogenic *Archaea* to generate methane. A considerable amount of microorganisms jointly participate in methane conversion when mixed with various substrate, because co-substrate can dilute inhibitory compounds (e.g. ammonia, VFAs, heavy metal) or balance micro/macro-nutrients (e.g. carbon to nitrogen ratio) (Jang et al., 2016). Similar growth patterns in both bacterial and archaeal gene numbers implied the well synergisms between two groups during AcoD process. The substantial FW-favorites microbial community was established in the desirable condition with FW addition (Jang et al., 2015a), thereby resulting in a higher methane productivity. From this perspective, the AcoD system should maintain a delicate balance between *Archaea* and *Bacteria* to support effective conversion of FW during AD process. This confirmed again that the critical influence of microbial adaptation should not be underestimated when evaluate AD status. The assessment of FW threshold should be considered as a flexibility goal that depend on reactor parameters and microbial dynamics, such as structure or richness. This work observed a strong positive correlation between archaeal ratios and methane yield, because these trends coupled well as FW load was further increased (Fig. 1-d). Decreases in archaeal ratio from 20–25% to 15–20% suggested that the reactor condition is already inhibited by excessive FW addition (as methane productivity decreased). While individual parameters, such as TVFAs/TA ratio or pH, are unable to timely indicate the deterioration. Thereby, the potential of archaeal numbers combined with AD parameters to evaluate reactor status was proposed in this study. Interestingly, a higher drop in gene copies of bacterial at stage 4 indicated that digestion of excessive FW was particularly unfavorable for *Bacteria* than *Archaea* population. This might suggest archaeal community having higher tolerance to excessive FW condition than bacterial community. A deeper investigation into why archaeal groups have high tolerance to stressful condition can improve our understanding of high-OLR digestion technology.

Table 4
Alpha-diversity indexes$_{(\text{stand deviation})}$ of microbial community at different digestion stage.

Sample	Shannon-Wiener index (H)	Observed species	Chao 1
D1	$9.42_{(0.43)}$	$7822_{(108)}$	$39080.5_{(2668.3)}$
D30	$8.08_{(0.08)}$	$7639_{(124)}$	$39984.0_{(1395.2)}$
D60	$8.19_{(0.55)}$	$7557_{(153)}$	$43178.5_{(5726.9)}$
D80	$8.17_{(0.42)}$	$7404_{(353)}$	$40719.0_{(3151.9)}$
D100	$7.81_{(0.27)}$	$6674_{(470)}$	$36397.8_{(2376.0)}$
D120	$7.49_{(0.10)}$	$5467_{(356)}$	$28691.4_{(3043.1)}$
D140	$7.66_{(0.47)}$	$5274_{(143)}$	$28362.8_{(3398.0)}$
D160	$7.36_{(0.24)}$	$4820_{(200)}$	$27949.3_{(2146.1)}$
D180	$6.99_{(0.33)}$	$4557_{(434)}$	$23480.5_{(2602.8)}$
D200	$6.07_{(0.81)}$	$4285_{(568)}$	$21246.5_{(1799.5)}$
D220	$5.21_{(0.17)}$	$4035_{(286)}$	$21266.4_{(1474.5)}$

3.3. HTS comprehensively revealed diverse microbial taxonomic composition

PCR-DGGE and RT-qPCR techniques collectively revealed the microbial dynamics were strongly influenced by FW addition. The detailed impacts of FW addition on comprehensive microbial diversity and richness require an in-depth investigation to determine whether some sensitive population (e.g. genus) can rapidly response to environmental disturbance. HTS is used to thoroughly reveal overall bacterial and archaeal taxonomic composition under

