



Cultured and uncultured microbial community associated with biogas production in anaerobic digestion processes

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Abstract

The search for sustainable development has increased interest in the improvement of technologies that use renewable energy sources. One of the alternatives in the production of renewable energy comes from the use of waste including urban solids, animal excrement from livestock, and biomass residues from agro-industrial plants. These materials may be used in the production of biogas, making its production highly sustainable and environmentally friendly. The present study aimed to evaluate the cultivated and uncultivated microbial community from a substrate (starter) used as an adapter for biogas production in anaerobic digestion processes. 16S rDNA metabarcoding revealed the domain of bacteria belonging to the phyla Firmicutes, Bacteroidota, Chloroflexi and Synergistota. The methanogenic group was represented by the phyla Halobacterota and Euryarchaeota. Through 16S rRNA sequencing of isolates recovered from the starter culture, the genera *Rhodococcus* (Actinobacteria phylum), *Vagococcus*, *Lysinibacillus*, *Niallia*, *Priestia*, *Robertmurraya*, *Proteiniclasticum* (Firmicutes phylum), and *Luteimonas* (Proteobacteria phylum) were identified, genera that were not observed in the metabarcoding data. The volatile solids, volatile organic acids, and total inorganic carbon reached 659.10 g kg⁻¹, 717.70 g kg⁻¹, 70,005.0 g kg⁻¹, respectively. The cultured groups are involved in the metabolism of sugars and other compounds derived from lignocellulosic material, as well as in anaerobic methane production processes. The results demonstrate that culture-dependent approaches, such as isolation and sequencing, and culture-independent studies, such as the Metabarcoding approach, are complementary methodologies that, when integrated provide robust and comprehensive information about the microbial communities involved in processes of the production of biogas in anaerobic digestion processes.

Keywords Culture-dependent · Culture-independent approaches · Anaerobic digestion processes · 16S rRNA · Metabarcoding

Introduction

Population growth has increased the demand for limited supplies of food and fuel by nations around the world. However, petroleum-derived fuels, which, in addition to being composed of highly polluting substances to the environment, are originated from finite sources of energy, factors that indicate the need to replace these fuels in the medium term with those produced from renewable sources. (Gielen et al. 2019). Innovative technologies are being developed to efficiently convert biomass into valuable products such as biogas (Vale et al. 2019). Biogas derived from animal manure and plant residues (lignocellulosic biomass) has been considered an alternative and renewable biofuel with ample energy capacity and can be

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a sustainable option over the use of fossil fuels (Anthony et al. 2019; Gulhane et al. 2018). One of the major limitations that biogas production still has is the lack of a higher yield in the anaerobic digestion (AD) process so that the technology can be transferred to large-scale biodigesters. This deficiency can be overcome with greater knowledge of the microbial communities involved in anaerobic digestion (Muturi et al. 2021). Biogas production from renewable and sustainable resources is becoming a prominent alternative in most developed and developing countries (Murunga et al. 2016), which has increased its use, a more viable option for modern society.

AD is a process derived from microbial metabolism that produces biogas (methane) from the conversion of organic matter (Orhoro et al. 2018). This process, which occurs in the absence of oxygen, involves different microbial groups, each one being responsible for the degradation of a category of organic compounds present in the system. In AD, methane (CH_4) production occurs over 4 stages, which are hydrolysis, acidogenesis, acetogenesis and methanogenesis (Vrieze and Verstraete 2016). In hydrolysis, carbohydrates, proteins and fats from animal manure and food residues are broken down into soluble compounds, such as monosaccharides, amino acids, and fatty acids, by the action of enzymes produced by hydrolytic bacteria of genera such as *Bacillus*, *Bacteroides* and *Eubacterium* (Soares et al. 2017). The products of hydrolysis are transformed into volatile fatty acids and alcohols by acidogenic bacterial genera (acidogenesis), including *Clostridium* and *Bacteroides* (Valijanian et al. 2018; Yan et al. 2020; Xu et al. 2021). The products from previous steps are transformed into acetic acid, hydrogen, and CO_2 by acetogenic bacteria, including *Desulfococcus* and *Desulfotomaculum*. Finally, methane formation (methanogenesis) occurs due to the presence of methanogenic archaea including *Methanosarcina* and *Methanobacterium* (Covey and Megonigal 2019; Knoblauch et al. 2018; Valijanian et al. 2018; Xu et al. 2021).

Therefore, the implementation of AD processes in biodigesters improves the production of biogas, since in these systems the conditions are controlled, and the processes can be standardized according to the market needs for this biofuel. In addition, the characterization of microbial communities in environmental samples using culture-dependent and culture-independent methods are widely used technologies (Arguita-Maeso et al. 2020; Wei et al. 2021), and essential for the optimization of the process. In this sense, this work aimed to analyze an inoculum used by the company CIBiogás—International Center for Renewable Energies, to optimize the anaerobic digestion tests developed by the corporation, using culture-dependent and independent methods for the taxonomic characterization of the communities present in the inoculum, as well as to identify the main groups involved in AD processes. Such information will be used to

optimize and develop strategies for a better understanding of biomethane production.

Materials and methods

Sampling and isolation

An inoculum sample (starter) produced in a biodigester was provided by the company CIBiogás located in the city of Foz do Iguaçu, Brazil and was used in the present work to assess the functional and taxonomic diversity of associated microorganisms. Sampling was carried out on 1/20/2020 in the laboratory of the company CIBiogás. Forty mL of the contents of the biodigester were collected, properly placed in 50 mL flasks under sterile conditions. The sample was homogenized in an automatic shaker and serial dilution (10^{-1} , 10^{-2} and 10^{-3}) was performed. Aliquots of 50 μL of each dilution were inoculated to the respective culture media, in triplicate, as described as follows:

