




# Enriching ruminal polysaccharide-degrading consortia via co-inoculation with methanogenic sludge and microbial mechanisms of acidification across lignocellulose loading gradients

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## Abstract

Using lignocellulosic materials as substrates, ruminal microbiota were co-inoculated with anaerobic sludge at different loading rates (LR) to study the microbial community in the semi-continuous mode. The results indicated that the highest CH<sub>4</sub> yield reached 0.22 L/g volatile solid at LR of 4 g/L/day, which obtained 56–58% of the theoretical value. In the steady stage with LR of 2–4 g/L/day and slurry recirculation, copies of total archaea increased. Especially the *Methanobacteriales* increased significantly ( $p < 0.05$ ) to  $3.30 \times 10^8$  copies/mL. The microbial communities were examined by MiSeq 16S rRNA sequencing. Enriched hydrolytic bacteria mainly belonged to *Clostridiales*, including *Ruminococcus*, *Ruminiclostridium*, and *Ruminofilibacter* settled in the rumen. High-active cellulase and xylanase were excreted in the co-inoculated system. Acid-producing bacteria by fermentation were affiliated with *Lachnospiraceae* and *Bacteroidales*. The acidogen members were mainly *Spirochaetaceae* and *Clostridiales*. Syntrophic oxidation bacteria mainly consisted of Synergistetes, propionate oxidizers (*Syntrophobacter* and *Pelotomaculum*), and butyrate oxidizers (*Syntrophus* and *Syntrophomonas*). There had no volatile fatty acid (VFA) accumulation and the pH values varied between 6.94 and 7.35. At LR of 6 g/L/day and a recirculation ratio of 1:1, the hardly degradable components and total VFA concentrations obviously increased. The total archaea and *Methanobacteriales* then deceased significantly to  $8.56 \times 10^5$  copies/mL and  $4.14 \times 10^3$  copies/mL respectively ( $p < 0.05$ ), which resulted in the inhibition of methanogenic activities. Subsequently, microbial diversity dropped, and the hydrolytic bacteria and syntrophic oxidizers obviously decreased. In contrast, the abundances of *Bacteroidales* increased significantly ( $p < 0.05$ ). Acetate, propionate, and butyrate concentrations reached 2.02, 6.54, and 0.53 g/L, respectively, which indicated “acidification” in the anaerobic reactor. Our study illustrated that co-inoculated anaerobic sludge enriched the ruminal function consortia and hydrogenotrophic methanogens played an important role in anaerobic digestion of lignocelluloses.

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**Keywords** Co-inoculated consortia · Lignocellulose digestion · Anaerobic acidification

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## Introduction

Ruminal microbiota are characterized as highly efficient players for converting lignocellulosic materials to volatile fatty acids (VFAs). Then, these VFA products are absorbed by rumen epithelial cells since that microbial community only contains a small quantity of hydrogenotrophic methanogens (Dai et al. 2015; Janssen and Kirs 2008; Morrison et al. 2009). Previous studies have indicated that rumen inoculated anaerobic digestion (RUDAD) enhances the hydrolytic efficiencies of corn stover (Hu and Yu 2005), cattail (Hu et al. 2007), wheat straw (Lazuka et al. 2015), and rice straw (Deng et al.

2017b; Zhang et al. 2016). The VFAs produced by RUDAD need to be further separated from the solid residues and transferred into the methanogenic fraction (Hu et al. 2007; Hu and Yu 2005). Thus, it would reduce the degradation efficiency of lignocelluloses. Addressing the problem of whether rumen microbiota are co-inoculated with methanogens to achieve lignocellulose hydrolysis and methanogenesis in the same reactor remains to be extensively elucidated. Except for previous investigations on enrichment of ruminal microbiota in the batch experiments (Deng et al. 2017b; Ozbayram et al. 2017), studies on the co-inoculum involved in the polysaccharide-degrading consortia are rare in a semi-continuous reactor.

Hydrolytic specialists in the rumen are mainly affiliated with *Ruminococcus* and *Fibrobacter* (Dai et al. 2015; Morrison et al. 2009; Nyonyo et al. 2014). However, these species have been less frequently reported to settle in batch tests (Azman et al. 2015; Deng et al. 2017b; Sun et al. 2016). In a long-term operation reactor with the co-inoculum, whether these functional bacteria can perform the hydrolytic activities needs to further be illustrated for more details. Subsequently, intermediates have been used to produce VFAs through acidogenesis in liquid fractions. The consumption of VFAs through syntrophic acetogenesis is indispensable since they are detrimental to anaerobic digestion. Microbial populations responsible for syntrophic cooperation need to be revealed. The overloading of lignocelluloses results in VFA accumulation and subsequent CH<sub>4</sub>-producing inhibition, which has been closely related with methanogens (Azman et al. 2015; Karthikeyan and Visvanathan 2013). Thus, detailed knowledge on the functional microbes and their interactions could be essential for uncovering the lignocellulosic digestion efficiencies in an anaerobic reactor. However, only a few investigations have studied the dynamics of the methanogenic community composition provoked by the organic overload rate (Lerm et al. 2012).

Therefore, this study aimed at evaluating digestion performance seeded with ruminal microbiota and anaerobic sludge in a semi-continuous reactor. The functional consortia, enriched by the application of different lignocelluloses loading rates and slurry recirculation, were investigated with MiSeq 16S rRNA sequencing. The copies of methanogenic archaea were further examined using absolute quantitative PCR (absolute Q-PCR). Thus, the mechanisms of acidification under lignocellulose overloading conditions were analyzed based on the changes of microbial communities.

## Materials and methods

### Inoculum and substrate

The rumen digesta were collected from a slaughterhouse (Wuxi, China) and immediately transferred to the laboratory. The rumen fluid was then recovered by straining the whole

digesta with two layers of cheesecloth, containing 2.28% total solid (TS) and 1.32% volatile solid (VS). Anaerobic sludge with 13.90% TS and 6.90% VS was obtained from an anaerobic reactor used to treat kitchen wastes at 39 °C (Jiangsu Clean Environmental Technology Co., Ltd., Suzhou, China). The rumen fluid was co-inoculated with anaerobic sludge into the reactor based on the equal VS content. Lignocellulosic feedstocks consisted of corn stalks, guinea grass, and rice straw. All the raw materials were collected and dried at 50 °C in an air-circulating oven. The dried feedstocks were ground with a pulverizer (Zhongcheng Pharmaceutical Machinery Co., Ltd., Changsha, China) to a size of 40-mesh. After mixing with an equal VS content, the substrates were then stored in a vacuum bag to prevent possible degradation. The average TS and VS were 95.30 and 88.59%, respectively.

