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Research Article

# Investigation of Bacterial and Methanogen Community Composition and Diversity in full-scale Anaerobic Manure Digesters

**Benoit St-Pierre[1*], André-Denis G. Wright[2]**
[1]Department of Animal Science, South Dakota State University, USA
[2]School of Animal and Comparative Biomedical Sciences, University of Arizona, USA

## Abstract

Anaerobic digestion of biopolymers into methane is accomplished by complex microbial communities that remain poorly characterized. Their composition tends to vary, likely as a result of many factors such as substrate composition, operating parameters, and microbial succession. In order to gain further insight, we performed a comparative analysis of microbial community composition amongst four mesophilic full scale anaerobic digesters, using a combined total of 133,789 high quality, non-chimeric bacterial and methanogen sequence reads from PCR-generated amplicons of the 16S rRNA gene. In three digesters that used manure as their main substrate, methanogen populations were composed predominantly of a common *Methanosarcina*-related Operational Taxonomic Unit (OTU). The composition of bacterial populations in these digesters was more diverse, with the most highly represented OTUs consisting of different combinations of *Bacteroidetes-Chloroflexi* or *Bacteroidetes-Firmicutes*-unclassified bacteria. In contrast, the dominant methanogen OTU in the remaining digester, whose substrate consisted of manure and off-farm lipid waste, was related to species of the genus *Methanoculleus*, and two bacterial OTUs (*Chloroflexi* and unclassified) together represented 83.7% of bacterial sequence reads. Our results suggest that while methanogen composition in anaerobic dairy manure digesters appeared to be limited to two main profiles, there was very limited convergence in bacterial phylogenetic composition. Since the major bacterial OTUs identified corresponded to uncultured species belonging to uncharacterized genera, future investigations will be required to determine their biochemical roles and assess the level of functional redundancy among them.

**Keywords**: 16s rRNA; Anaerobic Digestion; Methanogen; Bacteria.

## Introduction

In striving towards improved sustainability, anaerobic digestion represents an attractive solution to reducing the environmental impact of agricultural and industrial waste by its ability to metabolize organic by-products into methane, which can be harvested and used as a source of renewable energy [1]. It is a natural, multi-step process that is accomplished by complex communities of anaerobic microorganisms[2,3], whose composition can differ greatly amongst biogas plants depending on factors such as the chemical nature of the substrate(s), pH, and temperature. These consortia typically consist of thousands of different types of microorganisms, most of which are uncharacterized species whose function and metabolic activities still remain to be fully investigated.

Anaerobic microorganisms tend to be very specialized, and their organization into communities allows them to benefit from the complementary metabolic activities of other species [4]. Anaerobic consortia collectively perform four main functions [3]: fermentation, acidogenesis, acetogenesis, and methanogenesis. Fermenting bacteria typically hydrolyze biopolymers (polysaccharides, lipids, and proteins) into monomers, which they convert into short chain fatty acids (e.g. lactate, propionate), alcohols, hydrogen, and carbon dioxide. These can be metabolized by organic acid oxidizing bacteria during acetogenesis to generate hydrogen, acetate, formate, and carbon dioxide. The products of acetogenesis are themselves used as substrates by methanogenic archaea to synthesize methane [2]. Many metabolic reactions are only thermodynamically favorable when terminal molecules are maintained at low concentrations, requiring that they be continuously metabolized by other members of the community. Anaerobic microorganisms are thus each highly dependent on others, likely coordinating inter- and intra-species interactions through mechanisms such as cell-cell associations and quorum sensing [5,6].

Manure is a mixture of feces, urine, water and bedding material waste that is produced in very large quantities by livestock production systems [1]. It is a very suitable substrate or co-substrate for anaerobic digestion, because it contains a variety of biopolymers (polysaccharides, proteins and lipids), and it is already colonized by anaerobic microorganisms. Typically, most microorganisms identified in anaerobic digesters treating animal slurry correspond to uncultured species [7], which on

***Corresponding author:** Benoit St-Pierre, Department of Animal Science, South Dakota State University, USA. Tel: +1-605-688-5409; E-mail: Benoit.St-Pierre@sdstate.edu

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its own presents a serious challenge for devising microbiological manipulation strategies to increase methane production. In addition, reports to date have highlighted a high level of microbial diversity among different systems treating animal slurry as their main substrate [8-13], which has made defining a core microbiome for this substrate increasingly difficult. Thus, perhaps common microbial components in anaerobic digesters may only be defined within specific categories of animal slurry, according to factors such as host, diet and geographical location.

In this context, we have focused our efforts on characterizing the microbial communities of mesophilic anaerobic digesters treating manure from dairy farms operated in the state of Vermont (USA). From three sampled biogas plants using slurry from dairy cows fed similar diets as their primary substrate, we have previously found that one was populated with phylogenetically very distinct methanogen and bacterial groups compared to the others [14,15]. To further characterize the extent of microbial diversity and digester profiles in this region,we have investigated the bacterial and methanogen composition from four other mesophilic plants operated on dairy farms, which we describe in this report.

## Materials and Methods

### Anaerobic Manure Digester Sampling

Samples were collected from the effluent of each of four large-scale mesophilic anaerobic manure digesters operated on dairy farms during the months of August or October 2012. Each digester was sampled once by collecting 200 ml of digestate at a depth of approximately 0.5 meter. Digester samples were maintained on ice after collection, and frozen at -20°C within 2-4 hrs. Samples remained frozen until DNA extraction.

