Anaerobic Digestion Characteristics and Key Microorganisms Associated With Low-Temperature Rapeseed Cake and Sheep Manure Fermentation

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Abstract

Qinghai rural household biogas digesters were used to evaluate fermentation characteristics, including gas production and key microbial community changes, associated with low-temperature (15.2-17.8℃) mixed rapeseed cake and sheep manure anaerobic fermentations across 40 days using seven different ratios of material. Different raw material ratios resulted in significantly different effects on biogas yields and microbial community compositions. When the ratio of sheep manure to rapeseed cake was 1:2, the highest level of cumulative gas production was observed (122.92 $m^3 \cdot t^{-1}$). Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant bacterial phyla among the 29 digester samples (total relative abundances > 79.23%), followed by Synergistetes (4.09%-10.7%). *Lactobacillus* was the most abundant genus in the biogas digesters with high rapeseed cake contents (average relative abundances: 14.68%), while *Peptoniphilus* exhibited higher abundances (12.69%) in the mixed fermentation digester treatments. In addition, unclassified Synergistaceae abundances (6.64%) were positively associated with biogas production variation among treatments. *Bacteroides* (5.74%) and *Pseudomonas* (5.24%) both accounted for larger proportions of communities in the digesters that used more sheep manure. Methanomicrobiales (66.55%) was the most dominant archaeal group among digesters, with *Methanogenium* (41.82%) and *Methanoculleus* (16.55%) representing the main gas-producing archaeal genera; they were more abundant in biogas digesters with higher sheep manure contents and higher rapeseed cake contents, respectively. Regardless of the raw material ratios, *Methanoculleus* exhibited the highest abundances on the 4th day of fermentation. VFAs and pH were the main factors associated with differences in microbial communities among the 29 samples. Specifically, VFA concentrations were positively correlated with *Lactobacillus* and *Methanoculleus* abundances, while pH was positively correlated with *Bacteroides*, *Pseudomonas*, *Methanobrevibacter*, and *Methanobacterium* abundances.

Introduction

Rapeseed cake is a by-product that is obtained by pressing rapeseed to make oils. The cakes have abundant protein, amino acid, and other nutrient components and are agricultural solid wastes that can be reused as important resources. The annual global output of rapeseed cake is about 40 million tons (Stein et al. 2016). Concomitantly, the annual output of livestock and poultry manure in China is estimated to be 3.26 billion tons (Wang et al. 2019). The rampant accumulation and inefficient treatment of agricultural wastes like rapeseed cake and sheep manure have caused serious environmental pollution problems in China.

Anaerobic fermentation technology is a clean and efficient way to utilize agricultural waste resources. These techniques can effectively reduce the consumption of non-renewable energy and biomass resources in China, while also promoting the sound development of ecological agriculture (Wang et al. 2020; Zhai et al. 2020). Qinghai Province is located in the northeastern region of the Qinghai-Tibet Plateau and is situated at an average altitude of over 4,000 m and exhibits an average annual temperature of only 7.2°C. Low temperatures affect the development of the biogas industry in Qinghai, resulting in biogas utilization efficiency being far lower in this area compared to low altitude areas. Thus, numerous agricultural biogas digesters have been abandoned in the area and these wasted resources represent a serious problem (Tian et al. 2019; Yang *et al*. 2020; Han et al. 2020). However, the selection of easily degradable raw materials (Li et al. 2020), the fermentation of mixed substrate components (Shi et al. 2021) and fermentations with high solid contents (Elmitwalli et al. 2004) can adequately reduce environmental condition requirements for fermentation. Further, they can partially compensate for the low gas production rates caused by low temperatures. Consequently, research into high solid content and low temperature mixed anaerobic fermentation of rapeseed cake and sheep manure carries important significance for the development of biogas application technologies under the low temperature conditions present in Qinghai Province.

Anaerobic digestion is a complex microbiological process that is accomplished by a variety of anaerobic microorganisms, rendering it difficult to comprehensively evaluate these ecosystems using traditional culture-based microbiological analyses. However, 16S rRNA gene high-throughput sequencing can accurately assess the structural characteristics of various microbial communities using genetic techniques. High-throughput sequencing techniques have been widely used to study

complex microbial communities. Arelli et al. (2021), Bae et al. (2020), and Tao et al. (2021) have applied this technology to investigate the microbial community structural characteristics during co-digestion of meal waste and waste sludge. Acetyl compound catalysis is the primary metabolic pathway associated with digestion, and increased food waste levels leads to higher microbial abundances in digestors. Jang et al. (2014) used high-throughput sequencing technology to reveal the impact of different organic loads on the microbial community in the anaerobic digestion of wastewater.Likewise, high-throughput sequencing was used by Ndubuisi-Nnaji et al. (2020) to investigate changes in the microbial community structures during the co-digestion of corn stover, rice straw, and sheep manure, observing that the co-digestion microbial communities exhibited higher abundances. Yang et al. (2019) also used high-throughput sequencing and observed that the most favorable metabolic pathway during pig manure fermentation was the production of hydrogen in some communities, followed by the reduction of carbon dioxide to produce methane.

Previous studies have referred to anaerobic fermentation below 20°C as low-temperature fermentation, and fermentation conditions of rural household biogas digesters in Qinghai Province belong to this category (Han et al. 2020; Tiwari et al. 2021). In this study, we investigated rural household biogas digesters that were used as fermentation devices to conduct low-temperature (15.2-17.8°C) pilot experiments to specifically evaluate biogas production by mixed anaerobic fermentation of rapeseed cake and sheep manure using seven different ratios of high solid content waste. In addition, the microbial community structural dynamics were analyzed across the fermentation process. The results provide insights into the associations among microbial communities and environmental factors in digesters of this area, revealing relationships between the structures and functions of microbial communities, thus providing a theoretical basis for better resource utilization of agricultural wastes like rapeseed cake and sheep manure. These results also provide a technological framework that can be used to promote the application of agricultural wastes during low-temperature anaerobic fermentation treatment technology in Qinghai Province and other cold regions of northern China.

Materials And Methods

Experimental materials

Rapeseed cake was collected from the Farmers' Market at Duoba Town in Huangzhong County of Qinghai Province and ground to a particle size of less than 30 mm. Sheep manure was retrieved from Guinan County in Qinghai Province. The inoculum was taken from a rural household biogas digester fermented with sheep manure from Qinghai Province. Fermentation material and inoculum characteristics are shown in Table 1.