### Semi-continuous anaerobic digestion

The co-inoculum was added into a semi-continuous reactor with a 25% volume fraction. The CH<sub>4</sub> volume was directly measured with water column after CO<sub>2</sub> absorption with 4 mol/L NaOH saturated solution. The reactor was set at 39 ± 1 °C with a 2.5-L working volume. Just before the reactor started, a buffer solution including K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O (1.34 g/L), KH<sub>2</sub>PO<sub>4</sub> (0.51 g/L), and NaHCO<sub>3</sub> (5.00 g/L) was added to maintain the initial pH value between 6.80 and 7.10.

To keep the solid content ranging within a small scale, discharging volume of the digestate increased with the improvement of loading rate (LR). The digestate was extruded and filtered through two layers of 100-mesh nylon gauze prior to feeding substrates at intervals of 2 days. The squeezed residues were used to measure the chemical compositions. The liquid fractions were returned to the system after supplementing dH<sub>2</sub>O according to the corresponding slurry recirculation ratios. As shown in Table 1, the whole digestion process was set to six operation phases. They were divided into the start-up stage, the steady stage, and the overload stage based on the discrepancy of the digestion performance.

### Physicochemical analysis

The total organic carbon (TOC) and Kjeldahl nitrogen of the solid samples were measured with a TOC analyzer (SSM-5000, Shimadzu Co., Japan) and KDN-08 azotometer (Hangzhou Tuopu Instrument, Hangzhou, China) respectively and then used to calculate the C/N ratio. The cellulose, hemicellulose, and lignin content were analyzed according to a previously published principle with a slight modification of the residues being dried at 65 °C (Van Soest et al. 1991).

The slurry was centrifuged at 5000 r/min for 15 min, and then the supernatants were recovered for the triplicate assays after 10-fold dilution with dH<sub>2</sub>O. The pH values were measured with a DELTA 320 pH meter (Mettler Toledo, Shanghai,

**Table 1** Setting of the operating parameters in six operating conditions

Operating parameters	Start-up stage	Steady stage					Overload stage
	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6	
Loading rate (g/L/day)	1.5	2	3	4	4	6	
Slurry recirculation ratios	2:1	2:1	1.5:1	1.5:1	1:1	1:1	
Operation time (day)	0–24	25–48	49–72	73–96	97–130	131–166	

China).  $\text{NH}_4^+$  concentrations were analyzed with the Nessler reagent method (APHA 2005). Dissolved TOC in the liquid fractions was detected using a TOC analyzer (TOC-V<sub>CPH</sub>, Shimadzu Co., Japan). VFA concentrations were detected by a GC-2010 Plus (Shimadzu Co., Japan) according to the previous method (Deng et al. 2017a). Xylanase and cellulase activities were measured using the dinitrosalicylic acid (DNS) method and expressed as units per milliliter following a previous study (Deng et al. 2017b). One unit of enzymatic activity (U) was defined as the amount of enzyme by which 1  $\mu\text{g}$  of reducing sugar was produced per minute.

### MiSeq 16S rRNA gene sequencing

The microbiome was harvested in the start-up stage (the 1st, 12th, 24th day, denoted as 1-1, 1-2, 1-3), the steady stage (the 36th, 60th, 84th, 84th day, denoted as 2, 3, 4, 5), and the overload stage (the 132nd, 144th, 156th day, denoted as 6-1, 6-2, 6-3). All 10 samples were centrifuged at 5000 r/min for 10 min and the corresponding pellets were used to extract DNA using the Power soil® DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The amplified libraries were constructed with the V3-V4 regions of the 16S rRNA genes and then analyzed using the MiSeq Illumina platform (San Diego, CA, USA) by the paired-end protocol (Nelson et al. 2014). Subsequently, the raw sequences were pre-processed using the QIIME pipeline v1.8.0 (<http://qiime.org/>). After trimming the barcodes, the high-quality tags were classified to operational taxonomic units (OTUs) at a similarity of 97%, and subsequent statistical analysis was performed based on the OTUs. As shown in supplementary Figure S1, each sequence at the genus level was selected to a generated phylogenetic tree using MEGA 7.0 software (Kumar et al. 2016). All original sequences were deposited in the Sequence Read Archive of NCBI under the number: SRP119185.

### Absolute Q-PCR on methanogens

Three samples in each stage were selected for absolute Q-PCR analysis. The primer sets of total archaea, *Methanobacteriales*, and *Methanosarcinales* are used according to Steinmetz et al. (2016). The PCR program was as follows: 95 °C initial

denaturation for 3 min; followed by 40 cycles of 30 s denaturing at 95 °C, 30 s annealing at 60 °C, and 30 s extending at 72 °C; a final extension step of 72 °C for 30 s. The PCR products were analyzed electrophoretically in a 1% agarose gel and then gel-purified with an AxyPrep DNA gel-extraction kit (Axygen Corp., CA, USA). The target fragments were directly cloned into pMD19-T vector and then transformed into competent *E. coli* cells. The plasmid DNA were extracted and identified by sequencing results (Genewiz Inc., Suzhou, China).

Recombinant plasmid DNA concentrations were determined by an ND-1000 spectrophotometer (Thermo-Fisher, DE, USA), and copies of methanogen genes were then calculated according to Eq. 1. where MW represented genes sizes  $\times$  660 Da. DNA standards for quantification were 10-fold serially diluted with  $10^9$  to  $10^3$  copies/ $\mu\text{L}$  and run in triplicate alongside each Q-PCR assay.

methanogen(copies/ $\mu\text{L}$ )

$$= \text{genes concentration (g}/\mu\text{L}) \times 6 \times 10^{23} \frac{(\text{copies}/\text{mol})}{\text{MW}(\text{g}/\text{mol})} \quad (1)$$

The Q-PCR assay was set in SYBR Premix Ex Taq™ II (Takara Bio Inc., Dalian, China) with primer of a 0.20- $\mu\text{M}$  final concentration and a 2.00- $\mu\text{L}$  DNA template. The PCR reactions were performed in a One-Step Real-time PCR thermocycler (Applied Biosystems Inc. Carlsbad, CA, USA) following a previous study (Deng et al. 2017a). The copies of corresponding methanogens were calculated according to the Ct value and then expressed as copies per milliliter.

### Statistical analysis

SPSS 19.0 (IBM Statistics, Chicago, IL, USA) was used to evaluate the significant difference of three stages at a 0.05 *p* value. Alpha diversities including (Chao1 and Shannon index) were calculated based on OTU data as described previously (Nelson et al. 2014). The general discrepancy in the microbial community structure was determined by principal component analysis (PCA) using Canoco for Windows 4.5 software (Microcomputer Power, Ithaca, NY, USA).

