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BIOENERGY AND BIOFUELS

Straw- and slurry-associated prokaryotic communities differ during co-fermentation of straw and swine manure

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Abstract Anaerobic co-fermentation of straw and manure is widely used for waste treatment and biogas production. However, the differences between the straw- and slurry-associated prokaryotic communities, their dynamic changes throughout the co-fermentation process, and their correlations with bioreactor performance are not fully understood. To address these questions, we investigated the prokaryotic community compositions and the dynamics of prokaryotes attached to the straw and in the slurry during co-fermentation of wheat straw and swine manure using pyrosequencing technique. The results showed that straw- and slurry-associated prokaryotes were different in their structure and function. Straw-associated prokaryotic communities were overrepresented by the phyla *Spirochaetes* and *Fibrobacteres*, while *Synergistetes* and *Euryarchaeota* were more abundant in the slurry. The straw-associated candidate class TG3, genera *Fibrobacter*, *Bacteroides*, *Acetivibrio*, *Clostridium* III, *Papillibacter*, *Treponema*, *Sedimentibacter*, and *Lutispora* may specialize in substrate hydrolysis. Propionate was the most abundant volatile fatty acid in the slurry, and it was probably degraded through syntrophic oxidation by the genera *Pelotomaculum*, *Methanoculleus*, and *Methanosaeta*. The protein-fermenting bacteria *Aminobacterium* and *Cloacibacillus* were much abundant in the slurry, indicating that proteins are important substrates in the co-fermentation. This study provided a better understanding of the anaerobic co-fermentation process that is driven by spatially differentiated microbiota.

Keywords Co-fermentation · Straw · Swine manure · Prokaryotic community · 16S rRNA gene pyrosequencing

Jiabao Li and Junpeng Rui contributed equally to this study.

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Introduction

The anaerobic fermentation represents a prominent technology for waste treatment and bioenergy harvest (Werner et al. 2011). The process enriches microbial consortia that are mainly composed of prokaryotic communities to convert mixtures of organic matter including agricultural, animal, or food waste into biogas with methane content of 50-70 % (Luo and Angelidaki 2013; Town et al. 2014). Owing to the improvement of nutrient balance and buffer capacity, a variety of studies have demonstrated the enhancement effect of co-fermentation on the process performance (Ye et al. 2013; Ferrer et al. 2014). However, a lack of detailed understanding of the involved microbial communities has hindered the progress of current technique (Town et al. 2014). A full scenario of microbiota that specialize in each fermentation stage can be useful information to improve the process performance as well as troubleshooting issues with regard to reactor performance (Hanreich et al. 2013).

Microbiota are vital players for inorganic and organic matter transformation in the anaerobic fermentation system (Nelson et al. 2011; Werner et al. 2011). The key steps include substrate hydrolysis, fermentation to produce volatile fatty acids (VFAs), and methanogenesis. Most previous studies on microbial communities in this system primarily focus on the slurry-associated microbiota, which showed that bacterial phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Chloroflexi,* and methanogens such as *Methanomicrobiales* and *Methanosarcinales* were the most representative members (Nelson et al. 2012; Hanreich et al. 2013).

The hydrolysis is regarded as a rate-limiting step. The attachment of microbes to substrates is favorable for substrates hydrolysis. However, few studies have uncovered the tightly substrate-attached microbiota and their correlations with fermentation performance. The dynamics of substrate-attached *Clostridium* observed by fluorescence in situ hybridization indicated its dominant role in substrate solubilization in anaerobic digesters (O'Sullivan et al. 2005; Syutsubo et al. 2005). In the co-fermentation system, spatial distribution of microbiota with different function may aid microbial communities for substrate competition and responses to the changes in physicochemical conditions during fermentation process.

During anaerobic fermentation, slurry acidification caused by VFAs is detrimental for fermentation. To alleviate this effect, transformation of VFAs through syntrophic metabolism is essential. Syntrophy, an indispensable process linking fermentation and methanogenesis, can overcome unfavorable thermodynamics for the degradation of organic matter under methanogenic condition (McInerney et al. 2009). Most syntrophs are affiliated with the phyla *Firmicutes*, *Proteobacteria*, and *Synergistetes*, e.g., the genera *Pelotomaculum*, *Syntrophobacter*, *Syntrophus*, *Syntrophomonas*, *Desulfovibrio*, *Geobacter*, *Aminobacterium*, and *Aminomonas* (Sieber et al. 2012). In the co-fermentation system, niches responsible for syntrophic metabolism of specific VFA remain to be fully elucidated.

In this study, we investigated prokaryotic community compositions and structures associated to straw and slurry, and their dynamic changes in the co-fermentation process using pyrosequencing technique. The aims were to (i) examine the straw- and slurry-associated prokaryotic communities, (ii) determine the dynamic changes of prokaryotic communities during the co-fermentation process, and (iii) reveal the correlations among prokaryotic communities, fermentation performance, and environmental variables in the system.

