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ENVIRONMENTAL BIOTECHNOLOGY

Microbial community evolution in a lab-scale reactor operated to obtain biomass for biochemical methane potential assays

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Abstract

Biochemical methane potential (BMP) test is an important tool to evaluate the methane production biodegradability and toxicity of different wastes or wastewaters. This is a key parameter for assessing design and feasibility issues in the full-scale implementation of anaerobic digestion processes. A standardized and storable inoculum is the key to obtain reproducible results. In Uruguay, a local enterprise dedicated to design and install anaerobic digesters operated a lab-scale bioreactor as a source of biomass for BMP tests, using a protocol previously described. This reactor was controlled and fed with a mixture of varied organic compounds (lipids, cellulolytic wastes, proteins). Biomass was reintroduced into the reactor after BMP assays to maintain a constant volume and biomass concentration. The aim of this work was to evaluate how the microbial community evolved during this operation and the effect of storing biomass in the refrigerator. The composition of the microbial communities was analyzed by 16S rRNA amplicon sequencing using primers for Bacteria and Archaea. The methanogenic activity was determined, and the methanogens were quantified by *mcr*A qPCR. One sample was stored for a 5-month period in the refrigerator (4 °C); the activity and the microbial community composition were analyzed before and after storage. Results showed that applying the reported methodology, a reliable methanogenic sludge with an acceptable SMA was obtained even though the reactor suffered biomass alterations along the evaluated period. Refrigerating the acclimatized biomass for 5 months did not affect its activity nor its microbial composition according to the 16S rRNA gene sequence analysis, even though changes in the *mcr*A abundance were observed.

Key points

- *The applied methodology was successful to obtain biomass suitable to perform BMP assays.*
- *The microbial community was resilient to external biomass addition.*
- *Biomass storage at 4 °C for 5 months did not alter the methanogenic activity.*

Keywords Anaerobic digestion · Sludge storage · *mcr*A · qPCR · Specific methanogenic activity · Biochemical methanogenic potential

Introduction

Anaerobic digestion (AD) is a consolidated technology applied worldwide to obtain methane from wastes and wastewaters (Ampese et al. 2022; Grando et al. 2017). High organic content wastes and wastewaters can be converted into energy and fertilizers. The application of this technology is essential to move towards a circular economy. In Latin American countries whose economy is based on food production, the production of energy from the large amount of waste generated is essential to obtain more sustainable production (Moreda 2016).

The AD process includes four major microbial steps, i.e., hydrolysis, fermentation, acetogenesis, and methanogenesis, which are accomplished jointly by different types of microorganisms in tandem with their syntrophic interactions (Amin et al. 2020). In the acetogenic step, the acetogenic bacteria convert the organic acid products into acetate and hydrogen which become available for methanogenic archaea consumption (Schink 1997). Methanogenic archaea

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are critical for efficient degradation and play an important role in the syntrophic process. On the other hand, microbial composition of the inoculum source deterministically contributes in shaping the community structure and specific ecosystem function (De Vrieze et al. 2014; T. Liu et al. 2017; Perrotta et al. 2017). Thus, selecting an appropriate inoculum is important to ensure degradation of a wide variety of substrates to methane. Taking this process to full scale needs high knowledge of these complex process variables (Carballa et al. 2015). This knowledge allows us to monitor its proper functioning and therefore to obtain a predictable and stable product (Werner et al. 2011).

To apply this technology for the treatment of wastes and wastewaters, it is necessary to determine the potential to obtain methane using these wastes. BMP tests are simple and inexpensive procedures employed to evaluate the biodegradability and toxicity of different organic matter feed source to anaerobic treatment process and one of the key parameters for scaling up the process to further assessing design, economic, and managing issues for the full-scale implementation of anaerobic digestion processes (Angelidaki et al. 2009). In these tests, variations in the microbial community composition, different source, and origin inocula could lead to different biodegradability results (Raposo et al. 2011a, b). Thus, guaranteeing a proper microbial community acclimated to the test conditions or adapted to the substrate is necessary to obtain reliable and reproducible results for BMP tests. Insufficient activity or quality of the inoculum can lead to wrong results (Steinmetz et al. 2016).

The specific methanogenic activity (SMA) is another parameter of great importance which can be described as a measure of the specific chemical oxygen demand (COD) digestion or biotransformation rate. The degradation rate is higher when the inoculum is adapted to a substrate (Koch et al. 2017). Also, higher SMA results reflect higher capacity of the inoculum to degrade the organic matter into methane (Moreno-Andrade and Buitrón 2004).

Regular determination of the sludge SMA provides an idea of its methanogenic performance and evolution. This value is estimated from the methane production rate and the amount of inoculum added and it varies with substrate type, operating temperature, etc. (Hussain and Dubey 2014). It is reported that an appropriate inoculum should have a minimum SMA value (with acetate as substrate) between 0.10 and 0.15 g CH_4-COD/g VSS·day (Angelidaki et al. 2009; Hussain and Dubey 2014).

Additional information can be gained by studying the methanogenic community abundance inside the acclimatized and maintained reactor by qPCR (Oka et al. 2011). For this purpose, the *mcr*A gene was used as a key functional marker. This gene encodes part of the terminal enzyme complex in the methane production pathway and is thought to be unique and ubiquitous in methanogens which makes it perfect to target their detection (Cisek et al. 2023; Franchi et al. 2018; Wäge-Recchioni et al. 2020).

Obtaining an adapted inoculum to perform BMP tests is a common problem in laboratories and enterprises focused on testing the production of methane using different wastes, especially in Latin American countries where biogas plant availability is limited. For that, Steinmetz et al. (2016) proposed a protocol to enrich and maintain an active hydrolytic and methanogenic community to perform BMP tests. The authors propose to operate a lab-scale bioreactor inoculated with a mixture of different methanogenic sludge and fed with complex organic biomass easy to obtain for laboratories. As the objective was to obtain a microbial community with the capacity to degrade complex wastes, the feeding was carried out using organic matter balanced in its composition of lipids, proteins, and cellulose. For that, the authors use a mixture of dried grass (30% of VS), milk powder (25% of VS), swine feed (maize basis) (30% of VS), and vegetable oil (15% of VS). An adapted biomass with good methanogenic activity was obtained after 25 days of operation. BMP tests performed using the adapted inoculum and microcrystalline cellulose, maize silage, and dried distilled grain gave values similar to those reported in the bibliography indicating that the strategy was successful.

Once the sludge is acclimatized, the possibility of preserving it without altering its characteristics (microbial composition thus its metabolic performance) would represent an opportunity to have availability of a standardized inoculum for reproducible BMP results. This is the reason why many research groups have been studying different temperature long-term preservation methods (Astals et al. 2020; Bhattad et al. 2017; Castro et al. 2002; Heerenklage et al. 2019; Soldano et al. 2019).

Although these methodologies are frequently applied in laboratories, it is not yet known how the microbial community adapts during this acclimatization process and how much long-term storage at 4 °C affects the microbial community.

