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PCR–DGGE Analysis on Microbial Community Structure of Rural Household Biogas Digesters in Qinghai Plateau

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Abstract

To investigate contribution of environmental factor(s) to microbial community structure(s) involved in rural household biogas fermentation at Qinghai Plateau, we collected slurry samples from 15 digesters, with low-temperature working conditions (11.1–15.7 °C) and evenly distributed at three counties (Datong, Huangyuan, and Ledu) with cold plateau climate, to perform polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) and further sequencing. The bacterial communities in the total 15 digesters were classified into 38 genera with *Mangroviflexus* (12.1%) as the first dominant, and the archaeal communities into ten genera with *Methanogenium* (38.5%) as the most dominant. For each county, the digesters with higher biogas production, designated as HP digesters, exclusively had 1.6–3.1 °C higher fermentation temperature and the unique bacterial structure composition related, i.e., unclassified *Clostridiales* for all the HP digesters and unclassified *Marinilabiliaceae* and *Proteiniclasticum* for Ledu HP digesters. Regarding archaeal structure composition, *Methanogenium* exhibited significantly higher abundances at all the HP digesters and *Thermogymnomonas* was the unique species only identified at Ledu HP digesters with higher-temperature conditions. Redundancy analysis also confirmed the most important contribution of temperature to the microbial community structures investigated. This report emphasized the correlation between temperature and specific microbial community structure(s) that would benefit biogas production of rural household digesters at Qinghai Plateau.

Introduction

Biogas is a clean and renewable energy with a promising future [5, 13]. The extended application of biogas provides an effective approach to solve energy crisis, improving ecological environment and achieving sustainable development in rural areas [34, 37]. To optimize biogas production, previous studies predominantly investigated the operating processes and chemical/physical reaction conditions facilitating industrial biogas fermentation. Regarding this, the microbial community structures reported to date were mostly identified under large-scale engineering conditions, especially at thermodynamically favorable temperatures (>30 °C). However, little information has been reported concerning microbial community structures involved in biogas fermentation under natural/semi-natural conditions. The present study would concern about the microbial community structures in rural household biogas digesters (somewhat semi-natural biogas-producing systems) particularly under natural low-temperature conditions. Comparing to that at inland plain areas, the biogas fermentation at plateau has some special features such as high altitude, low atmospheric pressure, low

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temperature, and sharp temperature fluctuation between day and night. In this study, the sludge samples were collected from three counties in Qinghai Province which lies upon the Qinghai–Tibet Plateau with typical plateau environmental characteristics including low temperature (annually average −4 to 8 °C) and high altitude (over 2000 meters above sea level). Biogas production in rural household digesters has been developed as an important solution to providing energy for rural residents, especially for most rural residents at high plateau conditions such as Bolivian high plateau [2], Andean regions [20], Tibet communities [42], Qinghai–Tibetan Plateau [7], and Yunnan–Kweichow Plateau [12]. Rural household biogas fermentation has been considered as a complex microbial ecological system that works along with specific microbial community structure(s). The rural household biogas digesters, widely distributed in the Qinghai Province and most of which lying in Qinghai–Tibetan Plateau, provide desirable resources to investigate microbial community structures in the high plateau biogas fermentation.

Due to technical obstacle to isolate and cultivate most microorganisms in biogas digesters, many traditional methods might not work well to uncover the microbial community structures of such complicated ecological system [19]. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE), a culture-independent molecular biological method, with DNA molecule as study object, has succeeded in revealing microbial community structure in biogas fermentation process [39]. PCR–DGGE has been widely applied for microbial ecology research under various environments [6, 17, 38, 47, 49, 50]. To our knowledge, the microbial community structures to support rural biogas fermentation in high plateau environments (also cold climate areas) have only been elucidated in Yunnan regions [12]. In the present study, with samples from three different locations at Qinghai Plateau, we investigated the effect of plateau environmental factors (temperature and altitude) on microbial structures in rural household biogas digesters to reveal microbial structure characteristics at the specific plateau biogas fermentation conditions.