Materials and methods

Setup of co-fermentation system and sampling

The straw of wheat (*Triticum aestivum*) was collected from the agricultural field after harvest and air dried. It was cut into fragments less than 1 cm in length. The co-fermentation batch experiments were performed in 500 ml anaerobic flasks containing 400 ml fermentation slurry with final total solid (TS) content of 5 %. Fermentation slurry was a mixture of 10 g dry straw, 25 g fresh swine manure equivalent to 5 g dry manure, 100 ml inoculum equivalent to 5 g dry manure, and 265 ml tap water. The inoculum was obtained from an anaerobic fermenter actively producing biogas (more than 60 % CH_4) by digesting swine manure. A control using swine manure as single substrate was set up. All the experiments were conducted in triplicate.

The fermentation experiments were operated for 22 days under 35±2 °C. The fermentation content in a flask was mixed thoroughly twice a day by gyrating the flask with hand. The straw and slurry were sampled at day 0 (2 h after inoculation, representing the initial stage), day 3 (72 h after inoculation, peak I), and day 21 (peak II) based on the daily biogas production rate. Straw samples were washed three times in sterile sodium phosphate buffer (100 mM PBS buffer, pH 7.0). The slurry was carefully pipetted up and pelleted by centrifugation at 13,000×*g* for 5 min. All the processed samples were stored at -80 °C for DNA extraction.

Analytic methods

The biogas production was measured using a water replacement method. CH_4 and CO_2 in the biogas were measured by gas chromatography (GC, Agilent 7890A) equipped with a thermal conductivity detector (TCD). For the slurry NH_4^+-N and volatile fatty acids (VFAs) measurements, the slurry was centrifuged at 13,000×*g* and the supernatant was filtrated through a 0.22 μm filter (catalog no. SLGP033RS, Millipore, MA, USA). NH_4^+-N in the supernatant was quantified using indophenol blue method as previously described (Tzollas et al. 2010). VFAs were detected by a GC equipped with a polar capillary column (DB-FFAP, 30 m×0.25 mm×0.25 μm) and a flame ionization detector (FID) as previously described (Ishii et al. 2005). The chemical compositions of the straw at initiation stage and peak II were analyzed as described previously (Hess et al. 2011). The pH value of the slurry was measured with a pH meter.

DNA extraction, 16S rRNA gene amplification, and pyrosequencing

Total DNA was extracted according to the method described previously (Rademacher et al. 2012). For pyrosequencing, the 16S rRNA genes were amplified with primers 515F (5′-GTGYCAGCMGCCGCGGTA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) (targeting bacterial and archaeal V4–V5 region in 16S rRNA gene) with a 10-mer barcode at the 5′ end of primer 515F (Tamaki et al. 2011). In order to minimize PCR bias, two PCR reactions were set up for each

sample, and the PCR products in the replicate reactions were pooled. The details in PCR and related procedures were described previously (Li et al. 2014). The amplicons from each sample were pooled with an equimolar concentration and pyrosequenced with the GS FLX+ system (454 Life Sciences, Branford, CT).

Pyrosequencing data processing

The raw sequences were sorted based on the unique sample barcodes, trimmed for sequence quality, and denoised using QIIME pipeline (Caporaso et al. 2010). Chimera sequences were removed using UCHIME algorithm (Edgar et al. 2011). As the sequence number varied among samples, we randomly resampled the sequences to 5,600 reads per sample for further analysis. Then, the sequences were clustered by the complete-linkage clustering method in the QIIME pipeline. Operational taxonomic units (OTUs) were classified using 97 % identity of 16S rRNA gene sequence as a cutoff, and the OTUs table was generated for each sample and used for statistical analysis. Shannon index and Chao1 estimator were calculated at 97 % sequence identity in the Ribosomal Database Project pipeline (RDP) (http://pyro.cme.msu.edu/). The phylogenetic affiliation of each sequence was analyzed by RDP classifier at a confidence level of 80 %. To assure the accuracy of RDP classifier results, the representative sequences of dominant bacteria and archaea were subjected to BLAST homology search against the non-environmental sequences and non-metagenomes in the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov).

Statistical analysis

The general differences of prokaryotic community structure were evaluated by principal coordinates analysis (PCoA) in Fast UniFrac (http://bmf.colorado.edu/fastunifrac/) by using the relative abundances of the whole prokaryotic communities. Significance tests were conducted by three nonparametric multivariate statistical tests (ANOSIM, Adonis and MRPP) (Deng et al. 2012). One-way analysis of variance (ANOVA) with Duncan multiple range test was used to compare the differences between prokaryotic communities at different samplings from either straw or slurry samples. Based on the relative abundances of the top 208 genera (>0.0006 %), redundancy analysis (RDA) was performed using Canoco 4.5 software (Microcomputer Power, Ithaca, NY) to discern correlations between prokaryotic community structures and fermentation parameters. The pH, VFAs, and NH_4^+-N were the environmental variables. Response ratio analysis was conducted to detect significantly changed genera at peak II against peak I, and peak I against day 0 (Deng et al. 2012). Pearson's correlation analysis was conducted with SPSS Statistics 21.0 to examine the correlations between different genera, environmental variables, and fermentation performance.

Nucleotide sequence accession numbers

The original pyrosequencing data are available at the European Nucleotide Archive by accession no. PRJEB4791 (http://www.ebi.ac.uk/ena/data/view/PRJEB4791).

