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Comparison of the microbial communities in solid-state anaerobic digestion (SS-AD) reactors operated at mesophilic and thermophilic temperatures

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Abstract The microbiomes involved in liquid anaerobic digestion process have been investigated extensively, but the microbiomes underpinning solid-state anaerobic digestion (SS-AD) are poorly understood. In this study, microbiome composition and temporal succession in batch SS-AD reactors, operated at mesophilic or thermophilic temperatures, were investigated using Illumina sequencing of 16S rRNA gene amplicons. A greater microbial richness and evenness were found in the mesophilic than in the thermophilic SS-AD reactors. *Firmicutes* accounted for 60 and 82 % of the total *Bacteria* in the mesophilic and in the thermophilic SS-AD reactors, respectively. The genus *Methanothermobacter* dominated the *Archaea* in the thermophilic SS-AD reactors, while *Methanoculleus* predominated in the mesophilic SS-AD reactors. Interestingly, the data suggest syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis as an important pathway for biogas production during the thermophilic SS-AD. Canonical correspondence analysis (CCA) showed that temperature was the most influential factor in shaping the microbiomes in the SS-AD reactors. *Thermotogae* showed strong positive correlation with operation temperature, while *Fibrobacteres*, *Lentisphaerae*, *Spirochaetes*, and *Tenericutes* were positively correlated with daily biogas yield. This study provided new insight into the microbiome that drives SS-AD process, and the findings may help advance understanding of the microbiome in SS-AD reactors and the design and operation of SS-AD systems.

Keywords Illumina sequencing · 16S rRNA gene · Microbiome · Solid-state anaerobic digester

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Introduction

Anaerobic digestion (AD) has gained increasing attention in recent years because it is a technology that enables both waste reduction and bioenergy (as biogas) production. Based on total solid (TS) content of feedstock, AD can be categorized as liquid-state AD (L-AD) or solid-state AD (SS-AD) (Guendouz et al. 2010). Wastes that contain low TS content (<15 %), such as animal manure slurry, food-processing wastewater, and other high-strength organic wastewaters, are generally subjected to L-AD. On the other hand, SS-AD is advantageous and preferred for feedstocks with a high solid content (>15 %) because it eliminates the need to dilute the feedstocks to pumpable slurry and produces low-moisture digestate, which is much easier to handle. Crop residues, the organic fraction of municipal solid wastes (OFMSW), and food wastes with low water content are common examples of feedstocks that are particularly suitable for SS-AD. Additionally, compared to L-AD, SS-AD requires a smaller

reactor volume per unit mass of feedstock, thus requiring less capital to build and less energy for operation such as heating and mixing (Li et al. 2011). Therefore, although most of the AD plants currently in operation utilize L-AD, research on and implementation of SS-AD processes exceed those of L-AD, particularly in Europe (Yu et al. 2010).

The microbiomes in L-AD reactors, particularly those in constant stirred tank reactors (CSTR) and upper-flow anaerobic sludge blanket (UASB) reactors, have been extensively investigated, and much of the microbiological knowledge on AD processes was derived from such studies (Amani et al. 2010; Gerardi 2003; Narihiro and Sekiguchi 2007; O'Flaherty et al. 2006). Conceptually, the L-AD process has been divided into four stages: hydrolysis of polymeric feedstock components, acidogenesis from the hydrolysis products, syntrophic acetogenesis from fatty acids that contain more than two carbons, and methanogenesis. In addition to direct methanogenesis by acetoclastic methanogenic archaea, acetate can also be converted to methane through a co-metabolic pathway by acetate-oxidizing bacteria and hydrogenotrophic methanogens under certain conditions (Hattori 2008; Schnürer and Nordberg 2008; Shigematsu et al. 2004). Each of the above stages is carried out by a guild of diverse microorganisms, and successful and stable AD performance depends on a dynamic balance among these guilds. Unlike the microbiome in L-AD processes, the microbiome in SS-AD has not been well studied. The overall metabolic processes that occur during SS-AD are probably very similar to those in L-AD, but the unique physical and chemical conditions in SS-AD, such as low water activity, high cellulosic content in feedstock, limited mixing, restricted mass transfer, and retarded microbial cell translocation, probably select unique microbiomes therein. A few studies have examined the microbiomes in SS-AD processes using denaturing gradient gel electrophoresis (DGGE) (Shi et al. 2013), FISH (Montero et al. 2008, 2010; Zahedi et al. 2013a, b), clone libraries of 16S ribosomal RNA (rRNA) genes (Tang et al. 2011), 454 pyrosequencing of 16S rRNA gene amplicons of archaea only (Cho et al. 2013), and shotgun 454 pyrosequencing of metagenomic DNA (Li et al. 2013). Most of these studies analyzed the microbiomes of dry or SS-AD digesters fed with OFMSW or food wastes and did not identify detailed information on microbiome composition or diversity in SS-AD digesters. Thus, the major guilds and taxa of bacteria and methanogens involved in and important to SS-AD processes remain to be determined. The objective of the present study was to determine and compare the dynamic microbial compositions in two lab-scale SS-AD reactors fed with corn stover and operated at mesophilic and thermophilic temperatures. The correlation of microbial populations with environmental factors and SS-AD performance was also investigated. We present here new information on the bacteria and archaea that are potentially important to the SS-AD of corn stover, the physiochemical factors that shape the microbiome in the SS-AD reactors, and the populations (both bacterial and archaeal) that are correlated to digester performance.

Materials and methods

Sample information, metagenomic DNA extraction, and 16S rRNA gene sequencing

The DNA samples analyzed in the present study were prepared in a previous study (Shi et al. 2013), where the integrated AD (iAD, patent pending) process was used to digest corn stover at thermophilic and mesophilic temperatures in batch reactors. Detailed information on the iAD process and operation can be found in that previous paper. Here, the iAD experiment and samples were briefly described to help understand the samples and the results of the present study. Briefly, a portion of dewatered effluent from a mesophilic digester treating municipal sludge and food waste was pre-incubated anaerobically at 36 °C for 1 week, while another portion of the same dewatered effluent was pre-incubated at 55 °C for 2 weeks. Each of the temperature-adapted digested sludge was used as the inoculum for SS-AD at mesophilic or thermophilic temperatures in a respective manner. The inoculum was mixed with ground corn stover (~9 mm particles) at a 1:2 ratio (based on volatile solids) and placed in reactors (1-l working volume each, final TS about 20 %). The SS-AD digesters were maintained at mesophilic (36 °C) or thermophilic (55 °C) temperatures. At days 0, 4, 8, 12, and 38, two SS-AD digesters at each operation temperature were removed from incubation, and the contents of each digester were removed and mixed thoroughly using a handheld mixer prior to sample collection. Biogas production, methane content, reduction of TS and volatile solid (VS), degradation of cellulose and xylan, volatile fatty acids (VFA), pH, most probably numbers of cellulolytic bacteria, xylanolytic bacteria, and acetoclastic methanogens were determined (Shi et al. 2013).

