**Impact of different ratios of feedstock to liquid anaerobic digestion effluent on the
performance and microbiome of solid-state anaerobic digesters digesting corn stover**

Yueh-Fen Li^a, Jian Shi^b, Michael C. Nelson^c, Po-Hsu Chen^d, Joerg Graf^c, Yebo Li^{a,b},
Zhongtang Yu^{a,c,*}

^a Environmental Science Graduate Program,^d Department of Statistics,^e Department of
Animal Sciences, The Ohio State University, Columbus, OH 43210, USA;^cDepartment of
Molecular and Cell Biology, University of Connecticut, Storrs, CT 06269, USA. ^b
Department of Food, Agricultural and Biological Engineering, The Ohio State University/
Ohio Agricultural Research and Development Center, 1680 Madison Ave, Wooster, OH
44691, USA

* Correspondent footnote:

Zhongtang Yu

Department of Animal Sciences

The Ohio State University

2029 Fyffe Court

Columbus, OH 43210, USA

Tel: (614) 292-3057, fax: (614) 292-2929, e-mail: yu.226@osu.edu

20 **Abstract**

22 The objective of this study was to understand how the non-microbial factors of L-
AD effluent affected the microbiome composition and successions in the SS-AD digesters
using both Illumina sequencing and qPCR quantification of major genera of
24 methanogens. The SS-AD digesters started with a feedstock/total effluent (F/Et) ratio 2.2
(half of the effluent was autoclaved) performed stably, while the SS-AD digesters started
26 with a 4.4 F/Et ratio (no autoclaved effluent) suffered from digester acidification,
accumulation of volatile fatty acids, and ceased biogas production two weeks after startup.
28 Some bacteria and methanogens were affected by non-microbial factors of the L-AD effluent.
Alkalinity, the main difference between the two F/Et ratios, may be the crucial factor when
30 SS-AD digesters were started using L-AD effluent.

32 **Keywords:** Illumina sequencing, metagenomics, microbiome, 16S rRNA gene, succession

1. Introduction

The non-hazardous waste management hierarchy of the US EPA calls for reduce, reuse, and recycle (or Three R's) of wastes (<http://www.epa.gov/waste/nonhaz/municipal/hierarchy.htm>). Anaerobic digestion (AD) is one of the most attractive and practical technologies to achieve the three R's. Liquid anaerobic digestion (L-AD), which is suited for feedstock containing less than 15 % total solid (TS), is the traditional technology, while solid-state AD (SS-AD) is relatively new (Kothari et al., 2014; Li et al., 2011). Operated at TS content greater than 20%, SS-AD is the technology primarily used to digest feedstock of low moisture content, such as crop residues and the organic fraction of municipal solid waste (OFMSW). Compared to L-AD, SS-AD has several advantages over L-AD, including smaller digester volumes required for a given amount of feedstock, less capital to build, less energy requirement for operations such as heating and mixing, and easier to transport and use of the final digestate (Kothari et al., 2014; Li et al., 2011). Over the past decade, more SS-AD systems than L-AD systems were built in Europe to meet the demand for bioenergy. However, it has always been challenging and time-consuming to start up a SS-AD system. The limited or lack of mixing also retards mass transfer and translocation of microbial cells within the solid digestate matrix.

Several studies have demonstrated that effluent from L-AD reactors can expedite startup of SS-AD reactors and improve biogas production by providing the inoculum microbes, nitrogen, phosphorus, minerals, moisture, and buffering capacity needed for AD (Li et al., 2011). However, the ratio between feedstock and L-AD effluent (F/E) greatly

affects digester performance, especially length of the lag time before active biogas
56 production and process stability (Kusch et al., 2012; Xu & Li, 2012). A recent study
showed that, by including different portions of autoclaved L-AD effluent into the total
58 effluent, non-microbial factors present in L-AD effluent also affect the SS-AD process
with respect to pH, VFA concentration, biogas production, and process stability (Shi et al.,
60 2014). Community (both archaeal and bacterial) profiling using DGGE also revealed
considerable effects on the microbial populations by different portions of active and
62 autoclaved L-AD effluent. Canonical correspondence analysis (CCA) of the DGGE profiles
showed that some DGGE bands were correlated to SS-AD performance. However, the
64 DGGE profiling did not identify the specific bacterial or archaeal populations that were
affected by the treatments or correlated with digester performance. In the present study,
66 thus, Illumina sequencing of 16S rRNA amplicons was used to further examine the
microbiomedynamics in two SS-AD reactors in response to microbial and non-microbial
68 factors present in L-AD effluent. Population shifts of total methanogens and individual
genera of methanogens were also evaluated using quantitative PCR (qPCR). Plausible
70 correlations between microbial populations and the abiotic environmental factors in the
SS-AD reactors were assessed using CCA. The results of this study provide new insights
72 into the microbiological underpinning of SS-AD process and stability and may be helpful
to future research on SS-AD.

74 **2. Materials and Methods**

2.1 Sample information

The samples have been analyzed in a previous study (Shi et al., 2014). Briefly, two sets of batch SS-AD reactors (1.0-liter working volume each) were fed the same ground corn stover and inoculated with the same amount of non-autoclaved L-AD effluent to achieve the same feed/alive effluent (F/Ea) ratio of 4.4 (based on volatile solid content). Corn stover obtained from a farm operated by the Ohio Agriculture Research and Development Center in Wooster, OH was air dried to a moisture content of less than 10% and ground to pass through a 9 mm sieve. The L-AD effluent was collected from a mesophilic L-AD fed a mixture of municipal sewage sludge and food wastes (operated by the Quasar Energy Group, Cleveland, OH, USA). In addition to the non-autoclaved L-AD effluent, one set of the SS-AD reactors received an additional autoclaved L-AD effluent to achieve a feed/total effluent (F/Et) ratio of 2.2 (referred to as LFE digesters, while the other set of SS-AD reactors didn't receive the additional autoclaved effluent but sterile water, resulting in a F/Et ratio of 4.4 (referred to as HFE digesters). As a result, the two sets of reactors had the same F/Ea ratio of 4.4 but different F/Et ratios (4.4 vs. 2.2). In other words, the two sets of SS-AD reactors started with the same inoculum (or seed), but differed in non-microbial factors, such as pH, C/N ratio, alkalinity, and micronutrients (Shi et al., 2014). Water was added when needed to remain the TS content of 20%. All the batch SS-AD reactors were incubated at 36°C for 38 days. At predetermined time points (day 0, 2, 4, 6, 8, 10, 12, and 38), two replicate reactors from each set were terminated, and the content of each reactor was mixed thoroughly using a hand-held mixer and sampled for chemical and microbial analyses (Shi et al., 2014). In the present study, samples taken at day 0, 8, 12, and 38 for the LFE digesters (designated as samples LFE-t1, LFE-t2, LFE-t3, and LFE-t4, respectively)

