



Seasonal characterization of the prokaryotic microbiota of full-scale anaerobic UASB reactors treating domestic sewage in southern Brazil

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Abstract

Upflow Anaerobic Sludge Blanket (UASB) reactors are alternatives in the anaerobic treatment of sanitary sewage in different parts of the world; however, in temperate environments, they are subject to strong seasonal influence. Understanding the dynamics of the microbial community in these systems is essential to propose operational alternatives, improve projects and increase the quality of treated effluents. In this study, for one year, high-performance sequencing, associated with bioinformatics tools for taxonomic annotation and functional prediction was used to characterize the microbial community present in the sludge of biodigesters on full-scale, treating domestic sewage at ambient temperature. Among the most representative phyla stood out Desulfobacterota (20.21–28.64%), Proteobacteria (7.48–24.90%), Bacteroidota (10.05–18.37%), Caldiseericota (9.49–17.20%), and Halobacterota (3.23–6.55%). By performing a Canonical Correspondence Analysis (CCA), *Methanolinea* was correlated to the efficiency in removing Chemical Oxygen Demand (COD), Bacteroidetes_VadinHA17 to the production of volatile fatty acids (VFAs), and CI75cm.2.12 at temperature. On the other hand, *Desulfovibrio*, Spirochaetaceae_uncultured, *Methanosaeta*, Lentimicrobiaceae_unclassified, and ADurb.Bin063-1 were relevant in shaping the microbial community in a co-occurrence network. Diversity analyses showed greater richness and evenness for the colder seasons, possibly, due to the lesser influence of dominant taxa. Among the principal metabolic functions associated with the community, the metabolism of proteins and amino acids stood out (7.74–8.00%), and the genes related to the synthesis of VFAs presented higher relative abundance for the autumn and winter. Despite the differences in diversity and taxonomic composition, no significant changes were observed in the efficiency of the biodigesters.

Keywords Anaerobic digestion · Specific methanogenic activity · Microbial community · Co-occurrence network · picrust2

Introduction

The rational use of water resources and the proper treatment of wastewater originating from human activities have been widely stimulated in recent years, due to the scarcity of water in several parts of the world [1]. However, despite efforts, large volumes of untreated sewage still end up reaching water bodies—recent estimates suggest that only 56% of domestic sewage in the world is properly treated—causing damage to the delicate balance of aquatic ecosystems and socioeconomic impacts on the affect population [2]. In this context, the purification of sewage in Wastewater Treatment

Plants (WWTPs) is extremely important and several technologies are used to remove these waters from their polluting potential, including anaerobic digestion units [3].

The use of anaerobic digestion (AD) in the treatment of solid and liquid waste has been widely applied in several parts of the world for over a century [4, 5]. The word digestion is used in the context of stabilizing organic matter through the action of microorganisms in conditions that favor their growth and reproduction [6]. AD under ideal conditions is processed in 4 main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, where a wide diversity of species acts on the degradation of organic matter, generating methane gas (CH₄) and carbon dioxide (CO₂) as a final product [7]. These microbiological processes commonly occur in specialized units known as biodigesters or reactors. Among the range of biodigesters used, the UASB reactors stand out for their broad spectrum of use, showing a good capacity to remove Biochemical Oxygen Demand

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(BOD), COD, and Total Suspended Solids (TSS), presenting low sludge production and resistance to organic overload, requiring low operating and maintenance costs [8, 9]. Especially in tropical climates, they operate under ideal temperature conditions, requiring no additional heating spending, and are viable alternatives in developing countries [10, 11].

The UASB reactors are essentially characterized by the presence of a sludge blanket consisting of active biomass that forms granules, responsible for the degradation of the incoming organic matter, and a three-phase separator system, which enables solids retention, biogas collection, and consequent release of the liquid fraction of sewage, then properly treated [9]. UASB digesters enable the treatment of wastewater originating from several polluting sources, such as sugar, paper and cellulose, beverage, chemical, dairy, slaughterhouse, and sanitary sewage systems [12]. Sludge blanket reactors are widespread throughout the world. In Brazil, about 40% of WWTPs located only in the South, Southeast, and Midwest regions employ UASB reactor technology in the secondary treatment [10, 13, 14].

Used in the treatment of domestic sewage, UASB reactors are subject to a series of interferences that change the characteristics of the incoming wastewater, such as seasonal effects, socioeconomic level of the population served, infiltrations in the network, and type of sewage collection system (mixed or with separator) [15–17]. Such disturbances can mainly lead to changes in factors such as temperature, pH, flow, hydraulic retention time, and applied organic load, which can trigger imbalances in crucial stages of anaerobic digestion [18–20]. Under the influence of these variables, the microbiota present in these systems tends to suffer impacts, resulting in losses of efficiency and quality of the treated sewage [21].

Several studies have focused on the response of microbial communities to variations in the operating conditions of anaerobic reactors, notably using molecular biology and bioinformatics techniques [22–25]. However, these studies do not jointly address seasonal aspects, full-scale anaerobic reactors, systems with low influent organic load, and domestic sewage treatment. In this context, it is crucial to understand the dynamics of the microbiota subject to environmental contingencies in real-scale systems to provide subsidies for the elaboration of projects, monitoring, and more efficient operational control of these units in the process of removing associated pollutants.

From this perspective, the present study aimed to analyze and characterize the microbial community of UASB reactors for one year, using high-performance sequencing (NGS) and bioinformatics tools, to determine the taxonomic profile as well as to predict the main metabolic functions associated with the groups found, relating seasonal conditions of temperature, physical–chemical aspects and efficiency parameters evaluated in WWTPs.

Materials and methods

Study area

The collections of sludge, influent sewage, and effluent were carried out at three full-scale anaerobic reactors of the UASB-type (UASB-B, UASB-P, and UASB-T) while treating mixed sewage (rain and sewer) received by three different WWTPs (Fig. S1 and Table S1), located in the municipality of Caxias do Sul (51°10'06" W; 29°10'05" S), Brazil. The UASB-T reactor has a volume of 3990 m³, the others 2226 m³, and both were designed to operate continuously at ambient temperature and with a minimum hydraulic retention time (HRT) of 10 h. Considering that the reactors are inserted in the same context in the WWTPs where they are located and have similar characteristics regarding the sewage received, these were treated as replicates in the present study, noting that they were not subjected to significant changes in the operational process. The climate of the region is classified as Cfb (humid temperate) characterized by well-distributed rainfall and an average temperature of the hottest month below 22°C, considering the Köppen (1931) classification [26, 27].

Physicochemical analysis

Physicochemical analyses were carried out to characterize the influent and effluent sewage as well as the sludge used in the specific methanogenic activity (SMA) test during the period from October 2020 to October 2021. Measurements of Flow (Q), pH, COD, Total Kjeldahl Nitrogen (TKN), Ammoniacal Nitrogen (NH₃-N), and Total Phosphorus (PO₄-P) were carried out, referring to sewage and evaluation of Total Solids (TS), Fixed Solids (FS) and Volatile Solids (VS) related to the sludge collected from the biodigesters. The aforementioned analyses were performed according to the methods described in Standard Methods [28]. VFAs analyses were determined using a gas chromatograph (GC-Shimadzu – QP2010 Ultra) equipped with a DN–FFAP column (30 mm × 0.32 mm × 0.25), Flame Ionization Detector (FID), with Helium (He) gas as carrier, as well as synthetic air and nitrogen (N₂) as auxiliary gases [29]. The temperature assessment was performed daily and included measurements of the air, inlet, and outlet of the reactors. The SMA test was conducted according to the methodology proposed by Chernicharo [30]; however, the assembly of the apparatus to quantify the produced biogas followed the model suggested by Drogg et al. [31]. Data related to precipitation and other meteorological factors, referring to the study period, were acquired from the National Institute of Meteorology (INMET) [32].

Molecular analysis

Sampling, DNA extraction, and NGS sequencing

In all, 12 sludge collections were carried out for the months of January (summer), May (autumn), August (winter) and October (spring) of 2021 for analysis and characterization of the microbial community of the UASB reactors. The sludge was collected from valves installed on the side of the reactors at a height of 1.70 m and, when present, collected at the upper points of the blanket, eventually resulting in more than one sample per reactor and period, totaling 15 samples obtained. The samples were placed in sterilized vials and subsequently stored at 4 °C for one day before subsequent DNA extraction. DNA was extracted from the sampled sludge using the PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.) and later quantified with the Invitrogen Qubit Fluorimeter (Thermo Scientific), both processes being carried out according to the methodology proposed by the manufacturer.