Total metagenomic DNA was extracted from 0.5 g of each digestate sample using the repeated bead beating plus column purification (RBB + C) method (Yu and Morrison 2004). Following confirmation of DNA quality using agarose gel (0.8 %) electrophoresis and quantification using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), the DNA had been subjected to DGGE analysis (Shi et al. 2013). In the present study, the microbiome of the SS-AD samples was further analyzed by sequencing the V4 hypervariable region of the 16S rRNA gene (Caporaso et al. 2012). Briefly, the V4 region of 16S rRNA gene was amplified using the universal primers set 515F and 806R, which allows amplification of the V4 region of both bacteria and archaea. Both primers included the Illumina flowcell adapter sequences. The 806R primer also contained a unique barcode

sequence (12 nucleotides) for each of the sample for multiplexing. The amplicon libraries were sequenced on an Illumina MiSeq using a 2×250 bp paired end protocol (Nelson et al. 2014).

Sequencing data processing and analysis

The Illumina sequencing data was processed and analyzed using QIIME (v 1.7) (Caporaso et al. 2010) following the protocol developed by Nelson et al. (2014). Briefly, the paired reads were merged to form single contigs using SeqPrep (https://gtihub.com/jstjohn/SeqPrep), followed by removal of the phiX sequences. Sequences shorter than 300 bp were filtered out. After demultiplexing, the reads were assigned to species-equivalent operational taxonomic units (OTUs) (at 97 % sequence similarity) in a two-step process involving reference-based and de novo OTU clustering. Chimera checking was performed on the representative sequences of de novo OTUs using ChimeraSlayer (Haas et al. 2011) against the Greengenes database (gg_13_08) (DeSantis et al. 2006). OTUs representing less than 0.005 % of the total bacterial sequences or less than 0.5 % of the total archaeal sequences were filtered out prior to further analysis. Taxonomic classification of the remaining OTUs along with the calculation of alpha and beta diversity metrics was carried out as described by Nelson et al. (2014).

Canonical correspondence analysis (CCA) was performed using the Vegan Community Ecology package of R (http://cran.r-project.org/web/packages/vegan/) to examine correlations between OTUs and measures of reactor performance in terms of total biogas production, TS and VS reduction, and cellulose and xylan degradation. Relative abundance of the detected phyla and genera (percentage of total sequence each taxon represented) was used in the CCA analysis. The abundance (represented by number of sequences) of the OTUs that could be classified to known genera was log transformed for normalization. The distributions of these OTUs were visualized using a heatmap and clustering method implemented in the software GAP (http://gap.stat.sinica.edu.tw/Software/GAP/) as described in a previous study (Li et al. 2014). Pearson's correlation coefficients among the OTUs and among the samples were calculated to examine the similarity of the profiling. Hierarchical clustering trees were generated using the rank-two ellipse seriation method (Chen 2002; Wu et al. 2010).

Data availability

The sequencing data discussed in this study are available from the NCBI Sequence Read Archive (SRA) database under the accession number SRP039329.

Results

Summary of sequence data

Illumina-based 16S sequencing provides a greater than tenfold increase in depth coverage than 454 pyrosequencing, allowing for a more comprehensive examination of the complex microbial communities found in AD systems. Because Illumina data sets were recently shown to include low levels of contaminating reads that can potentially bias analysis (Nelson et al. 2014), we filtered out OTUs that were represented by less than 0.005 % (corresponding to about 60 sequences per OTU) of the total number of bacterial sequences or 0.5 % (about 25 sequences per OTU) of archaeal sequences. A higher percentage cutoff value was used to filter the archaeal sequences because much lower species richness was found in the samples and each archaeal OTU was represented by higher percentage of total archaeal sequences compared to bacterial OTUs. After filtering, over 1 million quality-checked sequences were obtained for the ten samples analyzed, with the number of sequences for each sample ranging from 44,774 to 188,268 (107,472 sequences per sample on average). Just over 99 % of all the sequences were assigned to the domain *Bacteria*, while about 0.4 % of the sequences were assigned to *Archaea*. All of the archaeal sequences and about 99 % of the bacterial sequences were classified to a phylum recognized in the Greengenes database. In total, 1,052 bacterial and 19 archaeal species-equivalent OTUs were found.

Comparison of community diversity and similarity

The microbial diversity of the mesophilic and the thermophilic SS-AD reactors was compared over the course of the 38 days of AD process using the Shannon diversity index, Simpson index, and phylogenetic distance (Table 1). Including the day 0 samples after the temperature-adapted pre-incubation period, nearly all the mesophilic SS-AD samples showed higher values of these diversity measurements than the thermophilic SS-AD samples, indicating a more diverse microbiome underpinning the mesophilic SS-AD process than that driving the thermophilic SS-AD process. In addition, as indicated by the evenness index, the microbial populations in the mesophilic SS-AD process had evener distribution than those in the thermophilic SS-AD process. During the first 4 days, the mesophilic SS-AD process had apparent increases in observed OTU richness, Shannon diversity index, phylogenetic distance, and evenness, while the thermophilic SS-AD process showed decreases in these three diversity indices and evenness from day 8.

Principal coordinate analysis (PCoA) was performed to examine the beta diversity of all the samples collected from both the mesophilic and the thermophilic SS-AD processes over time. Four different methods of distance matrix

Table 1 Alpha diversity indices of the SS-AD samples

Operation temperature (°C)	Sampling time (day)	Observed OTUs	Simpson (*D*)	Shannon (*H*)	Phylogenetic distance	Evenness (*E*)
36	0	586	0.95	6.10	54.20	0.96
	4	822	0.96	6.64	75.34	0.99
	8	846	0.96	6.46	76.28	0.96
	12	839	0.96	6.41	75.45	0.95
	38	808	0.96	6.34	73.66	0.95
55	0	685	0.92	5.93	67.15	0.91
	4	670	0.93	5.71	64.43	0.88
	8	667	0.93	5.68	63.75	0.87
	12	560	0.91	5.06	57.29	0.80
	38	592	0.93	5.48	57.55	0.86

calculation, namely Bray-Curtis, Ochiai, weighted UniFrac, and unweighted UniFrac, were used in the analysis, but they all generated very similar distributions of the samples on the PCoA plots (data not shown); thus, only the PCoA plot generated using weighted UniFrac was presented (Fig. 1). The samples were well separated based on SS-AD temperature along PC1 that explained about 55 % of total variation. The day 0 samples were plotted distantly along PC2 (19 % of total variation) from the rest of the samples collected at the later sampling dates for both the mesophilic and the thermophilic SS-AD processes. The samples collected from each SS-AD process also showed small but noticeable separation along PC2, particularly for the thermophilic SS-AD samples.

