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ENVIRONMENTAL BIOTECHNOLOGY

From mesophilic to thermophilic digestion: the transitions of anaerobic bacterial, archaeal, and fungal community structures in sludge and manure samples

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Abstract The shift of microbial communities during a transition from mesophilic anaerobic digestion (MAD) to thermophilic anaerobic digestion (TAD) was characterized in two treatments. One treatment was inoculated with sludge and the other was inoculated with manure. In this study, methane was produced both in MAD and TAD, but TAD has slightly more methane produced than MAD. A broad phylogenetic spectrum of bacterial, archaeal, and fungal taxa at thermophilic conditions was detected. *Coprothermobacter*, *Bacillus*, *Haloplasma*, *Clostridiisalibacter*, *Methanobacterium*, *Methanothermobacter*, *Saccharomycetales*, *Candida*, *Alternaria*, *Cladosporium*, and *Penicillium* were found almost exclusively in TAD, suggesting their adaptation to thermophilic conditions and ecological roles in digesting the organic compounds. The characterization of the lesser-known fungal community revealed that fungi probably constituted an important portion of the overall community within TAD and contributed to this process by degrading complex organic compounds. The shift of the microbial communities between MAD and TAD implied that temperature drastically affected the microbial diversity in anaerobic digestion. In addition, the difference in microbial communities between sludge and manure indicated that different source of inoculum also affected the microbial diversity and community.

Keywords Microbial community · Anaerobic digestion · Temperature · Activated sludge · Manure

Introduction

Anaerobic digestion is a common method to convert biodegradable organic matters to biogas, which is mostly composed of methane (Nallathambi Gunaseelan 1997). This process is widely used for industrial and domestic purposes because of its methane recovery potential and capability for sewage sludge stabilization (Gujer and Zehnder 1983; Parkin and Owen 1986). Thermophilic anaerobic digestion (TAD) is a well-known strategy to increase organic loading rates (OLR) and methane production rates, while simultaneously reducing solids and potential pathogens as compared to mesophilic systems (De la Rubia et al. 2013; Khemkhao et al. 2012). For example, mesophilic anaerobic digestion (MAD) took 30 to 40 days to eliminate an approximately 50 % of the initial mass of the sludge for methane production, whereas only 11 to 14 days were required for the same results under thermophilic conditions (Zbransk et al. 2000).

TAD, which is usually achieved by inoculating biomass from MAD at higher temperatures, is a microbiological process mainly mediated by thermophiles including thermophilic bacteria, archaea, and even eukaryotes (fungi) (De la Rubia et al. 2013). Previous studies have demonstrated that a wide diversity of organisms can be classified as thermophiles. A number of thermophilic bacteria and archaea clustering within

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the genera *Bacillus* (Darland and Brock 1971), *Thermus* (Brock and Freeze 1969), *Methanosarcina* (Zinder and Mah 1979), and *Methanosaeta* (Kamagata et al. 1992) have been identified previously. All these bulk studies have shown that there are dynamic microbial communities inhabiting the TAD process. An in-depth investigation of the microbial communities during the transition from mesophilic to thermophilic conditions can provide useful information for understanding and improving the performance of TAD. Although the community compositions of bacteria and archaea in anaerobic digestion processes have been investigated (Levén et al. 2007), the role of fungi has been largely overlooked. Furthermore, the information of the indigenous microbial communities that develop under thermophilic conditions, especially the community shifts resulting from the switch from mesophilic to thermophilic conditions is limited.

In the current study, we investigated the bacterial, archaeal, and fungal communities in activated sludge and manure digestion processes during a shift in temperature from 37 to 58 °C by high-throughput sequencing. A comparative community analysis was performed to investigate the microbial community dynamics during this transition. Shifts in bacterial and archaeal communities, as well as fungal communities were monitored and characterized. The overall goals of this study are (i) to reveal the shift in microbial communities during the transition from mesophilic to thermophilic conditions and (ii) to identify and interrogate the ecophysiology of the predominant thermophiles. It is anticipated that the systematic microbial community analysis obtained in this study will provide useful information to select the best strategy for the operation of TAD.

Materials and methods

Batch experiments

Two batch experiments were set up in the current study. The first experimental setup was inoculated from anaerobic sludge from a wastewater treatment plant in Michigan, USA. The second experimental setup was seeded from manure sampled from a biogas plant in Michigan, USA. Batch experiments were performed in 160-mL serum bottles filled with 80-mL sludge (49.5 g/L as total solids) or 80-mL manure (59.3 g/L as total solids). The headspace was replaced with nitrogen and carbon dioxide. Resazurin was added to each microcosm as anaerobic condition indicator. For each experimental setup, ten microcosms were established and incubated under 37 °C for 30 days, and then five of them were transferred to 58 °C for inoculation of thermophilic sludge and manure. The other five microcosms were still maintained at 37 °C as controls. A total of four treatments including thermophilic sludge, mesophilic sludge, thermophilic manure, and mesophilic manure were set up. Methane content in headspace was analyzed using a gas chromatograph system (Perkin Elmer, Waltham, MA, USA) equipped with a flame ionization detector and a capillary column (J&W Scientific, DB-624, diameter 0.53 mm; Folsom, CA, USA). Injector and detector temperatures were set at 200 °C and the column temperature was 120 °C. Total solids (TS) and volatile solids (VS) were measured in accordance with the standard methods (APHA et al. 2005).

DNA extraction, PCR amplification, and sequencing analysis

After 60 days incubation at two temperature conditions, total genomic DNA was extracted from three microcosms per treatment using the FastDNA® spin kit (MP bio, Santa Ana, USA) following the manufacturer's protocol. The extracted DNA was homogenized subject to PCR amplification. The V4 variable region for bacterial and archaeal 16S rRNA was amplified with primer pairs 515F/806R (Caporaso et al. 2011). Fungal internal transcribed spacer (ITS) was amplified using primer pair ITS1F-Bt1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4R-bt (TCCTCCGCTTATTGATATGC). Sequencing was performed at Mr. DNA (Shallowater, TX, USA) on a MiSeq platform following the manufacturer's guidelines. The sequences were denoised after depletion of the barcodes and removal of the ones that were less than 150 bp or having ambiguous bases. Operational taxonomic units (OTUs) were generated and chimeras were removed. OTUs were defined by clustering at 97 % similarity. Final OTUs were taxonomically classified using BLAST against a curated database derived from GreenGenes, RDPII, and NCBI (DeSantis et al. 2006).The reads were deposited into the NCBI short reads archive database (SRP059155).