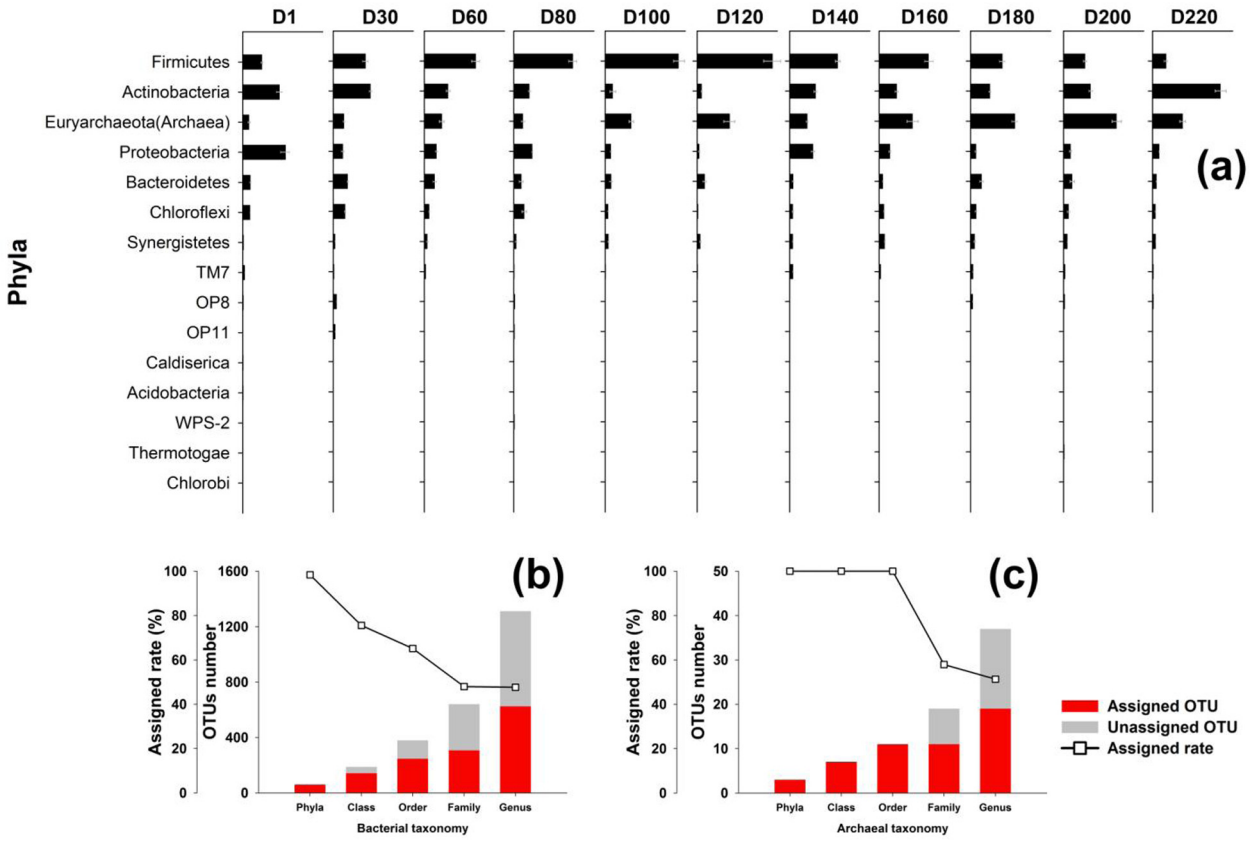

Fig. 3. Distribution and relative abundance of top 15 phyla within AcoD reactors libraries derived from Illumina HiSeq sequencing (a). Bar chart (b, c) represented OTUs assigned ratio of different taxonomic level for bacteria and archaea, respectively.

different FW co-digestion stage. In total, 1,877,211 clean sequences (98% of raw sequences with the averaged 482 bp length) were obtained with high-quality, resulting in a range of 44,395–74,955 sequences per sample. All samples approached an asymptote with Good's coverage over 98.0%, which means that the major population were identified with a reliable sequencing depth in each sample (3G data). Clean sequences were annotated into operational taxonomic units (OTUs) from phyla to genus level to estimate the phylotype richness and diversity, using a 97% sequence similarly cut-off value.

Alpha-diversity of microbial community indexes were calculated to compare inter-species diversity in each sample (Table 4) (Zheng et al., 2014). The highest Shannon-Wiener index (9.42 ± 0.43), observed species number (7822 ± 108), and Chao 1 index (43178.5 ± 5726.9) were observed at day 1, day 1, and day 60, respectively. A consistent decrease in these alpha-diversity indexes could be found with FW adding over time. Similar trend of substantially decreasing of alpha-diversity indicated that introduction of FW exerts a negative effect on the diversity of microbial population. One possible explanation is that substrate characteristic (FW-related substrate) formed a selective and strong competition. In this case, FW-favorites groups were enriched and dominated in AcoD system with FW further addition. A link between microbial diversity and process performance has been reported (Werner et al., 2011). Because biodiversity keeps AD systems from the declines in their functionality to ensure an adaptation to different status. The synergism between many populations guarantee that some will support a specific function when others fail (Wittebolle et al., 2009). From this perspective, diversity gradually decreases in biological systems might indicate the deterioration of reactor performance, such as methane productivity.