and at day 0, 4, 10, and 38 for the HFE digesters (designated as samples HFE-t1, HFE-t2, HFE-t3, and HFE-t4, respectively) were analyzed to examine microbial population shifts during the SS-AD process. More samples at the early stage of the SS-AD process were collected because of the dynamic changes in VFA concentration, pH, and biogas production rates during that period.

2.2 DNA extraction and 16S Illumina sequencing

Total metagenomic DNA was extracted from each sample using the repeated bead-beating plum column purification method (Yu & Morrison, 2004). The DNA quality was verified using agarose gel (1.0%) electrophoresis, and DNA concentrations were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The diversity and composition of the microbiome of the SS-ADs samples were examined by sequencing and analyzing the V4 hypervariable region of 16S rRNA gene (Caporaso et al., 2012). Briefly, the V4 region was first amplified with the primer set 515F and 806R targeting both bacteria and archaea. This primer set has been validated and used in the standard protocol of the Earth Microbiome Project (EMP) (<http://www.earthmicrobiome.org/emp-standard-protocols/16s/>). The 806R reverse primer also contained a barcode sequence unique for each sample for multiplexing. The amplicon libraries from all the samples were pooled at equal molar ratio and sequenced on an Illumina MiSeq system using the pair-ended 2 x 250bp protocol at University of Connecticut.

2.3 Sequence Data 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 pairedreads were merged to form single sequences using SeqPrep (<https://github.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 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 sequences were filtered out prior to further analysis(Bokulich et al., 2013). Taxonomic classification of the remaining OTUs and calculation of alpha and beta diversity indices or metrics were carried out as described by Nelson et al. (2014). Briefly, the sequences of each sample were rarefied before the diversity analysis. Alpha diversity measurements, including Shannon and Simpson indices, phylogenetic distance, and observed number of OTUs, were calculated for each sample. Beta diversity was calculated using the distance matrices generated using both the phylogenetic-based method UniFrac (Lozupone & Knight, 2005) and the non-phylogenic-based methods Ochiai and Bray-Curtis, and then visualized using principal coordinates analysis (PCoA).

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 environmental

factors important to AD, including pH, VFA concentration, and daily biogas production.

Relative abundance of the major OTUs (each accounting for $\geq 0.1\%$ of total sequences) and the 13 archaeal OTUs identified was used in the CCA analysis. Furthermore, the distribution of the major OTUs were further visualized using the generalized association plots with the clustering method implemented in the software GAP (<http://gap.stat.sinica.edu.tw/Software/GAP/>) as described elsewhere (Li et al., 2015a). Briefly, the abundance (represented by the number of sequences) of each major OTU was log transformed for normalization. Then the Pearson's correlation coefficients among the samples and among the OTUs 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).

2.4 Quantification of methanogens

Quantitative real-time PCR (qPCR) was used to quantify the common genera of methanogens in the SS-AD digesters using the genus-specific primer sets listed in Table S1. Individual sample-derived qPCR standard was prepared as described previously (Yu et al., 2005). Briefly, the DNA extracted from all the SS-ADs samples were pooled together (based on equal amount) and used as a template in PCR using each of the genus-specific primer set. The PCR was performed as follows: initial activation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 30s. The PCR reaction was ended with a final extension at 72°C for 10 min. Each of the PCR products was confirmed using agarose gel (1.5%) electrophoresis and then purified using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). The

purified PCR products were each quantified using a NanoDrop 1000 spectrometer (Thermo Scientific, Wilmington, DE), serially diluted, and used as the qPCR standards. The qPCR was carried out on an Mx3000p real-time PCR system (Stratagene, La Jolla, CA) as described previously (Nelson, 2011) using serial (1:10) dilutions ($10^1 - 10^7$ copies per qPCR reaction) of each qPCR standard ($R^2 > 0.99$ for all standard curves). The qPCR was performed using the following thermal profile: initial activation at 95°C for 5min, followed by 40 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min with the fluorescent signal detected at the end of the annealing/extension step. The Ct values were determined using the default setting of MxPro 4.10 software (Stratagene, La Jolla, CA). Samples with a Ct value greater than that of the no-template control were considered containing undetectable target.

2.5 Data availability

The sequences obtained in this study are available in the SRA database of NCBI under the BioProject ID PRJNA290510.

3. Results and Discussion

3.1 Comparison of digester performance

Significant differences in digester performance in terms of VFA concentration, pH, and biogas production were observed between two sets of SS-ADs with different F/Et ratios (Fig. 1). In the LFE digesters (with an F/Et ratio of 2.2), VFA concentration increased slightly in the first four days but decreased gradually thereafter to being almost undetectable until the end of the incubation. The daily biogas production peaked at day 8

after the startup of the digestion. Although the daily biogas production decreased gradually from 14 to 3 L/kg of VS fed, biogas was generated continuously throughout the operation, while the pH remained stable at around 8.8. The stable pH and longitudinally consistent conversion of VFA to biogas indicate balanced metabolic processes among different guilds of microorganisms that are responsible for acidogenesis (also syntrophic acetogenesis) and methanogenesis in the LFE digesters (referred to healthy digesters).