In the current study, therefore, we envisioned the opportunity to monitor a microbial community adapted within a reactor acclimatized with the main goal of producing biomass by the company NETUM S.R.L.

We aim to give light on how the microbial community is adapted during the acclimatization and storage procedures, a routine methodology performed in anaerobic digestion studies. For that purpose, we asked ourselves the following questions: (1) Does the sludge's activity sustain in time? (2) How does the microbial community evolve during acclimatization? (3) Does long-term storage at 4 °C affect the methanogenic activity and the microbial community composition?

Material and methods

Reactor operation to obtain an adapted sludge for BMP assays

Sludge acclimatization was accomplished by applying Steinmetz et al. (2016) procedure by NETUM S.R.L., Business Incubator Khem, a company specialized in designing anaerobic systems for industry in Uruguay. The company's goal was to obtain an anaerobic sludge with good methanogenic activity to carry out BMP tests. To achieve this, the reactor was operated in a nonconventional mode, primarily aimed at enriching and maintaining a biomass capable of producing methane while consuming various types of substrates. A 25-L capacity polyvinyl chloride (PVC) reactor with 22 L working volume was built (Supplementary Material: Fig. S1). The reactor was inoculated with different sources of inoculum: 4.5 L from anaerobic digester treating poultry wastewater; 0.5 L of biomass from BMP test performed using chicken feathers as substrate; 0.5 L from anaerobic digester treating pig production wastes; and a 16-L mixture of fresh cattle manure and biomass from an anaerobic digester from dairy farm wastewater treatment (a mixture composed globally of 25% inoculum from poultry origin and 75% of livestock origin) (Table 1). The resulting sludge mixture had the following characteristics: TS (g TS/L) (69.60 ± 1.19), VS (g VS/L) (22.75 ± 0.81), humidity (%) (93.04 ± 0.12), pH (7.00 ± 0.02). The reactor was operated without feeding for 16 days to consume all endogenous organic matter from the original seed sludge. To enrich the biomass within the reactor with microorganisms with a wide hydrolytic capability, a mixture of low cost and available substrates were used. The substrate mixture composition was designed according to Steinmetz et al. (2016) procedure as the following: yerba mate waste (refrigerated at 4 °C), a plant highly consumed as an infusion in Uruguay (Ferraz Júnior et al. 2020); milk powder; sunflower oil; crushed dog food (each substrate characterization is presented in Table 2). These substrates represent a very vast source of nutrients, from fiber to lipids, forcing the microbiome present in the sludge to adapt to a wide range of substrates. The reactor was operated in a nonconventional way as fed-batch (as illustrated in Supplementary Material: Fig. S1). It was fed every 48 h with a mixture of the above mentioned varied organic compounds based on an organic loading rate (OLR) of 0.30 kg VS/m^3·day, varying the amount of substrate added when needed considering the reactor's maximum volume and pH maintenance around 7.00. Periodic pH measurements were conducted, whereas operational temperature remained unmonitored. The operational temperature corresponded to room temperature, with mean temperatures of (11.07 ± 5.10) °C in winter and (21.55 ± 5.87) °C in summer (source: https://catalogodatos.gub.uy/dataset/inia-precipitacion-temps-extremas-lb) (Supplementary Material: Fig. S2). The reactor underwent manual agitation every 24 to 48 h. This involved two distinct movements: firstly, lifting and dropping it vertically, followed by repeatedly twisting it clockwise and counterclockwise. While operating the reactor to produce biomass for BMP tests, samples were collected from the reactor as needed (refer to Table 3). Consequently, in order to sustain the volume and biomass levels within the reactor, the biomass used to perform the tests was reintroduced after performing the experiments (Table 3). The frequency of

Table 1 Initial seed inoculum mixture characteristics (day 1 sample)

Mixture Composition	Volume (L)	% (v/v)
Sludge from anaerobic digester treating poultry slaughterhouse wastewater (two different poultry slaughterhouses)	4.5	21.0
Feather biochemical methane potential (BMP) tests digestate	0.5	2.3
Sludge from anaerobic digester treating piggery slaughterhouse wastewater	0.5	2.3
Dairy cattle manure and anaerobic digester sludge	16.0	75.0

Table 2 Physicochemical characteristics of organic substrates fed to the mother reactor according to Steinmetz et al. The values are means ± standard deviation (SD) from three experimental replicates

	SUBSTRATE			
	Yerba mate waste	Milk powder	Sunflower oil	Crushed dog food
TS (g TS/kg)	(215.67 ± 9.91)	(947.32 ± 4.77)	(912.29 ± 1.22)	(1,000.37 ± 0.37)
VS (g VS/kg)	(205.92 ± 15.14)	(866.46 ± 61.90)	(763.10 ± 54.21)	(994.71 ± 17.62)
Humidity (%)	(78.43 ± 0.99)	(5.27 ± 0.48)	(8.77 ± 0.12)	(-0.04 ± 0.04)
Feeding rate in VS (% w/w)	30	25	15	30

TS total solids, *VS* volatile solids

Table 3 Extraction and addition of liquid and biomass from the reactor. To maintain the reactor's working volume (22 L), from day 219 to day 294, 0.25 mL of water were added every 2 days. Samples taken from the biomass added to the reactor that were further analyzed by 16S rRNA gene sequence analysis were indicated as EB1 and EB2

Reactor operation day	Volume extracted from the reactor (L)	Volume returned to the reactor (L)	Seed inoculum description	% from total reactor volume
30	0.80			
40	4.00	2.0	Water	9.3
47		3.0	Cow manure + anaerobic digester sludge	14.0
61	2.50			
79		5.0	Anaerobic digester sludge	23.3
83	2.50			
84	2.50			
127		1.0	Round robin BMP tests digestate (substrates: gelatin, cornmeal, coffee, microcrystalline cellulose)	4.7
159	5.00			
161	2.50			
175	0.80			
203	0.03			
218	1.20			
219	2.10			
239	2.10			
279	1.20			
284		1.5	Recycled poultry manure and breeding bed BMP biomass (**EB1**)	7.0
321	0.03			
339		5.0	Recycled feather and hydrolyzed feather BMP biomass (**EB2**)	23.3
347	2.70			
362	3.00			

this introduction varied, contingent upon the decrease in reactor volume and the availability of biomass from tests in the laboratory. Total solids (TS%) were determined by drying samples at 105 °C and volatile solids (VS%) were determined by volatilizing the organic matter in a muffle oven for 2 h at 550 °C (APHA 2012).

Microbial community analysis by 16S rRNA gene amplicon sequencing

Samples (50 mL) were taken monthly for a year from the operating reactor to monitor the microbial community evolution using 16S rRNA amplicon sequencing. Each sample was named based on the reactor operation day. Additionally, two different analysis were performed for each sample (Bacteria and Archaea), we included a letter "B" when bacterial community was analyzed and a letter "A" when the archeal community was analyzed. For example, on the first day of operation (August 8th, 2018), a sample was taken and labeled as "B1" when the bacterial community was analyzed and "A1" when the archaeal community was analyzed. In the case of the sample taken after the storage experiment, we labeled this sample with the storage temperature (218 (4 °C)). Once extracted from the reactor, the samples were stored at − 20 °C until used. Two samples were extracted from the biomass utilized in different experiments following the completion of BMP tests. These biomass samples were introduced into the reactor and designated as external biomass: EB1 and EB2 (Table 3).

