Bioresource Technology 376 (2023) 128922

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Unraveling prevalence of homoacetogenesis and methanogenesis pathways due to inhibitors addition

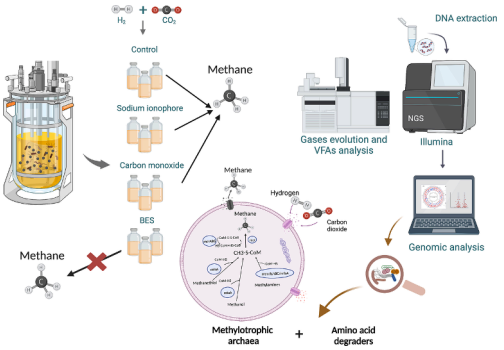
Rebecca Serna-García [a,*], Panagiotis Tsapekos [b], Laura Treu [c], Alberto Bouzas [a], Aurora Seco [a], Stefano Campanaro [c], Irini Angelidaki [b]

[a] CALAGUA – Unidad Mixta UV-UPV, Departament d'Enginyeria Química, Universitat de València, Avinguda de la Universitat s/n, 46100 Burjassot, Valencia, Spain
[b] Department of Chemical Engineering, Søltofts Plads 228A, Technical University of Denmark, DTU, 2800 Lyngby, Denmark
[c] Department of Biology, University of Padua, Via U. Bassi 58/b, Padova 35121, Italy

HIGHLIGHTS

- Methyl respiration was key pathway for CH_4 production in a biogas upgrading system.
- No CH_4 production was observed when adding 10, 25 or 50 mM of the inhibitor BES.
- A concentration of 60 μm of sodium ionophore (ETH2120) decreased acetate production.
- Homoacetogenic activity seems to be lower when applying 20 kPa CO.
- Amino acids fermenters and methylotrophic archaea dominated the microbial community.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biomethanation
Biogas upgrading
Methyl respiration
Bromoethanesulfonate
Anaerobic digestion microbiome

ABSTRACT

Three inhibitors targeting different microorganisms, both from Archaea and Bacteria domains, were evaluated for their effect on CO_2 biomethanation: sodium ionophore III (ETH2120), carbon monoxide (CO), and sodium 2-bromoethanesulfonate (BES). This study examines how these compounds affect the anaerobic digestion microbiome in a biogas upgrading process. While archaea were observed in all experiments, methane was produced only when adding ETH2120 or CO, not when adding BES, suggesting archaea were in an inactivated state. Methane was produced mainly via methylotrophic methanogenesis from methylamines. Acetate was produced at all conditions, but a slight reduction on acetate production (along with an enhancement on CH_4 production) was observed when applying 20 kPa of CO. Effects on CO_2 biomethanation were difficult to observe since the inoculum used was from a real biogas upgrading reactor, being this a complex environmental sample. Nevertheless, it must be mentioned that all compounds had effects on the microbial community composition.

* Corresponding author.
E-mail address: rebecca.serna@uv.es (R. Serna-García).

https://doi.org/10.1016/j.biortech.2023.128922
Received 22 January 2023; Received in revised form 13 March 2023; Accepted 15 March 2023
Available online 20 March 2023

1. Introduction

The increased use of fossil fuels has resulted in a significant rise in atmospheric CO_2 concentration, which is the primary cause of the greenhouse effect. Many recent studies have explored technologies to mitigate CO_2 production while producing renewable energy. In their study, Hashim et al. (2022) demonstrated that applying an emission trading scheme could be an effective mechanism for promoting a low-carbon economy transition, with benefits both in terms of the economy and the environment. Biomethane production is also a promising technology that offers solutions to the mentioned challenges, combining the generation of renewable energy while capturing CO_2 from biogas production as a potential solution for reducing GHG emissions and contributing to climate change mitigation (Cordova et al., 2022). In this regard, the abovementioned problems and the urgent need for gas imports in Europe after the Russian invasion of Ukraine in early 2022, prompted the European Commission to develop a plan: increasing biomethane production to 35 bcm by 2030 (European Commission, 2022). This would be key in the transition to a carbon–neutral energy system and to a circular bioeconomy.

Biomethanation is an attractive technology for carbon capture and utilization (CCU) and biogas upgrading that is encompassed in the methanogenesis process. There are several pathways in which microorganisms can grow and produce CH_4: acetoclastic (CH_4 production from acetate), carboxydotrophic (from CO), hydrogenotrophic (from $CO_2 + H_2$), methylotrophic (from methanol, methylamines, or methyl sulfides), and methyl respiration (from methylated compounds in the presence of H_2). In the case of CO_2 biomethanation, CO_2 (e.g. that present in biogas) can be converted to CH_4 via the CO_2-reductive pathway of hydrogenotrophic methanogens. There are also other reactions involved in methanogenesis process, but, it is still not clear how the involved microbial groups are establishing syntrophies to overcome the thermodynamics limitations associated with some metabolic pathways.

During methanogenesis, there are a lot of reactions in which microorganisms are interacting through symbiotic associations. In the last years, there has been special interest in unveiling syntrophic associations during methanogenesis, in which the metabolic processes of at least two microorganisms are coupled together. These associations frequently occur through electron transfer mediated by hydrogen or other carriers to facilitate metabolisms that otherwise would be thermodynamically unfavorable or unfeasible. The use of inhibitors or enhancers of certain pathways (such as homoacetogenesis or methanogenesis) could be useful to better understand the reactions happening during these complex symbiotic processes as CO_2 biomethanation.

Kottenhahn et al. (2018) observed a complete inhibition of acetate formation from H_2 and CO_2 in the acetogenic bacterium *Acetobacterium woodii* by the addition of the sodium ionophore III (ETH2120). This inhibitory effect is due to the fact that *A. woodi*i energetic metabolism is strictly dependent on a sodium ion gradient across the cytoplasmic membrane (Schmidt et al., 2009) which couples the ATP-synthesis to the Wood-Ljungdahl pathway (WLP). The addition of a sodium ionophore prevents that gradient, blocking the bacterium's energy metabolism. For this reason, using ETH2120 is a promising option for enhancing other processes, including formate-dependent H_2 production, or CH_4 production from H_2 and CO_2. The last alternative has not been studied yet.