Taxonomic classification of sequencing data can be used to assume the similar environmental functions that shared among the closely-affiliated communities (Rivière et al., 2009). In this study, detected OTU sequences were classified into 64 phyla (61 *Bacteria* + 3 *Archaea*) (Fig. 3). With the help of high-resolution technology, more and more OTUs were annotated from phyla to genus level. 195 class. Over 390 orders, 659 family and 1348 genus were detected based on HTS method. But the number of unassigned OTUs also increased constantly (Fig. 3-b, c). Almost half of *Bacteria* (47.7%) and *Archaea* (51.4%) have not been allocated into any established genus. Results confirmed that environmental samples usually contain numerous novel species in a highly diverse ecosystem. Current knowledge related with the observation and identification of diverse microorganisms is far from enough, not to mention how to evaluate their function pathway in AcoD process. Any advanced protocols in complex microbial population detecting require to be accompanied with corresponding visualization of these data. This study intended to combine genomics research toolkit *Circos* (Krzywinski et al., 2009) and statistical analysis (Vanwonterghem et al., 2014) to comprehensively visualize and identify the function/sensitive population at subgenus level using high-dimension sequencing datasets, which is not reported previously. Possible functions of the classified species were also highlighted in this study (Fig. 4).

3.4. Chang of microbial community indicated AcoD performance

Analysis of OTUs relative abundance showed that microbial communities in sludge mono-digestion stages were significantly different from those in FW co-digestion stages (Fig. 3a). The taxonomic summary of 15 top phyla composition showed that all