Table 1
Physical and chemical properties of raw materials and inocula used for digesters in this study

Test sample	pH	TS (%)	VS (%)	C (%)	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Crude protein (%)
Rapeseed cake	6.34±0.1	92.35±0.3	84.88±0.2	45.3±0.5	10.91±0.6	3.37±0.1	3.35±0.1	30.5±0.6
Sheep manure	7.87±0.0	38.48±0.0	30.07±0.1	38.24±0.4	33.73±0.4	14.50±0.3	9.81±0.5	11.63±0.2
Inoculum	7.31±0.1	2.37±0.1	1.28±0.2	27.41±0.9	ND	ND	ND	ND
Note: ND means not determined								

Experimental digesters

Rural household biogas digesters from Qinghai Province (36°3'11"N, 101°19'36"E) were used as fermentation devices. All biogas digesters exhibited identical structures and comprised underground hydraulic spherical biogas digesters with a

volume of 8 m^3 and a charging volume of about 5 m^3. Each biogas digester was connected to a biogas flow meter to record biogas production. A Rc-4 temperature recorder was connected to each biogas digester that automatically recorded fermentation temperatures every 2 h.

Experimental design

Rapeseed cake and sheep manure were mixed using seven ratios (Table 2) including 1) pure sheep manure (S), 2) pure rapeseed cake (R), 3) sheep manure and rapeseed cake mixed at a 1:1 ratio (S_1R_1), 4) sheep manure and rapeseed cake mixed in a 2:1 ratio (S_2R_1), 5) sheep manure and rapeseed cake mixed at a 3:1 ratio (S_3R_1), 6) sheep manure and rapeseed cake mixed at a 4:1 ratio (S_4R_1), and 7) sheep manure and rapeseed cake mixed at a 1:2 ratio (S_1R_2). The total dry matter used in each group was equivalent, and the solid content was adjusted to 16%, with two parallel experiments being used for each group. In each treatment, different raw materials were evenly stirred with a specified level of biogas slurry (with moisture content adjusted to 70%), covered with plastic film for heap retting over five days, and stirred every day during heap retting. Digester materials were mixed with 1,500 kg of inoculum (30% inoculum) and then biogas slurry was supplemented to a volume of 5 m^3. The biogas flow rate was recorded regularly every day starting on the second day after filling the biogas digesters. Fermentation liquid was collected from the biogas digester waters for sampling every three days with a self-constructed sampler to determine the physical and chemical properties of the fluids in addition to digester microbial community structures.

Preliminary data indicated that Qinghai rural household biogas digesters exhibited the highest fermentation temperatures from early to mid-August into mid-September, suggesting that these were the most suitable times for anaerobic fermentation[7]. The experiments of this study were consequently initiated on August 3, 2020 and ended on September 11, 2020, comprising a total of 40 days. The fermentation temperature range for each group was 15.2-17.8°C and the temperature difference before and after fermentation was 0.9-1.3°C following self-stabilization, indicating an essentially constant fermentation temperature within the biogas digesters.

Table 2
Amount of raw materials added to digesters used in this study

Treatment group	Rapeseed cake (kg)	Sheep manure (kg)
S	0.00	1,986.62
R	827.77	0.00
S_1R_1	413.89	993.31
S_2R_1	275.92	1,324.41
S_3R_1	206.94	1,489.96
S_4R_1	165.55	1,589.29
S_1R_2	551.85	662.21
Note: Weight was calculated based on dry matter in units of kg.		

Fermentation physicochemical properties

Total solids (TS) and volatile solids (VS) were determined using the drying method, wherein the total solids were dried in an oven at 105°C for 24 h and the volatile solids were burned in a muffle furnace at 550°C for 3 h. Ammonia nitrogen content was determined as previously described (Hu et al. 2017), along with volatile fatty acid (VFA) concentration measurements

(Shi et al. 2021). pH was measured with a pH meter (pHS-2F), and alkalinity content was measured with a potentiometric titrator (ZDJ-4A). Total carbon content was determined as previously described (Kainthola et al. 2020), as were lignin, cellulose, and hemicellulose content measurements (Li et al. 2020), in addition to crude protein content measurements (Bae et al. 2020).

Biogas production modeling

Dynamic models can express experimental results with more precise mathematical formulas, enabling a more in-depth understanding of complex anaerobic digestion processes and providing a corresponding theoretical framework (Zhang et al. 2016). The Gompertz equation (Eq. 1) was used to describe biogas production potential by fitting the model to cumulative biogas production from anaerobic fermentation of rapeseed cake and sheep manure:

$$P = P_m \cdot \exp\left\{-\exp\left[\frac{R_m \cdot e}{P_m}(\lambda - t) + 1\right]\right\}$$

In the equation, P is the cumulative methane production of VS corresponding to time T (in $m^3 \cdot t^{-1}$), Pm is the final cumulative VS biogas production (in $m^3 \cdot t^{-1}$), Rm is the maximum VS gas production rate (in $m^3 \cdot (t \cdot d)^{-1}$), λ is the gas production retention time (in d), T is the fermentation time (in d), and E is the constant exp (1) = 2.7183. The model fitting results were used to calculate the maximum VS gas production rate, Rm, and the gas production retention time, λ.

Sample collection

Seven biogas digesters with different raw material ratios were used in this study and biogas slurry samples were taken on the 4th, 16th, 28th, and 40th days of fermentation. Biogas slurry samples from the upper, middle, and lower layers of the biogas digesters were collected and mixed during sampling. Samples in each fermentation group were identified by the group and the fermentation time (e.g., for the S group: S_4, S_16, S_28, and S_40). Digesters inoculated with sheep manure as fermentation substrate were used as controls and identified as the CK samples.

Extractionof digester sludge genomic DNA

Sludge sample genomic DNA was extracted using a Qiagen QIAamp Fast DNA Stool Mini Kit and quality was evaluated with 1% agarose gel electrophoresis, wherein a clear primary DNA band without signs of degradation was required for further analysis. DNA concentrations and purities were then evaluated using a NanoDrop 2000 ultra-micro spectrophotometer prior to sequencing, with requirements of sample concentrations > 10 ng/μL, total sample volume > 500 ng, and $A_{260/280}$ values ranging from 1.8 to 2.0.

High-throughput sequencing

High-throughput sequencing of bacterial and archaeal 16S rRNA genes was conducted at Hangzhou Lianchuan Biotechnology Co., Ltd. Bacterial-specific PCR primers were used to amplify 16S rRNA gene fragments including 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), while the archaeal-specific primers, 'F' (5'-TGYCAGCCGCCGCGGTAA-3') and 'R' (5'-YCCGGCGTTGAVTCCAATT-3'), were used in separate reactions. PCR products were confirmed by 2% agarose gel electrophoresis. Amplicon library quality was evaluated with an Agilent 2100 Bioanalyzer (Agilent, USA) and Illumina (Kapa Biosciences, Woburn, MA, USA) library quantification kits, followed by paired-end sequencing on the NovaSeq PE250 platform.