Materials and Methods

Sample Collection

In this study, 15 rural household biogas digesters were selected from three counties Datong (DT), Huangyuan (HY), and Ledu (LD) in Qinghai Province of P.R. China. We selected five digesters for each county, designated as DT1–5, HY1–5, and LD1–5. Among the digesters of the same county, one digester with relatively lower fermentation temperature was set as control to compare with the other four digesters with 1.6–3.1 °C higher temperature regarding biogas production and microbial community structure related. The biogas fermentation conditions and environmental factors investigated in this work are listed in Table 1, including temperature (T) and height above sea level (H). Sludge samples collected were stored at 4 °C and immediately subjected to DNA extraction.

DNA Extraction and PCR Amplification

The total DNA was extracted from the microbes in the slurry sample using the Fast DNA Spin Kit for Soil (SK8233,

Table 1 Biogas production and environmental and working factors determined during rural biogas fermentation in the selected digesters

Sample	Biogas production (m^3/d)	Methane content (%)	T (°C)	pH	H (m)	TN (g/kg)	TP (g/kg)	TK (g/kg)	OM (g/kg)
DT1	0.98	57.8	13.5	8.24	2611.0	16.04	16.25	14.24	172.7
DT2	1.02	58.9	14.0	7.86	2608.9	16.34	14.10	16.29	141.2
DT3	0.96	58.6	13.3	8.28	2613.7	13.47	15.89	14.25	211.2
DT4	0.99	58.6	13.8	7.98	2612.0	14.32	14.08	16.11	163.8
DT5	0.61	57.7	11.7	7.74	2610.9	12.78	13.12	14.08	140.8
HY1	0.97	59.3	13.5	8.27	2685.5	14.39	16.00	14.51	151.2
HY2	0.96	60.7	13.3	8.41	2680.2	17.74	15.86	15.27	140.5
HY3	0.93	60.4	13.1	8.35	2687.5	15.12	16.34	15.68	166.1
HY4	0.92	61.4	13.0	8.14	2683.1	16.83	15.91	14.34	158.2
HY5	0.55	59.6	11.1	8.08	2683.6	13.87	14.00	14.01	135.4
LD1	0.99	57.1	14.8	7.48	2223.3	18.86	16.13	19.65	343.2
LD2	1.02	57.9	15.1	7.46	2224.3	17.07	16.14	20.67	283.0
LD3	1.08	58.1	15.7	7.43	2225.8	18.87	16.18	19.12	327.1
LD4	1.05	58.0	15.3	7.31	2228.3	19.52	16.10	19.88	346.4
LD5	0.67	57.5	12.6	7.25	2227.1	16.58	15.09	16.53	246.1

Sangon Biotech; Shanghai, China) and measured by agarose gel electrophoresis. PCR amplification of bacterial and archaeal 16S rDNA genes (V3 region) was conducted using the primers listed in Table A1. The PCR reaction design was according to standard PCR–DGGE protocol reported in previously successful works [3, 4].

DGGE Analysis

DGGE was performed on a D-Code Mutation System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products, all in ~200 bp range, were separated in a 8% (W/V) polyacrylamide gel with a denaturing gradient from 30 to 60% (100% denaturant corresponds to 7 M urea and 40% formamide). The gel was incubated in an ethidium bromide solution for 30 min and photographed under UV transillumination. The fingerprinting profile of DGGE was digitally processed using Quantity One software (Bio-Rad, USA). UPGMA method was applied to calculate phylogenetic tree based on comparing DGGE band patterns with 1/0 matrix constructs and thus to verify data reliability, as described in previous reports [14, 21].

DNA Sequencing and Analysis

Each band in the obtained DGGE gels was excised for DNA extraction using SanPrep Column DNA Gel Extraction Kit (SK8132, Sangon, Shanghai, China). The DNA isolated from corresponding DGGE band was PCR-amplified with no GC-clamp primers, and the PCR products were purified and ligated to pMD18-T vector (TaKaRa). The resulted plasmids were transformed into *E. coli* DH5α cells for DNA sequencing (Sangon Biotech Company Lid, Shanghai, China). Based on GenBank (http://www.ncbi.nl.nih.gov/BLAST/) and RDP (http://rdp.cme.msu.edu) databases, the sequencing results, containing desirable V3 region from bacterial or archaeal 16S rDNA genes, were subjected to homologous alignment to identify the taxonomic status of the obtained DGGE bands. These DNA sequences were deposited in the Sequence Read Archive database in NCBI with accession number PRJNA350392.

