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Effects of geographic area, feedstock, temperature, and operating time on microbial communities of six full-scale biogas plants

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HIGHLIGHTS

- Investigation on microbiomes of Parmigiano Reggiano and Grana Padano biogas plants.
- *Methanosarcina* abundance correlates with ammonium concentration.
- *Methanoculleus* more present under thermophilic conditions.
- *Thermotogales* correlates with hydraulic retention time.
- Acetate levels seems to influence *Methanosarcina* and *Methanosaeta* distribution.

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ABSTRACT

The objective of this study was to investigate the effect of different animal feedings operated in two distinct PDO (protected designation of origin) cheese production areas (Parmigiano Reggiano and Grana Padano) on the microbiome of six full-scale biogas plants, by means of Illumina sequencing and qPCR techniques. The effects of feedstock (cattle slurry manure, energy crops, agro-industrial by-products), temperature (mesophilic/thermophilic), and operating time were also examined, as were the relationships between the predominant bacterial and archaeal taxa and process parameters. The different feedstocks and temperatures strongly affected the microbiomes. A more biodiverse archaeal population was highlighted in Parmigiano Reggiano area plants, suggesting an influence of the different animal feedings. *Methanosarcina* and *Methanosaeta* showed an opposite distribution among anaerobic plants, with the former found to be related to ammonium concentration. The *Methanoculleus* genus was more abundant in the thermophilic digester whereas representation of the *Thermotogales* order correlated with hydraulic retention time.

1. Introduction

Anaerobic digestion (AD) is a well-known process whose optimization is capturing research attention because of the increasing demand for renewable energy sources, along with environmental problems concerning disposal of organic waste (such as livestock manure, agricultural and industrial by-products, wastewater, and municipal solid wastes). AD is the biological conversion of organic material into different end products including 'biogas', which is constituted by methane (55–70%) and carbon dioxide. The process involves a microbial suite that breaks down the organic compounds in four steps (i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis) (Appels et al., 2011). Understanding the makeup of this microbial assembly through quantification and identification of the key phylotypes would be useful for improving reactor performance (Koch et al., 2014) and could be achieved using real-time PCR and next-generation sequencing techniques, respectively.

Illumina platform use in microbial ecology is increasing (Caporaso et al., 2012) because of lower costs and greater coverage, allowing generation of many millions of partial 16S rRNA gene sequence reads (Bartram et al., 2011). Several studies have sought to define the core microbiome of AD and correlate it with process efficiency. A major microbial richness and evenness have already

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been highlighted in mesophilic compared to thermophilic digesters (Li et al., 2014; Sun et al., 2015; Sundberg et al., 2013; Theuerl et al., 2015). However, concerning the bacterial community, two main phyla, *Firmicutes* and *Bacteroidetes*, have proved to dominate in AD, among them the orders *Clostridiales* and *Bacteroidales* (Campanaro et al., 2016; De Vrieze et al., 2015; Li et al., 2014 and Li et al., 2016; Theuerl et al., 2015). These two orders are related to free and total ammonia concentration in the digester, respectively (De Vrieze et al., 2015). Concerning the archaeal community, the acetoclastic methanogens *Methanosarcina* and *Methanosaeta* are considered the most predominant genera in mesophilic digesters whereas *Methanoculleus* and *Methanothermobacter* are more present in thermophilic ones (Campanaro et al., 2016; Li et al., 2014). Relationships between hydrogenotrophic methanogens and methane production rate have been highlighted (Jang et al., 2014), as has their positive correlation with NH_3-N concentration (Theuerl et al., 2015). Despite the existing studies, a deeper and more robust characterization of a core AD microbiome, along with its relationship with the process efficiency, is still needed, especially considering well-operating plants under different operational conditions.

Based on EU policy evaluations, the prediction for the upcoming years is that at least 25% of all bioenergy can be originated from biogas (Holm-Nielsen et al., 2009). In Italy, especially in the Po Valley (one of the most productive agricultural areas), the 1.9 billion m^3 of biogas produced in 2012 were employed for electric energy generation (Mela and Canali, 2014). This area covers two distinct regions where two types of hard cheeses are produced, Parmigiano Reggiano (PR) and Grana Padano (GP), which are both protected designations of origin (PDOs). The products of these PDOs, the cheeses, follow different specifications concerning animal feeding (http://www.granapadano.com; http://www.parmigianoreggiano.com). In particular, cows for GP production can be fed with silage fodders, which are not allowed in PR production to limit *Clostridium* contamination and possible swelling defects in the cheese (Vliegher et al., 2015). This distinct animal feeding involves a different digestibility of the substrate, affecting the composition and the physico-chemical characteristics of cow manure (Aguerre et al., 2011; Amon et al., 2007; Climate changE-R project 2013–2016 – LIFE12 ENV/IT/000404, unpublished results). Such distinction could have an effect on the microbial composition of the slurry manure used to feed the digester and consequently influence AD process efficiency.

In this study, indexed Illumina sequencing was used to identify the key phylotypes of *Bacteria* and *Archaea* in digester samples from six full-scale anaerobic plants located in the PR and GP areas. Moreover, qPCR was used to quantify 16S rRNA gene copy numbers of total bacteria, archaea, *Clostridiales*, and a methanogen-specific gene (*mcrA*). These techniques were applied to investigate the effect of feed (cattle slurry manure alone or supplemented with energy crops and agro-industrial by-products), location area (difference in animal feeding in PR and GP), temperature (mesophilic and thermophilic plants), and time on the microbial community structure in well-operating plants. Furthermore, relationships among predominant bacterial and archaeal taxa and process parameters were also examined.

2. Materials and methods

2.1. Biogas plants and data collection

Six biogas plants (BGPs) were studied, all located in the north of Italy and each linked to respective cattle farms. BGP1, BGP3, and BGP5 were located in the production area of PR cheese, while BGP2, BGP4, and BGP6 were located in the production area of GP cheese. Experimental activities were carried out in collaboration with biogas plant owners, who provided data related to feeding substrates, electrical energy production, and process temperatures. All six full-scale biogas reactors were CSTRs (continuous stirred tank reactors), operated under mesophilic conditions (with the exception of BG5, which operated under thermophilic conditions), and had been running from 1 to 4 years, based on the year of construction (see Table 1 for details regarding substrate and operational conditions). All plants reported process stability at the time of sampling, and no major changes had occurred prior to sampling. The volume of CH_4 was calculated from the electrical energy produced by the biogas plants, considering the theoretical electrical efficiency equal for CHP (cogeneration heat and power) unit to 38% and the methane calorific value as 9.88 kWh/m^3 CH_4.

2.2. Sampling procedures

Biogas plants were sampled once a month during May, July, September, and November 2014, from the appropriate sampling valve. Prior to sampling, a thorough mixing of the digester was carried out to allow for retrieval of samples representative of the digester contents in its current state. The sampling valves and the equipment used to collect digestate samples were previously sterilized with 1.15% p/p NaOCl solution to limit exogenous bacterial contamination. About 350 mL of digestates was transferred in sterile bottles (500 mL; LP Italiana, Milan, Italy) and cooled at 4 °C to prevent further digestion. Samples for the microbiological analyses were then preserved in sterile tubes (50 mL; Sarstedt, Nurnbrecht, Germany) and stored at −20 °C until use. Microbiological analyses were carried out in triplicate, for a total of 72 analyzed samples.