Data optimization and analysis

DNA amplicons were sequenced on the Illumina NovaSeq platform according to the manufacturer's recommendations. Oligonucleotide barcodes were used to assign sequences to samples, followed by removal of barcode and primer sequences. The FLASH program was used to merge the paired-end reads. The fqtrim program (v0.94) was then used to filter the sequence data to obtain high-quality clean sequence tags. Finally, the Vsearch software program was used to identify and remove chimeric sequences (v2.3.4). The complexity of species diversity was evaluated in samples through five alpha diversity indicators including the Chao1, observed species, reads, Good’s coverage, and the Shannon diversity indices. Chao1 index was used to microbial richness. The higher Chao1 index indicate greater richness in the samples. Shannon index was used to measure microbial diversity. The greater Shannon index indicate higher diversity of the species in the sample. Sequence alignment was conducted with BLAST, and each representative sequence was taxonomically annotated using the SILVA database. Community composition within each sample was then summarized at the phylum and genus taxonomic classification levels for bacteria and at the order and genus levels for Archaea. The R language ‘Vegan’ package was used to construct a taxonomic classification heatmap and conduct RDA analysis.

Sequence accession numbers

Raw 16S rRNA gene sequences were deposited in the NCBI database under the BioSample accession Nos.SAMN21841898-SAMN21841926 for Bacteria and SAMN21842788-SAMN21842816 for Archaea.

Results And Discussion

Gas production among treatment groups

Changes in daily gas production and cumulative gas production among the treatment groups are shown in Fig. 1. Across 40 days, daily biogas production in each treatment group exhibited two to three peaks around 7–20 days. All of the mixed fermentation groups exhibited the highest peaks on the 7th day and they were higher than those for pure sheep manure fermentation (Fig. 1a). The S_1R_2 and S_1R_1 groups exhibited the highest peaks, reaching 6.897 and 7.193 m^3, respectively. Gas production ceased in the R group at 30 days, thereby completing the digestion process earlier than in the other treatments. The cumulative gas production in the six treatment groups (excluding the R group) clearly trended upwards over the first 20 days, followed by gradual increases in the treatments with higher rapeseed cake addition, and then a gradual slowing of the increasing trend (Fig. 1b).

Low temperatures considerably inhibit biogas production in fermentation systems, but rapeseed cake exhibits advantages of easy degradation and abundant nitrogen nutrient components that can balance C/N ratios during fermentation and improve biogas production under adverse conditions (Stein *et al*. 2008; Yang *et al*. 2020; Han et al. 2020). In this study, biogas production from R and S group treatments at low temperatures were 87.584 $m^3 \cdot t^{-1}$ and 42.663 $m^3 \cdot t^{-1}$, respectively. Comparison of the same conditions indicated that biogas production using rapeseed cake was 2.05 times that using sheep manure and it was also much higher than the 30 $mL \cdot g^{-1}$ VS from low-temperature pig manure biogas production that was observed by Yao et al. (2020). Thus, the methane production potential from rapeseed cake was much greater at low temperature than from livestock and poultry manure, suggesting that the former is a good material for fermentation. Compared with fermentation using rapeseed cake and sheep manure alone, mixed fermentation greatly improved methane production. The S_1R_2 group exhibited the largest cumulative gas production, reaching 122.92 $m^3 \cdot t^{-1}$, which was higher than the maximum methane production of 102.2 $mL \cdot g^{-1}$ observed by Kuang et al. (2020). Thus, mixed fermentation can balance the nutrient contents of fermentation raw materials and improve the low digestion efficiency of single-material fermentation, thereby increasing biogas production under adverse conditions (Zhao et al. 2018; Aworanti et al. 2017).

Dynamics of biogas production

The key parameters for fitting cumulative fermentation gas production using different ratios of raw materials were modeled with a modified Gompertz equation (Table 3). The R^2 of all treatment groups was greater than 0.99, indicating a good model fit. The P, P_m, and R_m parameters of the S_1R_2 group were the largest. The P, P_m, and R_m parameters in the mixed fermentation increased with increasing rapeseed cake addition, while λdecreased with increasing rapeseed cake addition. Thus, rapeseed cake was easy to degrade, and this process was synergistically associated with biogas production from sheep manure that improved the methane-producing rate and system stability.

Table 3
Parameters for the modified Gompertz model for biogas production among digester treatment groups

Treatment group	P ($m^3 \cdot t^{-1}$)	P_m ($m^3 \cdot t^{-1}$)	R_m ($m^3 \cdot (t \cdot d)^{-1}$)	λ (d)	R^2
S	42.663	43.081	1.981	2.362	0.999
R	87.584	89.233	5.134	0.729	0.997
S_1R_1	100.696	97.183	6.327	1.467	0.994
S_2R_1	88.068	87.398	4.854	1.474	0.998
S_3R_1	71.914	69.745	4.231	1.596	0.996
S_4R_1	67.614	66.187	4.057	2.108	0.998
S_1R_2	122.921	124.742	6.766	1.455	0.998

Changesin VFA contents, alkalinity, ammonia nitrogen, and pH during fermentation

Volatile fatty acids (VFAs), alkalinity (AK), ammonia nitrogen (AN) and pH are important parameters for evaluating the effects of anaerobic fermentation and can reflect the stability of fermentation systems and the effective operation of anaerobic digestion. Generally, excessive accumulation of VFAs, too low alkalinity levels, too high ammonia nitrogen concentrations, and too low pH may all lead to collapsed fermentation systems (Lin et al. 2011). These parameters exhibited very different values in the S and R groups (Table 4 and Fig. 1), indicating that these properties were influenced by substrate materials. In general, the VFA concentrations of the four samples in the R group were higher, while the pH was lower, leading to a somewhat unstable system. When rapeseed cake and sheep manure were included in a mixed fermentation, the key parameter properties of fermentation broths were improved and near normal.

Table 4
Physical and chemical properties of different digester treatment groups

Treatment group	VFAs (mg/L)	pH	AN (mg/L)	AK (mg/L)
S_4	2,781	7.50	830	6,332
S_16	2,482	7.51	853	7,135
S_28	2,795	7.44	827	6,591
S_40	2,321	7.44	800	5,915
R_4	8,981	5.54	768	4,970
R_16	3,167	6.32	619	5,039
R_28	8,677	5.70	602	3,157
R_40	9,547	6.04	532	2,829
S_1R_1_4	4,327	5.92	796	5,470
S_1R_1_16	3,553	6.49	760	5,627
S_1R_1_28	3,547	6.47	723	4,995
S_1R_1_40	3,245	6.52	680	4,500
S_2R_1_4	4,005	6.44	812	5,509
S_2R_1_16	3,009	6.58	831	6,093
S_2R_1_28	3,470	6.48	769	5,112
S_2R_1_40	3,096	6.50	718	5,341
S_3R_1_4	3,698	6.56	811	5,594
S_3R_1_16	3,144	6.61	809	6,286
S_3R_1_28	3,101	6.62	721	5,715
S_3R_1_40	3,000	6.60	752	5,565
S_4R_1_4	3,561	6.77	823	5,570
S_4R_1_16	2,828	6.71	839	5,834
S_4R_1_28	3,148	6.79	810	5,742
S_4R_1_40	2,875	6.79	750	5,629
S_1R_2_4	4,532	5.80	779	5,512
S_1R_2_16	3,422	6.49	740	5,665
S_1R_2_28	3,734	6.54	729	5,123
S_1R_2_40	3,683	6.47	677	4,140

Microbial diversity analysis

A total of 2,257,936 and 2,915,212 high-quality bacterial and archaeal gene sequences, respectively, were obtained from the 29 samples after quality filtering, resulting in a total of 18,868 bacterial OTUs and 5,485 archaeal OTUs (Table 5). Diversity coverage for all samples was estimated at higher than 0.994, suggesting adequate sampling of native sample diversity (Table 5). The Chao and Shannon index values for mixed fermentation communities (bacterial and archaeal) were both higher than those for single fermentation communities. Further, the 1:2 sheep manure and rapeseed cake mixture treatment group exhibited the highest bacterial and archaeal diversity. Thus, microbial community richness and diversity were highest in groups with higher gas production.