DNA extraction/PCR amplification and massive sequencing

For DNA extraction, each sample was thawed, and the biomass was separated by centrifugation (5000 rpm for 10 min at room temperature). DNA was recovered from approximately 250 mg from the pellet obtained using a commercial kit (ZR Soil Microbe DNA MiniPrep™, USA) following the manufacturer's protocol. 16S rRNA gene amplicons were obtained by PCR from the extracted DNA using adapters, barcodes, and the V4 Universal primers set 1 (520F 5′-AYTGGGYDTAAAGNG-3′ and 802R 5′-TACNNGGGTATCTAATCC-3′), as previously described (Claesson et al. 2009). A specific primer set 2 targeting the archaeal 16S rRNA gene region was also used to improve the recovery of methanogens (340F 5′-CCCTAHGGGGYGCASCA-3′ and 787R

5′-GGACTACVSGGGTATCTAAT-3′) (Pinto and Raskin 2012; Yu et al. 2005). PCR 16S rRNA gene amplicon size was confirmed on a 1% agarose gel electrophoresis. The amplicons were purified using a commercial kit (ZR Zymoclean™ Gel DNA Recovery Kit, USA).

Sequencing of the 16S rRNA gene amplicon libraries was carried out on the Ion Torrent Personal Genome Machine (PGM) in the platform at the Biological Research Institute "Clemente Estable" (Montevideo, Uruguay).

Data analysis

Multiplexed single-end sequencing reads for Bacteria and Archaea were imported and processed using Quantitative Insights Into Microbial Ecology' pipeline (QIIME2 2022.2 release) separately (2,613,476 in total for Bacteria and 1,214,784 for Archaea). The "divisive amplicon denoising algorithm" DADA2 (Callahan et al. 2016) plugin in QIIME2 was used to "denoise" sequencing reads. This step filters out noise and corrects errors in marginal sequences, removes chimeric sequences and singletons, and finally dereplicates the resulting sequences, resulting in high-resolution amplicon sequence variants (ASVs) for downstream analysis. For Bacteria, this resulted in 1,119,108 sequences ranging from 22,462 to 151,014 per sample (16 in total) representing 7509 ASVs. For Archaea, this resulted in 502,807 sequences ranging from 11,828 to 60,742 per sample (16 in total) representing 277 ASVs. The consensus sequences for the ASVs were classified with a classify-sklearn classifier trained against the most recent SILVA 16S rRNA gene reference (release 138.1) database (Quast et al. 2013). Archaea sequences were filtered from samples amplified with primer set 1, as well as Bacteria sequences were filtered from samples amplified with primer set 2. In order to complete downstream diversity and composition analyses, sequences were rarefied to the lowest number of sequences per sample (22,462 sequences for Bacteria and 7451 sequences for Archaea). When processing the data to construct the corresponding bar plots, letter B was used to assign the bacteria analysis and A for the archaeal analysis. The sequences were submitted to the NCBI (http://ncbi.nlm.nih.gov) under accession BioProject ID PRJNA694956.

Specific methanogenic activity and BMP tests

Specific methanogenic activity (SMA) was assessed by using acetate as substrate. Each biomass sample was characterized for their total solids (TS) and volatile solids (VS) according to Standard Methods for the Examination of Water and Wastewater (APHA 2012) techniques. Biomass samples were taken from the reactor and incubated at 37 °C without adding any substrate to degrade the residual organic matter prior to use. The experimental setup for these tests was according to the reported literature with some modifications (Soto et al. 1993). In 500-mL glass bottles, sodium acetate (2 g/L), 0.875 g VS/L acclimatized sludge, and distilled water were added to a final volume of 250 mL. Each bottle was flushed with N_2 (99.9%, Linde) to remove any oxygen. The experimental temperature was set to 37 °C and pH was measured at the beginning and set between 6 and 8. Before reaching the gas volume measuring device, biogas goes through a CO_2 absorption unit, a 3 M NaOH solution where CO_2 is neutralized. Methane volume produced is recorded automatically using the AMPTS II software at standard temperature and pressure conditions (0 °C and 1 atm.) and the SMA value was calculated from the slope of the curve of accumulated methane volume versus time. SMA results were expressed as g COD g VSS^{-1} day^{-1}.

One of the samples, taken at day 83, was used to perform BMP tests for a Latin-American anaerobic digestion interlaboratory study organized by Embrapa (Brazil) (Steinmetz et al. 2020; Holliger et al. 2016; Pham et al. 2013). Twenty-five laboratories participated voluntarily in this study. The validation BMP tests were carried out using three substrates: microcrystalline cellulose (synthetic sample), ground coffee beans (natural sample), cornmeal (natural sample). Beside those substrates, we performed a BMP test with gelatin as another substrate to evaluate the protein hydrolytic capacity of the inoculum.

Quantification of methanogens by mcrA qPCR

The methanogen abundance in the biomass from the reactor was quantified in samples taken monthly throughout a year. For that, DNA was extracted as explain before and *mcr*A gene copies was quantified in a Rotor-Gene 6000 (Corbett Life Science, USA) using the nonspecific fluorophore SYBR green I (Molecular Probes, Invitrogen™), with primers *mcr*A-F (5′-TTCGGTGGATCDCARAGRGC-3′) and *mcr*A-R (5′-GBARGTCGWAWCCGTAGAATCC-3′) (Denman et al. 2007) as described by Callejas et al. (2019). Each sample and standards were analyzed in triplicate. Real-time PCR efficiencies ranged from 74 to 88%, and the R^2 value for each standard curve line ranged 0.98–0.99.

Long-term storage experiment

The sample used for the storage experiment was taken at day 218 from the reactor and dispensed in a dark glass bottle sealed with a rubber septum and a plastic band. Pressure inside the bottle was released through a water trap system connected to the bottle septum. Prior to this experiment, the sample was pre-incubated at 37 °C for 10 days to ensure all endogenous organic matter consumption. Once degassed, the glass bottle was stored at 4 °C for a 5-month period. Samples were collected from this bottle before and after storage to

evaluate the effects in the inoculum activity (SMA) and the microbial community composition.

Statistical analysis

All the statistical analysis was performed in R version 3.5.1 (R Development Core Team 2013) with R Studio environment Version 1.3.1093. The biom files from QIIME2 for Bacteria and Archaea were imported and analyzed separately through phyloseq-modified workflow (McMurdie and Holmes 2013). Principal coordinate analysis (PCoA) using UniFrac distance metrics of non-transformed relative abundance diversity datasets were used in order to visualize the distribution of the bacterial and archaeal community composition. Alpha diversity was calculated using Shannon–Weaver richness estimator after rarefying. In order to determine significant differences between samples' medians in SMA and *mcr*A tests, the Shapiro–Wilk (to test normal distribution), one-way ANOVA (in cases when normal distribution and equal group variance applied), and Kruskal–Wallis tests (when distribution is not normal) were used. Differences among means with a p value below 0.05 were considered statistically significant differences (Supplementary Material: Table S1).

