BioEnergy Research (2026) 19:17
https://doi.org/10.1007/s12155-025-10950-9

RESEARCH

Microbial Responses to the Physicochemical Properties and Reactor's Operational Parameters in Vinasse Digestion in a Pilot-Scale Hybrid Anaerobic Reactor

Gabrielle Maria Fonseca Pierangeli[1] · Flávia Talarico Saia[1] · Valéria Del Nery[2] · Marcia Helena Rissato Zamariolli Damianovic[2] · Eloisa Pozzi[2] · Moacir Messias de Araújo Jr[3] · Gustavo Bueno Gregoracci[1]

Received: 22 September 2025 / Accepted: 31 December 2025

Abstract

Physicochemical and operational parameters affect microbial communities, impacting anaerobic digestion (AD) systems. Microbial dynamics in response to these parameters were temporally investigated by DNA and cDNA of the *16 S rrna* gene in a pilot-scale hybrid anaerobic reactor (HAnR) fed with sugarcane vinasse. Key microbes for biogas production, including *Bacteroidetes*-vadinHA17, *Paludibacteraceae*-H1, *Prolixibacteraceae*, *Desulfovibrio*, *Syntrophobacter* and *Methanospirillum*, were favored by the maintenance of volatile fatty acids (VFA) < 3700 mg.L^{-1}, pH ≥ 5.6, temperature ≥ 29.8 °C, chemical oxygen demand (COD) < 31.4 g.L^{-1} and organic loading rate (OLR) < 9.7 kg.COD.m^{-3}.d^{-1}. Temperature and pH also favored the redundancy of fermentation and H_2-mediated methanogenesis and the evenness and diversity indexes, all related to biogas quality. Concentrations up to 2,050 mg.SO_4^{2-}.L^{-1}, 3,270 mg.K.L^{-1}, 367 mg.Mg.L^{-1}, 0.4 mg.Cu.L^{-1} and 1.1 mg.Zn.L^{-1} led to adverse effects over microbial abundance, functional redundancy and ecology, but tolerant microorganisms, like *Paludibacteraceae*, *Anaerolineaceae* and *Methanosaeta*, kept fermentation and methanogenesis ongoing, although less efficiently. Microbial interactions were also affected by evaluated parameters beyond metabolic dependence. This study highlights the importance of parameter monitoring in scaled-up AD reactors to promote the adequate establishment of microbial communities and therefore contribute to system stability.

Highlights

- The microbiota evaluated by DNA and cDNA were affected by temperature, pH, VFA, COD and OLR.
- Higher pH and temperature and lower VFA, COD and OLR favored key-microbes to CH4 production.
- The temperature and pH also favored microbial ecology and metabolisms for CH4 production.
- Sulfate and metals harmed the microbiota, metabolisms and ecology in the reactor.
- Tolerant microbes kept the operation ongoing, but with less efficiency.
- The monitoring parameters have been shown to determine the microbial cooccurrence.

Keywords Anaerobic digestion · *16S rrna* · Microbiota · Temperature · PH · Organic · Loading rate · Metals

Introduction

Sugarcane vinasse, a pollutant residue generated from bioethanol distillation, can be treated and converted into biogas through anaerobic digestion (AD) by a consortium of hydrolytic, fermentative (acidogenic and acetogenic) and methanogenic microorganisms [1, 2]. The bioprocesses of vinasse digestion are strictly dependent on the environmental

✉ Flávia Talarico Saia
ft.saia@unifesp.br

[1] Institute of Marine Sciences, Federal University of São Paulo, Santos, SP, Brazil

[2] Biological Process Laboratory, São Carlos School of Engineering, University of São Paulo, São Carlos, SP, Brazil

[3] Brazil Bioproj Tecnologia Ambiental, São Carlos, SP, Brazil

Published online: 14 January 2026

conditions in the system, which can promote the structuring of a resistant microbial community disfavoring less adapted members [3]. Changes in the microbiota in anaerobic reactors occur according to operational conditions and physicochemical parameters [4, 5]. The occurrence of functional redundancy, characterized by distinct microorganisms performing similar functions, is also essential to keep a resilient community and to maintain the stability of the AD through operational disturbances [6, 7]. For adequate organic matter conversion into biogas, methanogenic archaea need to interact with bacteria that produce H_2, formate and acetate, main substrates for CH_4 production [8]. However, microbial groups of hydrolytic and fermentative bacteria and of methanogenic archaea that perform these processes may have distinct ideal conditions for growth [9]. Thereby, it is necessary to optimize the physicochemical and operational parameters (e.g. temperature, pH, organic load) that influence both bacteria and archaea as best as possible to ensure conversion of substrates into biogas [10–12].

The temperature of AD processes affects the microbial growth, metabolic activity, ecology and interactions in the reactor [12–16]. Once the vinasse is generated at temperatures between 65 and 107 °C, it may be advantageous to conduct the digestion of this residue at thermophilic conditions (45–55 °C), although several reactors were also operated at mesophilic temperature (20–40 °C) [2, 11]. The microbiota tends to be more diverse at mesophilic than thermophilic temperatures, which allow a greater reactor's resilience to overcome the operational disturbances and to keep a more stable system [11]. Changes in temperature can also generate synergic effects with other parameters, such as pH, organic load and concentrations of inhibitory compounds or nutrients, promoting different results of microbial tolerances in the reactor [16–18].

Anaerobic microbial communities may also be impacted by pH stress, which influences the growth rate and the metabolic pathways in the system [19]. Methanogens are particularly sensitive to pH alterations and can be inhibited in very acid or alkaline conditions, which affect gene expression, gene regulation and/or activity of methanogenic enzymes, being in general favored at neutral pH between 6.8 and 7.2 [11, 12, 20]. At pH lower than 6.0, the methanogenic community may be drastically reduced, while acidogenic bacteria dominate the microbial community [21]. Additionally, the volatile fatty acid (VFA) produced by those bacteria promote a low pH in the system, negatively interfering on AD and inhibiting the methanogenic activity [22].

Microbial communities of anaerobic reactors can also have distinct responses to the organic load rate (OLR) according to their tolerance and capacity of adaptation, which may be associated with further system decline [23–25]. High chemical oxygen demand (COD) and OLR, which characterize organic overloading, can change the acid-type fermentation in the system and harm the methanogenic community, causing an imbalance between hydrolysis/acidogenesis and methanogenesis processes [26, 27]. Therefore, overloading impairs the conversion of VFA to CH_4, leading to the acid accumulation and pH decrease [26, 28, 29]. Additionally, the continuous accumulation of VFA, especially propionic acid, can cause system failure [18]. On the other hand, the production of acetate promotes CH_4 production via acetoclastic or hydrogenotrophic methanogenesis in vinasse digestion, by direct consumption or by syntrophic oxidation to H_2 and CO_2, respectively [30]. The consumption of acetate and $H_2 + CO_2$ by methanogens maintain the bioprocesses of AD ongoing, since the interspecies H_2 transfer in syntrophic relationships reduces the H_2 partial pressure, allowing the occurrence of the acetogenesis step and keeping the anaerobic system in balance [18, 27].

The presence of recalcitrant and/or potentially inhibitory compounds in the vinasse, mainly sulfate (SO_4^{2-}) and metals, must also be considered in vinasse digestion [11, 31, 32]. The SO_4^{2-} in vinasse is reduced by sulfate reducing bacteria (SRB) along the AD process, decreasing biogas quality [29]. High concentration of SO_4^{2-} also impairs CH_4 production, through competition for substrates between methanogens and SRB, or direct inhibition of the methanogenic consortium by H_2S [4, 30, 33, 34]. In AD, the microbiota is also sensitive to high concentrations of metals, which may inhibit microbial activity and lead to reactor instability [9, 35]. The effects of metals on the anaerobic microbiota may synergize with other physicochemical properties, like carbon and sulfur concentration [36]. Copper, lead and iron may inhibit the cellulases of hydrolytic bacteria and also interfere with the acidogenic and acetogenic steps, impairing lignocellulosic compound degradation and VFA formation, while copper and zinc negatively affect the structure and function of microbial enzymes and the activity of methanogenic archaea, impacting the conversion of organic matter into biogas, thus decreasing the system efficiency [18, 35, 37].

Although it is important to evaluate monitoring parameters, it can be difficult to determine the relationship between those parameters and the microbiota to achieve the optimum operational condition, also affecting the reactor's scaling-up to industrial application. More studies are needed to optimize the operating conditions of anaerobic process, especially on scaled reactors [11]. Thus, we evaluate the influence of the physicochemical and operational parameters of a pilot-scale Hybrid Anaerobic Reactor (HAnR) over the abundant bacteria and archaea, functional redundancy and ecological parameters analyzed by the DNA and cDNA of the *16 S rrna* gene along the vinasse AD, identifying suitable conditions of the reactor that should favor key microorganisms in the system.

Materials and Methods

Reactor and Monitoring Parameters of the Vinasse Digestion

The pilot-scale HAnR (Fig. 1) had a bottom compartment (granular sludge blanket) and an upper compartment (fixed bed and support material Biobob®), with 6.88 m^3 of total volume, and it was used for the treatment of vinasse in a biorefinery plant in Brazil over 238 days during sugarcane harvest season [38]. Vinasse samples from the feed tank (affluent vinasse) were collected for physicochemical characterization, in parallel of the microbial analysis through amplicon sequence variant (ASV) using DNA and cDNA, and the evaluation of the HAnR's performance, previously investigated [38]. The microbial samples were collected from the sampling points P0 to P4 in the sludge blanket and pooled in a composed sample for each evaluation [38].

Twenty one temporal samples of affluent vinasse were analyzed for COD by colorimetric method [39], pH by potentiometric method [39] and VFA by direct titration [40], as well as for OLR and temperature. Seven samples of affluent vinasse were also analyzed for individual concentrations of alcohols (methanol - Meth, and ethanol - Eth) and acids (acetic - HAc, propionic - HPr, butyric - HBu, valeric – HVa, and caproic - HCa) by gas chromatography (GC) with flame ionization detector (FID), as previously described [41]. Additionally, six samples of vinasse were analyzed for SO_4^{2-} by photometric analysis (kit Visocolor, following the manufacturer instructions) and for metals (copper-Cu, lead-Pb, chromium-Cr, iron-Fe, magnesium-Mg, manganese-Mn, zinc-Zn, calcium-Ca, sodium-Na and potassium-K) by using an atomic absorption spectrophotometer. The measured values for the operational and physicochemical parameters are shown as Supplementary Information (Table S1).