Results

Fermentation performance and VFAs dynamics

During co-fermentation, daily biogas (CH_4 and CO_2) production rate showed two peaks (Fig. 1). The concentrations of CH_4 and CO_2 were approximately equal at peak I; thereafter, the concentration of CH_4 increased up to 70 % at peak II. The highest CH_4 production rate reached 485 ml l^{-1} day^{-1} at peak II. Total biogas yield in the 22 days of anaerobic fermentation was 500 ml when swine manure was the single substrate. However, the accumulative biogas production in the same period reached 4,400 ml in the co-fermentation of straw and swine manure. This indicated that most biogas possibly originated from straw degradation. Chemical analysis confirmed that the enhancement of biogas production was mostly due to the degradation of straw cellulose and hemicellulose (supplementary Table S1).

Propionate was the dominant VFA in the co-fermentation process. It reached a maximum of 50 mM at day 11 and dropped to a very low level after 19 days of fermentation (Fig. 2). Acetate decreased from the beginning until day 11, then it started to accumulate till day 19 (8.6 mM). It was likely

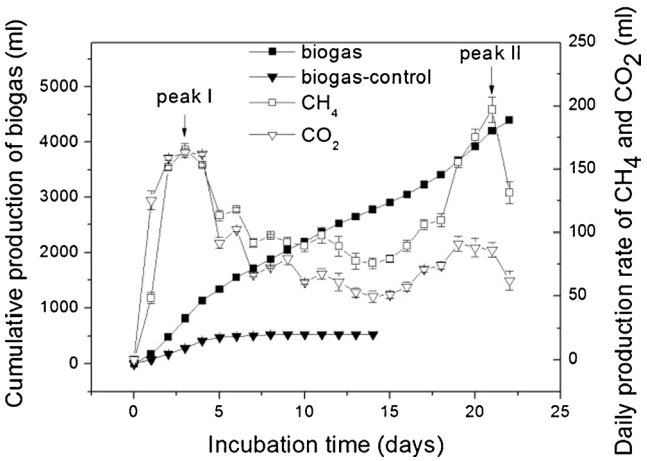

Fig. 1 The cumulative and daily production rate of biogas during 22 days of anaerobic co-fermentation under 35±2 °C. In the biogas-control, swine manure was used as single substrate for fermentation. Peaks I and II were at day 3 and 21, respectively. All the data were presented as means± standard deviations

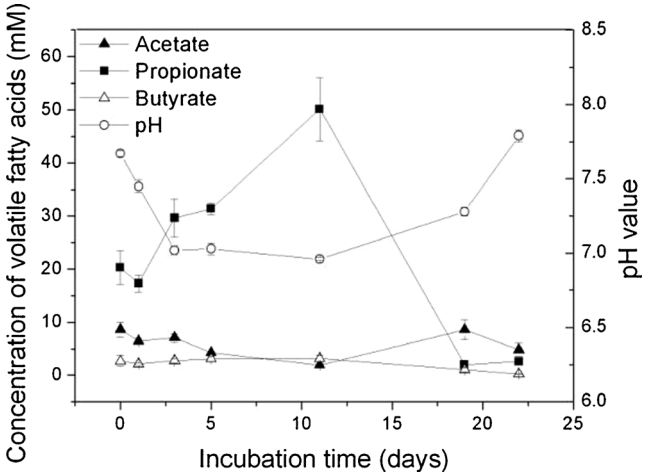

Fig. 2 The dynamic changes of VFAs concentrations (mM) and pH value during 22 days of anaerobic co-fermentation of straw and swine manure

Table 1 Prokaryotic diversity indices based on 97 % identity of 16S rRNA gene sequences and 5,600 reads per sample[a]

Samples	Chao1 estimator of richness	Observed species	Shannon's diversity index
S0	1520±24	517±10	6.190±0.053
S1	1431±53	459±5	6.156±0.072
S2	1979±298	590±40	6.222±0.067
L0	1257±715	531±177	6.639±0.193
L1	1249±577	507±100	6.543±0.182
L2	3044±435	840±38	6.754±0.111

[a] *S* straw samples, *L* slurry samples. *0*, *1*, and *2* represented the samplings at day 0, 3, and 21, respectively. All data were presented as means ± standard deviations. The differences between the straw and slurry samples, and among different samplings within either straw or slurry samples in the same column, were not significantly different at $p<0.05$ tested by ANOVA

that the accumulation of acetate during day 11–19 was mainly due to propionate transformation. Butyrate concentration was low throughout the whole process. Pearson's correlation test indicated that VFAs showed positive correlations between each other ($p<0.01$) (supplementary Table S2). CH_4 production was observed to be negatively correlated with acetate ($p<0.01$).

The pH value was 7.5 at start (Fig. 2), and it gradually decreased until the lowest value at day 11 (pH 7.0, corresponding to the highest propionate level). Then, it increased gradually until the end of co-fermentation process (pH 7.8). The concentration of NH_4^+ was relatively stable throughout the process (approximately 90 mg l^{-1}). The slurry pH was negatively correlated with propionate and butyrate, but positively correlated with NH_4^+, indicating the balance effect of VFAs and NH_4^+ on the pH value. Based on the CH_4 production rate and fermentation parameter changes, we collected straw and slurry samples at start, peak I, and peak II, respectively, for further prokaryotic community analysis.