Results

Bioreactor operation

The reactor was operated in a nonconventional way for 1 year in which samples were taken periodically (every 30 days on average) and biomass was reintroduced after its use in BMP or activity tests (Table 3). The addition of biomass after the experiments had two objectives: to maintain the amount of biomass in the reactor and to have a microbial community enriched in methanogenic microorganisms. The volume reintroduced into the reactor never exceeded 23% of the total working reactor volume (Table 3). Because of these variations, the biomass within the reactor varied between 5.98 and 25.79 g/L along this period of time (Supplementary Material: Fig. S3).

The OLR also presented variations; it was on average 0.15 varying between 0.05 and 0.26 kg SV/m^3·day (Supplementary Material: Fig. S3). The reactor's pH was measured periodically and was maintained around 7 (it varied between 6.77 and 7.62 with an average of 7.17). The reactor was operated at room temperature.

Methanogenic activity

To determine if it was possible to obtain biomass with good methanogenic activity maintained over time, the specific acetoclastic methanogenic activity was measured in selected samples. The results indicated an increase in activity during the operation. A statistically significant improvement in the specific methanogenic activity over time was observed with values ranging from 0.159 ± 0.003 to 0.241 ± 0.012 g CH_4-COD/g VSS·day (Fig. 1).

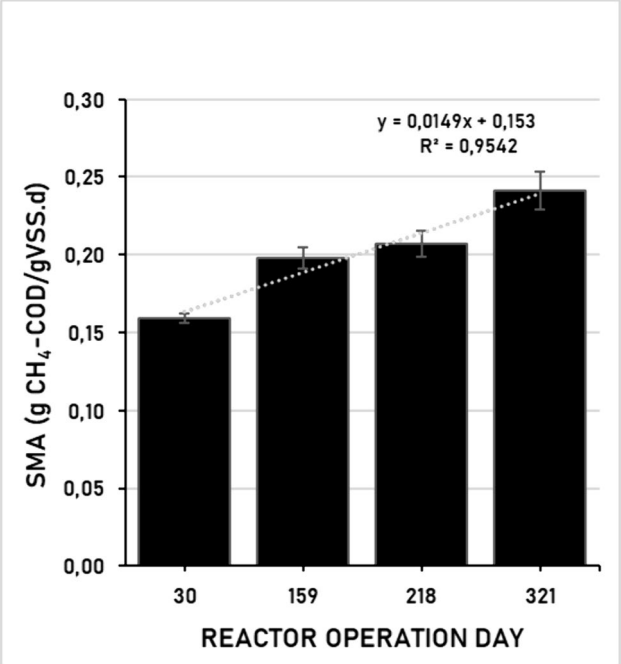

Fig. 1 SMA measured in samples taken from the reactor throughout a year. Numbers in the horizontal axis represent the operation day when the sample was extracted from the reactor. The values are means ± standard deviation (SD) from three experimental replicates. SD of means is represented by bars

Concurrently, the hydrolytic capacity of the sludge was determined in an interlaboratory study using four different substrates (Steinmetz et al. 2020). The BMP results for each of the substrates evaluated are shown in Table 4.

Table 4 Biochemical methane potential (BMP) (ml CH_4/gSV substrate) results from interlaboratory tests performed in two laboratories (Raposo et al. 2011a, b)

Substrate	IIBCE BMP	NETUM BMP	Reported BMP
Coffee	(322.8 ± 7.6)	(381.3 ± 23.3)	-
Cornmeal	(363.1 ± 5.9)	(300.7 ± 3.3)	-
Microcrystalline cellulose	(452.4 ± 11.0)	(450.0 ± 35.1)	(350 ± 29)
Gelatine	(348.3 ± 3.6)	(355 ± 16.4)	(380 ± 42)

IIBCE Biological Research Institute "Clemente Estable", *NETUM S.R.L.* Business Incubator Khem, a company specialized in designing anaerobic systems for industry in Uruguay

Quantification of methanogens by mcrA qPCR

One of the bottlenecks to obtain biomass with good methanogenic activity is establishing a community enriched in methanogens. To evaluate if this goal was achieved, a qPCR technique targeting the *mcr*A gene was applied. This method allows to determine the abundance of methanogenic Archaea within a sample in a rapid way compared to SMA tests or massive sequencing which require longer processing times.

The results showed that the community was enriched in methanogens and that they were maintained along the reactor operation. The values obtained in the samples taken monthly throughout a year ranged between 1.79×10^9 and 6.71×10^{10} copies/ng DNA (Fig. 2, Supplementary Material: Table S2). A gradual increase tendency was observed in the first 5 months (days 1 to 159) starting from 4.38×10^9 copies/ng DNA and increasing to 2.44×10^{10} copies/ng DNA. An order of magnitude decrease was observed in the next 3 months, but a recovery was observed from the sample taken on day 279 (5.52×10^{10} copies/ng DNA).

Microbial community analysis by 16S rRNA amplicon sequencing

The aim of this work was to evaluate if, with the applied reactor operation, it was possible to obtain and maintain a stable methanogenic community with good hydrolytic, fermentative, and methane-producing capacity. To verify whether this complex community was indeed selected and if it could be maintained over time, the microbial community composition was studied by 16S rRNA gene amplicon sequencing. As previous reports demonstrated that Universal primers underestimate archaeal population, we decided to use two sets of primers (Fischer et al. 2016). We also analyzed two samples from the external biomass prior to their introduction to the reactor (samples EB1 and EB2) to determine how this disturbance could affect the microbial community within the reactor.

The 16S rRNA analysis of the bacterial community at phylum level showed the predominance of Firmicutes, Synergistota, Actinobacteria, Chloroflexota, and Proteobacteria phylum (Fig. 3A). While the Archaea community presented a high dominance of Halobacterota (79.5–99.3%) and a lower dominance of Euryarcheota (1.6–20.5%) (Fig. 3B).

The bacterial composition at family level (Fig. 4A) showed the presence of *Synergistaceae*, *Anaerolineaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, and *Clostridiaceae*, and in lower abundance *Syntrophomonadaceae*, *Synrophorhabdaceae*, and *Smithellaceae*. The family *Desulfotomaculaceae* was detected only in 3 out of 14 samples analyzed (B61, B131, EB2), showing a very high relative abundance in sample B131 (25.8%) and B61 (8%). The bacterial diversity at lower taxonomic levels was very high, so we did not focus on these results (Supplementary Material: Fig. S4).

Due to the lower diversity, the analysis of the archaeal composition is presented at genus level. The results show that most of the samples were dominated by *Methanosaeta* (from 32.7 to 87.6% relative abundance), a genus nowadays reclassified as *Methanothrix* (Tindall 2014). A non-classified genus (represented by ASV 597) also stands out until day 279, but at lower abundance (from 8.1 to 12.4%) (Fig. 4B). To study the identity of this microorganism, ASV sequences classified in this unknown group were retrieved and compared with the database using BLAST search (https://blast.ncbi.nlm.nih.gov/). The sequence was classified within the *Methanotrichaceae* family, *Methanothrix* genus, with a similarity of 100% with the sequence from strain *Methanothrix soehngenii* (NR_102903).