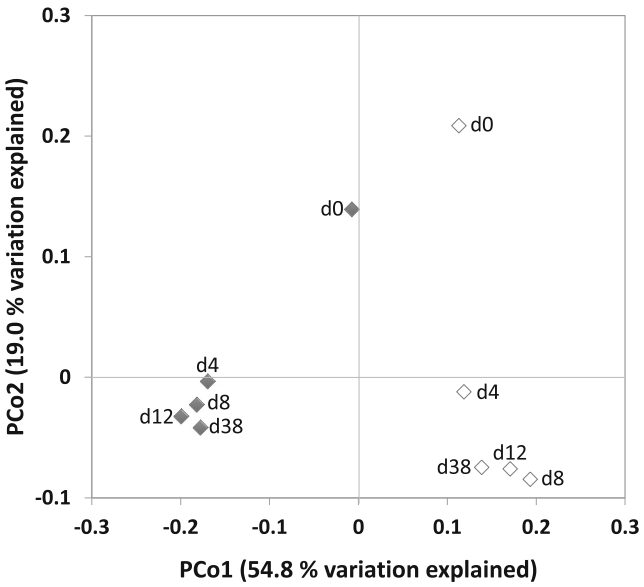

Fig. 1 Principal coordinate analysis (PCoA) of the microbial community in the mesophilic (*open diamond*) and the thermophilic (*filled diamond*) SS-AD processes based on weighted UniFrac distance matrix. Sampling days were indicated next to the *symbols*

Archaea

After the data processing and excluding the sequences that represented small OTUs (less than 0.5 % of total archaea sequences each), 4,671 sequences classified in the domain Archaea were obtained, and they were further clustered into 19 species-equivalent OTUs at 97 % sequence similarity. Accounting for 88 % of the archaeal sequences, 13 OTUs were classified to known genera, while the rest six OTUs could only be classified to the families *Methanospirillaceae*, WSA2 (in the order *Methanobacteriales*), and WCHD3-02 (in the class *Thermoplasmata*) (Table 2). A greater species richness was observed in the mesophilic than in the thermophilic SS-AD processes. *Methanothermobacter* dominated the archaea in the thermophilic SS-AD process (representing 66–93 % of total archaeal sequences of the thermophilic SS-AD) except at day 0 when *Methanosarcina*, *Methanosaeta*, *Methanosphaera*, and one OTU classified to the family *Methanospirillaceae* were found as the main archaeal groups and no *Methanothermobacter* was detected. However, compared to that of the thermophilic SS-AD, the archaeal community in the mesophilic SS-AD was found to contain more diverse and evenly distributed populations, except at day 0 when one *Methanosarcina* OTU (# 367815) dominated (accounting for 83 % of total archaeal sequences of the mesophilic SS-AD). Intriguingly, this *Methanosarcina* OTU became a minor member from day 4 when another *Methanosarcina* OTU (# 1129087) and a *Methanoculleus* OTU (# 840393) increased in relative abundance. In addition, from day 4, the mesophilic SS-AD methanogen community remained diverse and composed evenly of genera *Methanobacterium*, *Methanosphaera*, *Methanospirillaceae*, *Methanosarcina*, and the candidate family WCHD3-02. At the expense of abundance of other archaeal genera, *Methanoculleus* considerably increased its relative abundance from days 12 to 38. Overall, acetoclastic methanogens as a guild (both *Methanosarcina* and *Methanosaeta*) only

Table 2 Relative abundance of methanogens (shown as % of total archaeal sequence in each sample) in the SS-ADs

#OTU ID	Taxonomy assignment	Mesophilic					Thermophilic				
		0 day	4 days	8 days	12 days	38 days	0 day	4 days	8 days	12 days	38 days
None38466	g__*Methanobacterium*	5.1	5.0	1.9	3.3	2.0	5.6	1.9	1.5	1.5	1.1
551498	g__*Methanobacterium*	1.1	3.8	7.7	6.5	9.1	7.0	4.3	0.6	–	0.3
101553	g__*Methanobacterium*	1.7	5.2	2.1	1.1	0.8	7.4	3.7	1.6	0.9	1.2
167356	g__*Methanobrevibacter*	3.4	1.2	1.0	0.4	0.3	0.9	1.2	0.5	–	–
589886	g__*Methanosphaera*	–	13.1	7.5	5.8	1.5	17.7	6.2	1.6	2.6	0.5
227	g__*Methanothermobacter*	–	–	–	0.7	–	–	66.7	83.8	91.7	93.4
27098	f__*WSA2*	–	1.8	0.2	0.7	0.2	2.3	3.7	1.3	0.2	–
4027691	g__*Methanoculleus*	–	–	1.5	0.7	3.0	1.4	0.6	–	–	0.8
108784	g__*Methanoculleus*	–	–	–	–	–	–	1.9	6.0	0.7	1.4
840393	g__*Methanoculleus*	–	6.6	25.8	27.6	53.4	1.9	1.2	0.5	0.2	–
263121	f__*Methanospirillaceae*	1.1	13.3	7.1	8.4	2.6	17.2	1.2	1.3	0.4	0.3
584426	g__*Methanosaeta*	1.7	13.1	11.3	9.5	2.6	17.2	4.9	1.0	1.7	0.5
62890	g__*Methanomethylovorans*	–	6.6	2.1	–	–	3.3	–	–	–	–
367815	g__*Methanosarcina*	83.0	0.2	–	0.7	0.2	1.4	–	–	–	–
1129087	g__*Methanosarcina*	1.1	12.7	20.0	19.3	11.4	16.3	2.5	0.3	–	0.2
None52289	f__*WCHD3-02*	1.1	9.7	7.7	9.8	8.8	–	–	–	–	–
136107	f__*WCHD3-02*	–	4.0	2.1	1.5	0.5	–	–	–	–	–
282634	f__*WCHD3-02*	0.6	2.4	1.0	1.8	1.6	–	–	–	–	–
570725	f__*WCHD3-02*	–	1.4	1.0	2.2	2.0	0.5	–	–	–	0.6

"–" indicates the presence of OTU in that sample that is less than 0.1 %, *g* genus, *f* family

accounted for a relatively small portion of the total archaeal community, and this guild was less predominant in the thermophilic SS-AD process (0.7–7.4 % of total archaeal sequences of the thermophilic SS-AD) than in the mesophilic SS-AD process (14–31 % of total archaeal sequences of the mesophilic SS-AD) from days 4 to 38.