Statistical analyses

The similarity of microbial communities among different treatments was determined using UniFrac. QIIME calculated both weighted and unweighted UniFrac. Unweighted pair group method with arithmetic mean (UPGMA) clustering were conducted on weighted UniFrac (Kuczynski et al. 2012). The open-source software Cytoscape 2.816 was employed to visualize the 33 most abundant prokaryotic genera and 22 most abundant fungal taxa based on their relative abundances.

Results

Methane production

In the current study, the values of TS and VS were summarized in Table S1. As demonstrated in Fig. 1, methane was produced in both thermophilic and mesophilic treatments

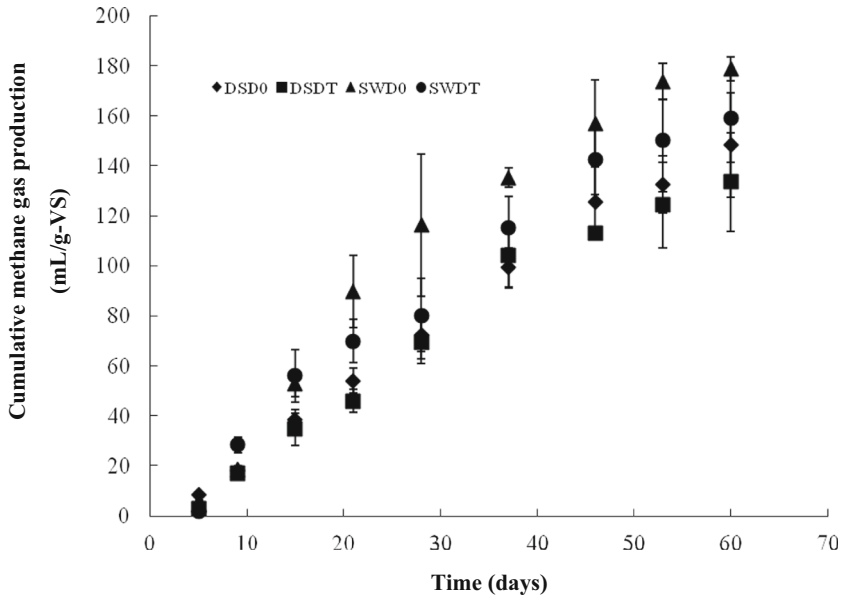

Fig. 1 Methane production yield during the inoculation period

throughout the incubation periods, indicating the activity of methanogens in mesophilic and thermophilic conditions.

General analyses of the Illumina-derived dataset

A total of eight sequencing libraries were retrieved including four prokaryotic (bacterial and archaeal) sequencing libraries and four fungal sequencing libraries. Detailed information of sequencing libraries was summarized in Table 1. After filtering the low-quality reads, chimeras and trimming the adapters, barcodes and primers, a total of 132,431 valid reads (average read length 280 bp) were identified from four prokaryotic sequencing libraries and 168,219 valid reads (average read length 250 bp) were identified from four fungal ITS sequencing libraries through Illumina MiSeq sequencing. The number of Chao 1, ACE, and Shannon at a cutoff level of 3 % were summarized in Table 2. Thermophilic conditions may not decrease the microbial diversity as indicated from non-parametric indicators. For example, SWDT has higher Chao 1 and ACE than SWD0. For fungal sequencing libraries, both DSFT and SWFT have higher Chao 1 and ACE than DSF0 and SWF0, respectively, indicating that fungal communities at higher temperature may be more diverse than at mesophilic conditions. The Venn diagram, as calculated at a distance of 0.03, with shared and unique OTUs was used to describe the similarity and difference among different libraries (Fig. 2). The prokaryotic sequencing libraries shared 152 out of 22, 254 OTUs (0.68 % of the total) that have been observed in the four treatments. The number of OTUs unique to individual sequencing library was 7048 (DSD0), 3252 (DSDT), 4306 (SWD0), and 5027 (SWDT), accounting for 88.2 % of the total observed OTUs. The fungal sequencing libraries shared 39 out of 7345 OTUs (0.53 % of the total) that have been observed in the four treatments. The number of OTUs unique to individual sequencing library was 598 (DSF0), 1645 (DSFT), 1527 (SWF0), and 2860 (SWFT), accounting for 90.3 % of the total observed OTUs. The small number of OTUs shared by all four prokaryotic and fungal libraries indicated diverse microbial communities inhabiting in these mixed microbial systems.

Table 1 Different Illumina sequencing libraries and their corresponding inoculation conditions

Sample name	Targeted genes	Inoculum	Temperature (°C)
DSD0	Bacterial and archaeal 16S rRNA	Sludge	37
DSDT	Bacterial and archaeal 16S rRNA	Sludge	58
SWD0	Bacterial and archaeal 16S rRNA	Manure	37
SWDT	Bacterial and archaeal 16S rRNA	Manure	58
DSF0	Fungal ITS	Sludge	37
DSFT	Fungal ITS	Sludge	58
SWF0	Fungal ITS	Manure	37
SWFT	Fungal ITS	Manure	58

Table 2 Summary of Illumina sequencing data and microbial diversity based on OTUs at 97 % sequence similarity

Sequencing library	Qualified reads	No. of OTU	Coverage	Chao1	Ace	Shannon	Simpson
DSD0	43293.0	8832.0	0.8	41403.8	90368.1	7.0	7.3
DSDT	24398.0	4838.0	0.8	23339.4	53544.7	6.4	6.8
SWD0	29972.0	5589.0	0.8	23707.3	54815.8	6.3	6.7
SWDT	34768.0	6400.0	0.8	30089.6	63493.5	6.1	6.5
DSF0	10127.0	991.0	0.9	2353.4	4398.5	4.9	0.0
DSFT	38916.0	2176.0	1.0	4895.9	6762.3	4.6	0.0
SWF0	56704.0	1951.0	1.0	3310.2	4054.9	3.0	0.2
SWFT	62472.0	3326.0	1.0	6152.0	8328.0	4.9	0.0

Prokaryotic taxonomic profile

We taxonomically grouped the Illumina sequencing libraries according to Ribosome Database Project (RDP). The prokaryotic OTUs fell into 32 different bacterial phyla and 2 archaeal phyla. *Proteobacteria* accounted for 45.6 and 25.2 % of the total valid reads in DSD0 and DSDT, respectively (Figure S1). However, *Firmicutes* was the most abundant phylum in sequencing libraries derived from manure samples, accounting for 46.5 and 69.3 % in SWD0 and SWDT, respectively. It is notable that sequences related to *Firmicutes* were more abundant at thermophilic than mesophilic conditions. As indicated by a previous study, the 515F/806R primer pair is nearly universal to archaea and bacteria (Walters et al. 2011). In accordance with this observation, our current study also identified a large number of archaeal sequences in four libraries. Archaeal reads accounted for 6.0 % of the total reads, indicating a relatively small amount of archaeal reads occurring in the libraries compared to bacterial reads. *Euryarchaeota* was the most abundant archaeal phylum in all libraries. *Crenarchaeota* only constituted a minor fraction in archaeal reads with relative abundances less than 1 %. Notably, *Euryarchaeota* increased in abundances at thermophilic conditions, especially in sludge samples.