Carbon monoxide (CO) is a highly toxic gas for most living organisms since it inhibits cellular metabolisms by binding to metalloproteins such as hemoglobin (Wu and Wang, 2005). However, acetogens and methanogens have been reported to form syntrophies to process CO into CH_4. Homoacetogenic bacteria consume CO through the WLP and their tolerance to CO is markedly different among species (Mayer et al., 2018). CO acts as a competitive inhibitor of several hydrogenases that play a key role in electron transfer. The hydrogen-dependent CO_2 reductase (HDCR) of *A. woodi*, or the formate dehydrogenase (FDH) of *Clostridium autoethanogenum* were found to be sensitive to CO (Schuchmann and Müller, 2013; Wang et al., 2013). Bertsch et al. (2015) reported that the addition of 5 kPa of CO to the H_2-CO_2 gas phase of an *A. woodii* culture reduced acetate production, and partial pressures above 10 kPa led to a complete inhibition of acetate production from H_2 and CO_2.

The capacity to use CO as an energy source for carboxidotrophic growth is interesting for syngas-based microbiology and has been demonstrated in a few methanogenic species in pure cultures, but, it has to be considered that adaptation is quite long, and microbial growth is very slow (Schöne and Rother, 2018). Scarce information can be found in the literature regarding the amount of CO that can be used as a carbon source for methane production, without causing toxic effects. Research is needed to elucidate the effects of CO on the anaerobic digestion process.

Sodium 2-bromoethanesulfonate (BES) is a well-known methanogenic inhibitor used in several studies and is useful for studying alternative methanogenic reactions such as homoacetogenesis. BES competes as a structural analog with the cofactor methyl-coenzyme M in the methanogenic pathway inhibiting the final step of methane formation (Liu et al., 2011). BES is a methanogenic-specific inhibitor whose effective inhibition concentration is different depending on the application systems. In some cases, a BES concentration of 10 mM was sufficient to achieve inhibition, while in others a concentration of 50 mM was required (Liu et al., 2011). Logroño et al. (2022) studied the effect of 50 mM BES addition on hydrogenotrophic metabolism in a biomethanation process using different cultures (both mixed and pure cultures). The authors observed a complete inhibition of methane production when working with hydrogenotrophic enrichment cultures, while transient inhibition was observed with a more complex community of anaerobic granules.

The main goal of this work was to determine the impact of toxicants during CO_2 biomethanation and to demonstrate their effects on microbial composition. To the best of authors knowledge, ETH210 has only been studied in pure cultures, and while BES inhibition has been observed in several studies, the required concentration for complete inhibition and its effect on bacterial communities has yet to be clarified. CO effect on both CH_4 production and homoacetogenesis has yet to be completely clarified. These three inhibitors, targeting different microorganisms associated with both archaeal and bacterial domains, were used in the present study to reveal their effects on CO_2 biomethanation; a specific inoculum rich in hydrogenotrophic archaea was considered as the best target. High-throughput 16S rRNA sequencing was performed to unveil the microorganisms involved in the different metabolic pathways. The overall target was to gain better insight into the biological response of the anaerobic digestion microbiome to these compounds, focusing on the effects on the different syntrophies or associations formed between microorganisms.

2. Material and methods

The study conducted in this paper was carried out at lab-scale. The following paragraphs summarise the methodology used in the study.

2.1. Experimental set-up and design

To determine biogas production and anaerobic biodegradability, batch assays were carried out in 118-mL glass serum bottles with 40-mL working volume following the guidelines of the biochemical methane potential (BMP) protocol (Angelidaki et al., 2009). The headspace/liquid volume ratio was set to 3 for all experiments. Prior to incubation, the bottles were flushed with nitrogen gas for 10 min to ensure anaerobic conditions and then immediately sealed with rubber stoppers and aluminium caps. All bottles were incubated at a temperature of 55 ± 1 °C in an orbital shaker (IKA® KS 4000i control, Germany) with a constant agitation of 150 rpm and a horizontal position to maximize gas–liquid transfer. Experiments were conducted in 3 biological

replicates during the days necessary to reach a pseudo steady-state in terms of gas production.

All bottles were inoculated with the reactor content of a thermophilic biogas upgrading reactor enriched with hydrogenotrophic methanogens, described in Treu et al. (2018). The biogas upgrading reactor was a continuous stirred tank reactor originally inoculated from a thermophilic biogas reactor treating raw cattle manure (Bassani et al., 2015). Three different groups of experiments were performed (Table 1) to test the effect of different compounds on homoacetogenesis and methanogenesis processes. In all experiments, H_2 and CO_2 were added to the inoculum as feeding gases in a volumetric proportion of 4:1, respectively, keeping an initial overpressure of 1 atm. A control bottle with inoculum and gases was included for all assays. In the first experiment, named "ETH2120", different concentrations of sodium ionophore III (ETH2120, Sigma Aldrich, DK): 20 μM, 30 μM and 60 μM were tested. In the second experiment, named "CO", the gases CO_2 and H_2 were injected at the beginning of the experiment, and, after H_2 was consumed, the bottles were flushed again with N_2, and H_2 and CO_2 were fed with the same proportion (4:1 of H_2:CO_2). During the second gas feeding, CO was fed in different proportions in order to obtain initial CO partial pressures of 5, 10 and 20 kPa. Pressure in the bottles was measured using a portable manometer-thermometer HD2124.2 (Delta Ohm, Italy). In the third experiment, named "BES", different concentrations of 2-bromoethanolsulfonate (BES, Sigma Aldrich, DK) (10, 25 and 50 mM) were tested. All chemicals used in the present study were high purity (above 98 %).

2.2. Analytical methods

Gas from batch bottles headspace was collected using lock gas tight syringes inserted through the rubber stoppers. Percentage of different gases in the headspace was obtained through gas chromatography and gas volumes were calculated and normalised to conditions of 1 atm and 0 °C of pressure and temperature, respectively. The CH_4•and CO_2•content in biogas were measured with a gas chromatograph (GC) (Thermoscientific GC-8A, Japan) equipped with a Thermo (P/N 26004–6030) column (30 m, 20 mm OD, 0.32 mm inner diameter (ID)) with a thermal conductivity detector (TCD) and helium as carrier gas. The H_2•gas was determined with a GC (Shimadzu GC-11A, Tokyo-Japan) equipped with a glass column (2 m, 5 mm OD, 2.6 mm ID) with a flame ionization detector (FID).