Table 5
Bacterial and Archaeal community richness and diversity metrics for biogas digesters using different raw material ratios

Sample	Bacteria					Archaea				
	Reads	OTUs	Chao1	Shannon	Coverage	Reads	OTUs	Chao1	Shannon	Coverage
S_4	75,892	528	550.10	6.09	1	100,323	52	52.00	0.47	1
S_16	70,431	365	382.00	5.80	1	108,495	50	50.00	0.44	1
S_28	75,477	276	282.38	5.60	1	106,798	51	53.00	0.49	1
S_40	74,162	367	387.00	5.45	1	98,301	42	42.00	0.47	0.997
R_4	77,687	630	643.32	6.00	1	121,037	80	81.50	2.39	1
R_16	78,843	503	513.88	5.48	1	109,752	55	55.00	1.44	1
R_28	84,161	393	405.35	4.91	1	107,095	58	58.00	1.47	1
R_40	82,191	408	414.25	5.03	1	126,276	67	67.00	1.11	1
S_1R_1_4	80,948	762	791.81	6.79	1	86,500	275	276.91	3.92	1
S_1R_1_16	84,053	1031	1,034.58	7.73	1	118,312	261	261.67	4.39	1
S_1R_1_28	80,515	1056	1,088.44	7.67	1	113,685	351	351.00	4.79	1
S_1R_1_40	75,257	988	1,015.51	7.55	1	99,030	328	328.46	4.80	1
S_2R_1_4	80,499	558	575.07	5.66	1	90,458	222	222.60	3.76	1
S_2R_1_16	84,928	637	680.50	5.62	1	94,405	198	198.00	4.23	1
S_2R_1_28	78,095	559	589.64	5.75	1	96,694	226	226.60	4.20	1
S_2R_1_40	82,061	628	664.18	5.64	1	108,885	283	286.21	4.70	1
S_3R_1_4	70,415	529	545.59	5.69	1	102,956	222	122.00	2.54	1
S_3R_1_16	79,185	313	313.00	5.98	1	85,881	264	164.50	2.46	1
S_3R_1_28	79,954	589	600.47	6.01	1	71,215	249	257.00	3.28	1
S_3R_1_40	76,032	747	769.55	6.50	1	80,346	189	189.00	3.11	1
S_4R_1_4	67,787	561	582.21	6.12	1	76,973	106	106.41	1.47	0.995
S_4R_1_16	77,419	629	635.00	6.26	1	113,560	170	173.50	1.56	1
S_4R_1_28	77,615	554	555.31	6.07	1	91,488	167	167.50	1.97	1
S_4R_1_40	74,240	378	387.46	5.26	1	99,334	149	149.00	1.93	1
S_1R_2_4	83,654	793	809.92	7.17	1	110,863	360	361.15	5.18	1
S_1R_2_16	75,892	1,235	1,274.38	8.08	1	108,000	466	479.36	5.70	1
S_1R_2_28	75,851	1,050	1,079.56	7.81	1	100,617	273	273.00	3.97	1
S_1R_2_40	78,821	1,196	1,222.91	8.11	1	93,274	190	190.00	3.09	1

Sample	Bacteria					Archaea				
	Reads	OTUs	Chao1	Shannon	Coverage	Reads	OTUs	Chao1	Shannon	Coverage
CK	75,871	405	552.70	5.37	0.994	94,659	81	87.56	1.94	1

Bacterial community analysis

A total of seven bacterial phyla and seven bacterial genera with relative abundances > 1% were identified in the biogas digesters (Fig. 2). Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant phyla among all samples, comprising total relative abundances of > 79.23% (Fig. 2a). The proportions of these taxa were relatively similar among biogas digesters and did not considerably differ with different raw material treatments. Thus, these groups are likely the most generally important taxa in digester fermentation systems and likely play important roles in hydrolyzing macromolecules, producing acid, and maintaining the stability of fermentation systems (Ng et al. 2016; Ariesyady et al. 2007; Regueiro et al. 2012; Li et al. 2015). The four aforementioned phyla accounted for the highest proportions (92.16%) in the S group communities and the lowest proportions (79.23%) in the CK communities. Synergistetes were also prominent members of communities, exhibiting relative abundances of 7.30%, and their abundances varied with gas production. Synergistetes were most abundant in the S_1R_2 group (10.7%) and least abundant in the S group (4.09%). Synergistetes and methanogens exhibit mutualistic metabolic relationships and typically are some of the core microbial groups in anaerobic digestion that contribute to methane production in digester systems (Baena et al. 2000). Temporal variation was also observed for bacterial taxa within each biogas digester treatment. For example, Synergistetes exhibited higher relative abundances on the 16th day in each group. Gas production in each group was relatively abundant before and after fermentation (Fig. 1), indicating that Synergistetes could play a critical role in gas production. Firmicutes exhibited higher relative abundances on the 4th day in the biogas digesters of the R, S_1R_1, and S_1R_2 groups, while Actinobacteria exhibited increased relative abundances starting on the 16th day. Firmicutes degrade acidic substances that are produced in the early stage of fermentation, consistent with the above temporal variation in their abundances. The presence of Actinobacteria in the digesters could be due to the high sulfide content in rapeseed cake that would be degraded in the medium term (Ariesyady et al. 2007). Proteobacteria relative abundances rapidly increased with increased fermentation time in the biogas digesters of the S, S_3R_1, and S_4R_1 groups, peaking on the 28th day (44.27%, 30.75%, and 29.77%, respectively). Lastly, Bacteroidetes exhibited their highest relative abundances on the 4th day of fermentation in the above groups (35.08%, 23.24%, and 36.09%, respectively).