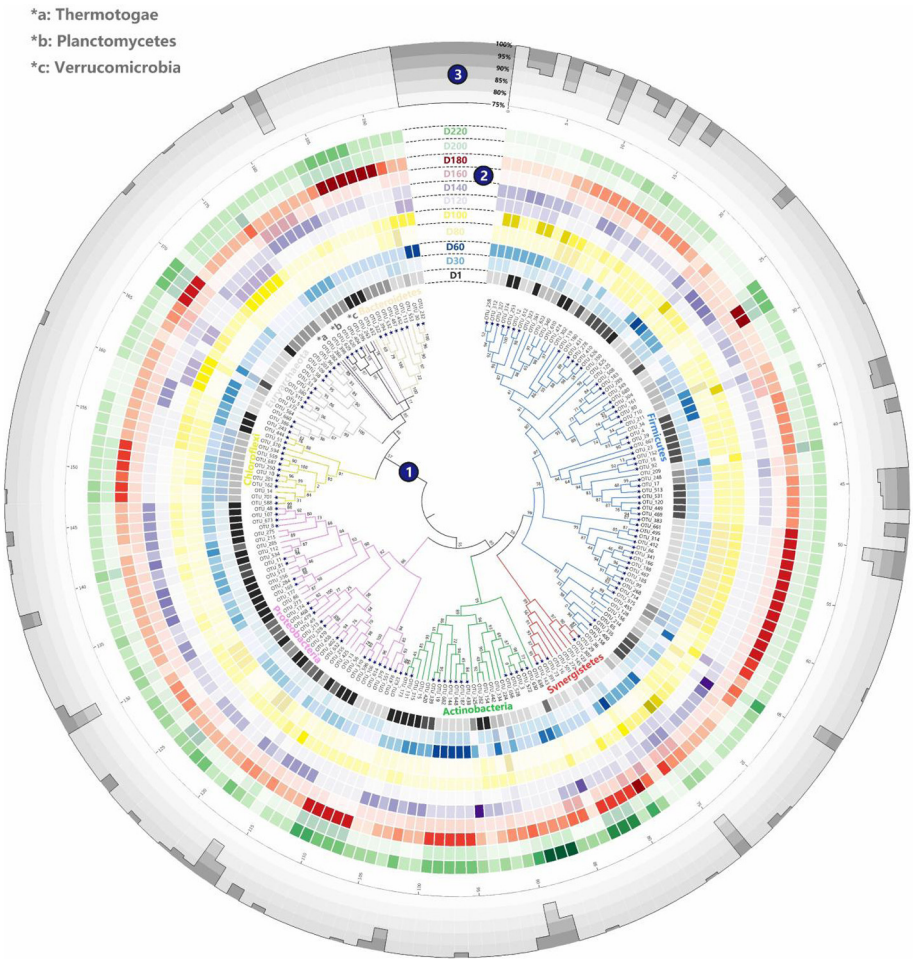

Fig. 4. The phylogenetic tree and relative abundance of top genus in different digestion stage, visualized by *Circos*. Ring 1 presents the evolution relationship of different OTUs using a phylogenetic tree. Ring 2 displays the relative abundance of each OTU using a heatmap. Ring 3 labels the confidence level of identified OTUs. All OTUs ID and corresponding affiliations are summarized in supplemental data Table S2. For interpretation of the high-resolution figure, the reader is referred to the web version of this article.

stages were typically dominated by the phyla *Firmicutes*, *Actinobacteria*, *Euryarchaeota*, *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, and *Synergistetes*. However, the change microbial population under FW addition could also be observed. Abundance of the phylum *Firmicutes* increased from 14.5 ± 0.2% (stage 0) to the highest 56.5 ± 2.3% (stage 2), then gradually decreased to 10.4 ± 0.7% (stage 4). The variation pattern showed a significant similarity with methane yield (Fig. 1-d). This can be explained that *Firmicutes* has the ability to degrade metabolic byproducts (such as proteins or polysaccharides) to provide necessary substrate for methane production (Jang et al., 2015a). Because higher concentrations of polysaccharides, proteins, and humic acid substances were also obtained from day 100 to day 140 (stage 2). Thus, a higher methane productivity was achieved. Moreover, the predominance of *Firmicutes* confirmed its high tolerance to high-strength FW condition. Because *Firmicutes* are reported to have a thicker cell wall with many peptidoglycan, which can produce endospores to resistant extreme environments (Zhang et al., 2011). The second phylum *Actinobacteria* presents an opposite trend. The lowest abundance of *Actinobacteria* was observed at stage 2, but increased even over 50% at stage 4. The predominance of *Actinobacteria* has been reported to efficiently convert organic matters to organic acids (Ventura et al., 2007). It is assumed that *Actinobacteria* was particularly favorable to high FW-OLR stress, but its growth pattern suggested the possibility of somewhat time delay. The third phylum in each stage was the archaeal phylum *Euryarchaeota*. Although *Euryarchaeota* was not a major phylum in stage 0, but its abundance consistently increased from 4.8 ± 0.4% to 39.7 ± 1.2% accompany with FW addition and finally dominated in stage 4, which highly coincided with RT-qPCR results. Besides, the phylum *Proteobacteria* was found to be the most abundant in D1 sample with 32.2 ± 2.2%, followed by 17.6 ± 1.9% in D140 sample. But its growth trend in the whole process was messy and disorder. Similarly, other predominant phyla (e.g. *Bacteroidetes*, *Chloroflexi*, *Synergistetes*, *TM7* or *Acidobacteria*) were observed with relative abundance ranging from 0.1% to 10.0% without a specific rule can be followed.

Fig. 4 presented the distribution of top 100 genus (represented by 203 OTUs at subgenus level) in each stage, mainly consisted of 3 circular data. Ring 1 presented the evolution relationship of different OTUs by a phylogenetic tree. Genetic distance was used to evaluate possible property or novel function of the affiliated OTUs. Corresponding phylogenetic affiliation of each OTU were summarized in Supplementary data. Ring 2 displays the relative abundance of each OTU at different stages using heatmap. Results showed that the averaged abundance of each OTU was less than 3.2 ± 0.2%, except for the genus *Methanosaeta* (14.7 ± 0.7%) and *N09* (9.8 ± 0.5%). Above rare species created great challenges in sequences detection and analysis. Ring 3 labeled the confidence level of identified OTUs. Although each subgenus accounted for a

