Anaerobic digestion characteristics and key microorganisms associated with low-temperature rapeseed cake and sheep manure fermentation

Li Liu · Rongbo Xiong · Yi Li · Laisheng Chen · Rui Han

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Abstract
In this study, gas production from mixed anaerobic fermentation of rapeseed cake and sheep manure at low temperature (15.2–17.8 °C) was investigated in Qinghai rural household biogas digesters to understand the temporal dynamics of key microbial populations involved in fermentations. Different raw material ratios resulted in significantly different effects on biogas yields and microbial community compositions over 40 days. When the dry weight ratio of sheep manure to rapeseed cake was 1:2, the highest level of cumulative gas production was observed (122.92 m³·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 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. 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, Methanoculleus and Methanothrix abundances, while pH was positively correlated with Bacteroides, Pseudomonas, and Methanobacterium abundances.

Keywords Rapeseed cake · Sheep manure · Low temperature · Anaerobic digestion · Microbial analysis

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 such as 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). However, temperature is an important factor that affects anaerobic fermentation. Specifically, low temperature (< 20 °C) can severely inhibit the activity and metabolism of methanogens, thereby restricting the application of anaerobic fermentation technologies (Hu et al. 2017).

Qinghai Province is located in the northeastern region of the Qingha–Tibet Plateau and is situated at an average altitude of over 4000 m and exhibits an average annual temperature of only 7.2 °C. The fermentation conditions of rural household biogas digesters in this area represent typical low-temperature fermentation (Han et al. 2020; Tiwari et al. 2021). 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 rural household biogas digesters have been abandoned in the area and these wasted resources represent a serious problem (Tian et al. 2019; Yang et al. 2019b; 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. Rapeseed cake is an easily degradable, high-quality, fermentation raw material. Mixed fermentation with rapeseed cake and livestock manure with high solid contents (≥ 10%) can balance nutrient levels of fermentation systems, while also improving the efficiency of low-temperature fermentation with single raw materials. Further, mixed fermentation can also effectively solve problems including the substantial energy consumption and large biogas slurry discharge involved in low solid content (< 10%) fermentation (Jabłoński et al. 2015). In addition, anaerobic digestion is a complex microbial process that is coordinated by multiple microorganisms. Understanding the temporal dynamics of microbial populations in biogas digesters will help identify the interactions between various microbial groups during anaerobic fermentation and inform strategies to improve the efficiency of biogas production (Jang et al. 2014; Ndubuisi-Nnaji et al. 2020).

High-throughput sequencing of 16S rRNA genes can effectively illuminate the structural characteristics of anaerobic fermentation microbial communities. High-throughput sequencing techniques have been widely used to study complex microbial communities (Arelli et al. 2021; Bae et al. 2020; Tao et al. 2021; Yang et al. 2019a). In this study, a pilot experiment with rural low-temperature (15.2–17.8 °C) household biogas digesters was conducted to understand the temporal dynamics of fermentation microbial communities. Specifically, biogas production was evaluated from seven treatments of high solid content mixed anaerobic fermentations containing rapeseed cake and sheep manure. Microbial community structural dynamics were concomitantly investigated during fermentation and the relationships between microbial community structures and functions were particularly analyzed. This research provides a theoretical framework for optimal resource utilization from agricultural wastes such as rapeseed cake and sheep manure. Moreover, these results provide a technical reference for promoting the application of low-temperature anaerobic fermentation of agricultural wastes in the 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.

| 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 |

ND not determined
about 5 m³. 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 (S₁R₁), (4) sheep manure and rapeseed cake mixed in a 2:1 ratio (S₂R₁), (5) sheep manure and rapeseed cake mixed at a 3:1 ratio (S₃R₁), (6) sheep manure and rapeseed cake mixed at a 4:1 ratio (S₄R₁), and (7) sheep manure and rapeseed cake mixed at a 1:2 ratio (S₁R₂). 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 5 days, and stirred every day during heap retting. Digester materials were mixed with 1500 kg of inoculum (30% inoculum) and then biogas slurry (with moisture content adjusted to 70%), covered with plastic film for heap retting over 5 days, and stirred every day during heap retting. Digester materials were mixed with 1500 kg of inoculum (30% inoculum) and then biogas slurry was supplemented to a volume of 5 m³. 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 3 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 (Han et al. 2020). 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.

**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). Lignin, cellulose, and hemicellulose contents were measured as previously described (Li et al. 2020), in addition to crude protein content measurements (Bae 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:

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

In the equation, \( P \) is the cumulative methane production of VS corresponding to time (in m³·t⁻¹), \( P_m \) is the final cumulative VS biogas production (in m³·t⁻¹), \( R_m \) is the maximum VS gas production rate (in m³·(t·d)⁻¹), \( \lambda \) 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, \( R_m \), and the gas production retention time, \( \lambda \).