Overall difference in prokaryotes associated with straw and slurry

Prokaryotic community compositions associated with straw and slurry samples collected at day 0, 3, and 21 were analyzed using pyrosequencing method. A total of 129,340 high-quality sequences were obtained (ranging from 5,600 to 10,446 per sample, data not shown); all sequences were aligned and clustered to calculate operational taxonomic units (OTUs) using 97 % sequence identity as a cutoff, resulting in 459 to 840 OTUs at a sequencing depth of 5,600 reads per sample (Table 1). The Shannon's diversity indices were higher in the slurry samples than that in the straw samples at all fermentation stages (Table 1).

The differences in prokaryotic communities between straw and slurry samples were also demonstrated by principal coordinates analysis (PCoA). It showed a distinct community structure between straw and slurry samples (Fig. 3), indicating significant variations (Adonis, $F=0.337$, $p=0.001$; ANOSIM, $R=0.672$, $p=0.001$; MRPP, $\delta=0.55$, $p<0.001$) between straw- and slurry-associated prokaryotic communities. Samples from peaks I and II were separated in both straw and slurry samples. The straw samples were clustered relatively closer than those from slurry samples, which indicated a more dynamic change of prokaryotic communities in the slurry than that associated to straw.

Fermentation process-related dynamics of prokaryotic community structure

Prokaryotic community structure at phylum rank was analyzed in detail for all the samples (supplementary Table S3).

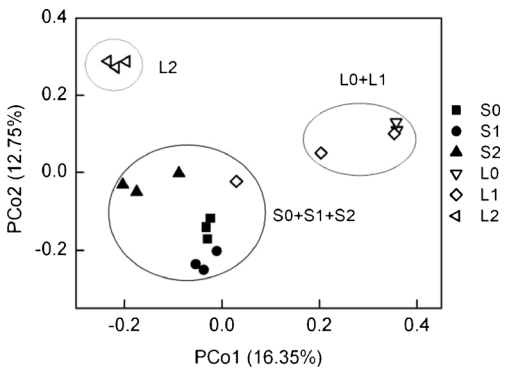

Fig. 3 The principal coordinates analysis (*PCoA*) based on the whole prokaryotic communities at genus level in the co-fermentation of straw and swine manure. *S* straw samples, *L* slurry samples. *0*, *1*, and *2* represented the samplings at day 0, 3, and 21, respectively

In general, *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, *Synergistetes*, and *Fibrobacteres* were most abundant (relative abundances >4.90 %, Table 2), while each of the other phyla was less than 1.31 %. The phyla *Spirochaetes* and *Fibrobacteres* predominated in straw samples. However, *Synergistetes* and *Euryarchaeota* were more abundant in the slurry. *Firmicutes* were evenly present in both samples.

A considerable fraction (55 %) of pyrosequencing reads were unclassified at genus rank (supplementary Table S4), indicating that a large proportion of prokaryotes and their functional traits in the co-fermentation system were still not well recorded at genus level. Nonetheless, the dominant bacterial genera *Treponema*, *Clostridium* III, *Alkaliflexus*, *Fibrobacter*, and *Sedimentibacter* overrepresented in straw samples (Table 2), while *Clostridium* XI, *Pelotomaculum*, *Syntrophomonas*, *Cloacibacillus*, *Aminobacterium*, *Methanoculleus*, and *Methanosaeta* were more abundant in the slurry samples.

Based on the relative abundances, process-related dynamics of prokaryotic communities were observed (supplementary Table S4). Straw-associated *Fibrobacter*, *Bacteroides*, *Acetivibrio*, *Sedimentibacter*, *Clostridium* III, and *Lutispora* increased significantly at peak I (95 % confidence interval) (Fig. 4a), indicating their significance in straw hydrolysis. While *Fibrobacter* decreased at peak II and was replaced by the uncultured candidate order TG3-1 (affiliated to class TG3, phylum *Fibrobacteres* in RDP database) (Table 2), the relative abundances of *Firmicutes* (*Bacillus*, *Papillibacter*, *Syntrophobotulus*, and *Syntrophomonas*), *Spirochaetes* (*Spirochaeta* and *Treponema*), and methanogens (*Methanoculleus*) increased significantly at peak II compared to those at peak I (Fig. 4b).

In the slurry samples, the relative abundances of genera *Treponema*, *Clostridium* XIVa, *Clostridium* III, *Acinetobacter*, *Pyramidobacter*, *Tissierella*, *Clostridium* XVIII, and *Lactobacillus* increased significantly at peak I (Fig. 4c), while *Aminobacterium* kept stable (Table 2). Compared to peak I, *Methanoculleus*, *Methanosaeta*, *Pelotomaculum*, *Dehalogenimonas*, *Acetivibrio*, *Pyramidobacter*, *Cloacibacillus*, and *Alkaliflexus* increased significantly at peak II (Fig. 4d). In addition, Pearson's correlation analysis revealed positive correlation between the dominant methanogens and these bacterial genera ($p<0.05$) (Table 3).