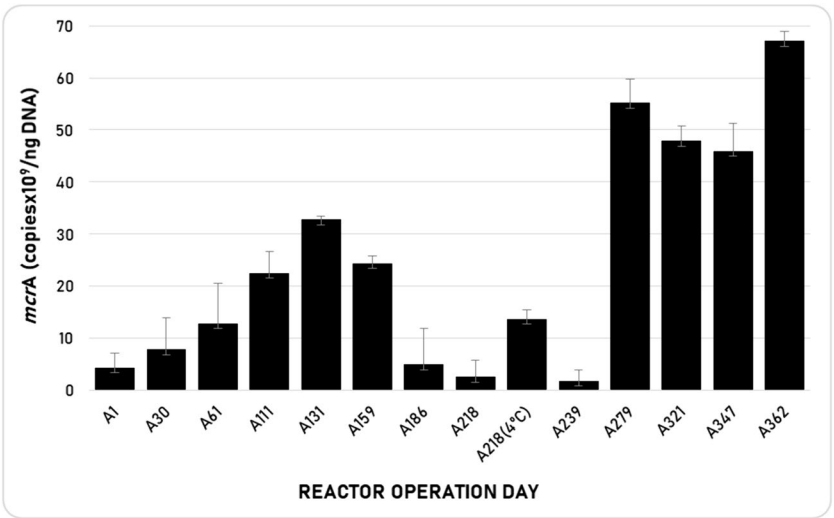

Fig. 2 Graphic representation of *mcr*A gene quantification results by qPCR method in samples taken from the reactor throughout a year. The values are means and the standard deviation comes from three experimental replicates

Fig. 3 **A** Bacteria bar plot representing the relative abundance at phylum level present in each sample according to 16S rRNA analysis. **B** Archaeal bar plot representing the relative abundance at phylum level present in each sample according to 16S rRNA analysis. Triangles represent the events of external volume addition (reinoculation) to the mother reactor, as described in Table 3. The columns on the right display the external biomass composition prior to its introduction to the reactor (EB1 and EB2), along with the composition analysis of the storage experiment (218 and 218 (4 °C)). Herein we specify the sample name together with its sampling month: *Sample 1*: August; *Sample 30*: September; *Sample 61*: October; *Sample 111*: November; *Sample 131*: December; Sample 159: January; Sample 186: February; *Sample 218*: March; *Sample 239*: April; *Sample 279*: May; *Sample 321*: June; *Sample 347*: July; *Sample 362*: August

Additionally, to determine the effect of the biomass addition into the reactor, two samples from the biomass added to the reactor (EB1 and EB2) were aleatory chosen to be analyzed (Table 3). These two samples showed different microbial composition compared to the samples taken from the reactor, with a high prevalence of Firmicutes at phylum level and *Planocaccaceae* at family level in sample EB1. The results also showed that samples B61, B111, B131, B321, and B347 taken shortly after the addition of external biomass presented a different microbial composition. Samples B61 and B131 presented a higher dominance of Firmicutes compared to other samples, while in sample B321 the low abundance phyla were observed to increase their proportion

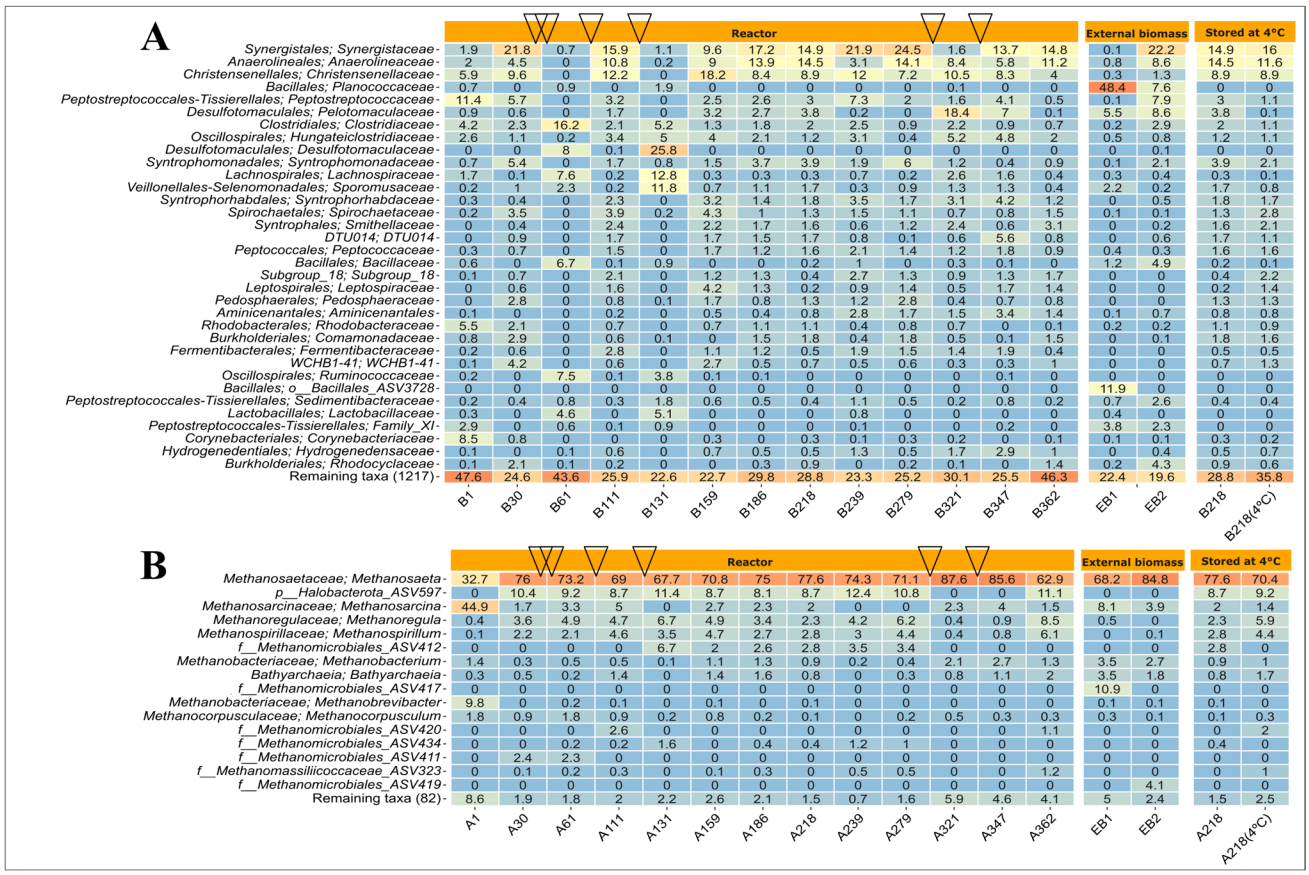

Fig. 4 **A** Heat map describing bacterial composition at family level. **B** Heatmap describing archeal composition at genus level. In heat map legend p represents phylum; o, order; c, class; and f, family