2.3. Physico-chemical analyses

The physico-chemical characteristics of plant effluent are reported in Table 2. Total solids (TS), volatile solids (VS), and ammonium concentration (NH_4^+-N) were measured as described in standard methods (APHA, 2005), whereas total volatile fatty acids (VFAs) and total alkalinity were determined as described by Nordmann (1977) through an automatic titrator (TIM 840, Hach Lange). The pH value was measured by a bench pH meter (XS Instruments) whereas CH_4 content in biogas was measured monthly by a portable biogas analyzer (GA2000 PLUS, Geotechnical Instruments, UK) during data collection activities on biogas plants. TS and VS of feedstocks were also measured monthly to calculate the VS degradation efficiency as described by Koch (2015).

2.4. Microbiological analyses

2.4.1. DNA extraction

DNA extraction was carried out on 100 mg of each replicate with the Fast DNA™ SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH) according to the manufacturer's protocol. Concentrations of double-stranded DNA in the extracts were determined using the Quant-iT dsDNA HS assay kit and the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). The DNA was then stored at −20 °C for further analyses.

2.4.2. DNA amplification and Illumina high-throughput sequencing

PCR amplification of the bacterial V3-V4 regions of the 16S rRNA gene was carried out as detailed in Połka et al. (2014), except that 0.25 μM of each primer and 1 ng of DNA were used. PCR amplification of the archaeal V3-V4 regions of the 16S rRNA gene was carried out using the KAPA HiFi Hot Start (2X) (Kapa Biosystems, Inc., Wilmington, MA, USA) and the primer pair 344F (5′-ACGGGGYGCAGCAGGCGCGA-3′) (Raskin et al., 1994) and

Table 1
Process parameters of the biogas plants analyzed in this study.

Biogas plant	Volume (m^3)	Feedstock	Area	Temperature (°C)	CHP (kWel)	OLR[a] ($kg\ VS\ m^{-3}\ day^{-1}$)	HRT (days)	SMP* ($m^3\ kg\ VS^{-1}\ day^{-1}$)	CH_4* (%)	VS degradation efficiency[a] (%)
1	8000	CE	PR	44.0	999	2.60 ± 0.10	48	0.30 ± 0.01	50.3 ± 0.6	62.47 ± 1.25
2	4600	CEA	GP	45.2	750	3.04 ± 0.21	66	0.35 ± 0.06	51.6 ± 0.5	71.59 ± 2.03
3	3800	C	PR	39.0	330	2.16 ± 0.11	32	0.18 ± 0.01	57.4 ± 1.7	33.66 ± 3.93
4	7500	CEA	GP	42.5	999	2.49 ± 0.21	92	0.34 ± 0.03	66.3 ± 2.4	72.71 ± 2.02
5	2900	CEA	PR	50.0	526	3.17 ± 0.08	67	0.34 ± 0.02	53.1 ± 0.2	67.62 ± 1.33
6	1800	C	GP	42.0	99	1.57 ± 0.10	44	0.22 ± 0.01	56.0 ± 0.1	43.41 ± 1.92

C: Cattle slurry manure; CE: Cattle slurry manure and energy crops; CEA: Cattle slurry manure, energy crops, and agro-industrial by-products. PR: Parmigiano Reggiano area; GP: Grana Padano area.
[a] Average values from May to November 2014.

Table 2
Physico-chemical characteristics of plant effluent (average values from May to November 2014).

Biogas plant	TS ($g\ L^{-1}$)	VS ($g\ L^{-1}$)	Total VFAs ($mg\ L^{-1}$)	Total alkalinity ($mg\ CaCO_3\ L^{-1}$)	NH_4^+-N ($mg\ L^{-1}$)	pH
1	66.7 ± 2.2	49.5 ± 1.7	2743 ± 88	12809 ± 877	1823 ± 83	7.86 ± 0.10
2	82.7 ± 11.0	64.1 ± 8.9	3268 ± 346	13695 ± 1611	2418 ± 347	7.98 ± 0.09
3	66.7 ± 2.8	48.5 ± 2.9	2909 ± 152	13363 ± 208	1928 ± 130	7.85 ± 0.10
4	40.1 ± 3.4	29.9 ± 2.1	1743 ± 208	7810 ± 1462	1353 ± 450	7.60 ± 0.13
5	94.1 ± 4.5	74.4 ± 3.6	3795 ± 217	13763 ± 435	2224 ± 188	7.94 ± 0.08
6	62.7 ± 4.9	46.6 ± 3.5	3064 ± 534	12954 ± 817	1852 ± 236	7.69 ± 0.03

806R (5′-GGACTACVSGGGTATCTAAT-3′) (Takai and Horikoshi, 2000). The amplification was carried out in a 25 μL reaction volume, containing 10 ng genomic DNA and 0.25 μM of each primer, using the following touch-down PCR conditions: initial denaturation at 94 °C for 3 min, followed by 28 cycles of annealing beginning at 67 °C and ending at 55 °C for 15 s, and extension at 72 °C for 20 s. The annealing temperature was lowered 1 °C every cycle until reaching 55 °C, which was used for the remaining cycles. Forward primers were indexed throughout a 9 nucleic acid–base extension at their 5′ end as for the bacterial 16S rRNA analyses. Equimolar PCR products of the digester DNA templates were multiplexed into two separate pools, one for Bacteria and one for Archaea, which were then purified with the Agencourt® AMPure® XP kit (Beckman Coulter, Italy). The two pools were finally sequenced at the Parco Tecnologico Padano facilities (PTP, Lodi, Italy) with a MiSeq Illumina instrument (Illumina Inc., San Diego, CA) operating with V3 chemistry and producing 300 bp paired-end reads.

2.4.3. Real-time PCR

Bacteria, Archaea, *Clostridiales*, and methanogen populations in the digester samples were quantified by real-time PCR using primers and conditions as described in Table 3.

The 20 μL reaction mixtures contained 10 μL KAPA SYBR® FAST qPCR Kit Master Mix 2X (Biolab Scientifics Instruments SA, Switzerland), 0.4 μM of each primer (0.3 μM for *Clostridiales* and methanogens), and 2 μL of DNA. For Archaea amplification, 10 μL of KAPA PROBE® FAST qPCR Kit Master Mix 2X (Biolab Scientifics Instruments SA, Switzerland) was used together with 0.1 μM of labeled probe.

Templates for standard curves were represented by 10-fold dilutions of genomic DNA (for Bacteria, *Clostridiales*, and methanogens) obtained by reference strains as listed in Table 3. The standard curve for the Archaea assay was represented by 10-fold dilutions of the 16S *M. smithii* (DSM 861) plasmid DNA, which was cloned into *E. coli* using a TOPO-TA vector cloning kit (Invitrogen, Carlsbad, CA, USA). All DNA samples were tested in duplicate using the StepOnePlus™ Real-Time PCR System (Applied Biosystems Japan, Tokyo, Japan).

2.5. Bioinformatics and statistical analyses

High-throughput sequencing data filtering, multiplexing, and preparation for concomitant statistical analyses were carried out as previously detailed (Połka et al., 2014). Statistical analyses on operational taxonomic units (OTUs) and taxonomy matrixes were performed in Mothur v.1.32.1 (Schloss et al., 2009) and R (http://www.R-project.org/) and included hierarchical clustering with the average linkage algorithm at different taxonomic levels, principal component analysis (PCA) to assess the unconstrained sample grouping, and canonical correspondence analysis (CCA) to assess the significance of feed, location area, temperature, and time on the analyzed diversity. Statistical analyses on calculated indices related to microbial alpha-diversity and qPCR results were carried out in R, with ANOVA and Tukey (HSD) post hoc test ($\alpha < 0.05$). Where ANOVA was not applicable, the Kruskal–Wallis

Table 3
Real-time PCR primers and conditions used in this study.