On the contrary, the HFE digesters (with an F/Et ratio of 4.4) resulted in accumulation of VFA, pH drop, and inhibition to biogas production (Fig. 1). Accumulation of VFA and corresponding drop of pH started at day 2 after the startup. Although daily biogas production reached 8 L/kg VS fed at day 4, it rapidly decreased when the pH value dropped to below 6 at day 8. One week after the pH dropped to about 6, biogas production completely ceased. The L-AD effluent inoculum and autoclaved L-AD effluent contained less VS but more nitrogen and alkalinity than the corn stover. Therefore, compared to the LFE digesters, the HFE digesters had a higher organic loading and higher C/N ratio, resulting in organic overloading, rapid acidogenesis, pH decline, and eventually acidified digesters (referred to as sour digesters). Furthermore, the alkalinity of the HFE digesters was lower than that of the LFE digesters (1,036 vs. 1,731 mg CaCO₃/kg digester content) (Shi et al., 2014). Because adequate alkalinity is essential for stable AD process, especially during the startup (Gerardi, 2003), the failure of the HFE digesters was probably attributable, at least in part, to their low alkalinity. These results are in concordance with previous findings that feedstock/effluent (leachate) ratios primarily determined the success of the startup of AD process (Raposo et al., 2009; Xu & Li, 2012).

3.2 Summary of Illumina sequence data

Illumina sequencing allows for comprehensive analysis of complex microbiome, but minor crossover between flow cells in the same run and carryover contamination between runs can artificially inflate diversity (Nelson et al., 2014; Salipante et al., 2014). Sequencing artifacts also results (Nelson et al., 2014). Thus, after the quality check, chimera filtration, and OTUs clustering, the resulting OTUs represented each by $\leq 0.005\%$ of total sequences were filtered out to minimize potential diversity inflation. Eventually, 762,816 sequences were obtained from the 8 samples, and they were subsequently clustered into 1,039 OTUs at 97% sequence similarity, of which 1,026 OTUs were classified as bacteria and 13 were classified as archaea. About 97.6% of the total sequences could be assigned to a known bacterial phylum in the Greengenes database, with *Firmicutes* (represented by 61.0% of total sequences) being the most abundant one, followed by *Bacteroidetes* (17.9%), *Proteobacteria* (8.2%), *Synergistetes* (2.9%), *Spirochaetes* (2.0%), and candidate phylum OP9 (1.5%) (Fig. 2). Other phyla were each represented by $\leq 1\%$ of total sequences, including *Actinobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Planctomycetes*, *Lentisphaerae*, *Fibrobacteres*, *Tenericutes*, *Chlamydiae*, *Armatimonadetes*, *Acidobacteria*, *Thermotogae*, the candidate phyla NKB19, TM6, WS1, OD1, and TM7. Archaeal sequences were only represented by 0.4% of the total sequences, and all of them were assigned to the phylum *Euryarchaeota*.

3.3 Comparison of overall microbiome between the healthy and the sour SS-AD digesters

Beta diversity was calculated and used to examine the similarity of the microbiome between the healthy (LFE) and the sour (HFE) SS-AD digesters. The

clustering patterns of PCoA plots generated from the three different methods were very similar, and only the one generated from weighted UniFrac was presented (Fig. 3). The samples were clustered into three groups, with the first group corresponding to day 0 samples of both types of digesters, the second group containing the samples collected from the sour digesters at days 4 and 10, and the third group containing the samples collected at day 8 (t2), 12 (t3), and 38 (t4) from the healthy digesters and the samples collected at day 38 (t4) from the sour digesters. Explaining 65.6 % of total variation, PC1 separated the samples taken at day 0 from the samples collected at the later time points. On the other hand, PC 2, which explained 20.1 % of total variation, separated the samples collected at day 4 (t2) and 10 (t3) of the sour digesters from other samples. The similar microbiomes at day 0 but different microbiomes at the later days between the two F/Et ratios are expected. Interestingly, the microbiomes converged by day 38 by the end of the SS-AD process. The alpha diversity analysis results showed that the microbiome had similar species richness and Simpson and Shannon indices between the two F/Et ratios and over time (Table 1), suggesting microbiome successions but not dramatic change in species richness or evenness.

3.4 Bacterial community

3.4.1 Bacterial community composition and structure at phylum level

Ten major bacterial phyla, each represented by $\geq 1\%$ total sequences in at least one sample, together accounted for 96.1% of the total sequences obtained (Fig. 2). Bacterial sequences that were unable to be assigned to a known phylum accounted for 1-3% of total sequences. During incubation, microbiome successions at phylum level were highly similar

for both the healthy and the sour digesters, except for the phyla *Spirochaetes*, *Verrucomicrobia*, and *Fibrobacteres*. In accordance with other studies on L-AD digesters (Li et al., 2015a) and SS-AD digesters (Li et al., 2013; Li et al., 2015b; Yue et al., 2013) fed cellulosic feedstock and operated at mesophilic temperature, *Firmicutes* and *Bacteroidetes* were the two most predominant phyla in all the digesters at all times. However, the phylum-level distribution differs from that reported for the global diversity of L-AD digesters (Nelson et al., 2011), where the largest phylum was *Chloroflexi*, followed by *Proteobacteria*, *Firmicutes*, and *Bacteroides*. Considering that most of the sequences in the previous study (Nelson et al., 2011) were obtained from digesters fed municipal sludge, the differences in feedstock chemical composition (corn stover rich in cellulose vs. municipal sludge rich in microbial cells) may be a major factor contributing to the above discrepancies in phylum distribution. *Firmicutes* accounted for 56-65% of the total sequences in each sample and its proportion only fluctuated slightly. However, the second most predominant phylum, *Bacteroidetes*, increased its relative abundance from 7-8% at day 0 to 21-22% when the daily biogas production peaked (t₂) and gradually reached 25% relative abundance at the end of the operation irrespective of F/Et ratio. The opposite held true for the relative abundance of the third most predominant phylum *Proteobacteria*, decreasing from about 15% (the healthy digesters) and 17% (the sour digesters) at day 0 to about 2% (the healthy digesters) and 4% (the sour digesters) by day 38. Other smaller phyla including *Synergistetes*, *Chloroflexi*, *Actinobacteria*, and OP9 decreased their relative abundance, from about 2-5% each at day 0 to about 0.2-3.5% each by day 38.