Hydrolytic culture media (HM), composed by the inorganic salts (SI) KH_2PO_4 10 $\text{g}\cdot\text{L}^{-1}$; $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ 6.6 $\text{g}\cdot\text{L}^{-1}$; NaCl 8 $\text{g}\cdot\text{L}^{-1}$; Na_2SO_4 0.28 $\text{g}\cdot\text{L}^{-1}$; NH_4Cl 8 $\text{g}\cdot\text{L}^{-1}$ and $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 1 $\text{g}\cdot\text{L}^{-1}$ plus the trace elements $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.1 $\text{g}\cdot\text{L}^{-1}$; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ 0.03 $\text{g}\cdot\text{L}^{-1}$; H_3BO_3 0.3 $\text{g}\cdot\text{L}^{-1}$; $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ 0.2 $\text{g}\cdot\text{L}^{-1}$; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.01 $\text{g}\cdot\text{L}^{-1}$; $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ 0.02 $\text{g}\cdot\text{L}^{-1}$ and agar 20 $\text{g}\cdot\text{L}^{-1}$. For each 1000 mL of the hydrolytic culture media, the following reagents were added, separately for each analysis: (i) for isolation of cellulase-producing bacteria (CMC): CMC (carboxymethyl cellulose) 0.2%; (ii) for isolation of amylase-producing bacteria (AM): soluble starch 0.2%; (iii) for isolation of ligninase-producing bacteria (GUA and RBBR): guaiacol (99%) and 425 μL and RBBR (Remazol Brilliant Blue R) 1000 $\text{mg}\cdot\text{L}^{-1}$, separately; (iv) for isolation of lipase-producing bacteria (OL): olive oil 1%; (v) for isolation of protease-producing bacteria (LE): skimmed milk 10%; (vi) for isolation of distinct bacteria (NA-nutrient agar): meat extract 3 $\text{g}\cdot\text{L}^{-1}$; peptone 5 $\text{g}\cdot\text{L}^{-1}$, pH 6.8. The different culture media containing the sample inoculum were incubated at 37 °C for 5 to 7 days. Morphologically distinct colonies were purified and preserved at -80 °C in 20% glycerol.

Acidogenic culture medium (ACD)—basic medium—composed by (a) glucose 1 $\text{g}\cdot\text{L}^{-1}$; inorganic salts KH_2PO_4 10 $\text{g}\cdot\text{L}^{-1}$; $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ 6.6 $\text{g}\cdot\text{L}^{-1}$; NaCl 8 $\text{g}\cdot\text{L}^{-1}$; Na_2SO_4 0.28 $\text{g}\cdot\text{L}^{-1}$; NH_4Cl 8 $\text{g}\cdot\text{L}^{-1}$ and $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 1 $\text{g}\cdot\text{L}^{-1}$, plus the trace elements $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ 0.1 $\text{g}\cdot\text{L}^{-1}$; $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ 0.03 $\text{g}\cdot\text{L}^{-1}$; H_3BO_3 0.3 $\text{g}\cdot\text{L}^{-1}$; $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ 0.2 $\text{g}\cdot\text{L}^{-1}$; $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.01 $\text{g}\cdot\text{L}^{-1}$; $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ 0.02 $\text{g}\cdot\text{L}^{-1}$ and cysteine 0.05 $\text{g}\cdot\text{L}^{-1}$; (b) vitamin solution 5 ml (100 mL): PP vitamin 2 mg; B12 vitamin 1 mg; B6 vitamin 5 mg; C vitamin 2.5 mg; pantoic acid 0.5 mg; Bc vitamin 1 mg; biotin 3.5 mg; B2 vitamin 2.2 mg; Choline 2.5 mg; p-aminobenzoic acid 1 mg;

(c) Na_2HCO_3 (5%) 0.05 mL; Na_2S (1%) 0.05 mL; resazurin 0.5 g.L^{-1} 2 mL (Agustini 2014; Ren et al. 2007).

Acetogenic culture medium (ACT): (a) basal medium, composed of NH_4Cl 1 g.L^{-1} ; MgCl_2 0.1 g.L^{-1} ; KH_2PO_4 0.4 g.L^{-1} ; cysteine hydrochloride 0.5 g.L^{-1} ; Na_2SO_4 0.5 g.L^{-1} ; NaHCO_3 7 g.L^{-1} ; CaCO_3 10 g.L^{-1} ; yeast extract 2 g.L^{-1} ; (b) vitamin solution (5 mL): biotin 2 mg.L^{-1} ; folic acid 2 mg.L^{-1} ; pyridoxine hydrochloride 10 mg.L^{-1} ; riboflavina 5 mg.L^{-1} ; thiamine 5 mL; nicotinic acid 5 mg.L^{-1} ; pantothenic acid 5 mg.L^{-1} ; B12 vitamin 0.1 mg.L^{-1} ; p-aminobenzoic acid 5 mg.L^{-1} ; thioethic acid 5 mg.L^{-1} ; pH 6.7; resazurin 0.5 g.L^{-1} 2 mL (Agustini 2014; Manimegalai et al. 2014).

Methanogenic culture medium (MET): (a) K_2HPO_4 0.023 g.L^{-1} ; KH_2PO_4 0.023 g.L^{-1} ; $(\text{NH}_4)_2\text{SO}_4$ 0.023 g.L^{-1} ; NaCl 0.046 g.L^{-1} ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.009 g.L^{-1} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.006 g.L^{-1} ; yeast extract 0.2 g.L^{-1} ; Na_2CO_3 0.4 g.L^{-1} ; cysteine hydrochloride 0.025 g.L^{-1} ; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ 0.025 g.L^{-1} . (b) vitamin solution (5 mL): biotin 2 mg.L^{-1} ; folic acid 2 mg.L^{-1} ; pyridoxine hydrochloride 10 mg.L^{-1} ; riboflavin 5 mg.L^{-1} ; thiamine 5 mg.L^{-1} ; nicotinic acid 5 mg.L^{-1} ; pantothenic acid 5 mg.L^{-1} ; B12 vitamin 0.1 mg.L^{-1} ; p-aminobenzoic acid 5 mg.L^{-1} ; thioethic acid 5 mg.L^{-1} ; pH 7.2. (c) enriched medium: $\text{C}_7\text{H}_5\text{NaO}_2$ 0.2 g.L^{-1} ; NH_4Cl 0.075 g.L^{-1} ; K_2HPO_4 0.04 g.L^{-1} ; MgCl_2 0.01 g.L^{-1} ; Na_2CO_3 0.15 g.L^{-1} ; pH 7.2; resazurin 0.5 g.L^{-1} 2 mL (Agustini 2014; Manimegalai et al. 2014, modified).

For the ACD, ACT and MET culture media, 10% of the substrate (starter) was added. The three media were distributed in 10 mL penicillin vials in the presence of flow of nitrogen and the vials were sealed and sterilized. The sample (500 μL) was added to the vials with the aid of a 1 mL syringe and incubated at 37 °C for 30 days (Ferry et al. 1974; Manimegalai et al. 2014;). After incubation, 50 μL aliquots were added to the solid culture media (ACD, ACT and MET—added 20 g of agar per liter), placed in an anaerobic jar with CO_2 atmosphere, incubated at 37 °C for 15 days. The isolated strains were purified and preserved at – 80 °C in 20% glycerol. The inoculum temperature on the day of collection was determined and was around 37.7 °C.