The DNA samples resulting from the extraction process were submitted to the library preparation and sequencing steps. The preparation of libraries was carried out by PCR amplification of the V3/V4 gene regions of 16S rRNA, using primers 341F (CCTACGGGGRSGCAGCA G) [33] and 806R (GGACTACHVGGGTWTCTAAT) [34]. In addition to the target region, a partial Illumina adapter, based on the TruSeq structure, was attached to the amplicons. After, the sequencing of the libraries was conducted using paired-end runs of 500 cycles, using V2 × 500 sequencing kits (Illumina, USA) with 100,000 coverage reads. The readings generated from the raw data were deposited in the database of the National Center for Biotechnology Information (NCBI) using the Sequence Read Archive (SRA) platform under the code BioProject PRJNA821214.

Bioinformatics analysis of the generated data

The bioinformatics analyses were initially processed using the program FastQC v.0.11.9 [35] to evaluate the quality of the sequenced nucleotides. The import and processing of the obtained sequences were conducted through the Quantitative Insights Into Microbial Ecology pipeline (QIIME2 version 2021.2) [36]. Sequences from primers and adapters were removed using the q2-cutadapt plugin with subsequent removal of noise, chimeras, and other sequencing errors as well as the merge of forward and reverse reads—considering the overlapping regions—using the Divisive

Amplicon Denoising Algorithm (DADA2) [37] via the q2-dada2 plugin. Any singletons obtained in the process were discarded through the q2-feature-table plugin. The resulting Amplicon Sequence Variant (ASVs), fasta sequences, and the relative abundance values obtained were used to perform the taxonomic signature, functional prediction, construction of rarefaction curves, alpha, and beta diversity analyses.

The taxonomic annotation was processed using the feature-classifier plugin from the classify-sklearn method, using the SILVA database (v. 138) [38] obtained, processed, and trained for use in the QIIME2 program—considering the Naïve Bayes method—using the RESCRIPt plugin [39]. The taxonomic classification for the phylum category was based on the nomenclature proposed by Whitman et al. [40].

Analysis of alpha, beta diversity, rarefaction curves as well as PERMANOVA and Principal Coordinates (PCoA) were designed using the q2-phylogeny and q2-diversity plugins. For that, the Shannon–Wiener and Pielou Evenness indices were applied for alpha diversity, and Weighted Unifrac considered beta diversity [41–44].

Functional inferences were made using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software [45], in the predefined configurations (default), using the ASVs generated in the previous step for sequence alignment (HMMER) and insertion into a phylogenetic tree of reference [46, 47]. The generated data were normalized by the 16S rRNA copy number, while the prediction of genes, enzymes, and metabolic functions was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) [48], Enzyme Commission Number (EC number), and MetaCyc [49]. The identified gene families were classified, considering the second and third functional level of classification (KEGG BRITE) and their respective relative abundance values obtained through the online software BURRITO [50].

Statistical analysis

The taxonomic data generated were submitted to differential abundance analysis using the R DESeq2 package [51], based on the extreme seasons (winter and summer). The resulting matrices were used to generate a Volcano-type plot, built using the ggplot2 package [52]. The representative identified taxa (equal to or greater than 1%) and their respective relative abundance values were used to generate a correlation matrix—using Spearman's metric— and the subsequent construction of a network (graph), respectively, using the Past (version 4.06b) [53] and Gephi (version 0.9.2) software [54]. The taxonomic composition was also crossed with the physicochemical data and correlated through Canonical Correspondence Analysis (CCA) using the Past program (version 4.06b). The metabolic functions and the abundance values

predicted in the PICRUST2 and BURRITO software from the generated ASVs were used for the analysis of differential abundance, considering all seasons, using the Stamp software (version 2.1.3) [55], applying the one-way ANOVA statistical test, post-hoc test Tukey–Kramer. Other statistical analyses, including normality tests (Kolmogorov–Smirnov and Shapiro–Wilk), descriptive statistics, ANOVA, and correlation, were conducted in the SPSS program (version 20) [56].

Results and Discussion

Physicochemical analyses and meteorological conditions

Influent sewage, effluent, and sludge from UASB reactors were monitored from October 2020 to October 2021. The values obtained for the different physical–chemical parameters analyzed as well as the performance characteristics of the bioreactors are presented in Table 1, divided by seasons. The bioreactors operated at ambient temperature in the psychrophilic (< 20 °C) or mesophilic (20–40 °C) [57, 58] range during the study period, as verified by the temperature values of the influent sewage and the sludge from the bioreactors (see Table 1). It was verified that the ambient temperature was statistically different between the

seasons (Kruskal–Wallis $p=0.00$; significance ≤ 0.01), promoting significant changes in the temperature of the influent sewage (ANOVA $p=0.00$; significance ≤ 0.01), except for autumn and winter periods, which did not differ (Dunnett T3 $p=0.745$; significance ≤ 0.05). Temperature values in the psychrophilic range pose challenges for anaerobic digestion, since chemical reactions, which would normally take place in mesophilic conditions under lower energy expenditure, demand higher costs under these circumstances, thus some microorganisms, normally psychrotrophic, tend to stand out for thermodynamic reasons, such as archaea and hydrogenotrophic bacteria, usually sulfate-reducing [59].

The evaluation of the parameters COD, PO₄-P, TKN, and NH₃-N (see Table 1) allowed us to classify the influent sewage from the bioreactors as low to medium concentrated, and the results are compatible with the usual concentration ranges found for domestic sewage [60–62]. Despite the low influent organic load during the study period, the reactors showed a good COD removal efficiency between 48.89 and 88.84% (above 80% during autumn and below 50% during summer). These results are satisfactory considering that UASB reactors were designed to treat wastewater with a high organic load [9]. In monitoring full-scale UASB reactors, Gaur et al. [63] found good COD removal capacity ($51 \pm 13.56\%$) treating domestic sewage with low organic loading rate (OLR); however, the values obtained were, on average, lower than those presented here.

Table 1 Mean and standard deviation of the physicochemical operational parameters and performance characteristics of the analyzed UASB reactors divided by seasons

Parameters	Spring		Summer		Autumn		Winter	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
T _{air} (°C)	19.24	± 4.76	21.62	± 3.18	16.23	± 5.15	14.48	± 5.64
T _{influent} (°C)	22.34	± 1.46	24.23	± 1.42	18.97	± 2.73	17.60	± 1.73
T _{sludge} (°C)	20.45	± 1.89	24.00	± 1.00	18.60	± 0.84	17.90	± 1.78
Flow (L/s)	29.45	± 12.58	33.07	± 9.44	34.55	± 10.39	36.89	± 10.94
HRT ^a (h)	29.31	± 11.76	23.66	± 5.81	22.76	± 5.89	21.63	± 6.58
pH	7.55	± 0.18	7.49	± 0.13	7.53	± 0.12	7.64	± 0.21
COD _{influent} (mg/L)	257.50	± 66.06	154.33	± 48.85	253.00	± 2.82	302.80	± 67.12
NH ₃ -N (mg/L)	39.23	± 15.40	27.16	± 5.45	44.45	± 4.87	40.96	± 16.37
TKN ^b (mg/L)	51.24	± 29.45	32.70	± 3.54	23.77	± 26.20	47.21	± 8.85
PO ₄ -P (mg/L)	3.81	± 0.66	2.28	± 2.86	3.63	± 0.83	5.61	± 1.87
OLR ^c	0.26	± 0.14	0.27	± 0.20	0.29	± 0.11	0.47	± 0.33
SMA ^d	0.03	± 0.00	0.04	± 0.03	0.03	± 0.01	0.03	± 0.00
COD removal efficiency (%)	68.76	± 10.08	58.36	± 9.09	81.48	± 10.40	66.77	± 13.26
Acetate (mg/L)	154.11	± 44.74	107.15	± 2.23	105.81	± 1.14	101.04	± 3.01
Butyrate (mg/L)	121.56	± 1.88	–	–	121.10	± 1.95	121.37	± 0.27
Propionate (mg/L)	100.8	± 4.23	93.89	± 0.99	93.17	± 0.87	104.70	± 4.31

Values are expressed as mean ± standard deviation (SD)

^aHydraulic Retention Time

^bTotal Kjeldahl Nitrogen

^cOrganic Loading Rate expressed in kgDQO.m⁻³ d⁻¹

^dSpecific Methanogenic Activity of the sludge sampled (gDQO_{CH₄}.gSV⁻¹.d⁻¹)

The temperature and other variables related to seasonal changes did not promote significant changes in the efficiency of the reactors, in terms of COD removal (ANOVA $p=0.215$; significance ≤ 0.05) between different seasons. In a study based on pilot-scale UASB reactors, Zhang et al. [22] also did not find significant changes in COD removal efficiency, operating at temperatures between 12 and 20 °C, thus obtaining average efficiency of 60%. The same author found a loss of efficiency, due to insufficient methanogenic capacity, only when the temperature decreased to 10°C.