Bacteria

Twelve major phyla (represented by greater than 1 % of total bacterial sequences each in at least one sample) were found (Fig. 2). The percentage of sequences that could not be assigned to any known phyla ranged from 0 to 2 % across the samples. Differences in relative abundance of these phyla were observed between the mesophilic and the thermophilic SS-AD processes (Fig. 2). Being the most predominant phylum, *Firmicutes* sequences accounted for more than 60 % of total bacterial sequences among the mesophilic SS-AD samples and more than 80 % among the thermophilic SS-AD samples except the thermophilic SS-AD sample collected at day 0 when *Firmicutes* sequences were 67 % of total bacterial sequences. *Bacteroidetes* was the second largest phylum in the mesophilic SS-AD (13 % and about 20 % of total bacterial sequences at day 0 and the later sampling days, respectively), but among the thermophilic SS-AD samples, it only represented 6 % of total bacterial sequences at day 0 and about 1 % at the later sampling days. Although the phyla *Proteobacteria*, OP9, *Synergistetes*, *Chloroflexi*, and *Actinobacteria* were found in both the SS-AD processes, several phyla showed temperature-dependent occurrence. Specifically, *Thermotogae* sequences increased from being undetected at day 0 to about 6 % of the total bacterial sequences at day 38 in the thermophilic SS-AD process. On the contrary, the relative abundance of *Spirochaetes*, *Lentisphaerae*, and *Verrucomicrobia* grew gradually over the course of the mesophilic SS-AD process, with *Spirochaetes* sequences increasing from being undetected to 8 % and *Lentisphaerae* and *Verrucomicrobia* increasing from 0.3 to 1 % of total bacterial sequences at day 38.

After filtering out the OTUs represented by less than 0.005 % (60 sequences each) of total bacterial sequences, 1,052 species-level OTUs (defined at 3 % sequence dissimilarity) were assigned to the domain *Bacteria*. Fourteen OTUs were predominant, and each was represented by more than 1 % total bacterial sequences (Table 3). These 14 OTUs together accounted for 55.3 % of the total bacterial sequences. The largest four OTUs were all classified to the class *Clostridia* and accounted for about one third of the total bacterial sequences. The most predominant OTU (accounting for 12 % of total bacterial sequences) could not be classified to any existing genus or family but to the candidate order SHA-98, while the second most predominant OTU (10.8 % of total

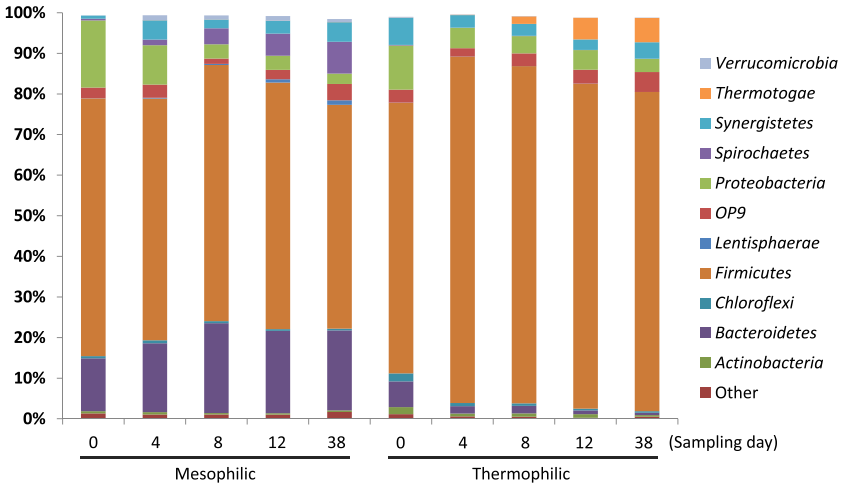

Fig. 2 Major bacterial phyla (each represented by >1 % total sequences in at least one sample) observed in the SS-AD processes

bacterial sequences) could only be assigned to the order *Natranaerobiales*. Both of these two OTUs were observed in both the mesophilic and the thermophilic digesters, but they were about twice as predominant in the mesophilic digester as in the thermophilic digester. The third most predominant OTU (7.2 % of the total bacterial sequences) was assigned to the order *Clostridiales*, and the majority of the sequences representing this OTU were obtained from the mesophilic digester. On the contrary, the fourth largest OTU (5.3 % of the total bacterial sequences) was assigned to the family *Halanaerobiaceae*, which contains known cellulolytic bacteria (Simankova et al. 1993). This OTU was mostly observed in the thermophilic digester. The remaining ten major OTUs had a relative abundance ranging 1–3 % of the total bacterial sequences, and they could be divided into three groups based on their distribution in the SS-ADs. The first group included three OTUs assigned to the order *Bacteroidales* (with one further assigned to the family *Marinilabiaceae* and another to family *Porphyromonadaceae*) and two OTUs assigned to the genus *Treponema* in the phylum *Spirochaetes*. This group of OTUs was mostly observed in the mesophilic digester. The second group included two OTUs with one assigned to the family *Haloplasmataceae* and the other to the unclassified *Clostridia* group OPB54 in the phylum *Firmicutes*, and they

Table 3 Relative abundance (% of total bacterial sequences in each sample) of the major OTUs (representing >1 % of the total sequences) in the SS-AD systems