Correlation Analysis

In the present study, we exploited Vegan 2.2 package of R language to correlate microbial community structure(s) with some particular environmental factors that are shown in Table 1. The detrended correspondence analysis (DCA) is proposed to measure the length of gradient along the ordination axis that determines a suitable method [CCA for >3.0 or redundancy analysis (RDA) for <2.0] to finally produce simulated graphic plots.

Results and Discussion

DGGE Fingerprint and Diversity Analysis of Rural Household Biogas Digesters

The DGGE fingerprint analysis based on 16S rDNA PCR amplification was conducted for bacteria (Fig. 1) and archaea (Fig. 2) in the slurry samples from 15 rural household biogas digesters (DT1–5, HY1–5, and LD1–5), generally exhibiting diversified profiles of bacterial and archaeal community structures. The indexes to evaluate microbial diversity and the related community evenness are summarized in Table A2.

Sequencing Analysis of Bacterial Community Structures

Figure 1 displayed a total of 130 bands that constituted current community structures for DT bacteria (DTB), HY bacteria (HYB), and LD bacteria (LDB). All these bands, including DTB-1–DTB-43 (Fig. 1a), HYB-1–HYB-38 (Fig. 1b), and LDB-1–LDB-49 (Fig. 1c), were separated, recovered, and sequenced to achieve homology alignment using GenBank and RDP database. The sequence alignment revealed highly diversified bacterial community structures for the rural household biogas digesters, exhibiting desirably high sequence similarity (74.2–100%) and consisted of ten phyla with Bacteroidetes, Firmicutes, and Proteobacteria as the most abundant (Table A3). The bacterial species belonging to these three phyla, usually isolated from anaerobic environments and identified by both DGGE and deep sequencing analysis [1, 10, 29, 35], have been documented to constitute dominant bacterial community in thermodynamics-optimized biogas digesters at both industrial and experimental scales, functioning in carbohydrate catabolism [30], cellulose degrading [36], and protein hydrolysis [24]. Our PCR–DGGE results also suggested the importance of the three bacterial phyla for rural biogas fermentation at Qinghai Plateau, presumably indicating common predominant bacterial community structures to support industrial and natural/semi-natural biogas fermentations.

Taken into account all the three counties (DT, HY, and LD), the bacteria deduced from PCR–DGGE–sequencing results of 15 rural biogas digesters were classified into 38 genera with *Mangroviflexus* (12.1%) as the dominant (Fig. 3a). Compared to DT and HY samples, an obviously larger proportion of *Mangroviflexus* was observed in LD (Fig. 3b), suggesting a diversity regarding prominent bacteria and their abundances in the three regions. The genus *Mangroviflexus*, usually combined with the members of