VFA concentrations were determined from the liquid phase using a GC (Shimadzu GC-2010, Kyoto, Japan), which was equipped with an FID and an FFAP fused-silica capillary column (30 m, 0.53 mm ID, film thickness 1.0 mm) using nitrogen as a carrier gas. Prior to VFA determination with the GC, samples were pretreated diluting them, adding orthophosphoric acid (85 %, Sigma Aldrich, DK) 34 % v/v to acidify the samples and then centrifuging them at 15000 rpm for 10 min to remove suspended solids. The supernatant was transferred to a GC glass vial and mixed with 4-methyl-valeric acid (99 %, Sigma Aldrich, DK), that was used as internal standard.

Table 1
Experimental conditions for the different experiments carried out.

Experiment	Compound added	Gases fed	Expected microorganism affected	Microbial samples
Control		H_2, CO_2		x
Sodium ionophore (ETH2120)	ETH2120 20 μM	H_2, CO_2	Homoacetogens	
	ETH2120 30 μM	H_2, CO_2		
	ETH2120 60 μM	H_2, CO_2		x
Carbon monoxide	CO 5 kPa	H_2, CO_2, CO	Homoacetogens and/or methanogens	
	CO 10 kPa	H_2, CO_2, CO		
	CO 20 kPa	H_2, CO_2, CO		x
BES	BES 10 mM	H_2, CO_2	Methanogens	
	BES 25 mM	H_2, CO_2		
	BES 50 mM	H_2, CO_2		x

ETH2120: Sodium ionophore III; CO: carbon monoxide; *BES: 2-bromoethanesulfonate*.

2.3. DNA extraction and microbial community composition

Triplicate samples were taken from the bottles' liquid media at the end of each experiment. The bottles in which the maximum concentration of each inhibitor was tested (Table 1) were chosen for the microbial analysis.

Genomic DNA isolation and purification was carried out using the PowerSoil® DNA Isolation Kit (MO BIO laboratories, Inc.) following the instructions of the manufacturer's protocol with an initial step of purification with phenol:chloroform:isoamylalcohol with (25:24:1) pH 8 (Sigma-Aldrich, DK). The quality and quantity of DNA extractions were defined with NanoDrop 2000 (Thermo Fisher Scientific, USA) and Qubit fluorometer (Life Technologies, Carlsbad, CA, USA).

The hypervariable V4 regions of 16S rRNA genes were amplified by PCR using universal primers 515F/806R and subsequently, the Illumina MiSeq sequencing platform was used to sequence 16S rRNA gene amplicons using the 500-cycle chemistry. The raw reads were uploaded in Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under the BioProject PRJNA911060 with accession number from SRR22681396 to SRR22681385. The bioinformatic analysis was conducted using the software CLC Genomics Workbench (V.21.0.4) with microbial genomics module plug in (QIAGEN Bioinformatics, Germany) as previously described in Treu et al. (2018). The resulting filtered sequences were clustered into Operational Taxonomic Units defined at 3 % dissimilarity ($OTU_{0.97}$). Manual verification of the taxonomic assignment was performed with nucleotide BLAST search against NCBI 16S rRNA database (bacteria and archaea).

2.4. Statistical analysis

Data were analysed by principal co-ordinate analysis (PCoA) based on the Bray-Curtis distances matrix to explore the beta diversity of the different samples. Comparisons between the three different conditions tested and the control (two-groups comparison) were also carried out using the Statistical Analysis of Metagenomic Profiles (STAMP) software, version 2.1.3, applying a Welch's *t* test (P-value < 0.05) (Parks et al., 2014). Canonical correspondence analysis (CCA) was performed using the R functions implemented in VEGAN v2.4-4 (Hammer et al., 2001).

3. Results and discussion

In the present work, use of three compounds (ETH2120, CO and BES) to understand homoacetogenesis or methanogenesis prevalence in a biogas upgrading reactor was investigated. Effects on both biological performance (Section 3.1) and microbial population (Section 3.2) were evaluated.

3.1. Biological performance

The results of the biological process evaluation for the three experiments performed are shown in Fig. 1. Gas production or consumption in the first experiment (ETH2120) was similar under the three conditions tested (Fig. 2) and also very similar to the control (Fig. 1). H_2 was completely consumed after about 50 h while only part of the CO_2 was consumed. CH_4 was produced at a similar rate in all three assays, and, the maximum production achieved (around 10 mL) was also the same as in the control. The CH_4 production rate was higher in the control during the first few hours. Acetic and butyric acids were accumulated, while no accumulation of propionic acid was detected (Fig. 2). Literature indicates that ETH2120 can block acetate production from H_2 and CO_2 by inhibiting homoacetogens, as reported by Kottenhahn et al. (2018). During the 80 h that the experiment lasted, acetate was produced when adding 20 and 30 μM ETH2120, but a clear production was not observed when adding 60 μM ETH2120 (Fig. 2). Comparing acetate production to that observed in the control or to the other experiments at the same time point (80 h), it is clear that acetate was not produced at the same rate (Fig. 1). If the experiments had been conducted using a continuous reactor, it is likely that a more pronounced effect would have been observed. Kottenhahn et al. (2018) observed a complete inhibition of acetate production using ETH2120, but the authors worked with pure cultures of *A. woodii*, whereas in the present study, the inoculum was composed of a complex microbial community, making it difficult to observe a strong effect in the biological process. Nevertheless, a difference in the microbial population was observed between the different experiments, as described in Section 3.2, so, ETH2120 affected the microbial population, but apparently this had limited impact on homoacetogenesis.

In the second group of assays, CO was completely consumed in all experiments. The higher the CO concentration, the longer it took to be consumed. The CO_2 was not totally consumed, but the CO_2 production due to CO consumption (Eq. (1)) has to be considered. The hydrogen was completely consumed after about 20 h when 5 or 10 kPa of CO were fed and after 40 h when 20 kPa were applied (Fig. 3). The acetate produced was higher compared to the control or the other conditions tested. The acetogenic bacteria used CO for acetate production (Eq.1) as previously reported for anaerobic sludge mixtures (Grimalt-Alemany et al., 2020). Among the three tested CO partial pressures, a lower amount of acetate was produced when 20 kPa was applied (see Fig. 3). This may indicate an effect of CO on acetogenic bacteria, as previously observed by Mayer et al. (2018). They reported a strong inhibition of growth and acetate formation in *Clostridium aceticum* when CO was fed with increasing partial pressures ranging from 10 to 50 kPa. Propionic acid and butyric acid were also produced simultaneously. It should be highlighted that CO was the only carbon source that led to propionic acid accumulation, either because there was excess production or because the conversion rate to acetate was very low (see Section 3.2 for microbiological details).