## Results

### Reactor performances

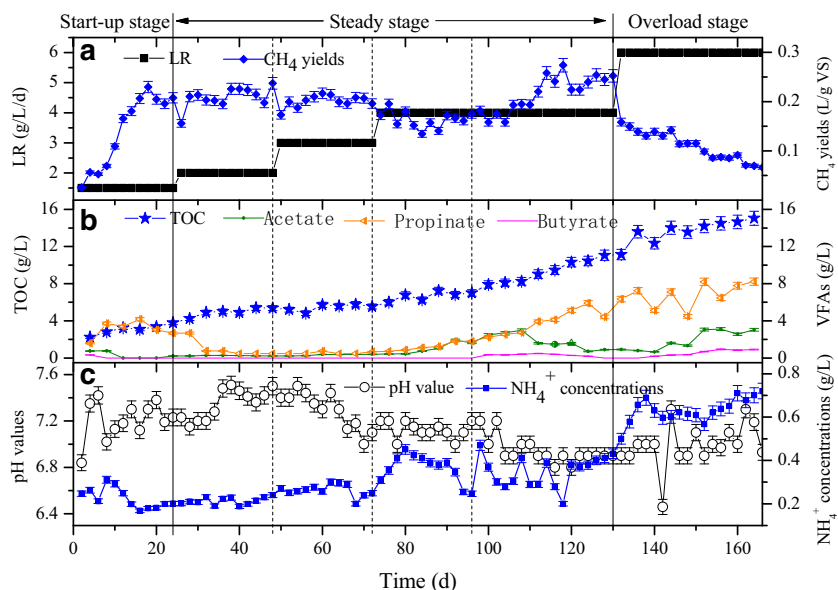
#### CH<sub>4</sub> production

The CH<sub>4</sub> yields of the six operation phases are shown in Fig. 1a. In the start-up stage with 1.5 g/L/day LR, the CH<sub>4</sub> yields were on a gradual increment. However, the average yield only reached 0.14 L/g VS of lignocellulosic materials, which was attributed to the longer retention times of the methanogens in the artificial reactor (Demirel and Scherer 2008). In the steady stage, there were relatively stable CH<sub>4</sub> yields, ranging from 0.16 L/g VS to 0.27 L/g VS. At phase 4 with 4 g/L/day LR, the average CH<sub>4</sub> yield dropped to 0.16 L/g VS. However, the average value reached a maximum of 0.22 L/g VS when the slurry recirculation ratio was set to 1:1 from 1.5:1 at phase 5. In the overload stage, the average CH<sub>4</sub> yield dropped sharply to 0.11 L/g VS.

#### The changes of TOC and VFA concentrations

As shown in Fig. 1b, the concentrations of TOC in the liquid digestate increased to 15.05 g/L from the original 2.24 g/L at the end of the experiment, especially reaching 13.67 g/L of the average concentration at the overload stage (shown in Table 2). The accumulated VFAs, mostly including acetate, propionate, and butyrate, were influenced both by the LR and recirculation ratios in this study. At phase 4, acetate began to accumulate with a concentration of 1.04 g/L. When the recirculation ratios reduced at phase 5, the acetate concentration decreased to 0.90 g/L from the initial 2.49 g/L, which was utilized through acetotrophic methanogenesis. The propionate concentration reached an average of 2.70 g/L at phase 1.

**Fig. 1** The dynamic changes of digestion performance at different operating conditions



Subsequently, propionate was consumed through syntrophic oxidation in the whole steady stage.

In the overload state with an LR of 6 g/L/day, the equilibrium between acidogenesis and methanogenesis was broken. Individual VFA also began to accumulate. As shown in Table 2, the average concentrations of acetate, propionate, and butyrate reached 2.02, 6.54, and 0.53 g/L, respectively. This indicated that “acidification” occurred in the anaerobic reactor.

#### The changes of pH values and NH<sub>4</sub><sup>+</sup> concentrations

As shown in Fig. 1c, the accumulation of VFAs lead to a decline in the pH values. The initial pH value was 7.21, and the values varied between 6.94 and 7.35 in the steady stage. The average pH value of the final stage was 6.99, which was still within the appropriate methanogenesis range of 6.5 to 8.5 (Azman et al. 2015). It was also shown that the pH values could not reflect the VFA accumulation timely in the anaerobic reactor, and NH<sub>4</sub><sup>+</sup> could also influence the pH values. The shifts of NH<sub>4</sub><sup>+</sup> concentrations had little effect in the first two stages, ranging from 0.21 to 0.36 g/L on average. In the overload stage, the values changed between 0.50 and 0.72 g/L with an average value of 0.63 g/L.

#### Characterizations of biodegradability and hydrolytic activities

Because CH<sub>4</sub> production originated from the lignocellulose biodegradability, it would be inevitable to analyze the composition changes of the solid residues in every phase. As shown in Fig. 2, the proportions of cellulose and hemicellulose of the six phases decreased, whereas those of lignin increased compared with the raw materials. In the steady stage, the



**Table 2** Summary of digestion parameters at different operating conditions<sup>a</sup>

Digestion parameters	Start-up stage	Steady stage				Overload stage
	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
CH <sub>4</sub> yields (L/g VS)	0.14 ± 0.07	0.21 ± 0.02	0.20 ± 0.01	0.16 ± 0.02	0.22 ± 0.04	0.11 ± 0.03
TOC concentrations (g/L)	3.17 ± 0.36	4.97 ± 0.38	5.45 ± 0.33	6.69 ± 0.43	9.32 ± 1.12	13.67 ± 1.10
Acetate concentrations (g/L)	0.29 ± 0.34	0.25 ± 0.03	0.34 ± 0.08	1.04 ± 0.57	1.81 ± 0.78	2.02 ± 0.97
Propionate concentrations (g/L)	2.70 ± 0.85	0.57 ± 0.86	0.08 ± 0.12	0.81 ± 0.43	3.50 ± 1.25	6.54 ± 1.34
Butyrate concentrations (g/L)	0.06 ± 0.12	0	0	0	0.31 ± 0.14	0.53 ± 0.34
pH values	7.21 ± 0.16	7.35 ± 0.13	7.30 ± 0.15	7.13 ± 0.07	6.94 ± 0.11	6.99 ± 0.18
NH <sub>4</sub> <sup>+</sup> concentrations (g/L)	0.22 ± 0.04	0.21 ± 0.02	0.26 ± 0.03	0.36 ± 0.06	0.35 ± 0.07	0.63 ± 0.05
Xylanase activities (U/mL)	6.02 ± 1.48	6.13 ± 0.71	5.50 ± 0.55	4.13 ± 0.22	6.45 ± 0.72	4.50 ± 0.70
Cellulase activities (U/mL)	18.04 ± 0.03	37.08 ± 0.66	41.72 ± 1.14	36.44 ± 0.49	50.06 ± 10.09	21.32 ± 1.31

<sup>a</sup> Values are means ± SD.  $n = 12, 12, 12, 12, 17,$  and  $18$  in six phases for CH<sub>4</sub> productivities, pH, and NH<sub>4</sub><sup>+</sup> concentrations respectively;  $n = 6, 6, 6, 6, 8,$  and  $9$  in six phases for DOM and VFA concentrations respectively, and  $n = 3$  for xylanase and cellulase activities

proportions of hemicellulose and cellulose were 20.38–4.85 and 26.78–29.74%, respectively. The values of the two compositions increased to 25.18 and 31.88% in the overload stage. Correspondingly, cellulase and xylanase activities were used to assess the hydrolytic potential of the anaerobic co-inoculum (Deng et al. 2017b). The values were 36.44–50.06 and 4.13–6.45 U/mL in the steady stage, which was higher than those from the samples in the overload stage (shown in Table 2).