Correlations between prokaryotic communities and environmental variables and fermentation performance

To explore correlations between prokaryotic communities and environmental variables and fermentation performance, redundancy analysis (RDA) was applied using the top 208 genera (relative abundance >0.0006 %). The results showed that 63 and 14 % of the variations at genus level can be explained by *x* and *y*-axes, respectively (Fig. 5). Spatially distributed genera showed differential correlations with environmental variables and fermentation performance.

In the straw samples, two dominant genera (*Clostridium* III and *Treponema*) showed positive and significant correlations ($p<0.05$) with CH_4 (supplementary Table S5). It was noted that only genera *Desulfovibrio*, *Ruminococcus*, and *Cellulosilyticum* were positively correlated with acetate ($p<0.05$). However, propionate and butyrate were significantly and positively correlated with more genera ($p<0.05$), e.g., *Fibrobacter*, *Curtobacterium*, *Lutispora*, *Ruminococcus*, *Acetivibrio*, and *Cellulosilyticum*. The results indicated that these bacteria may play roles in VFAs production.

In the slurry samples, acetate was only observed to be negatively ($p<0.05$) correlated with *Methanosaeta*, *Pyramidobacter*, *Cloacibacillus*, and several syntrophic bacteria (supplementary Table S6), suggesting that they were possibly involved in acetate consumption. Propionate and butyrate were positively correlated with genera *Clostridium* XI, *Turicibacter*, *Anaerococcus*, and *Gelria* ($p<0.05$); however, it was negative for *Methanoculleus*, *Methanosaeta*, and *Pelotomaculum*, implicating the roles of slurry-associated prokaryotes in the production and consumption of the major VFAs. Genera *Methanoculleus*, *Methanosaeta*, *Pelotomaculum*, *Cloacibacillus*, and *Syntrophus* were positively ($p<0.05$) correlated with CH_4 (supplementary Table S6), which indicated their positive contributions to methanogenesis. All above results indicated that straw- and slurry-associated prokaryotic communities differed in VFAs production, consumption, and CH_4 production.

Discussion

In this study, process-related dynamics of prokaryotic communities associated to both straw and slurry were observed in the co-fermentation of straw and swine manure with pyrosequencing technique. Due to the difference in physicochemical nature of straw and slurry, prokaryotic community composition, structure, and diversity throughout the process differentiated. These results revealed the spatial distribution of microbiota that participated in the co-fermentation of plant substrates and manures.

Straw contributed remarkably to biogas production during the co-fermentation process in this study, which demonstrated the enhancement effect of co-fermentation on the performance in anaerobic digesters (Ye et al. 2013). Straw-associated microbiota may be mainly responsible for the straw degradation and provide substrates for other microbes. The abundances of phylum *Synergistetes*, genera *Pelotomaculum* and *Syntrophomonas* were detected much higher in the co-fermentation system in this study than those in manure-only fermentation system (Liu et al.

Table 2 The relative abundance (%) of dominant bacteria and archaea at phylum or genus level[a]