$$4CO + 2H_2O \leftrightarrow 1CH_3COO^- - + H^+ + 2CO_2 \quad \Delta G^o = -165.4\,kJ/mol \qquad (1)$$

The CH_4 production was similar under all tested conditions (approximately 10 mL), although there was a slight increase (around 12 mL) when 20 kPa of CO was added (Fig. 3). This increase could be attributed to the impact on acetogenic bacteria, as previously mentioned, which can enhance methanogenesis, or it could be due to the

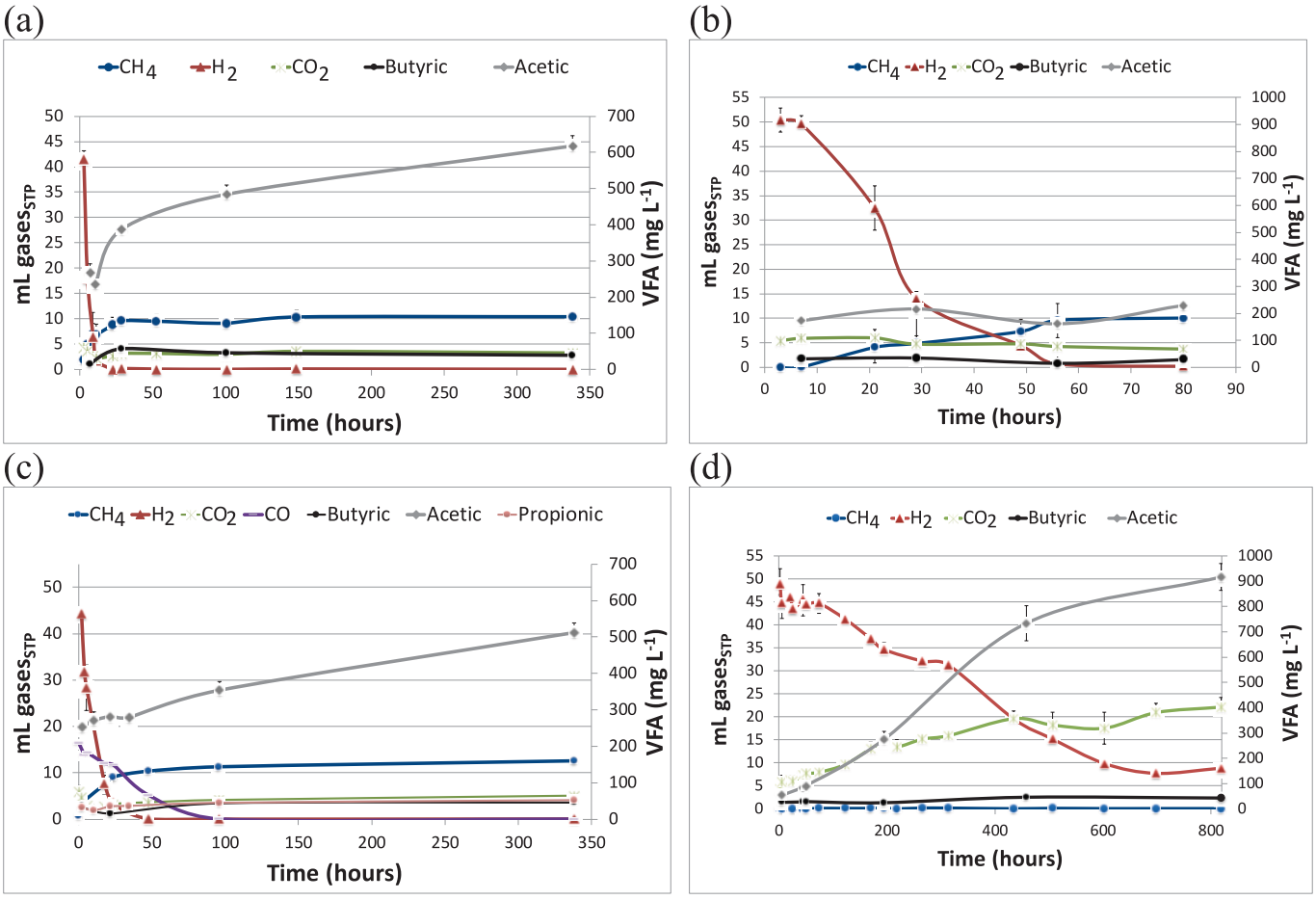


Fig. 1. Gas composition and volatile fatty acids (VFA) evolution in the control (a), sodium ionophore (ETH2120) 60 μM experiment (b) carbon monoxide (20 kPa) experiment (c) and BES (2-bromoethanesulfonate) 50 mM experiment (d).