The results obtained for the SMA test at 37°C revealed a low sludge activity of CH_4 (0.017–0.096 $\text{gCODCH}_4\cdot\text{gVS}^{-1}\cdot\text{d}^{-1}$) compared to average values for sludge from UASB digesters fed with domestic sewage situated between 0.10 to 0.20 $\text{gCODCH}_4\cdot\text{gVS}^{-1}\cdot\text{d}^{-1}$ [64]. However, other works found numbers close to those described in the present study for UASB reactors. Bertolino et al. [65], treating sanitary sewage and using volatile suspended solids (VSS) values in the calculation, obtained a mean SMA of 0.0579 $\text{gCODCH}_4\cdot\text{gVSS}^{-1}\cdot\text{d}^{-1}$, Agrawal et al. [66] numbers equivalent to 0.040 $\text{gCODCH}_4\cdot\text{gVSS}^{-1}\cdot\text{d}^{-1}$ and employing a solution containing different organic acids—acetic, propionic, and butyric acid—Alves et al. [67] reached values in the range of 0.040 $\text{gCODCH}_4\cdot\text{gVS}^{-1}\cdot\text{d}^{-1}$ for sludge from swine manure.

Regarding rainfall (Fig. S2), despite the reactors receiving mixed sewage—domestic sewage plus rainwater—no significant correlations were found between the average inflow of the system and the accumulated precipitation for the study period (Spearman = 0.495; $p=0.086$; significance: ≤ 0.05), which suggests that the effects triggered by precipitation were punctual and not lasting to the point of influencing the average flow, HRT, as well as the efficiency in the COD removal process; However, it should be noted that the accumulated precipitation for months of study was low by regional standards. Punctual changes promoted by precipitation were also observed by Oliveira et al. [68], triggering a slight increase in the influent and effluent BOD, changes in pH, and alkalinity, although such changes did not impair the treatment efficiency, similar to the results found here. Other authors, however, have verified negative effects of precipitation, such as changes in the influent organic load, oxidation–reduction potential of the medium, conductivity and temperature decrease, parameters equally relevant for the anaerobic digestion process [69].

Characterization of the prokaryotic microbiota present in UASB biodigesters

The NGS sequencing analyses of the sludge samples collected from the 3 UASB reactors operating in full scale, from amplicons from the V3 and V4 regions of the 16S rRNA gene, resulted in a total of 1,451,602 reads recovered after pre-processing and denoising. In total, 3,384 ASVs,

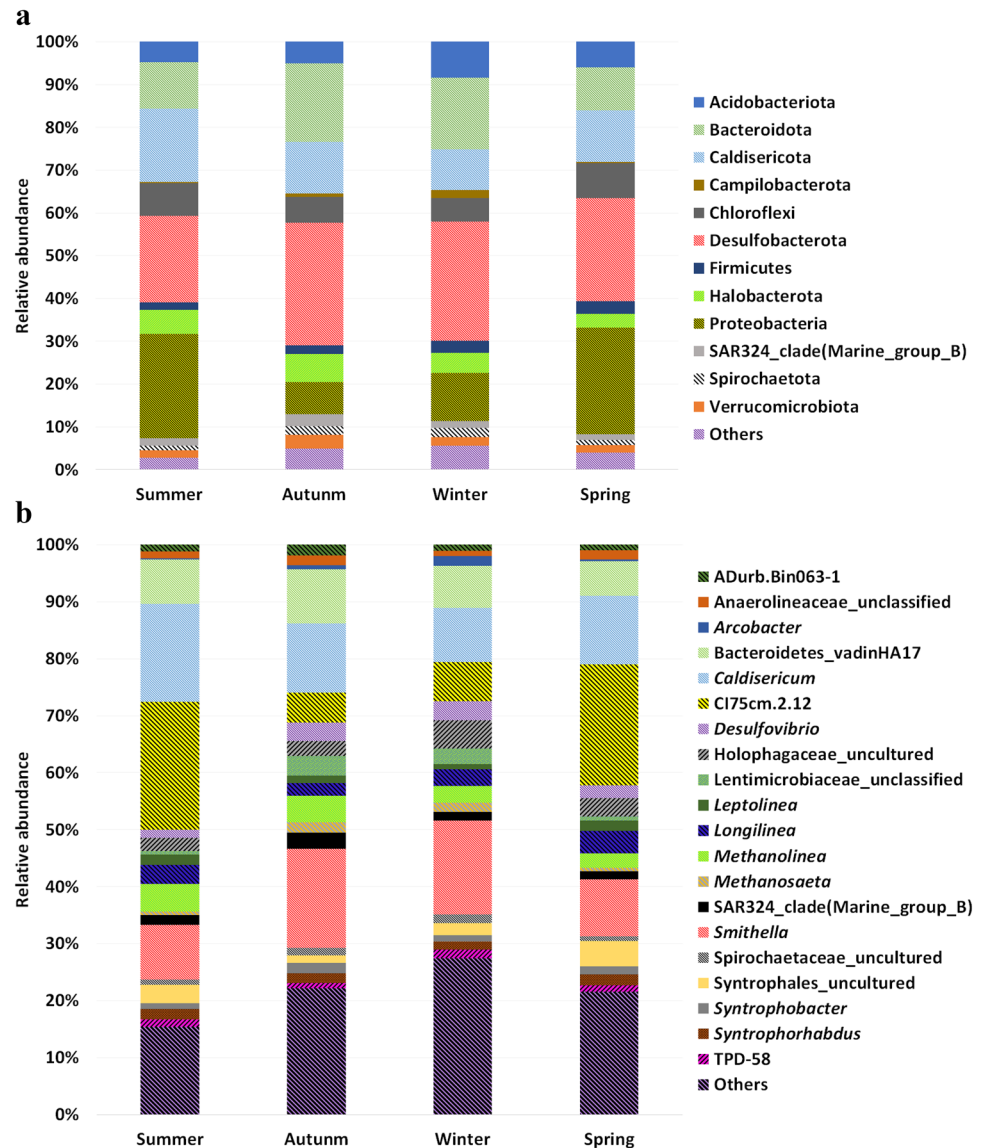
represented by more than one sequence, were found after applying the DADA2 algorithm, thus achieving stabilization in the rarefaction curves for all sample areas (Fig. S3). The number of ASVs obtained was higher than that found in other studies carried out in anaerobic reactors, operating at full scale and treating domestic sewage [70].

The taxonomic annotation from all ASVs recovered allowed for the identification of 50 phyla, of which 10 did not present a defined taxonomy (phylum candidate). Of the total number of phyla found, only 12 presented relative abundance greater than or equal to 1% in at least one season of the year (Fig. 1a). Some phyla showed visible seasonal variation in relative abundance, highlighting: Desulfobacterota (20.21–28.64%), Proteobacteria (7.48–24.90%), Bacteroidota (10.05–18.37%), and Caldisericota (9.49–17.20%). The only representative phylum inserted in the Archaea domain with an abundance greater than or equal to 1% was Halobacterota (3.23–6.55%) for the analyzed periods.