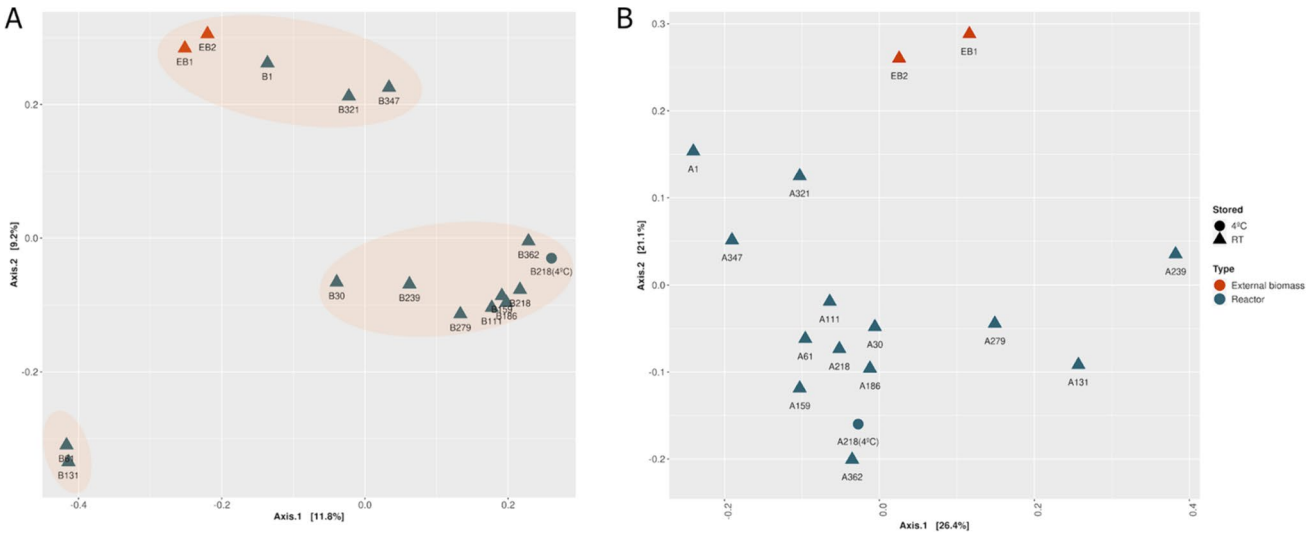

Fig. 5 Unifrac distance matrix PCoA for bacterial (**A**) and archaeal (**B**) community. Samples were differentiated by color (orange: external biomass, green: reactor samples). Circle shaped spots represent 4 °C stored samples while triangles represent samples stored at room temperature

(Fig. 3A). No correlation was found between the operational parameters (OLR, VS) and this observation.

The changes produced by the addition of external biomass were clearly observed when Unifrac distance matrix PCoA analysis was performed. Samples were grouped into three groups (Fig. 5A). Group 1 included samples B321 and B347 and samples from the biomass introduced to the reactor during the evaluated period (EB1 and EB2) suggesting that the biomass composition inside the reactor was affected by the introduced biomass. Group 2 included samples B61 and B131 taken after the addition of anaerobic digester sludge waste and round robin BMP test digestate. Finally, group 3 was composed of samples taken far after the addition of any external biomass.

On the other hand, the PCoA performed using data from the archaeal community did not show a clear tendency, as observed in the bacterial community. Nevertheless, it could be noted that samples taken after the addition of biomass (samples A321 and A347) were separated from the rest of the samples (Fig. 5B).

Shannon alpha diversity indexes were determined to evaluate the diversity within the bacterial and archaeal communities (Supplementary Material: Table S3). The results showed that samples taken after external biomass addition were the least diverse (3.9–4.5), except for sample B321 (5.7). The addition of external biomass may have enriched certain groups present in the reactor causing a decrease in the diversity within these samples. The remaining samples showed Shannon index values between 4.6 and 6.0. Archaeal Shannon diversity index analysis showed lower values when compared with bacteria communities. In this case, we also observed that samples taken after external biomass addition had lower diversity (1.1 and 1.3) while the remaining samples showed an index between 1.8 and 2.6 (Supplementary Material: Table S3).

Even though these results demonstrate a significant alteration resulting from external biomass reintroduction, further investigation is required to confirm whether this effect stems from the biomass addition rather than changes in reactor operation. To ascertain this, it would be necessary to analyze samples taken both before and after the addition of external biomass, ensuring no other variations occur in the reactor operating conditions. Yet, the focus of this study was to examine how the operational conditions of this reactor influenced microbial communities, encompassing both the introduction of biomass and the varying operational conditions of the reactor.

Effect of storage at 4 °C in the microbial community composition

Refrigerating sludge samples is a very frequent practice in laboratories performing BMP tests to have sludge availability whenever it is needed. To determine how this practice affects the microbial composition and its methanogenic activity, one sample was stored at the refrigerator for 5 months and afterwards the SMA and the microbial community analysis were performed. Methanogens were also quantified by qPCR before and after storage.

No significant statistical difference was observed between SMA medians. SMA for sample 218 was 0.207 ± 0.009 g CH_4-COD/g VSS·day and for the sample stored at low temperature for 5 months (sample 218 (4 °C)) was 0.184 ± 0.011 g CH_4-COD/g VSS. d (Table 5). Therefore, the sludge methanogenic activity remained practically unchanged after being stored at 4 °C for 5 months.

Q-PCR results showed a one order increase in the *mcr*A copy number/ng DNA after storage. This difference was statistically significant according to Kruskal–Wallis analysis ($p < 0.05$).

Results from the bacterial community analysis showed that the predominant phyla were the same for both samples (B218 and B218(4 °C)): Firmicutes, Proteobacteria, Synergistota, and Chloroflexota (Fig. 3A). Moreover, PCoA analysis showed that both samples grouped together indicating that the small differences between the relative abundance of the microorganisms detected was less than the community variation over time. Similar results were obtained for the Archaea community (Fig. 3B). This was also reflected in PCoA archaeal analysis where the samples taken before and after the storage clustered together (Fig. 5B).

Discussion

Biomass with good methanogenic activity was obtained and sustained

Both the SMA and the qPCR results showed that the built-up community was enriched in methanogens and that they were maintained during the reactor operation. The acetoclastic methanogenic activity reached values of 0.241 g CH_4-COD/gVSS·day which is considered an appropriate methanogenic activity for a sludge (Angelidaki et al. 2009; Callejas et al. 2022, 2019; Hussain and Dubey 2014). The proportion of

Table 5 Specific methanogenic activity (SMA) assay results and *mcr*A copy numbers for samples taken before and after storage at 4 °C for a 5-month period

Reactor operation day	SMA (gCH_4-COD/gVSS.d)	*mcr*A(copies/ng DNA)	gVSS/L inoculum
218	(0.207 ± 0.009)	2.54×10^9	25.6
218 (4 °C)	(0.184 ± 0.011)	13.7×10^9	25.6

COD chemical oxygen demand, *VSS* volatile suspended solids

methanogens determined by qPCR also showed values similar to those obtained in previous studies for functional reactors. Callejas et al. (2019) reported similar values for a full-scale UASB reactor designed for dairy wastewater treatment where its microbiome was monitored during the startup and operation processes applying the same qPCR technique.