Target gene	Primers	Thermal cycles	Reference strain	Reference
16S rRNA Bacteria	Uni331F (5′-TCCTACGGGAGGCAGCAGT-3′) Uni797R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′)	95 °C 3 min; 95 °C 10 s, 60 °C 40 s (35 cycles)	*E. coli* ATCC 700926 D-5	Nadkarni et al. (2002)
16S rRNA Archaea	ARC787F (5′-ATTAGATACCCSBGTAGTCC-3′) ARC1059R (5′-GCCATGCACCWCCTCT-3′) ARC915 Probe (FAM-5′-AGGAATTGGCGGGGGAGCAC-3′-TAM)	94 °C 3 min; 94 °C 10 s, 60 °C 20 s (45 cycles)	*M. smithii* DSM 861	Yu et al. (2005)
16S rRNA Clostridiales	Clostridiales F (5′-GGAMGAWAATGACGGTAC-3′) Clostridiales R (5′-CTAGTARRCATCGTTTACGGC-3′)	95 °C 3 min; 95 °C 10 s, 56 °C 20 s (35 cycles)	*C. perfringens* DSM 756	This study
mcrA methanogens	qmcrA F (5′-TTCGGTGGATCDCARAGRGC-3′) qmcrA R (5′-GBARGTCGWAWCCGTAGAATCC-3′)	95 °C 3 min; 95 °C 10 s, 60 °C 40 s (40 cycles)	*M. smithii* DSM 861	Denman et al. (2007)

non-parametric test for significant differences estimation and the Nemenyi–Damico–Wolfe–Dunn joint ranking test (for confidence intervals of 99%) were performed, along with a Tukey post hoc analysis for pairwise comparisons.

3. Results and discussion

3.1. Biogas plants performance

Monitored process parameters (e.g., temperature, pH, feed, organic loading rate (OLR)) of BGPs was unvaried during the months of sampling (Tables 1 and 2). Thus, specific methane production (SMP), volatile solid degradation efficiency, ammonium concentration, total VFAs, total alkalinity, and methane content in biogas were kept stable throughout the process for each analyzed plant. ANOVA (Table S1, Supplementary data) and post hoc analysis (Fig. S1, Supplementary data) of these parameters showed that BGP3 and BGP6 (only cattle slurry manure fed) were significantly different from the other plants for SMP and VS degradation efficiency, exhibiting the lowest values. This lower efficiency could be linked to the type of feedstock, which resulted in less easy degradability and balance in nutrient composition compared to co-digestion with crop wastes (Lehtomaki et al., 2007). In contrast, BGP4 (which was fed also with energy crops and agro-industrial by-products) showed the highest methane content in biogas and the lowest total VFAs and NH_4^+-N accumulation, which are two of the causes of AD inhibition (Kumar et al., 2016; Town and Dumonceaux, 2016; Zhang et al., 2014). In addition, we note that BGP2, which differed from BGP4 in hydraulic retention time (HRT), OLR, and the ratio between substrates, exhibited an opposite trend. These results suggest an effect of feed composition and mixture ratio on VS degradation efficiency and methane production yield, as Lin et al. (2011) previously highlighted. In fact, they pointed out an effect of the substrates ratio on the reactor stability and performance, as they affect the accumulation of VFAs and NH_3.

3.2. Bacterial communities

3.2.1. Illumina sequencing analyses

Assembled sequences of the amplicons pool resulted in 1,374,899 reads, which was reduced to 1,206,900 after size screening (fragments >370 bp), alignment screening (V3-V4 region), and chimera and taxonomy (non-bacterial sequences) filtering. As a result, a total of 429,939 high-quality sequences were analyzed (after downscaling to the lowest populated sample: 6231 reads per sample, retaining 69 samples).

3.2.1.1. OTU-based analyses. A total of 126 major OTUs were found to cover 99.9% of total bacterial diversity (Fig. S2, Supplementary data). All BGPs were clearly separated from one another, and the two predominant OTUs accounted for 40% of BGP5 bacterial diversity but less than 2% in BGP4. This result showed an evident species richness diversity between the two plants, which differed by location area (PR and GP, respectively) and process temperature (thermophilic and mesophilic, respectively). In particular, Otu00001 represented the MBA08 order (belonging to the *Clostridia* class), which was previously found in thermophilic digesters and whose dominant presence was confirmed by Sun et al. (2015). Otu00002 represented the *Adhaeribacter* genus (*Sphingobacteria* class), whereas Otu00003 (SMB53 genus, belonging to the *Clostridia* class), Otu00004 (*Turicibacter* genus, belonging to *Bacilli* class), and OTU00005 (*Clostridium sordellii*), were mainly present in BGP1, BGP3, and BGP6, accounting for 22% of bacterial diversity.

3.2.1.2. α-Diversity indexes. The achieved coverage of the identified bacterial total diversity was over 90% (Fig. S3, Supplementary data). α-Diversity analysis testing the BGP effect (Fig. 1a) showed that BGP5 (the thermophilic plant) was less diverse, OTU-rich, and even, in relation to the other mesophilic plants. This behavior was also observed by Li et al. (2014), Sun et al. (2015), Theuerl et al. (2015), and Sundberg et al. (2013), who showed major bacterial selection under higher temperature conditions. On the contrary, BGP4 and BGP6 (sharing the same location area of GP, as well as the mesophilic condition) exhibited the highest bacterial diversity, OTU-richness, and evenness. Surprisingly, BGP2, which differed from BGP4 for the ratio between substrates, showed a behavior more similar to BGP5.

3.2.1.3. β-Diversity analyses. Microbiome similarity among BGPs for bacterial communities was first tested by means of PCA. The resulting plot (Fig. 2a) separated all plants along PC1 (which explained 51.5% of the total variation), with the exception of BGP3 and BGP4. These last BGPs were instead differentiated along PC2 (which explained 18.1% of the total variation). BGP1 and BGP2 were found to be partly overlapping, even if they differ for feed and location area. CCA was used to test the significance of time (Fig. S4, Supplementary data), feed, area, and temperature effects (Fig. 3a). As we expected, time had no effect on plant microbiomes; in fact, no disturbance occurred in all BGPs during the period considered in this study. In contrast, a significant effect of feed, location area, and temperature was found. Thus, the different animal feeding, regulated by PDO specifications and operated in the geographically separated GP and PR areas, seemed to have an impact on plant microbiomes.

3.2.1.4. Taxonomy-based analyses. Taxonomic assignment (Fig. S5, Supplementary data) showed that 99.7% of sequences were correctly classified at the phylum level, and 96% and 93.9% at the class and order levels, respectively. Percentages were decreased going to the family (69.8%), genus (56.7%), and species (16.7%) levels. A clear clustering of BGPs emerged early at the phylum taxonomic level (Fig. S6, Supplementary data). Among the different BGP feeds, the three most predominant phyla were highlighted (in decreasing order of abundance): *Firmicutes* (ranging from 50% to 73%), *Bacteroidetes* (6–27%), and *Proteobacteria* (3–8%). The same bacterial phyla predominance and relative abundances were found by Li et al. (2016) in solid-state anaerobic digesters digesting corn stover.