The phyla *Spirochaetes*, *Verrucomicrobia*, and *Fibrobacteres* showed difference in population shifts between the two F/Et ratios (Fig. 3). *Spirochaetes* was barely detected at day 0 but it expanded its population from day 8 (t2) in the healthy digesters, while in the sour digesters it only reached a sizeable relative abundance at the end of the incubation (day 38). *Spirochaetes* was detected as a ubiquitous group of bacteria in anaerobic digesters (Lee et al., 2013). The increased proportion of this phylum in the healthy digesters (except at day 0) and in the sour digesters only at day 38 corroborates its suggested ability to ferment glucose in digesters (Delbès et al., 2000). The results suggest that this phylum may contribute to acidogenesis by fermenting the sugars released from corn stover hydrolysis in the healthy digesters. *Verrucomicrobia* was mostly observed when the daily biogas production peaked and persisted in the healthy digesters, but its proportion was probably too small to make an impact to the SS-AD process. Most of the *Fibrobacteres* sequences obtained in this study were from the samples collected at day 38 (t4) from the sour digesters, where they accounted for 2% of total the sequences. Although members of this phylum can be highly cellulolytic, this phylum probably contributes little to the digestion corn stover in the SS-AD digesters.

3.4.2 Predominant bacterial OTUs

Collectively, 1,026 species-level OTUs (clustered at 97% sequence similarity) were obtained, of which 131 predominant OTUs each was represented by $\geq 0.1\%$ of total sequences. These major OTUs, together accounting for 81.4% of the total sequences, were further analyzed and the results were presented as generalized association plots (Fig. 4). Consistent with the PCoA analysis, the clustering of the microbiome based on Pearson's correlation

coefficients showed that the digester microbiome underwent temporal successions, and the successions were affected by F/Et ratios. Based on their distribution pattern and relative abundance in the samples, the major OTUs were clustered into three groups (Fig. 4c and 4d). Group I contained 48 OTUs that were most predominant in the t1 samples (day 0) from both the healthy and the sour digesters. Apparently, the Group I OTUs were from the active L-AD effluent and they did not survive well in any of the SS-AD digesters. About 50% of the Group I OTUs were classified to the class *Clostridia*, especially to the family *Ruminococcaceae*, genus *Syntrophomonas*, the candidate family ML1228J-1, and candidate order SHA-98. The OTUs classified to *Ruminococcaceae*, a family containing known bacteria capable of hydrolyzing and fermenting carbohydrates, might have played an important role in the digestion of corn stover. The genus *Syntrophomonas* contains syntrophic acetogens, and the *Syntrophomonas* OTUs could be important in the syntrophic acetogenesis from other fatty acid, such as butyrate. The two candidate taxa were both previously reported in SS-AD digesters also fed corn stover, and it appeared to be positively correlated to VFA concentration (Li et al., 2015b). Although candidate family ML1228J-1 and candidate order SHA-98 are classified within *Clostridia* that contain many known fermenting bacteria, their actual metabolic functions remain to be determined. OTUs belonging to *Rhodobacteraceae* and *Pseudomonadaceae* of the class *Gammaproteobacteria* were also found in Group I. One OTU was classified to the genus *Anaerobaculum* (in the phylum *Synergistetes*), which contains known species capable of utilizing peptides and amino acids in addition to sugars (Maune & Tanner, 2012).

The Group II OTUs, 62 in total, were further divided into three subgroups. Subgroup IIa contained 12 OTUs that were overall found more predominant in the healthy than in the sour digesters. This subgroup OTUs included OTUs classified as *Syntrophomonas*, Order *Bacteroidales* (two out of three OTUs further assigned as *Porphyromonadaceae*), and *Dethiosulfovibrionaceae* in the phylum *Synergistetes*. These OTUs may participate in the process of biogas production from corn stover in SS-AD digesters at normal organic loading. The 19 OTUs in subgroup IIb were found most predominant in the t2, t3, and t4 samples of the healthy digesters and the t4 sample of the sour digesters (the cluster in quadrant I of Fig. 2). Such distribution patterns suggest that this subgroup of OTUs were favored by the SS-AD conditions in the healthy digesters but not in the sour digester. However, they might have become adapted to the conditions in the sour digesters. The OTUs in this subgroup were mainly classified to the class *Clostridia* with further taxonomic assignment to the family *Clostridiaceae* and the candidate order MBA08, and to the order *Bacteroidales* with further assignment to the families *Marinilabiaceae* and *Porphyromonadaceae*. Two OTUs assigned to the genus *Treponema*, which contains known homoacetogens (Graber & Breznak, 2004), were also observed in this subgroup. The dearth of this subgroup in the t1 samples of both digesters and the increased relative abundance in all the later samples of the healthy digesters and the t4 sample of the sour digesters suggests their involvement in the AD process. Subgroup IIc consisted of 31 OTUs with their relative abundance found the highest in the sample t2 and t3 of the healthy digester, but they were also observed in the t2 and t3 samples of the sour digester. This subgroup of OTUs thrived when the highest daily biogas production

occurred in both digesters, suggesting that they were important and active in the AD process under both healthy and sour conditions. The most predominant OTUs in this subgroup were all classified to the phylum *Bacteroidetes*, with most of them being further classified to *Marinilabiaceae* and *Porphyromonadaceae*, while others were mostly classified to *Clostridiaceae* and *Lachnospiraceae* in the phylum *Firmicutes*. One OTU each in subgroup IIc was classified to *Erysipelothrix*, which contains known homoacetogens, and to *Anaerostipes*, which contains known butyrate-producing bacteria. These two genera were also found in a previous study where corn stover was digested in mesophilic SS-AD digesters (Li et al., 2015b).