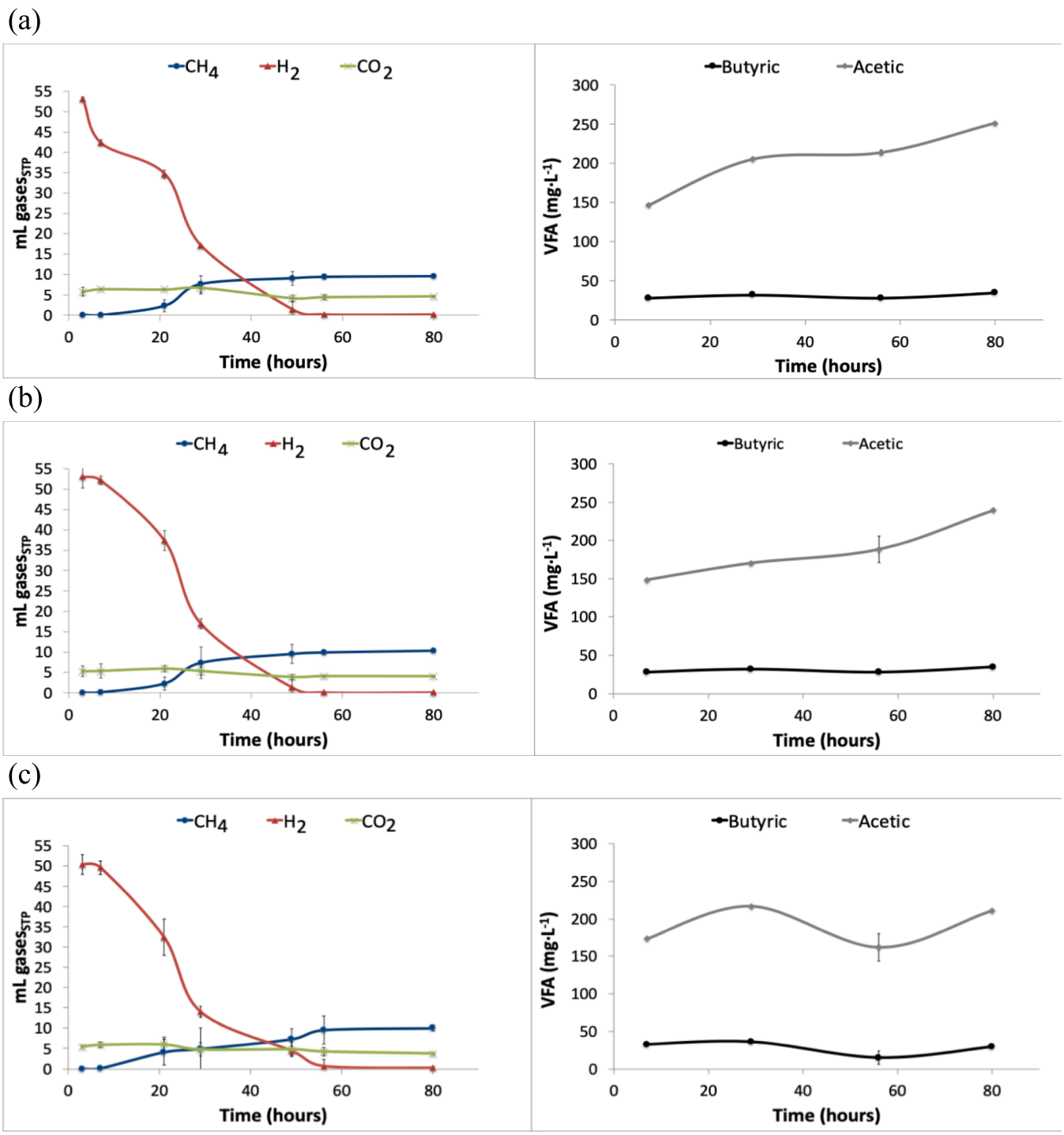


Fig. 2. On the left side, gas composition; on the right side, volatile fatty acids (VFA) production in the sodium ionophore (ETH2120) experiment for concentrations of 20 $\mu g \cdot L^{-1}$ (a), 30 $\mu g \cdot L^{-1}$ (b) and 60 $\mu g \cdot L^{-1}$ (c).

use of CO as a carbon source for CH_4 production. That CH_4 production increase would have been higher if the ratio $\%H_2 + \%CO_2/(\%CO_2 + \%CO)$ would have been closer to 4 (Asimakopoulos et al., 2021), since this index indicates the stoichiometrically (theoretically) ideal gas composition for the full conversion of CO and CO_2 into CH_4. In the current experiment that index was decreasing when the CO fed to the culture was increasing, so, the index for 20 kPa of CO was around 2.3. Previous studies at mesophilic conditions (Navarro et al., 2016) tested the effects of different CO partial pressures (from 10 to 152 kPa) on a microbial community dominated by aceticlastic methanogens and found the highest CH_4 yield when CO partial pressure was 10 kPa, while inhibition of aceticlastic methanogens was detected with CO partial pressures above 20 kPa. As will be described in Section 3.2, no aceticlastic methanogens were observed in the present work since the via for methanogenesis production was a different one. This could explain why in the present study an enhancement of CH_4 production was not observed when applying 10 kPa of CO.

CH_4 production was not observed with the three different BES concentrations tested. Despite this finding, the relative abundance of archaea in BES samples was similar to the control and to the other conditions tested (see Section 3.2). This finding suggests that the inhibitor was forcing the archaea in a metabolically inactive state. The