Lineage	Mesophilic					Thermophilic				
	0 day	4 days	8 days	12 days	38 days	0 day	4 days	8 days	12 days	38 days
Firmicutes; *Clostridia*; *SHA-98*	10.7	11.3	8.1	9.6	10.0	15.0	11.1	11.1	11.2	8.7
Firmicutes; *Clostridia*; *Natranaerobiales*; *ML1228J-1*	–	15.4	7.5	8.7	8.0	21.1	10.2	8.1	7.4	2.0
Firmicutes; *Clostridia*; *Clostridiales*	17.3	2.0	13.4	11.1	8.5	0.1	0.1	–	–	–
Firmicutes; *Clostridia*; *Halanaerobiales*; *Halanaerobiaceae*	–	–	–	–	–	–	18.1	19.3	24.0	7.9
Bacteroidetes; *Bacteroidia*; *Bacteroidales*; *Marinilabiaceae*	–	–	7.9	6.9	4.2	–	–	–	–	–
OP9; *OPB46*; *OPB72*; *TIBD11*	2.2	2.5	1.0	1.9	3.2	2.4	1.5	2.5	3.2	4.0
Bacteroidetes; *Bacteroidia*; *Bacteroidales*	0.1	0.7	1.2	2.5	7.0	2.9	0.6	0.6	0.3	0.2
Firmicutes; *Clostridia*; *Clostridiales*; *Ruminococcaceae*	–	2.1	1.2	1.5	1.4	7.1	2.7	2.0	2.1	1.3
Firmicutes; *Bacilli*; *Haloplasmatales*; *Haloplasmataceae*	–	–	–	–	–	–	8.3	7.9	3.9	2.9
Bacteroidetes; *Bacteroidia*; *Bacteroidales*; *Porphyromonadaceae*	2.2	2.4	1.8	2.2	2.1	1.3	0.6	0.7	0.3	0.2
Firmicutes; *Clostridia*; *OPB54*	–	–	–	–	–	–	–	1.2	4.7	21.3
Spirochaetes; *Spirochaetes*; *Spirochaetales*; *Spirochaetaceae*; *Treponema*	0.2	0.5	1.9	2.8	4.0	–	–	–	–	–
Spirochaetes; *Spirochaetes*; *Spirochaetales*; *Spirochaetaceae*; *Treponema*	0.2	0.8	1.8	2.4	3.5	–	–	–	–	–
Synergistetes; *Synergistia*; *Synergistales*; *Anaerobaculaceae*; *Anaerobaculum*	–	1.3	0.5	1.0	2.0	1.8	0.7	0.6	0.6	0.9

"–" indicates relative abundance <0.05 % in the sample

were mostly observed in the thermophilic digester. The third group was found in both the mesophilic and the thermophilic digesters, and it included three OTUs with one each assigned to the candidate family TIBD11 (in the candidate phylum OP9), the family *Ruminococcaceae*, and the genus *Anaerobaculum* (in the phylum *Synergistetes*).

Distribution of OTUs assigned to known genera

To further understand the SS-AD process in each digester, the OTUs that were classified to known genera were further analyzed. In the present study, 259 OTUs (about 24 % of the total bacterial OTUs that represented about 17 % of the total bacterial sequences) with varying relative abundance were able to be classified to existing genera (Online Resource 1). These OTUs were clustered into seven groups based on their distribution pattern and relative abundance in the samples (Fig. 3). Group I contained 27 OTUs that were all found abundant in the thermophilic SS-AD. Most of these OTUs were classified to the class *Clostridia*. Interestingly, these OTUs exhibited temporal shifts over the course of the thermophilic SS-AD. Specifically, three of these OTUs assigned to the genus *Tepidmicrobium*, which contains species with both cellulolytic and xylanolytic abilities (Niu et al. 2009; Phitsuwan et al. 2010) or protein and amino acid degradation abilities (Slobodkin et al. 2006), were found more abundant at day 0. However, at days 4 and 8, OTUs assigned to the genera *Clostridium*, *Dethiobacter*, and *Tepidanaerobacter* all of which contain syntrophic acetate-oxidizing bacteria, along with other OTUs became abundant. From days 8 to 16, OTUs belonging to the genus *Thermacetogenium*, which contained known acetate-utilizing species producing hydrogen (Hattori et al. 2000), were found more predominant than the OTUs in other genera. The OTUs representing the candidate genus S1 in the family *Thermotagaceae*, which contains known acetate oxidizers in the genus *Thermotoga*, gradually increased in relative abundance over the course of the SS-AD, and S1 became the most predominant genus at day 38 in the thermophilic SS-AD.