In contrast to Group II OTUs, Group III contained 22 OTUs that were mostly found in the sour digesters except the t1 sample thereof. Most of Group III OTUs were classified to the order *Clostridiales*. These OTUs may be more adapted to the low pH in the sour digesters than the other OTUs. Three of the OTUs were classified to *Porphyromonadaceae*, which contains strains producing lactate, acetate, and butyrate from glucose fermentation (Jabari et al., 2012). The OTUs that were classified to the genus *Ethanoligenens* (in the family *Ruminococcaceae*) and the genus *Ruminococcus* (OUT ID #114575) were only found in the t3 sample from the sour digesters. The only species in the genus *Ethanoligenens* is *Ethanoligenens harbinense*, which is a sublineage within the *Clostridium cellulosi* rRNA cluster. *E. harbinense* was isolated from a continuous stirred-tank reactor fed molasses wastewater, and it was found to ferment glucose to ethanol and acetate (Xing et al., 2006). It is not certain if these *Ethanoligenens* OTUs play a similar role in the sour digesters. Group III contained all the sequences classified to the phylum

Fibrobacteres, and they were further classified to the candidate family TSCOR003-O20.

Interestingly, they were detected only in the t4 sample of the sour digesters. Sequences that were assigned to TSCOR003-O20 were initially obtained from rice paddy soil (Ishii et al., 2009). However, no cultured representative is available for this candidate family, and the characteristics or the functions of this bacterial group remain unknown.

3.5 Archaeal community

3.5.1 Archaeal community composition and structure

In total, 3,395 quality-checked archaeal sequences were obtained, and these sequences were clustered into 13 species-level OTUs (at 97% sequence similarity) (Table 2). Eight major OTUs accounted for 81% of the total archaeal sequences, and all these OTUs were classified to known genera, while the remaining five OTUs were classified to the family *Methanospirillaceae* (2 OTUs) and the genus *Methanomassiliicoccus* within the class *Thermoplasmata* (3 OTUs). The genus *Methanosarcina* was represented by the most predominant OTUs (representing 25% of the total archaeal sequences), followed by *Methanoculleus* (14%), *Methanosaeta* (12%), *Methanosphaera* (10%), and the family *Methanospirillaceae* (9%). In both the healthy and the sour SS-AD digesters, OTUs assigned to *Methanosphaera*, *Methanospirillaceae*, *Methanosaeta*, and *Methanomethylovorans* were found most predominant in the t1 samples at day 0, and they decreased gradually over the SS-AD process. On the contrary, the *Methanomassiliicoccus* OTUs, which were barely observed at day 0, populated during the SS-AD process in both the healthy and the sour digesters and became a predominant group by day 38, especially in the healthy digesters (representing up to 21% of the total sequences). Similarly,

Methanoculleus grew more predominant in both the healthy (29%) and the sour (14%) digesters by day 38. *Methanosarcina* was predominant in both digesters at day 0 (20% and 24% in the healthy and the sour digesters, respectively); however, its predominance varied over the SS-AD process, and by day 38, it decreased in the healthy digesters while it became the most predominant OTU (accounting for 46% of the archaeal sequences) in the sour digesters. The archaeal community in the sour digesters became less even over time, with *Methanosarcina*, *Methanoculleus*, and two OTUs classified as *Methanobacterium* together representing nearly 90% of the archaeal sequences. Such reduced community evenness probably reflects the selective pressure from the high VFA concentration and the low pH in the sour digesters.

3.5.2 Abundance of methanogen genera

The archaeal sequences accounted for only a small portion (0.4%) of total sequences obtained from the Illumina sequencing. To better understand their population dynamics during the SS-AD processes in the healthy and the sour digesters, four of the seven methanogen genera found in the SS-AD digesters, including *Methanobacterium*, *Methanoculleus*, *Methanosaeta*, and *Methanosarcina*, which are generally considered important to biogas production, were quantified using qPCR (Fig. 5). Overall, the abundance determined by qPCR was in general agreement with the predominance represented by the Illumina sequencing data for all the genera quantified except *Methanosaeta*. *Methanosarcina* was the most abundant genus in all the samples, ranging from $1.2 - 6.1 \times 10^6$ copies of 16S rRNA gene per μg of metagenomic DNA except a lower abundance (7.4×10^5 copies) in sample t4 of the healthy digesters. Moreover, consistent

with the sequencing data, *Methanosarcina* was more abundant in the sour than in the healthy digesters. Because little biogas was produced in the sour digester, the role and importance of this genus in the sour SS-AD digesters remains to be determined. *Methanoculleus* was the second most abundant genus, which appeared at lower abundance in the early than in the later SS-AD process. This genus grew in abundance towards the end of the SS-AD process, especially in the healthy digesters where it reached an abundance of 1.8×10^6 copies per μg of metagenomic DNA by day 38. *Methanobacterium* was the third most abundant genus with a gradually increased population during the SS-AD process in both digesters. Its abundance increased from 2.4×10^5 to 8.5×10^5 in the healthy digesters but decreased from 5.4×10^6 to 1.3×10^6 in the sour digesters. *Methanosaeta* was not detected at day 0 of the healthy digester or at day 38 of both digesters, but it was detected in other samples though at much lower abundance (1-3 logs) than other major methanogenic genera. Being a major genus of L-AD, the significance of this genus in SS-AD, at least SS-AD of corn stover, remains to be determined.