Further comparison of the dominant phyla down to class, order, and genus levels was performed to reveal the microbial community evolution in response to temperature change. At the class level, *Clostridia* was the most abundant bacterial class accounting for 28.5 % of the total reads, followed by the classes *Deltaproteobacteria* (8.68 %), *Actinobacteria* (7.74 %) and *Betaproteobacteria* (7.11 %). A total of ten archaeal classes were identified and *Methanobacteria* was the most abundant in thermophilic libraries (DSDT and SWDT) (Figure S2). In contrast, *Methanomicrobia* demonstrated relatively high abundances in mesophilic libraries (DSD0 and SWD0). Diverse microbial communities were also demonstrated at order levels (Figure S3A). For instance, in thermophilic libraries, *Thermoanaerobacterales*, *Actinomycetales*, *Syntrophobacterales*, and *Methanobacteriales* were the most dominant orders seeded from sludge, while *Clostridiales*, *Bacillales*, *Haloplasmatales*, *Bacteroidales*, and *Thermoanaerobacterales* were the dominant orders derived from manure samples. Notably, *Thermoanaerobacterales* became the dominant orders in two thermophilic libraries, indicating that the members of *Thermoanaerobacterales* may contain many thermophilic species. Furthermore, *Methanobacteriales* was the most abundant archaeal order in two thermophilic

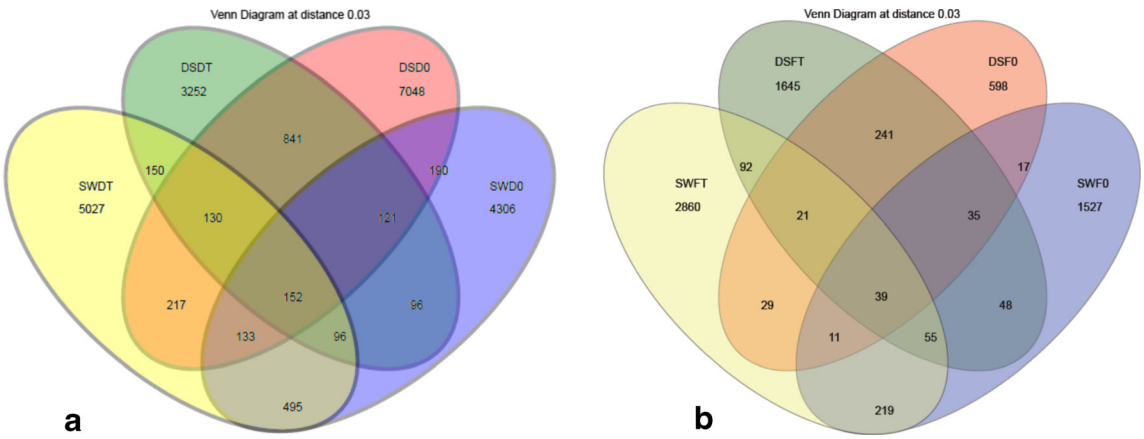

Fig. 2 Venn diagrams (calculated in MOTHUR v.1.28.0 at a distance of 0.03) from Illumina prokaryotic sequencing data (**a**) and fungal sequencing data (**b**). Central overlap shows the 152 OTUs in core community shared between the four prokaryotic sequencing libraries and 39 OTUs in core community shared between the four fungal ITS sequencing libraries

libraries, suggesting that *Methanobacteriales*-affiliated methanogen may play a central role in thermophilic methane production.

Fungal taxonomic profile

Fungal phylogenetic affiliation was determined by fungal ITS. A total of seven fungal phyla were detected (Figure S4). About 82.7 % of the total fungal reads could be assigned to the phylum *Ascomycota*, which was the most abundant phylum in libraries of DSFT, SWF0, and SWFT. Nevertheless, *Basidiomycota* was the most dominant phylum in DSF0 (79.4 %). Five other fungal phyla, including *Glomeromycota*, *Chytridiomycota*, *Monoblepharidomycota*, *Neocallimastigomycota*, and *Blastocladiomycota* occurred only sparsely. At the class level, the difference among fungal communities inoculated from different biomasses is distinguished. *Dothideomycetes* was the most abundant class in libraries derived from manure samples. In libraries derived from sludge, the abundance of *Saccharomycetes* increased along with temperature increasing. Conversely, the abundance of *Basidiomycota* decreased when the temperature increased. At the fungal order level, *Saccharomycetales* became the most dominant fungal order in two thermophilic libraries, accounting for 22.89 % in SWFT and 61.56 % in DSFT (Figure S3B). *Basidiomycota* and *Paraglomerales* were other dominant fungal orders in two thermophilic libraries. It is noteworthy that all these three fungal orders only occurred with low levels in two mesophilic libraries (<2 %), suggesting that they might be enriched by the temperature increase.

Microbial community evolution in response to the temperature increase

Differences between four prokaryotic libraries and four fungal libraries were demonstrated from the 16S rRNA and ITS amplicon data as shown in Fig. 3. Unifrac PoCA analysis indicated that temperature may have a significant impact on the microbial communities, where PoCA axis 1 showed 55.1 and 81.9 % of the variation and PoCA axis 2 showed 31.3 and 14.6 % variation in prokaryotic and fungal communities, respectively. These results were supported by the observed changes in the representative abundance in the major clades, especially at the genus level within the prokaryotic and fungal communities. Libraries of DSD0 and DSDT were clustered, while SWD0 and SWDT were distantly related (Fig. 3a). A similar trend was observed in the fungal communities (Fig. 3b). DSF0 and DSFT were grouped together, whereas SWFT and SWF0 were not clustered. High temperature is a primary factor to shift the abundance of the major taxonomic groups, especially at genus level. Therefore, the analyses based on the genus level will allow us to compare and further verify the functional evolution of the community in details.