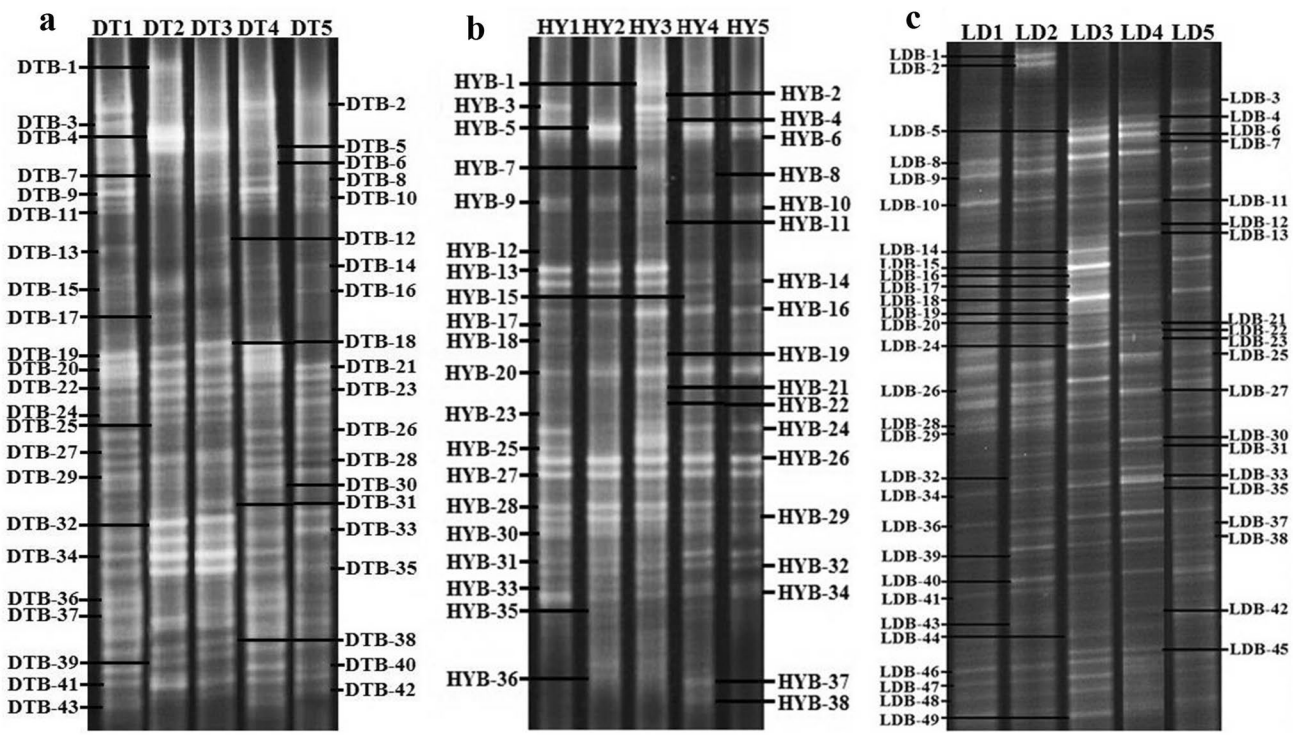

Fig. 1 DGGE fingerprints represented for bacterial community in the selected rural household biogas digesters of DT County (**a**), HY County (**b**), and LD County (**c**)

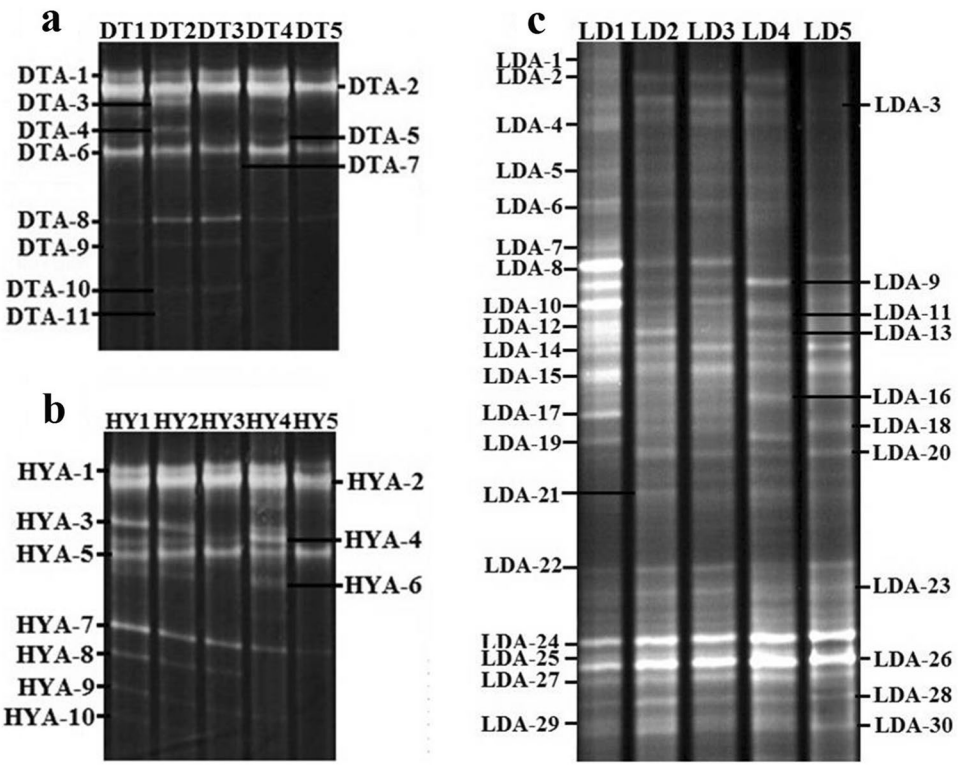

Fig. 2 DGGE fingerprints represented for archaeal communities in the selected rural household biogas digesters of DT County (**a**), HY County (**b**), and LD County (**c**)