In anaerobic digesters, operating at full-scale and treating domestic sewage, other studies have identified the phyla Proteobacteria, Chloroflexi, Firmicutes, and Bacteroidota (Bacteroidetes) among the most abundant [70–73]. The exceptions presented were the phyla Acidobacteriota, Caldisericota and Desulfobacterota. The phylum Acidobacteriota is made up of members with an important role in the degradation of carbohydrates as well as in the acetogenic step of anaerobic digestion, notably, using aromatic compounds for this purpose [74–76]. Concerning the phylum Caldisericota, it currently has only one representative genus (*Caldisericum*) related to the reduction of sulfur compounds [77, 78]. Desulfobacterota is a new nomenclatural proposition, considering that some members of this group had phylogenetic inconsistencies, which did not support their classification in the previous phylum, Proteobacteria [79]. Representatives of this group are associated with the reductive activity of sulfur compounds, but some members subsist in anaerobic environments, where they produce acetate and butyrate from propionate in syntrophy with hydrogenotrophic methanogenic archaea [80, 81]. Regarding the phylum Halobacterota, recent studies reassessing phylogenetic aspects within the Archaea domain found problems in maintaining the phylum Euryarchaeota as a monophyletic clade, proposing its division into 3 new phyla, where the phylum Halobacterota (Halobacteriota) was created [82]. Members of this phylum are associated with methanogenesis (hydrogenotrophic, acetoclastic, and methylotrophic metabolic pathways), sulfur-reducing activity and some groups are present in hypersaline environments [83–86].

Within the taxonomic level of the genus, 655 distinct groups were found, 641 belonging to the Bacteria domain and 14 to the Archaea domain. Evaluating the total number of groups found, 46.87% received a taxonomic signature of the genus, and only 20 presented relative abundance greater

Fig. 1 Relative abundance of representative taxa divided by seasons. **(a)** Distribution by phyla and **(b)** genera



than or equal to 1%, 10 of which were unclassified or not cultivable, considering at least one season of the year (Fig. 1b). Among the most abundant genera prevailed CI75cm.2.12 (5.34–22.45%), *Smithella* (9.60–17.36%), *Caldisericum* (9.49–17.20%), *Bacteroidetes_vadinHA17* (6.12–9.46%), *Methanolinea* (2.51–4.81%) and *Longilinea* (2.21–3.93%).

CI75cm.2.12 (Family Nitrosococcaceae) was present in all samples, but with higher relative abundance for the summer and spring periods. The Nitrosococcaceae family is related to the nitrogen cycle, which in anaerobic environments may indicate the incidence of denitrification processes with reduction of NO_3^- to N_2 [87, 88]. Considering that the studied reactors are followed by Submerged Aerated Biofilters (SABs) units—to oxidize the ammonia (NH_3) present in the sewage to nitrate (NO_3^-) and that the sludge produced is recycled to the UASB reactors—the prevalence of the group in question could be explained, due to the availability of the

electron acceptor nitrate for anaerobic respiration by these microorganisms. However, the group in question decreased for the autumn and winter periods, a fact that may be related to temperature since biological processes of nitrification are impaired with lower values for this variable, thus making NO_3^- unavailable for use by this organism [89–93].

Bacteroidetes_vadinHA17 belongs to the order Bacteroidales and is related to the metabolism of proteins and amino acids, in addition to being of great importance in UASB reactors in the degradation of complex organic compounds from different types of effluents, playing a relevant role in the stages of hydrolysis and acidogenesis [94–97].

Other important genera found, *Longilinea* and *Leptolinea* (0.95–1.77%), metabolize carbohydrates, the former in syntrophy with hydrogenotrophic archaea, both producing acetate, a substrate for the production of CH_4 by the acetoclastic pathway [98, 99]. In addition to this fundamental association,

Longilinea (Anaerolineaceae) seems to contribute to the formation of granules in reactors, together with acetoclastic archaea of the genus *Methanosaeta* (0.61–1.80%), helping in the efficiency, maintenance of sludge blankets and the anaerobic digestion process as a whole [95, 96, 100]. Also, acetogenic, the genera *Syntrophorhabdus* (1.46–1.98%) and *Syntrophobacter* (1.06–1.83%) were identified in the samples. However, the first is linked to the degradation of phenolic compounds, and the second uses propionic acid as substrate, both in syntrophy with hydrogenotrophic archaea [101, 102].

In the methanogenic stage, only the genera *Methanolinea* and *Methanosaeta* presented relative abundance above 1%, standing out from the other archaea found: *Methanospirillum*, *Methanobacterium*, *Methanoregula*, *Methanosphaera*, *Methanomethylovorans*, and *Methanomassiliicoccus*. The genus *Methanolinea* is associated with syntrophic systems where there is the degradation of propionic acid and production of H₂ [103], corroborated by the strong presence of the *Smithella* acetogenic bacterium that metabolizes this substrate producing acetate and H₂ [104]. *Methanosaeta*, on the other hand, is related to the production of methane by the acetoclastic pathway [105]. Zhang et al. [81] also reported the predominance of these genera in samples collected from reactors operating at full-scale treating domestic sewage. The prevalence of these genera is related to the syntrophic associations already reported. However, the more expressive abundance of a hydrogenotrophic genus (*Methanolinea*) can be explained by the greater susceptibility of acetotrophic archaea to environmental disturbances, often acting as indicators of potential failures in the anaerobic digestion process when in small numbers, since most of the CH₄ produced in these systems occurs, under optimal conditions, by the acetoclastic pathway [6]. In addition, lower temperatures tend to favor the occurrence of hydrogenotrophic groups [25, 106, 107] and McKeown et al. [108] also reported the formation of microbial consortia in psychophilic bands, where there is propionic acid oxidation in syntrophy with this class of archaea, in agreement with the results observed here. In the present work, a low occurrence of the genus *Methanosaeta* was observed, with greater abundance in the winter period, indicating a possible suppression of this taxon by factors not linked to temperature, such as: competition for substrate (acetate) or by the high concentration of inhibitory chemical compounds (H₂S, NH₃⁺, and Na⁺) [109]. It is worth mentioning that despite the low proportion of methanogenic organisms and the reduced values of SMA, no extreme changes were observed in the maintenance of the efficiency of COD removal from the system, indicating that organic matter removal occurred through other metabolic pathways.

The analyses also revealed the presence of other groups that are important for the operation of the biodigestion units. The genera *Desulfovibrio* (1.44–3.33%) and *Caldisericum*,

which presents sulfidogenic activity, were found in considerable abundance in the analyzed samples. In a study conducted in UASB reactors, verifying the dynamics of the microbial community in the methanogenesis-sulfidogenesis transition process, Fernández-Palacios et al. [110] found that *Desulfovibrio* seems to be linked to a considerable impairment of methanogenesis via the acetoclastic pathway, promoting an accumulation of acetate in the biodigesters in the presence of sulfate. However, Borja [111] explained that the genus, in the absence of SO₄²⁻ is associated with acetogenic activity, therefore not causing damage but rather contributing to the production of CH₄. Sulfate-reducing bacteria can compete for carbon sources with methanogenic archaea. This process occurs when the COD/SO₄²⁻ ratio is equal to or less than 1 or through the generated hydrogen sulfide (H₂S), which is toxic to these organisms, hindering their growth and reducing the contribution of methane (CH₄) to the biogas produced [112–114]. In addition to the biological aspect, excess H₂S, as it is a highly corrosive gas, compromises pipes, structures, and equipment used in the construction of treatment units and eventual energy use of the biogas produced [64].

Differential abundance of representative groups

Regarding the differential abundance, considering the extreme seasons (summer and winter), Fig. 2 highlights the groups at the genus/ASV level that showed significant changes in their relative abundances through the use of the DESeq2 algorithm. Among the representative groups (Fig. 1b) that were statistically different, *Methanolinea*, CI75cm.2.12, *Caldisericum*, Lentimicrobiaceae_unclassified, and *Arcobacter* are present. Except for *Arcobacter* and Lentimicrobiaceae, whose abundance was higher in the winter, the other groups showed higher abundance in the summer.