Four Hills Farms (FHF) is located in Bristol, Vermont (USA). Its anaerobic digester is of mixed plug-flow design (DVO Inc, Chilton, WI, USA), with a capacity of 5.78 X $10^6$ liters, operating temperature of 38.3 °C, and manure is the only substrate used. The FHF anaerobic digester had been in operation for three months at the time of sampling, and had generated 2.87 X $10^5$ kWh of electricity during the month of October 2012.

Kane Scenic River Farms (KSR) is located in Enosburg Falls, Vermont (USA). Its anaerobic digester is of mixed plug-flow design (DVO Inc, Chilton, WI, USA), with a capacity of 2.64 X $10^6$ liters, operating temperature of 38.3 °C, and manure is the only substrate used. The anaerobic digester had been in operation for 12 months at the time of sampling, and had generated 8.3 X $10^4$ kWh of electricity during the month of October 2012.

Monument Farms (MF) is located in Middlebury, Vermont (USA). Its anaerobic digester is a mixed plug-flow design (DVO Inc, Chilton, WI, USA), with a capacity of 1.46 X $10^6$ liters and operating temperature of 38.3 °C. The MF anaerobic digester had been in operation for 14 months at the time of sampling. While dairy cattle manure is the main substrate, whey from a local cheese processing plant is also added as secondary substrate. The MF digester generated 5.6 X $10^4$ kWh of electricity during the month of August 2012.

Terryland Farms (TLD) is located in St-Eugene, Ontario (Canada). Its anaerobic digester is of complete-mix design (CH Four Biogas, Ottawa, ON, Canada), with a capacity of 1.00 X $10^6$ liters, and is operated at 40 °C. Manure represents approximately 50% (v/v) of the biomass treated by the digester, with the remainder consisting of off farm waste, such as animal fat from slaughterhouses or meat processing facilities (~ 25%), lipid-rich by-products from food manufacturing plants (~ 20%) and grease trap waste (~ 10%). At the time of sampling, the TLD digester had been in operation for four years. TLD had generated 1.64 X $10^5$ kWh of electricity during the month of August 2012.

### Microbial DNA Isolation and PCR Amplification of 16S rRNA Gene Sequences

Microbial DNA from the effluent of anaerobic manure digester samples was isolated as described by Yu and Morrison [16]. Briefly, the method consisted of lysing samples by repeated bead beating, followed by purification of microbial genomic DNA using the QIAamp DNA stool mini kit (QIAGEN). Hypervariable V1-V3 regions of methanogen and bacterial 16S rRNA genomic sequences were amplified from purified digester microbial DNA by PCR using a specific universal primer pair for each type. The Met86F forward primer [17] and Met 471 reverse primer (5'-GWRTTACCGCGGCKGCTG-3', modified from the 519R primer) were used to target methanogens, while the 27F forward primer[18] and 519R reverse primer [19] were used for bacteria. PCR reactions were performed using the Phusion DNA polymerase (ThermoFisher Scientific) on a C1000 Thermal Cycler (BioRad) under the following conditions: hot start (3 min, 98°C), followed by 30 cycles of denaturation (30 s, 98°C), annealing (30 s, 50°C) and extension (30 s, 72°C), and ending with a final extension period (10 min, 72°C). PCR products were separated by agarose gel electrophoresis, and amplicons of the expected size (~ 380 bp or ~ 500 bp for methanogen or bacterial 16S rRNA genes, respectively) were excised for DNA extraction using the QiaexII Gel extraction kit (Qiagen). For each digester sample, approximately 400 ng of amplicons from each primer pair were submitted to Molecular Research DNA Lab (MRDNA) (Shallowater, TX, USA) for next generation sequencing. Sequencing of methanogen 16S rRNA gene amplicons was performed using the Illumina MiSeq 300 platform to generate overlapping paired end reads, while bacterial 16S rRNA gene amplicons were sequenced using the Roche 454 platform.

### Computational Analysis of Methanogen and Bacterial 16S rRNA Gene Amplicons

Sequences for methanogen 16S rRNA gene V1-V3 amplicons,

assembled as contigs from overlapping MiSeq 300 paired-end reads from the same flow cell clusters, were screened for the presence of both Met86F (forward) and Met471R (reverse) primer nucleotide sequences. Full length reads were then screened for quality using custom Perl-written scripts (available upon request). Only full length reads with a minimal Phred quality score of 25 (base call accuracy of 99.7%) at each nucleotide position were used for population structure analysis.

Bacterial 16S rRNA sequence reads were screened for quality using MOTHUR [20]. Reads containing the 27F primer sequence were first selected using trim.flows [21]. The command shhh.flows, MOTHUR's implementation of PyroNoise [22], was then used to screen for high quality sequence reads according to default threshold values, and to create consensus sequences. Since a very limited number of bacterial sequence reads covered the entire 27F-519R targeted region of the bacterial 16S rRNA gene, the conserved sequence GTGTATGAAG, located at nucleotide positions 403 – 412 of the *Escherichia coli* 16S rRNA gene, was selected as the 3' end limit of the target region. Thus, for bacterial 16S RNA gene sequences, bioinformatics analysis was performed using the V1-V2 hypervariable regions.