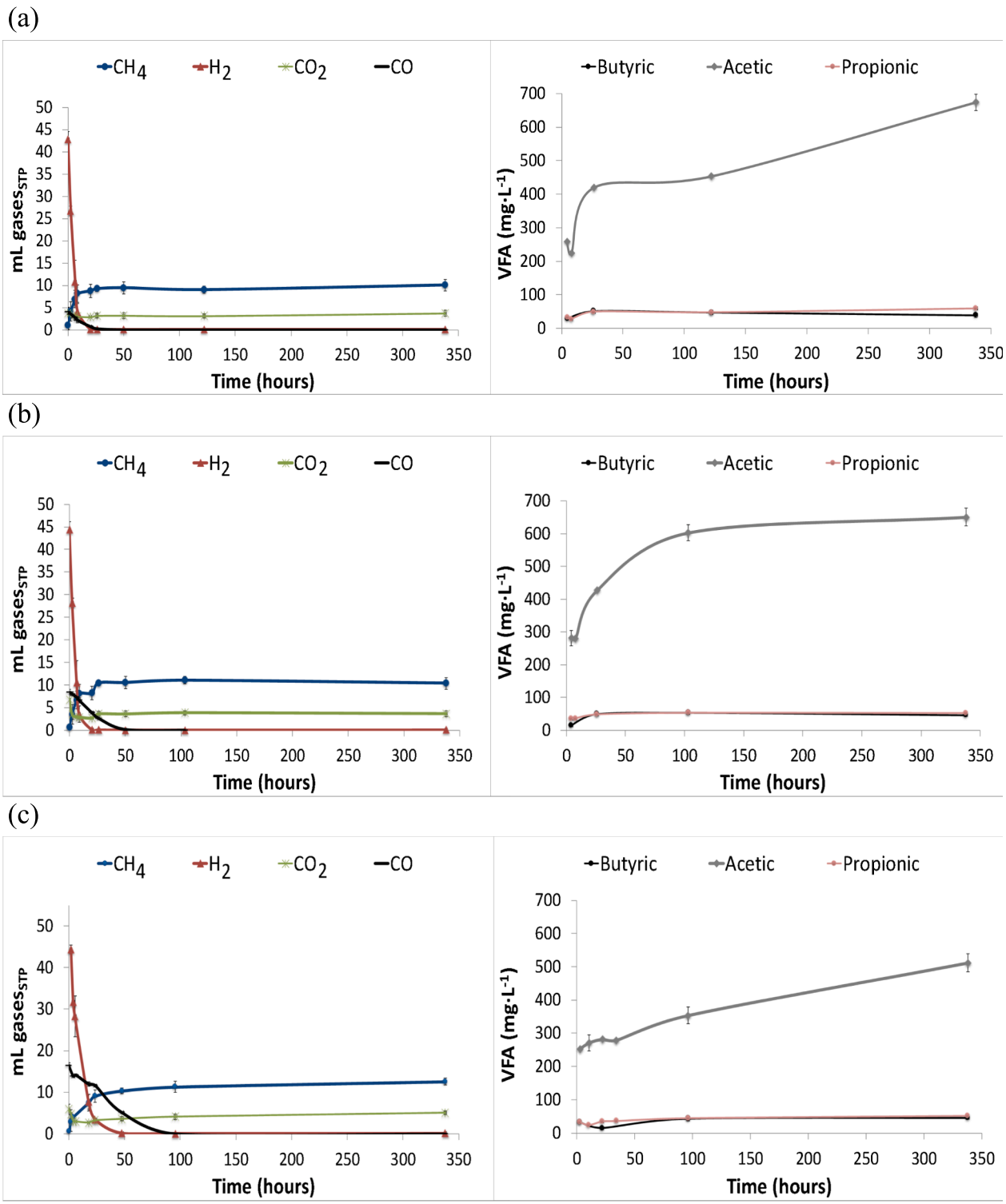


Fig. 3. On the left side, gas composition; on the right side, volatile fatty acids (VFA) production in the carbon monoxide (CO) experiment. Partial pressures for CO used were 5 kPa (a), 10 kPa (b) and 20 kPa (c).

higher the BES concentration, the lower the H_2 consumption rate. In BES samples, complete H_2 depletion lasted longer than in the control or the other samples: it took around 250 h at 10 mM BES concentration, and around 300 h at 25 mM. At 50 mM BES, H_2 was not totally consumed as it was in the other two concentrations tested, and it took more than 400 h to consume the half of the total amount fed. Webster et al. (2016) found that inhibitors targeting archaea were also affecting the activity of syntrophic bacteria, according to this, the higher the BES, the lower the H_2 that both archaea and bacteria can consume, leading in less energetically favorable conditions for the acetogenic syntrophic bacteria. This finding is also corroborated by microbiological data in Section 3.2 where it is reported that relative abundance of hydrogenotrophic microorganisms belonging to Rikenellaceae family was lower in BES samples.

Except for the methane production inhibition caused by BES addition, no enhancement or inhibition of other reactions was clearly

observed from monitoring the biochemical parameters working with batch reactors in the present biomethanation study. The addition of CO and ETH210 at their maximum concentrations (20 kPa and 60 μm, respectively) resulted in a minor decrease in acetate production. In the case of CO addition, that decrease was observed alongside a slight increase in CH_4 production while in the case of ETH2120 addition, no enhancement of CH_4 production was observed. The use of these inhibitors in a continuous system would be helpful to understand the effect of these compounds in the biological process. Moreover, when performing biomethanation with mixed cultures inoculated with digestate from a thermophilic reactor, as in the present work, a set of reactions is expected to take place. Alternative reactions that compete with methanogenesis, such as homoacetogenesis, are difficult to control. Indeed, Voelklein et al. (2019) observed that proliferation of homoacetogens can be associated with 40 % utilization of the added H_2 leading to a subsequent increase in acetate production and consequently, methane formation by the aceticlastic methanogens. Since in the present work acetate production was occurring in all experiments, it can also be assumed that homoacetogenesis from H_2 was not inhibited. This finding is in accordance with previous studies (Agneessens et al., 2018) where homoacetogenesis was the dominant pathway for utilization of exogenous H_2 added in a microbiome fed with manure (same feedstock as the one used for the reactor took as inoculum in this work).

3.2. Microbial composition

The microbial community found in the four conditions was largely dominated by a small number of species since only 18 OTUs were representing 89 % of the microbiome, and 29 OTUs in total were found above 0.5 % of relative abundance in this study (see supplementary materials). The unevenness of the microbiome can be related both to the lack of biodiversity in the inoculum, which undergoes a strong selection