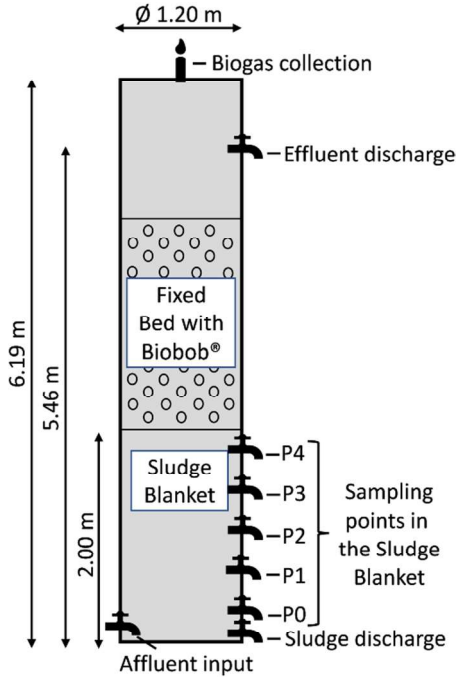

Fig. 1 Pilot-scale hybrid anaerobic reactor scheme

Identification of Parameters that Affect the Microbiota in Vinasse Digestion

The effect of physicochemical and operational parameters was investigated over the abundant microbiota of the bottom compartment (sludge blanket) of the HAnR, which had a greater COD removal and a more stable microbial community than the upper compartment. Abundant microorganisms included ASVs of *Bacteroidetes*, *Firmicutes*, *Cloacimonetes*, *Chloroflexi*, *Synergistetes*, *Deltaproteobacteria*, *Methanosarcinales*, *Methanobacteriales*, *Methanomicrobiales* and *Methanomassiliicoccales*, and some ASVs were identified as key microorganisms to CH_4 production [38].

Multivariate regression trees (MRT) were constructed to verify the changes in the ASVs abundance in response to the monitoring parameters, employing the composition of abundant ASVs by DNA and cDNA and the parameters of temperature, pH, VFA, COD and OLR. The individual concentrations of alcohols, acids, metals and SO_4^{2-} were not applied in MRT, since these parameters were not measured in all samples. The MRT were elaborated using the mvpart package v.1.6–2.6 [42] in R v.4.0.2 [43], with up to 4 divisions and at least 3 samples in each group. Graphs of the microbial abundance were associated to each leaf of MRT, and the significance between groups was investigated by the Kruskal-Wallis' test and Dunn's test followed by a Benjamini-Hochberg correction ($p \leq 0.025$), employing the dunn.test package v.1.3.5 [44] in R v.4.0.2 [43].

To complement the results, analysis of Spearman's rank correlation coefficients (SCC) were done using the corrplot package v.0.92 [45] in R v.4.0.2 [43]. In this analysis, the abundant ASVs, the individual metabolic redundancy and the ecological indexes of richness (Chao1), evenness (Pielou) and diversity (Neff-Shannon), previously reported [38], were correlated with the physicochemical and operational parameters. The individual concentrations of alcohols, acids, metals and SO_4^{2-} of vinasse were included in the SCC analysis, and the correlations were considered significant when $p \leq 0.05$.

Microbial Interactions in the Vinasse Digestion

The interactions among the abundant ASVs were analyzed through the time series of samples, to identify the patterns

of microbial co-occurrence by the DNA and cDNA along the HAnR operation and how the ASVs influence each other with time delay. The microbial interactions were evaluated by the SCC using the extended Local Similarity Analysis (eLSA) [46] with the Python script, and employing a time delay of 0–1, corresponding between 0 and 21 days of the HAnR operation. This analysis was conducted with the ASV relative abundance, and the co-occurrence interactions were considered significant when $p \leq 0.05$. Finally, Cytoscape v.3.10.1 [47] was employed to construct the interaction networks of ASVs evaluated by DNA and cDNA. The correlations between the microorganisms were discussed as a potential syntrophic association in the sludge blanket of the HAnR.

Results and Discussion

Effects of Monitoring Parameters Over Microbial Abundance, Metabolisms and Ecology

Microbial communities evaluated by both DNA and cDNA of the *16 S rrna* gene are affected by the monitoring parameters in AD process [13, 48]. By the DNA profile in the HAnR, the microbial abundance was mainly splitted by VFA concentration (3,700 mg.L^{-1}) in the affluent vinasse, followed by temperature (30.7 °C) and pH (5.6) (Fig. 2a). Conditions of VFA ≥ 3,700 mg L^{-1}, temperature < 30.7 °C and pH ≥ 5.6 increased the abundance of 5716-*Paludibacteraceae*-H1 and 109-*Methanospirillum*, positively correlated to $\%CH_4$ in biogas during HAnR's operation [38]. Meanwhile, the abundance of microbiota evaluated by cDNA was splitted by temperature (29.8 °C), COD (31.4 g.L^{-1}) and OLR (9.7 kg.COD.m^{-3}.d^{-1}) in the HAnR (Fig. 2b). The maintenance of temperature ≥ 29.8 °C, COD < 31.4 g L^{-1} and OLR < 9.7 kg COD m^{-3} d^{-1} promoted the greatest abundance of 2442-*Bacteroidetes*-vadinHA17, 6826-*Prolixibacteraceae*, 24,995-*Desulfovibrio* and 27,908-*Syntrophobacter* by cDNA, all positively correlated to $\%CH_4$ [38].

Additionally, the effects of temperature, pH, VFA, COD and OLR over microbiota were reinforced by the SCC analysis, also pointing out the impacts of sulfate and metals over the microbial abundance (Fig. 3a), metabolisms (Fig. 3b) and ecological indexes (Fig. 3c) by both DNA and cDNA.

Temperature

The temperature in the HAnR was between 24 and 32 °C and affected some microorganisms. Higher abundance of *Bacteroidetes* by increasing temperature from 25 °C to 35 °C occurred in AD of potato peel [16], similar to 2442-vadinHA17 (Fig. 2b) and 6826-*Prolixibacteraceae*, favored by increased mesophilic temperatures (≥ 29.8 °C) in the HAnR (Figs. 2b and 3a). Meanwhile, temperature change from mesophilic to thermophilic conditions in AD led to lower abundance of *Bacteroidetes*, including vadinHA17 and *Prolixibacteraceae* [13, 17, 49, 50], which may impact the hydrolysis and acidogenesis steps in vinasse AD. Mesophilic temperature seems to also be adequate for the development of *Deltaproteobacteria*, since the increase of temperature from 37 °C to 40 °C reduced its abundance in AD of sludge [17]. In the HAnR, while 24,995-*Desulfovibrio* and 27,908-*Syntrophobacter* were favored by temperature increase until 32 °C, 26,623-*Smithella* was less tolerant to this condition (Fig. 2b), suggesting different responses to temperature within the *Deltaproteobacteria* class. For the *Firmicutes* phylum, members are abundant in anaerobic reactors both under mesophilic and thermophilic conditions [51], which explains the absence of significative effects of temperature over 16,739-*Dehalobacterium* and 18,644-*Ruminococcaceae* in the HAnR (Figs. 2b and 3a). During AD of sludge, the abundance of *Ruminococcaceae* was also not affected by temperatures between 37 and 53 °C [50]. In contrast, members of *Clostridia* decreased their abundance after temperature reduction from 35 °C to 20 °C in co-digestion of cow manure and corn straw [15], suggesting less tolerance to lower temperatures.

Concerning the members of the *Cloacimonetes* phylum, lower abundance was reported at thermophilic AD systems [17, 50]. In the HAnR, 11,341 and 11,361-*Candidatus* Cloacimonas were positively correlated with temperature up to 32 °C (Fig. 3a). Additionally, positive correlation between *Candidatus* Cloacimonas and temperature up to 45 °C was recently reported in AD [52], suggesting some members of *Cloacimonetes* may resist to high temperatures. In the *Chloroflexi* phylum, 9443-*Anaerolineaceae* was negatively affected by temperature up to 32 °C in the HAnR (Fig. 3a). In contrast, changes in temperature from 37 to 44 °C kept stable the abundance of *Anaerolineaceae*, but at 52 °C their abundance also decreased [17, 50], evidencing distinct tolerances of *Anaerolineaceae* to temperature changes. For the *Synergistetes* phylum, members of *Synergistaceae* increased their abundance at temperatures ranging between 37 and 43 °C [50], similarly to 30,759-Syner-01 with temperature increase in the HAnR (Figs. 2b and 3a).

Regarding the methanogenic archaea, *Methanosaeta* was more abundant at 37 °C, and decreased its abundance with the temperature increase to 41–53 °C [50, 52]. In the HAnR, the ideal conditions for the abundance of *Methanosaeta* were < 29.8 °C (Fig. 2b), being negatively affected by higher temperature (Fig. 3a). Additionally, *Methanomassiliicoccus* were more abundant at 15 °C than at 35 °C during the digestion of potato peel [16], contrasting with the positive correlation between 409-*Methanomassiliicoccaceae* and the temperature