Interestingly, the *Chloroflexi* phylum was mostly present in BGP3 and BGP4, OP9 was mostly absent in BGP2 (even if this plant had the same characteristics of BGP4), and *Thermotogae* was found only in BGP5 (as it was the only thermophilic plant). *Actinobacteria* accounted for less than 5% in all BGPs, and the low abundance of this last phylum in biogas plants has already been reported (Campanaro et al., 2016). At lower taxonomic levels, the pattern of the dominant taxa was conserved. In fact, within the *Firmicutes* phylum, *Clostridia* (accounting for 40–60%) and *Bacilli* (3–14%) were the major classes (Fig. 4), in accordance with De Vrieze et al. (2015). Worth noting, BGP4 (the plant that exhibited the lowest total VFAs and ammonium accumulation) showed the lowest presence of *Bacilli* (3–4%). Regarding the *Bacteroidetes* phylum, the *Bacteroidia* (2–15%) and *Sphingobacteria* (2–20%) classes were mainly found, even though they showed a differential distribution among BGPs. In particular, *Sphingobacteria* were most abundant in BGP2 and BGP5, but they were almost absent in BGP3 and BGP4, whereas *Bacteroidia* showed the lowest percentage in BGP5 and the highest percentage in BGP3. In the *Chloroflexi* phylum, the *Anaerolineae* class retained the same distribution. In relation to order level (Fig. S7, Supplementary data), *Clostridiales* retained its dominant role, which would be expected from its involvement in

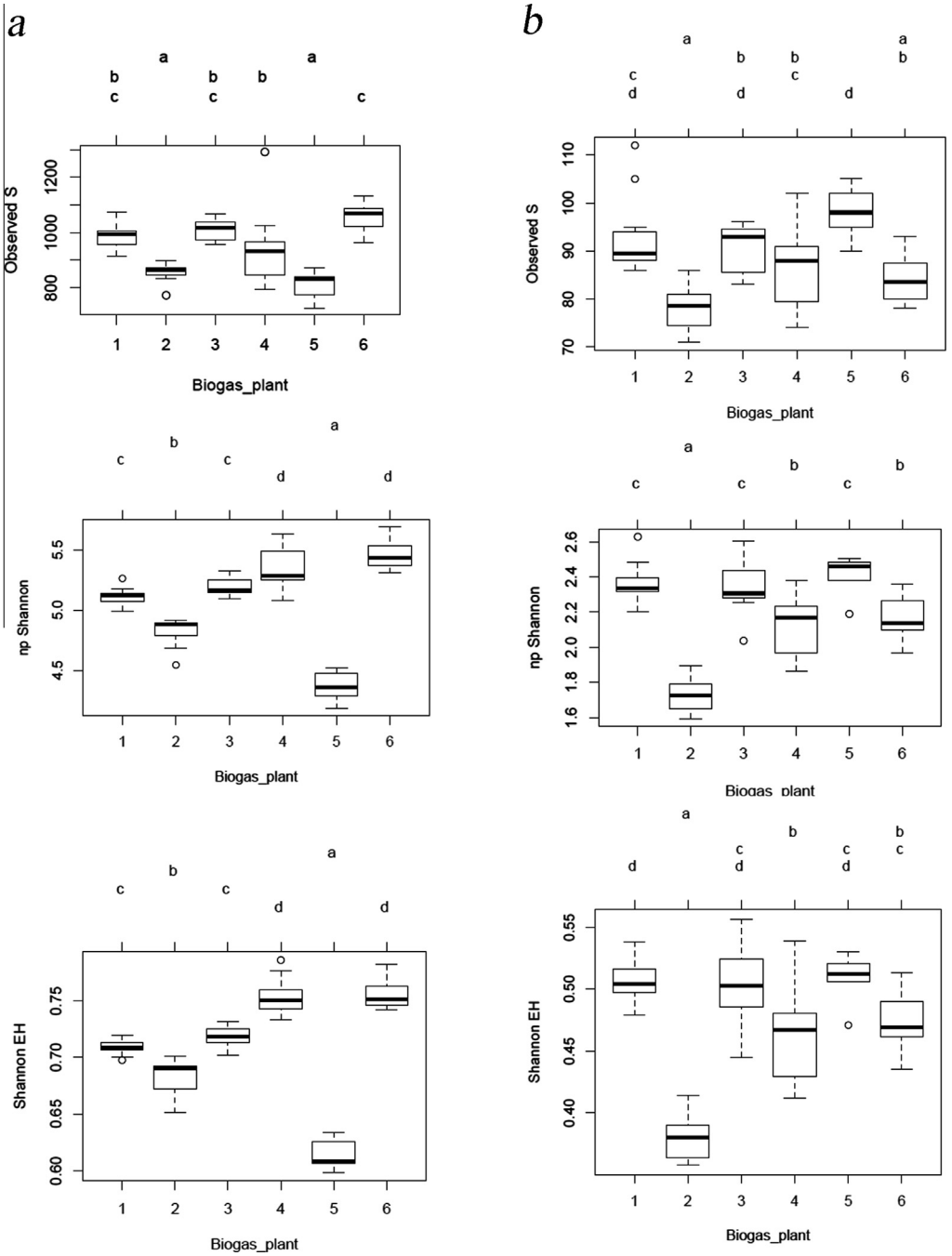

Fig. 1. Boxplots of the observed richness (S), the np Shannon, and the Shannon EH for the bacterial (a) and archaeal (b) analyzed datasets, considering the digester effect. Significant differences among BGPs are indicated by different letters, according to the performed ANOVA and Tukey (HSD) test ($P < 0.05$).

many metabolic activities preceding the methanogenesis step of AD (Hanreich et al., 2013), including syntrophic acetate oxidation reactions in conditions of inhibition by ammonia concentration (Lü et al., 2013). The six BGPs plants were separated also at the family level (Fig. S8, Supplementary data). In the *Clostridiales* order, *Clostridiaceae* remained the dominant family (accounting for 15–32%), but the *Ruminococcaceae* family was also present (although in low percentages in all plants and almost absent in BGP5). Within the *Bacillales* order, *Turicibacteraceae* was found in all plants (accounting for 3–8%), with a lower abundance in BGP4 and BGP5. Interestingly, *Streptococcaceae* was present mostly in BGP2 (2–8%). Concerning *Bacteroidales*, *Cytophagaceae* accounted for 2–14% in all plants, with the exception of BGP3 and BGP4, where it was almost absent. *Porphyromonadaceae* represented less than 7% in all BGPs except BGP2, whereas uncultured SB-1 was found only in BGP3 (10%). At this taxonomic level, the most different plants were BGP2 and BGP3, differing for feed (cattle slurry manure supplemented with energy crops and agro-industrial by-products and cattle slurry manure alone, respectively) and location area (GP and PR, respectively). Moreover, the microbial differences found between BGP2 and BGP4 (which shared the same location area of GP, mesophilic conditions, and type of feedstock) could be related to the different HRT, which was much shorter in BGP2 than in BGP4. Lower HRT can cause a shift in the bacterial community structure, affecting reactor performance (Baek et al., 2016); in fact, BGP2 showed higher total VFAs and ammonium accumulation