Taxa rank	S0	S1	S2	L0	L1	L2	Average	Closest reference strains[b]	% Identity	References
Phylum *Spirochaetes***	17.4±0.28^{b}	16.65±0.44^{b}	20.98±1.15^{a}	0.18±0.06^{b}	4.90±2.32^{ab}	7.08±0.49^{a}	11.84			
*Treponema***	11.64±0.80^{b}	13.51±1.05^{b}	17.08±2.02^{a}	0.09±0.07^{b}	1.71±1.27^{a}	1.17±0.42^{ab}	7.97	*T. zuelzerae* (NR_104797)	96	
Phylum *Firmicutes*	38.71±0.74^{b}	41.72±0.80^{a}	39.42±0.84^{ab}	81.23±4.31^{a}	64.03±7.94^{b}	29.47±0.53^{c}	47.21			
Clostridium III**	7.22±0.26^{c}	12.26±1.03^{b}	14.6±0.66^{a}	0.28±0.16^{a}	3.60±3.62^{a}	1.73±0.09^{a}	6.99	*R. bacterium* (JN656278)	99	
Clostridium sensu *strict*	7.27±0.60^{a}	1.66±0.20^{b}	1.95±0.09^{b}	7.19±1.34^{a}	4.61±3.13^{ab}	1.74±0.46^{b}	3.89	*Clostridium* sp. (JQ607647)	98	
Clostridium XI*	1.67±0.21^{a}	0.64±0.15^{b}	0.77±0.22^{b}	11.46±3.18^{a}	3.77±1.26^{b}	1.00±0.13^{b}	2.73	*Clostridium* sp. (JF693898)	99	
Pelotomaculum	0.17±0.12^{a}	0.11±0.03^{a}	0.21±0.12^{a}	0.23±0.21^{b}	0.34±0.16^{b}	5.84±0.70^{a}	1.20	*P. schinkii* (X91169)	95	(de Bok et al. 2005)
Syntrophomonas	1.23±0.15^{a}	0.37±0.12^{c}	0.94±0.08^{b}	0.51±0.32^{b}	0.81±0.82^{b}	2.21±0.53^{a}	1.04	*S. wolfei* (DQ666176)	98	(Wu et al. 2007)
*Sedimentibacter***	0.54±0.18^{b}	1.16±0.19^{a}	1.49±0.31^{a}	0.30±0.25^{a}	0.35±0.27^{a}	0.72±0.22^{a}	0.79	*Sedimentibacter* sp. (AB598276)	97	(Imachi et al. 2011)
Phylum *Synergistetes***	2 .00±0.20^{a}	1.03±0.09^{b}	1.45±0.12^{b}	7.89±3.30^{b}	6.21±1.85^{b}	22.96±1.49^{a}	6.87			
*Cloacibacillus**	0.79±0.14^{a}	0.20±0.08^{b}	0.30±0.13^{b}	3.98±2.59^{b}	3.51±1.99^{b}	17.08±2.15^{a}	4.33	*C. bacterium* (EU728779)	99	
*Aminobacterium***	0.89±0.15^{a}	0.46±0.03^{b}	0.81±0.09^{a}	3.42±1.78^{a}	1.88±1.31^{a}	2.03±0.03^{a}	1.47	*A. colombiense* (CP001997)	98	(Chertkov et al. 2010)
Phylum *Bacteroidetes*	30.72±1.08^{a}	27.41±0.87^{b}	15.20±0.65^{c}	1.66±0.20^{b}	15.78±8.1^{ab}	21.15±0.29^{a}	19.65			
*Alkaliflexus***	4.40±2.16^{a}	6.23±1.59^{a}	6.90±1.05^{a}	0.11±0.07^{b}	0.20±0.11^{b}	0.50±0.21^{a}	3.23	*R. xylanolyticum* (DQ141183)	95	
Phylum *Fibrobacteres***:	0.52±0.07^{c}	9.85±0.45^{b}	14.32±0.41^{a}	0.05±0.01^{a}	2.12±1.18^{a}	0.92±0.08^{a}	4.90			
Order TG3-1	0.25±0.04^{b}	0.39±0.08^{b}	14.12±0.79^{a}	0.04±0.03^{b}	0.10±0.06^{b}	0.87±0.09^{a}	2.78			
Fibrobacter	0.23±0.06^{b}	8.46±0.81^{a}	0.17±0.07^{b}	0.01±0.01^{a}	2.01±1.97^{a}	0.05±0.05^{a}	1.93	*F. succinogenes* (GU999989)	90	
Phylum *Euryarchaeota**	0.43±0.10^{b}	0.26±0.02^{b}	0.91±0.02^{a}	0.77±0.10^{b}	0.49±0.26^{b}	4.85±0.22^{a}	1.31			
Methanoculleus	0.16±0.05^{b}	0.13±0.02^{b}	0.51±0.07^{a}	0.05±0.02^{b}	0.00±0.00^{b}	2.37±0.53^{a}	0.57	*M. receptaculi* (NR_043961)	99	(Cheng et al. 2008)
Methanosaeta	0.06±0.02^{b}	0.04±0.01^{b}	0.20±0.03^{a}	0.09±0.07^{b}	0.07±0.05^{b}	1.96±0.50^{a}	0.42	*M. concilii* (NR_102903)	100	(Barber et al. 2011)

[a] *S* straw samples, *L* slurry samples. *0*, *1*, and *2* represent the sampling time at day 0, 3, and 21, respectively. All data were presented as means ± standard deviations. Values in a row with different letters from either straw or slurry samples meant significant difference at $p<0.05$. The asterisks in taxa rank showed the significant difference between straw and slurry samples. ** Significant at $p<0.01$, * significant at $p<0.05$

[b] The representative sequences of the dominant prokaryotes were subjected to BLAST homology search against the non-environmental sequences and non-metagenomes in the NCBI nucleotide database. The accession number of closest sequence was provided in the parenthesis