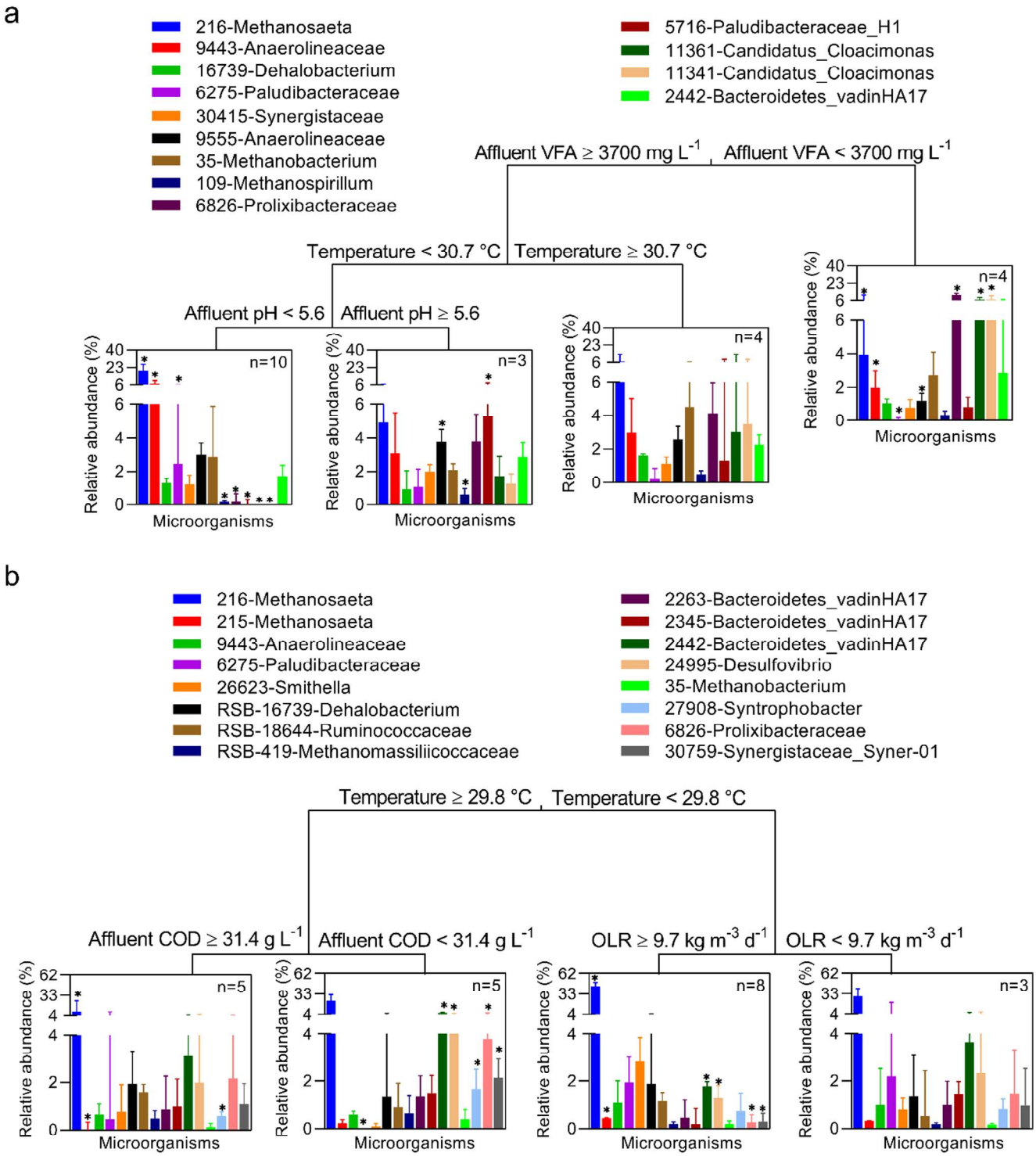

Fig. 2 Multivariate regression tree (MRT) of monitoring parameters that influenced abundant microbial ASV in the sludge blanket in the HAnR's operation. **(a)** MRT of abundant microorganisms by DNA analysis of the *16 S rrna* gene. **(b)** MRT of abundant microorganisms by cDNA analysis of the *16 S rrna* gene. COD = chemical oxygen demand; OLR = organic loading rate; VFA = volatile fatty acid. * $p \leq 0.25$ by the Kruskal-Wallis and Dunn's test with Benjamini-Hochberg correction

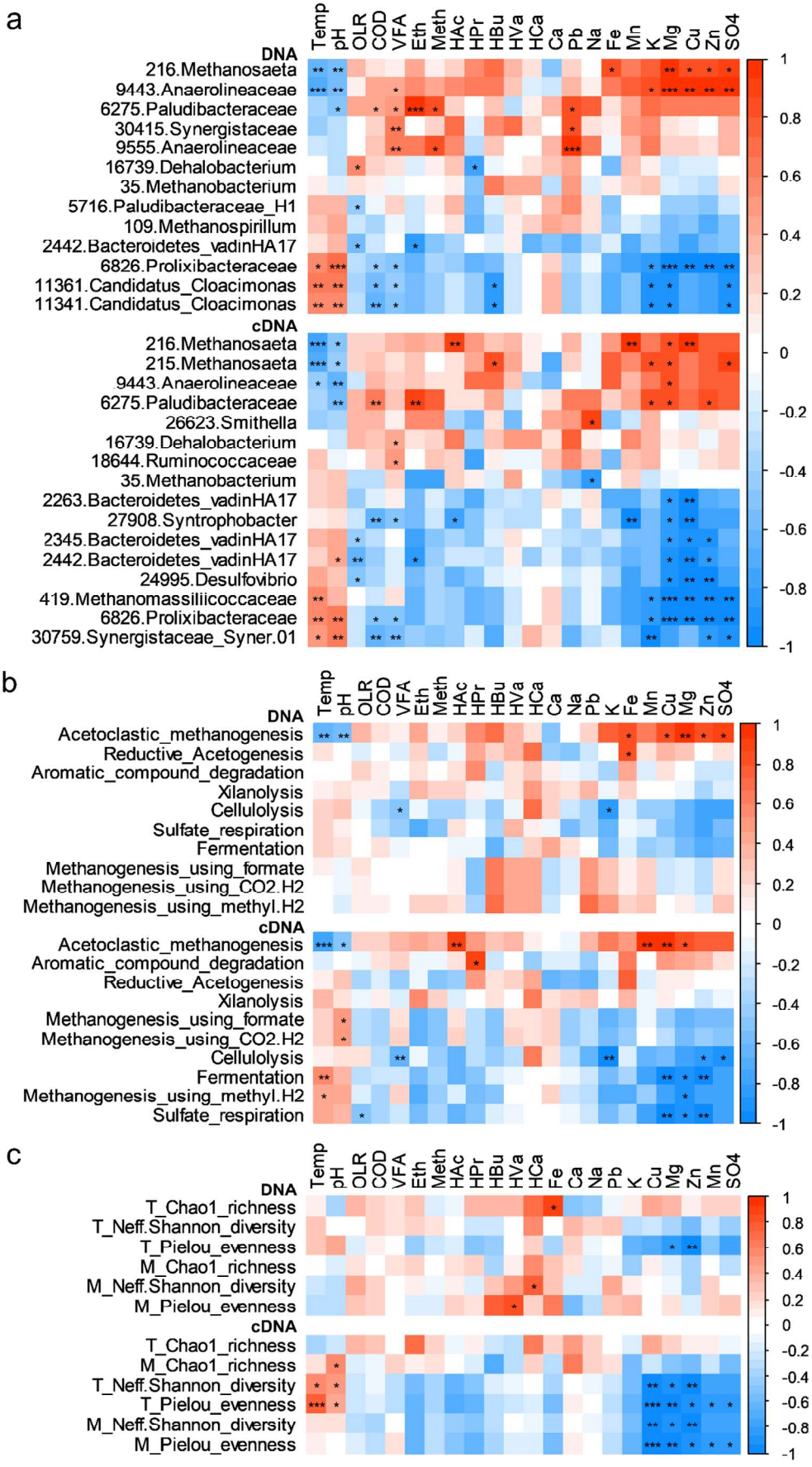

Fig. 3 Matrix of Spearman's rank correlation coefficient (SCC) between parameters and **(a)** abundant microorganisms, **(b)** microbial functional redundancy, and **(c)** microbial ecology, by DNA and cDNA analysis of *16 S rrna* gene. Temp = temperature. OLR = organic loading rate. COD = chemical oxygen demand. VFA = volatile fatty acids. Eth = ethanol. Meth = methanol. HAc = acetic acid. HPr = propionic acid. HBu = butyric acid. HVa = valeric acid. HCa = caproic acid. T = taxonomic. M = metabolic * $p \leq 0{,}05$; ** $p \leq 0{,}01$; *** $p \leq 0{,}001$

in the HAnR (Fig. 3a). Therefore, different responses to temperature may occur among methanogens at the genus level.

Temperature was positively correlated to fermentation redundancy by cDNA analysis (Fig. 3b). In the co-digestion of cow manure and corn straw, the fermentation was lower at 20 °C than at 35 °C, possibly affecting the activity of microbial intracellular enzymes of acidogenesis [15]. Compared to high temperatures, there was a decrease in the fermentation of propionic and butyric acid at 10 °C and 20 °C, respectively, suggesting a negative effect of low temperatures over acetogenesis in the co-digestion of cattle slurry and maize straw [53]. On the other hand, acetogenesis was stable at 35 °C, but was negatively affected at 55 °C in the digestion of food waste, as a result of microbial alterations at thermophilic conditions [49]. Therefore, fermentation processes can be impacted depending on the microbial tolerance to temperature, and mesophilic temperatures up to 32 °C in the HAnR were suitable for this metabolism. Redundancy of acetoclastic methanogenesis was negatively affected by temperature, while methanogenesis using methyl + H_2 was favored by temperature through cDNA (Fig. 3b). Hydrogenotrophic/methylotrophic methanogenesis were also favored by increased temperatures, although the acetoclastic pathway prevailed at 37 °C in AD of sludge [50]. In another study, acetoclastic methanogenesis mediated by *Methanosarcina* was abundant even at 45 °C, while the hydrogenotrophic pathway mediated by *Methanobacteriaceae* prevailed mainly at lower temperatures [53], evidencing distinct responses of methanogenic pathways to temperature according to specific archaeal tolerances.

The temperature of vinasse AD in the HAnR was positively correlated with the taxonomic evenness and diversity by cDNA (Fig. 3c). Other studies reported higher microbial diversity at 37 °C than at thermophilic conditions [17, 52]. Additionally, both the evenness and diversity were greater at 20–37 °C, decreasing at lower or higher temperatures [53]. This indicates that the temperature of vinasse digestion up to 32 °C in the HAnR was adequate to maintain the taxonomic evenness and diversity of microbes evaluated by cDNA, both positively correlated with %CH_4 in the biogas [38]. At mesophilic temperatures, the microbial community tends to be more diverse than at thermophilic conditions, allowing greater resilience to surpass disturbances in the operation and keeping the stability of vinasse digestion [11], which was also observed in the HAnR.

pH and VFA

Some microorganisms in the HAnR were influenced by pH ranging from 4.2 to 9.7. In the AD of activated sludge, *Bacteroidetes* phylum dominated the microbial community at pH 5.0 [21]. Only 6275-*Paludibacteraceae* was favored at pH < 5.6 (Fig. 2a) and was negatively correlated to pH values (Fig. 3a), while 6826-*Prolixibacteraceae* and 5716-*Paludibacteraceae*-H1 were less abundant under those conditions (Fig. 2a) and 6826-*Prolixibacteraceae* and 2442-vadinHA17 had positive correlation to pH in the HAnR (Fig. 3a). Similarly, at pH 7.0–7.8.0.8 in the AD of manure, positive and negative correlations were reported between the pH and *Bacteroidetes* members evaluated by DNA or cDNA [13]. A decrease in the abundance of *Bacteroidetes* was observed by reducing the pH from 8.1 to 7.3 also in the digestion of manure [54] and from 7.0 to 5.0 in the digestion of sugar refinery wastewater [19]. Additionally, there was a positive correlation between alkaline pH and *Bacteroidetes* members in AD of potato peel [16]. Clearly, *Bacteroidetes* microbes respond distinctly to pH variability, although the reactor operation at alkaline conditions could be adequate for the occurrence of this phylum [51].