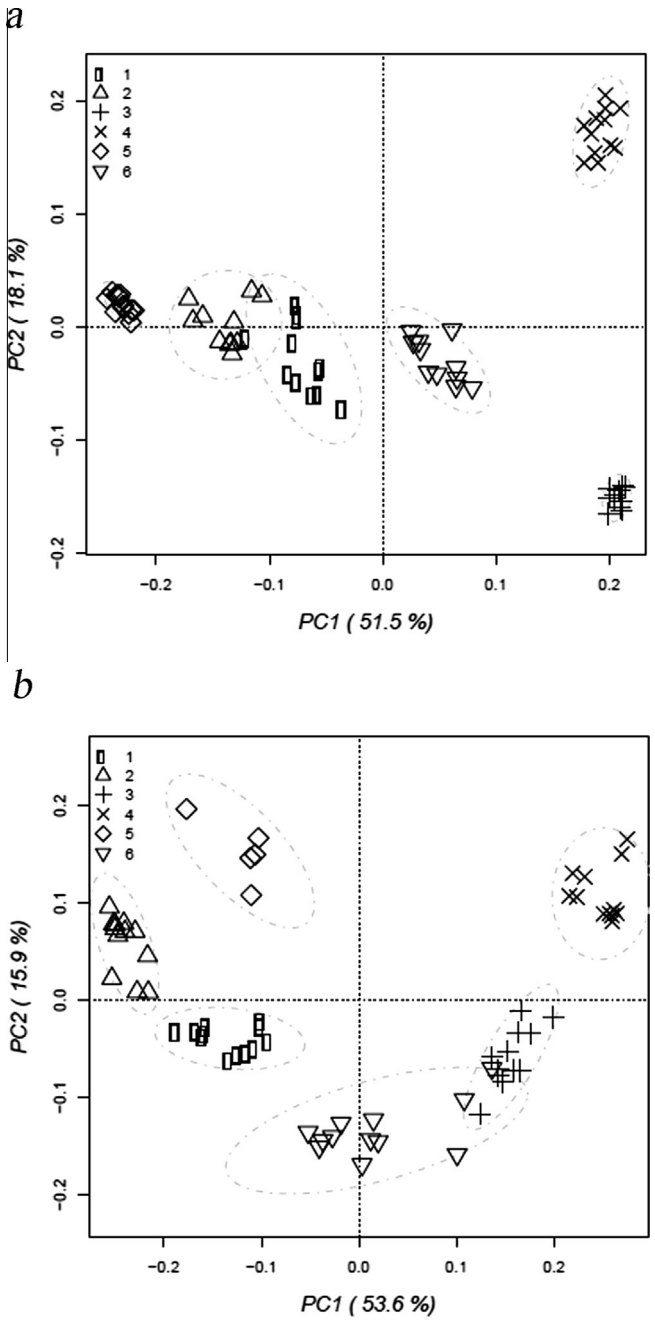

Fig. 2. Principal component analysis (PCA), with ordination ellipses, of the bacterial (a) and archaeal (b) population of the six full-scale biogas plants. Data are classified by digester.

than BGP4 as well as a lower methane content in the biogas produced by the plant.

3.2.1.5. Relationships between process parameters and bacterial communities. Interaction between process parameters and bacterial orders was investigated throughout a tripartite CCA (Fig. 5a). After verifying that time had no effect on the plant microbiome, we tested this interaction considering four values for each process parameter analyzed (corresponding to the four sampling months) as replicate. The first and second canonical axes represented 57.0% and 17.9% of the variance, respectively. The microbial communities were separated considering the digester effect. BGP1, BGP2, BGP3, and BGP6 were distinguished by the first canonical axis and BGP4 and BGP5 by the second axis. The bacterial community distribution agreed with the taxonomic distribution (Fig. S9, Supplementary data). A CCA triplot (Fig. 5a) shows that the order of *Thermotogales* was clearly related to the HRT. Briones et al. (2007) also found a dominance of this population in biogas reactors at high HRT.

3.2.2. Real-time quantification of total Bacteria and Clostridiales

To understand the internal microbial dynamics of BGPs, qPCR quantification of total *Bacteria* and *Clostridiales* (the most abundant order found in all plants) was carried out. Mean 16S rRNA gene copies of bacterial and *Clostridiales* populations ranged from 9.3 to 9.6 and from 8.5 to 8.8 $\log_{10}$ copies per gram of sample, respectively. ANOVA of the results showed no significant differences in relation to time, considering each plant separately (data not shown), whereas slight but significant differences were found between BGPs, identified by the Tukey (HSD) post hoc test (Table 4). In particular, differences among bacterial populations were found in the thermophilic PR plant BGP5 (significantly higher than the other plants). *Clostridiales* differed in the mesophilic GP plant BGP2 (fed with cattle slurry manure supplemented with energy crops and agro-industrial by-products), which exhibited significantly lower amounts than BGP5 and BGP6 (only cattle slurry manure fed); however, on the whole, there was not a significant difference between PR plants and GP plants, so the effect of the location area on bacterial diversity highlighted by the CCA (Fig. 3a) cannot be attributed to the order of *Clostridiales*.

3.3. Archaeal communities

3.3.1. Illumina sequencing analyses

Assembled sequences of the amplicons pool resulted in 1,190,445 reads, which was reduced to 670,139 after size screening (fragments between 380 bp and 430 bp), alignment screening (V4–V5 region), and taxonomy (excluding bacterial sequences) filtering. As a result, a total of 670,108 high-quality sequences were analyzed (after downscaling to the lowest populated sample: 2599 reads, retaining 65 samples).

3.3.1.1. OTU-based analyses. Thirty-seven major OTUs were found to cover the 99.9% of total archaeal diversity (Fig. S10, Supplementary data). All of the BGPs were highly differentiated, and 8 OTUs were predominant (covering more than 80% in all BGPs but BGP4, which ranged from 55% to 85%). In decreasing order of abundance, it was found at the genus level (for some at the candidate species), as follows: *Methanosarcina*, *Methanobrevibacter* (among which *M. thaueri* was at 80%), *Methanobacterium* (among which *M. petrolearium* was at 99% and *M. beijingense* was at 97%), and *Methanosaeta*. The most different (in terms of OTU relative abundance composition) were the thermophilic PR plant BGP5 (which was fed with cattle slurry manure supplemented with energy crops and agro-industrial by-products) and the mesophilic GP plant BGP6 (only cattle slurry manure fed); in the former, the *Methanobacterium* genus (including *M. beijingense*) was dominant; and in the BGP6, *Methanosarcina*, *Methanobrevibacter*, and *Methanobacterium petrolearium* prevailed. Moreover, OTU00001 (*Methanosarcina* genus) accounted for 40% and 60% in the mesophilic plants BGP1 and BGP2 (differing for feed and area), respectively, where very low percentages of OTU00004 (*Methanosaeta* genus) were found. Conversely, OTU00004 accounted for 35% and 55% in the mesophilic plants BGP3 and BGP4 (which also differ for feed and area), respectively, where very low percentages of OTU00001 were found. Thus, it seemed that these two genera followed an opposite trend in digesters.