scarce ratio, their alignment results were still reliable with a confidence level over 80%. OTUs belong to the phylum *Firmicutes* showed a larger number than other phyla (73/203), followed by *Proteobacteria* (40/203), *Actinobacteria* (24/203), and *Euryarchaeota* (16/203). As for the combination of abundance results in Fig. 3-a, the less abundant phylum *Proteobacteria* (rank 4th) appeared to show a higher diversity than the major phyla *Actinobacteria* and *Euryarchaeota* at sub-taxonomy. Particularly, many efforts have been made to isolate various members belong to *Firmicutes*, such as the genus *Clostridium* and *Streptococcus*. Related research on the metabolic characteristics together suggested a wide range of substrates can be utilized directly for their growth, even including the complex high-molecular proteins or polysaccharides (Wiegel et al., 2006). Thus, such flexibility may explain the highest diversity of *Firmicutes* in many cases of AD systems. Moreover, the addition of FW to stage 2 occurred simultaneously with the significant enrichment of genus *Ruminococcus* and *Streptococcus* from 0.2 ± 0.01% to 10.2 ± 0.2% and 0.1 ± 0.01% to 12.6 ± 0.3%, respectively. But these predominant members decreased to minor levels and finally faded away at stage 4. Previous efforts reported that *Ruminococcus* and *Streptococcus* are moderate *Bacteria* in AD systems. A significant variation in two members suggested that FW addition as well as increasing loading of FW might be critical impacts inhibiting their growth in FW co-digestion reactors. On the contrary, the genera *N09* became the major genus after stage 2. At the end of stage 4, relative abundance of *N09* accounted for 62.16 ± 0.51% in bacterial population.

From the results of RT-qPCR (Fig. 2-b) and OTUs classification (Fig. 3-a), the richness and diversity of archaeal taxonomic distribution was much less than those of *Bacteria*. Fig. 4 revealed a markedly different composition of *Archaea* (*Euryarchaeota*). OTUs classification showed that the genera *Methanosaeta* was predominant methanogen group throughout the overall digestion process (over 35%). Particularly, *Methanosaeta* showed an optimum growth and enriched with FW addition, which was increased from 3.77 ± 0.16% on day 1 to the highest 35.92 ± 0.51% on day 200. Notably, changing patterns of the genus *Methanosaeta* and *Methanosarcina* suggested their important role in methanogenesis stage with FW addition. *Methanosaeta* has been known to be strictly aceticlastic for methane production. While *Methanosarcina* is metabolically versatile (Garcia et al., 2000). This study observed a much lower growth rate of *Methanosarcina* (3%) than *Methanosaeta* (35%) at stage 4. *Methanosarcina* are more tolerant to harsh conditions (low pH, high VFAs) as compared to *Methanosaeta*. However, *Methanosaeta* have a high substrate (acetate) affinity as compared to *Methanosarcina* (Venkiteshwaran et al., 2017). Therefore, *Methanosaeta* will dominate over *Methanosarcina* in conditions of neutral pH and low TVFAs (acetate) concentrations as observed in this study. Besides, A significant increase in the hydrogenotrophic genera *Methanospirillum* from 0.22 ± 0.01% to 1.54 ± 0.01% occurred by day 120, suggesting a preference for higher FW condition. Similarly, the genera *Methanosarcina* increased from 0.14 ± 0.01% to 3.02 ± 0.02% in FW co-digestion stage 2 and 3. These archaeal members could tolerate high FW loading suggest that they are more resistant than previously thought. In previous researches, specific members belong to *Archaea* accounted for no more than 20% with similar digestion conditions (Jang et al., 2015b; Wang et al., 2013). Further work should be continued to understand the biological mechanisms that why methanogens groups have high tolerance to excessive FW in AcoD systems. Results can be applied to enhance AD technology under high-strength FW conditions.

3.5. Identification of function species

The changes in digestion conditions (e.g. source of wastewater sludge, type of food waste etc.) will result in the appearance of different populations. In many cases, different growth/reduction of particular members were roughly explained as a preference/inadaptation to reactor conditions (Jang et al., 2016, 2015a). Moreover, these researches mainly focused on the limited members that usually dominated in AcoD systems for a long time, such as *Methanosaeta*, *Firmicutes*, *Bacteroidetes*, *Clostridia* (De Vrieze et al., 2016; Jang et al., 2016; Silvestre et al., 2011; Yang et al., 2016), etc. Less attention has been paid to the rare species that detected in digestion samples, such as *Thermotogae*, *Planctomycetes*, and *Verrucomicrobia* found in this study. It is suggested that some species with the relative low abundance (e.g. *Archaea*) possibly formed critical methanogenic phylotypes which were underestimated (De Vrieze et al., 2016), as suggested in Section 3.4. More contradictory observation and limited novel functional pathway would be developed from these studies, if we only evaluate the change of microbial composition and relative abundance involved in AD systems. This can be interpreted as the likely biases derived from digestion indicators, as mentioned before. In the following part, the hypothesis of underlying association between digestion functions and whole community populations were proposed by multivariant analysis. In total, all 1348 genus detected in this study, regardless of their