Members of *Synergistaceae* were affected by pH in the digestion of sugar refinery wastewater and were less abundant at pH 7 than at acid conditions [19]. In contrast, the positive correlation between pH and 30,759-Syner-01 in the HAnR (Fig. 3a) indicates that the increased pH favored this bacterium and evidence adaptability of the *Synergistetes* phylum to pH ranges. The *Chloroflexi* phylum was also affected by pH in anaerobic reactors and was more abundant at acid pH than at neutral ones in the digestion of activated sludge [21], which was also observed for members of *Anaerolineaceae* class in the digestion of sugar refinery wastewater [19]. Similarly, 9443-*Anaerolineaceae* was abundant at pH < 5.6 (Fig. 2a) and it was negatively correlated with pH (Fig. 3a). However, in sludge fermentation, *Anaerolineaceae* was abundant even at pH 10 [55], suggesting flexibility to pH variations within this family. For the *Firmicutes* phylum, a great tolerance to pH from acid to alkaline occurs in anaerobic reactors [51], as also reported in the biorefinery wastewater digestion [19] and in the HAnR, by the absence of significative effects of pH on 16,739-*Dehalobacterium* and 18,644-*Ruminococcaceae* (Figs. 2 and 3a). On the other hand, *Ruminococcaceae* decreased its abundance with pH ranging from 6.5 to 8.5 in digestion of manure [56] and was negatively correlated with pH 7.0–8.0 in co-digestion of vinasse and manure [57].

Methanosaeta can resist to acid conditions in AD [51], explaining its high abundance under pH < 5.6 (Fig. 2a), and its negative correlation to pH (Fig. 3a). In contrast, *Methanosaeta* was positively correlated to pH ranging from 6.1 to 8.4 in digestion of potato peel, being favored at alkaline conditions [16], and it was more abundant at pH 6.0–6.5.0.5 than at lower values [19, 21]. Those results suggest different tolerances of *Methanosaeta* to pH in the system. Concerning other methanogens, *Methanospirillum* and a member of *Methanomassiliicoccaceae* were

positively correlated with pH 6.1–8.4 in AD of food waste [16]. However, these archaea did not significative correlate with pH in the HAnR (Fig. 3a), although 109-*Methanospirillum* showed more abundance at pH ≥ 5.6 (Fig. 2a), reinforcing the preference of hydrogenotrophic methanogens to alkaline conditions. The pH of vinasse also affected the redundancy of methanogenesis, being positively correlated with hydrogenotrophic pathways and negatively correlated to the acetoclastic one (Fig. 3b). By reducing the pH from 7.0 to 5.0, hydrogenotrophic methanogenesis was totally suppressed in AD of sugar refinery wastewater, while acetoclastic methanogenesis persisted [19], similar to the correlation results in the HAnR.

Concerning the ecological indexes, the taxonomic evenness and diversity by cDNA, previously related to high CH_4 in biogas [38], had positive correlation to pH, which also occurred for the metabolic richness by cDNA (Fig. 3c). In the digestion of sugar refinery wastewater, microbial diversity and richness were also affected by pH, and they were higher at neutral pH than at acid conditions [19], similar to what occurred in vinasse digestion.

The microbiota in the HAnR was also affected by VFA, varying from 2,073 to 7,369 $mg.L^{-1}$. The *Bacteroidetes* phylum have hydrolytic and acidogenic bacteria that contribute to the VFA production through degradation of complex organic matter [13, 21, 50, 58]. Despite that, bacteria of this phylum had distinct responses to the VFA in the HAnR (Figs. 2 and 3a), including 6826-*Prolixibacteraceae* with high abundance at VFA < 3,700 $mg.L^{-1}$ (Fig. 2a) and negatively correlated to VFA in vinasse through the DNA and cDNA analysis (Fig. 3a), pointing out a low tolerance to acids. In contrast, the low abundance of 6275-*Paludibacteraceae* when VFA < 3,700 $mg.L^{-1}$ (Fig. 2a) and the positive correlation to VFA in vinasse by DNA (Fig. 3a) suggest that high acid concentration benefits this bacterium. Moreover, the abundant ASVs of vadinHA17 did not show significant correlations to VFA in affluent vinasse, similar to reported in the co-digestion of manure and vinasse for produced VFA at concentrations up to 13,000 $mg.L^{-1}$ [57], reflecting both tolerance and a high contribution to acids production. From the *Firmicutes*, *Ruminococcaceae* had positive correlation to the VFA concentrations higher than 12,500 $mg.L^{-1}$ in the co-digestion of vinasse and manure [57], as also observed in the HAnR for 18,644-*Ruminococcaceae* by cDNA (Fig. 3a). In the digestion of cow manure, positive correlation also occurred between this microorganism and the production of HAc (73.9 $mg.L^{-1}$), HPr (106.7 $mg.L^{-1}$) and HBu (114.5 $mg.L^{-1}$) [56], although 18,644-*Ruminococcaceae* did not correlate to the individual concentrations of these acids up to 36.7 $mg.HBu.L^{-1}$ and 201.5 $mg.HAc.L^{-1}$ in vinasse (Fig. 3a). Those results indicate high tolerance of *Ruminococcaceae* to acids in the residue or produced in the system.

In the co-digestion of pig manure and vinegar, the acidogenic bacteria *Candidatus* Cloacimonas did not correlate to the total VFA concentrations up to 2,850 $mg.L^{-1}$ produced in the system, or to the individual concentrations of HBu or HPr, although there was a tendency to negative correlation [59]. In fact, ASVs of this genus had higher abundance at VFA < 3,700 $mg.L^{-1}$ in vinasse (Fig. 2a) and were negatively correlated to both total VFA and HBu (Fig. 3a). On the other hand, in the AD of sludge, *Candidatus* Cloacimonas were positively correlated with VFA, but at concentrations up to 500 $mg.L^{-1}$ [52]. These observations indicate that *Candidatus* Cloacimonas is favored at low VFA concentrations, but it is harmed by the increase of acids in the reactor. *Chloroflexi* phylum is associated to VFA production by fermentation in AD [18, 55]. At VFA > 12,500 $mg.L^{-1}$, negative correlation occurred between *Anaerolineaceae* and the acids in the co-digestion of vinasse and manure [57]. However, positive correlations between ASVs of *Anaerolineaceae* and VFA in the HAnR (Fig. 3a) suggest these bacteria may resist the acid concentrations in vinasse reaching 7,300 $mg.L^{-1}$. In the same study of co-digestion, *Synergistaceae* was associated to VFA conversion into H_2, and it had negative correlation with VFA up to 13,000 $mg.L^{-1}$ [57], similar to observed to 30,759-Syner-01 by cDNA and contrasting to 30,415-*Synergistaceae* by DNA (Fig. 3a). Those results evidence distinct responses of abundant *Synergistetes* in the system, related to acid production or consumption and to different tolerances.

Concerning the archaeal community, a positive correlation was reported between *Methanosaeta* and the concentrations of VFA up to 500 $mg.L^{-1}$ in the digestion of sludge [52]. In the HAnR, the lower abundance of *Methanosaeta* occurred with VFA < 3,700 $mg.L^{-1}$ (Fig. 3a), and this archaeon also had positive correlation to HAc and HBu (Fig. 4a). Additionally, the absence of correlations between 35-*Methanobacterium* and 109-*Methanospirillum* and several monitoring parameters suggests adaptability of the hydrogenotrophic archaea to the conditions of vinasse treatment in the HAnR, contributing to the resilience of the system. Finally, the negative correlation of cellulolysis and total VFA (Fig. 3b) could reflect the inhibition of cellulose degradation at VFA over 2,000 $mg.L^{-1}$, as previously discussed [59].

COD and OLR

Other parameters influencing the abundant microbiota included COD and OLR, related to organic matter. In the HAnR, COD values were between 23.2 and 42.4 $g.L^{-1}$, while OLR varied between 4.5 and 16.3 $kg.COD.m^{-3}.d^{-1}$. Low OLR during AD contributed to the establishment of *Bacteroidetes*, with high abundance at 8 kg.COD.