### The diversities of bacterial communities

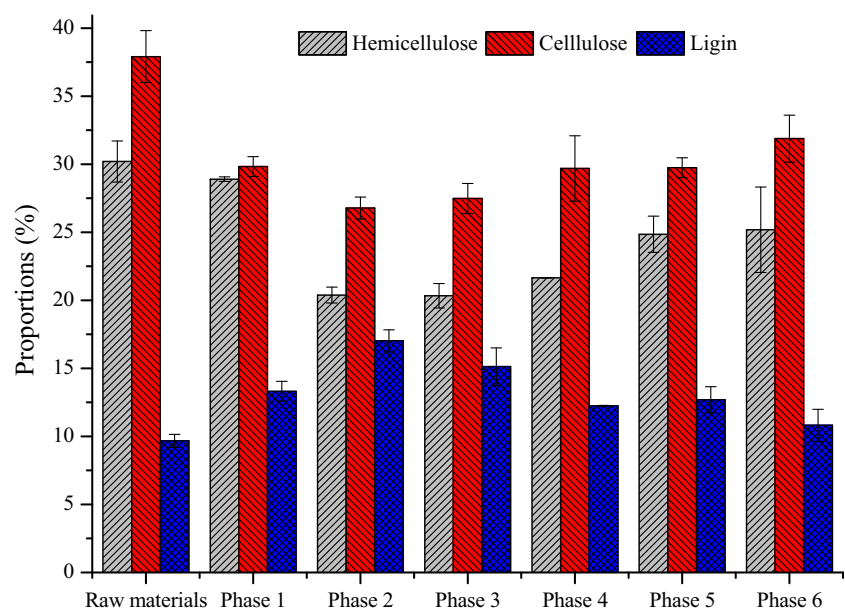
A total of 530,243 chimera-eliminated tags were obtained by sequence analysis with an average length of 360–480 bp. Alpha diversity analysis showed that the chaol index in the three phases changed slightly. However, the Shannon index

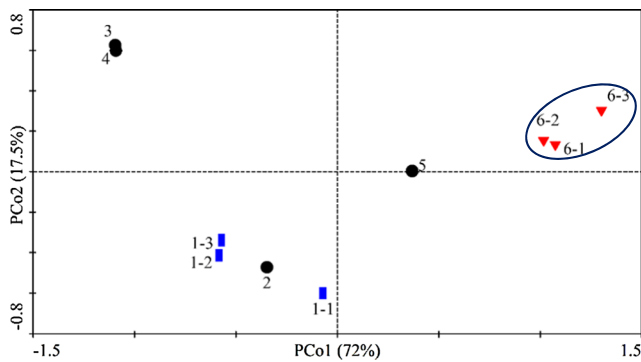
decreased obviously, being 5.25, 4.36, and 2.59 respectively (shown in Supplementary Table S1).

As shown in Fig. 3, PCA was evaluated based on the abundances of 505 common OTUs. In the PCo 1 coordinate, a high LR led to the failure of digestion and obvious changes of the microbial communities (6-1, 6-2, and 6-3) compared with those in the start-up stage, accounting for a 72% discrepancy. In the PCo 2 coordinate, the microbial communities of phases 3 and 4 were distinguished by 17.5% compared with those in the initial stage. However, the diversity from phase 2 was similar to those in the start-up stage, whereas the sample of phase 5 was close to those in the overload stage.

The OTUs were annotated by blasting the Silva 16S rRNA database, showing that the phyla of *Bacteroidetes*, *Firmicutes*, *Synergistetes*, *Chloroflexi*, *Cloacimonetes* (WWE1), *Actinobacteria*, *Fibrobacteres*, *Spirochaetae*, and

**Fig. 2** The dynamic changes of lignocellulosic compositions at the initial and following six phases





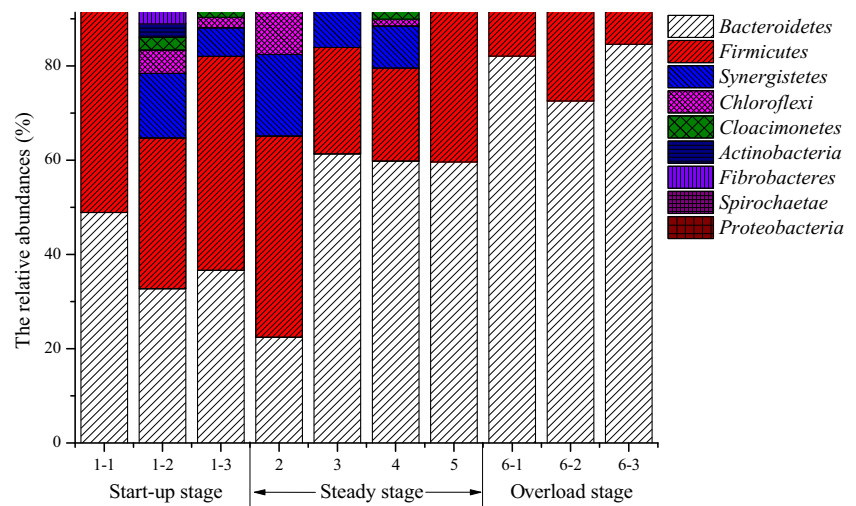
**Fig. 3** The principal component analysis (PCA) based on the abundances of 505 common OTUs

*Proteobacteria* were the predominant microbial populations. The relative abundances of the phyla are shown in Fig. 4, and the total coverage was greater than 97.16% in each sample. In the steady stage, the relative abundances of *Synergistetes*, *Chloroflexi*, and *Cloacimonetes* increased. The *Proteobacteria* numbers changed slightly, while *Actinobacteria*, *Fibrobacteres*, and *Spirochaetae* declined in abundance. In the overload stage, the relative abundance of *Firmicutes* decreased from 41.44 to 18.61%, whereas the proportions of *Bacteroidetes* increased from 39.42 to 79.75%. Other phyla dropped obviously.