We observed an inconsistent relationship between the SMA and the *mcr*A copy number/ng DNA in some of the samples (Morris et al. 2014). While the SMA increases in time, a one order decrease in magnitude was observed in the *mcr*A abundance in samples taken at days 186, 218, and 239. This drop in *mcr*A abundance could be due to a decrease in the proportion of hydrogenotrophic methanogens (detected by *mcr*A quantification but not detected by the acetoclastic methanogenic activity), or to the difficulty of the PCR methods to detect all the methanogens. The lack of consistency between both techniques was previously reported (Dellagnezze et al. 2023) and it is a problem caused by the bias of the different methods.

The hydrolytic and fermentative capacity of the biomass was tested in a Latin American interlaboratory assay using four compounds with different chemical composition: microcrystalline cellulose, ground coffee beans, cornmeal, and gelatin (Steinmetz et al. 2020). The results obtained in our laboratory were satisfactory according to the statistical analysis evaluation (Supplementary Material: Fig. S5). These results on this variety of complex substrates tested the entire community performance indicating a well-functioning hydrolytic and methanogenic community.

A microbial community with hydrolytic and methanogenic microorganisms was enriched and maintained during reactor operation

Both the bacterial and archaeal microbial community analysis showed a community with a taxonomic composition of a well-established methanogenic community. The Firmicutes, Synergistota, Actinobacteria, Chloroflexota, and Proteobacteria phyla dominated the bacterial community. These phyla are key for degrading complex organic matter as carbohydrate, lipids, protein, etc., to simpler compounds more accessible to methanogens (Li et al. 2015; Liu et al. 2009). Their presence could be an indicator of a well-adapted to a wide variety of substrates sludge. At the same time, microorganisms syntrophic to hydrogen-consuming methanogen, such as those belonging to Synergistota phylum, were also detected in these samples (Ito et al. 2011; Militon et al. 2015). These results confirm that the chosen strategy to obtain an adapted biomass to perform BMP tests was successful.

When the analysis was performed at Family level (Fig. 4A), we observed a microbial community composed mainly of hydrolytic and fermentative microorganisms (*Synergistaceae*, *Anaerolineaceae*, *Christensenellaceae*, *Peptostreptococcaceae*, *Clostridiaceae*), along with syntrophic oxidation microorganisms (*Syntrophomonadaceae*, *Synrophorhabdaceae*, *Smithellaceae*) (Rosenberg et al. 2013; McInerney et al. 2008).

The family *Desulfotomaculaceae*, composed of sulfate-reducing bacteria, was detected only in 3 out of 14 samples analyzed showing a very high relative abundance in sample B131 (25.8%) and B61 (8%) (Barton and Hamilton 2007), indicating that sulfate may be present in the complex digested substrates. Whenever sulfate is present, it competes with methanogenesis in an anaerobic ecosystem, but, on the other hand, in its absence, species from this family are also known to perform syntrophic oxidation of organic acids, an important role for methanogenesis. In the present case study, sulfate content in the reactor was not measured; then, this genus role cannot be predicted from the 16S rRNA gene sequence alone. De Francisci et al. (2015) detected an increase in *Desulfotomaculum* in a reactor overfed with proteins, in our case the increase in this family occurred after the addition of BMP tests digestate using as substrates: gelatin, cornmeal, coffee, microcrystalline cellulose (at day 127).

Within the archaeal community, the genus *Methanosaeta*, acetoclastic methanogens, predominated showing high abundance in some samples (from 32.7 to 87.6%). This genus was reclassified as *Methanothrix* and it is a known acetoclastic methanogen (Carr et al. 2018; Chen and He 2015). Several previous studies have noted the dominance of *Methanosaeta* suggesting that this occurrence might be widespread in the anaerobic digestion process (Moertelmaier et al. 2014; van Haandel et al. 2014; Yilmaz et al. 2014).

Another microorganism, represented by ASV 597, exhibited a high level of dominance. This ASV could not be classified using the QIIME analysis; however, using the BLAST tool, we found a high-sequence homology with the sequence corresponding to *Methanothrix soehngenii*, which is known as an acetoclastic methanogen (Ferry 2020). According to Fig. 4, the following genera from hydrogenotrophic archaea were observed: *Methanoregula*, *Methanospirillum*, *Methanobacterium*, *Methanobrevibacter*, *Methanocorpusculum*, along with several unknown genera within the *Methanomicrobiales* order (Garcia et al. 2022). The relative abundance of each of these genera was less than 10%. Therefore, hydrogenotrophic methanogenesis can occur but in lower dominance.

These results showed that acetoclastic methanogenesis predominates in all samples, which is important for a stable process performance as according to literature; this population accounts for most of the methane production in anaerobic digestion (Chen and He 2015).

Disturbances occurred during the external biomass addition

As the reactor was operated to generate biomass stock to be used in BMP tests, the reactor was periodically disturbed by biomass extraction. Consequently, to stabilize the biomass level, we reintroduced the biomass back into the reactor after BMP tests were conducted and whenever it was available in the lab. Different substrates were used in each test, so it was reasonable to expect that the new biomass adapted to the different substrate digestion. To evaluate the effect of these perturbations in the microbial communities, we also analyzed two of the added to the reactor biomass samples (EB1 and EB2). These two samples showed different microbial composition according to the 16S rRNA gene analysis (Fig. 3A, B). As these biomass samples were taken from BMP tests performed with different substrates, we expected a different selection of microorganisms during each batch test. Sample EB1 was taken from a test performed using poultry manure and breading bed wastes, and presented high abundance of Firmicutes, while sample EB2 was taken from a BMP test performed with chicken feathers and enzymatically hydrolyzed chicken feathers. The microbial composition in sample EB2 was similar to the biomass composition within the reactor.