3.6 Correlation between environmental factors and microbial populations

To better understand the roles and importance of individual groups of microbes in the SS-AD process, plausible relationship between microbial populations and the environmental and performance measurements, including pH, daily biogas production, and VFA concentrations, was evaluated using CCA. Based on the assumption that AD process is most likely driven by predominant bacteria, we performed CCA analysis using the 131 major OTUs that were each represented by at least 0.1% of the total sequences and using 13 major archaeal OTUs (Fig. 6). CCA1 and CCA2 together explained 82 % of total variance,

which indicate that these two coordinates could represent the CCA results. Two groups of
426 OTUs showed positive correlation to the factors included in the CCA analysis. One group
was located along the direction of the biogas vector and was positively correlated with
428 biogas production. This group included the OTUs classified to the genus *Sporanaerobacter*,
which contains species capable of utilizing peptides and amino acids in addition to
430 sugars (Hernandez-Eugenio et al., 2002), and the genus *Oligosphaera* in the phylum
Lentisphaerae. The other group of OTUs showed strong positive correlation with VFA
432 concentration. This small group included OTUs assigned to the genera *Mycobacterium* (in
the phylum of *Actinobacteria*), *Rhodobacter* (in the phylum of *Proteobacteria*), and
434 *Erysipelothrix* (in the phylum *Firmicutes*), the order *Clostridia*, and the
families *Ruminococcaceae* and *Lachnospiraceae*. This group of OTUs might be involved in
436 either production or consumption of VFA, or they can adapt to high VFA concentration
and low pH. For the archaeal OTUs, four OTUs that were classified to the new genus
438 *Methanomassiliicoccus* showed positive correlation to the daily biogas production. The
remaining nine major archaeal OTUs showed no strong correlation to either the
440 environmental factors or the performance measurements. *Methanomassiliicoccus* sequences
were found in mesophilic SS-AD digesters fed corn stover (previously designated as
442 candidate family WCHD3-02, (Li et al., 2015b)). However, only two characterized species
within this genus were recently reported, and both (*M. intestinalis* and *M. luminyensis*)
444 were from human feces. Its repeated detection in SS-AD digesters, at least in SS-AD
digesters fed corn stover, warrants further investigation.

Extra caution is needed when it comes to interpretation of the mathematical correlation between microbial composition and environmental/performance measurements because correlation does not necessarily mean causality. A potential cause of this scenario is that 16S rRNA amplicons that are sequenced can be amplified from both live and dead or inactive bacteria and archaea and that only limited functionality information can be inferred from 16S rRNA gene sequences. Future research using functional metagenomics, metatranscriptomics and metaproteomics can help understand the role and importance of individual bacteria and archaea in SS-AD process. Nevertheless, this exploratory research of identifying microbial composition is surely needed to serve as a foundation for future studies of SS-ADs system.

4. Conclusion

Organic loading (expressed as feedstock/total effluent, F/Et, ratio) affected SS-AD of corn stover, with a F/Et ratio of 2.2 leading to stable operation, while a 4.4 F/Et ratio resulted in organic overloading. The microbiome underwent considerable temporal successions during SS-AD, and organic loading dramatically affected such successions. The alkalinity of L-AD effluent may be another important factor that can affect the stability of SS-AD. Some bacterial OTUs were positively correlated to biogas production, while another group was positively correlated to VFA concentration. Interestingly, four OTUs in the new genus *Methanomassiliicoccus* were positively correlated to daily biogas production.

Acknowledgements

This study was partially supported by a Department of Energy (DOE) award (DE-FG36-05GO85010) and a United States Department of Agriculture/National Institute of Food and Agriculture (USDA/NIFA) award (2012-10008-20302).

References

1. Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods*, **10**(1), 57-9.
2. Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*, **7**(5), 335-6.
3. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *Isme Journal*, **6**(8), 1621-1624.
4. Chen, C.H. 2002. Generalized association plots: Information visualization via iteratively generated correlation matrices. *Statistica Sinica*, **12**(1), 7-29.
5. Delbès, C., Moletta, R., Godon, J.J. 2000. Monitoring of activity dynamics of an anaerobic digester bacterial community using 16S rRNA polymerase chain reaction--single-strand conformation polymorphism analysis. *Environmental Microbiology*, **2**(5), 506-15.
6. DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*, **72**(7), 5069-72.

7. Franke-Whittle, I.H., Goberna, M., Insam, H. 2009. Design and testing of real-time PCR primers for the quantification of *Methanoculleus*, *Methanosarcina*, *Methanothermobacter*, and a group of uncultured methanogens. *Can J Microbiol*, **55**(5), 611-6.
8. Gerardi, M.H. 2003. *The Microbiology of Anaerobic Digesters*. John Wiley & Sons, Inc.
9. Graber, J.R., Breznak, J.A. 2004. Physiology and nutrition of *Treponema primitia*, an H₂/CO₂-acetogenic spirochete from termite hindguts. *Appl Environ Microbiol*, **70**(3), 1307-14.
10. Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S.K., Sodergren, E., Methe, B., DeSantis, T.Z., Human Microbiome, C., Petrosino, J.F., Knight, R., Birren, B.W. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res*, **21**(3), 494-504.
11. Hernandez-Eugenio, G., Fardeau, M.L., Cayol, J.L., Patel, B.K., Thomas, P., Macarie, H., Garcia, J.L., Ollivier, B. 2002. *Sporanaerobacter acetigenes* gen. nov., sp. nov., a novel acetogenic, facultatively sulfur-reducing bacterium. *Int J Syst Evol Microbiol*, **52**(Pt 4), 1217-23.
12. Ishii, S., Yamamoto, M., Kikuchi, M., Oshima, K., Hattori, M., Otsuka, S., Senoo, K. 2009. Microbial Populations Responsive to Denitrification-Inducing Conditions in Rice Paddy Soil, as Revealed by Comparative 16S rRNA Gene Analysis. *Applied and Environmental Microbiology*, **75**(22), 7070-7078.
13. Jabari, L., Gannoun, H., Cayol, J.L., Hedi, A., Sakamoto, M., Falsen, E., Ohkuma, M., Hamdi, M., Fauque, G., Ollivier, B., Fardeau, M.L. 2012. *Macellibacteroides fermentans* gen. nov., sp. nov., a member of the family Porphyromonadaceae isolated from an upflow anaerobic filter treating abattoir wastewaters. *Int J Syst Evol Microbiol*, **62**(Pt 10), 2522-7.
14. Kothari, R., Pandey, A.K., Kumar, S., Tyagi, V.V., Tyagi, S.K. 2014. Different aspects of dry anaerobic digestion for bio-energy: An overview. *Renewable & Sustainable Energy Reviews*, **39**, 174-195.
15. Kusch, S., Oechsner, H., Jungbluth, T. 2012. Effect of various leachate recirculation strategies on batch anaerobic digestion of solid substrates. *Int. J. of Environment and Waste Management*, **9**, 69-88.
16. Lee, S.H., Park, J.H., Kang, H.J., Lee, Y.H., Lee, T.J., Park, H.D. 2013. Distribution and abundance of Spirochaetes in full-scale anaerobic digesters. *Bioresour Technol*, **145**, 25-32.
17. Li, A., Chu, Y., Wang, X., Ren, L., Yu, J., Liu, X., Yan, J., Zhang, L., Wu, S., Li, S. 2013. A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. *Biotechnol Biofuels*, **6**(1), 3.
18. Li, Y.B., Park, S.Y., Zhu, J.Y. 2011. Solid-state anaerobic digestion for methane production from organic waste. *Renewable & Sustainable Energy Reviews*, **15**(1), 821-826.
19. Li, Y.F., Abraham, C., Nelson, M.C., Chen, P.H., Graf, J., Yu, Z. 2015a. Effect of organic loading on the microbiota in a temperature-phased anaerobic digestion