Morphological and biochemical characterization

Morphological analysis of the microbial isolates was performed by sowing each strain on plates containing the same culture media HM, ACD, ACT and MET. The characteristics of cell structures were analyzed using the Gram stain technique. Biochemical assays were performed with the isolates using the following media: *i*) CLED (BD) culture medium containing casein peptone 4.0 g.L^{-1} ; gelatin peptone 4.0 g.L^{-1} ; meat extract 3.0 g.L^{-1} ; lactose 10.0 g.L^{-1} ; L-cystine 0.128 g.L^{-1} ; agar 15.0 g.L^{-1} and bromothymol blue 0.02 g.L^{-1} ; *ii*) MacConkey (BD) containing peptide casein 1.5 g.L^{-1} ;

meat peptone 1.5 g.L^{-1} ; gelatin peptone 17.0 g.L^{-1} ; bile salts (mixture) 1.5 g.L^{-1} ; lactose 10.0 g.L^{-1} ; sodium chloride 5.0 g.L^{-1} ; neutral red 0.03 g.L^{-1} ; crystal violet 0.001 g.L^{-1} ; agar 13.5 g.L^{-1} . Bacteria from ACT and MET media were cultivated in the respective media, without the addition of other substances, under anaerobic conditions.

Physicochemical analysis

The physicochemical analyses were performed by evaluating the following parameters: total solids, fixed solids, volatile solids, volatile organic acids (VOA), total inorganic carbon (TIC), temperature and pH. All these parameters were determined in the CIBiogás laboratory (APHA 2017).

Molecular analysis

DNA extraction

For DNA extraction, performed based on the protocol of Aamir et al. (2015), 10 morphologically distinct isolates were selected, 1 from each different culture medium and 3 from the methanogenic medium (MET). Cells were extracted with 900 μL of phenol in a tube containing a small amount of glass beads followed by incubation at 65 °C for 20 min. Samples were centrifuged at 16,000 $\times g$ for 10 min at 4 °C. The supernatants were added with 800 μL of phenol, briefly homogenized and centrifuged at 16,000 $\times g$ for 5 min at 4 °C. Phenol, in a 1:1 ratio, was added to the supernatant, followed by brief homogenization and centrifugation at 16,000 $\times g$ for 5 min at 4 °C. A volume of 600 μL of isopropanol was added to the supernatants, followed by homogenization and incubation at – 20 °C for 20 min. Samples were centrifuged at 16,000 $\times g$ for 10 min at 4 °C and supernatants were discarded. A volume of 100 μL of 70% ethanol was added to the pellets and, after 1 min, the ethanol was discarded. Pellets were dried at room temperature and then suspended in 50 μL of sterile MilliQ water. The extracted DNA was quantified in 0.8% agarose gel and visualized in a photo documenter.

PCR and purification

The DNAs of the isolates were subjected to PCR for amplification of the 16S rRNA gene. Reactions were performed with Buffer Solution 1 X, MgCl_2 solution 1.5 mM, primer pair 0.5 μM , dNTP's 0.2 mM, Taq DNA polymerase 2.0 U and genomic DNA 2–25 ng, for a total volume of 25 μL . PCR conditions were: an initial cycle of 5 min at 95 °C; 40 sequential amplification cycles of 30 s of 95 °C, 30 s of 63 °C and 60 s of 72 °C; plus 1 final cycle of 10 min at 72 °C. Amplicons were purified using the GFX Gel Band Purification Kit column kit and were visualized in 0.8% agarose gel (Aamir et al. 2015). The set of primers used was

16S 10f (AGTTTGATCCTGGCTC) e 1100r (GGGTTG CGCTCGTTG) (Belgini et al. 2014).

Sequencing and phylogenetic analysis

Amplified products purified were sequenced using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems™) for ABI 3500 Genetic Analyzer (Applied Biosystems™), according to the manufacturer's guideline. Partial gene sequences obtained from isolates were assembled in a consensus sequence using the BioEdit program and further compared to sequences obtained from reference and type strains in the public database GenBank (www.ncbi.nlm.nih.gov). Sequence alignment was performed using the BioEdit program and analyzed with MEGA X software by using the Kimura Evolutionary distances substitution model (Kimura 1980). Phylogenetic reconstruction was performed using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987) with bootstrap values calculated from 1000 replicate runs.

Sequence accession numbers of the 16S rDNA strains

The sequences obtained from the isolates sequencing were deposited in GenBank under the following accession codes: D2 (OK570084), AM1 (OK570085), RB1 (OK570086), C3 (OK570087), OL2 (OK570088), D1 (OK570089), L3 (OK570090), D3 (OK570091), CMC1 (OK570092) and NA1 (OK570093). D1-D3: methanogenic medium.

Metagenomic DNA extraction and 16S ribosomal gene sequencing.

Metabarcoding analyses were performed by an outsourced company, MicrobiomeX. DNA extraction was performed using the DNeasy Powersoil Pro kit (Qiagen), following the manufacturer's recommendations. The integrity and purity of the extracted DNA were verified in 0.8% agarose gel electrophoresis and in a NanoDrop1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA), respectively. The hypervariable V4 region of the 16S gene was amplified using primers 515F (5' GTGYCAGCMGCCGCG GTAA) and 806R (5' GGACTACNVGGGTWTCTAAT) (Caporaso et al. 2010) and submitted to large-scale sequencing using the Illumina MiSeq platform (2 × 250 bp).

Bioinformatic analysis

The quality of raw sequences was checked using the FASTQ program version 0.11.5 (Andrews 2010). Primer sequences were removed by the Cutadapt tool (Martin 2011). Microbiome analyses were performed using the DADA2 tool version 1.18.0 (Callahan et al. 2016), comprising: removal of low-quality reads (phread < 20) and noise (denoising), joining

of R1 sequences (forward) and R2 (reverse) removal of chimeras (using the consensus method) and assembly of representative sequences based on amplicon sequence variants (ASVs). Subsequently, the taxonomic classification was assigned using the SILVA ribosomal RNA gene database version 138 (Quast et al. 2013).