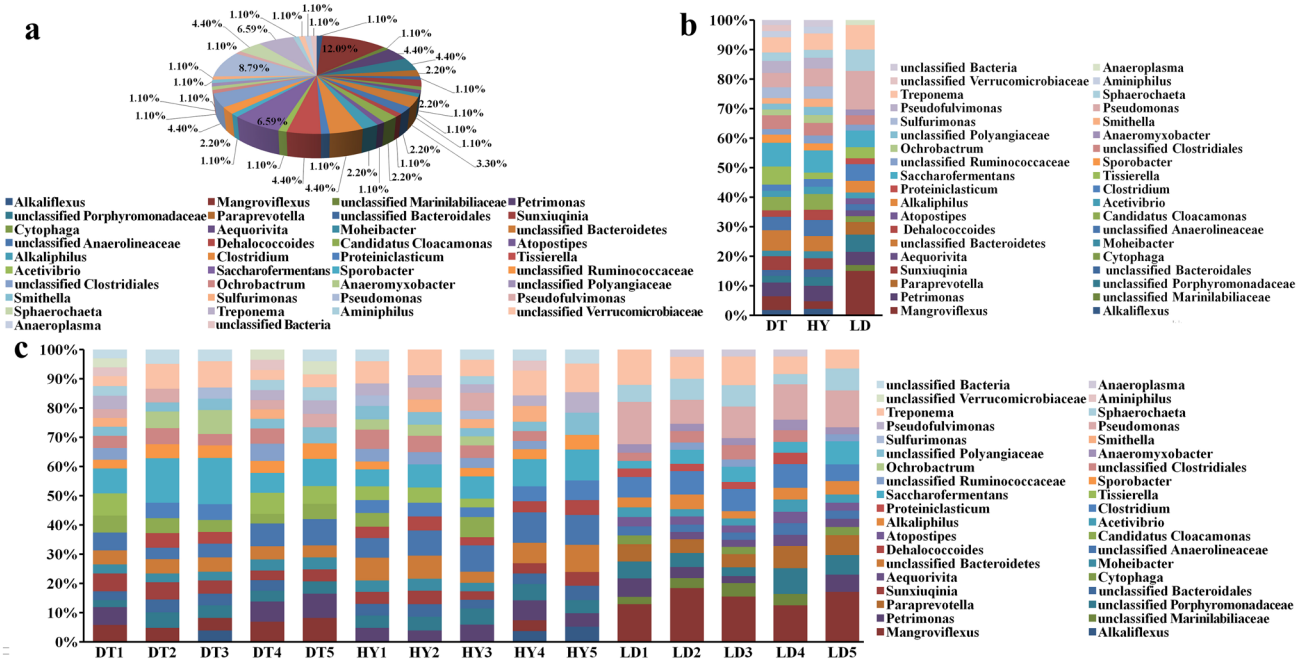

Fig. 3 Analysis of bacterial community structures for rural household biogas digesters selected from DT, HY, and LD counties. **a** General bacterial community structures in all the selected digesters of three counties (DT, HY, and LD), **b** comparative analysis of bacterial community structures in DT, HY, and LD counties to evaluate H effects, and **c** comparative analysis of bacterial community structures of all the 15 biogas digesters to evaluate T effects

family Marinilabiliaceae, played a crucial role in reducing and oxidizing reactions to bio-degrade organic matters [11, 53, 54]. Specifically we noted the coordination of genera *Mangroviflexus* and unclassified *Marinilabiliaceae* at relatively higher fermentation temperatures and lower altitude (Fig. 3b, c). These two genera, mainly distributed in Mangrove ecosystems typically as the South China Sea [9], often shared unusually close links in evolution and function [53]. The observed predominance of these two genera and their responses to temperature and altitude might come into being on geological timescales and exert considerable impacts on semi-natural biogas fermentation under cold climate conditions.