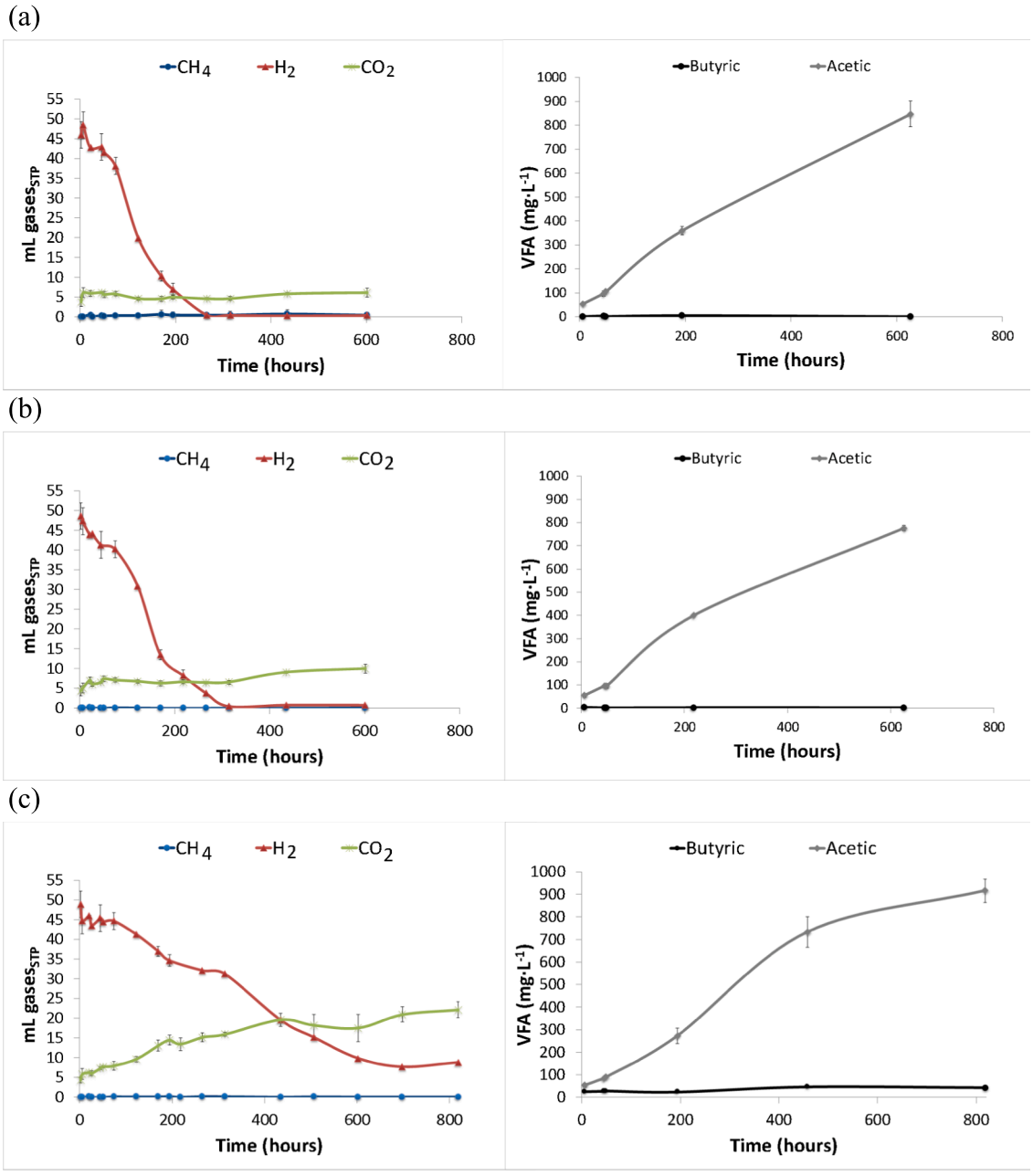


Fig. 4. On the left side, gas composition; on the right side, volatile fatty acids (VFA) production in the BES (2-bromoethanesulfonate) experiment for concentrations of 10 mM (a), 25 mM (b) and 50 mM (c).

during adaptation to the simplified feedstock represented by CO_2-H_2 (Bassani et al., 2015), and/or to the same process (H_2 and CO_2 feeding) but in the current experiment. The 29 OTUs were taxonomically assigned to eight different phyla, being Firmicutes, Euryarchaeota and Bacteroidetes the most represented (Fig. 5).

The most abundant bacterial group in all conditions studied was the family Synergistaceae (Synergistetes phylum), with abundances above 26 % in all samples (Fig. 5). Most of the microbiota was represented mainly by three groups: *Aminovibrio* sp., *Rikenellaceae* sp. and *Aminipila* sp., with relative abundances of 27 %, 18 % and 19 %, respectively, in the control sample, 26 %, 17 %, and 18 % in the ETH2120 samples, 28 %, 17 %, and 17 % in the CO samples and 34 %, 16 % and 11 % in the BES samples (see supplementary materials). The species belonging to the three taxa are involved in amino acids degradation. *Aminovibrio* sp. is an anaerobic bacterium that can ferment amino acids and organic acids that was isolated from an anaerobic propionate-oxidizing enrichment culture (Honda et al., 2013). The genus *Aminipila* was proposed by Ueki et al. (2018) and belongs to the class Clostridia. The BLASTn similarity search against NCBI database of the OTU revealed 95 % similarity with *Aminipila butyrica*, which is one of the few species included in that genus. In the work of Ueki et al. (2018), the microorganism was isolated from a reactor digesting cattle manure and it was classified as an amino acid fermenter ($_L$-arginine, $_L$-lysine and $_L$-serine) producing acetate and butyrate. As shown in Figs. 2, 3 and 4, around 30–50 mg·L^{-1} of butyric acid and 250–700 mg·L^{-1} of acetic acid were produced in all conditions. These short-chain fatty acids (mainly acetic, propionic and butyric