### The characterizations of functional bacteria

The common polysaccharide-degrading bacteria are shown in Table 3. The main hydrolytic bacteria belonged to the *Clostridiales* order within *Firmicutes*, including *Ruminococcus*, *Ruminiclostridium*, and *Ruminofilibacter*. Except for *Ruminococcus flavefaciens*, the other hydrolytic bacteria had a similar change tendency. There was high abundances in the first two stages. The proportions decreased obviously in the overload stage, especially *Fibrobacter* disappearing from the reactor, although there was no

**Fig. 4** The changes in the relative abundances of bacteria at the phylum level with > 97.16% total coverage in each sample



significant difference in the steady and overload stage ( $p < 0.05$ ). VFA-producing bacteria by fermentation mainly included *Lachnospiraceae* and *Bacteroidales*. In the overload stage, the relative abundance of *Lachnospiraceae* gradually diminished, whereas *Bacteroidales* increased significantly ( $p < 0.05$ ), accounting for 79.75%. The members of *Clostridiales* (*Caldicoprobacter*, *Sedimentibacter*, *Anaerovorax*) and *Spirochaetae* were regarded as acidogens (Azman et al. 2015; FitzGerald et al. 2015; Wang et al. 2010). The relative abundance was higher in the first two stages than in the overload stage, although there was a slight discrepancy. In the steady stage, the abundances of the *Synergistetes* phylum increased to 9.45%. Propionate oxidizers belonged to *Syntrophobacterales* in the *Proteobacteria* phylum, including *Syntrophobacter* and *Pelotomaculum*. *Syntrophus* and *Syntrophomonas*, attaching to *Syntrophomonadaceae* of *Firmicutes* phylum, were the butyrate oxidizers.

### The changes of methanogenic community composition in the three stages

The proportion of methanogens was relatively low in the functional microbiota because sequencing primer sets were not designed specifically for the methanogenic community. Therefore, Q-PCR analysis was performed for the total archaea, *Methanobacteriales*, and *Methanosarcinales*, according to Steinmetz et al. (2016). The actual fragment sizes were identified by sequencing and had 100% similarity with the known methanogenic populations in the GenBank database (shown in Supplementary Figure S2). The standard regression equation was constructed with 10-fold serially diluted plasmid DNA as follows. Total archaea:  $Y = -3.436 \lg X + 41.072$  ( $R^2 = 0.9986$ ); *Methanobacteriales*:  $Y = -3.472 \lg X + 40.885$  ( $R^2 = 0.9958$ ); *Methanosarcinales*:  $Y = -3.1351 \lg X + 39.172$  ( $R^2 =$

**Table 3** The changes on abundances of the function bacteria in the three stages (%)

Functional bacteria	Start-up stage	Steady stage	Overload stage	References
<b>Hydrolytic bacteria</b>				
p_Firmicutes				
o_Clostridiales	39.11 ± 0.07 <sup>a</sup>	24.85 ± 0.06 <sup>bc</sup>	18.30 ± 0.04 <sup>bc</sup>	Sun et al. (2016)
s_Ruminococcus flavefaciens	2.98 ± 0.02 <sup>a</sup>	2.34 ± 0.02 <sup>a</sup>	2.72 ± 0.01 <sup>a</sup>	Dai et al. (2015); Morrison et al. (2009)
g_Ruminiclostridium	0.47 ± 0.00 <sup>ab</sup>	0.26 ± 0.00 <sup>bc</sup>	0.02 ± 0.00 <sup>bc</sup>	Kröber et al. (2009)
g_Ruminofilibacter	3.04 ± 0.02 <sup>ab</sup>	0.85 ± 0.01 <sup>bc</sup>	0.10 ± 0.00 <sup>bc</sup>	Kröber et al. (2009)
s_Butyrvibrio fibrisolvens	0.12 ± 0.00 <sup>a</sup>	0.09 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	Dai et al. (2015); Morrison et al. (2009)
p_Fibrobacteres				
g_Fibrobacter	0.21 ± 0.00 <sup>a</sup>	0.10 ± 0.00 <sup>a</sup>	0	Dai et al. (2015); Morrison et al. (2009)
<b>VFA-producing bacteria</b>				
f_Lachnospiraceae	8.19 ± 0.01 <sup>a</sup>	5.46 ± 0.01 <sup>bc</sup>	4.25% ± 0.01 <sup>bc</sup>	Nyonyo et al. (2014)
p_Bacteroidetes				
o_Bacteroidales	38.92 ± 0.07 <sup>bc</sup>	50.59 ± 0.16 <sup>bc</sup>	79.75 ± 0.05 <sup>a</sup>	Hatamoto et al. (2014)
f_Prevotellaceae	18.80 ± 0.06 <sup>a</sup>	3.33 ± 0.01 <sup>bc</sup>	5.05 ± 0.04 <sup>bc</sup>	Fondevila and Dehority (1996); Lazuka et al. (2015)
f_Rikenellaceae	14.01 ± 0.03 <sup>c</sup>	43.18 ± 0.17 <sup>b</sup>	72.57 ± 0.05 <sup>a</sup>	Sun et al. (2016)
<b>Acetogens</b>				
o_Clostridiales				
g_Caldicoprobacter	0.15 ± 0.00 <sup>a</sup>	0.20 ± 0.00 <sup>a</sup>	0.02 ± 0.00 <sup>a</sup>	Azman et al. (2015)
g_Sedimentibacter	1.12 ± 0.00 <sup>a</sup>	1.55 ± 0.01 <sup>a</sup>	0.44 ± 0.00 <sup>a</sup>	Wang et al. (2010)
g_Anaerovorax	0.08 ± 0.00 <sup>a</sup>	0.07 ± 0.00 <sup>a</sup>	0.07 ± 0.00 <sup>a</sup>	Wang et al. (2010)
p_Spirochaetae				
f_Spirochaetaceae	1.22 ± 0.01 <sup>a</sup>	0.49 ± 0.00 <sup>a</sup>	0.08 ± 0.00 <sup>a</sup>	FitzGerald et al. (2015)
<b>Syntrophic oxidizers</b>				
p_Synergistetes				
p_Synergistetes	7.16 ± 0.05 <sup>a</sup>	9.45 ± 0.05 <sup>a</sup>	0.18 ± 0.00 <sup>a</sup>	Liu et al. (2009)
p_Proteobacteria				
o_Syntrophobacterales				
o_Syntrophobacterales	0.04 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	0	Müller et al. (2010)
g_Syntrophobacter	0.002 ± 0.000 <sup>a</sup>	0.002 ± 0.000 <sup>a</sup>	0	
g_Pelotomaculum	0.20 ± 0.00 <sup>a</sup>	0.35 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	Müller et al. (2010)
s_Pelotomaculum schinkii	0.13 ± 0.00 <sup>a</sup>	0.30 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	de Bok et al. (2005)
g_Syntrophus	0.04 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	0	
p_Firmicutes				
f_Syntrophomonadaceae	0.78 ± 0.01 <sup>a</sup>	0.57 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	Müller et al. (2010)
g_Syntrophomonas	0.48 ± 0.00 <sup>a</sup>	0.36 ± 0.00 <sup>a</sup>	0.01 ± 0.00 <sup>a</sup>	