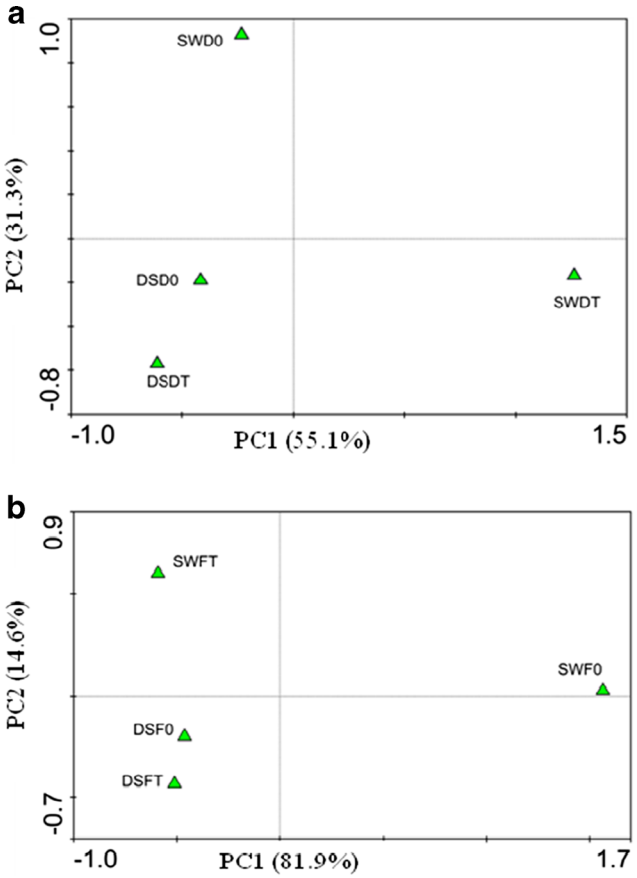

Fig. 3 PCoA plot based on the sequencing data. The microbial community of eight libraries (four prokaryotic and four fungal libraries) were compared and clustered. **a** Comparison of four prokaryotic sequencing libraries.**b** Comparison of four fungal libraries. The axes are the percentage of variation explained by the components

As illustrated from Fig. 4, sequences related to genera *Coprothermobacter*, *Dermatophilus*, *Syntrophus*, and *Azoarcus* showed relatively high abundances (>5 % relative abundances) in DSDT. Among these four genera, *Coprothermobacter* and *Dermatophilus* were frequently detected in TAD. In SWDT, *Bacillus*, *Haloplasma*, *Proteiniphilum*, *Clostridiisalibacter*, and *Caldicoprobacter* occurred at high levels (>5 % relative abundances). In addition, the abundances of *Bacillus*, *Haloplasma*, *Clostridiisalibacter*, and *Caldicoprobacter* increased notably at thermophilic conditions, whereas the number of *Proteiniphilum* dropped significantly with a temperature increase. At the thermophilic condition, *Methanobacterium* became the most dominant archaeal genus in DSDT (>90 % of the total archaeal reads) (Fig. 5), while the relative abundances of *Methanosaeta*, *Methanomicrobium*, and *Methanothermobacter* were less than 5 % of the total archaeal reads in DSDT. *Methanothermobacter* and *Methanomicrobium* were prevalent in SWDT with relative abundances greater than 20 % of the total archaeal reads. Sequences assigned to genera *Methanosphaera*,

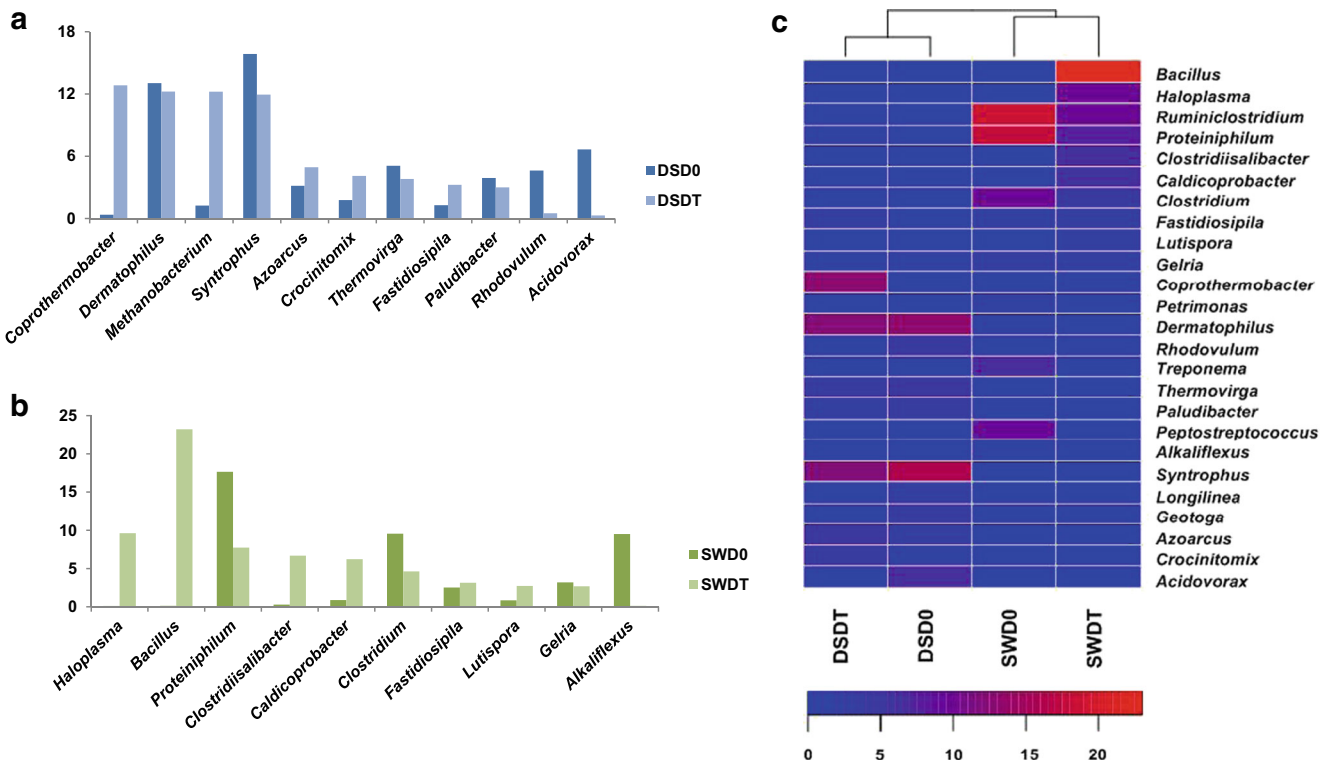

Fig. 4 Comparison of the relative abundance (%) of the most common bacterial genera (>1 %) in four prokaryotic sequencing libraries in the **a** sludge microcosms and **b** manure microcosms. **c** Heatmap of the dominant genera in each sequencing library. Please note the unit of y-axis of **a** and **b** is %