Samples B61, B111, B131, B321, and B347 were taken shortly after the addition of external biomass. Samples B61 and B131 presented a higher dominance of Firmicutes compared to other samples, while in sample B321 low abundance phyla increased their proportion (Fig. 3A). It has been postulated that substrate type determines differences in the reactor microbial phylogenetic structure (Zhang et al. 2014). Therefore, the relationship between the external biomass sludge characteristics and the affected samples from the present case study was analyzed. In the case of the initial seed sludge mixture prepared on day 1 (Table 1), it was composed globally of 25% sludge from poultry wastes origin and 5% of cattle wastes origin. The next external biomass, added on day 47 and 79 to the reactor, was basically cattle manure and anaerobic manure digester sludge. Firmicutes and Bacteroidota were reported as dominant in most manure digesters as expected coming from cattle ruminal content (Liu et al. 2009; Narihiro et al. 2015; Pitta et al. 2010). Consequently, this could explain Firmicutes predominance in samples B1, B61, and B131. On the other hand, Bacteroidota phylum was practically absent in all samples. The latest biomass addition to the reactor was poultry wastes origin samples (poultry manure, poultry litter, feathers). According to Zhang et al. (2018), chicken manure contains diverse gut microbes, mainly species derived from Proteobacteria. On the other hand, poultry litter, which is primarily a mixture of bedding materials and bird excreta, are reported to contain, depending on the number of reuse cycles, members of the Proteobacteria phylum (Cressman et al. 2010). Moreover, feathers are high content protein structures (dry feathers contain 91% protein) (Mézes and Tamás 2015), and substrates with these characteristics could be contributing with microorganisms related to protein degradation (Javůrková et al. 2019; Shawkey et al. 2005). Firmicutes was the predominant phylum in samples 321 and 347 (Fig. 3A) even though Proteobacteria is known to be one of the dominant phyla in anaerobic digesters (Li et al. 2015; Bovio-Winkler et al. 2021) and the protein-rich characteristics of the poultry biomass reinoculated into the reactor on days 284 and 339 (Table 3).

In summary, a highly diverse bacterial community dominated by fermenting and hydrolyzing bacteria was observed throughout the studied period. Despite noticing variations in relative abundance during the evaluated period, the sludge's methanogenic activity did not decline (Fig. 2), which indicates that the acetoclastic methanogenic population remained active.

The results obtained demonstrated resilience and robustness of the microbial community to the disturbance produced by the biomass addition episodes. Disturbance and community stability are necessarily related, as stability is defined as the community's response to disturbance (Shade et al. 2012). Probable functional redundancy within the community may explain sludge's activity maintenance throughout the whole evaluated period (Allison and Martiny 2009).

Effect of the storage at 4 °C

Previous studies have been reported for long-term preservation methods at room temperature, refrigerating, freezing, and lyophilization of inoculum samples prior to its use in anaerobic batch tests (Astals et al. 2020; Bhattad et al. 2017; Castro et al. 2002; Heerenklage et al. 2019; Soldano et al. 2019). Results from these experiments demonstrated high biogas or methane production recovery for room temperature and refrigerated (4 °C) samples while biogas production and the lag phase were drastically affected in the case of frozen or lyophilized samples. Contrary to this, another report suggested a drop in methane yields and slower production kinetics after 1-month storage for both refrigerated and frozen samples (4 °C and − 20 °C respectively) (Hagen et al. 2015).

From the abovementioned reports, only a few employed SMA tests to evaluate storage impact in the inoculum activity (Astals et al. 2020; Bhattad et al. 2017; Castro et al. 2002). Instead, most of them evaluated inoculum's quality based on BMP tests results which can be less sensitive to changes in the methanogenic activity than metabolic assays (e.g., SMA) as they comprise the entire degradation process while metabolic assays directly target a specific biological step (e.g., methanogenesis) (Astals et al. 2020).

Castro et al. (2002) analyzed the entire anaerobic population during an SMA test using glucose as the substrate and specifically targeted acetoclastic methanogens by employing acetate as the substrate. After a 5-month storage experiment, they observed lower SMA results for the experiments tested with glucose than for those with acetate. From this, it could be concluded a higher resistance from the acetoclastic methanogens to different storage methods. The lower SMA results obtained with glucose might be attributed to the lower sensitivity previously mentioned to changes in the inoculum activity than metabolic assays. This group argued that the selection of the preservation method and the storage characteristics are inoculum specific. According to our results, the microbial community was not affected, but, as this analysis was based on DNA, we cannot infer the activity was not affected.

The results from the present work were consistent with those reported by several groups. Acetoclastic activity remained stable after a 5-month storage at 4 °C (Zitomer et al. 2007). To our knowledge, there are no previous studies monitoring an acclimatization to produce methane microbial community sludge at this storage temperature for a 5-month period combining SMA, qPCR, and massive sequencing tools.

The increase in the methanogens proportion after storage could be explained by the higher sensitivity to low temperature of the hydrolytic and fermentative microorganisms. If their degradation rate is higher, their DNA will not persist in the sample; consequently, *mcr*A copy number versus total DNA proportion will increase. Despite the increase shown in the methanogens quantification after storage by qPCR, the SMA maintained. A possible explanation for this fact is that, as the SMA is determined by VSS gram even though DNA was degraded, the amount of biomass remains, or the change is undetectable by the applied method. Further work is needed to confirm this hypothesis.

The abovementioned results contradict those reported by Hagen et al. who argue a decay in the sludge activity after a 1-month 4 °C storage (Hagen et al. 2015). In the cited article, the most abundant phyla were hydrogenotrophic Methanomicrobiales (genus; *Methanoculleus*) and "Miscellaneous Crenarchaeota Group" (MCG) (clone GrfC26) the first 2 months of storage, but longer storage led to a shift in dominance to acetoclastic methanogens, Methanosarcinales (genus; *Methanosarcina*). In the present case study, acetoclastic *Methanosaeta* (now reclassified as *Methanothrix*) was the predominant archaeal genus before and after storage (Fig. 4B). From these observations, acetoclastic community resistance to refrigeration could be argued explaining why no archaeal community differences were observed.

On the other hand, as we did not test the BMP of complex substrates after their storage, we cannot affirm other steps in the anaerobic chain were not affected; this should be further investigated.

In this work, the effect of storage at 4 °C was evaluated as it is a common practice in laboratories which perform BMP tests. It would be interesting to evaluate other sludge storage conditions such as preservation at room temperature, freeze-drying, among others, therefore, selecting the best storage condition which does not affect the microbial community composition in time. Deeper work is necessary to evaluate how the different storage methods affect the microbial communities.

In summary, applying the reported methodology to produce an appropriate inoculum guarantees a microbial community able to perform BMP tests on a wide variety of substrates. The microbial community composition was affected by external biomass addition episodes; despite this, the sludge's activity as well as its methanogens abundance maintained over the evaluated period. The microbial profile changed predominating different microorganisms according to the external sludge origin, returning to its initial structure rapidly. Storing an acclimatized inoculum in the refrigerator for 5 months did not affect its methanogenic activity nor its microbial composition although changes in *mcr*A abundance were observed through qPCR, likely attributable to the degradation of DNA from other bacteria.

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Author contribution CE and GZ conceived the project. CE and GZ designed and guided the study. VS and GZ prepared and characterized each inoculum. VS performed the laboratory experiments and processed the samples for massive sequencing. PB-W performed bioinformatics analyses. CE, PB-W, and VS analyzed the data and PB-W prepared the figures and tables related to the bioinformatics analysis results. VS prepared the other data tables. CE, PB-W, and VS drafted the manuscript. All authors edited the manuscript and approved the final manuscript.

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Data availability The sequences were submitted to the NCBI (http://ncbi.nlm.nih.gov) under accession BioProject ID PRJNA694956.

Declarations

Conflict of interest The authors declare no competing interests.

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