At the genus level, *Lactobacillus* (relative abundances of 14.68%), *Peptoniphilus* (12.69%), unclassified Synergistaceae(6.64%), *Bacteroides* (5.74%), and *Pseudomonas* (5.24%) were dominant among all samples (Fig. 2b). *Lactobacillus* are members of the Firmicutes and have strong tolerance to acid, growing well under initial acidic conditions in digesters (Yang et al. 2019). *Lactobacillus* relative abundances decreased across fermentation time, with highest abundances in the R group (61.9%), followed by the S_1R_2 (35.88%) and S_1R_1 (21.2%) groups. *Peptoniphilus* are often found in feces (Ryu et al. 2021; Tiezzi et al. 2020) and degrade many substances including proteins, fats, and carbohydrates, but can also convert acetic acid and lactic acid into H_2 and CO_2(Jang et al. 2014; Luo *et al.* 2014). *Peptoniphilus* were relatively abundant in the mixed fermentation treatment groups. Mixed fermentation may balance the nutrients in the fermentation systems, rendering it more suitable for the growth of these and other bacterial taxa (Kainthola et al. 2020; Zhao et al. 2018). Unclassified Synergistaceae are members of the Synergistetes and exhibited relative abundance distributions consistent with those of Synergistetes, wherein increased methane contents corresponded to their higher relative abundances. *Bacteroides* and *Pseudomonas* are members of Bacteroidetes and Proteobacteria, respectively, and play important roles in the efficient degradation of organic matter, often representing the dominant taxa in fermentation systems that use manure as raw materials (Ndubuisi-Nnaji et al. 2020). The relative abundances of *Pseudomonas* were higher in the S and CK groups (35.78% and 12.41%, respectively) relative to the other groups. When sheep manure and rapeseed cake were used in mixed fermentation, *Pseudomonas* abundances sharply dropped to almost zero. The relative abundances of *Bacteroides* were

higher in the S_2R_1, S, S_3R_1, S_4R_1, and CK groups (10.19%, 9.79%, 9.35%, 7.12%, and 7.05%, respectively), and their abundances all peaked on the 4th day of fermentation, followed by sharp decreases.

Archaeal community analysis

A total of five archaeal orders and 10 genera (relative abundances > 1%) were identified in biogas digesters with different raw material components (Fig. 3). At the order level, Methanomicrobiales was the most dominant group, with average relative abundances of 66.55% among the digesters. Methanosarcinales was the second most dominant group, with average relative abundances of 20.63% across digesters (Fig. 3a). Methanomicrobiales are often dominant in low-temperature biogas fermentation environments (McKeown et al. 2012) and exhibited increased abundances with increased rapeseed cake addition, such as observed in the R (71.18%-74.22% relative abundances) and S_1R_2 (69.16%-72.13%) groups. The proportions of Methanobacteriales in each group fluctuated to some extent, but overall trends were not obvious, and thus, substrate ratios did not obviously affect their abundances.

Among the nine archaeal genera identified in the 29 samples, *Methanogenium* was the most dominant (average relative abundances of 41.82%), followed by *Methanoculleus* and *Methanobrevibacter* (16.55 and 14.33%, respectively), and then *Nitrososphaera* and *Methanocorpusculum* (7.99% and 7.57%, respectively). The above methanogens exhibit hydrogenotrophic methanogenesis pathways (Fig. 3b). *Methanogenium*, *Methanoculleus*, and *Methanocorpusculum* are all members of the Methanomicrobiales, with *Methanogenium* and *Methanocorpusculum* being common in low-temperature environments. The former has a wide distribution range, with the lowest growth temperatures being observed at 0°C. In the low temperature environments of Qinghai, *Methanogenium* are often dominant (Han et al. 2021; Asakawa *et al*. 2003; Chong et al. 2002), consistent with the rural household biogas digesters in this study (15.2–17.8°C), where they were also dominant. Likewise, *Methanocorpusculum* often appear in low-temperature anaerobic digestion systems that use feces as raw materials (Han et al. 2021; Gao et al. 2014). Among the above methanogens, *Methanogenium* exhibited the highest relative abundances (56.57%) in the S group that used sheep manure as raw material, while the abundances of *Methanocorpusculum* were relatively equivalent among groups. *Methanoculleus*are often present in fermentation systems with high organic loads and exhibit high tolerance to environmental conditions with high VFA concentrations (Bae et al. 2020; Mathai et al. 2020). *Methanoculleus* exhibited abundances as high as 35.86% in the R group that had average VFA concentrations of 7,593 mg/L. In addition, *Methanoculleus* exhibited their highest relative abundances on the 4th day of fermentation, regardless of materials used in the treatment, and their abundances were consistent with variation in VFAs (Table 4). *Methanobrevibacter* are members of the Methanobacteriales. Although this genus often appears in low-temperature environments, its existence may be more dependent on the characteristics of fermentation materials, especially in anaerobic fermentation systems using livestock manure as raw materials, where it exhibits high relative abundances (Rodriguez-Sanchez et al. 2020; Shanmugam et al. 2014). The relative abundances of *Methanobrevibacter* were highest in this study in the mixed fermentation groups that had relatively high contents of sheep manure, including the S_4R_1 and S_3R_1 groups (17.13% and 17.12%, respectively), followed by the S_1R_1 and S_1R_2 groups (14.83% and 13.69%, respectively). Thus, it is likely that *Methanobrevibacter* is more adapted to the mixed anaerobic environments that were dominated by feces and may play important roles in methane production (Shanmugam et al. 2014). Although *Nitrososphaera* did not participate in the methanogenic pathway, it often appears in high nitrogen environments such as feces (Enebe *et al*. 2021; Pizzeghello et al. 2021). The relative abundances of *Nitrososphaera* were positively correlated with high contents of sheep manure in this study, which was reflected in the group with more sheep manure, the higher its relative abundances.

OTU distributions

To understand the overall differences in communities across biogas digesters, the distributions of the 30 most abundant OTUs were evaluated (Fig. 4). Among the 30 bacterial genera and OTUs with the highest abundances, shared genera primarily originated from seven phyla. Firmicutes (46.74%) exhibited the highest relative abundances, followed by the

Proteobacteria (15.76%), Bacteroidetes (13.24%), Actinobacteria (11.3%), and Synergistetes (7.3%) (Fig. 4a). The abundances of genera among biogas digesters with different starting material ratios were quite different. For example, *Lactobacillus* abundances were as high as 56.65%, 27.13%, and 19.95% in the R, S_1R_2, and S_1R_1 group biogas digesters, respectively, but their abundances were extremely low in the biogas digesters with higher sheep manure contents.

Shared archaeal genera among the digesters mainly derived from 14 orders, with the highest relative abundances corresponding to the Methanomicrobiales (66.55%) (Fig. 4b). A total of 14 genera could not be classified at the genus level, including those that could only be classified as Euryarchaeota, Archaea, and Nitrososphaeria. As observed for the bacterial communities, archaeal genera abundances widely varied among different biogas digesters.