Statistical analysis

Statistical analyses were performed in the R statistical environment (v. 3.6.1) (R Development Core Team, 2014). The taxonomic table containing the count was imported along with the "metadata" file for analysis in the R Phyloseq package (McMurdie and Holmes 2013). Sequencing coverage was assessed by rarefaction analysis. Alpha diversity indices based on the Chao1 richness estimator (Chao 1984), the observed species and the Shannon–Wiener H' index were calculated by the R Phyloseq package. The microbial composition was expressed in relative abundance for all taxonomic levels.

Accession numbers

The metabarcoding raw sequence data are deposited in the European Nucleotide Archive under accession numbers: Sample ERS7624265 (SAMEA9945945) Inoculum.

Results and discussion

Isolation and phylogenetic analysis

The results of the present study showed the existence of a multiple bacterial community in the studied sample. A total of 30 bacteria were isolated from all culture media used (Table 1) except for the guaiacol-containing (which did not show bacterial growth). Among the culture media to isolate hydrolytic bacteria, the NA medium had the highest number of bacterial colonies ($n=10$), followed by the LE medium ($n=5$). Regarding the culture medium for isolation of anaerobic bacteria, the acidogenic medium (ACD) did not recover any bacteria, while the acetogenic (ACT) and methanogenic (MET) recovered 2 and 3 isolates, respectively. Biochemical analyses in CLED culture medium and the use of the 4 distinct groups of culture media, simulating the 4 phases of anaerobic digestion (hydrolytic, acidogenic, acetogenic and methanogenic), suggested the presence of 16 distinct ribotypes from the 30 isolates recovered from the starter, with the vast majority being Gram-positive bacteria (Table 1).

Ten of the 30 isolates recovered were sequenced. From the hydrolytic culture media, the following genera and/or species were identified: *Lisinibacillus capsici*., *Luteimonas*

Table 1 Microbial groups recovered from the inoculum (starter) derived from the anaerobic digestion process

Bacteria recovered from hydrolytic medium									
Code	Substrate	N° colonies	Enzyme	Lactose Ferm	Morphotype (n = n° iso- lates)	Morfology	Gram	16S rDNA ID	Culture Medium CLED
LE3	Milk	5	protease	–	1 (n=5)	Rod	+	<i>Lysinibacillus</i> sp.	Bright and transparent colony /blue medium
NA1	NA	10	–		2 (n=4)		–	<i>Luteimonas</i> sp.	Bright yellow colony/ blue medium
NA2					3 (n=3)		+	N.A	Opaque White colony/ blue medium
NA3					4 (n=3)		–	N.A	Bright greenish colony/ blue medium
RBBR1	RBBR	1	ligninase		5 (n=1)			<i>Priestia megaterium</i>	Opaque yellow colony/ blue medium
AM1	Starch	4	amylase	+	6 (n=2)		+	<i>Bacillus</i> sp.	Dark cream colony with exsudate/yellow medium
AM4					7 (n=2)			N.A	Opaque yellow colony/ yellow medium
CMC1	CMC	3	cellulase	–	8 (n=1)	Cocos	+	<i>Rhodococcus</i> sp.	Opaque light brown colony/blue medium
CMC2					9 (n=2)			N.A	Bright white colony/ blue medium
OL1	Olive oil	2	lipase	+	10 (n=1)	Rod		N.A	Opaque yellow colony/ yellow medium
OL2				–	11 (n=1)			<i>Niallia circulans</i>	Greenish colony/blue colony
Bacteria recovered from acetogenic medium									
ACT2	ACT	2	N.A	N.A	12 (n=1)	Cocos	+	N.A	ACT Cream colony
ACT3					13 (n=1)	Rod		<i>Robertmurraya siralis</i>	Cream colony
Bacteria recovered from methanogenic medium									
MET1	MET	3	N.A	N.A	14 (n=1)	Rod	+	<i>Vagococcus acidifermentans</i>	MET Cream colony
MET2					15 (n=1)			<i>Bacillus</i> sp.	Yellowish colony
MET3					16 (n=1)			<i>Proteinclasticum</i> sp	Cottony colony

N.A not analyzed

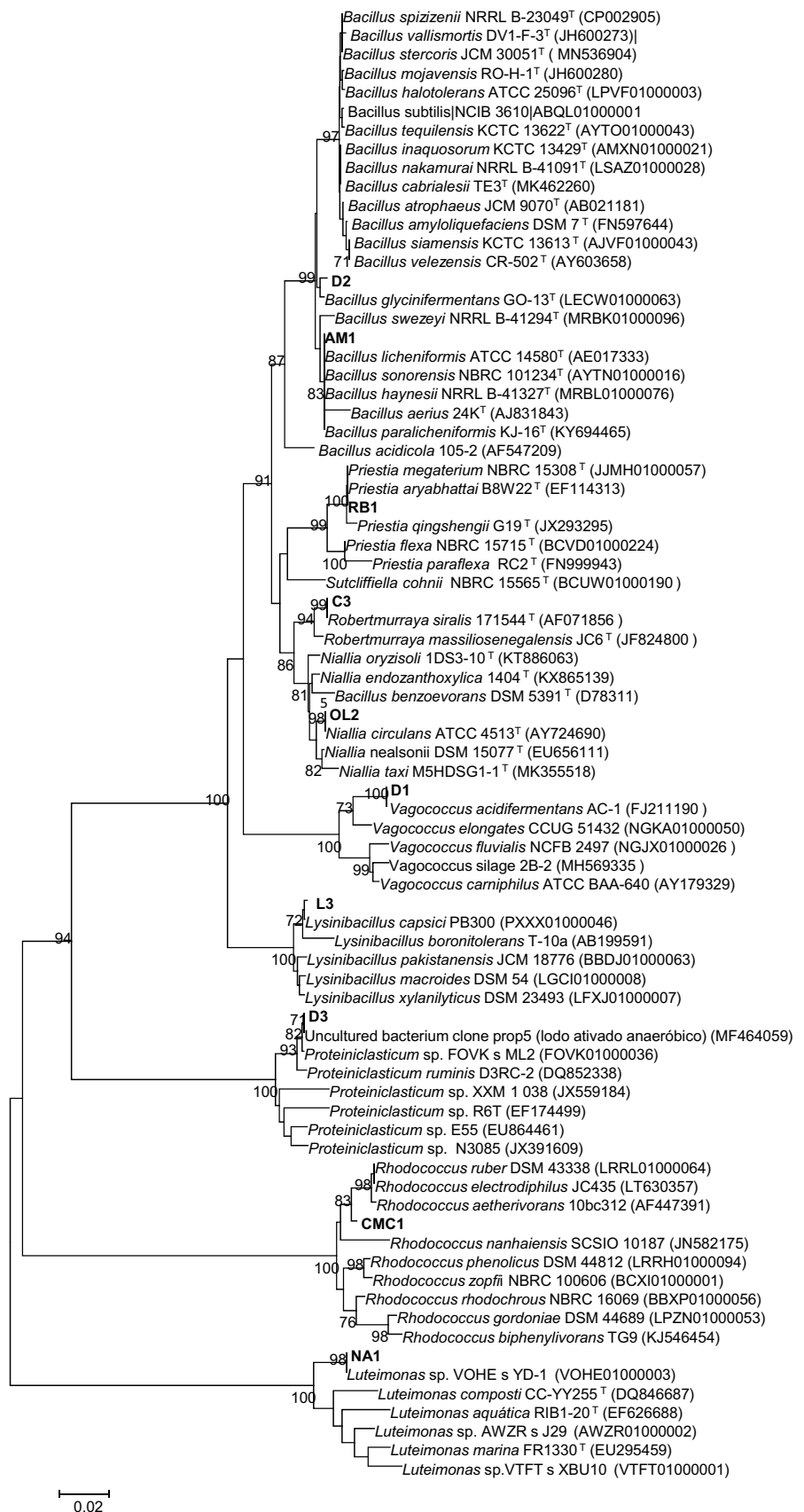
sp., *Priestia* sp., *Bacillus* sp., *Rhodococcus* sp., *Niallia circulans*, for the mediums LE, NA, RBBR, AM, CMC and OL, respectively. Regarding the culture medium presenting acetogenic conditions (ACT), a *Robertmurraya siralis* strain was identified, while the culture medium showing methanogenic conditions (MET) recovered the strains *Vagococcus acidifermentans*, *Bacillus* sp., and *Proteinclasticum* sp. (Table 1, Fig. 1).