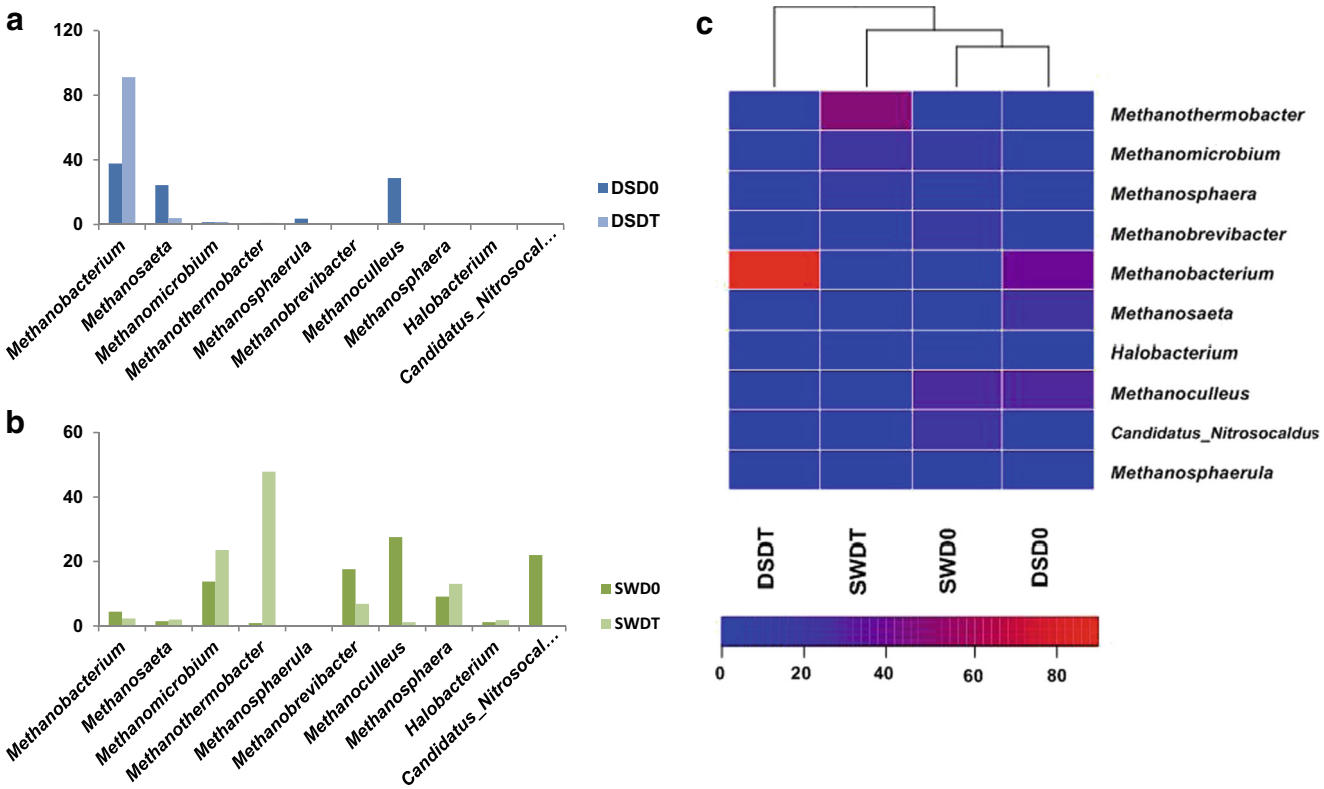

Fig. 5 Comparison of the relative abundance (%) of the most common archaeal genera (>1 %) in four prokaryotic sequencing libraries in the **a** sludge microcosms and **b** manure microcosms. **c** Heatmap of the dominant genera in each sequencing library. Please note the unit of y-axis of **a** and **b** is %

Methanobrevibacter, *Methanobacterium*, *Methanosaeta*, *Halobacterium*, and *Methanoculleus* demonstrated their relative abundances greater than 1 % of the total archaeal reads in SWDT.

A number of fungal genera increased their abundances at thermophilic conditions compared to mesophilic conditions (Fig. 6). For instance, *Alternaria*, *Cladosporium*, *Penicillium*, *Hypocrea*, *Epicoccum*, *Pseudocosmospora*, *Aspergillus*, *Pichia*, and *Trichosporon* were abundant (>3 %) in SWFT. However, their relative abundances in SWF0 were less (<1 %) as compared with that in SWFT. *Candida*, *Dipodascus*, *Geotrichum*, *Galactomyces*, *Exophiala*, *Cyberlindnera*, *Trichoderma*, *Peyronellaea*, *Engyodontium*, *Rhodotorula*, *Paraconiothyrium*, and *Cyclothyrium* increased their relative abundances significantly with the increase in temperature. In contrast, the genus of *Leptosphaerulina* (81 % in SWF0), which dominated in mesophilic conditions, decreased its abundance notably at the elevated temperatures.

Furthermore, a profile clustering network analysis was applied to compare the differences of the microbial communities at mesophilic and thermophilic conditions in a straightforward way. One can determine the change of abundances of a certain genus in different libraries based on the size of corresponding node. The network analysis generated by a Cytoscape network showed the most abundant prokaryotic and fungal genera and highlighted their relative distribution and abundances. At the bacterial network (Fig. 7), *Syntrophus*, *Dermatophilus*, *Coprothermobacter*, *Methanobacterium*, *Proteiniphilum*, *Ruminiclostridium*, and *Bacillus* were the most abundant genera based on the size of the corresponding nodes. For example, *Syntrophus* was the most abundant in DSD0 and DSDT. *Coprothermobacter* was more abundant in DSDT based on the size of purple node than other nodes. *Bacillus* was exclusively abundant in SWDT. Other genera with smaller nodes, such as *Paludibacter* and *Fastidiosipila*, were ubiquitous in four libraries.

At the fungal Cytoscape network, the sizes of nodes representing *Leptosphaerulina* and *Basidiomycota* were larger than other taxa (Fig. 8), indicating their dominance in SWF0 and DSF0. A number of purple and yellow nodes demonstrated that the corresponding genera were exclusively enriched in thermophilic conditions. In accordance with such observation, many of such genera contained thermophilic fungal species.