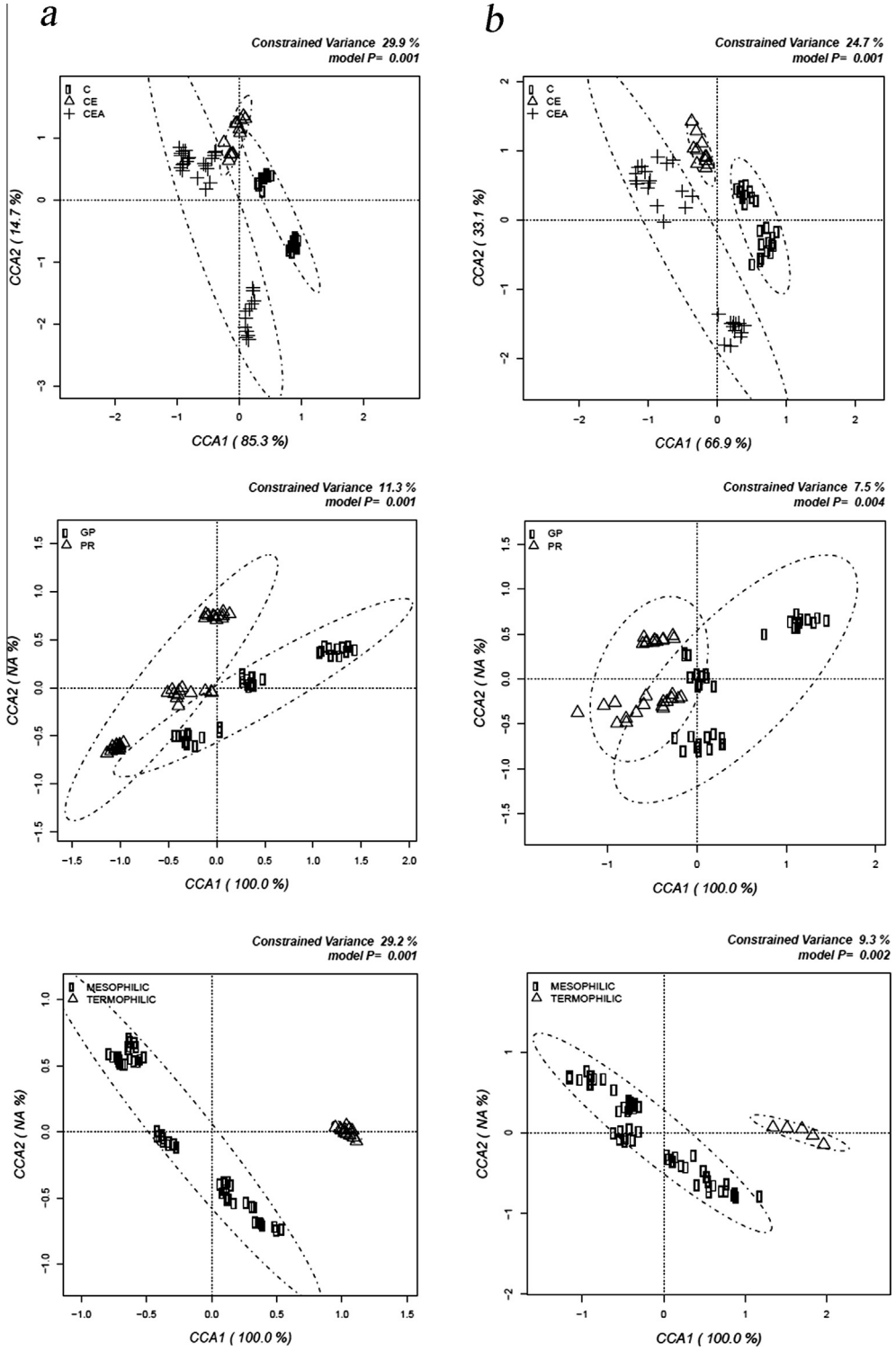

Fig. 3. Hypothesis-driven canonical correspondence analysis (CCA) model testing the significance of feed, area, and temperature effects on bacterial (a) and archaeal (b) communities. C: cattle slurry manure; CE: cattle slurry manure and energy crops; CEA: cattle slurry manure, energy crops, and agro-industrial by-products. GP: Grana Padano area; PR: Parmigiano Reggiano area.

3.3.1.2. α-Diversity indexes. The achieved coverage of the identified archaeal total diversity was over 98% (Fig. S3, Supplementary data). α-Diversity analysis considering BGP effect (Fig. 1b) showed that BGP1, BGP3, and BGP5 (sharing the same location area of PR) were more diverse, OTU-rich, and even. In contrast, BGP2 showed the opposite trend and, as for the bacterial community, it was significantly different from BGP4 (which run under the same conditions).

3.3.1.3. β-Diversity analyses. Microbiome similarity among BGPs for the archaeal population was tested by means of PCA. The resulting plot (Fig. 2b) separated BGP4 and BGP3 from BGP1, BGP2, and BGP5 along PC1 (which explained 53.6% of the total variation). BGP6 was instead differentiated along PC2 (which explained 15.9% of the total variation), even if it was found to be partly overlapping with BGP3 (which was also cattle slurry manure fed). CCA was used to test the significance of time (Fig. S4, Supplementary

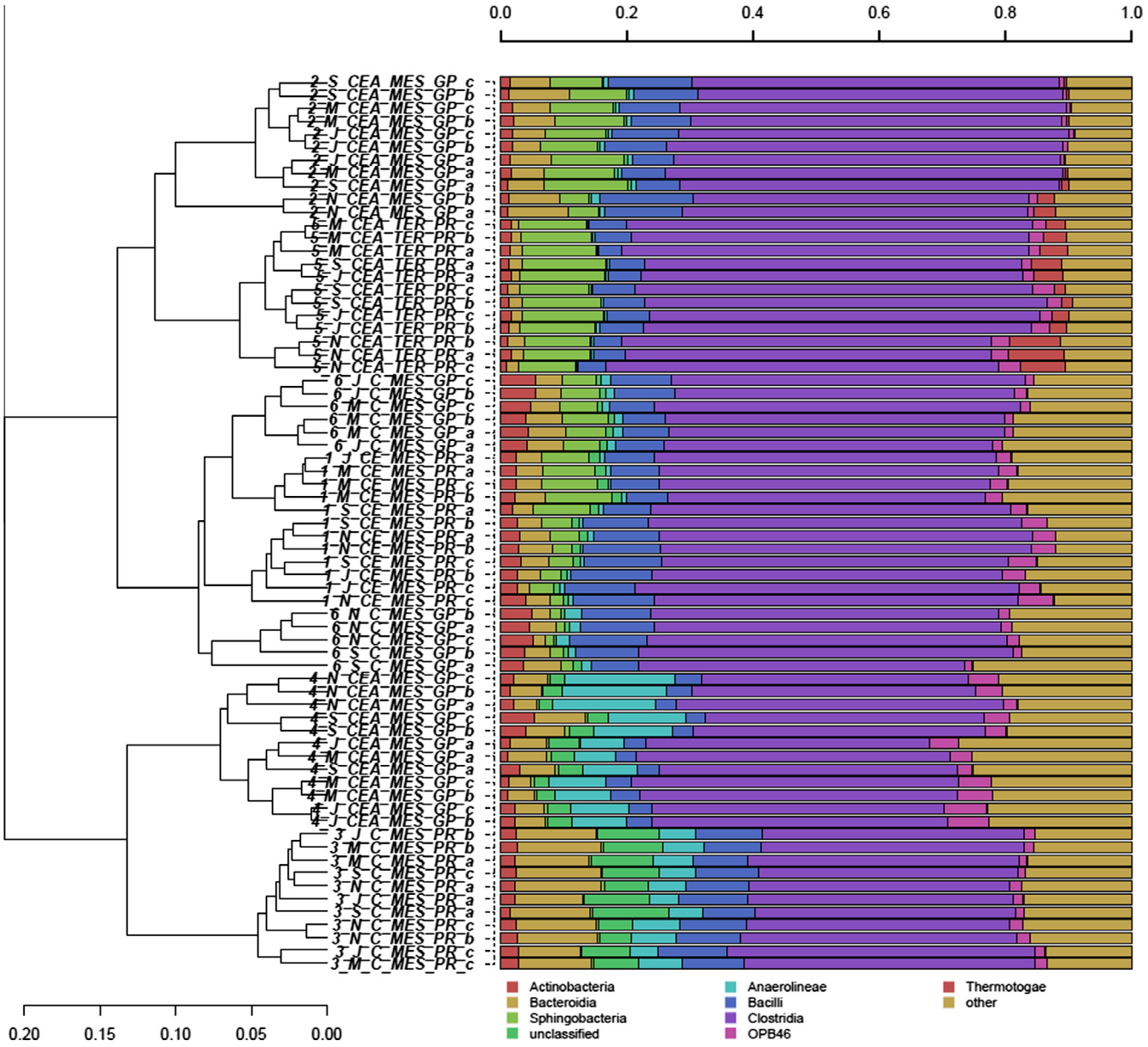

Fig. 4. Hierarchical clustering of bacterial classified sequences using the average linkage algorithm according to the class classifications for taxa participating with ⩾5% in at least one sample. Taxa with lower participation were added to the "other" sequence group. 1, 2, 3, 4, 5, 6: biogas plants. M: May; J: July; S: September; N: November. C: cattle slurry manure; CE: cattle slurry manure and energy crops; CEA: cattle slurry manure, energy crops, and agro-industrial by-products. MES: mesophilic; TER: thermophilic. GP: Grana Padano area; PR: Parmigiano Reggiano area. a, b, c: replicates.

data), feed, area, and temperature effects on microbiome (Fig. 3b). As for the bacterial population, feed, location area, and temperature showed a significant effect. A major separation was noted among the type of feed in relation to the bacterial CCA (Fig. 3a).