Operational Taxonomic Unit (OTU) clustering for methanogen and bacterial sequence datasets was performed as separate analyses using the same steps and commands from the open-source software MOTHUR (commands and functions are underlined below) [20]. Alignments were generated by the align.seqs function, and further optimized using custom Perl-written scripts (available upon request) and user assessment. Chimeric sequences were identified using the chimera-slayer and uchime functions, and subsequently removed from further analysis. Aligned chimera-free digester sequence reads were then combined with aligned representative sequences from methanogen or bacterial OTUs identified in previous studies [14,15] to generate genetic distance using dist.seqs, which were provided as input to the function cluster to group sequence reads into OTUs. For methanogens, OTU clustering was performed at a genetic distance cutoff of 2%, which was determined from known methanogen species as a representative limit of genetic variation in 16S rRNA gene sequences between methanogen species of the same genus [14]. For bacteria, a genetic distance cutoff of 5% was used, which was determined to be representative of the genetic variation in 16S rRNA gene sequences for the V1-V2 hypervariable regions between bacterial species of the same genus, using *Clostridium*, *Prevotella*, and *Streptococcus* as representative genera [15]. Phylogenetic assignment of OTUs was performed using a combination of the web tool RDP classifier [23], and the BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI nucleotide sequence database[24].

For alpha diversity analysis, the command collect.single from MOTHUR was used to generate Shannon and Simpson indexes. To correct for differences in sequence read numbers among samples, these tests were performed on ten independent random samplings of 10,000 reads from each bacterial data set and on ten independent random samplings of 7,000 reads from each methanogen dataset. For beta diversity analysis, separate OTU shared files were generated for bacteria and methanogens, each file including the four digesters from this study and three from previous studies [14,15]. These shared files were used as input for the command pca in MOTHUR. Principal Components 1 (PC1) and 2 (PC2), representing the highest level of variation, were plotted using Microsoft Excel.

### Accession Numbers for Next Generation Sequencing Data

Sequence data is available from the NCBI Sequence Read Archive as experiments-runs SRX703251-SRR1584246 (FHF-bacteria), SRX708834-SRR1584248 (KSR-bacteria), SRX708956-SRR1584396 (MF-bacteria), SRX708957-SRR1584398 (TLD-bacteria), SRX708964-SRR1584417 (FHF-methanogens), SRX708965-SRR1584418 (KSR-methanogens), SRX708966-SRR1584419 (MF-methanogens), and SRX708967-SRR1584420 (TLD-methanogens).

## Results

### Analysis of Methanogen Populations in Anaerobic Manure Digesters

The methanogen composition of four large-scale dairy manure digesters was investigated using a combined total of 44,089 non-chimeric sequence reads, corresponding to 8,968 unique sequences that clustered into a total of 587 species-level OTUs using a genetic distance threshold of 2% (Table 1). Of the methanogen OTUs identified in this study, 24 OTUs had previously been identified in the effluent of other dairy manure digesters [14], including VT-Met-1, VT-Met-6 and Vet-Met-22. OTUs belonging to the order Methanosarcinales were overall the most highly represented, followed by the orders Methanomicrobiales and Methanobacteriales (Table 2).

The FHF, KSR and MF digesters had similar methanogen profiles, with diversity in the range of 142 - 186 OTUs, and the predominance of *Methanosarcina*-related archaea (Figure 1). PCA analysis also supported this observation (Figure 3). VT-Met-1 was the most highly represented OTU in these digesters (67.0 – 85.6%), and showed species-level sequence identity to *Methanosarcina thermophile* (99.2%). In contrast, methanogens related to species belonging to the genus *Methanoculleus* (order Methanomicrobiales) were found to be the most abundant in the TLD digester. Its most highly represented OTU, VT-Met-308, displayed 99.4% sequence identity to *Methanoculleus bourgensis*. It has recently been reported from a microbiological survey of co-digestion of brown water and food waste [25].

**Table 1.** General characteristics of the bacterial and methanogen profiles in an aerobic manure digesters

| | Digester | Reads[a] | OTUs | Single-read OTUs | Shannon[b] | Simpson[b] |
|---|---|---|---|---|---|---|
| **Bacteria** | FHF | 20,232 | 486 | 225 | 2.31 ± 0.01 | 0.227 ± 0.002 |
| | KSR | 41,888 | 2,874 | 1,616 | 3.51 ± 0.02 | 0.126 ± 7E-4 |
| | MF | 14,446 | 438 | 219 | 1.64 ± 0.02 | 0.481 ± 0.003 |
| | TLD | 13,135 | 306 | 142 | 1.68 ± 0.01 | 0.374 ± 0.002 |
| **Methanogens** | FHF | 7,329 | 186 | 118 | 1.69 ± 0.01 | 0.471 ± 0.002 |
| | KSR | 7,771 | 154 | 88 | 1.24 ± 0.01 | 0.585 ± 0.002 |
| | MF | 10,461 | 142 | 83 | 0.85 ± 0.02 | 0.739 ± 0.003 |
| | TLD | 18,528 | 357 | 195 | 1.74 ± 0.02 | 0.460 ± 0.004 |

[a]. Number of chimera-free sequence reads used for population composition analysis

[b]. Values shown are the averages and standard deviations from 10 samplings of 10,000 reads for bacteria and from 10 samplings of 7,000 reads for methanogens

**Table 2.** Taxonomic distribution of methanogen OTUs and their relative abundance in anaerobic manure digesters.

| | FHF | | KSR | | MF | | TLD | |
|---|---|---|---|---|---|---|---|---|
| **Order** | **OTUs** | **%[a]** | **OTUs** | **%[a]** | **OTUs** | **%[a]** | **OTUs** | **%[a]** |
| *Methanosarcinales* | 80 | 83.05 | 55 | 81.42 | 61 | 89.29 | 160 | 25.59 |
| *Methanomicrobiales* | 32 | 8.75 | 21 | 4.86 | 21 | 3.79 | 126 | 73.02 |
| *Methanobacteriales* | 65 | 7.60 | 74 | 13.64 | 55 | 6.83 | 26 | 0.60 |
| *Methanoplasmatales* | 1 | 0.46 | 1 | 0.04 | 1 | 0.05 | 1 | 0.00 |
| unclasssified | 7 | 0.14 | 3 | 0.04 | 4 | 0.04 | 44 | 0.78 |

[a]. Percentage of digester reads belonging to each order.