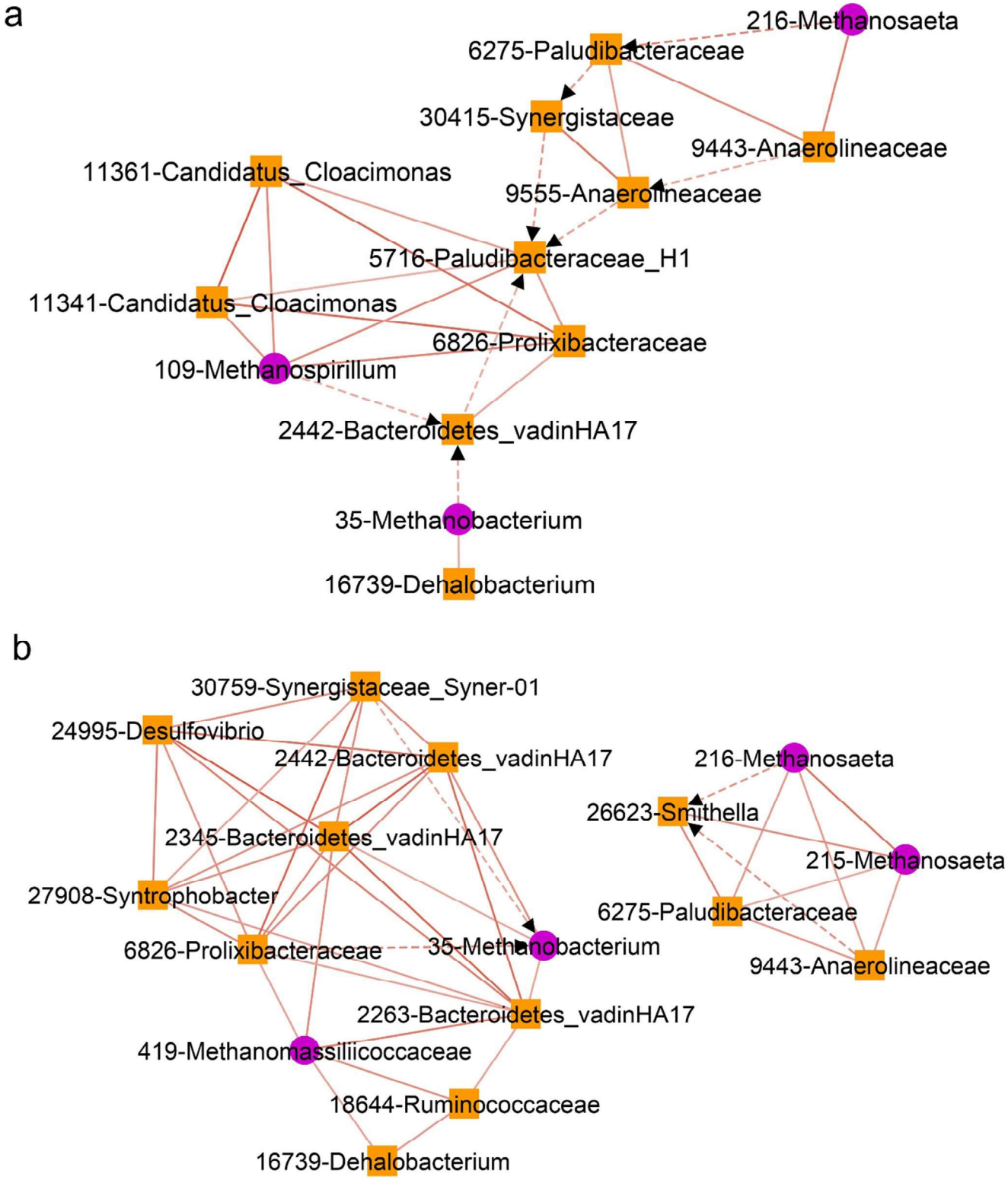

Fig. 4 Network of significative ($p \leq 0.05$) and positive microbial interactions based on the temporal cooccurrence between abundant archaea (purple circle) and bacteria (orange square) in the sludge blanket during HAnR's operation, according to the Spearman's rank correlation coefficient (SCC) identified by the eLSA analysis. **(a)** Microbial interaction network through DNA analysis of *16 S rrna*. **(b)** Microbial interaction network through cDNA analysis of *16 S rrna*. The arrows on the dashed lines indicate the direction of time delay between the nodes (one sample period, between 7 and 21 days)

$m^{-3}.d^{-1}$ [51], which explains the decreased abundance of 6826-*Prolixibacteraceae* and 2345 and 2442-vadinHA17 at OLR ≥ 9.7 kg.COD.$m^{-3}.d^{-1}$ (Fig. 2b) and the negative correlation between 2345 and 2442-vadinHA17 and 5716-*Paludibacteraceae*-H1 and OLR (Fig. 3a). In co-digestion of vinasse and glycerol, members of *Bacteroidetes* were also abundant at 10 kg.COD.$m^{-3}.d^{-1}$ [60]. Moreover, during the digestion of sugar refinery wastewater, the higher abundance of *Bacteroidetes* was observed at OLR 54 kg.COD.$m^{-3}.d^{-1}$, compared to lower OLR [27]. Therefore, although high organic matter can be harmful to *Bacteroidetes*, some populations might be more tolerant, as observed to 6275-*Paludibacteraceae* positively correlated to COD in the HAnR both by DNA and cDNA (Fig. 3a). Regarding *Deltaproteobacteria*, *Desulfovibrio* was more abundant than *Smithella* at 10 kg.COD.$m^{-3}.d^{-1}$ in the vinasse co-digestion with glycerol [60]. In the HAnR, 26,623-*Smithella* was more abundant at OLR ≥ 9.7 kg.COD.$m^{-3}.d^{-1}$, while 24,995-*Desulfovibrio* was less abundant in those conditions (Fig. 2b) and was negatively correlated to OLR (Fig. 3a), revealing distinct tolerance of SRB to OLR values that contributes to microbiota adaptability and functional redundancy in the reactor under different environmental conditions.

In a study of vinasse digestion, one ASV of *Clostridiales* dominated the *Firmicutes* community and was more abundant at OLR 4.4 kg.COD.$m^{-3}.d^{-5}$ than at lower values [7]. Similarly, *Clostridia* showed high tolerance to OLR in AD of sugar refinery wastewater, being more abundant at 54 kg.COD.$m^{-3}.d^{-1}$ than at lower OLR [27]. In the HAnR, 16,739-*Dehalobacterium* presented positive correlation with OLR by the DNA analysis (Fig. 3a), corroborating that conditions of high OLR might favor the establishment of microorganisms related to *Firmicutes* [51]. For the *Synergistaceae* family, a reduction of abundance was reported at OLR 4.4 kg.COD.$m^{-3}.d^{-5}$, compared to lower OLR, which was associated to the vinasse toxicity [7], similar to the decrease of 30,759-Syner-01 at OLR ≥ 9.7 kg.COD.$m^{-3}.d^{-1}$ in the HAnR (Fig. 2b). Another study of biorefinery residue digestion also reported the decrease of *Synergistia* abundance by the increase of OLR from 12 to 54 kg.COD.$m^{-3}.d^{-1}$, reinforcing its lesser tolerance to organic overload [27]. Regarding methanogenic archaea, *Methanosaeta* showed tolerance to high OLR in the AD, being abundant even at OLR values of 28 kg.COD.$m^{-3}.d^{-1}$ [51]. Similarly, in the digestion of biorefinery wastewater, *Methanosaeta* increased its abundance at OLR up to 36 kg.COD.$m^{-3}.d^{-1}$ [27]. These observations explain the higher abundance of 215 and 216-*Methanosaeta* at OLR ≥ 9.7 kg.COD.$m^{-3}.d^{-1}$ seen here (Fig. 2b) and reflect a high adaptability of this archaeon to increased OLR. Lastly, the COD and OLR values in HAnR did not have great effects over the functional redundancy and the ecological indexes (Fig. 3b and c), possibly contributing to the reactor's stability and reflecting a resilient microbiota.

Sulfate and Metals

Sulfate and metals also influenced the microorganisms in the HAnR. High concentrations of sulfate and metals can impair the microbial community of AD and affect the efficiency of treatment and biogas production, although some microorganisms are more resistant or tolerant than others, which keeps the system's stability [9]. In the HAnR, 216-*Methanosaeta* had considerable resistance to sulfate and metals evidenced by positive correlations (Fig. 3a), prevailing over the others even at adverse conditions and keeping its high abundance. However, it did not reflect the greater %CH_4 in biogas by *Methanosaeta* [38], which can be possibly explained by the toxic effect caused by sulfate and metals over the hydrolytic and fermentative bacteria in AD [9, 61].

In fact, some bacteria evaluated by DNA or cDNA in the HAnR were negatively correlated with sulfate and/or metals (Fig. 3a), reinforcing that these microorganisms were harmed, possibly decreasing substrate production for methanogenesis. Nonetheless, potentially tolerant bacteria, like 6275-*Paludibacteraceae* and 9443-*Anaerolineaceae*, correlated positively to some metals and/or sulfate (Fig. 3a), still allowed the performance of HAnR together with 216-*Methanosaeta*. Methanogens related to *Methanosaetaceae* were also resistant to sulfate over 1,600 mg.L^{-1} in other studies of vinasse digestion [33, 62], similar to what we observed for the maximum values of 2,050 mg.SO_4^{2-}.L^{-1} during the HAnR's operation. Sulfate concentrations higher than 3,000 mg.L^{-1} may inhibit methanogenic archaea in vinasse digestion [4], which possible occurred in the HAnR for the less tolerant methanogen 419-*Methanomassiliicoccaceae*, negatively correlated to SO_4^{2-} (Fig. 3a). However, sulfate was positively correlated with acetoclastic methanogenesis, and did not affect the redundancy of other methanogenesis pathways in general (Fig. 3b), explaining the CH_4 production in the HAnR even under high SO_4^{2-} concentration. Moreover, the taxonomic and metabolic evenness, important to increase the %CH_4 in the biogas during the HAnR's operation [38] were negatively correlated to sulfate, potentially impacting the biogas quality. Other study shows that evenness was also impacted by increased SO_4^{2-} in the vinasse AD, which was related to a toxic effect to microorganisms in the system [33], as occurred in the HAnR.

Metals are important micronutrients that contribute to bacterial and archaeal activity in anaerobic consortia to degrade vinasse organic matter [63]. Metallic ions

are present in enzymes and act as cofactors for metabolic pathways in AD [10], which likely has favored the ASVs of *Methanosaeta* and the acetoclastic methanogenesis in the HAnR, shown by positive correlations to some metals, including K, Mg, Cu, Zn, Fe and Mn (Fig. 3a and b). Although important to microbial growth and enzymatic activity, metals concentration above the tolerance limit can lead to toxicity for the microbiota, impairing the anaerobic system [37]. It was reported that bacteria are more tolerant than archaea to the concentrations of metals as trace-element [64], contrasting to observed in the HAnR for the metals in sugarcane vinasse (Fig. 3a).

Regarding potassium concentration, values up to 3,270 mg.K.L^{-1} in vinasse were within the moderate inhibitory range for AD [31], which may explain the negative effect of this metal over 6826-*Prolixibacteraceae*, 11,341 and 11,361-*Candidatus* Cloacimonas, 30,759-Syner-01 and 419-*Methanomassiliicoccaceae* (Fig. 3a). In the HAnR, 6826-*Prolixibacteraceae* and 419-*Methanomassiliicoccaceae* likely contributed to the %CH_4 in biogas [38], indicating that K concentration may have harmed those microorganisms and consequently impaired the CH_4 production. The redundancy of cellulolysis was also negatively affected by K (Fig. 3b), corroborating the adverse effect of this metal in the HAnR. However, the potential tolerance of some microbes to K concentrations possibly contributed to the stability of COD removal in the HAnR. In fact, other study of vinasse digestion showed that microbiota was resilient to concentrations up to 6,000 mg.K.L^{-1}, sustaining COD removal [65].

