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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