Discussion

Previous studies indicated that temperature had a remarkable impact on microbial communities in many microbial systems, such as enhanced biological phosphorus removal system (Panswad et al. 2003), hot springs (Skirnisdottir et al. 2000), acid mine drainage (Sun et al. 2015), and activated sludge (Wang et al. 2012). Therefore, we proposed that a change of temperature from 37 to 58 °C may result in the shift of

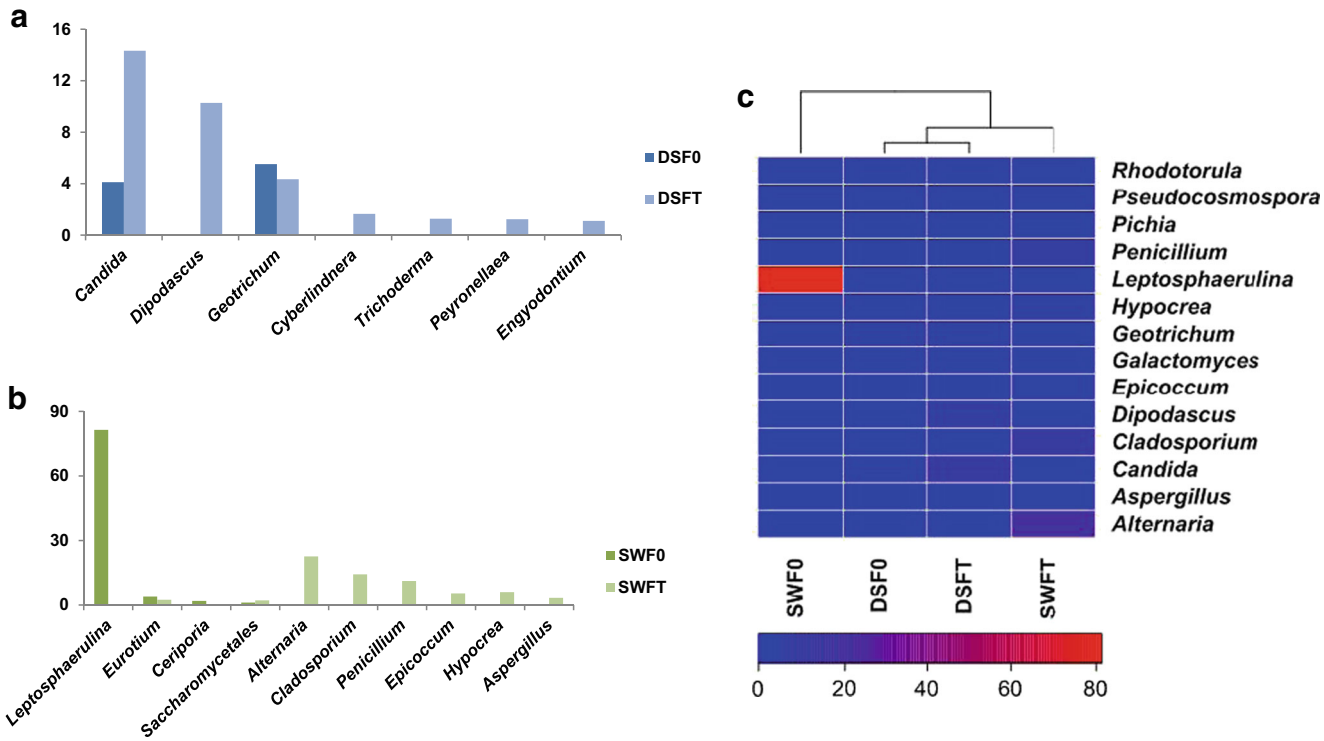

Fig. 6 Comparison of the relative abundance (%) of the most common fungal genera (>1 %) in four fungal sequencing libraries in the **a** sludge microcosms and **b** manure microcosms. **c** Heatmap of the dominant genera in each sequencing library. Please note the unit of y-axis of **a** and **b** is %

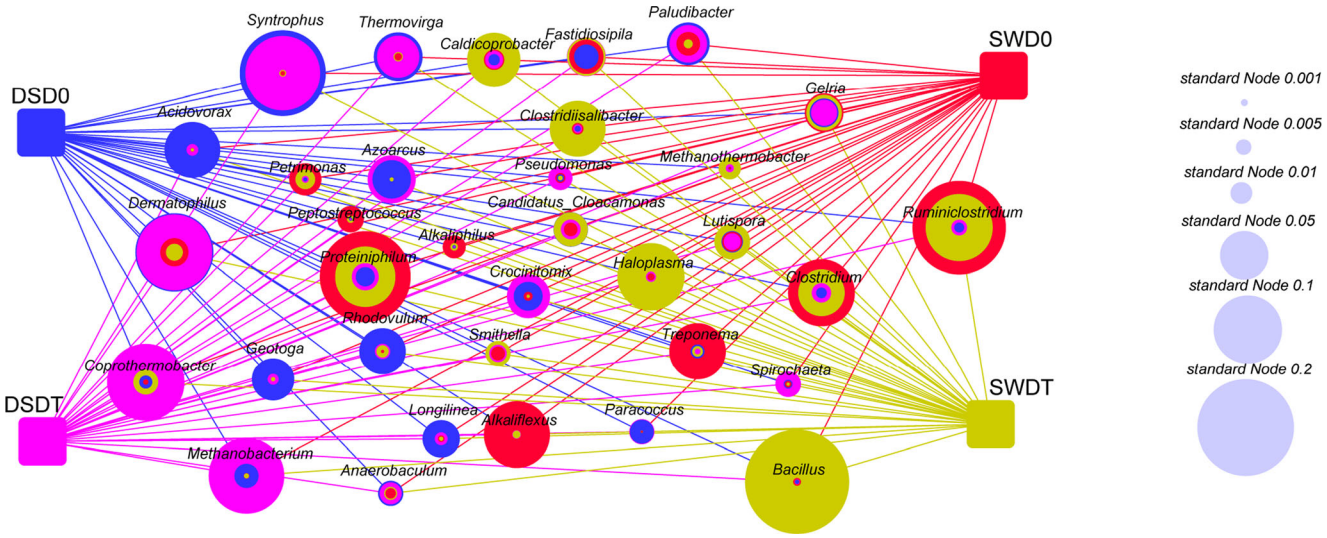

Fig. 7 Profile clustering Cytoscape network visualizing the 33 most abundant bacterial and archaeal genera and their associations with four prokaryotic sequencing libraries. A comparative node (*blue*) indicates the size of a node that would represent the relative abundance in a group. For example, standard node 0.01 shows that this size would represent 1 % of the corresponding genus in a group. Standard node 0.2 shows that this size would represent 20 % of the corresponding genus in a group

microbial communities by selecting these microorganisms able to adapt to the thermal environment. These enriched microorganisms may play an important role in TAD and investigation of these microorganisms may provide critical information for operating and optimizing the performance of TAD. In addition, methane production rate in thermophilic conditions is slightly higher than mesophilic conditions. This discrepancy in methane production might be due to the change of major methanogens, as discussed later.