At certain concentrations, copper acts as an enzymatic cofactor and is essential to microbial activity of protein and carbohydrate hydrolysis in anaerobic reactors [37]. This metal promotes the activity of bacterial cellulase enzyme and the production of VFA, but high concentrations inhibit enzymatic activity and methanogenic growth, with the ideal range between 5 and 30 mg.Cu.L^{-1} for biogas generation [35]. Concentrations lower than 2.2 mg.Cu.L^{-1} did not cause inhibitory effects on the vinasse digestion [31]. Moreover, the addition of 0.5 mg.Cu.L^{-1} in waste food digestion improved the production of biogas, while higher concentrations impaired this process [66]. In the HAnR, vinasse had concentrations up to 0.4 mg.Cu.L^{-1}, and some microorganisms, metabolisms and ecological indexes were negatively correlated with this metal, including 6826-*Prolixibacteraceae*, 2263, 2345 and 2442-*Bacteroidetes*-vadinHA17, 27,908-*Syntrophobacter*, 24,995-*Desulfovibrio*, 419-*Methanomassiliicoccaceae*, the fermentative metabolism and the taxonomic and metabolic evenness and diversity (Fig. 3), all previously positively correlated with the %CH_4 in the biogas [38]. This reflects a negative impact of Cu on the release of methanogenic substrates through fermentation, by harming the acidogenic and acetogenic microorganisms in AD [37]. Therefore, the effect of Cu even at low concentration over microbiota, metabolisms and microbial ecology potentially impaired the biogas production in the HAnR. However, 9443-*Anaerolineaceae* and 216-*Methanosaeta* were tolerant to Cu concentrations evidenced by the positive correlation (Fig. 3a), likely contributing to the HAnR's performance even at adverse conditions.

Inhibitory effects on the AD were not detected at concentrations of 2.2 mg.Zn.L^{-1} in vinasse [31]. In the HAnR, vinasse had lower concentrations, adding up to 1.1 mg.Zn.L^{-1}, but some microbes positively correlated to %CH_4, such as 6826-*Prolixibacteraceae*, 2263, 2345 and 2442-*Bacteroidetes*-vadinHA17, 24,995-*Desulfovibrio*, 27,908-*Syntrophobacter* and 419-*Methanomassiliicoccaceae*, besides the metabolic and taxonomic diversity and evenness [38], were disfavored by zinc, showing negative correlation (Fig. 3a) and suggesting an adverse effect of this metal over the biogas quality. The addition of 1 mg.Zn.L^{-1} also had a high inhibitory effect to biogas production and harmed the occurrence of *Methanomassiliicoccaceae* in AD of synthetic wastewater [67]. Moreover, the redundancy of cellulolysis and fermentation by cDNA, the last one positively correlated with %CH_4 [38], were also negatively correlated to Zn (Fig. 3b). On the other hand, concentrations between 0.2 and 7.0 mg.Zn.L^{-1} did not limit microbial growth and CH_4 production in the vinasse AD elsewhere [68], and values up to 5 mg.Zn.L^{-1} favored the fermentation in another study [35], which reflect distinct microbial responses to Zn. In the HAnR, tolerant microorganisms included 6275-*Paludibacteraceae*, 9443-*Anaerolineaceae* and 216-*Methanosaeta*, positively correlated to Zn (Fig. 3b). The tolerance of *Methanosaeta* to 1 mg.Zn.L^{-1} was previously reported, although promoting less biogas production under this condition [67], similar to what was observed in this study.

Concentrations over 1,000 mg.Mg.L^{-1} normally characterize a moderate inhibition of AD, but it did not affect vinasse digestion in a previous study [31]. In contrast, some microorganisms positively correlated with %CH_4 in the HAnR, like 6826-*Prolixibacteraceae*, 2345 and 2442-*Bacteroidetes*-vadinHA17, 24,995-*Desulfovibrio*, 27,908-*Syntrophobacter* and 419-*Methanomassiliicoccaceae* [38], were negatively correlated to this metal (Fig. 3a) which added up to 367 mg.Mg.L^{-1}, pointing out a potentially harmful effect of magnesium over CH_4 generation. Additionally, the redundancy of fermentation and methanogenesis using methyl and H_2, as well as the taxonomic and metabolic evenness and diversity, also positively correlated to the %CH_4 [38], were also disfavored by Mg through cDNA (Fig. 3b and c), reinforcing that this metal impairs the biogas quality. As observed for other metals, 6275-*Paludibacteraceae*, 9443-*Anaerolineaceae*, and 215 and 216-*Methanosaeta* had

tolerance to Mg evidenced by positive correlations (Fig. 3a), potentially contributing to the system's maintenance.

In vinasse, concentrations of 7 mg.Fe.L^{-1} increased abundance or activity of key microorganisms of anaerobic processes, allowing stable operational conditions and greater efficiency in the treatment and CH_4 production [63]. On another study, concentrations between 2.0 and 22.0 mg.Fe.L^{-1} in the vinasse digestion were also favorable to microbial growth and CH_4 production [68]. Similarly, microbiota was not harmed by concentrations up to 18.9 mg.Fe.L^{-1} in the HAnR, suggesting adequate concentrations of iron for vinasse digestion. For lead, concentrations below 0.2 mg.Pb.L^{-1} were shown to favor AD [37], which also occurred in the HAnR for 6275-*Paludibacteraceae*, 9555-*Anaerolineaceae* and 30,415-*Synergistaceae* by DNA, favored by concentrations up to 0.3 mg.Pb.L^{-1} (Fig. 3a). For calcium, concentrations in the HAnR added up to 2,800 mg.Ca.L^{-1}, being within the range of moderate inhibition of AD [31]. However, the evaluated microorganisms, metabolisms and ecological indexes in the HAnR were tolerant to this metal (Fig. 3). Regarding sodium, values of 182.5 mg.Na.L^{-1} in vinasse digestion did not cause inhibitory effects to the microbiota [31], as observed for the concentrations up to 161 mg.Na.L^{-1} in the HAnR, that only impaired 35-*Methanobacterium* by cDNA (Fig. 3a). Moreover, concentrations of 0.1 mg.Mn.L^{-1} in the AD of rice straw favored AD, but increasing up to 1,000 mg.Mn.L^{-1} modified the microbiota and negatively affected the hydrolysis and methanogenesis, causing an irreversible effect on the system [64]. The maximum manganese concentration of 5.4 mg.Mn.L^{-1} in the HAnR favored 216-*Methanosaeta* and the redundancy of acetoclastic methanogenesis by cDNA, only harming 27,908-*Syntrophobacter* and the taxonomic and metabolic evenness (Fig. 3).

In general, although the adverse conditions in the HAnR have impaired some microorganisms that contribute to organic matter degradation and biogas production, other microbial groups that also act on those processes were favored and kept the AD and the CH_4 generation ongoing, even with lower efficiency. These results reinforce that the stability of AD in scaled-up reactors also depends on the occurrence of functional redundancy and a resilient microbial community [7, 33].

Microbial Interactions in the Vinasse Digestion

The synergy between the bacterial and archaeal communities cooccurring in the AD are essential to the adequate performance of reactors [62]. Although methanogens are important as CH_4 productors in anaerobic systems, they must interact in parallel with hydrolytic and fermentative bacterial groups that convert the complex organic matter into H_2, CO_2, formate and acetate, substrates for the methanogenesis [8, 69]. Concerning the interactions between microorganisms in the sludge blanket of the HAnR explored by SCC analysis through time series, *Candidatus* Cloacimonas can participate in syntrophic consortia with microorganisms that utilize H_2, including the hydrogenotrophic methanogenic archaea and acetogenic or sulfate reducing bacteria [70], explaining its interaction with *Methanospirillum* (Fig. 4a). Similarly, the enrichment of *Candidatus* Cloacimonas was reported with hydrogenotrophic methanogens in the mesophilic co-digestion of cow and sheep manure [71]. Meanwhile, *Candidatus* Cloacimonas and *Methanosaeta*, responsible for the acetate production and consumption, respectively, correlated to each other in vinasse digestion [62]. Those microorganisms did not interact in the HAnR possibly due to their occurrence under different conditions of temperature, pH and organic load, besides their distinct tolerances to sulfate and metals concentrations.

Members of vadinHA17 could have contributed to the H_2 production by protein and amino acids degradation in the system, as pointed out previously [72], justifying the interaction between these bacteria and the hydrogenotrophic or H_2-dependent methylotrophic methanogens 35-*Methanobacterium*, 109-*Methanospirillum* or 419-*Methanomassiliicoccaceae* (Fig. 4a and b). Additionally, members of *Bacteroidetes* may also form a syntrophic relationship with SRB for organic matter degradation, as observed in the vinasse co-digestion with dairy effluent [5]. Such interactions occurred in the HAnR among 6275-*Paludibacteraceae* and 26,623-*Smithella* and ASVs of vadinHA17, 6826-*Prolixibacteraceae* and 24,995-*Desulfovibrio* and 27,908-*Syntrophobacter* (Fig. 4b), suggesting that the H_2 produced by *Bacteroidetes* phylum is also used by SRB microbes. The benefit for 2442-vadinHA17 mediated by 35-*Methanobacterium* and 109-*Methanospirillum* with time delay (Fig. 4a) reinforces that H_2 consumption by hydrogenotrophic methanogenic archaea may improve the establishment of syntrophic bacteria by reducing the H_2 partial pressure in the system [73]. On the other hand, the benefit for 35-*Methanobacterium* promoted by 6826-*Prolixibacteraceae* with time delay (Fig. 4b) seems to reflect the distinct conditions of microbial growth for bacteria and archaea [9, 73]. Additionally, interaction between *Synergistaceae* and *Methanobacteriaceae* were reported in the co-digestion of vinasse and manure, mediated by H_2 production and consumption, respectively, which was also observed between 35-*Methanobacterium* and 30,759-Syner-01 with time delay in the HAnR (Fig. 4b).

In the co-digestion of vinasse and glycerol, the acetate production by *Desulfovibrio* and *Smithella* favored the establishment of *Methanosaeta* [60], which seemed to

occur only by 26,623-*Smithella* in the HAnR, considering its interaction with 215-*Methanosaeta* (Fig. 4b). It is possible that 24,995-*Desulfovibrio* did not interact with *Methanosaeta* due to conflicting environmental conditions that favored each of these microorganisms. In contrast, other ASV interacting with *Methanosaeta* mainly through the cDNA analysis, including 6275-*Paludibacteraceae* and 9443-*Anaerolineaceae* (Fig. 4b), cooccurred at the same environmental conditions in the HAnR. These bacteria potentially produced acetate by organic matter degradation, which was later converted into CH_4 by *Methanosaeta*. In fact, it was suggested that members of *Anaerolineaceae* can release simple carbon sources, such as acetate, through complex organic matter fermentation, as pointed out in the co-digestion of vinasse with hemicellulose or manure [57, 74].