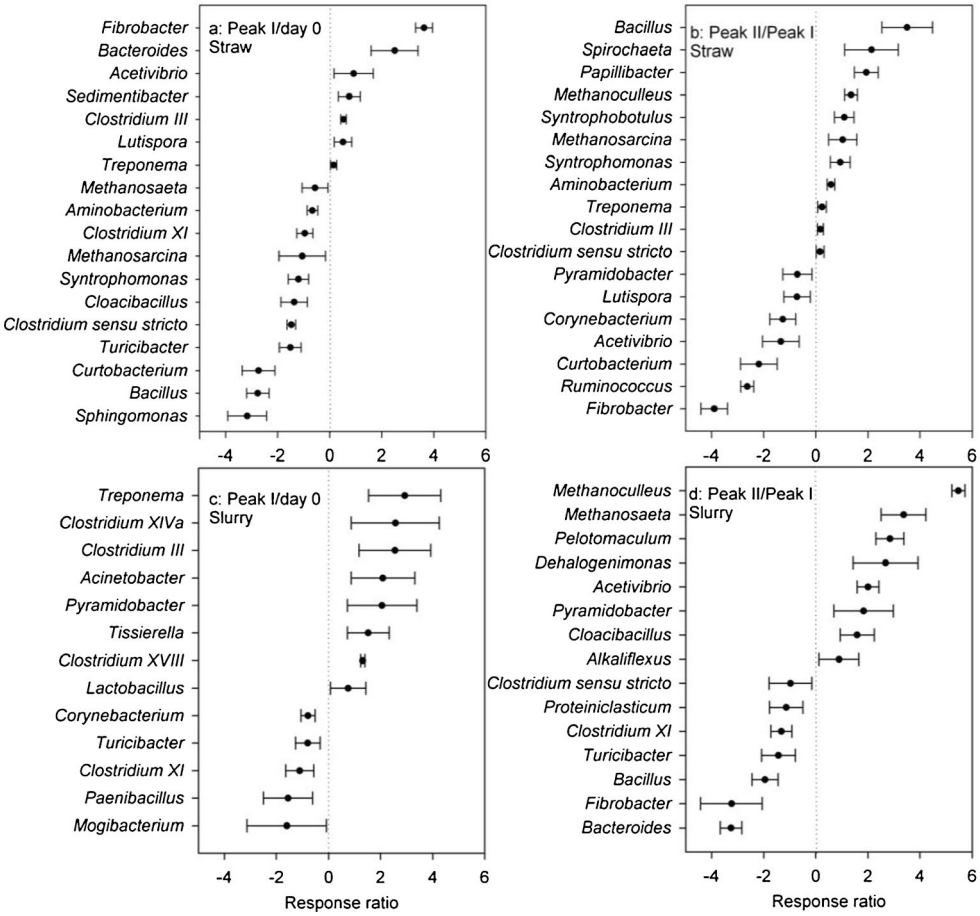

Fig. 4 Significantly changed genera in straw (**a**, **b**) and slurry (**c**, **d**) samples determined using the response ratio method at a 95 % confidence interval based on the relative abundances of the top 208 genera. **a**, **b** Straw samples, **c**, **d**, slurry samples. **a**, **c** Peak I versus day 0, **b**, **d** peak II versus peak I

Table 3 Pearson's correlation of dominant methanogens and dominant bacteria in the slurry samples[a]

	Methanoculleus	*Methanosaeta*
Treponema	0.083	0.005
Clostridium III	−0.128	−0.100
Clostridium XI	−0.629	−0.619
Pelotomaculum	0.983**	0.948**
Syntrophomonas	0.731*	0.869**
Cloacibacillus	0.906**	0.953**
Aminobacterium	−0.191	−0.220
Alkaliflexus	0.731*	0.721*
Fibrobacter	−0.405	−0.386
Curtobacterium	−0.682	−0.679
Bacillus	−0.824*	−0.842**
Acetivibrio	0.935**	0.912**
Dehalogenimonas	0.986**	0.853**
Pyramidobacter	0.918**	0.958**

[a] **Significant at $p<0.01$, * significant at $p<0.05$

2009), where these bacteria were not detected. The occurrence of high abundances of syntrophs suggested that the high efficient performance may be achieved through syntrophic interactions between fermenting bacteria and methanogens.

In anaerobic digesters, the hydrolysis is a rate-limiting step, and the attachment of microbes to substrates is favorable for hydrolysis. In this study, RDA analysis, response ratio, and correlation analysis suggested that members of phyla *Spirochaetes*, *Fibrobacteres*, as well as *Firmicutes* may be the main substrates degraders, e.g., straw-attached TG3 (one candidate class of the phylum *Fibrobacteres*), and those genera significantly increased at peaks I and II. The TG3 bacteria were mainly found in rumen and wood-feeding termite gut (Hongoh et al. 2005; Hongoh et al. 2006; Hess et al. 2011), which are efficient in transforming plant polymers into sugars and VFAs. Thus, the prevalence of these bacteria associated to straw indicated their dominant roles in the breakdown of plant polymers and supplying soluble substrates for the fermentation and methanogenesis occurring in the slurry.

Propionate was the most abundant VFA in the slurry, which agreed well with previous findings that propionate