Interestingly, as shown in Fig. 3b, the genera *Anaeromyxobacter*, unclassified *Marinilabiliaceae* and *Proteiniclasticum* were only detected in LD region with relatively lower altitude, whereas the genera *Sporobacter*, unclassified *Bacteroidetes* and *Sunxiuqinia* were only detected in higher-altitude regions DT and HY. These unique genera exclusively occupied rather small proportions (1.1–2.2%), but their response to specific altitude might reflect the effects of Qinghai Plateau environment on bacterial community structure(s). These genera members extensively participated in anaerobic biological processes [8, 11, 27, 33, 44, 52], presumably giving certain functional compensation to support biogas fermentation at changing altitude conditions. As shown in Table 1, for each county, rural household biogas production had positive correlation with fermentation temperature. Interestingly, the unclassified *Marinilabiliaceae*, the unique genus in LD region, only appeared in bacterial community structure at relatively higher fermentation temperature (Fig. 3c, LD1–4). A similar response to the temperature increasing was also observed for genus *Proteiniclasticum* (Fig. 3c, LD1–5). In contrast, the genus unclassified *Clostridiales* appeared in all the higher fermentation temperature digesters from DT, HY, and LD counties (Fig. 3c, DT1–4, HY1–4, and LD1–4), corresponding to higher biogas production (Table 1). The results suggested that fermentation temperature did modulate bacterial community structures that contributed to plateau rural household biogas production. Moreover, almost all the predominant bacterial communities observed in our study have been recorded in the latest reports on industry-scale biogas plants (working temperature >30 °C) in northeastern China [18] and central Germany [29], indicating some common metabolic processes to generate methane via bacteria activities under different thermal conditions.

Sequencing Analysis of Archaeal Community Structures

Figure 2 displayed a total of 51 bands that constituted current community structures for DT archaea (DTA), HY archaea (HYA), and LD archaea (LDA). All these bands, including

DTA-1–DTA-11 (Fig. 2a), HYA-1–HYA-10 (Fig. 2b), and LDA-1–LDA-31 (Fig. 2c), were separated, recovered, and sequenced to achieve homology alignment using GenBank and RDP database. The sequence alignment revealed diversified archaeal community structures for the rural biogas digesters sampled in the present study, exhibiting desirably high sequence similarity (74.4–91.4%) and consisted of 6 orders with Methanomicrobiales as the most abundant (Table A4). As shown in Fig. 4a, the archaea detected in the three counties were classified into ten genera with *Methanogenium* as the most dominant (38.5%). Through PCR–DGGE and metagenomics cues, Methanomicrobiales have been usually found at anaerobic digestive conditions including pilot- and industry-scale biogas fermentations [12, 18, 28, 29, 43]. However, these Methanomicrobiales-involved biogas fermentations were mostly processed under optimal thermodynamics conditions, in agreement to the mesophilic characteristics of most members of Methanomicrobiales. Dong et al. have described considerable proportions of *Methanogenium* in Yunnan rural biogas digesters at 10 and 18 °C fermentation conditions [12]. Here, using DT, HY, and LD digester samples, we also observed a major occupation of *Methanogenium* in archaeal community structures at notably lower fermentation temperatures (11.1–15.7 °C) (Fig. 4b). *Methanogenium*, a genus of Methanomicrobiales, has been documented to comprise specific members as *Methanogenium frigidum* to adapt to low or extremely low growth temperatures [16], suggesting a crucial contribution of this genus to low-temperature biogas fermentation at plateau environment. According to the report of Dong et al. [12], a sharp decrease of *Methanosaeta* abundance, accompanied by a significant increase of *Methanogenium* abundance, well suggested an adaptively change in archaea-mediated methanogenic pathways, i.e., from aceticlastic to hydrogenotrophic methanogens, which was in agreement with our results from 11.1 to 15.7 °C biogas-producing environment. Further, regardless of height altitudes, the abundance of *Methanogenium* at each county was increased with 2–3 °C temperature increasing (Fig. 4c), well corresponding to the enhancing of biogas production (Table 1). The obtained results suggested a promoting effect of fermentation temperature on *Methanogenium* abundance that regulated rural biogas production. *Methanogenium* members including *Methanogenium marinum* have been demonstrated as the dominant methanogenic population in household anaerobic digesters [15, 32]. The present study also documented the similar results and further