The groups reported with the highest occurrence for the summer period have some species whose optimal growth conditions are related to mesophilic and thermophilic environments. CI75cm.2.12, as already discussed, a possible link with nitrogen metabolism (denitrification), occurring in environments with NO₃⁻ availability, therefore dependent on nitrification processes that operate in aerobic environments under higher temperatures [88]. On the other hand, the groups reported with higher abundance for winter are linked to psychophilic-mesophilic conditions, with species of the genus *Arcobacter* being described as pathogenic to humans [115, 116], but recent studies have revealed that some members of the group are related to acetate consumption and H₂S oxidation in anaerobic environments and psychophilic conditions [117]. The other representative genera (Fig. 1b), which did not show significant differential abundance, indicate that the UASB reactors studied are capable

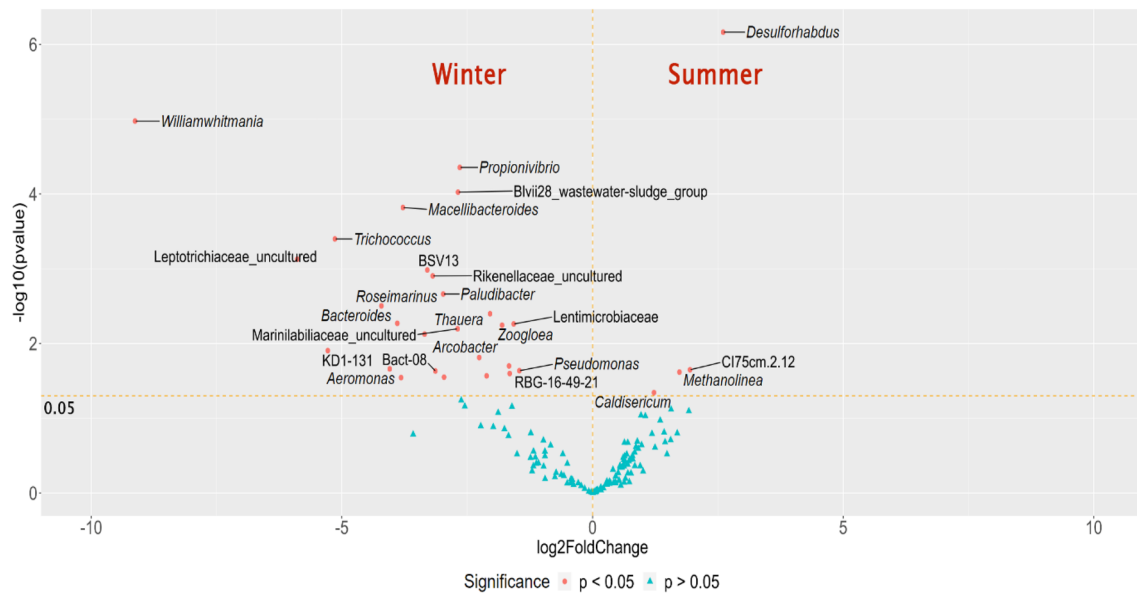


Fig. 2 Volcano plot showing differential abundance by genus/ASV considering winter and summer seasons. The red circles represent the groups that presented significant differential abundance ($p < 0.05$), while the triangles non-significant groups ($p > 0.05$). The values

of the y-axis are expressed in $-\log_{10}$ of the p value and the x-axis in \log_2 FoldChange, relative to the abundance of the genera, considering the winter and summer period

of maintaining a relatively stable microbial community throughout the year, even under the influence of environmental variations imposed by seasonal factors.

Relationship between representative taxonomic groups in the community

In anaerobic systems, the co-occurrence of certain groups of microorganisms can indicate the degree of mutual dependence to which they are subjected. Figure 3 presents the most representative genera and their correlations in the samples from the different UASB reactors. In the presented network, it is possible to observe interesting associations between members of the Anaerolineaceae family (phylum Chloroflexi), highlighted by the positive correlation between the *Longilinea*, *Leptolinea*, and Anaerolineaceae_unclassified, which play a relevant role in the structuring of sludge granules in anaerobic digesters [118, 119]. This family has been associated with the presence of the methanogenic genus *Methanosaeta* in anaerobic systems [120]; however, in the present study no significant correlations were identified, with the before-mentioned taxa.

The genus *Methanosaeta* was correlated with *Syntrophobacter*, *Smithella* (with expressive abundance in the samples), and Lentimicrobiaceae_unclassified – relevance in the production of acetate and VFA fermenting carbohydrates [121]—but highlighting the strong correlation with *Desulfovibrio* (Spearman = 0.935; $p = 0.000$; significance ≤ 0.01) which in the absence of sulfate can ferment several amino

acids to acetate [122]. *Methanosaeta* still correlates with Spirochaetaceae_uncultured, a family which has some members with possible carbohydrate fermentative activity—producing acetate in syntrophy with hydrogenotrophic archaea—but groups with oxidative acetate metabolism have also been reported [123–125].

Among the hydrolytic and acidogenic groups, establishing several connections in the network, Bacteroidetes_VadinHA17 stands out, showing a correlation with several acetogenic groups in this system. As for the association observed between the *Smithella* genus and Bacteroidetes_vadinHA17, both with expressive abundance in the samples, it is feasible to consider that the fermentative capacity of the latter would result in the production of substrates capable of being used by the former, for example, the fermentation of some amino acids, such as methionine and threonine, which can be metabolized to propionic acid, a substrate used by some species of the *Smithella* genus [79].

Regarding the genus *Caldisericum* and the absence of significant correlations in the network with other groups, despite its expressive abundance, it is imperative to point out that its role in the metabolism of organic compounds in anaerobic systems remains unclear; however, some studies propose its participation in the amino acid degradation, ammonia assimilation, butyric acid consumption and even autotrophic growth using enzymes linked to the reverse citric acid cycle [126–128].

Another group with outstanding abundance in the study, CI75cm.2.12 (family Nitrosococcaceae), presents a positive

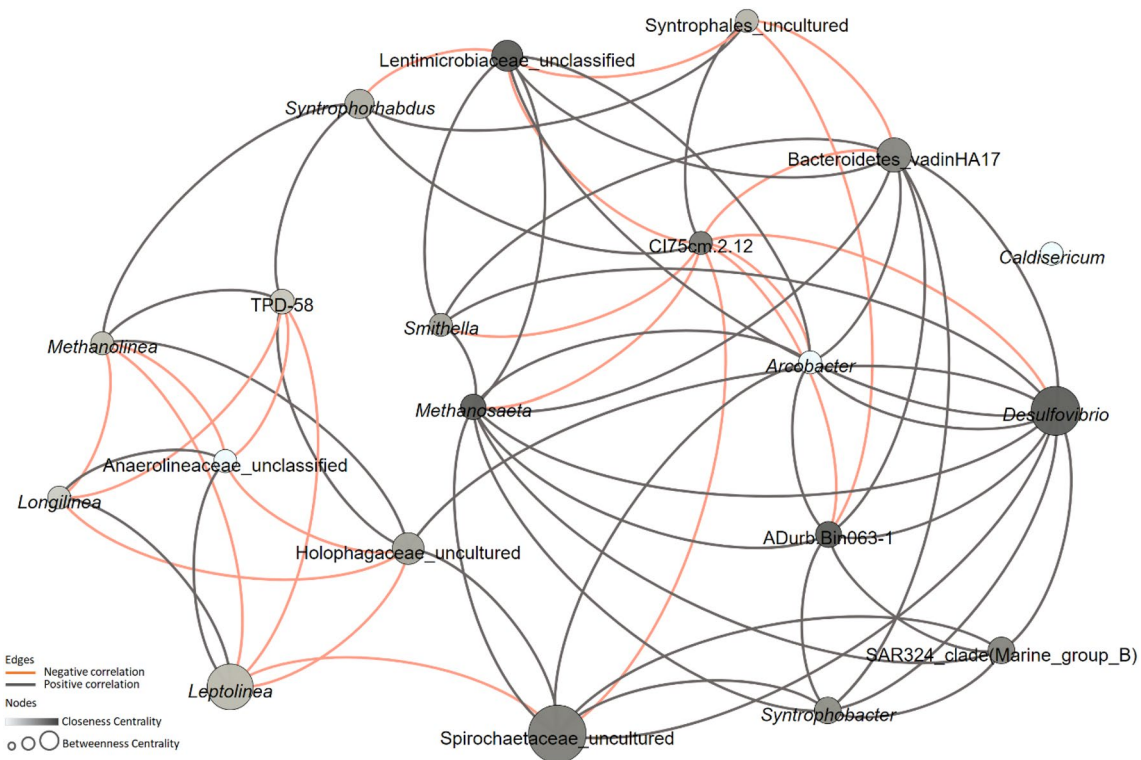


Fig. 3 Co-occurrence network showing the correlation between the most representative groups present in the sludge samples. The network was built using data from a Spearman correlation matrix and presents only connections whose significance level was ≤ 0.05 . The connections in grey represent positive correlations, while in orange,

negative correlations. Dark grey nodes have higher values for Closeness Centrality, while larger nodes have higher Betweenness Centrality. Correlation analyses were performed using the Past 4.06 program, and the network was built using the Gephi 0.9.2 program

correlation with the acetogenic elements *Syntrophorhabdus* and *Syntrophales_uncultured*, but several negative associations with other groups of importance for the functioning of the reactors, including *Methanosaeta* (Spearman = -0.728 ; $p=0.002$; significance ≤ 0.01), which would show possible inhibitory effects. Wang et al. [129] highlighted that some denitrifying groups could use acetate as an electron donor, which corroborates the negative relationships observed.