<sup>a</sup> Values are means ± SD. In the start-up, overload stage,  $n = 3$ ; in the steady stage,  $n = 4$ . Mean values with different superscript letters in the same row indicate a significant difference at  $p < 0.05$

0.9873), where  $X$  represents the plasmid DNA concentrations with  $10^9$ – $10^6$  copies/ $\mu$ L, and  $Y$  is the Ct value. Consequently, the copies of the methanogenic archaea were calculated and shown in Table 4. In the steady stage, the total archaea and *Methanosarcinales* increased to  $6.20 \times 10^8$  and  $1.75 \times 10^7$  copies/mL, respectively. The *Methanobacteriales* increased significantly ( $p < 0.05$ ), being  $3.30 \times 10^8$  copies/mL. In the overload stage, the total archaea and *Methanobacteriales* decreased significantly ( $p < 0.05$ ). The copies of *Methanosarcinales* dropped to  $2.08 \times 10^4$  copies/mL.

## Discussion

### The changes of digestion performance

The theoretical  $\text{CH}_4$  yield of cellulose is 0.415 L/g VS based on the structural formula of  $(\text{C}_6\text{H}_{10}\text{O}_5)_n$ , and the theoretical value of hemicellulose is 0.424 L/g VS with the structural formula of  $(\text{C}_5\text{H}_8\text{O}_4)_n$  (Karthikeyan and Visvanathan 2013). Thus, the feedstock achieves 0.35–0.36 L/g VS of theoretical values based on the proportions of cellulose and hemicellulose

**Table 4** The copies of different methanogenic archaea at the three stages

Copies/mL	Total archaea	<i>Methanobacteriales</i>	<i>Methanosarcinales</i>
Start-up stage	$2.75 (\pm 3.29) \times 10^{8bc}$	$2.22 (\pm 2.65) \times 10^{7bc}$	$1.42 (\pm 1.56) \times 10^{7a}$
Steady stage	$6.20 (\pm 1.08) \times 10^{8ab}$	$3.30 (\pm 1.60) \times 10^{8a}$	$1.75 (\pm 2.10) \times 10^{7a}$
Overload stage	$8.56 (\pm 12.1) \times 10^{5c}$	$4.14 (\pm 1.40) \times 10^{3c}$	$2.08 (\pm 2.23) \times 10^{4a}$

<sup>a</sup> Values are means  $\pm$  SD,  $n=3$ . Mean values with different superscript letters in the same column indicate a significant difference at  $p < 0.05$

in this study. The maximum yield at phase 5 reached 56–58% of the theoretical value, which had obvious advantages compared with the lignocellulose digestion after physicochemical pretreatments (Mussoline et al. 2013). The higher CH<sub>4</sub> yields could be explained from three respects. First, mixed substrates, including easily digestible corn stalks and guinea grass, were beneficial to lignocellulose hydrolysis. In a previous study, Fuma et al. (2012) confirmed that bean husks facilitated the ability of the hydrolytic bacteria to colonize within the residues, to stimulate the fibrolytic enzymes of *Fibrobacter succinogenes* and then accelerate the biodegradation of recalcitrant rice straw. Second, the ruminal microbiota contained high amounts of lignocellulose-degrading consortia, which benefitted the hydrolytic efficiency (Dai et al. 2015). In addition, the methanogenic sludge enhanced the beneficial cooperation since the microbial interactions were involved in the hydrolysis of lignocelluloses (Azman et al. 2015; Miller et al. 2000; Rychlik and May 2000). Our previous study on the rumen co-inoculated anaerobic digestion of rice straw indicated that co-inoculation enhanced the hydrolysis and CH<sub>4</sub> yields, making digestion more efficient (Deng et al. 2017a).

In a previous study on single corn stalk degradation with recirculation, Hu et al. (2014) found that refractory degradable TOC components accumulating in the digestate resulted in the malfunction of the anaerobic digester. In addition, excessively high concentrations of VFAs will inhibit methanogenic activities (Hu et al. 2014; Nordberg et al. 2007; Shakeri Yekta et al. 2012; Xiao et al. 2013; Zhang et al. 2016). The concentrations over 8 g/L were noted as the threshold value of inhibition in mesophilic conditions (Karthikeyan and Visvanathan 2013). In the present study, the total VFA concentration of 9.09 g/L was accompanied by the inhibition of methanogenesis in the overload stage, making the CH<sub>4</sub> yield drop sharply (shown in Table 2).

Acetate composition performed a “multi-faceted” role, not only as the substrate of the acetotrophic methanogenesis but also as the inhibitor of methanogens when it is higher than the inhibition threshold. The investigation by Xiao et al. (2013) indicated that the threshold value was 3 g/L, which completely inhibited CH<sub>4</sub>-producing activity. In the present study, the acetate concentration was lower than the inhibition value in the whole digestion process. This also indicated that the acetotrophic methanogenesis was enhanced by co-