3.3.1.4. Taxonomy-based analyses. Taxonomic assignment (Fig. S5, Supplementary data) showed that 99.9% of sequences were correctly classified at the class level. Percentages were slightly reduced going to the order (99.7%), family (98.8%), and genus (97.9%) levels, with a strong decrease to the species level (39.4% of sequences correctly classified). As shown from the phylum classification (Fig. S11, Supplementary data), all BGPs were almost totally represented by *Euryarchaeota*. Going to a deeper level, two main classes were highlighted (Fig. S12, Supplementary data), *Methanobacteria* (accounting for 35–90% of archaeal diversity) and *Methanomicrobia* (accounting for 11–70%), among which *Methanobacteriales* and *Methanosarcinales* were respectively the main orders (Fig. S13, Supplementary data).

Continuing with the taxonomic classification, the most abundant archaeal families (present at ⩾5% in at least one sample) were represented (Fig. S14, Supplementary data). A high clustering of BGPs was found. The predominant family was assigned to *Methanobacteriaceae* (hydrogenotrophic methanogens), which accounted for more than 50% in all BGPs but the mesophilic GP plants BGP2 and BGP4 (both running under the same conditions). *Methanosarcinaceae* (mixotrophic methanogens) prevailed in BGP2 (accounting for more than 50%), followed by BGP1 and BGP6 (which differ for feed and location area), whereas *Methanosaetaceae* (obligate acetoclastic methanogens) prevailed in BGP3 and BGP4 (accounting for 7–58%), which also differ for feed and area. *Methanomicrobiaceae* was mostly present in the thermophilic plant BGP5 (reaching 30% of abundance). A high differentiation among BGPs was retained, also representing the most

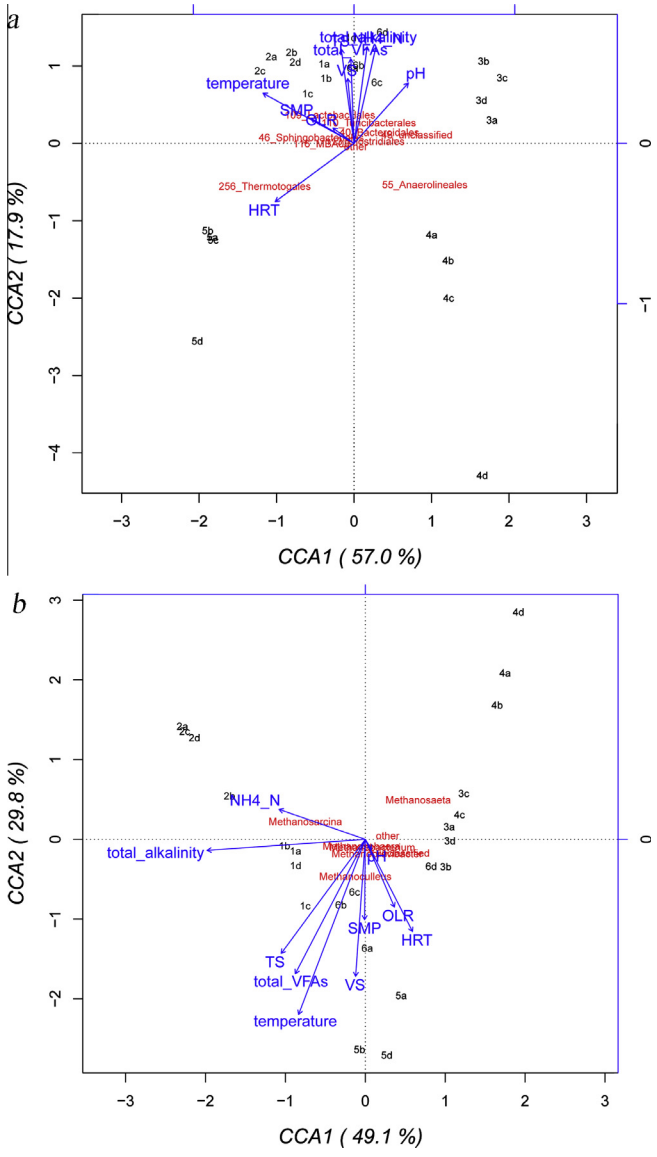

Fig. 5. Canonical correspondence analysis (CCA) triplot to investigate the relationship between relative abundance of bacterial order (a) and archaeal genus (b) and reactor performance data. The percentages on each axis indicate the variation in the samples. Straight arrows indicate the direction of increase of each variable, and lengths are proportional to their strength on the microbial communities. Letters next to BGPs are related to the four replicates considered. 1, 2, 3, 4, 5, 6: biogas plants. a, b, c, d: replicates.

abundant archaeal genus (Fig. 6). Among the predominant family of *Methanobacteriaceae*, the major genera were *Methanobacterium* and *Methanobrevibacter*, together accounting for 40–85% of archaeal diversity. *Methanosphaera* and *Methanothermobacter* were present in a very low percentage (less than 10%), mostly in the mesophilic PR plant BGP1 (fed with cattle slurry manure and energy crops) and only in the thermophilic PR plant BGP5, respectively. *Methanosarcina*, *Methanosaeta*, and *Methanoculleus* retained the same trend showed by family clustering. It is known that acetate (together with H_2) is the main product of the VFA acetogenesis step preceding methanogenesis in AD; in addition, as already observed by Demirel and Scherer (2008), *Methanosaeta* prefers a low acetate level whereas *Methanosarcina* needs a higher acetate concentration but then shows faster growth kinetics. Thus, the major abundance of *Methanosaeta* in BGP4 and *Methanosarcina* in BGP2 could be related to the total VFA levels; in fact, BGP4 and BGP2 presented the lowest and highest VFA accumulation, respectively, considering the mesophilic condition. Moreover, as Campanaro et al. (2016) already reported, the hydrogenotrophic methanogen genus of *Methanoculleus* was mostly present in thermophilic digesters (as we found in BGP5), which indicates a preference for the hydrogenotrophic pathway under high temperature processes.

3.3.1.5. Relationships between process parameters and archaeal communities. Interactions between process parameters and archaeal genus were also tested throughout CCA (Fig. 5b). The first and second canonical axes represented 49.1% and 29.8% of the variance, respectively. The microbial communities were separated considering digester effect. BGP1, BGP5, and BGP6 were distinguished by the first canonical axis and BGP2, BGP3, and BGP4 by the second axis. The archaeal community distribution agrees with the taxonomic one (Fig. S9, Supplementary data). The CCA triplot (Fig. 5b) shows that *Methanosarcina* was related to the ammonium concentration in the plant. This genus was in fact more abundant in BGP2 where a higher NH_4^+-N concentration was found. The dominance of *Methanosarcinaceae* at high total ammonia, salt and/or volatile fatty acid concentrations was also highlighted by other full-scale studies (De Vrieze et al., 2015; Williams et al., 2013). The fact that this correlation was not found in BGP4 (which run under the same conditions of BGP2 but fed with different ratio between substrates), can underline the linkage between the feedstock characteristics and nutritional imbalances in the biogas plants.