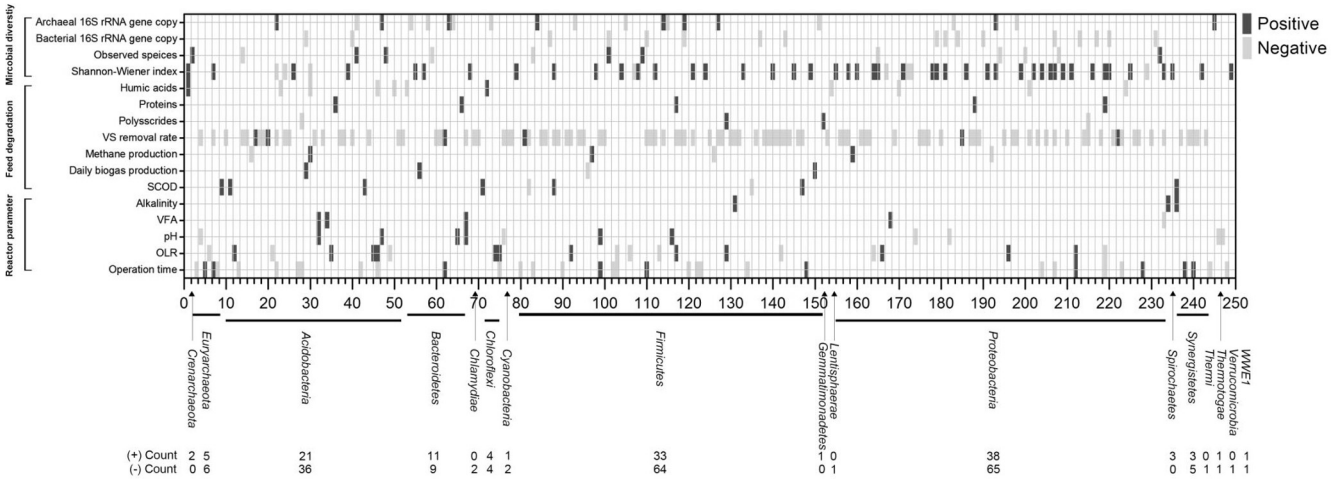

Fig. 5. Person correlation map of 16 selected environmental functions and 249 highly correlated genera ($r > 0.8$ and P-value <0.05). Black and grey plots represent the positive and negative correlations, respectively.

abundance, were directly used to calculate the Pearson correlation indexes with possible digestion function. Only those items with $r > 0.8$ and P-value <0.05 were screened out. As showed in Fig. 5, there were 249 core genera strongly correlated with 16 digestion functions (mainly grouped into 3 parts: reactor parameter, feed degradation and microbial diversity). Among them, 124 and 198 genera were positively and negatively correlated with these possible functions, which confirmed that diverse microorganisms showed heterogenous functions. Most of them showed a positive correlation with Shannon-Wiener index, while negatively correlated with VS removal rate. The orders of correlated phyla counts were *Proteobacteria* (103), *Firmicutes* (97), *Actinobacteria* (57), and *Bacteroidetes* (20). But the dominant members belong to *Euryarchaeota* was only found to be 11 counts (rank 5th). Results also agreed with previous findings that the phyla *Firmicutes* and *Bacteroidetes* are likely responsible for the degradation of proteins and polysaccharides for fermentation in typical digestion systems (Jang et al., 2015a; Wiegel et al., 2006).