Microorganisms can perform several types of metabolisms, and microbial syntrophic relationships are mediated by metabolic co-dependence in organic matter conversion into biogas [13, 75]. Besides metabolic co-dependence, our results suggest that bacteria and archaea also interact respecting environmental conditions that favor their cooccurrence, such as temperature, pH, organic load and concentrations of sulfate and metals. Thus, even exchanging metabolites with each other, some microorganisms cannot interact if they prevail under different conditions in the reactor due to their specific tolerances. Temperature variation and organic overload in AD were reported to promote distinct microbial interaction, also reflecting different tolerance of microorganisms to disturbances in the system [13, 26]. Moreover, microbes may have distinct interactions under different nutritional conditions, affecting bioprocesses in the reactor [76], which potentially also occurred in the HAnR. Finally, further studies must focus on improving the knowledge about uncultured or unclassified microorganisms, allowing the deep exploration of functional profiles and microbial interactions [26].

Conclusions

The effects of physicochemical and operational parameters over microbiota were temporally evaluated in a pilot-scale HAnR during AD of sugarcane vinasse. Microbial abundance, functional redundancy and ecology were affected by temperature, pH, VFA, COD and OLR, besides sulfate and metal concentrations. Tolerant bacteria and archaea contributed to the functional redundancy and methane production even under adverse conditions in the system, but less efficiently. The findings revealed a complex scenario for structuring the microbial communities as a response to monitoring parameters during the bioconversion of organic matter into biogas, an important knowledge for further improvement of scaled-up AD reactors.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12155-025-10950-9.

Acknowledgements The authors thank São Paulo Research Foundation - Brasil (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) for supporting this study.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and formal analysis were performed by Gabrielle Maria Fonseca Pierangeli, Gustavo Bueno Gregoracci and Flávia Talarico Saia. The first draft of the manuscript was written by Gabrielle Maria Fonseca Pierangeli and all authors commented on previous versions of the manuscript and approved the final manuscript.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). This work was financed by the São Paulo Research Foundation - Brasil (FAPESP), Process Number #2017/24509-0, and by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, Process Number #88887.629454/2021-00.

Data Availability The data presented in this study is available in online repository: https://www.ncbi.nlm.nih.gov/, SRA PRJNA1020541, and in Supplementary Information.

Declarations

Competing interests The authors declare no competing interests.

References

1. Weiland P (2010) Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85:849–860. https://doi.org/10.1007/s00253-009-2246-7
2. Parsaee M, Kiani Deh Kiani M, Karimi K (2019) A review of biogas production from sugarcane vinasse. Biomass Bioenergy 122:117–125. https://doi.org/10.1016/j.biombioe.2019.01.034
3. Almeida WA, Ratusznei SM, Zaiat M, Rodrigues JaD (2017) AnSBBR applied to biomethane production for vinasse treatment:

effects of organic loading, feed strategy and temperature. Braz J Chem Eng 34:759–773. https://doi.org/10.1590/0104-6632.20170343s20150584
4. España-Gamboa EI, Mijangos-Cortés JO, Hernández-Zárate G et al (2012) Methane production by treating vinasses from hydrous ethanol using a modified UASB reactor. Biotechnol Biofuels 5:82. https://doi.org/10.1186/1754-6834-5-82
5. Siqueira JCde, Assemany P, Siniscalchi LAB (2022) Microbial dynamics and methanogenic potential of co-digestion of sugarcane vinasse and dairy secondary effluent in an upflow anaerobic sludge blanket reactor. Bioresour Technol 361:127654. https://doi.org/10.1016/j.biortech.2022.127654
6. Paulo LM, Castilla-Archilla J, Ramiro-Garcia J et al (2020) Microbial community redundancy and resilience underpins high-rate anaerobic treatment of dairy-processing wastewater at ambient temperatures. Front Bioeng Biotechnol. https://doi.org/10.3389/fbioe.2020.00192
7. Callejas C, López I, Bovio-Winkler P et al (2022) Temporal analysis of the microbiota involved in the anaerobic degradation of sugarcane Vinasse in a full-scale methanogenic UASB reactor. Biomass Conv Bioref 12:3887–3897. https://doi.org/10.1007/s13399-021-01281-8
8. Maman LG, Palizban F, Fallah Atanaki F et al (2020) Co-abundance analysis reveals hidden players associated with high methane yield phenotype in sheep rumen microbiome. Sci Rep 10:4995. https://doi.org/10.1038/s41598-020-61942-y
9. Paulo LM, Stams AJM, Sousa DZ (2015) Methanogens, sulphate and heavy metals: a complex system. Rev Environ Sci Biotechnol 14:537–553. https://doi.org/10.1007/s11157-015-9387-1
10. Goswami R, Chattopadhyay P, Shome A et al (2016) An overview of physico-chemical mechanisms of biogas production by microbial communities: a step towards sustainable waste management. 3 Biotech 6:72. https://doi.org/10.1007/s13205-016-0395-9
11. Kiani Deh Kiani M, Parsaee M, Safieddin Ardebili SM et al (2022) Different bioreactor configurations for biogas production from sugarcane vinasse: a comprehensive review. Biomass Bioenerg 161:106446. https://doi.org/10.1016/j.biombioe.2022.106446
12. Li G, Xu F, Yang T et al (2023) Microbial behavior and influencing factors in the anaerobic digestion of distiller: a comprehensive review. Fermentation 9:199. https://doi.org/10.3390/fermentation9030199
13. Lin Q, De Vrieze J, Li J, Li X (2016) Temperature affects microbial abundance, activity and interactions in anaerobic digestion. Bioresour Technol 209:228–236. https://doi.org/10.1016/j.biortech.2016.02.132
14. Shaw GT-W, Weng C-Y, Chen C-Y et al (2019) A systematic approach re-analyzing the effects of temperature disturbance on the microbial community of mesophilic anaerobic digestion. Sci Rep 9:6560. https://doi.org/10.1038/s41598-019-42987-0
15. Wang S, Ma F, Ma W et al (2019) Influence of temperature on biogas production efficiency and microbial community in a two-phase anaerobic digestion system. Water 11:133. https://doi.org/10.3390/w11010133
16. Chen T, Zhang L, Guo W et al (2024) Temperature drives microbial communities in anaerobic digestion during biogas production from food waste. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-024-32698-z
17. Westerholm M, Isaksson S, Karlsson Lindsjö O, Schnürer A (2018) Microbial community adaptability to altered temperature conditions determines the potential for process optimisation in biogas production. Appl Energy 226:838–848. https://doi.org/10.1016/j.apenergy.2018.06.045
18. Harirchi S, Wainaina S, Sar T et al (2022) Microbiological insights into anaerobic digestion for biogas, hydrogen or volatile fatty acids (VFAs): a review. Bioengineered 13:6521–6557. https://doi.org/10.1080/21655979.2022.2035986
19. Zhang L, Ban Q, Li J, Wan C (2019) Functional bacterial and archaeal dynamics dictated by pH stress during sugar refinery wastewater in a UASB. Bioresour Technol 288:121464. https://doi.org/10.1016/j.biortech.2019.121464
20. Qiu S, Zhang X, Xia W et al (2023) Effect of extreme pH conditions on methanogenesis: methanogen metabolism and community structure. Sci Total Environ 877:162702. https://doi.org/10.1016/j.scitotenv.2023.162702
21. Latif MA, Mehta CM, Batstone DJ (2017) Influence of low pH on continuous anaerobic digestion of waste activated sludge. Water Res 113:42–49. https://doi.org/10.1016/j.watres.2017.02.002
22. Naspolini BF, Machado AC, de O, Cravo Junior WB et al (2017) Bioconversion of sugarcane Vinasse into High-Added value products and energy. Biomed Res Int 2017:8986165. https://doi.org/10.1155/2017/8986165
23. Siqueira LM, Damiano ESG, Silva EL (2013) Influence of organic loading rate on the anaerobic treatment of sugarcane vinasse and biogás production in fluidized bed reactor. J Environ Sci Health Tox Hazard Subst Environ Eng 48:1707–1716. https://doi.org/10.1080/10934529.2013.815535
24. Caillet H, Adelard L (2020) Start-up strategy and process performance of semi-continuous anaerobic digestion of raw sugarcane vinasse. Waste Biomass Valor. https://doi.org/10.1007/s12649-020-00964-z
25. Borges AdoV, Fuess LT, Takeda PY et al (2022) Co-digestion of biofuel by-products: enhanced biofilm formation maintains high organic matter removal when methanogenesis fails. J Environ Manage 310:114768. https://doi.org/10.1016/j.jenvman.2022.114768
26. Braz GHR, Fernandez-Gonzalez N, Lema JM, Carballa M (2019) Organic overloading affects the microbial interactions during anaerobic digestion in sewage sludge reactors. Chemosphere 222:323–332. https://doi.org/10.1016/j.chemosphere.2019.01.124
27. Zhang L, Ban Q, Li J (2018) Microbial community dynamics at high organic loading rates revealed by pyrosequencing during sugar refinery wastewater treatment in a UASB reactor. Front Environ Sci Eng 12:4. https://doi.org/10.1007/s11783-018-1045-8
28. Leite AF, Janke L, Lv Z et al (2015) Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: effects of increasing organic loading rate on methanogenic community dynamics. Int J Mol Sci 16:23210–23226. https://doi.org/10.3390/ijms161023210
29. Cabrera-Díaz A, Pereda-Reyes I, Oliva-Merencio D et al (2017) Anaerobic digestion of sugarcane vinasse through a methanogenic UASB reactor followed by a packed bed reactor. Appl Biochem Biotechnol 183:1127–1145. https://doi.org/10.1007/s12010-017-2488-2
30. Kiyuna LSM, Fuess LT, Zaiat M (2017) Unraveling the influence of the COD/sulfate ratio on organic matter removal and methane production from the biodigestion of sugarcane vinasse. Bioresour Technol 232:103–112. https://doi.org/10.1016/j.biortech.2017.02.028
31. Ramos-Vaquerizo F, Cruz-Salomón A, Ríos-Valdovinos E et al (2018) Anaerobic treatment of Vinasse from sugarcane ethanol production in expanded granular sludge bed bioreactor. J Chem Eng Process Technol 9:1–8. https://doi.org/10.4172/2157-7048.1000375
32. Borges AdoV, Fuess LT, Alves I et al (2021) Co-digesting sugarcane vinasse and distilled glycerol to enhance bioenergy generation in biofuel-producing plants. Energy Convers Manag 250:114897. https://doi.org/10.1016/j.enconman.2021.114897