The taxonomic groups identified in this study have already been reported in the literature, with strains involved in hydrolysis processes of compounds present in the metabolism and production of biogas. The genus *Lysinibacillus* have already been identified in a study of the characterization of the methanogenic microbial community in brewery wastewater samples (Murunga et al. 2016) as well as in samples of digestate associated with digestion processes using

animal manure and food waste (Sun et al. 2020). The specie *Lysinibacillus sphaericus* has been reported as a strain capable of breaking down the complex structure of lignin (Persinoti et al. 2018; Rashid et al. 2017). *Luteimonas* species have been reported with the activity of Esterase (C4), β -Galactosidase, α and β -Glucosidase as well as strains were present in samples of biogas waste and organic manure (Pu et al. 2018; Roh et al. 2008).

Several *Bacillus* species including *B. megaterium* (currently known as *Priestia megaterium*), *B. licheniformis*, *B. pumilus*, *B. brovis* and *B. alvei*, have already been recovered from samples obtained of anaerobic digestion processes for biogas production (Biedendieck et al. 2021; Rabah et al. 2010). *Bacillus* genus is represented by mandatory or facultative aerobic species, and the species *B. halodurans* has been reported as a carbohydrate fermenter in

Fig. 1 Phylogenetic analysis based on partial bacterial 16S rRNA sequences of isolates from starter sample. Bootstrap values (1000 replicate runs, shown as %) greater than 70% are listed. GenBank accession numbers are listed after species names



high-temperature environments in an anaerobic biodigestion process in acidic phases (Ali Shah et al. 2014).

Yoon (2021), suggested a new potential species of the genus *Bacillus* (or proposed new genus *Niallia*), with the new species *Niallia circulans*, and no reports were found of the association of this new species with processes of anaerobic digestion and biogas production. Likewise, according to Gupta et al. (2020), representatives of the new genera *Niallia* gen. nov., *Priestia* gen. nov., *Robertmurraya* gen. nov., were reclassified from several *Bacillus* species after strong phylogenetic and molecular evidence using multiple phylogenetic trees on a genomic scale. *Proteiniclasticum* sp. and *Clostridium* sp. were observed in a study involving the use of peat soil, digested sludge, and ruminal fluid for simultaneous consumption of carbon dioxide and production of acetic acid in a biogas production process (Chaikitkaew et al. 2021).

The species *Rhodococcus opacus* PD630 has catabolic pathways and tolerance mechanisms for aromatic compounds present in ligninocellulosic material, including hexoses and pentoses, and can be considered a good candidate for hydrolysis of the material found in the starter (Anthony et al. 2019). Representatives of the genus *Vagococcus* were identified in a study addressing the genomic analysis of 16S rRNA in anaerobic digestion processes and were correlated with ammonia inhibition (Poirier et al. 2020). In addition, the first-time reported species *Vagococcus acidifermentans* was isolated from an acidogenic fermentation bioreactor in

Naju province, South Korea, with the ability to ferment different sugars (Wang et al. 2011).

Metabarcoding analysis

The initial metabarcoding analyses of the sample resulted in a total of 20,652 reads and, after quality processing (filtering, denoising, reads merging, and chimera removal), 16,377 final sequences were obtained. The rarefaction curve of the observed ASVs richness showed that it reached saturation, indicating that the sampling was efficient and capable of revealing almost all the prokaryote microbial species of the samples (Fig. 2). Shannon and Simpson's index values 97 and 0.96, respectively, indicated great diversity in the samples.

Metabarcoding results revealed great prokaryotic diversity and showed the presence of representatives of 16 different phyla, 14 from Bacteria and 2 from Archaea domain (Fig. 3). The most abundant phylum was Firmicutes (42.60%), followed by Bacteroidota (32.41%), Chloroflexi (11.38%) and Synergistota (4.07%). The archaea domain was represented by Halobacterota (77.71%) and Euryarchaeota (22.29%). The low archaeal diversity observed may be a result of the inability of prokaryotic primers to amplify archaea, as they are specific for the bacterial rRNA genes.