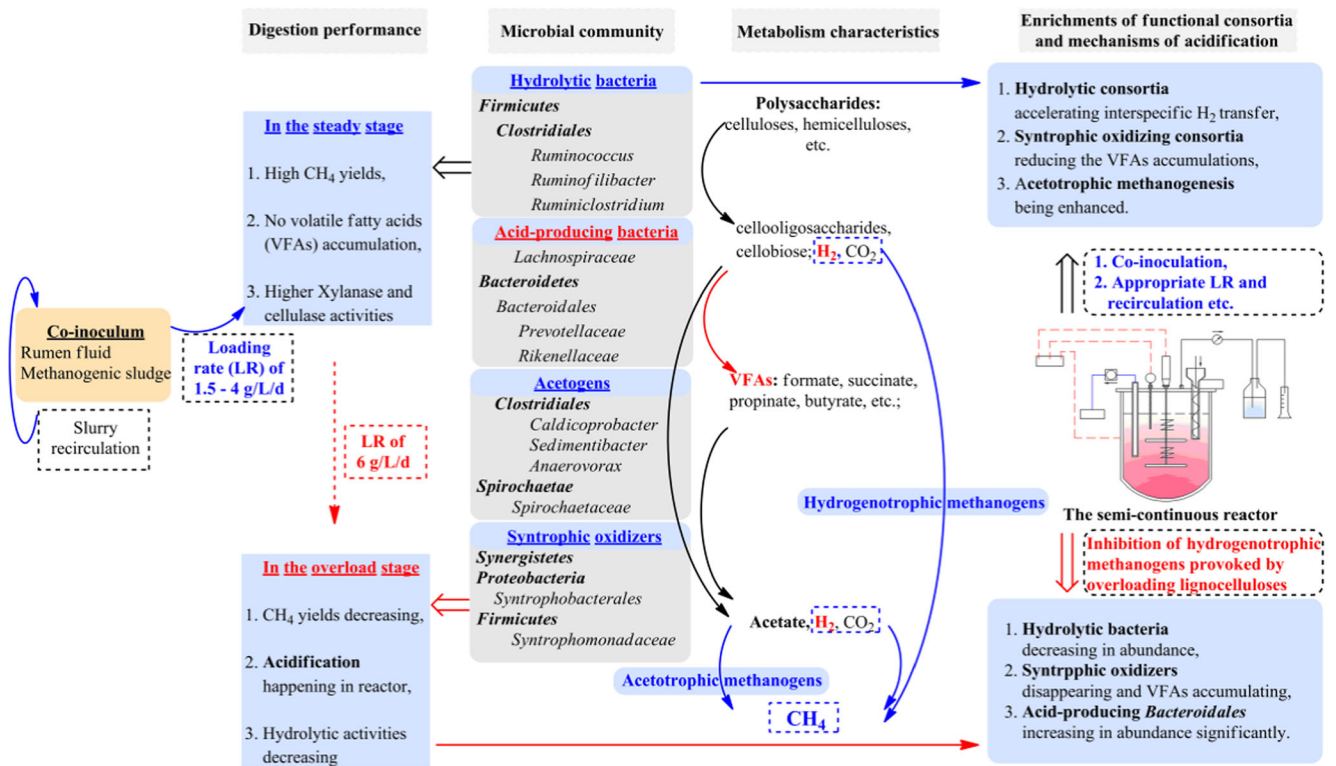
inoculation. In addition, Lerm et al. (2012) believed that syntrophic oxidation accelerated with increment of LRs. Once exceeding the metabolic capacities of the methanogens, the reducing H<sub>2</sub> could not be consumed and then metabolized via propionate and butyrate accumulation. In the present study, the average concentrations of propionate and butyrate reached 6.54 and 0.53 g/L respectively in the overload stage, which indicated that anaerobic digestion was inhibited (shown in Table 2).

Nitrogen is indispensable to microbial synthesis and metabolism. The NH<sub>4</sub><sup>+</sup> concentration with 0.20 g/L was of vital importance for anaerobic bacteria (Karthikeyan and Visvanathan 2013). However, the slurry recirculation could also cause an accumulation of NH<sub>4</sub><sup>+</sup>, as well as the TOC components, which eventually affected digestion performance. In a previous study on the anaerobic digestion of alfalfa silage, NH<sub>4</sub><sup>+</sup> inhibition eventually appeared at 100% liquid recirculation (Nordberg et al. 2007). It is generally believed that the NH<sub>4</sub><sup>+</sup> concentration at more than 1.50 g/L has obvious toxicity effects on the anaerobic reactor (Karthikeyan and Visvanathan 2013). In the present study, the initial nitrogen source mainly came from the co-inocula. The raw materials hardly brought nitrogen into the reactor because the lignocelluloses had a high C/N ratio of 46.82. The accumulated NH<sub>4</sub><sup>+</sup> was not the inhibition factor because of the low concentration. The increment of concentrations just reflected the organic nitrogen releasing from dying microbes in the overload stage.

### Biodegradability of lignocellulosic compositions

It is generally believed that CH<sub>4</sub> is produced from the degradation of cellulose and hemicellulose rather than lignin composition in the anaerobic digestion process (Lazuka et al. 2015). This is similar to the shifts of lignocellulosic compositions witnessed in the present study. As shown in Table 2, the corresponding hydrolytic activities in the steady phase were higher than those of the other two stages. The xylanase and cellulase activities at phase 5 had the highest values of 6.45 and 50.06 U/mL respectively, which were consistent with the CH<sub>4</sub> production rate. It also suggested that the hydrolysis process was still the rate-limiting step regulating lignocelluloses degradation, and the high hydrolytic activities were beneficial to the CH<sub>4</sub>-producing efficiency.





**Fig. 5** Enrichment of functional consortia under the semi-continuous conditions and mechanisms of acidification

The composition degradation rate dropped significantly in the overload stage, which illustrated that the hydrolytic activities were restrained. This phenomenon was explained from two respects. On the one hand, hydrolytic bacteria were inhibited by high VFA concentrations. In a previous study, Romsaiyud et al. (2009) found that high acetate concentration of over 1.80 g/L would affect the enzymatic cellulose hydrolysis, which led to the inhibition of the hydrolytic bacteria (Romsaiyud et al. 2009). In the present study, the acetate concentration of this stage was 2.02 g/L, higher than the inhibitory threshold (shown in Table 2). On the other hand, the methanogens were more easily inhibited by high VFA concentrations than cellulolytic microbes, resulting that H<sub>2</sub> produced during the hydrolysis process could be consumed by hydrogenotrophic methanogenesis. This would decrease hydrolytic efficiencies of lignocelluloses (Azman et al. 2015; Miller et al. 2000; Rychlik and May 2000).

### The characterizations of microbial community composition

The phyla *Bacteroidetes* and *Firmicutes* mostly dominated in the samples, which agreed with previous studies on the anaerobic digestion of lignocelluloses (Lazuka et al. 2015; Sun et al. 2016). Within the *Firmicutes*, *Clostridiales* members served predominant roles in polysaccharide digestion (Azman et al. 2015). In the steady stage, higher abundances of *Synergistetes* indicated that efficient syntrophic oxidations

were achieved (Liu et al. 2009). *Chloroflexi* populations were known to metabolize carbohydrate and microbial detritus (Miura and Okabe 2008; Sun et al. 2016). The *Cloacimonetes* bacteria were discovered to hydrolyze the celluloses or utilize fermentation products (Limam et al. 2014). The phylum *Proteobacteria* contained the genes coding for cellulolytic enzymes (Azman et al. 2015). Thus, the analysis of the dominant community composition accompanied with high CH<sub>4</sub> yields and hydrolytic activities suggested that these bacteria played an important role in the process of lignocellulosic hydrolysis and fermentation metabolism (shown in Table 2).