**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

| Treatment group | Rapeseed cake (kg) | Sheep manure (kg) |
|-----------------|-------------------|-------------------|
| S               | 0.00              | 1986.62           |
| R               | 827.77            | 0.00              |
| S₁R₁            | 413.89            | 993.31            |
| S₂R₁            | 275.92            | 1324.41           |
| S₃R₁            | 206.94            | 1489.96           |
| S₄R₁            | 165.55            | 1589.29           |
| S₁R₂            | 551.85            | 662.21            |

Weight was calculated based on dry matter (TS) in units of kg.
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.

**Extraction of 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′-GAC TACHVGGGTATCTAATCC-3′), while the archaeal-specific primers, ‘F’ (5′-TGYCAGCGCCGCCCGTAA-3′) and ‘R’ (5′-YCCGGCGGTGAVTCCTAAATT-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 (Han et al. 2020). Shannon index was used to measure microbial diversity. The greater Shannon index indicate higher diversity of the species in the sample (Li et al. 2015). 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 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 m³, 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. 2016; Yang et al. 2019b; Han et al. 2020). In this study, biogas production from R and S group treatments at low temperatures were 87.584 m³·t⁻¹ and 42.663 m³·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·g⁻¹ VS from low-temperature pig manure biogas production that was observed by Yao et al. (2020). Thus, the biogas 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,
cake and sheep manure alone, mixed fermentation greatly improved biogas production. The S1R2 group exhibited the largest cumulative gas production, reaching 122.92 m³·t⁻¹. 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 S1R2 group were the largest. The $P$, $P_m$, and $R_m$ parameters in the mixed fermentation increased with increasing rapeseed cake addition, while $\lambda$ decreased 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 biogas-producing rate and system stability.

### Changes in 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 S1 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.

### Microbial diversity analysis

It can be seen from Table S2, 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 5485 archaeal OTUs. Diversity coverage for all samples was estimated at higher than 0.994, suggesting...
adequate sampling of native sample diversity. 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.

**Bacterial community analysis**

A total of seven bacterial phyla and seventeen 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 S1R2 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

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**Fig. 2** Bacterial community structures in digesters at the **a** phylum and **b** genus levels

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gas production. Firmicutes exhibited higher relative abundances on the 4th day in the biogas digesters of the R, S₃R₁, and S₄R₂ 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, S₃R₁, and S₄R₂ 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. 2019a). Lactobacillus relative abundances decreased across fermentation time, with highest abundances in the R group (61.9%), followed by the S₄R₂ (35.88%) and S₃R₁ (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 H₂ and CO₂ (Jang et al. 2014; Luo and Angelidaki 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 biogas 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 S₃R₁, S₃R₂, S₄R₁, 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 ten 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 S₄R₂ (69.16–72.13%) groups. The proportions of Methanobacteria 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 and Nagaoka 2003; Chong et al. 2021), 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 7593 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 S1). *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 S1R1 and S3R1 groups (17.13% and 17.12%, respectively), followed by the S1R2 and S3R2 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 and Babalola 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 further investigate bacterial and archaeal community dynamics, the taxonomic classifications were analyzed at the sub-division level. Therefore, the distributions of the 30 most abundant OTUs were evaluated (Fig. 4). In the heatmap, the color gradient and similarity reflect the similarity and difference of the composition of different samples, and the red color indicates the highest relative abundance. 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, the communities from the four periods of rapeseed cake single fermentation and the fourth day communities of S, R, and S, R, clustered together, likely due to the dominance of Lactobacillus that are acid-resistant bacteria. The communities from the four periods of sheep manure single fermentation also clustered together, likely due to Pseudomonas being the dominant genus of these communities. Pseudomonas is thus a representative member of single-fecal fermentation systems.

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. Methanogenium was detected in all samples, although the four time periods of sheep manure single fermentation and CK contained the highest abundances. These samples all clustered together, suggesting that methane production in these systems is dominated by Methanogenium. The communities from the four periods of rapeseed cake single fermentation and that of the fourth day of S, R, exhibited the lowest Methanogenium abundances, but the highest abundances of Methanoculleus, indicating that increased addition of rapeseed cake led to more acidic environments that were more suitable for the growth of acid-tolerant taxa. Nevertheless, Methanogenium and Methanoculleus were the most abundant genera overall, indicating that the low-temperature conditions of Qinghai rural household biogas digesters selected for the dominance of hydrogenotrophic methanogens.

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 S1). Thus, VFAs likely controlled the abundances

**Fig. 4** Heatmap showing the 30 most abundant a bacterial and b archaeal OTUs among biogas digester communities
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 S1). 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 *Methanotrichis* abundances. In anaerobic fermentation systems with highly variable VFA concentrations, the relative abundances of *Methanoculleus* and *Methanotrichis* also differ (Guo et al. 2021; Li et al. 2021). Further, *Methanobacterium* abundances were positively correlated with pH, consistent with previous studies (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**

The results from this study indicated that the use of different raw material ratios significantly affected biogas yields and microbial community characteristics. (1) The investigation of low-temperature mixed anaerobic fermentations within seven biogas digesters using different raw material ratios indicated that 1:2 dry matter ratios of sheep manure and rapeseed cake led to the highest cumulative biogas production (122.92 m$^3$·t$^{-1}$). (2) *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.

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