Correlations between digester microbial community compositions and environmental factors

RDA was conducted to evaluate the relationships between microbial community compositions and environmental factors among samples (Fig. 5). Bacterial community structures were mainly related to VFA concentrations and pH (Fig. 5a). Indeed, VFA concentration was the most important factor associated with bacterial community variation among biogas digesters with different raw material ratios. In addition, VFA concentrations were positively correlated with *Lactobacillus* abundances. *Lactobacillus* were abundant in the biogas digesters with high VFA concentrations. VFA concentrations were relatively highest on the 4th day of digester fermentations due to the accumulation of acid in the pre-fermentation period, consistent with *Lactobacillus* abundances that were highest on the 4th day, followed by subsequent gradual decreases (Table 4). Thus, VFAs likely controlled the abundances of *Lactobacillus*, consistent with previous studies (Shi et al. 2021). pH also concomitantly exhibited apparent effects on the distributions of bacterial groups and was positively correlated with the abundances of *Bacteroides* and *Pseudomonas*. These two bacterial taxa exhibited highest abundances in biogas digesters with pH > 6.5 (Table 4). Thus, pH significantly affected *Bacteroides* and *Pseudomonas* abundances, consistent with the results of Choure and Al-Mur (Choure et al. 2021; Al-Mur et al. 2021). Among the environmental factors, AK and AN exhibited non-significant correlations with bacterial community structures.

Similar to the bacterial community structures, archaeal community structures were also primarily related to VFA concentrations and pH (Fig. 5b). In addition, VFAs were also the most important factor contributing to differences in archaeal communities among biogas digesters and were positively correlated with *Methanoculleus* and *Methanothrix* abundances. In anaerobic fermentation systems with highly variable VFA concentrations, the relative abundances of *Methanoculleus* and *Methanothrix* also differ (Lim et al. 2020). Further, both *Methanobrevibacter* and *Methanobacterium* abundances were positively correlated with pH, consistent with previous studies (Zhao et al. 2020; Fones et al. 2021). As observed for the bacterial communities, other environmental factors, including AK and AN, also did not exhibit significant effects on archaeal communities.

Conclusions

Anaerobic fermentation is a clean and efficient way to utilize agricultural waste resources like rapeseed cake and sheep manure. The evaluation of low-temperature mixed anaerobic fermentation within seven biogas digesters using different raw material ratios indicated that a dry matter ratio of sheep manure and rapeseed cake of 1:2 corresponded to the highest cumulative biogas production (122.92 $m^3 \cdot t^{-1}$). High-throughput sequencing was used to investigate the microbial communities associated with the fermentation process using different digester material ratios. *Lactobacillus*, *Peptoniphilus*, unclassified Synergistaceae, *Bacteroides*, and *Pseudomonas* were the most dominant bacterial taxa among the 29 digester samples analyzed here, and their abundances varied with the ratios of raw materials. Concomitantly, *Methanogenium* and *Methanoculleus* were the main gas-producing archaea identified in the digesters. Specifically, *Methanogenium* exhibited higher abundances in biogas digesters with higher sheep manure contents, while *Methanoculleus* exhibited higher abundances in biogas digesters with higher rapeseed cake contents. VFA concentrations and pH were the primary factors

associated with differences in archaeal and bacterial community structures among all digesters of this study. This study provides a theoretical basis for the utilization of agricultural waste resources like rapeseed cake and sheep manure, while also providing a framework for the promotion and application of low-temperature anaerobic fermentation treatment of agricultural waste in Qinghai Province.

Declarations

Declarations

There is no conflict of interest.