In accordance with our hypothesis, the microbial community results showed significant differences between mesophilic and thermophilic conditions as indicated by PCoA and UPGMA analyses. At phylum level, a remarkable observation was that the abundances of *Firmicutes* increased in both manure and sludge samples when temperature increased to 58 °C. The dominance of *Firmicutes*-related bacteria in thermophilic conditions could be related to their ability of adaptation to living in thermal environments. Our

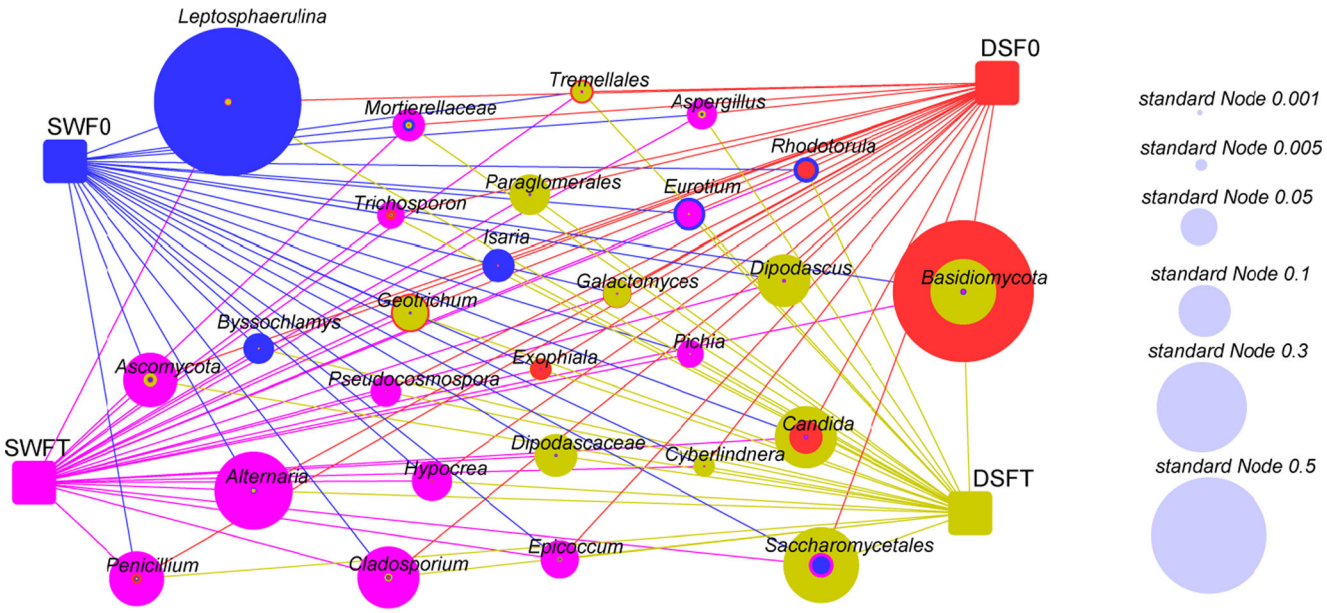

Fig. 8 Profile clustering Cytoscape network visualizing the 22 most abundant fungal genera and their associations with four fungal sequencing libraries. A comparative node (*blue*) indicates the size of a node that would represent the relative abundance in a group. For example, standard node 0.01 shows that this size would represent 1 % of the corresponding genus in a group. Standard node 0.5 shows that this size would represent 50 % of the corresponding genus in a group

observations were supported by previous studies where *Firmicutes* has also been detected in the extreme thermophilic environments (Emmerich et al. 2012; Orcutt et al. 2010).

In this study, we have been able to capture broader phylogenetic taxa than in any previous studies of TAD, including sequences of several taxa that were rarely detected before. Several fungal phyla, including *Glomeromycota*, *Chytridiomycota*, *Monoblepharidomycota*, *Neocallimastigomycota*, and *Blastocladiomycota* were seldom reported in TAD. These fungal phyla only constituted a minor fraction in the total fungal reads (2.3 % overall). However, we could not rule out the possibility that they played an ecological role in TAD. *Ascomycota* was the dominant fungal phylum in two thermophilic sequencing libraries (DSFT and SWFT) and one mesophilic sequencing library (SWF0), accounting for more than 90 % of the total reads in manure sequencing libraries and 70 % in DSFT. The dominance of *Ascomycota*-related fungi suggested their importance in TAD. In agreement with this observation, *Ascomycota* has been reported as important decomposers (Osono 2003) for breaking down organic compounds (Štursová et al. 2012).

Deeper sequencing at the genus level revealed that some genera were enriched atthermophilic conditions, indicating these microorganisms were able to survive or even thrive under thermophilic conditions. Remarkably, different genera were enriched in manure and sludge samples, indicating that the inocula were another important factor in determining the microbial community compositions. Some of the enriched genera have been identified as thermophiles. However, several genera have rarely been detected in thermophilic conditions, especially fungal genera, suggesting that our current understanding of microbial communities in TAD is still very limited. Although microorganisms have not been isolated in the present study, it may be possible to predict their metabolic capabilities based on those phylogenetically closely related isolates (Bond et al. 2000).

Important bacterial genera and species

Coprothermobacter was the most abundant genus in DSDT, but this phylotype demonstrated much less relative abundance in DSD0. *Coprothermobacter*-related species have been isolated from sludge previously. For instance, *Coprothermobacter platensis* was an anaerobic proteolytic thermophilic bacterium isolated from anaerobic mesophilic sludge (Etchebehere et al. 1998). In another study, Wrighton et al. (2008) isolated *Coprothermobacter* from thermophilic microbial fuel cells. In this study, *Coprothermobacter* was closely related to *Coprothermobacter proteolyticus*, which was isolated from biokitchen wastes digested by a dry anaerobic composting process (DRANCO) under thermophilic conditions (55 °C) (Kersters et al. 1994). The optimal temperature for this species was 65–70 °C. *C. proteolyticus* was able to utilize proteinaceous materials (Sasaki et al. 2011) and oleate under thermophilic conditions (Menes et al. 2001). All these observations indicated *Coprothermobacter* species may play a significant role for digesting organic compounds in TAD. Other genera including *Syntrophus*, *Dermatophilus*, *Azoarcus*, and *Crocinitomix* occurred at relatively high abundances in DSDT.