- (TPAD) system co-digesting dairy manure and waste whey. *Appl Microbiol Biotechnol*.
20. Li, Y.F., Nelson, M.C., Chen, P.H., Graf, J., Li, Y., Yu, Z.T. 2015b. Comparison of the microbial communities in solid-state anaerobic digestion (SS-AD) reactors operated at mesophilic and thermophilic temperatures. *Applied Microbiology and Biotechnology*, **99**(2), 969-980.
 21. Lozupone, C., Knight, R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol*, **71**(12), 8228-35.
 22. Maune, M.W., Tanner, R.S. 2012. Description of *Anaerobaculum hydrogeniformans* sp. nov., an anaerobe that produces hydrogen from glucose, and emended description of the genus *Anaerobaculum*. *Int J Syst Evol Microbiol*, **62**(Pt 4), 832-8.
 23. Nelson, M.C. 2011. An integrated investigation of the microbial communities underpinning biogas production in anaerobic digestion systems, Vol. PhD, The Ohio State University.
 24. Nelson, M.C., Morrison, H.G., Benjamino, J., Grim, S.L., Graf, J. 2014. Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. *PLoS One*, **9**(4), e94249.
 25. Nelson, M.C., Morrison, M., Yu, Z. 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. *Bioresour Technol*, **102**(4), 3730-9.
 26. Raposo, F., Borja, R., Martin, M.A., Martin, A., de la Rubia, M.A., Rincon, B. 2009. Influence of inoculum-substrate ratio on the anaerobic digestion of sunflower oil cake in batch mode: Process stability and kinetic evaluation. *Chemical Engineering Journal*, **149**(1-3), 70-77.
 27. Salipante, S.J., Kawashima, T., Rosenthal, C., Hoogestraat, D.R., Cummings, L.A., Sengupta, D.J., Harkins, T.T., Cookson, B.T., Hoffman, N.G. 2014. Performance comparison of Illumina and ion torrent next-generation sequencing platforms for 16S rRNA-based bacterial community profiling. *Appl Environ Microbiol*, **80**(24), 7583-91.
 28. Shi, J., Xu, F., Wang, Z., Stiverson, J.A., Yu, Z., Li, Y. 2014. Effects of microbial and non-microbial factors of liquid anaerobic digestion effluent as inoculum on solid-state anaerobic digestion of corn stover. *Bioresource Technology*, **157**, 188-96.
 29. Shigematsu, T., Tang, Y., Kawaguchi, H., Ninomiya, K., Kijima, J., Kobayashi, T., Morimura, S., Kida, K. 2003. Effect of dilution rate on structure of a mesophilic acetate-degrading methanogenic community during continuous cultivation. *J Biosci Bioeng*, **96**(6), 547-58.
 30. Wu, H.M., Tien, Y.J., Chen, C.H. 2010. GAP: A graphical environment for matrix visualization and cluster analysis. *Computational Statistics & Data Analysis*, **54**(3), 767-778.
 31. Xing, D., Ren, N., Li, Q., Lin, M., Wang, A., Zhao, L. 2006. *Ethanoligenens harbinense* gen. nov., sp. nov., isolated from molasses wastewater. *Int J Syst Evol Microbiol*, **56**(Pt 4), 755-60.
 32. Xu, F., Li, Y. 2012. Solid-state co-digestion of expired dog food and corn stover for methane production. *Bioresour Technol*, **118**, 219-26.

33. Yu, Z., Michel, F.C., Jr., Hansen, G., Wittum, T., Morrison, M. 2005. Development and application of real-time PCR assays for quantification of genes encoding tetracycline resistance. *Appl Environ Microbiol*, **71**(11), 6926-33.
34. Yu, Z., Morrison, M. 2004. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol*, **70**(8), 4800-6.
35. Yue, Z., Chen, R., Yang, F., MacLellan, J., Marsh, T., Liu, Y., Liao, W. 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. *Bioresour Technol*, **128**, 65-71.

Table and Figure Legends

Table 1 Alpha diversity indices of the SS-AD samples.

Table 2 Archaeal relative abundance (percentage of total sequence in each sample) in the SS-ADs samples.

Fig. 1. Total VFA concentration (A), pH (B), and daily biogas production (C) in the two sets of SS-AD reactors with different F/Et ratios. Open squares, the healthy (or LFE) digesters; filled triangles, the sour (or HFE) digesters. The time points analyzed in this study are indicated by *.