The main phyla found in our work have already been reported in other studies that evaluated the diversity in

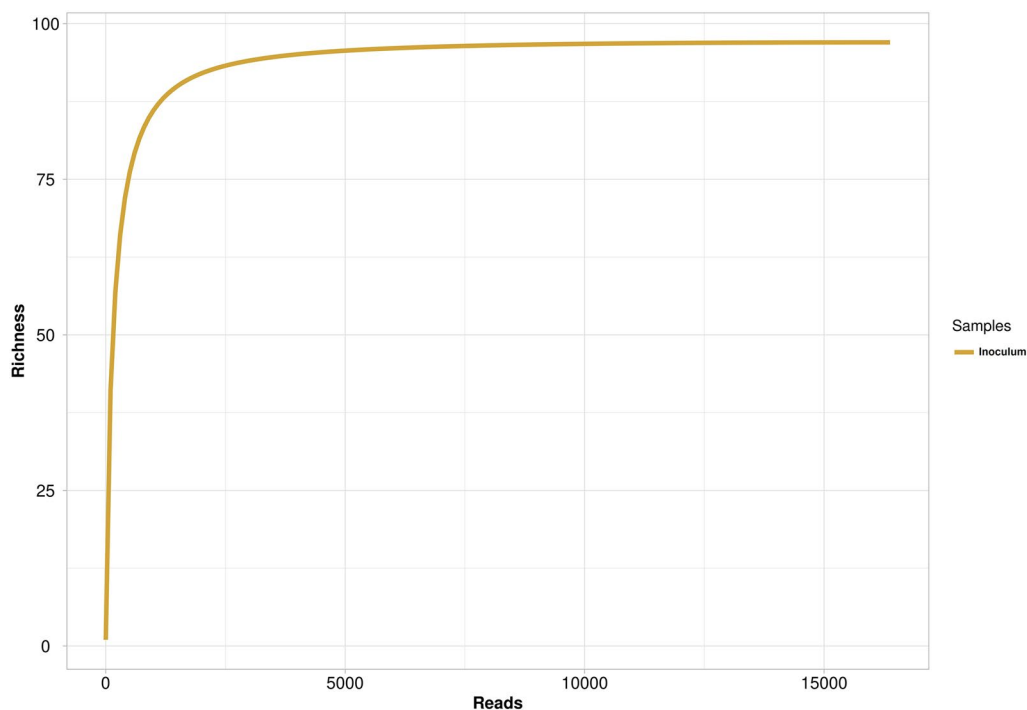


Fig. 2 Rarefaction curve of the prokaryote 16S rRNA gene sequences obtained from inoculum (starter) from CIBiogás anaerobic processes using the metabarcoding method. ASVs calculated at 99% identity

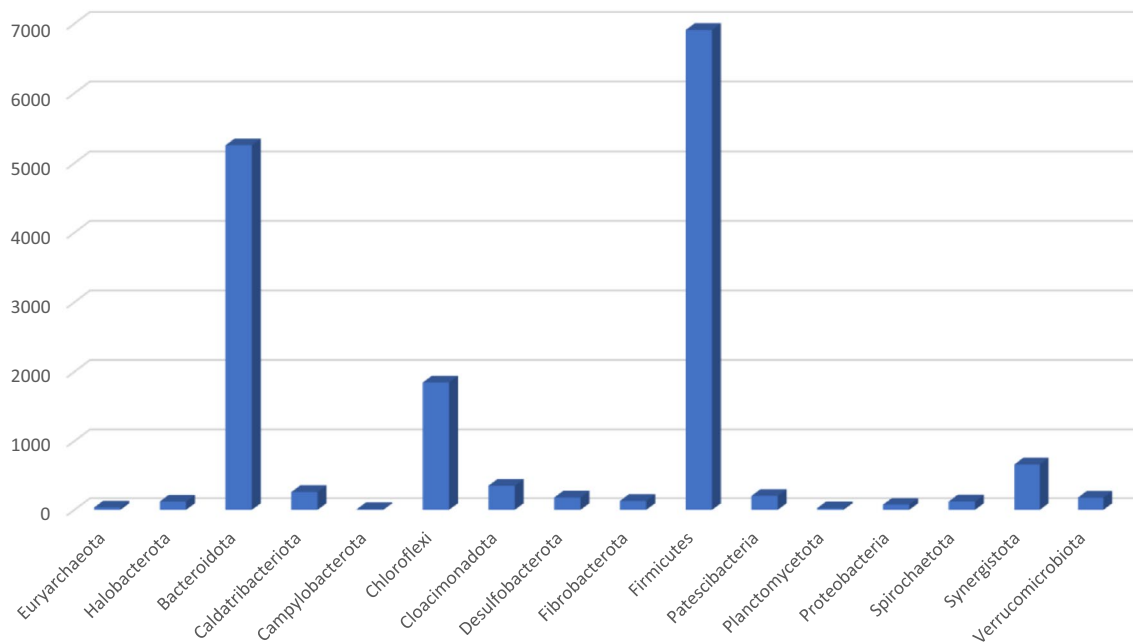


Fig. 3 Number of bacterial and archaeal phyla obtained through metabarcoding method from inoculum (starter) from CIBiogás anaerobic processes

anaerobic digestion processes. In the work carried out by Nordgård et al. (2017), the authors observed that the Firmicutes phylum was more abundant in swine manure samples. Brandt et al. (2020), observed a greater abundance of representatives of the phyla Bacteroidota and Firmicutes in complex microbial communities associated with anaerobic digestion processes in different biogas and wastewater treatment plants. Representatives of the phylum Chloroflexi were found in large-scale anaerobic digesters with excess sludge capture from wastewater treatment plants (Petriglieri et al. 2018), while in processes used to understand the regulatory role of H_2 in methane production in anaerobic digestion processes the presence of members of the phylum Synergistota was described (Kakuk et al. 2021). Representatives of methanogenic archaea are well known in biogas production processes. The archaeal phyla found in our study, Halobacterota and Euryarchaeota, have also been reported in other works involving anaerobic digestion and biogas production (Heitkamp et al. 2021; Zhang et al. 2020).

Firmicutes phylum has been well described in the literature as an important taxonomic group associated with anaerobic digestion processes as its representatives can express metabolic pathways involved in this process, such as at acetogenic phase, in the degradation of cellulosic compounds, with the formation of volatile acetic acid, CO_2 and hydrogen (Mukhuba et al. 2020; Nordgård et al. 2017; Zhou et al. 2017). The most abundant genera belonging to the Firmicutes phylum observed in the sample were *Enterococcus* (17.27%), HN-HF0106 (15.32%), *Clostridium sensu stricto*

1 (15.28%), *Syntrophomonas* (4.4%) and a large abundance of unaffiliated bacteria NA (23.67%) (Fig. 4).