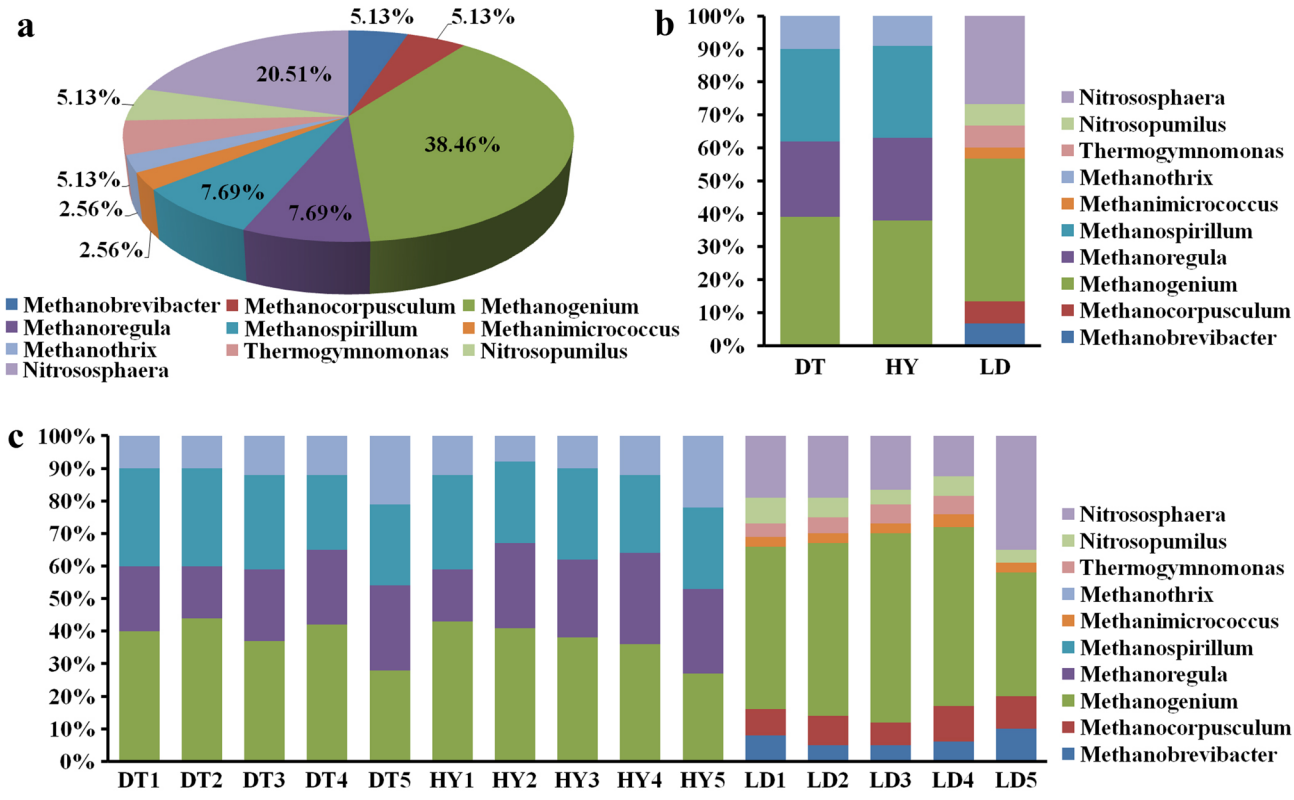

Fig. 4 Analysis of archaeal community structures for rural household biogas digesters selected from DT, HY, and LD counties. **a** General archaeal community structures in all the selected digesters of three counties (DT, HY, and LD), **b** comparative analysis of archaeal community structures in DT, HY, and LD counties to evaluate H effects, and **c** comparative analysis of archaeal community structures of all the 15 biogas digesters to evaluate T effects