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Figures

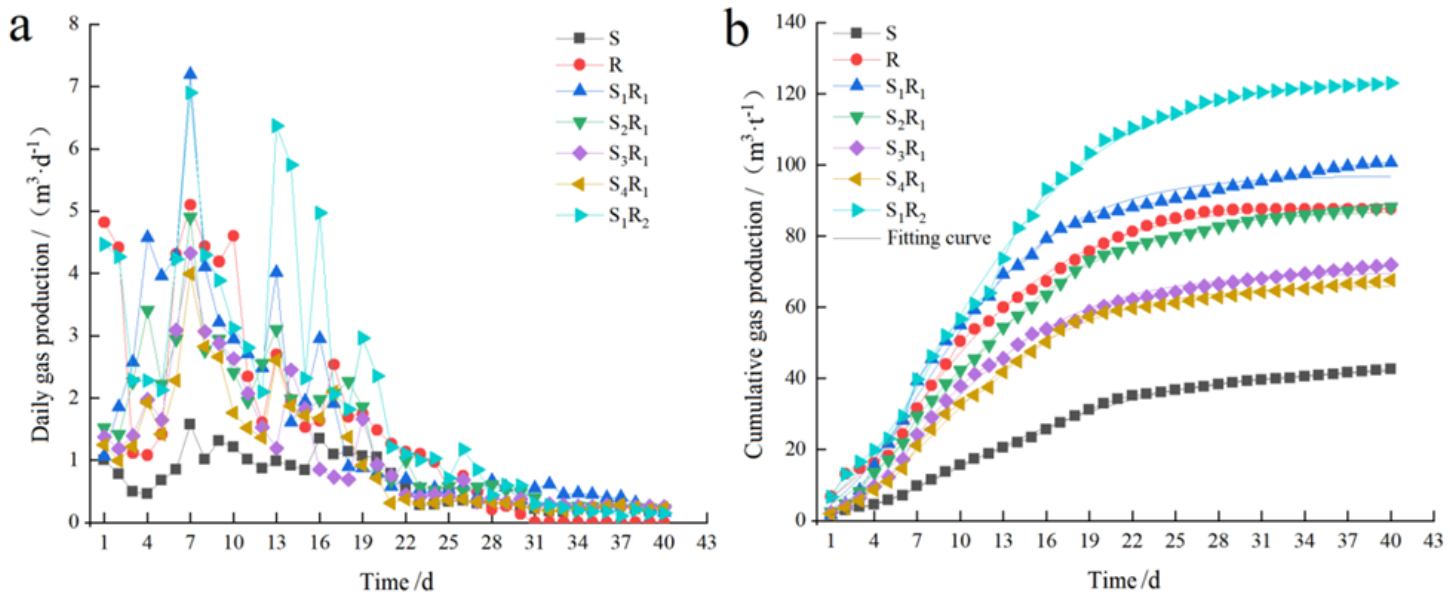

Figure 1

Variation in gas production among different digester treatment groups

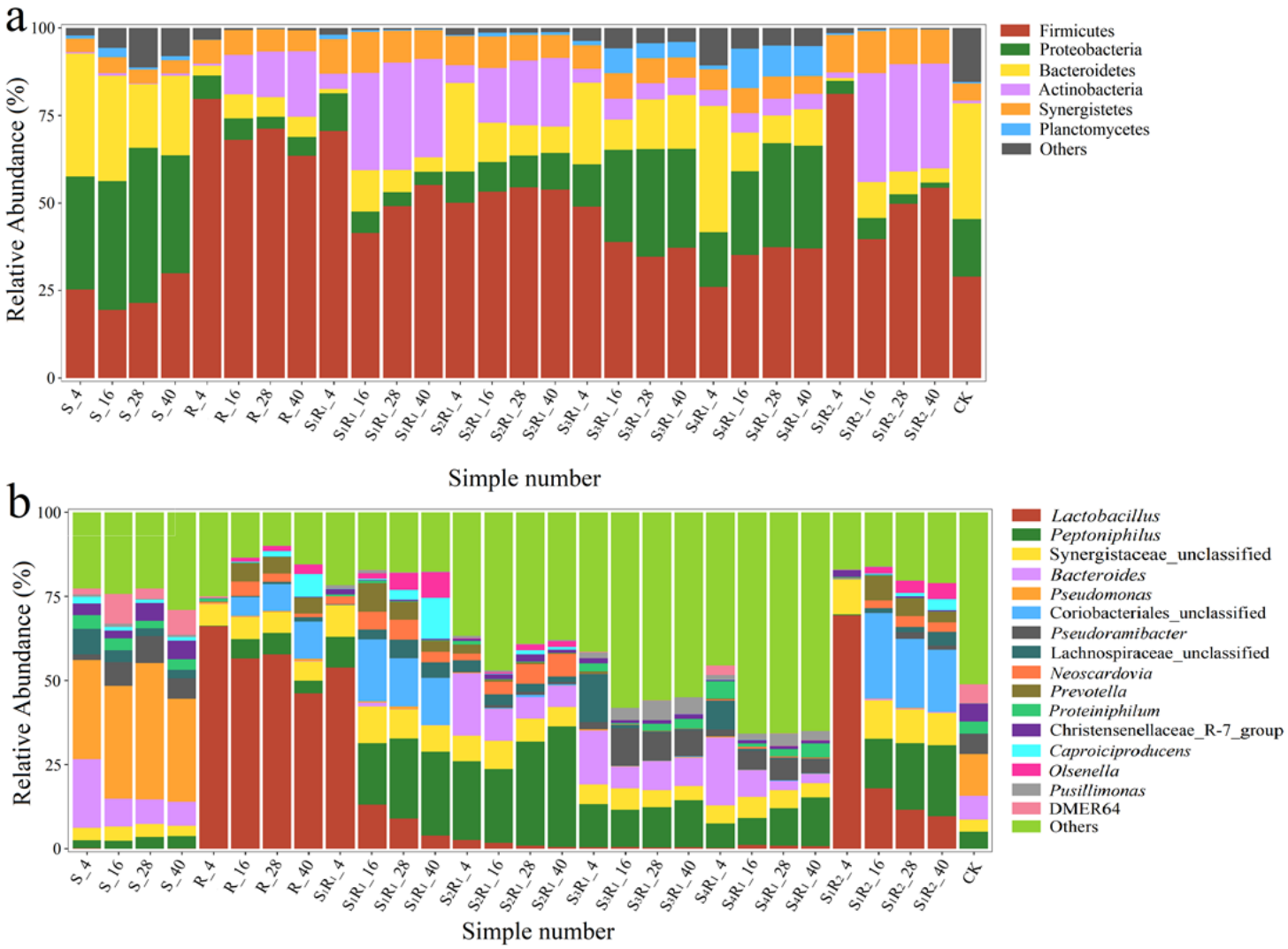

Figure 2

Bacterial community structures in digesters at the a) phylum and b) genus levels

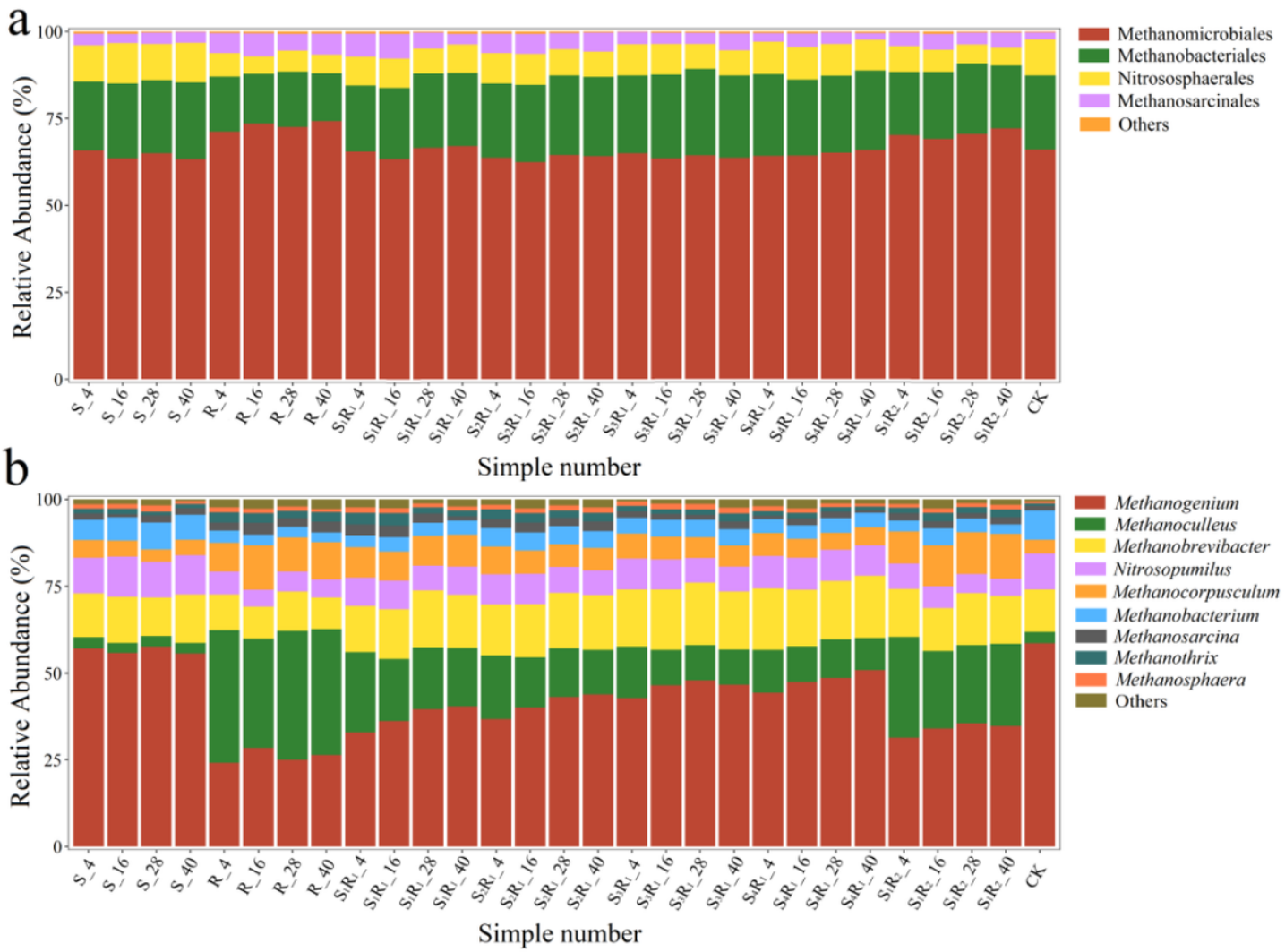

Figure 3