Watcharasukarn et al. (2009), performed a study where the ability to reduce pathogens from biogas plants was evaluated. In this study, *Enterococcus* species were used as biological indicators in treatments where the temperature exceeds 55 °C. The authors concluded that *Enterococcus* spp. can be resistant to different types of waste treatments, serving as biological indicators in biogas plants. Regarding the genus HN-HF0106, members of this group have been associated with cellulolytic activity, being able to use cellulose as a substrate with the production of H_2 and acetate (Xie et al. 2021). In a work by Hahnke et al. (2014), carried out from a biogas production reactor fed with corn silage and wheat straw, found a new anaerobic hydrogen-producing mesophilic bacterium affiliated to the genus *Clostridium sensu stricto* (cluster I of the clostridia). This strain, cultivated in the presence of glucose, was able to produce H_2 , CO_2 , formate, lactate, and propionate, which are intermediate compounds to produce methane. In another work, the structures of the microbial community in biogas digesters with different types of waste, including cow, pig, sheep manure and human feces, were evaluated. *Clostridium sensu stricto* 1 represented the highest abundance in the digester with mixed raw materials including dairy cattle manure, sheep manure, and human feces (Han et al. 2021).

Wongfaed et al. (2020) evaluated the effect of the presence of oil and its derivatives (long-chain fatty acids) in palm oil factory effluent destined for methane production,

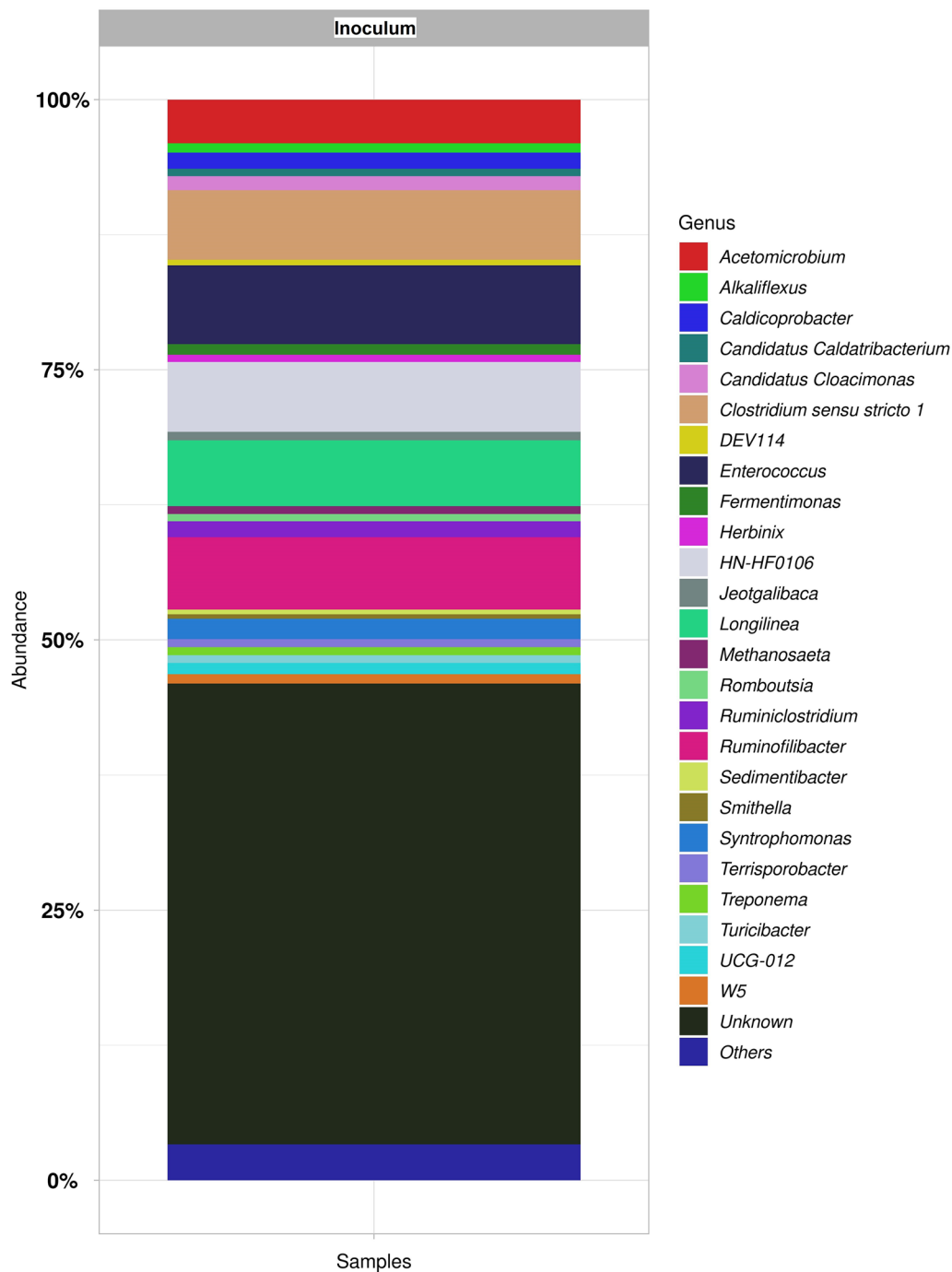


Fig. 4 Genera distribution associate in the starter by metabarcoding analyses

as well as the structure of the microbial community. The authors observed cooperation between fatty acid degrading bacteria including *Syntrophomonas* sp. (strain capable of using long-chain fatty acids with more than 12 carbon atoms) and *Acinetobacter* sp., with H_2 consuming methanogenic bacteria, including *Methanococcus* sp. and *Methanogenium* sp. The authors point out that the occurrence of this association in the normal AD process plays an important

role in the degradation of oil and derivatives present in palm oil mill effluent.

The most abundant genus belonging to the phylum Bacteroidota was *Ruminofilibacter* (20.87%), while the main Chloroflexi genus was *Longilinea* (54%) and, for the phylum Synergistota, the most abundant group was *Acetomicrobium* (100%). There are no reports in the literature on the association between these genera in the production of

biogas. In a work developed by Dong et al. (2019), genes from representatives of the genus *Ruminofilibacter* (related to cellulose degradation) were found in large quantities in the digestate after anaerobic digestion of cattle manure for biogas production. Yıldırım et al. (2017) evaluated the effects of bioaugmentation using anaerobic ruminal fungi on biogas production in anaerobic digesters fed with animal manure. In the study, the genera *Clostridium* and *Longilinea* were some of the most abundant observed in digesters, and the genus *Clostridium* has been reported to be important in the production of butanol, butyric acid, acetone and isopropanol, intermediate compounds in this bioprocess. The authors also reported that these two genera were the ones with the greatest capacity to degrade animal waste, which provided higher methane yields.