## Analysis of Bacterial Populations in Anaerobic Manure Digesters

For bacteria, a combined total of 89,701 non-chimeric sequence reads was obtained. These corresponded to 17,826 unique sequences, which clustered into 3,748 OTUs at a 5% genetic distance cutoff (Table 1). Taxonomic assignment revealed that 2,571 OTUs belonged to 14 bacterial phyla, while 1,177 OTUs were designated as unclassified (Table 3). With 1,641 OTUs, bacteria belonging to the phylum *Firmicutes* were the most diverse, followed by those belonging to the phyla *Bacteroidetes* (660 OTUs), *Proteobacteria* (121 OTUs) and *Chloroflexi* (102 OTUs). The remaining bacteria with taxonomic affiliations belonged to a wide range of phyla: *Actinobacteria* (20 OTUs), *Synergistetes* (11 OTUs), *Fusobacteria* (4 OTUs), TM7 (4 OTUs), *Verrucomicrobia* (2 OTUs), *Fibrobacteres* (2 OTUs), *Acidobacteria* (1 OTU), *Planctomycetes* (1 OTU), *Tenericutes* (1 OTU) and *Lentisphaerae* (1 OTU). Of the 3,748 manure digester OTUs found in this study, 483 OTUs had previously been identified in the effluent of other dairy manure digesters [15].

In the FHF digester, bacteria belonging to the phylum Bacteroidetes were the most prevalent group, representing 62.7% of its bacterial sequence reads (Figure 2). The two most abundant FHF OTUs in this phylum, VT-Bac-2 and VT-Bac-7, belonged to class Bacteroidia and have been identified in three other anaerobic manure digesters [15]. They each show limited sequence identity to their closest valid taxon, with respectively 84% to *Bacteroides coprocola*, originally isolated from human feces [26], and 88% to *Marinilabilia salmonicolor*, originally isolated from a marine environment [27]. VT-Bac-7 was also identified in a biogas plant using maize silage, green rye, and chicken manure as co-substrates [28].Well represented FHF Bacteroidetes also included OTUs specific to this digester, such as VT-Bac-2180, VT-Bac-2182, VT-Bac-2183 and VT-Bac-2184, which together represented 9.7% of FHF bacterial sequence reads.

Bacteria belonging to the phylum *Chloroflexi* were the second most prevalent group in the FHF digester, representing 29.9% of sequence reads, the vast majority belonging to VT-Bac-12 (15.0%) and VT-Bac-2158 (14.2%). Both OTUs have previously been identified in the effluent of the Green Mountain Dairy manure anaerobic digester, but in lower abundance (3.7% and 0.01%, respectively) [15]. They were assigned to the class Anaerolinea of the phylum *Chloroflexi,* whose valid species so far have been exclusively identified in anaerobic digesters [29]. In the FHF