Furthermore, 28 core genera and 10 environmental variations were screened out for RDA exam (Fig. 6). The selected members include the most 13 frequently correlated genus, top 5 archaeal genus and top 10 bacterial genus. Multivariate constrained gradient analysis can further explain the putative interactions among species, functions and samples (Vanwonterghem et al., 2014). RDA results showed that the first two axes explained 71.9% and 23.0% of total variation for microbial communities. Unexpected, most of selected digestion variations were clustered closely, such as methane yield, TVFAs concentrations, polysaccharides, proteins, humic acid substances, indicating a complex cooperation networks between substrate degradation and biogas conversion during the AD process. Also, the adverse impacts of FW loading can be also revealed in RDA results, due to the negative association among FW-OLR and methane yield, metabolic by-products or microbial diversity, which was coincided with above discussion. Distribution of samples showed a significant evolution. Samples from effective digestion performance stages (D80-D160) were clustered closely. When FW increased to stage 4, digested samples D200 and D220 were clearly separated from other samples. This confirmed again that although digester had a good pH and TVFAs concentration, function microbial community has already changed (even worse) under FW addition of 4 kg VS m^{-3} d^{-1}, then a decrease in methane productivity was observed. This contradiction can be explained by the delay of operation indicators. *Archaea* as well as bacterial community also showed an obvious adaptation to different environmental conditions, in order to maintain ecological function. Co-occurrences were observed among different species (e.g. *Methanobrevibacter*, *Ruminococcus*, and *Hyphomicrobium*), confirming they worked together to complete the complex AcoD chain-reaction. Because the potential of microbial population to respond to stressful AD conditions highly depended on the formation of well cooperation networks between partner members (De Vrieze et al., 2016). These results suggested that management to improve reactors stability or methane productivity will highly depend on the impacts of AD microbial population. Particularly, most of archaeal species (*Methanosarcina*, *Methanospirillum*, *Methanobacterium*) have an important function correlated to TVFAs concentrations. It is possible that such groups have a higher activity to convert continuous feeding of high organic substrates because they were enriched at stage 4, as revealed in Figs. 2 and 4. Moreover, methane production is conducted via aceticlastic or hydrogenotrophic methanogenesis pathways (Garcia et al., 2000). From day 100 to day 200, the dominant genera of methanogen transferred from *Methanosarcina* (metabolically versatile, decreased from 0.37% to 0.07%) for methane production to *Methanosaeta* (strictly

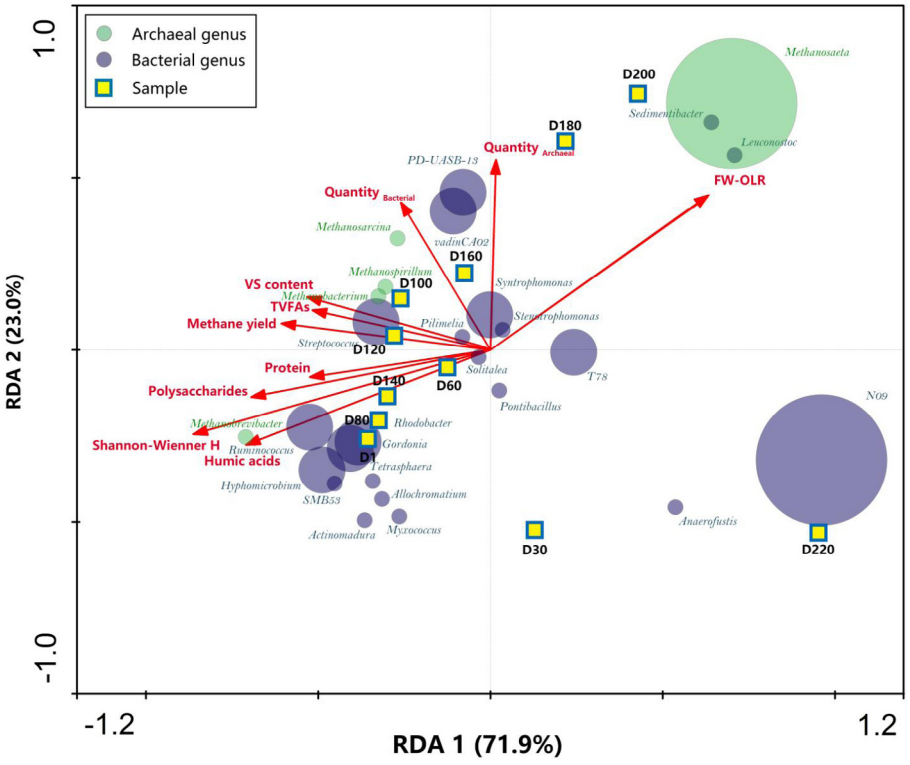

Fig. 6. RDA triplot based on the representative digestion functions, the most frequently correlated and abundant genus data in each stage. Archaeal genus and bacterial genus are represented as green and purple circles. Circular size represents the relative abundance of each genus. The yellow square represents the digestion sample. Each red arrow represents the environmental gradient in the RDA model. Arrow length indicates the strength of correlation with a given ordination axis. Angle between two plots indicates the degree to which the environmental variables are correlated.

aceticlastic, increased from 13.46% to 20.87%), suggesting a change in system condition (e.g. toxic effect of excessive addition of FW in this study).