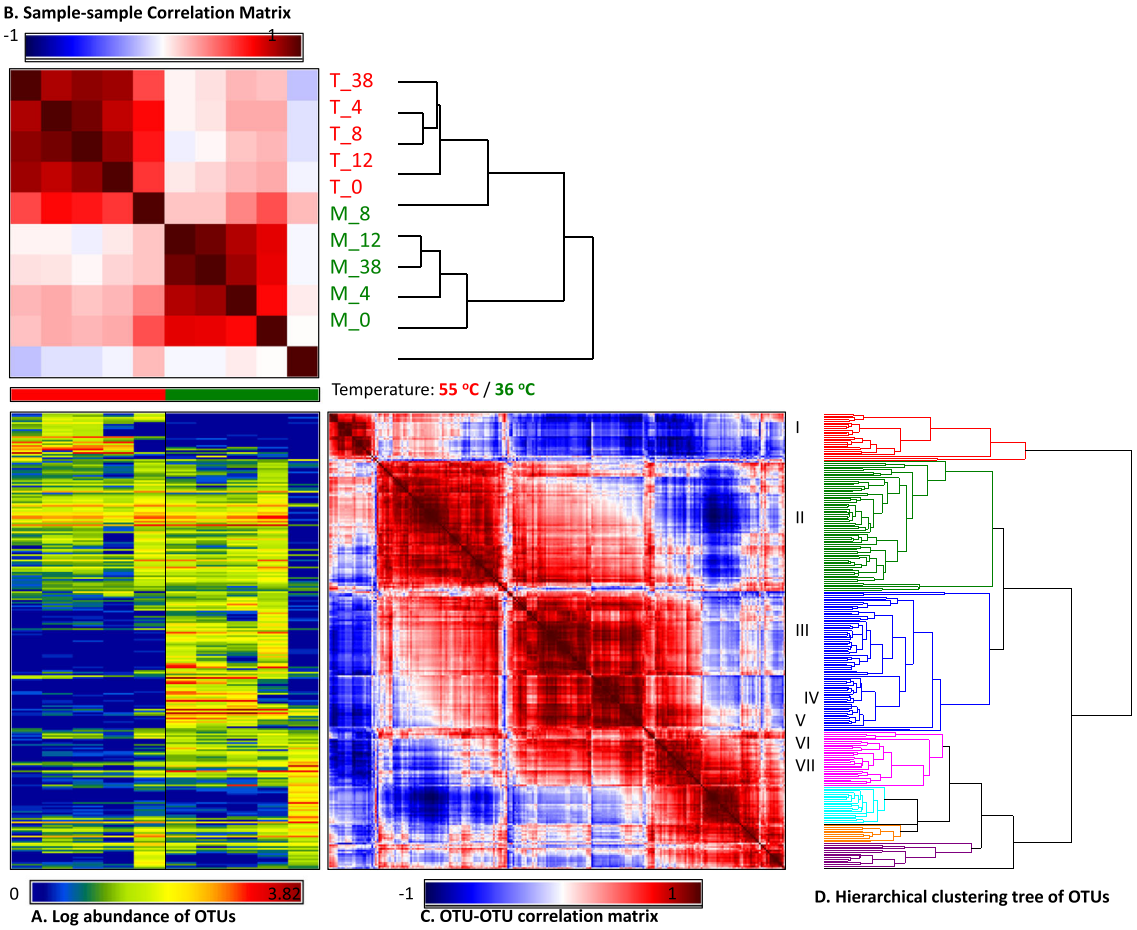

Fig. 3 Generalized association plots for the 259 OTUs identified to genus level from the ten data sets in the two SS-AD reactors. **a** The log abundance heatmap of the 259 OTUs, **b** the sample-sample correlation map among ten data sets, **c** the OTU-OTU correlation map among the 259 OTUs, and **d** the hierarchical clustering tree for sorting the correlation map in **c**. The ten data sets and the 259 OTUs were sorted based on corresponding OTU-OTU correlation and sample-sample correlation, respectively

Group II contained 74 OTUs that were ubiquitous in all but the first sample (day 0) in both the mesophilic and the thermophilic SS-AD processes. However, these OTUs were more predominant in the samples collected at the early stage of the SS-AD processes (day 4 for the mesophilic SS-AD and day 0 for the thermophilic SS-AD). The OTUs in group II were diverse and were assigned to genera that are known to utilize a variety of substrates, including sugars, peptides, and amino acids. Some OTUs were classified to the genera *Anaerobaculum* and *Sporanaerobacter*, both of which contain species capable of utilizing peptides and amino acids in addition to sugars (Hernandez-Eugenio et al. 2002; Maune and Tanner 2012). The genus *Pyramidobacter* and bacteria closely related to clone vadinCA02 in the phylum *Synergistetes*, which also contain amino acid-degrading bacteria (Vartoukian et al. 2007), were represented by six and four predominant OTUs, respectively. Five OTUs in this group were assigned to the genus *Syntrophomonas*. Interestingly, these OTUs showed co-occurrence with hydrogenotrophic methanogens in the genera *Methanobacterium* and *Methanosphaera*. Besides, some OTUs were assigned to the genus *Rhodobacter*, a genus containing species with versatile metabolic capacities, and to the candidate genus T78 (in the phylum *Chloroflexi*) with potential ability to degrade carbohydrates (Ariesyady et al. 2007; Sekiguchi et al. 2001). The ubiquity of these OTUs in this group might be contributed to their versatile function.

Groups III, IV, and VI most likely contained mesophilic species because they were barely observed in the thermophilic SS-AD. Group III contained 80 OTUs and were mostly observed in the mesophilic SS-AD starting from day 4. Among the group III OTUs, 30 were classified to *Clostridium* and two to *Anaerostipes*, a genus containing butyrate-producing bacteria. These OTUs were found predominant especially at days 4 and 8. Another six predominant OTUs each were assigned to the genera *Erysipelothrix* and *Treponema*, both of which contain known homoacetogens (Graber and Breznak 2004). Two predominant OTUs were classified to *Sedimentibacter*, a genus containing amino acid- and pyruvate-utilizing bacteria. In contrast to group III, group IV OTUs were more abundant at day 0 of the mesophilic SS-AD, but they diminished gradually thereafter. In group IV, nine OTUs were assigned to the genus *Clostridium* and ten large OTUs were assigned to the genus *Syntrophomonas*. The OTUs in group V were assigned to *Amaricoccus*, a genus closely related to *Rhodobacter* but with uncertain metabolism, and they were found predominant at day 0 in the mesophilic SS-AD.

The OTUs in groups VI and VII were only observed in the samples collected at day 0, and they went almost undetectable after day 4 in both digesters. Most of the OTUs in these two groups were assigned to the phylum *Proteobacteria*. These OTUs might be outcompeted by other guilds in the digesters, or they could not survive under the SS-AD conditions.

Correlation of microbial groups with environmental factors and SS-AD performance

The physicochemical conditions and performance data of the digesters were collected and reported previously (Shi et al. 2013). To better understand the roles of the microbes in the SS-AD processes, we examined plausible relationship between microbial populations and abiotic environmental variables, including operation temperature, pH, acetate and propionate concentrations, and digester performance, including daily biogas yield, daily fiber reduction, and daily TS destruction, using CCA. Considering that the SS-AD process is most likely driven by predominant bacteria, we performed CCA analysis using the 221 major OTUs that were each represented by at least 0.05 % of the total sequences, the genera, and the phyla represented by these major OTUs (Fig. 4). At any of these three taxonomic levels (OTU, genus, phyla), the results of CCA analysis showed that the environmental variables we included in the analysis explained more than 72 % of the variations of relative abundance, suggesting that the environmental variables explained much of the distribution of the major taxa. Furthermore, CCA1 and CCA2 together explained more than 69 % of the constrained inertia in all the CCA analyses, which indicates that these two coordinates are adequate to represent the CCA results.

The SS-AD temperatures affected the distribution of the detected phyla, major OTUs, and genera the greatest ($p<0.05$ in Mantel test). The temperature and the acetic acid concentration were positively correlated, but they were correlated inversely with the pH. Daily fiber (cellulose and xylan) degradation, daily TS removal, and daily biogas yield pointed in the same general direction, which implies that they were positively correlated in both of the SS-AD processes. At phylum level (Fig. 4a), *Thermotogae* showed strong positive correlation with temperature, while *Fibrobacteres*, *Lentisphaerae*, *Spirochaetes*, and *Tenericutes* were positively correlated with daily biogas yield. At genus level (Fig. 4b), *Tepidanaerobacter* and *Thermoacetogenium*, both of which contain known acetate-oxidizing bacteria, and *Methanothermobacter* were positively correlated with temperature. *Halanaerobiaceae* and *Haloplasmataceae*, both containing known fermenting bacteria, were also highly correlated positively with temperature and positively correlated with acetic acid concentration. The class *Clostridia* and the genus *Methanobrevibacter* were found correlated positively with daily biogas yield. At OTU level (Fig. 4c), a group of OTUs showed strong positive correlation with acetic acid concentration. This group of OTUs included two OTUs assigned to the family *Haloplasmataceae*, two OTUs assigned to the family *Halanaerobiaceae*, two OTUs assigned to the order *Clostridia*, one OTU assigned to the family *Clostridiaceae*, one OTU assigned to the genus *Clostridium*, one OTU assigned to the candidate family ML1228 in the