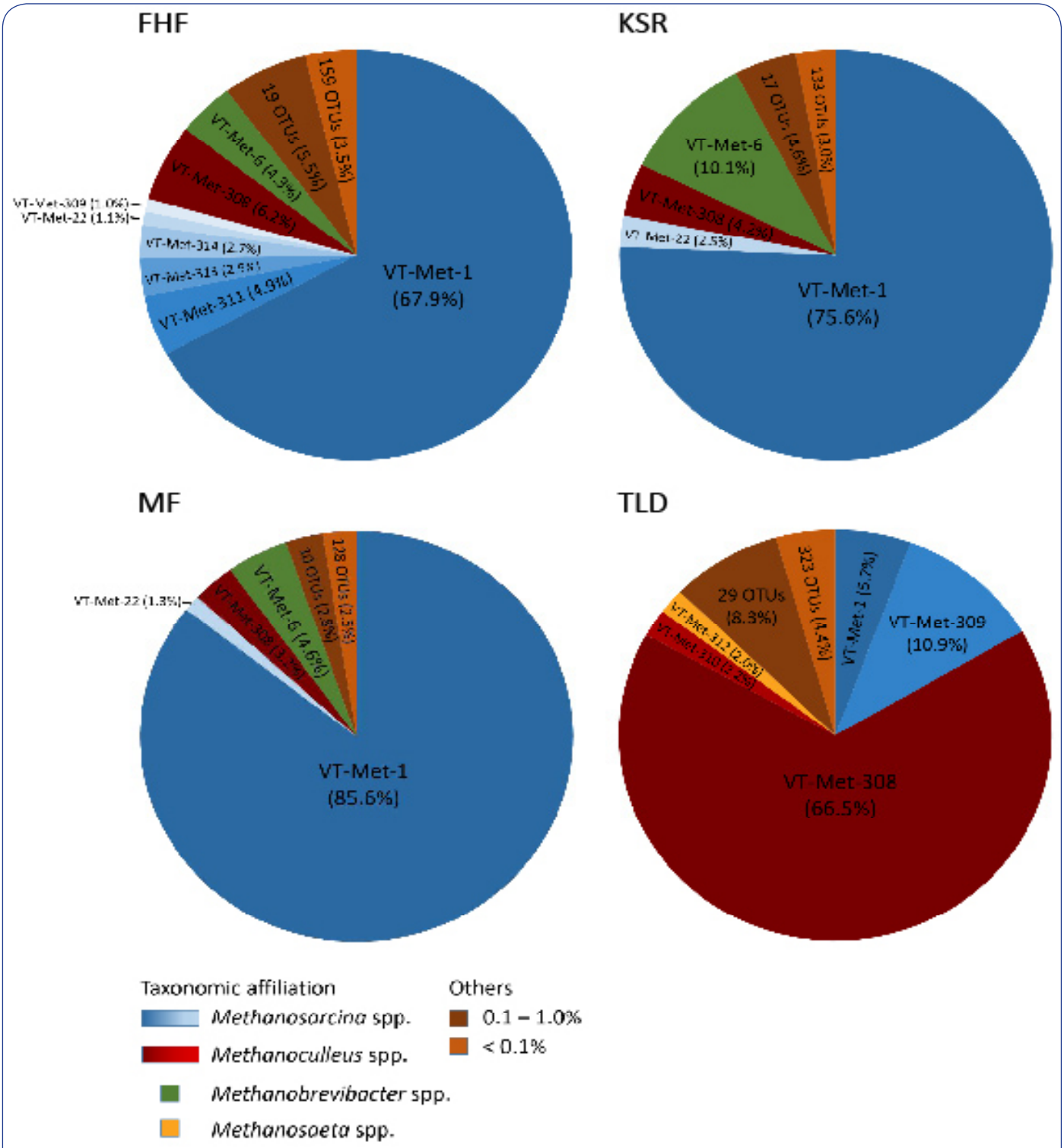


**Figure 1.** Methanogen OTU profiles in mesophilic anaerobic dairy manure digesters. Pie-chart diagrams show the methanogen OTU representation in the anaerobic manure digesters investigated: FHF (Four Hills Farms), KSR (Kane Scenic River Farms), MF(Monument Farms), and TLD (Terryland Farms). The taxonomic assignment (genus of closest related taxon) of each OTU is also indicated.

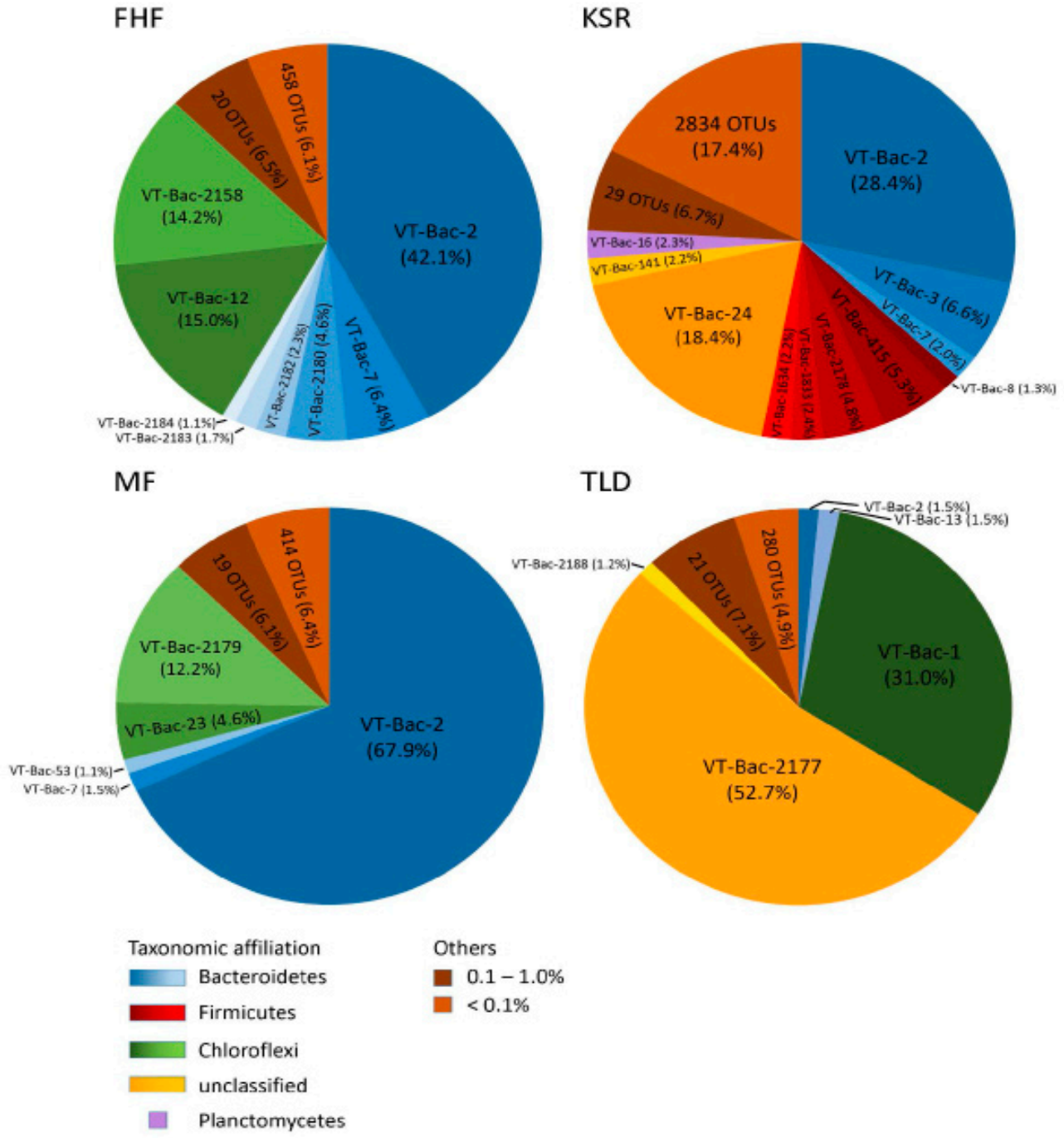


**Figure 2.** Bacterial OTU profiles in mesophilic anaerobic dairy manure digesters. Pie-chart diagrams show the bacterial OTU representation in the anaerobic manure digesters investigated: FHF (Four Hills Farms), KSR (Kane Scenic River Farms), MF (Monument Farms), and TLD (Terryland Farms). The taxonomic assignment (predicted phylum or bacterial group) of each OTU is also indicated.