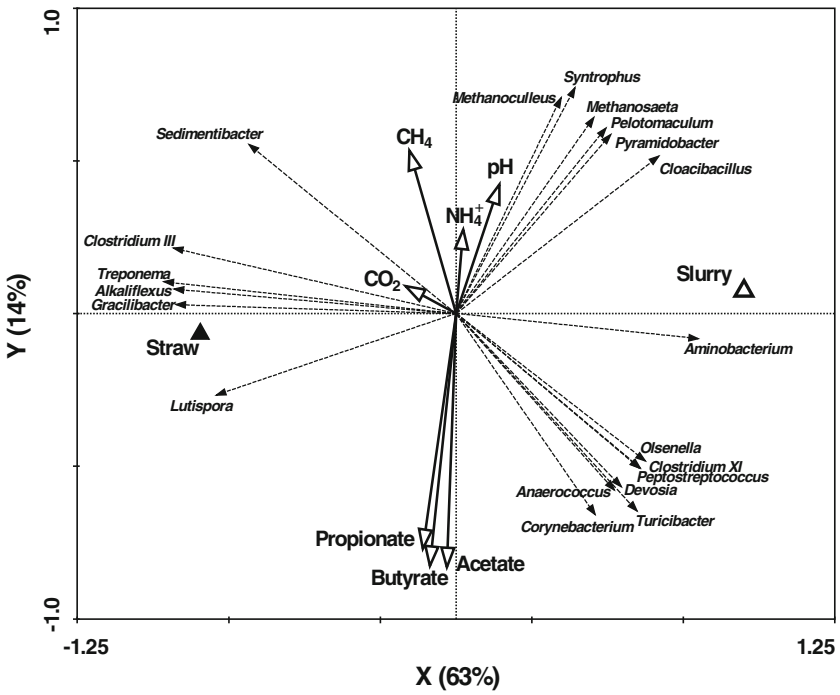

Fig. 5 Redundancy analysis (*RDA*) among fermentation performance (CH_4, CO_2), environmental variables (VFAs, pH, NH_4^+), and prokaryotic community compositions (top 208 genera) at genus level. According to the species fit range of 60–100 %, the top 20 genera were listed in the map. Values on the axes indicated the percentages of total variation explained by each axis

accumulated to 6.2 g l^{-1} (84 mM) during restart of a full-scale anaerobic biowaste digester (Gallert and Winter 2008). The accumulation of propionate was likely due to the activities of dominant straw-attached *Fibrobacter* and slurry-associated *Clostridium* XI, which were positively correlated with propionate. Other less abundant genera may also be involved.

It is important for propionate to be consumed efficiently, as it could potentially cause acidification in anaerobic fermentation. Microbiota may take some time to succeed and acclimate to the high propionate level, thus a lag period occurred between propionate (day 11) and CH_4 peak (day 21). Transformation of propionate into acetate and H_2 can only be realized via syntrophic metabolism under methanogenic condition (Stams and Plugge 2009). Some pure cultures including genera *Pelotomaculum*, *Syntrophobacter*, *Smithella*, and *Desulfotomaculum* are the hitherto identified propionate oxidizers (Muller et al. 2010). In this study, a positive ($p<0.05$) correlation between genus *Pelotomaculum* and *Methanoculleus* further suggested that the *Pelotomaculum* was likely to be involved in H_2 and acetate production. Nonetheless, the turnover rate of acetate and butyrate was faster than propionate, which could be due to the presence of acetoclastic *Methanosaeta* and *Syntrophomonas*, respectively (Sieber et al. 2012). This demonstrated that the degradation rate of propionate may be a limiting factor for CH_4 production at least in a certain fermentation period.

Phylum *Synergistetes* dominated in the slurry samples, in which genera *Cloacibacillus* and *Aminobacterium* were most abundant. They are able to ferment proteins or amino acids to produce VFAs, H_2, and CO_2 (Ganesan et al. 2008; Looft et al. 2013). It was found that *Aminobacterium* played a dominant role in a methanogenic digester containing bovine serum albumin as a sole carbon source (Tang et al. 2005). Though *Aminobacterium* was not significantly correlated with the dominant hydrogenotrophic methanogens in this study, its stable and high relative abundance throughout the fermentation process implicated its constitutive role in the degradation of amino acids. Besides, the positive correlation of genus *Cloacibacillus* with the two dominant methanogens ($p<0.01$) also suggested a strong synergistic metabolism between amino acid-oxidizers and methanogens in the slurry.

CH_4 at peak I could be transformed from acetate that was brought from the inoculum or produced by fermenting bacteria, which was favored by the presence of acetoclastic *Methanosaeta*. However, *Methanoculleus* and *Methanosaeta* probably accounted for the CH_4 production at peak II. The differences in CH_4 production rate between the two samplings were partially but not proportionally consistent with the relative abundances of methanogens.

Previous studies showed that archaea accounted for up to 20 % in anaerobic digesters (Zakrzewski et al. 2012; Sundberg et al. 2013). In this study, though the primer set should cover most methanogens, the highest relative abundance of *Euryarchaeota* only accounted for 4.85 % of total reads. One explanation might be that our co-fermentation system was not at optimal condition. Some studies showed that hydrogenotrophic pathway prevailed in the co-digesters of agricultural waste (Jaenicke et al. 2011). Acetoclastic methanogenesis may be a major pathway in some systems (Supaphol et al. 2011), or both pathways were utilized for methanogenesis (Li et al. 2013). In our system, the equal relative abundance of *Methanoculleus* and *Methanosaeta*

indicated that both pathways for CH_4 production were most likely co-occurred in the mesophilic co-fermentation of straw and swine manure.

In summary, process-related dynamics of prokaryotic communities associated to both straw and slurry was observed in the co-fermentation of straw and swine manure with pyrosequencing technique. Plant material degraders were abundant in the straw. In contrast, syntrophic bacteria involved in propionate and amino acids degradation were highly abundant in the slurry, which supported hydrogenotrophic and acetoclastic methanogenesis. Above findings built the linkages among fermentation performance, microbial structure, and environmental variables. This provided a better understanding of the anaerobic co-fermentation process that is driven by the specific and different spatially distributed microbiota in the system.

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

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