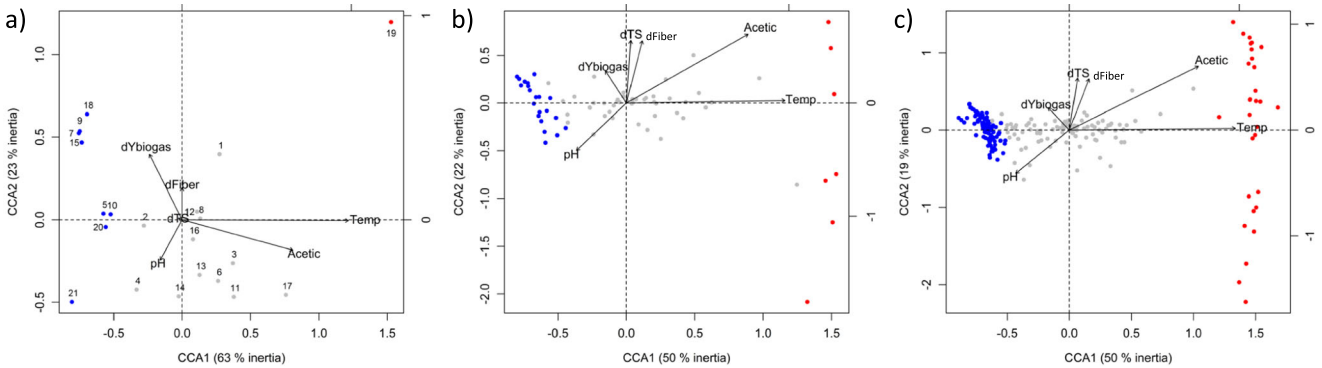

Fig. 4 Results of canonical correspondence analysis (CCA) at **a** phyla level, **b** genus level (only the genera that contained abundant OTUs (each representing ≥0.05 % of total sequences), and **c** OTU level (only the abundant OTUs). *Dots* in the figures represent individual taxa/OTUs. *Dots in blue* represent the taxa/OTUs that were ≥ten times more abundant in the mesophilic than in the thermophilic SS-AD, while *dots in red* represent the taxa/OTU that were ≥ten times more abundant in the thermophilic than in the mesophilic SS-AD. *Gray dots* represent taxa/OTUs whose abundance did not differ by ≥tenfold. The taxa/OTUs represented by the *blue*, *red*, and *gray spots* were included in Online Resource 2. The *arrows* represent the direction of increase of the variables: *Temp* temperature, *dYbiogas* daily biogas yield, *dFiber* daily fiber (cellulose and xylan) reduction rate, *dTS* daily TS reduction rate, *Acetic* acetic acid concentration. The length of the *arrows* indicates the degree of correlation along the ordination axes. Corresponding phyla in **a**: *Euryarchaeota* (*1*), unclassified bacteria (*2*), *Actinobacteria* (*3*), *Armatimonadetes* (*4*), *Bacteroidetes* (*5*), *Chloroflexi* (*6*), *Fibrobacteres* (*7*), *Firmicutes* (*8*), *Lentisphaerae* (*9*), Candidate division NKB19 (*10*), Candidate division OD1 (*11*), Candidate division OP9 (*12*), *Planctomycetes* (*13*), *Proteobacteria* (*14*), *Spirochaetes* (*15*), *Synergistetes* (*16*), Candidate division TM6 (*17*), *Tenericutes* (*18*), *Thermotogae* (*19*), *Verrucomicrobia* (*20*), and Candidate division WS1 (*21*) (color figure online)

phylum *Firmicutes*, and one OTU assigned to the candidate order SHA in the phylum *Firmicutes*. This group of OTUs might be involved in either acetate production or oxidization.

Discussion

AD as a technology has been evolving rapidly in the past 10 years from being primarily a conventional waste treatment process to being a major bioenergy production process. Cost-effective, efficient, and reliable AD technologies remain the "holy grail" of the AD industry, for which SS-AD is considered a promising area of development. This study is one of the few that have investigated the microbiomes driving SS-AD. Besides comprehensive revelation of the bacteria and archaea present in the SS-AD processes operated at mesophilic and thermophilic temperatures, the present study also revealed plausible correlations between microbial groups and measurements of AD performance. Unlike L-AD, SS-AD processes, such as the iAD (Shi et al. 2013) process and the SMARTFERM process (http://www.smartferm.com/), are often operated in batch mode. During batch operation, the microbiome underpinning the AD typically undergoes temporal successions. In this study, the same source of seed sludge and identical feedstocks was used to establish two SS-AD reactors operated at different temperatures, mesophilic vs. thermophilic. This provided an opportunity to assess the temporal successions arose from selection and adaptation of the original seed community. Overall, after a 38-day period, the microbial communities of the two SS-AD reactors diverged and formed stable and unique communities. Consistent with the finding from other studies on L-ADs (Levén et al. 2007; Pycke et al. 2011), the microbiome in the mesophilic SS-AD was more diverse than that in the thermophilic SS-AD.

Differences in physicochemical conditions (e.g., water activity, TS and VS content, mass transfer, and microbial translocation) and operation (feeding and mixing) exist between SS-AD and L-AD processes. In the literature, one study reported similar methane yields from SS-AD and L-AD of the same feedstock (Brown et al. 2012), but no study has been reported that compares the microbiomes of SS-AD and L-AD fed with the same feedstock. A recent study examined the bacterial and archaeal communities in a mesophilic L-AD fed with slurry of corn stover using clone libraries (Qiao et al. 2013). Comparison between that study and the present study revealed some characteristics of the microbiome in the mesophilic SS-AD. At phylum level, the mesophilic SS-AD microbiome had a greater proportion of *Firmicutes* (more than 60 vs. 48.3 %) and *Bacteroidetes* (about 20 % vs. less than 8 %) but a smaller proportion of *Chloroflexi* (0.5 vs. 20.1 %) and *Actinobacteria* (about 1 % vs. 9.1 %) than the mesophilic L-AD microbiome. Comparison of our results to the results of Qiao et al (2013) at any taxonomic level below phylum was difficult because of limited information reported in that study. However, one striking difference is the lack of syntrophic acetate-oxidizing bacteria in the L-AD of corn stover and detection of several genera of such bacteria in the SS-AD of corn stover. With respect to methanogens, the L-AD and the mesophilic SS-AD had similar ratio of hydrogenotrophic methanogens and acetoclastic methanogens, but the mesophilic SS-AD samples collected at days 8 and 12 had a