**Table 3.** Taxonomic distribution of bacterial OTUs by phyla and their relative abundance in anaerobic manure digesters.

| | FHF | | KSR | | MF | | TLD | |
|---|---|---|---|---|---|---|---|---|
| **Phylum** | **OTUs** | **%[a]** | **OTUs** | **%[a]** | **OTUs** | **%[a]** | **OTUs** | **%[a]** |
| *Acidobacteria* | 0 | 0.00 | 1 | 0.01 | 0 | 0.00 | 0 | 0.00 |
| *Actinobacteria* | 0 | 0.00 | 20 | 0.07 | 0 | 0.00 | 0 | 0.00 |
| *Bacteroidetes* | 195 | 62.74 | 338 | 40.66 | 193 | 75.83 | 55 | 4.95 |
| *Chloroflexi* | 32 | 29.92 | 13 | 0.20 | 41 | 17.73 | 35 | 33.82 |
| *Fibrobacteres* | 0 | 0.00 | 2 | 0.01[b] | 0 | 0.00 | 0 | 0.00 |
| *Firmicutes* | 126 | 2.85 | 1478 | 28.21 | 78 | 1.80 | 74 | 2.65 |
| *Fusobacteria* | 3 | 0.02 | 2 | 0.01[b] | 0 | 0.00 | 0 | 0.00 |
| *Lentisphaerae* | 0 | 0.00 | 1 | 0.01[b] | 0 | 0.00 | 0 | 0.00 |
| *Planctomycetes* | 1 | 0.67 | 2 | 2.23 | 0 | 0.00 | 0 | 0.00 |
| *Proteobacteria* | 11 | 0.13 | 86 | 0.56 | 11 | 0.10 | 18 | 0.21 |
| *Synergistetes* | 0 | 0.00 | 10 | 0.07 | 1 | 0.01[b] | 0 | 0.00 |
| *Tenericutes* | 0 | 0.00 | 1 | 0.03 | 0 | 0.00 | 0 | 0.00 |
| TM7 | 1 | 0.01[b] | 4 | 0.01[b] | 0 | 0.00 | 0 | 0.00 |
| *Verrucomicrobia* | 0 | 0.00 | 1 | 0.01[b] | 0 | 0.00 | 1 | 0.02 |
| unclassified | 117 | 3.67 | 915 | 27.87 | 114 | 4.53 | 123 | 58.36 |

[a]. Percentage of digester reads belonging to each phylum.

[b]. Frequency less than or equal to 0.01%

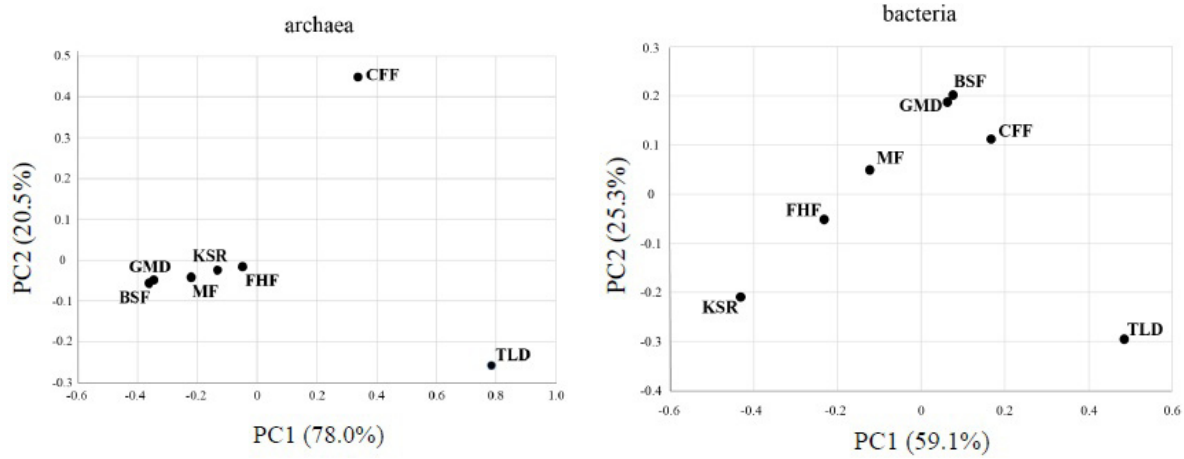


**Figure 3**. Comparison of bacterial and archaeal communities from anaerobic manure digesters by Principal Component Analysis. Principal Components were generated based on the OTU distribution of the samples described in this study [FHF (Four Hills Farms), KSR (Kane Scenic River Farms), MF(Monument Farms), and TLD (Terryland Farms)] as well as from previous reports [BSF (Blue Spruce Farms), GMD (Green Mountain Dairy), and CFF (Chaput Family Farms)]. Principal Components 1 (PC1) and 2 (PC2), representing the highest level of variation, are shown as scatter plots.

digester, *Firmicutes* and unclassified sequences were found at 2.8%, and 3.7%, respectively.