In the network, it is still possible to verify a positive link between the archaea *Methanolinea* with the genus *Syntrophorhabdus*, TPD58, and *Holophagaceae_uncultured* (family that has some genera with outstanding syntrophic importance in anaerobic systems, such as *Holophaga* and *Geothrix*). *Holophaga* is related to the production of acetate from aromatic compounds through the homoacetogenic pathway, while *Geothrix* is related to the oxidation of acetate, using the iron ion as an electron acceptor [75]. TPD58 (family Thermoanaerobaculaceae) is possibly associated with carbohydrate fermentation [130] and related to syntrophy with the mentioned archaea.

The calculations of the topological properties of the network allowed us to verify the importance of some taxa in the network (see Table S2). Considering the Closeness

Centrality (CC), Betweenness Centrality (BC), and degree together, *Bacteroidetes_vadinHA17* (CC = 0.8; BC = 6.25; degree = 9), *Spirochaetaceae_uncultured* (CC = 0.83; BC = 19.16; degree = 8), and *Desulfovibrio* (CC = 1; BC = 14.25; degree = 11) stood out. Besides these taxa, other groups showed high values for degree and low values for BC: *ADurb.Bin063-1* (BC = 1.66; degree = 8), *Methanosaeta* (BC = 1.25; degree = 10), *Lentimicrobiaceae_unclassified* (BC = 4.41; degree = 8), and *CI75cm.2.12* (BC = 0; degree = 10). In this context, groups that present higher numbers for the three coefficients may present synergistic or competitive relevance to others in the microbial community, while taxa showing lower values for BC and higher for degree may be considered keys, determining the structure of the network and promoting an increase or decrease in the diversity of the system [131–134].

Correspondence of abiotic factors with representative groups

Analyzing the graph obtained through the CCA (Fig. 4), it is possible to verify that the *Spirochaetaceae_uncultured*, *Smithella*, *Methanosaeta*, *Desulfovibrio*, and *Arcobacter* are

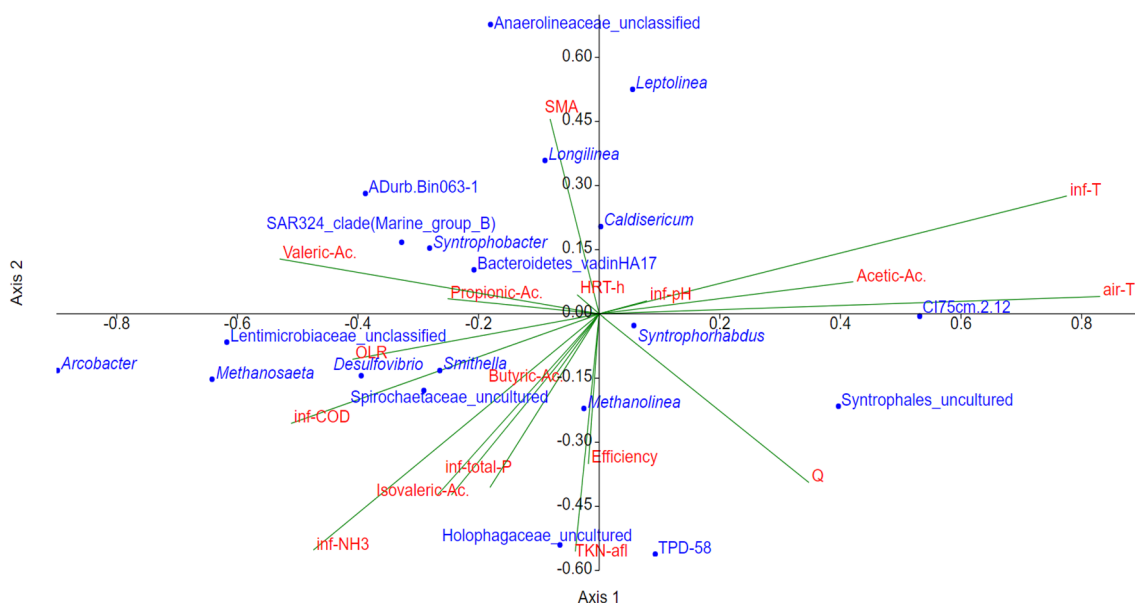


Fig. 4 CCA between environmental variables and representative taxonomic groups. SMA: Specific Methanogenic Activity. HRT: Hydraulic Retention Time. OLR: Organic Loading Rate. COD: Chemical Oxygen Demand. TKN: Total Kjeldahl Nitrogen. Q: Flow. inf-T: Influent Temperature (°C). air-T: Air Temperature (°C). Envi-

ronmental variables are shown in red and green arrows to illustrate the weight of correlation with microbial genera, while taxonomic groups are represented in blue. The proximity between environmental variables and microorganisms reflects their correlation

associated with an increase in COD and OLR values. In anaerobic systems where low-concentrated sewage predominates, small increases in organic load would probably be able to sustain a greater diversity of species due to the addition of resources and consequent decrease in interspecific competition as well as a more favorable C/N ratio [135]. Reshmi et al. [136], studying estuarine environments, reported that a greater contribution of organic matter favored the occurrence of acetotrophic groups, such as the genus *Methanosaeta*, similar to the data presented in the present study.

The genus *Methanolinea*, the most abundant archaea in the samples, is correlated with an increase in the efficiency of the COD removal process and with higher levels of TKN. According to Bandara et al. [137], hydrogenotrophic archaea play a significant role in COD removal in biodigesters operating under psychrophilic conditions, and Ruiz-Sánchez et al. [138] also found an association between this group of archaea with increased nitrogen concentration. This taxon is extremely relevant, considering that secondary treatment in WWTPs aims to remove organic matter from the sewage [12].

The *Longilinea*, *Leptolinea* and *Anaerolineaceae_unclassified* showed a close correlation with the sludge SMA, contrary to what was expected if we consider the methanogenic groups. This factor may be related to the ability that members of the *Anaerolineaceae* family have in the granular structuring of the sludge. They form filamentous

colonies, allowing syntrophic relationships to be processed more efficiently, in addition to acting in the production of acetate from carbohydrate fermentation [99, 100, 139].

As for VFAs, it is possible to identify that SAR324, *Syntrophobacter*, and *Bacteroidetes_vadinHA17* are correlated to the concentration of propionic and valeric acids. *Syntrophorhabdus* and CI75cm.2.12 are related to acetic acid. In this context, the importance of *Bacteroidetes_vadinHA17* in the production of VFAs is evident, mainly its hydrolytic and acidogenic role in the metabolism of proteins, amino acids, and carbohydrates. *Syntrophobacter* has a relevant acetogenic role, metabolizing propionic acid and SAR324 is linked to the degradation of VFAs and oxidation of sulfur compounds [97, 140, 141]. *Syntrophorhabdus* is also related to the production of acetic acid [102] and CI75cm.2.12 may be linked to the use of this substrate as an electron donor for NO_3^- reduction reactions [129], which would represent a potential competitor in methanogenic systems.

The increase in temperature (air and influent), an important factor in seasonal conditions, did not seem to exert a preponderant influence on the abundance of most of the identified taxa, with the exception of the CI75cm.2.12, which explains its massive presence in the summer period (Fig. 1b). Conversely, lower temperatures are correlated with a higher richness of representative taxa found, as highlighted in the CCA.