Archaeal community structures in digesters at the a) order and b) genus levels

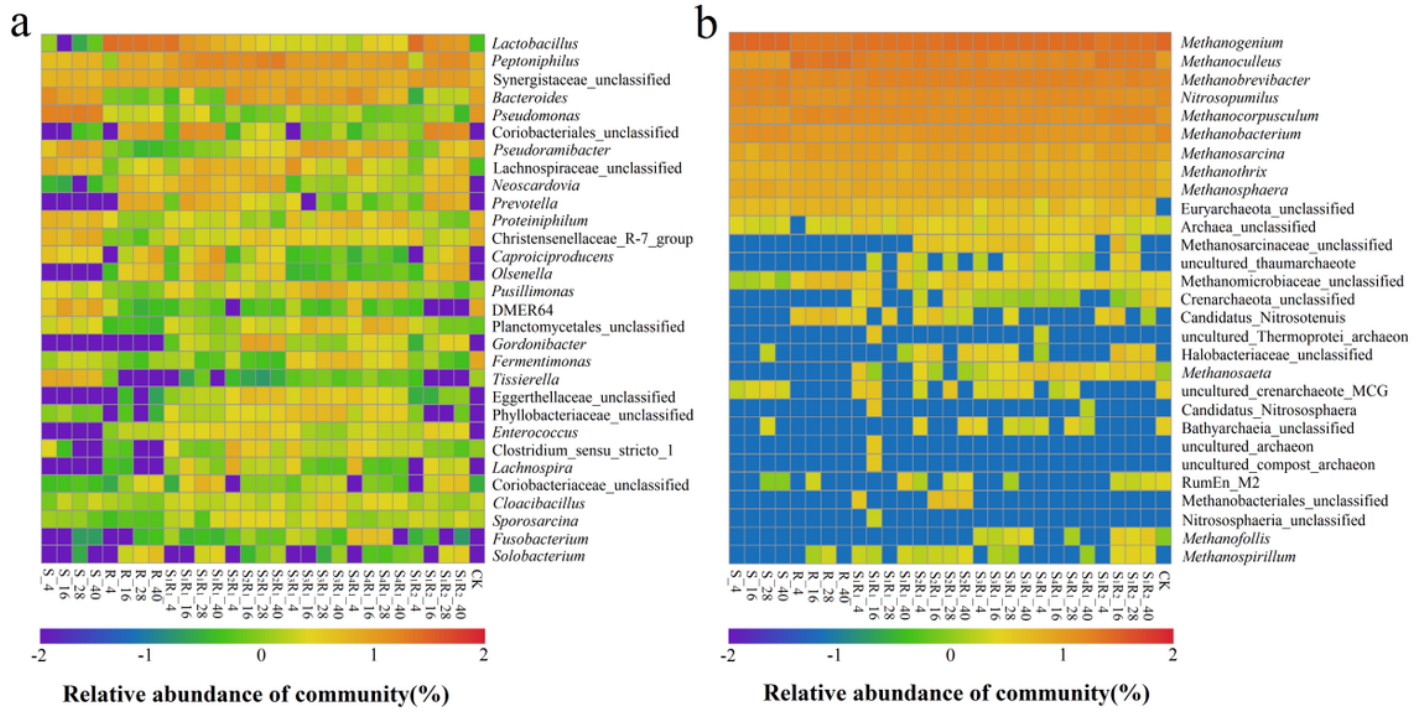

Figure 4

Heatmap showing the 30 most abundant a) bacterial andb) archaeal OTUs among biogas digesters

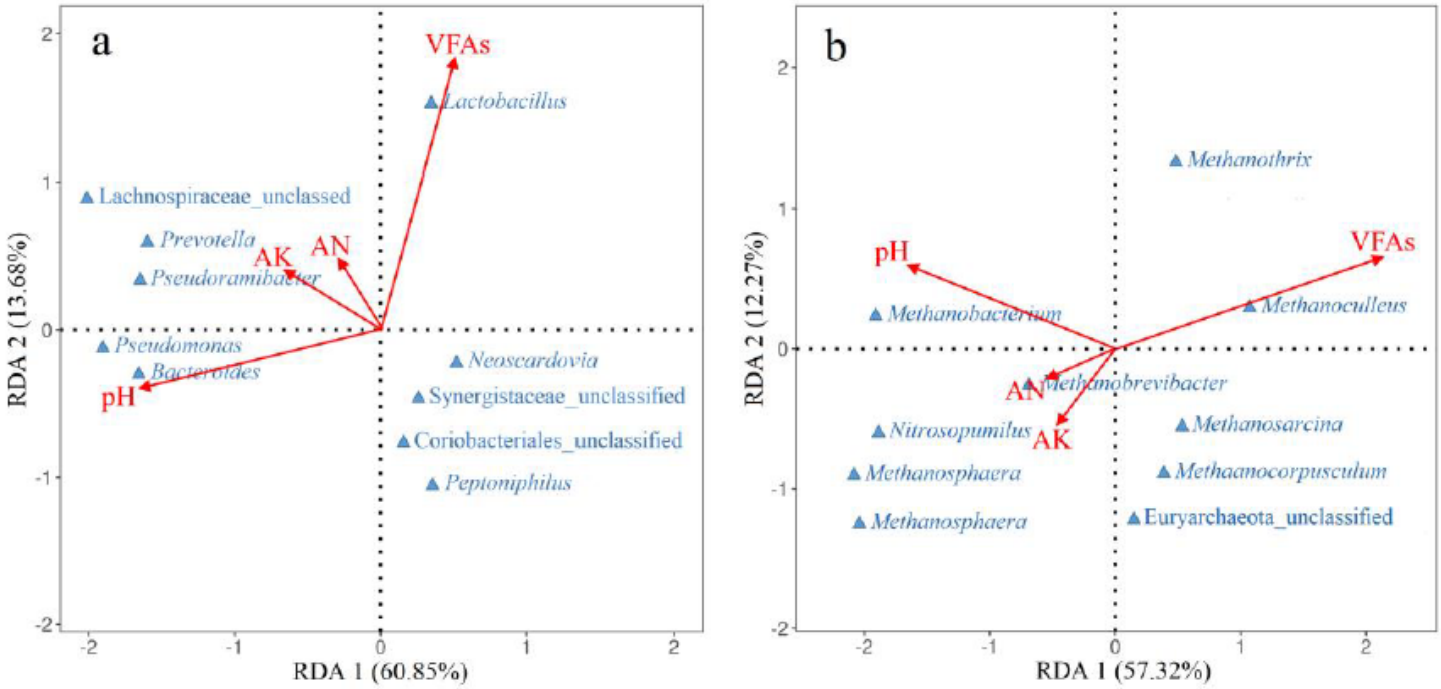

Figure 5

RDA analysis of the a) bacterial and b) archaeal community composition and relevant environmental factors within biogas digesters.