Interestingly, two genera with the highest relative abundance (*N09* and *Methanosaeta*, ca. 9.8% and 14.7%, respectively) did not show strong correlations with any of selective functions in this study (Figs. 5 and 6). Although *Methanosaeta* is regarded to rely on partner bacterial members to provide them with metabolic substrate (e.g. acetate), the dynamics of *Methanosaeta* is only related with one digestion parameter (FW-OLR) over time. The reason why *N09* and *Methanosaeta* formed the most dominant groups without direct associations with digestion process is still unclear. Again, these results confirmed that using major population to explain possible functions are likely biased, as conducted in many previous works. On the contrary, a considerable number of rare members (<1%) have strong correlation with digestion process were observed in this study. Most of them were strongly related with methane production, polysaccharides breakdown, substrate conversion, such as the phylum *Crenarchaeota* (*Archaea*), *Chlamydiae*, *Cyanobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Spirochaetes*, *Thermi*, *Verrucomicrobia*, and *WWE1* (Fig. 5); the genus *Hyphomicrobium* (1.0%), *Gordonia* (0.9%), *Methanobacterium* (0.4%), and *Methanospirillum* (0.2%) (Fig. 6), etc. However, most of them are uncultured clones lacking detailed phylogenetical information, thus, limited reference is available to discuss their possible functions during AD operation. For instance, recent studies reported the distribution of critical roles of *Crenarchaeota* in ammonia oxidation environment (Treusch et al., 2005), but this may not be able to explain their function in AD system due to the lack of oxygen in anaerobic environment. Except for methane production, the presence of excessive *Gordonia* is reported to result in digestion foaming (Suhartini et al., 2014). *Gordonia* also has been drawn much attention due to their ability of biodegradation of several chemical compounds in AD systems (Singh et al., 2016). The genus *Methanobacterium* is suggested to present ubiquitously in anaerobic environment and dominate in other AD reactors (Lee et al., 2017). It is reported that *Methanobacterium* can grow at a wide pH range from 4.7 to 9.9 and can use CO_2 and H_2 as substrates for methane production. However, more than 25 species belong to *Methanobacterium*, without validly named yet, were found from various habitats (Cadilloquiroz et al., 2014). The broad range of genus *Methanobacterium* members created challenge in identification of detailed function in AD process. This study proposed that such rare groups but highly contributed to AD process can be also considered as important driving force behind AD process, which were frequently covered by the rich groups before (e.g. *Proteobacteria*, *Firmicutes*, *Actinobacteria* or *Bacteroidetes*), mainly because of the limits of detecting resolution or statistical methods. Emerging data from increased HTS technology is challenging our understating of how rare members as well as partner groups are driven by different digestion parameters in biological treatment, in terms of biodiversity and functionality. Similarly, Ziels et al. proposed that using rare species *Syntrophomonas* to predict the capacity of FW-degradation, because the development of *Syntrophomonas* was found to be positively correlated with specific methane productivity and methanogenic activity in AD reactor (Ziels et al., 2015).

Lastly, the results from correlation hypotheses should be carefully tested with complementary methods, such as metagenomic or single cell genomics techniques. Effective identification and multivariate analysis on diverse species can advance our understanding on microbial diversity and functional complexity, though it is hard to explain in highly complex environments. In such case, the measurements of pH, VFAs, alkalinity are currently regarded as substitutes due to the convenience. Further work towards to develop the online/*in situ* monitoring for microbial community as well as digestion parameters, can be used as an early warning to accurately, objectively and timely reveal the underlying pathways occurring in AD, such as reactor stability or FW threshold.

4. Conclusions

This study investigated how a continuous AcoD process responded to increased addition of FW during a long-term operation. A serious methane restriction was observed when FW added to 4 kg VS m^{-3} d^{-3} without any acidification at such high OLRs. The FW-induced performance perturbation was associated with significant shifts of microbial community. Decrease of biodiversity and sensitive species can be considered as early warnings for the deterioration of reactors. A wide spread of function members with low abundance but strongly correlated with digestion process, such as *Hyphomicrobium*, *Gordonia*, *Methanospirillum*, etc., which were also crucial driving forces behind AcoD.

Acknowledgements

This work was supported by National Natural Science Foundation of China (NSFC, Grant No. 51378189, 51578223 and 51521006). A great appreciation is extended to Yang Bai for proofing this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.07.056.

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