Diversity analysis for the sample periods studied

The alpha diversity analysis using Shannon index and Pielou evenness revealed greater diversity for winter, followed by spring/autumn and summer, showing significant differences between all seasons in relation to summer (Fig. S4). The greater diversity for the colder periods can be explained by the decrease in dominant taxa (greater equitability) that could inhibit or suppress competition other groups in a favorable climate situation (temperature), either by dispute related to resources or by the generation of by-products with potential toxicity [142]. From this perspective, toxic compounds, such as H₂S, can act as growth inhibitors for many microorganisms [143] and a decrease in the relative abundance of the sulfidogenic taxon *Caldisericum*, as well as CI75cm.2.12 can be observed during the winter period, which could have promoted the increase in diversity for the colder season.

These results contradict other studies, which commonly associate the increase in temperature with a greater richness of Operational Taxonomic Units (OTUs) or ASVs [91]. However, the increase in alpha diversity related to the decrease in sludge temperature could be attested by the strong negative correlation (Spearman = 0.787; $p = 0.000$; significance ≤ 0.01) between the values obtained for the Shannon index and the variable in question. Another relevant aspect refers to the progressive increase in the number of rarer ASVs (summer to winter), verified by the greater presence of taxa with abundance lower than 1% (Others) (Fig. 1b), contributing to the increase in richness and consequently of the diversity in the samples [91]. In general, higher levels of diversity are desirable in biological systems since this would represent a greater repertoire of available redundant metabolic pathways, allowing the microbiota to adapt to eventual disturbances and maintain the integrity of the system [144].

As for the beta diversity, the application of the Weighted Unifrac metric, concomitant with the Principal Coordinate Analysis (PCoA), evaluating the proximity relationships between the microbial communities of the four seasons of the year, revealed more dissimilarity between winter-summer and autumn-spring, most of the difference explained by the x-axis (43.87%) of the PCoA graph. The mentioned similarity relations were supported by the Permutation Analysis of Variance (PERMANOVA), using 999 permutations and a significance level ≤ 0.05 (Fig. S5). These combined analyses showed differences between the microbial communities of the UASB reactors in detriment to seasonal conditions, especially considering the extreme seasons (summer and winter). This fact, possibly related to temperature, was also verified by other authors studying microbial communities in WWTPs [16, 91, 145]. However, despite the differences observed, there were no significant changes in the efficiency of the

biodigesters, possibly indicating an overlap in the functions performed by taxa within the community. This fact was also verified by Resende et al. [146], but evaluating the microbiota from the biodigestion of bovine manure, considering pilot scale reactors, under ambient temperature conditions and aspects related to seasonal changes.

Functional prediction

The functional prediction analysis using the PICRUSt2 software, by comparing with the KO, EC, and MetaCyc databases, revealed the presence of 6,853 genes, 2,085 enzymes, and 373 metabolic pathways associated with sequences of ASVs obtained from sludge samples from UASB reactors for the periods evaluated. Considering the total number of genes identified (KO) 92.57%, 89.63%, 96.11%, and 95.40% were found for summer, autumn, winter, and spring.

The prediction evaluation was assessed using the Nearest Sequenced Taxon Index (NSTI) values calculated using the PICRUSt2 software for each ASV, with the numbers obtained being in the range of 0.352 to 0.434 (weighted NSTI) considering the seasons. Of the total number of ASVs obtained (3,384), only two were removed because they exceeded the cutoff limit stipulated as standard by the program (2.0). The NSTI values achieved are within the standard range configured in the PICRUSt2 software. However, they suggest low coverage – since poorly characterized environments present less availability of genomes in the database for phylogenetic comparisons performed by the program, thus not resulting in more accurate approximations [147].

From the genes found, using the BURRITO software and the KEGG BRITE classification hierarchy, it was possible to identify the main functions associated with the microbial community for the period studied. The most representative functions obtained for the SuperPathway category are linked to cellular mechanisms of translation (16.94–17.59%), membrane transport (14.48–14.93%), amino acid metabolism (7.74–8.00%), as well as DNA replication and repair (6.76–6.82%). In general, at this level of classification, there were no evident differences in the functions identified between the seasons. However, when considering more specific categories (SubPathway) differential abundance was observed (Fig. S6). The Alanine, aspartate, and glutamate metabolism and Transport functions were more abundant for the winter and autumn periods, differing statistically from the other seasons of the year. DNA replication proteins, Glyoxylate and dicarboxylate, and Terpenoid backbone biosynthesis showed differential abundance only between autumn and spring, while Protein folding and associated processing showed a statistical difference between spring-winter and spring-autumn.

Table 2 Correlation between representative taxonomic groups and metabolic functions with differential abundance for seasons

Metabolic functions	Taxonomic groups	Spearman	p-value ^a
Alanine, aspartate and glutamate metabolism	<i>Arcobacter</i>	0.700	0.016
	<i>Desulfovibrio</i>	0.618	0.043
	Lentimicrobiaceae_unclassified	0.791	0.004
	<i>Methanosaeta</i>	0.709	0.015
	<i>Smithella</i>	0.700	0.016
	<i>Spirochaetaceae_uncultured</i>	0.627	0.039
	Bacteroidetes_VadinHA17	0.709	0.015
Glyoxylate and dicarboxylate metabolism	CI75cm.2.12	0.918	0.000
	Syntrophales_uncultured	0.818	0.002
Protein folding and associated processing	CI75cm.2.12	0.809	0.003
Terpenoid backbone biosynthesis	Lentimicrobiaceae_unclassified	0.700	0.016
	<i>Smithella</i>	0.764	0.006
	Bacteroidetes_VadinHA17	0.855	0.001
Transport	<i>Arcobacter</i>	0.627	0.039
	Lentimicrobiaceae_unclassified	0.964	0.000
	VadinHA17	0.627	0.039

^aSignificance level 0.05

From these data, it was possible to identify that some taxonomic groups were correlated with the above metabolic functions, possibly promoting the differences observed between the seasons (see Table 2). Thus, the correlation between *Desulfovibrio*, *Methanosaeta*, and Spirochaetaceae_uncultured with amino acid metabolism is highlighted, and *Arcobacter*, Bacteroidetes_VadinHA17, Lentimicrobiaceae_unclassified and *Smithella* in addition to this function, also associated with membrane transport and structural biosynthesis of terpenes. Except for *Arcobacter*, Lentimicrobiaceae_unclassified, *Smithella*, and *Methanosaeta*, the others have already been reported in the literature for presenting fermentative activity from amino acids [97, 122, 125]. Concerning terpene synthesis, there is a need to study the role of these taxa in their metabolism, assuming that these compounds have biotechnological applications, mainly in the production of drugs, insecticides, antimicrobials, and alternative fuels [148]. In the present work, the PWY-5910, PWY-922, and PWY-6174 pathways (mevalonate pathway) were found associated with the identified genes, related in prokaryotes to the synthesis of essential components of cell walls, membranes, electron transporters, and compounds used in the conversion from light into energy [149].

Syntrophales_uncultured and CI75cm.2.12 were correlated to the glyoxylate and dicarboxylate cycle. The glyoxylate cycle in prokaryotes is linked to the synthesis of carbohydrates from fatty acids and acetate – mainly under conditions of oxidative stress, infections, and antibiotic resistance – and has even been associated with cellular respiration and denitrification processes [150]. In the present study, the aceA (K01637) and aceB/glcB (K01638) genes which, respectively, encode the enzymes isocitrate lyase and

malate synthase, were found in greater abundance for the summer and spring samples, periods in which the taxa were described in greater prevalence. These results, added to the data presented in Fig. 4, demonstrated that the CI75cm.2.12 probably uses acetate as an electron donor or carbon source, competing with acetoclastic archaea for this substrate and performing some type of function related to nitrogen metabolism, possibly acting in denitrification processes.