Within the main hydrolytic bacteria of start-up and steady stages, *Ruminococcus*, *Butyrivibrio fibrisolvens*, and *Fibrobacter* were highly efficient hydrolytic bacteria that settled in the rumen niche (Dai et al. 2015; Morrison et al. 2009; Nyonyo et al. 2014). *Ruminiclostridium* displayed anaerobic hydrolytic activities while some species of *Ruminofilibacter* (e.g., *Ruminofilibacter xylanolyticum*) processed hemicellulolytic activities (Azman et al. 2015; Kröber et al. 2009). Higher activities of cellulase and xylanase were then achieved in the reactor. Within the VFA-producing bacteria, many unclassified species of *Lachnospiraceae* belonged to soluble polysaccharide-degrading bacteria (Nyonyo et al. 2014). *Bacteroidales*, usually present in the anaerobic reactor, degraded various polysaccharides with the production of VFAs (Hatamoto et al. 2014). *Prevotellaceae* numbers, which were affiliated with the *Bacteroidales* order, can utilize pectin

to produce succinate or butyrate. It was suggested that the degradation was beneficial to the exposure of the cellulose portions (Fondevila and Dehority 1996; Lazuka et al. 2015). A significant increment of *Bacteroidales* in the abundance indicated that this order was involved in the acidification in the overload stage. In contrast, the higher abundances of acetogens (*Clostridiales* and *Spirochaetaceae*) illustrated efficient fermentation activities in the first two stages (FitzGerald et al. 2015).

It was regarded that syntrophic oxidation accelerated with the increment of LR within a certain range (Lerm et al. 2012). In the steady stage, the appearance of *Synergistetes* indicated that efficiently acetotrophic methanogenesis was attained (FitzGerald et al. 2015; Liu et al. 2009). Syntrophic oxidizers relieved the accumulation of propionate and butyrate. This also explained the performances of individual VFA in the co-inoculated system. In the overload stage, the proportions of propionate and butyrate oxidizers significantly decreased, especially *Syntrophobacter* disappeared from the reactor. This was attributed to the inhibition of oxidization and associated methanogenesis because of the overloading of lignocelluloses (Lerm et al. 2012; Müller et al. 2010).

The shifts of methanogenic compositions were used to illustrate how the operation parameters influenced the methanogenic community structure and bioconversion pathways (Steinmetz et al. 2016). Hydrogenotrophic *Methanobacteriales*, including *Methanobacterium*, *Methanobrevibacter*, and *Methanospaera*, were the most abundant methanogens in the rumen (Dai et al. 2015; Janssen and Kirs 2008). The members within this order could form functional consortia with hydrolytic bacteria and syntrophic oxidizers to accelerate the interspecies H<sub>2</sub> transfer (Miller et al. 2000; Rychlik and May 2000). The methylotrophic and aceticlastic *Methanosarcinales* (*Methanosarcina* and *Methanosaeta*) were indispensable for anaerobic digestion due to the rapid consumption of acetate (Demirel and Scherer 2008; FitzGerald et al. 2015). The increments of methanogen copies were achieved for the enhancement of methanogenic activities in the steady stage.

### Enrichments of functional consortia and mechanisms of acidification

As shown in Fig. 5, the ruminal microbiota co-inoculated with anaerobic sludge were enriched under the LR of 1.5–4 g/L/day and slurry recirculation. In view of the strengthening mechanism, co-inoculum by increasing the proportion of methanogens was beneficial to the formation of the functional consortia. First, hydrogenotrophic methanogens consumed the H<sub>2</sub> produced during the lignocellulosic digestion process, which made the NAD<sup>+</sup> of ruminal hydrolytic bacteria (*Ruminococcus*, *Fibrobacter*) regenerate, and then more ATP was produced by the cell. As a result, the digestion

efficiency of the hydrolytic consortia was enhanced (Miller et al. 2000; Rychlik and May 2000). In a study on the switchgrass digestion in the bovine rumen, Piao et al. (2014) found that *Methanobrevibacter* was inevitable for cellulolytic activities in situ. On the investigation of digestion performance enhanced by sheep rumen microbiota, microbial profiles suggested that enhancement cultures increased the abundances of *Ruminococcaceae* and hydrogenotrophic *Methanobacteriales*, which could enhance the CH<sub>4</sub> yields from wheat straw in batch reactors (Ozbayram et al. 2017). In our previous study of rumen-derived digestion of rice straw, it was indicated that microbial cooperation involving *Methanobrevibacter* and *Ruminococcus* was pivotal for digestion activities (Deng et al. 2017a). Thus, the high hydrolytic activities and CH<sub>4</sub> yields would contribute to the effect of the methanogenic sludge co-inoculated into the artificial reactor in the present study. Consequently, the predominant ruminal bacteria involving *Ruminococcus* and *Fibrobacter* settled down in the start-up and steady stages, which was different from other studies in biogas plants (Azman et al. 2015; Sun et al. 2016).

In the liquid phase, VFAs were consumed only when syntrophic oxidizing consortia formed because the methanogens could overcome the unfeasible thermodynamics by lowering the H<sub>2</sub> concentrations. Hydrogenotrophic *Methanospirillum* and *Methanobacterium* were usually regarded as syntrophic partners (Müller et al. 2010). In the present study, the obvious increments of *Methanobacteriales* could accelerate H<sub>2</sub> transfer and then made the syntrophic oxidations more efficient. This also explained the reactor performance of no propionate and butyrate accumulation in the steady stage.

In the overload stage with 6 g/L/day LR, the TOC concentrations in the slurry increased rapidly to 13.67 from 9.28 g/L, and the total VFA concentration reached 9.09 g/L. Subsequently, the copies of total archaea, especially *Methanobacteriales*, decreased significantly ( $p < 0.05$ ), and the CH<sub>4</sub> yield dropped to 0.11 L/g VS. Lerm et al. (2012) also found that hydrogenotrophic populations were especially important for the reactor, and the VFAs accumulated along with disappearance of *Methanobacterium*. As a result, the unconsumed H<sub>2</sub> then suppressed the hydrolytic activities and syntrophic oxidations, making the corresponding functional bacteria decline in abundance. At the same time, *Bacteroidales* increased significantly ( $p < 0.05$ ), and the *Prevotellaceae* and *Rikenellaceae* members within the order could produce excessive VFAs. These acid-producing bacteria were found to be influenced slightly by the inhibiting components in the liquid fractions (Hu et al. 2007; Hu and Yu 2005; Lerm et al. 2012; Zhang et al. 2016). Thus, the inhibition of hydrogenotrophic methanogens provoked by overloading lignocelluloses eventually decreased the digestion efficiency in the semi-continuous reactor.

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## Compliance with ethical standards

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

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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