Members of the genera *Bacillus*, *Haloplasma*, *Ruminiclostridium*, *Proteiniphilum*, *Clostridiisalibacter*, and *Caldicoprobacter* occurred at high levels in SWDT. Among these genera, *Bacillus*, *Haloplasma*, *Clostridiisalibacter*, and *Caldicoprobacter* increased their relative abundances in SWDT as compared to SWD0. *Bacillus* was the most abundant genus (23.22 %) in SWDT but only accounted for 0.1 % in SWD0, indicating that a temperature increase remarkably facilitated the growth of *Bacillus* species. In the current study, *Bacillus*-related sequences were closely related to *Bacillus infernus*. *B. infernus* was able to reduce Fe(III) with formate and lactate as electron donor at temperatures from 40 to 65 °C (Boone et al. 1995). In addition, *B. infernus* has been frequently detected in TAD (Cheon et al. 2007; Merlino et al. 2013; Park et al. 2008). The dominance of *B. infernus* in thermophilic manure samples implied its important role in TAD. Other genera, such as *Haloplasma*, *Clostridiisalibacter*, and *Caldicoprobacter* increased their abundances with temperature increase, suggesting that they may contain thermophilic microorganisms. Consistently, *Haloplasma* spp. were previously detected in thermophilic biogas reactors fed with different agricultural waste materials (Ziganshin et al. 2013), two-phase biogas reactor (Rademacher et al. 2012), a thermophilic anaerobic digester (Hori et al. 2014), and aerated cattle manure composting piles (Maeda et al. 2010). *Clostridiisalibacter paucivorans* have been detected in deep-sea hydrothermal environments (Jiang et al. 2015).*Caldicoprobacter* contained several thermophilic species, such as *Caldicoprobacter oshimai* (Yokoyama et al. 2010), *Caldicoprobacter algeriensis* (Bouanane-Darenfed et al. 2011) and *Caldicoprobacter guelmensis* (Bouanane-Darenfed et al. 2013). The detection of various microorganisms indicated that TAD might be mediated by a suite of thermophiles.

Important archaeal genera and species

Methanobacterium was the most abundant (91.2 % of total archaeal reads) archaeal genus in DSDT but reduced its abundance to 37.7 % in DSD0. The remarkable enrichment under thermophilic conditions indicated that the high temperature might facilitate the growth of *Methanobacterium*-related archaea. Most *Methanobacterium*-related sequences were closely related to *Methanobacterium subterraneum*. *M. subterraneum* was an alkaliphilic, eurythermic, and halotolerant methanogen, which was first isolated from deep granitic groundwater (Kotelnikova et al. 1998). *Methanobacterium* also contained some thermophilic species such as *Methanobacterium*

thermoautotrophicus isolated from sludge (Zeikus and Wolee 1972), *Methanobacterium wolfei* isolated from sludge and river sediments (Winter et al. 1984) and *Methanobacterium thermoalcaliphilum* isolated from a biogas plant (Blotevogel et al. 1985). All these observations suggested that *Methanobacterium* species have been frequently encountered in sludge under thermophilic conditions.

A different scenario was observed in manure samples. *Methanothermobacter* spp. significantly raised their abundances from 0.9 % in SWD0 to 47.9 % in SWDT. This remarkable difference indicated *Methanothermobacter* might adapt to thermophilic conditions and outcompete other methanogens. The genus *Methanothermobacter* was proposed early in 2000 (Wasserfallen et al. 2000). Our experimental results indicated that most *Methanothermobacter*-related sequences were closely related to *Methanothermobacter thermoautotrophicum*, accounting for 47.5 % in SWDT. *M. thermoautotrophicum* (formerly *Methanobacterium thermoautotrophicum*) is a lithoautotrophic and thermophilic archaeon (Zeikus and Wolee 1972), which was first isolated from sewage sludge in 1971. It has been frequently detected in anaerobic digestion processes (Luo and Angelidaki 2013), including some thermophilic digestion studies (Tada et al. 2006; Ueno and Tatara 2008). Overall, the enrichment of *Methanobacterium* and *Methanothermobacter* species in thermophilic conditions suggested their importance in producing methane in TAD.

Important fungal phylotypes

Compared to bacterial and archaeal communities, fungal communities in TAD have been less addressed in the previous studies. In the current study, Illumina sequencing offered an opportunity to systematically characterize the shift of fungal communities from 37 to 58 °C in anaerobic digestion. *Ascomycota* was the most dominant phylum in thermophilic samples, accounting for 82.5 % of total reads of DSFT and SWFT. Ritari et al. (2012) utilized pyrosequencing analysis to characterize the fungal communities in MAD and TAD. Only two phyla including *Ascomycota* and *Basidiomycota* were detected in that study and the *Ascomycota*-related sequences represented almost 99 % of the fungal sequences (Ritari et al. 2012). All these observations indicated *Ascomycota* probably contained members that were able to thrive in thermophilic conditions.

A phylogenetically wide spectrum of different fungal genera significantly increased their relative abundances in thermophilic conditions, suggesting their adaption to thermal environments. Another notable observation was that various fungal genera were enriched from different inocula. In sludge samples, *Candida* and *Dipodascus* seemed to prefer elevated thermophilic temperatures. In manure samples, *Alternaria*, *Cladosporium*, *Penicillium*, *Hypocrea*, and *Epicoccum* were found mostly or exclusively in thermophilic conditions. Numerous other fungal genera increased their abundances at elevated temperatures, but to a lesser extent. Among the enriched genera, many have been identified as thermophilic fungi such as *Candida* (Shin et al. 2001), *Dipodascaceae* (Hultman et al. 2010), *Alternaria* (Craveri et al. 1972), and *Cladosporium* (Ghazifard et al. 2001).

It is notable that thermophilic microcosms contained more fungal diversity than mesophilic microcosms. In accordance with our observation, Ritari et al. (2012) also found a more diverse fungal community in thermophilic digesters than in mesophilic digesters. Unlike bacteria, fungi are often able to thrive under extreme environments such as low nutrient availability, low pH, and low moisture (Buchan et al. 2003; de Boer et al. 2005; Margesin et al. 2005). Among these enriched fungal genera, many were capable of degrading organic compounds. For instance, *Candida* and *Penicillium* contained species that were able to degrade lignin and cellulose (Kirk and Farrell 1987; Kuhar et al. 2008; Wood and Garcia-Campayo 1991). This observation indicated that fungi might play an important ecological role in thermophilic digestion by degrading more complex organic compounds.

In summary, high-throughput Illumina sequencing was employed to characterize the shift in microbial communities during a transition from mesophilic digestion to thermophilic digestion. Our results showed that the bacterial, archaeal, and fungal community compositions changed dramatically, indicating that temperature was an important factor in shaping the microbial communities inhabiting the AD process. In addition, microbial community compositions differed significantly between samples inoculated from sludge and manure, suggesting that source of inoculum was probably another important factor in shaping the thermophilic microbial communities. A phylogenetically wide spectrum of phylotypes was enriched in thermophilic conditions, indicating that TAD might be mediated by a suite of thermophiles. Among them, many have been classified as thermophilic microorganisms. However, others have rarely been detected in thermophilic conditions, indicating the relatively broad phylogenetic coverage of high-throughput sequencing. Also, our experimental results demonstrated that a more diverse fungal community was found in TAD than in MAD. Further investigations are needed to isolate thermophiles to test their metabolic capabilities in TAD, as well as to profile the metagenomics for understanding the metabolic potentials of bacteria, archaea, and fungi in thermophilic conditions.

Compliance with ethical standards

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

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

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