The expressive number of genes with functions related to the metabolism of amino acids and proteins (Fig. S6a), which in addition to having an essential cellular function, reflects the composition of domestic sewage, since these biomolecules are present in high concentration, reaching together with fibers, the largest portion of the organic matter present in these residues [151, 152]. This characteristic highlights another problem, the excess of hydrogen sulfide, considering that a large portion of the H₂S generated in anaerobic systems treating domestic effluents derives from the metabolism of proteins and amino acids that have the thiol group (-SH) in their constitution [153]. On the other hand, the low relative abundance of functions associated with lipid metabolism may be related to the small concentration of these macromolecules in the domestic effluent that supplies the reactors, due to the installation of fat retaining boxes in homes and commercial establishments in the city, which prevent the arrival of excess of these compounds to the sewage system, consequently reducing the proliferation of microorganisms capable of processing these substrates. There is also a large number of unpredicted or unknown functions associated with the genes found, an important fact, as it is indicative that despite the high number of studies addressing microbial communities in anaerobic systems, it

is still necessary to know the functions performed by these microorganisms in the environment.

The data generated also made it possible to determine the presence of several genes linked to the production of the main VFAs found, acetate, butyrate, and propionate. Regarding acetate synthesis, was verified the occurrence of *folD* (K01491) (Wood–Ljungdahl pathway), *metF* (K00297), and *pta* (K13788) genes, respectively, related to the enzymes methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase, and phosphate acetyltransferase [154–156]. About propionate, were found the genes PCCB (K01966), *sucC* and *sucD* (K01902, K01903), *MUT* (K01847), and *MCEE* (K05606) responsible for encoding the enzymes propionyl-CoA carboxylase, succinyl-CoA synthetase, methylmalonyl-CoA mutase and methylmalonyl-CoA epimerase [157]. And for butyrate, the genes *buk* (K00929), *bcd* (K00248), *fadB* (K00074), *abfD* (K14534) were predicted, in order, linked to the enzymes butyrate kinase, butyryl-CoA dehydrogenase, 3-hydroxybutyryl-CoA dehydrogenase and 4-hydroxybutyryl-CoA dehydratase/vinylacetyl-CoA-Delta-isomerase [158]. Except for genes *sucC* and *sucD*, which presented higher values for the autumn and summer periods, the others were found in greater abundance for the autumn and winter periods (Fig. 5), coinciding with the greater presence of taxonomic groups linked to acidogenic and acetogenic processes as well as a greater diversity of ASVs verified (Fig. 1b, e Fig. S4).

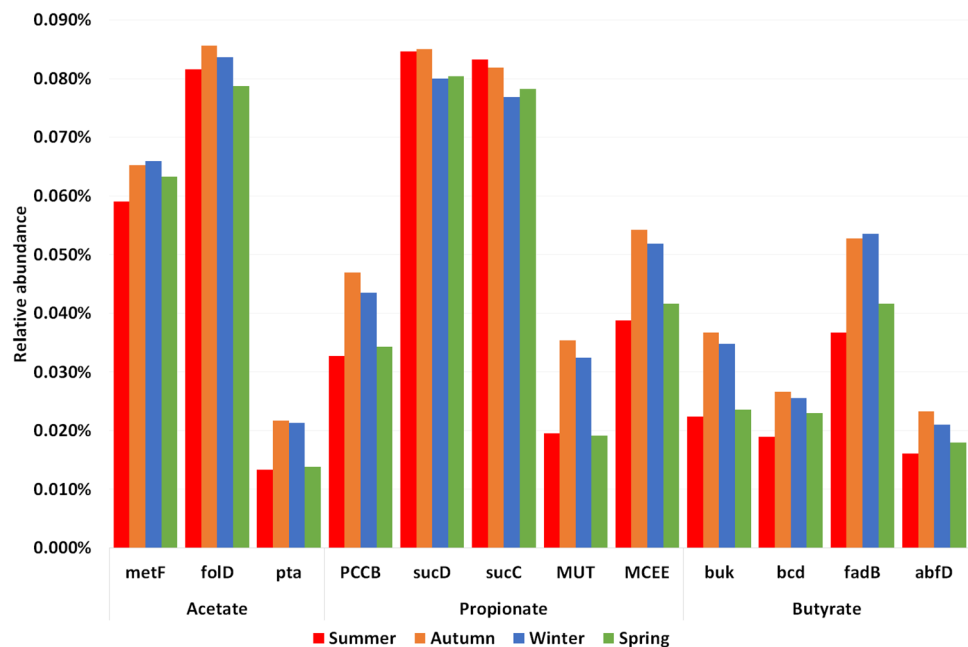
Considering the methanogenic step, the main genes identified were associated with the acetotrophic (METH-ACETATE-PWY) and hydrogenotrophic (METHANO-GENESIS-PWY) pathways, respectively presenting relative abundances in the range of 0.215–0.290% and 0.049–0.092%

for the periods evaluated, the first being more abundant for the autumn–winter period and the second autumn–summer. These periods also coincide with the highest values of relative abundance recorded for the Methane and Energy metabolism functions (Fig. S6a) linked to CH₄ biosynthesis. It is surprising that the acetotrophic methanogenic pathway was more abundant, bearing in mind that the most representative methanogenic taxon identified was the genus *Methanolinea* (hydrogenotrophic). This fact was also verified by other authors [159] and may be linked to the overlap of functions presented by some taxa that have genes coding for enzymes related to both pathways, such as Formylmethanofuran dehydrogenase (K11261 gene; *fwdE*), identified in the present study.

Conclusion

The seasonal study of the sludge from the UASB reactors revealed the presence of a diverse microbial community represented by members related to the different stages of the anaerobic digestion process. Among them stood out *Bacteroidetes_VadinHA17*, with hydrolytic and acidogenic importance, the acetogenic bacterium *Smithella* and the methanogenic hydrogenotrophic archaea *Methanolinea*. The correlation between taxa revealed some already known syntrophic interactions and possible inhibitory effects caused by interspecific competition within the community. *Methanolinea* was linked to reactors efficiency and TKN; Anaerolineaceae family members to SMA, and other groups related to the increase of influent organic matter. However, some groups need to be better characterized to confirm their real

Fig. 5 Relative abundance of genes related to VFA synthesis divided by seasons



function within the microbiome, among them *Caldisericum*, described in the literature as sulfidogenic, but not showing significant correlations with any taxon in the constructed co-occurrence network (Fig. 3) and CI75cm. 2.12 (family Nitrosococaceae) related to the nitrogen cycle, identified in this work, strongly related to temperature increase, acetate concentration and negatively correlated to several members of the microbial community. The present study also showed that the microbiota present in the digesters varied between seasons, showing high alpha diversity, including higher levels for the winter period.

The prediction of metabolic pathways revealed stability of the main functions at the SuperPathway categorical level and small variations in SubPathway for the seasons. Functions linked to amino acid and protein metabolism, membrane transport, glyoxylate cycle, and terpene synthesis showed differential abundance. The expressive number of genes linked to the metabolism of amino acids and proteins highlighted the importance of groups capable of metabolizing these substrates due to the composition of the sewage, highlighting the *Bacteroidetes_VadinHA17*. Surprisingly, a more fraction of the genes linked to the synthesis of the main VFAs found in the samples showed higher relative abundance for the autumn and winter periods.

The sludge from the reactors showed a low methane production capacity ($< 0.10 \text{ gDQOCH}_4 \cdot \text{gSV}^{-1} \cdot \text{day}^{-1}$); however, the efficiency in COD removal remained stable even under psychrophilic circumstances. These facts allowed us to glimpse potential applications of sludge as inoculum in biodigesters, used in the treatment of domestic sewage and subjected to similar climatic conditions, although they do not support its viability for the production of CH_4 , for energy purposes.

Studies of this nature are important for understanding the dynamics of the microbiota under unfavorable situations and its relationship with the functioning of biodigesters, mainly to guide operational processes and possible modulations in these microbiomes. The importance of further studies with similar themes in anaerobic systems operated on a real scale is highlighted, remembering that in uncontrolled environments, abiotic conditions are heterogeneous and are subject to daily, monthly, and annual changes, unlike simulated experiments in laboratories. Thus, given the historical differences in seasonal characteristics in the region, further research addressing this issue should be carried out to confirm or refute the patterns and results obtained in the present work.

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Author contributions JG designed the study, carried out the experiments, analyzed the data, and wrote the paper; NLL carried out the experiments; JI carried out the experiments; FEM supervised the research; SP supervised the research. All authors read and approved the final manuscript.

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Data availability The data used to support the findings of this study is available upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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