Zhao et al. (2013) evaluated the dynamics of the microbial community in composting systems using biogas slurry compost and cow manure compost for biogas production. The authors adopted the denaturing gradient gel electrophoresis (DGGE) and gene clone library approaches, finding sequences associated with the *Acetomicrobium* genus after sequencing the clones. Representatives of the *Acetomicrobium* genus were reported as dominant in a dark fermentation process of fats and protein, using proteins as substrate (Litti et al. 2020). However, it is important to highlight that a large quantity of bacteria was not affiliated to any taxonomic group (NA = 42.16%), showing that a lot of information remains unknown and reinforcing the need for further studies to characterize the taxonomic groups associated with the starter studied here.

Regarding the archaeal sequences, representatives were found for the genera *Methanosaeta* and *Methanobacterium*, respectively, of the phyla of the Halobacterota and Euryarchaeota phyla in the starter, which have already been related to other processes of anaerobic digestion and biogas production. Representatives of the *Methanosaeta* genus maintained their dominance over other methanogenic groups in a study where acetoclastic methanogen groups able to act at low pH were acclimated to replace the use of NaOH to regulate buffer pH, a procedure that can inhibit methanogenic microorganisms (Ali et al. 2019). The acetoclastic methanogenic genus *Methanosaeta* has also been observed in other studies to improve biogas production (Zamorano et al. 2020; Chen et al. 2017). Concerning the genus *Methanobacterium*, representatives of this group were reported in a study that evaluated the production of biogas containing hydrogen and methane using Microbial Electrolysis Cell (He et al. 2021). In this work, the authors observed that through hydrogenotrophic methanogenesis, the group could synthesize CH₄ using H₂ and CO₂.

The diversity of the microbial community found in anaerobic digestion processes is very diverse, and a large group of bacteria can be found in the organic substrates used in the

system. From the beginning of the process, with the anaerobic degradation of organic substances, to the formation of biogas, there is the participation of a diverse microbial consortium, which includes fermentative bacteria, hydrogen-producing acetogenic bacteria, hydrogen-consuming acetogenic bacteria, carbon dioxide-reducing methanogens and acetoclastic methanogenic archaea (Lohani and Havukainen 2018).

The hydrolytic metabolism performed by enzymes such as amylases, lipases, ligninases, cellulases and proteases breaks down organic matter into simpler compounds, including sugars, amino acids, fatty acids, and peptides. This hydrolysis is generally carried out by the metabolic activity of anaerobic bacteria associated with the genera *Streptococcus* and enterobacteria (Kunz et al. 2019; Ali Shah et al. 2014), and these groups were found in our work, *Enterococcus* representing the most abundant enterobacteria, and *Streptococcus* in lesser abundance (0.42% of Firmicutes).

Metabolites formed by enzymatic hydrolysis are converted to other compounds in the acidogenic step. Glucose can be converted into lactic acid by *Lactobacillus*, and fatty acids can be degraded by *Acetobacter* species via β -oxidation, forming acetate. Likewise, amino acids are degraded by *Clostridium* species to form acetate, ammonia, carbon dioxide and hydrogen sulfide (Kunz et al. 2019). In our study, we found *Clostridium*, but it was not possible to observe *Lactobacillus* and *Acetobacter*. However, a relative abundance of *Acetomicrobium* was found, which can ferment glucose to acetate, CO₂ and H₂ (Hania et al. 2016), as well as the genus gene HN-HF0106 (Xie et al. 2021).

During the acidogenic step, further short-chain organic acids can be formed including formic, acetic, propionic, butyric and pentanoic acids, as well as alcohols (methanol, ethanol), aldehydes, carbon dioxide and hydrogen (Ali Shah et al. 2014). In our work, it was possible to isolate 3 distinct lactose fermenting morphotypes, two isolates recovered from the culture medium enriched with starch (01 *Bacillus* sp.) and one isolated from the culture medium enriched with olive oil, which proves that they are bacteria capable of fermenting simpler sugars and lipids via enzymatic hydrolysis. According to Westerholm and Schnürer (2019), the degradation of proteins and amino acids in anaerobic digesters has been shown to be carried out by several genera within the Firmicutes phylum (predominant in our work), which include Gram-positive bacilli.

In the methanogenesis stage (strictly anaerobic), the carbon contained in the biomass is converted into carbon dioxide and methane by methanogenic archaea. Acetoclastic methanogenic archaea, such as the genus *Methanosarcina*, convert acetate to methane, and the hydrogenotrophic methanogenic archaea, such as the genus *Methanobacterium* and *Methanospirillum*, convert hydrogen and carbon dioxide to methane (Kunz et al. 2019). Our findings

corroborate those reported by Kunz et al. (2019) in view of the methanogenic representatives, including *Methanobacterium* in the inoculum sample studied in the present work.

The analysis of parameters found for volatile solids, volatile organic acids (FOS) and total inorganic carbon (TAC) show the rich nutritional composition of the evaluated substrate (carbon sources) for the development of the microbial community studied (Cerqueira et al. 2011). The concentrations of volatile solids, FOS, and TAC, found in the inoculum were 659.10 g kg⁻¹, 717.70 g kg⁻¹, 70,005.0 g kg⁻¹, respectively, which correspond to a large amount of material, including volatile organic acids (acetic, propionic, and butyric acids) and inorganic carbon (Cerqueira et al. 2011). pH can influence microbial growth inside the biodigester. On the day of inoculum collection, the pH was 7.6, which may favor the growth of methanogenic archaea, whose optimal pH for development is 6.7 to 7.5. However, fermentative bacteria can adapt to pH variations between 4.0 and 8.5 (Ali Shah et al. 2014).

Thus, we can say that the methodology adopted in this study was able to recover hydrolytic bacteria, such as proteolytic, ligninolytic, amylolytic and cellulolytic bacteria, capable of hydrolyzing protein, lignin, starch, and cellulose that may be present in the inoculum composition, as well as bacteria of the acetogenic phase. However, it was not possible to isolate methanogenic archaea using the media defined for this purpose. This limitation was overcome by using the combination of culture-dependent (enrichment and isolation) and culture-independent (meta-barcoding) methods, which allowed access to a greater amount of information about the microbial diversity associated with the anaerobic digestion process (starter). The methods were complementary, as with culture-dependent methods it was possible to isolate representative strains of AD, which were not observed in the culture-independent method and vice versa. Thus, we can conclude that the adoption of both approaches to characterize the microbial community in samples of AD processes is integrative and provides information of great relevance for understanding the microbial function and dynamics in the different stages of biogas production.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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