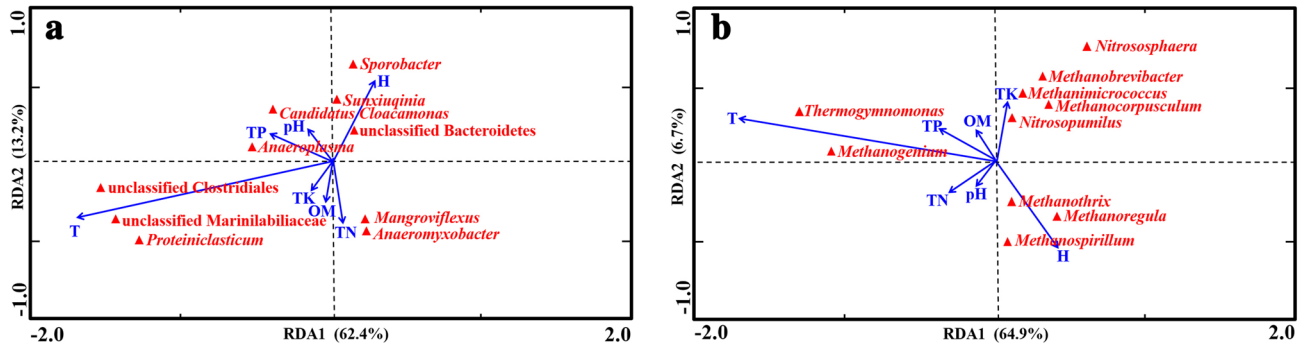

Fig. 5 RDA results of bacterial (**a**) and archaeal (**b**) community structures of the selected rural household biogas digesters in this study

revealed a tight correlation of *Methanogenium* abundance with methanogenic temperatures.

Except *Methanogenium*, the other nine archaea genera could be classified into two groups, each corresponding to a specific height altitude. One group was only detected at LD region with relatively lower height altitude, including *Nitrososphaera, Nitrosopumilus, Thermogymnomonas, Methanimicrococcus, Methanocorpusculum*, and *Methanobrevibacter* (Fig. 4b). Among them, the genus *Thermogymnomonas* appeared only at higher-temperature digesters LD1–4 (Fig. 4c), agreeing to the reported thermophilic characteristics for this genus [31]. *Thermogymnomonas* members extensively function in waste water anaerobic reactions [22, 48], mine drainage water processes [40, 45], hydrometallurgy-oriented bioleaching operations [41], and methanogenic communities associated with anaerobic biodegradation [51]. Here, we at first time reported the contribution of *Thermogymnomonas* to high plateau biogas production, and the result from PCR–DGGE well agreed to the latest Illumina sequencing of archaeal compositions in mesophilic biogas fermentation [23]. Another group was only detected at DT and HY regions with relatively higher height altitude, including *Methanothrix, Methanospirillum*, and *Methanoregula* (Fig. 4b). All these genera members have been found in anaerobic bio-digestion systems related to the organic acid utilization [25, 26, 46], to some extent indicating a functional response to higher altitudes. Regarding biogas production, the difference between five digesters selected at the same county was much more than that between the three counties at different elevations, suggesting the bigger impact of temperature than height altitude on plateau rural biogas fermentation.

Redundancy Analysis (RDA)

RDA results indicted 1.59 and 1.23 gradient length for bacteria and archaea, respectively (Fig. 5). Figure 5a illustrated 75.6% of bacterial community variation in sludge samples, and the bacterial structures in samples were well correlated with T and H. RDA analysis also indicated a close correlation of T with the specific bacterial composition including unclassified *Clostridiales*, unclassified *Marinilabiliaceae*, and *Proteiniclasticum* (Fig. 5a). The similar RDA pattern was obtained in the archaeal community variation analysis. Figure 5b also indicated the significant effects of T and H on *Thermogymnomonas, Methanogenium, Methanothrix, Methanoregula*, and *Methanospirillum*. In contrast, the other environmental factors including TP, TK, TN, OM, and pH had little effects on bacterial and archaeal community structures.

In summary, the present study investigated microbial community structures of rural household biogas digesters at Qinghai Plateau, revealing the first important contribution of temperature to particular bacteria and archaeal community structures in the digesters with higher production at such plateau lower-temperature conditions.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

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