greater proportion of *Methanosarcina* than the samples from the L-AD. Among common hydrogenotrophic methanogens, *Methanosphaerula* (29 %), *Methanospirillum* (19 %), and *Methanobacterium* (13 %) were the most abundant genera in the L-AD, while in the SS-AD, *Methanoculleus* (53 %) was the most predominant genus followed by *Methanobacterium*, *Methanospirillaceae*, and *Methanosphaera* from days 8 to 38. It should be pointed out that besides the physical state of the digester matrix (liquid vs. solid), other operational factors such as mixing, seeding, feeding (continuous vs. batch), and organic loading rate could also have affected the microbiome in these two types of AD of corn stover. However, it is not possible to operate an L-AD and an SS-AD on the same type of feedstock with identical parameters (such as the same water content, mixing). Therefore, the above differences in microbiome between L-AD and SS-AD may reflect some of the real-world differences in microbiome between L-AD and SS-AD digesters fed with corn stover.

Previous studies have reported that hydrogenotrophic methanogens were more predominant than acetoclastic methanogens in thermophilic AD (Briones et al. 2007; Hori et al. 2006; Sasaki et al. 2011; Tang et al. 2011). In the present study, acetoclastic methanogens (i.e., *Methanosarcina* and *Methanosaeta*) were detected at very low abundances in the thermophilic SS-AD, with an exception at day 0. In addition, many genera containing known homoacetogens, some of which are known to oxidize acetate to CO_2 and H_2 in syntrophy with hydrogenotrophic methanogens (Hattori 2008), were found in the thermophilic SS-AD. These two findings together suggest that the non-acetoclastic oxidative pathway coupling syntrophic acetate oxidation and hydrogenotrophic methanogenesis might be important for biogas production in thermophilic SS-AD processes. This alternative pathway for biogas production has previously been reported for an SS-AD reactor fed with waste paper (Tang et al. 2011) and an L-AD reactor fed with artificial garbage (Sasaki et al. 2011), but this is the first study suggesting this pathway in SS-AD of corn stover. As *Methanothermobacter* was the most abundant genus of methanogens in the thermophilic SS-AD (except at day 0 when SS-AD started), members of this genus likely play an important role in biogas production in this system. The abundance of *Methanothermobacter* increased over time, and it remains to be determined if such increase can be attributed to its greater affinity for hydrogen (Zinder 1993) and/or its ability to grow at higher pH (Wasserfallen et al. 2000) than other hydrogenotrophic methanogens. Indeed, the pH of the two SS-AD processes in the present study was relatively high, ranging from 7.4 to 8.9 (Shi et al. 2013). In the mesophilic SS-AD, *Methanosarcina* was the predominant genus of methanogens in the early stage (up to day 8) but declined rapidly as hydrogenotrophic methanogens, particularly the genus *Methanoculleus*, grew in abundance. Several studies have observed such a dynamic succession in L-AD of solid feedstock operated at thermophilic conditions (Krakat et al. 2010; Liu et al. 2009; Sasaki et al. 2011). The succession of methanogens observed in the mesophilic SS-AD in the present study might be due to the low concentrations of acetate at these time points and the high affinity of *Methanoculleus* for hydrogen as a substrate for methanogenesis (Goberna et al. 2009; Hori et al. 2006; Sasaki et al. 2011). This premise is consistent with the observations of low acetate concentrations in the mesophilic SS-AD (Shi et al. 2013) and the higher abundance of *Methanosarcina* in a mesophilic SS-AD fed with readily digestible food waste (Cho et al. 2013).

Firmicutes was found more predominant in the thermophilic SS-AD than in the mesophilic SS-AD (about 60 % vs. about 80 % of total bacterial sequences). Dominance of this phylum (about 90 % of total bacteria) was also found in a thermophilic digester treating waste paper (Tang et al. 2011). On the other hand, *Bacteroidetes* had a greater proportion in the mesophilic SS-AD than in the thermophilic SS-AD, which is consistent with the finding in a recent study comparing microbial compositions in 21 different L-AD digesters operated at either mesophilic or thermophilic temperatures irrespective of the types of feedstock (Sundberg et al. 2013). Members of *Firmicutes* might play a more significant role in thermophilic digesters than in mesophilic digesters of both liquid state and solid state.

The bacterial candidate phylum OP9 was represented by 1–5 % total bacterial sequences in all the digester samples of both SS-AD digesters. This phylum was first discovered in the hot springs in the Yellowstone National Park (Hugenholtz et al. 1998), and ever since, it has been found in other anaerobic environments, such as geothermal areas, wastewater treatment plants, and anaerobic digesters (Rivière et al. 2009; Tang et al. 2011; Vick et al. 2010). Both the genome of an isolate in this phylum and metagenomic contigs assigned to this phylum from an anaerobic cellulolytic microbiome suggest capability of hydrolyzing cellulose and hemicellulose and fermenting sugars via glycolysis to hydrogen, acetate, and ethanol (Dodsworth et al. 2013). Such cellulolytic and hemicellulolytic capabilities might help explain the presence of the phylum OP9 in the SS-ADs of corn stover. Further research is needed to confirm their metabolic capability and to what extent this new phylum contributes to fiber degradation in SS-AD.

Multivariate analyses have been widely used in community ecology of plants, fish, and bacteria to help elucidate the relationship between abundance of groups of organisms and the habitat environments (see review by Ramette 2007). In the present study, the relationship between the presence and abundance of microbial taxa and some important operational factors/performance indicators in the SS-AD processes, including temperature, pH, acetate concentration, degradation of feedstock (solid content), and biogas production, was

revealed through CCA. As expected, temperature was found to be the most influential factor shaping the microbiome of SS-ADs. Some microbial groups and guilds were highly correlated with acetate concentrations (such as the phylum *Synergistetes*, families *Haloplasmataceae* and *Halanaerobiaceae*), which might indicate their role in acetate production in SS-ADs. On the other hand, the positive correlation between the biogas yield and the genus *Methanobrevibacter* and the class *Clostridia* could reflect the importance of these two taxa in biogas production in SS-AD. Although the causality between microbes and other factors or indicators could not be confirmed at this stage, the correlations between microbes and environmental variables and performance measurements can guide future studies and improvement of design and operation of SS-AD. More research studies need to be done to confirm the correlations and establish the causality. Targeted quantitative analysis of the microbial groups that have correlations with environmental or performance measurements will help establish possible causality and eventually help optimize SS-AD process.

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Conflict of interest The authors declare that they have no conflict of interest.

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