Fig. 2. Major bacterial phyla (each represented by $\geq 1\%$ of total sequences in at least one sample) found in the SS-AD samples.

Fig. 3. Principal coordinates analysis (PCoA) of the microbiome of the SS-AD digesters fed two different F/Et ratios. The PCoA plot was based on weighted UniFrac analysis. Open squares, healthy digesters (F/Et = 2.2); filled triangles, sour digesters (F/Et = 4.4). Respectively, t1 and t4 represent day 0 and 38 for both the healthy and the sour

digesters; t2 represents day 4 (for sour digesters) or day 8 (for healthy digesters), and t3
represent day 10 (for sour digesters) or day 12 (for healthy digesters).

Fig. 4. Generalized association plots for the major OTUs (each represented $> 0.1\%$ of the
total sequences). (a) Heatmap of relative abundance of the OTUs, (b) the sample-
sample correlation map among the 8 samples, (c) the OTU-OTU correlation map
among the OTUs, and (d) the hierarchical clustering tree for sorting the correlation map
in (c). In (a) and (b) and in (c), samples and OTUs were sorted based on corresponding
sample-sample correlation and OTU-OTU correlation, respectively. The OTUs included
in this figure are listed in Table S2.

Fig. 5. Abundance of four genera of methanogens detected in the SS-AD digesters.

Fig. 6. Canonical correspondence analysis of the major OTUs of bacteria and archaea. Dots
in the figure represent individual OTUs.

Fig 1. Total VFA concentration (A), pH (B), and daily biogas production (C) in the two sets of SS-AD reactors with different F/Et ratios. Open squares, the healthy (or LFE) digesters; filled triangles, the sour (or HFE) digesters. The time points analyzed in this study are indicated by *.

Fig. 2. Major bacterial phyla (each represented by $\geq 1\%$ of total sequences in at least one sample) found in the SS-AD samples.

Fig. 3. Principal coordinates analysis (PCoA) of the microbiome of the SS-AD digesters fed two different F/Et ratios. The PCoA plot was based on weighted UniFrac analysis. Open squares, healthy digesters (F/Et = 2.2); filled triangles, sour digesters (F/Et = 4.4). Respectively, t1 and t4 represent day 0 and 38 for both the healthy and the sour digesters. t2 represents day 4 (for sour digesters) or day 8 (for healthy digesters), and t3 represent day 10 (for sour digesters) or day 12 (for healthy digesters).

(b) Sample-sample correlation matrix

(a) Relative abundance of OTUs (log)

(c) OTU-OTU correlation matrix

Group

(d) Hierarchical clustering tree of OTUs

Fig. 4 Generalized association plots for the major OTUs (each represented > 0.1% of the total sequences). (a) Heatmap of relative abundance of the OTUs, (b) the sample-sample correlation map among the 8 samples, (c) the OTU-OTU correlation map among the OTUs, and (d) the hierarchical clustering tree for sorting the correlation map in (c). In (a) and (b) and in (c), samples and OTUs were sorted based on corresponding sample-sample correlation and OTU-OTU correlation, respectively. The OTUs included in this figure are listed in Table S2.

Fig. 5. Abundance of four genera of methanogens detected in the SS-AD digesters.

Fig. 6. Canonical correspondence analysis of the major OTUs of bacteria and archaea. Dots in the figure represent individual OTUs.

Table 1. Alpha diversity indices of the SS-AD samples

Digester	Sampling time point	Observed OTUs	Simpson	Shannon	Phylogenetic distance
LFE	t1	725	0.93	6.91	352.63
	t2	876	0.96	7.01	252.10
	t3	882	0.96	7.64	364.42
	t4	841	0.96	7.20	324.79
HFE	t1	730	0.94	7.31	415.28
	t2	876	0.98	7.72	261.60
	t3	917	0.97	7.65	351.49
	t4	829	0.97	7.21	254.44

Table 2. Archaeal relative abundance in the SS-ADs samples^a

OTU ID	Taxonomic Lineage	LFE				HFE			
		t1	t2	t3	t4	t1	t2	t3	t4
101553	<i>Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobacterium</i>	2	2	1	1	2	1	-	3
N38466	<i>Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobacterium</i>	3	3	2	7	2	10	5	10
551498	<i>Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanobacterium</i>	1	6	13	20	2	19	6	20
589886	<i>Methanobacteria; Methanobacteriales; Methanobacteriaceae; Methanosphaera</i>	10	7	5	2	21	19	5	1
840393	<i>Methanomicrobia; Methanomicrobiales; Methanomicrobiaceae; Methanoculleus</i>	16	18	24	29	2	4	26	14
N55432	<i>Methanomicrobia; Methanomicrobiales; Methanospirillaceae</i>	3	-	1	-	2	1	-	-
263121	<i>Methanomicrobia; Methanomicrobiales; Methanospirillaceae</i>	15	8	4	1	12	10	7	1
584426	<i>Methanomicrobia; Methanosarcinales; Methanosaetaceae; Methanosaeta</i>	21	8	2	3	20	18	2	1
62890	<i>Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanomethylovorans</i>	6	1	-	-	13	2	-	-
1129087	<i>Methanomicrobia; Methanosarcinales; Methanosarcinaceae; Methanosarcina</i>	20	19	29	9	24	16	36	46
136107	<i>Thermoplasmata; Methanomassiliicoccales; Methanomassiliicoccaceae; Methanomassiliicoccus</i>	2	6	3	2	-	-	1	-
282634	<i>Thermoplasmata; Methanomassiliicoccales; Methanomassiliicoccaceae; Methanomassiliicoccus</i>	1	6	4	5	-	-	6	4
N52289	<i>Thermoplasmata; Methanomassiliicoccales; Methanomassiliicoccaceae; Methanomassiliicoccus</i>	-	18	12	21	-	-	6	1

^a “-” representing a relative abundance less than 0.5%.