The MF digester showed an overall bacterial profile more similar to the FHF digester than to other samples (Figures 2-3). Indeed, bacteria belonging to the *Bacteroidetes* (75.8% of sequence reads) and *Chloroflexi* (17.7%) were the most abundant phyla and, similarly, bacteria belonging to the *Firmicutes* (1.8%) and those that were unclassified (4.5%) only represented a small percentage of sequence reads. While its most highly represented *Bacteroidetes* OTUs (VT-Bac-2 and VT-Bac-7) were the same as the FHF digester, the MF digester had different *Chloroflexi* OTUs, which consisted mainly of VT-Bac-23 and VT-Bac-2179. VT-Bac-23 had previously been identified in the effluent of the Green Mountain

Dairy digester [15], while VT-Bac-2179 was found to be specific to the MF digester.

The KSR bacterial populations were by far the most diverse amongst digesters sampled in this study, with 2874 OTUs and three main groups identified: *Bacteroidetes* (40.7%), *Firmicutes* (28.2%), and unclassified bacteria (27.9%) (Figures 2-3). Its most abundant *Bacteroidetes* OTUs (VT-Bac-2, VT-Bac-3, and VT-Bac-7) were the same as found in other manure digesters [15]. Very distinctive features of the KSR digester were lowest abundance of *Chloroflexi* (0.2%), and highest of *Firmicutes* amongst the samples collected in this study. Three of the main *Firmicutes* OTUs (VT-Bac-415, VT-Bac-1634 and VT-Bac-1833) had been previously identified in other anaerobic manure digesters, but in much lower abundance (< 0.1%). VT-Bac-2178 was the second most abundant *Firmicutes* OTU in the KSR digester, and was specific to that sample. Another distinct feature of the KSR digester's profile was the high prevalence of VT-Bac-24 (18.4%), whose taxonomic affiliation is currently undetermined, and it may belong to a bacterial phylogenetic group that has yet to be characterized. VT-Bac-24 had previously been found in the Blue Spruce Farms digester, but at a lower frequency (3.2%).

The bacterial populations identified in the TLD digester were the least diverse of the samples analyzed in this study (Figure 3; Table 1). They consisted primarily of unclassified bacteria (58.4%) and *Chloroflexi* (33.8%). VT-Bac-2177, the most abundant OTU in this sample, had not previously been identified in other manure digesters, and could not be assigned to known bacterial taxonomic groups. The second most abundant OTU, VT-Bac-1, belonged to the phylum *Chloroflexi*, and had previously been identified as the most abundant OTU in the Chaput Family Farms digester (26.0%) [15]. *Bacteroidetes* (4.9%) and *Firmicutes* (2.6%) were found in low abundance in the TLD digester.

## Discussion

For methanogens, two major composition profiles have been observed in our studies of dairy manure digesters: predominance of either *Methanosarcina*-related or *Methanoculleus*-related archaea. In comparison with reports from other groups on anaerobic digestion of manure, from either large scale- or laboratory scale-bioreactors, predominance of Methanosarcinales [30-32], predominance of Methanomicrobiales [33,34] or co-existence in similar proportions have been described [8,35,36]. In digesters with high representation of Methanosarcinales (FHF, KSR and MF), we found VT-Met-1 to be the most abundant methanogen OTU, representing at least 67.9% of archaea. The same OTU was also the most highly represented in the Blue Spruce Farms (BSF) and Green Mountain Dairy (GMD) digesters [14]. Accordingly, these five digesters were found to be clustered together by PCA (Figure 3).

We also observed that the representation of VT-Met-1 in *Methanosarcina*-predominant samples was higher in digesters that had been in operation for longer periods of time: 67.9% at 3 months (FHF), 75.6% at 12 months (KSR), 85.6% at 14 months (MF), 98.5% at 4 years (GMD) and 99.7% at 5 years (BSF). This could be indicative of succession for methanogen populations in digesters, perhaps transitioning from a "gastrointestinal composition", such as found in manure, to a "digester composition". Evidence of succession or transitions in methanogen composition has been reported in anaerobic digester environments, either from laboratory-scale experiments [37,38] or from sampling full-scale biogas reactors [39, 40]. Microbial succession could be explained from our current understanding of host-microbiota interactions in herbivores. By absorbing various nutrients such as volatile fatty acids, fatty acids, amino acids and saccharides, hosts such as dairy cows can act as competitors for substrates with their microbiota. In the absence of competing gastro-intestinal host cells in a digester environment, these nutrients would become more available, allowing certain types of microorganisms to thrive. For these opportunistic microorganisms to establish themselves as residents in a digester, they would likely need to maintain their populations in spite of continuous losses to effluent and competition from the input manure, which is already colonized with a gastrointestinal microbial community at very high density. After a certain time, the microorganisms with the best adaptations for a digester environment would remain established and would not easily be outcompeted under usual operating conditions [41]. While more in depth analyses are required to validate this model, our observations suggest that microbial succession should be taken into consideration when anaerobic digester microbial communities are investigated.