3.3.2. Real-time quantification of total Archaea and Methanogens

To understand the internal microbial dynamics of BGPs, qPCR quantification of total Archaea and methanogens (responsible for methane production) was carried out. Mean 16S rRNA gene copies of the archaeal population ranged from 9.4 to 9.8 $\log_{10}$ copies per gram of sample, whereas the *mcrA* gene of the methanogen population ranged from 8.2 to 8.4 $\log_{10}$ copies per gram of sample. ANOVA of the results showed significant differences among BGPs concerning only the archaeal population; in particular, the GP plant

Table 4
Abundance of total Bacteria, Clostridiales, total Archaea, and methanogen populations and statistically significant differences among BGPs.

Microbial population	Biogas plant						ANOVA P value
	1	2	3	4	5	6	
	Gene copies/g of sample*						
Bacteria	9.4 ± 0.2^{a}	9.4 ± 0.1^{a}	9.3 ± 0.1^{a}	9.4 ± 0.1^{a}	9.6 ± 0.1^{b}	9.4 ± 0.2^{a}	0.000629
Archaea	9.7 ± 0.2^{ab}	9.7 ± 0.2^{ab}	9.5 ± 0.2^{a}	9.8 ± 0.2^{b}	9.4 ± 0.3^{a}	9.6 ± 0.2^{ab}	0.00146
Clostridiales	8.7 ± 0.2^{ac}	8.5 ± 0.2^{a}	8.6 ± 0.2^{ab}	8.5 ± 0.2^{ab}	8.7 ± 0.2^{bc}	8.8 ± 0.1^{c}	0.000569
Methanogens	8.3 ± 0.2	8.3 ± 0.1	8.3 ± 0.2	8.2 ± 0.1	8.3 ± 0.1	8.4 ± 0.1	0.133

ANOVA significance: $P < 0.05$.
Post-hoc pairwise comparison: Tukey HSD ($\alpha < 0.05$). Significant pairwise differences are indicated with different letters (biogas plant sharing at least one letter, are not significantly different).
* Average values (expressed as $\log_{10}$) from May to November 2014.

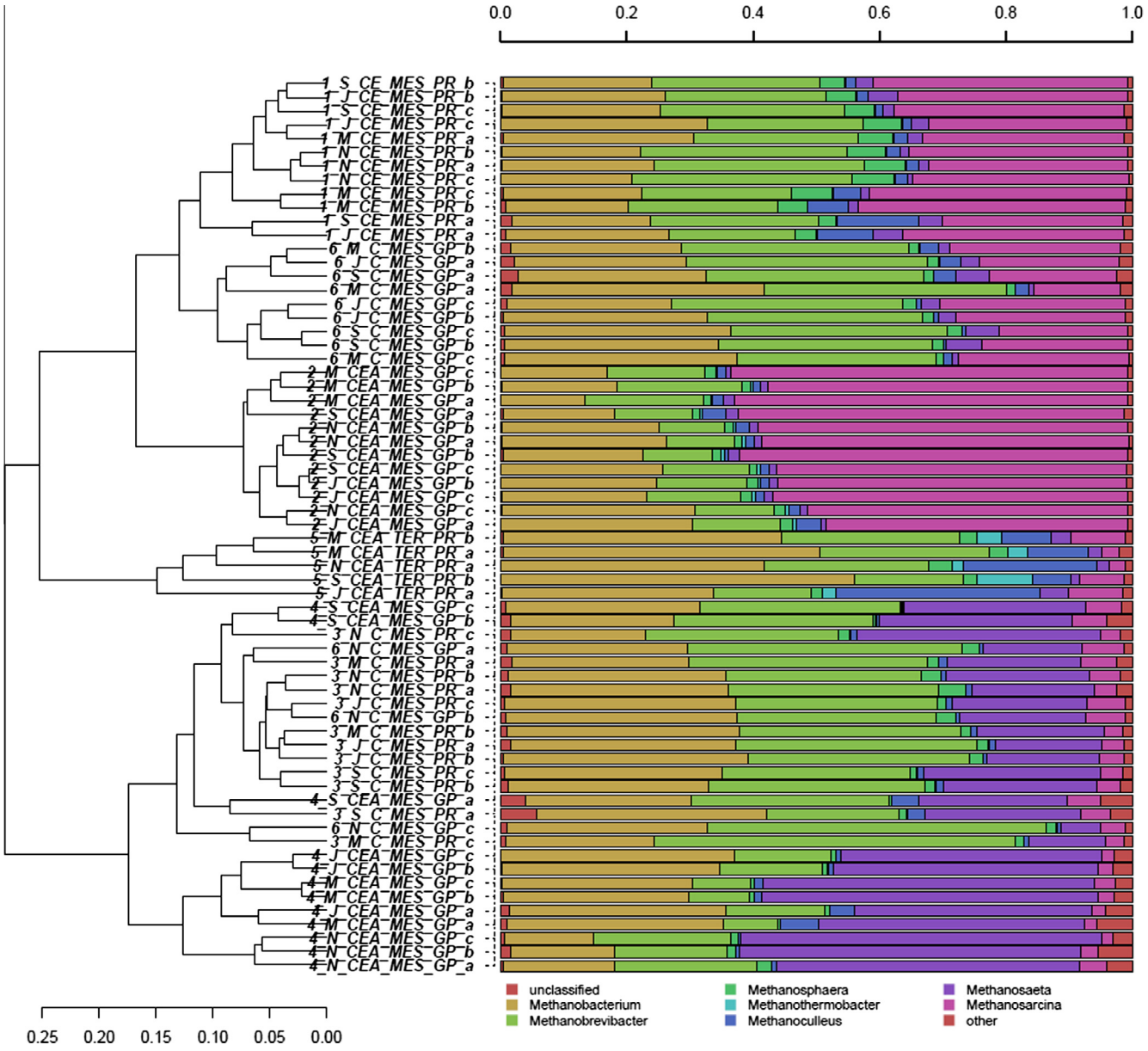

Fig. 6. Hierarchical clustering of archaeal classified sequences using the average linkage algorithm according to the genus classifications for taxa participating with ⩾5% in at least one sample. Taxa with lower participation were added to the "other" sequence group. 1, 2, 3, 4, 5, 6: biogas plants. M: May; J: July; S: September; N: November. C: cattle slurry manure; CE: cattle slurry manure and energy crops; CEA: cattle slurry manure, energy crops, and agro-industrial by-products. MES: mesophilic; TER: thermophilic. GP: Grana Padano area; PR: Parmigiano Reggiano area. a, b, c: replicates.

BGP4 (which exhibited the lowest total VFA and ammonium accumulation, and the highest methane content in biogas) was significantly higher in Archaea consortia than were the two PR plants BGP3 (only cattle manure fed) and BGP5 (the only plant under thermophilic conditions) (Table 4). This difference could be mostly due to the type of feedstock, and, particularly, to the different animal feeding operated in the two geographic areas considered. The fact that no significant differences in methanogen populations among BGPs were highlighted suggests that reactor performance is mainly related to the different taxonomic distribution of methanogens and not to their overall number.

4. Conclusions

Different feedstocks and temperatures had significant effects on the microbial communities of BGPs. Furthermore, a geographic influence was also highlighted, probably due to the different animal feedings operated in PR and GP areas; in particular, PR BGPs resulted in more biodiversity in terms of the archaeal population. The acetoclastic methanogens *Methanosarcina* and *Methanosaeta* clearly showed an opposite distribution among BGPs, which can be correlated with competitiveness for acetate consumption. The *Methanosarcina* genus was related to ammonium concentration, but the *Methanoculleus* genus was more present in the thermophilic digester, and the *Thermotogales* order correlated with HRT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.07.058.

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