33. Jiménez J, Barrera EL, De Vrieze J et al (2018) Microbial community dynamics reflect reactor stability during the anaerobic digestion of a very high strength and sulfate-rich vinasse. Journal of Chemical Technology & Biotechnology 93:975–984. https://doi.org/10.1002/jctb.5449
34. Peruzzo VV, Sachet FH, Torres APR et al (2018) Influence of sulfide on the evaluation of methane production through the degradation of sugarcane vinasse. Sci Cum Ind 6:1–6
35. Guo Q, Majeed S, Xu R et al (2019) Heavy metals interact with the microbial community and affect biogas production in anaerobic digestion: a review. J Environ Manage 240:266–272. https://doi.org/10.1016/j.jenvman.2019.03.104
36. Pierangeli GMF, da Silva KMR, Coelho LHG et al (2023) Effects of metal contamination with physicochemical properties on the sediment microbial communities in a tropical eutrophic-hypereutrophic urban reservoir in Brazil. Environ Sci Pollut Res Int 30:54961–54978. https://doi.org/10.1007/s11356-023-26114-1
37. Kadam R, Khanthong K, Jang H et al (2022) Occurrence, fate, and implications of heavy metals during anaerobic digestion: a review. Energies 15:8618. https://doi.org/10.3390/en15228618
38. Pierangeli GMF, Gregoracci GB, Del Nery V et al (2024) Long-term temporal dynamics of total and potentially active microbiota affect the biogas quality from the anaerobic digestion of vinasse in a pilot-scale hybrid anaerobic reactor. Bioresour Technol Rep 26:101822. https://doi.org/10.1016/j.biteb.2024.101822
39. APHA (2012) Standard methods for the examination of water and wastewater, 22nd edn. American Water Works Association, Water Environment Federation, Washington, D. C., New York, American Public Health Association
40. DiLallo R, Albertson OE (1961) Volatile acids by direct titration. J (Water Pollution Control Federation) 33:356–365
41. Adorno MAT, Hirasawa JS, Varesche MBA (2024) Development and validation of two methods to quantify volatile acids (C2-C6) by GC/FID: headspace (Automatic and Manual) and Liquid-Liquid extraction (LLE). https://doi.org/10.4236/ajac.2014.57049. ResearchGate
42. De'ath G, Thermeau TM, Atkinson B, Ripley B (2014) mvpart: Multivariate partitioning
43. R Core Team (2020) R: A Language and environment for statistical computing. R Foundation for Statistical Computing
44. Dinno A (2017) dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums
45. Wei T, Simko V, Levy M et al (2021) corrplot: Visualization of a Correlation Matrix
46. Xia LC, Steele JA, Cram JA et al (2011) Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates. BMC Syst Biol 5:S15. https://doi.org/10.1186/1752-0509-5-S2-S15
47. Shannon P, Markiel A, Ozier O et al (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 13:2498–2504. https://doi.org/10.1101/gr.1239303
48. De Vrieze J, Regueiro L, Props R et al (2016) Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. Biotechnol Biofuels 9:244. https://doi.org/10.1186/s13068-016-0652-5
49. Kim M-S, Kim D-H, Yun Y-M (2017) Effect of operation temperature on anaerobic digestion of food waste: performance and microbial analysis. Fuel 209:598–605. https://doi.org/10.1016/j.fuel.2017.08.033
50. Steiniger B, Hupfauf S, Insam H, Schaum C (2023) Exploring anaerobic digestion from mesophilic to thermophilic temperatures—operational and microbial aspects. Fermentation 9:798. https://doi.org/10.3390/fermentation9090798
51. Xu R, Fang S, Zhang L et al (2021) Distribution patterns of functional microbial community in anaerobic digesters under different operational circumstances: a review. Bioresour Technol 341:125823. https://doi.org/10.1016/j.biortech.2021.125823
52. Sudiartha GAW, Imai T, Chairattanamanokorn P, Reungsang A (2024) Unveiling the impact of temperature shift on microbial community dynamics and metabolic pathways in anaerobic digestion. Process Saf Environ Prot. https://doi.org/10.1016/j.psep.2024.04.121
53. Hupfauf S, Plattner P, Wagner AO et al (2018) Temperature shapes the microbiota in anaerobic digestion and drives efficiency to a maximum at 45°C. Bioresour Technol 269:309–318. https://doi.org/10.1016/j.biortech.2018.08.106
54. Kampmann K, Ratering S, Kramer I et al (2012) Unexpected stability of bacteroidetes and firmicutes communities in laboratory biogas reactors fed with different defined substrates. Appl Environ Microbiol 78:2106–2119. https://doi.org/10.1128/AEM.06394-11
55. Di Leto Y, Capri FC, Mineo A et al (2022) Initial pH conditions shape the microbial community structure of sewage sludge in batch fermentations for the improvement of volatile fatty acid production. Microorganisms 10:2073. https://doi.org/10.3390/microorganisms10102073
56. Castro-Ramos JJ, Solís-Oba A, Solís-Oba M et al (2022) Effect of the initial pH on the anaerobic digestion process of dairy cattle manure. AMB Express 12:162. https://doi.org/10.1186/s13568-022-01486-8
57. Qin Y, Huang L, Jiang Q et al (2022) Anaerobic co-digestion of molasses vinasse and three kinds of manure: a comparative study of performance at different mixture ratio and organic loading rate. J Clean Prod 371:133631. https://doi.org/10.1016/j.jclepro.2022.133631
58. Chen S, Cheng H, Wyckoff KN, He Q (2016) Linkages of firmicutes and bacteroidetes populations to methanogenic process performance. J Ind Microbiol Biotechnol 43:771–781. https://doi.org/10.1007/s10295-016-1760-8
59. Chen L, Meng X, Zhou G et al (2022) Effects of organic loading rates on the anaerobic co-digestion of fresh vinegar residue and pig manure: focus on the performance and microbial communities. Biochem Eng J 183:108441. https://doi.org/10.1016/j.bej.2022.108441
60. de Menezes CA, de Souza Almeida P, Camargo FP et al (2023) Two problems in one shot: vinasse and glycerol co-digestion in a thermophilic high-rate reactor to improve process stability even at high sulfate concentrations. Sci Total Environ 862:160823. https://doi.org/10.1016/j.scitotenv.2022.160823
61. Nguyen Q-M, Bui D-C, Phuong T et al (2019) Investigation of heavy metal effects on the anaerobic co-digestion process of waste activated sludge and septic tank sludge. Int J Chem Eng 2019:1–9. https://doi.org/10.1155/2019/5138060
62. Iltchenco J, Peruzzo V, Eva Magrini F et al (2021) Microbiota profile in mesophilic biodigestion of sugarcane vinasse in batch reactors. Water Sci Technol 84:2028–2039. https://doi.org/10.2166/wst.2021.375
63. de Barros VG, Duda RM, Vantini JdaS et al (2017) Improved methane production from sugarcane vinasse with filter cake in thermophilic UASB reactors, with predominance of methanothermobacter and methanosarcina archaea and thermotogae bacteria. Bioresour Technol 244:371–381. https://doi.org/10.1016/j.biortech.2017.07.106
64. Cai Y, Zheng Z, Zhao Y et al (2018) Effects of molybdenum, selenium and manganese supplementation on the performance of anaerobic digestion and the characteristics of bacterial community in acidogenic stage. Bioresour Technol 266:166–175. https://doi.org/10.1016/j.biortech.2018.06.061

65. López I, Borzacconi L, Passeggi M (2018) Anaerobic treatment of sugar cane vinasse: treatability and real-scale operation. J Chem Technol Biotechnol 93:1320–1327. https://doi.org/10.1002/jctb.5493
66. Mary CT, Swarnalatha K, Harishma SJ (2024) Experimental analysis on the effects of trace metals as micronutrients in enhancing biomethane production. Sustainable Energy Research 11:3. https://doi.org/10.1186/s40807-023-00093-w
67. Zhang X, Yang H, Wei D et al (2023) Tolerance of anaerobic digestion sludge to heavy metals: COD removal, biogas production, and microbial variation. J Water Process Eng 55:104157. https://doi.org/10.1016/j.jwpe.2023.104157
68. Santana Junior AE, Duda RM, Oliveira RAde (2019) Improving the energy balance of ethanol industry with methane production from vinasse and molasses in two-stage anaerobic reactors. J Clean Prod 238:117577. https://doi.org/10.1016/j.jclepro.2019.07.052
69. Pierangeli GMF, Domingues MR, de Jesus TA et al (2021) Higher abundance of sediment methanogens and methanotrophs do not predict the atmospheric methane and carbon dioxide flows in eutrophic tropical freshwater reservoirs. Front Microbiol. https://doi.org/10.3389/fmicb.2021.647921
70. Pelletier E, Kreimeyer A, Bocs S et al (2008) Candidatus cloacamonas acidaminovorans": genome sequence reconstruction provides a first glimpse of a new bacterial division. J Bacteriol. https://doi.org/10.1128/JB.01248-07
71. Li Y, Achinas S, Zhao J et al (2020) Co-digestion of cow and sheep manure: performance evaluation and relative microbial activity. Renew Energy 153:553–563. https://doi.org/10.1016/j.renene.2020.02.041
72. Mei R, Nobu MK, Narihiro T, Liu W-T (2020) Metagenomic and metatranscriptomic analyses revealed uncultured Bacteroidales populations as the dominant proteolytic amino acid degraders in anaerobic digesters. Front Microbiol. https://doi.org/10.3389/fmicb.2020.593006
73. Braga Nan L, Trably E, Santa-Catalina G et al (2022) Microbial community redundance in biomethanation systems lead to faster recovery of methane production rates after starvation. Sci Total Environ 804:150073. https://doi.org/10.1016/j.scitotenv.2021.150073
74. Adarme OFH, Baêta BEL, Torres MC et al (2022) Biogas production by anaerobic co-digestion of sugarcane biorefinery byproducts: comparative analyses of performance and microbial community in novel single-and two-stage systems. Bioresour Technol 354:127185. https://doi.org/10.1016/j.biortech.2022.127185
75. Zhu X, Campanaro S, Treu L et al (2020) Metabolic dependencies govern microbial syntrophies during methanogenesis in an anaerobic digestion ecosystem. Microbiome 8:22. https://doi.org/10.1186/s40168-019-0780-9
76. Nakasaki K, Nguyen KK, Ballesteros FC et al (2020) Characterizing the microbial community involved in anaerobic digestion of lipid-rich wastewater to produce methane gas. Anaerobe 61:102082. https://doi.org/10.1016/j.anaerobe.2019.102082