The TLD digester had in contrast a population profile with a higher frequency of *Methanoculleus*-related methanogens than *Methanosarcina* –related methanogens. In this respect, the TLD methanogen composition was reminiscent of the Chaput Family Farms (CFF) digester profile [14], with 48.0% representation of Methanomicrobiales. However, while manure digesters with high representation of *Methanosarcina*-related methanogens shared the same prominent OTU (VT-Met-1), two different *Methanoculleus*-related OTUs, VT-Met-2 and VT-Met-308, were identified in the CFF and TLD digesters, respectively. This distinction was well represented by PCA (Figure 3). The closest valid taxon to both OTUs was *Methanoculleus bourgensis*, but each showed a different degree of sequence identity (97.7% and 99.4%, respectively). Based on a 2% species-level genetic distance cutoff, VT-Met-2 may have represented a novel species of the genus *Methanoculleus.*

Anaerobic digestion of cattle manure typically results in high concentrations of acetate and ammonia. Species belonging to the genus *Methanosarcina* have been found to be prevalent in these conditions [30-32], due to their distinctive ability to use acetate as a substrate for methanogenesis and their greater robustness to conditions that would be too restrictive for most other methanogens

[42]. The identification of two dairy manure digesters with a predominance of *Methanoculleus*-related methanogens, CFF [14] and TLD (this study), was thus quite intriguing. These digesters are of different design, operated at distinct temperatures (36.1 °C vs 40.0 °C), and have distinct operating periods (10 months vs 4 years, at their respective time of sampling), but their common feature that was distinct from the other digesters sampled was the use of lipids as a co-substrate with manure from dairy cows. Perhaps Methanomicrobiales enrichment in manure digesters that added lipids as a co-substrate was due to their ability to metabolize distinct downstream by-products of lipid catabolism generated by upstream fermenting, acidogenic and / or acetogenic bacteria. Interestingly, while known *Methanoculleus*-related archaea use carbon dioxide and hydrogen gas as their main substrates for methanogenesis, they also have the distinctive ability to metabolize secondary alcohols into methane [1,43,44]. Alternatively, these methanogens may be resistant to currently undefined conditions that are detrimental to the survival of *Methanosarcina*-related archaea. Whether due to substrate or other factor, it would be of great interest to determine which conditions were favorable to Methanomicrobiales in the TLD and CFF digesters, and if they were also found in biogas plants treating cattle manure with plant biomass [33,34], swine manure [45-47] or chicken manure and plant biomass [9], where these methanogens have been reported to be abundant.

In contrast to digester methanogens, bacterial populations were far more complex, with the vast majority of bacterial OTUs identified corresponding to uncharacterized species whose functions and metabolic activities were difficult to predict based on phylogenetic assignments [7]. Since manure includes a variety of biopolymers such as soluble and structural polysaccharides, proteins and lipids, it has the potential to support a wide variety of bacterial metabolic activities, increasing the difficulty in assigning functions to unknown digester species. For instance, while the FHF, KSR, and MF digesters (this study), as well as the BSF and GMD digesters [14], all supported methanogen populations that were predominantly related to species of the genus *Methanosarcina*, they shared a very limited number of bacterial OTUs. Indeed, only VT-Bac-2 and VT-Bac-7 were identified in all dairy manure digesters, with representation in the range of 1.5% - 67.9% and 0.7% - 6.4%, respectively. Thus, there appeared to be very limited convergence in bacterial OTU composition despite the same type of substrate being used by dairy manure digesters. This is well illustrated by PCA, as these digesters were not grouped into a well-defined cluster (Figure 3). The diversity of profiles remained complex even when defined taxonomically, as they included co-dominance of *Bacteroidetes* and *Chloroflexi* (FHF and MF), co-dominance of *Firmicutes* and *Bacteroidetes* (BSF and GMD), and co-dominance of *Bacteroidetes*, *Firmicutes* and unclassified bacteria (KSR).

A similar situation was observed with *Methanoculleus*-predominant digesters, with very distinct TLD and CFF bacterial profiles. However, VT-Bac-1, found at respectively 31.0% and 21.0% compared to 0 – 0.4% in the other five other digesters, may represent a possible link between these two digesters. While other *Chloroflexi* OTUs (VT-Bac-12, VT-Bac-23, VT-Bac-2158 and VT-Bac-2179) were well represented in certain digesters (FHF and MH), perhaps VT-Bac-1 was found in greater abundance in TLD and CFF as a result of a particular metabolic activity or tolerance that was well adapted to their conditions. Alternatively, major *Chloroflexi* OTUs may perform similar functions, and their higher abundance in particular digesters is the result of a stochastic process rather than selection from specific factors. In addition to OTU functional redundancy and stochastic effects, microbial succession may also have influenced the bacterial composition of manure digesters. For instance, VT-Bac-2158 was found at 14.2% in the FHF digester compared to 0.01% in the GMD digester. Since the FHF digester had only been in operation for two months at the time the samples were collected, compared to four years for the GMD digester, perhaps VT-Bac-2158 corresponded to a species whose frequency would become reduced as the FHF digester continues to run.

To take full advantage of renewable biomass through anaerobic digestion, a better understanding of the microbiology responsible for this technology should continue to be pursued. As a result of our combined investigations of seven anaerobic manure digesters, we have observed two main methanogen profiles when dairy manure is used as primary substrate. Based on a comparison of operational parameters among digesters, the use of lipids as a co-substrate may be an important factor in determining whether a methanogen profile would be *Methanoculleus*-predominant rather than *Methanosarcina*-predominant. Due to its high energy content, lipid waste is a co-substrate of great interest to increase methane production [1]. While in depth functional investigations are required in order to gain further insights, we anticipate that different methanogens may need to be targeted for microbiological manipulations depending on whether lipids are used as a co-substrate.

With regards to the composition of bacterial populations in manure digesters, we have been unable to define a common core consortium for dairy manure based on phylogenetic predictions. Only two of the most abundant bacterial OTUs were identified in all samples, with other abundant OTUs overlapping between subsets of digesters. We hypothesize that functional redundancy amongst bacterial OTUs is one of the major reasons for this observation, and why convergence of bacterial populations to a common profile was not observed. However, from the perspective of improving biomethanation from agricultural waste, functional redundancy may hold the promise of increased potential and versatility of the pool of microorganisms that can be manipulated to fulfill biotechnological needs.

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