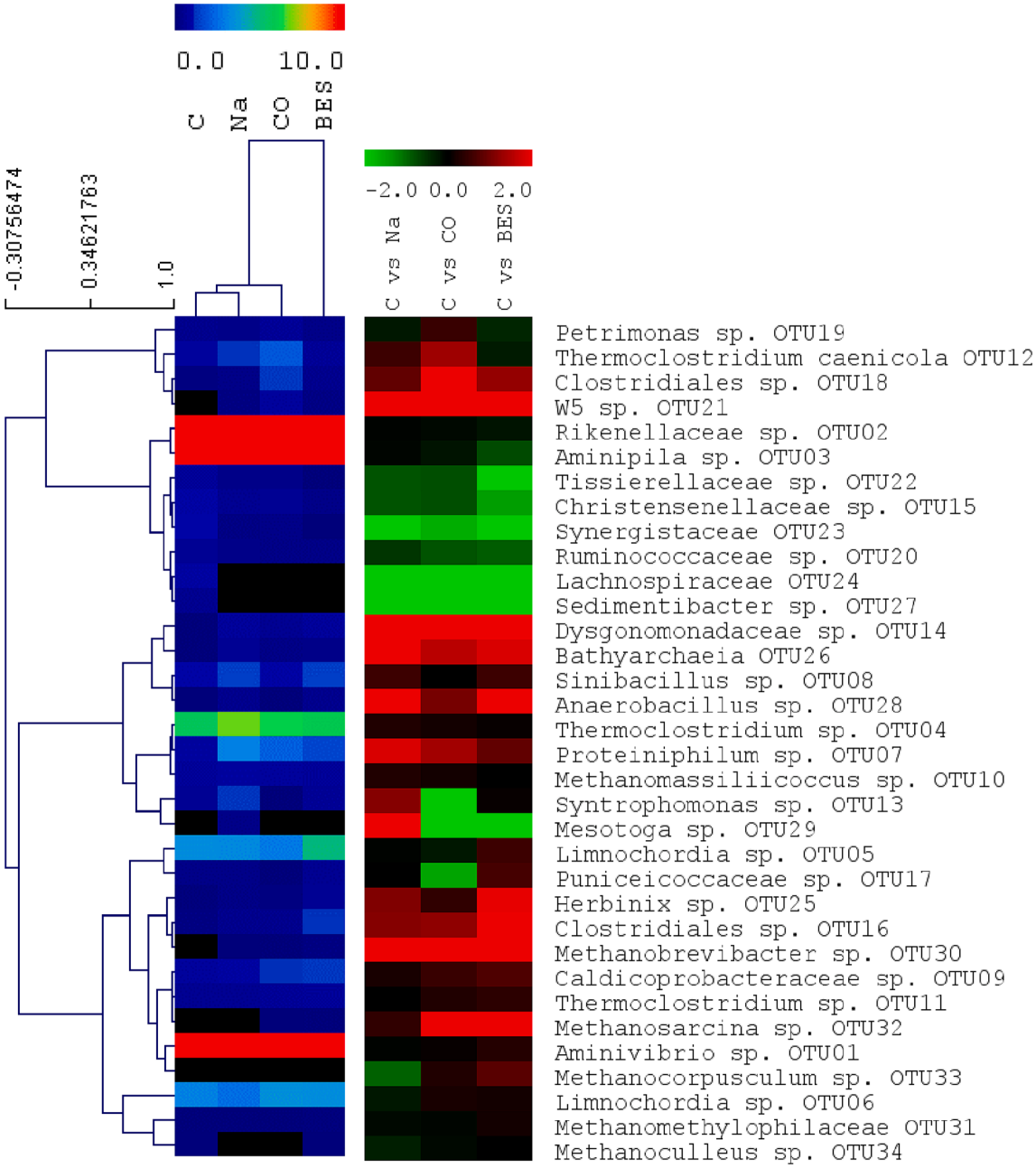


Fig. 5. Heatmap representing the relative abundance of the 34 most abundant OTUs; On the left side, abundances values range from 0% (color black) to 10% (color red), being saturated the higher values (color scale shown in the upper part). Only OTUs with an average relative abundance higher than 0.5% are reported. On the right side of the graphic, the red-colored part represents the increase in relative abundance at fold change while the decrease is shown in green. The heatmap on the left also shows clustering based on Pearson correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acids) are known as the major-end products from amino acids reductive deamination (Boudry et al., 2016). Additionally, in this study, cattle manure was utilized as the feedstock for the reactor inoculation, and this substrate is known to have a high protein content (Tasaki, 2021). Therefore, the substantial presence of an amino acid-degrading community in the system can be attributed to the high protein content of the cattle manure.

Amino acids-degrading microorganisms being the most abundant groups could be linked to the presence of methylotrophic methanogens that generate/conserve energy from protein degradation metabolism (Lloyd et al., 2013). During methylotrophic methanogenesis, methyl-coenzyme M can be produced via transfer of a methyl group from methylamines as mono-, di-, and trimethylamine to be later transformed into CH_4 (Borrel et al., 2013) and these methylamines are produced by anaerobic bacteria that decarboxylate nitrogenous organic matter as glycine betaine (Nayak and Marx, 2014). Order Methanomassiliicoccales, including *Methanomassiliicoccus* sp., which was the most abundant archaeon in all samples, and *Methanomethylophilaceae* sp. (also found in all samples in a lower abundance), have been described as a group of obligate H_2-dependant methylotrophic archaea able to use a wide panel of methylated compounds (Li et al., 2016). *Methanosphaera* sp., an archaeon found in the three conditions tested, has also been described as a methylotrophic methanogen that can only grow on methyl compounds when H_2 is present. It must be emphasized that in these groups of methanogens, the genes involved in the conventional CO_2-reduction/methyl-oxidation pathways are not present (Borrel et al., 2013). Due to the lack of these genes, all these microorganisms use the methyl-respiration pathway for CH_4 production (Eq. (2)); in this metabolism the H_2 is being used as electron donor, while in the conventional methylotrophic pathway (Eq. (3)) the carbon source itself is being used as electron donor (Buan, 2018). This corroborates the biological process results, that showed a fast H_2 consumption in all conditions, while the CO_2 was not consumed (Section 3.1).

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \quad \Delta G^o = -78.6 \tag{2}$$

$$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H_2O + H^+ \Delta G^o = -112.5 \tag{3}$$

The second most abundant archaea in the current work belong to Bathyarchaeia family (phylum Crenarchaeota), which is also encoding methyl-CoM reductase-like enzymes and genes involved in methanogenesis from methylated amines (Loh et al., 2021). The presence of this pathway evidenced a similarity of Bathyarchaeia with the order Methanomassiliicoccales and a possible link with bacteria involved in protein hydrolysis and amino acid utilization.

Other less abundant archaea observed in the current work were *Methanoculleus*, a hydrogenotrophic methanogen (Kougias et al., 2017) that act in syntrophy with syntrophic acetate-oxidizing bacteria (SAOB) to generate methane, *Methanobrevibacter*, which is also a hydrogenotrophic methanogen, and *Methanosarcina*, which is a generalist methanogen able to grow using any of the known methanogenic pathways, including methyl respiration. The presence of *Methanobrevibacter* could be related with the inoculum characteristics. Treu et al. (2018) found that *Methanothermobacter* sp. was the most abundant microorganism, and they also found *Methanobrevibacter* in the community present in the sludge used as inoculum in the present work. These two last genera belong to the same family (Methanobacteriaceae).

Comparison of the abundance profiles of the different microorganisms in the three conditions tested, revealed some differences reported in the PCoA shown in Fig. 6. The PCoA plot shows that the first and the second component account for a large fraction of the overall variability (PC1 = 70 %, PC2 = 11 %). PCoA revealed significant differences in microbial community composition between the control and the other samples, and also between the different conditions tested. BES samples were clearly separated from the other samples. Two-sided Welch's *t* test also showed significant differences ($P < 0.05$) between the control and the rest of the groups (see supplementary material).

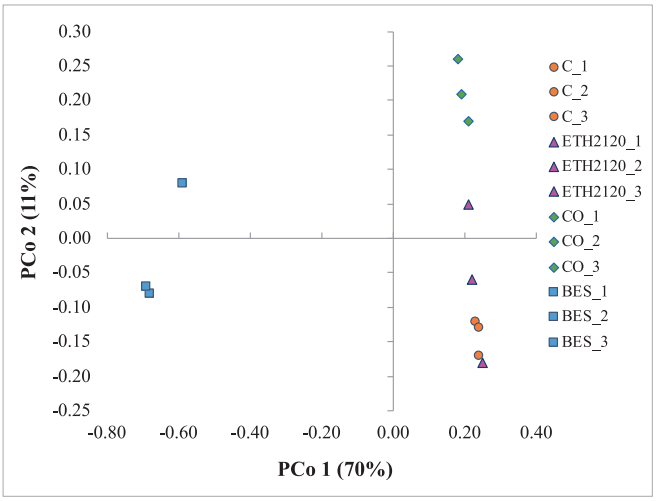


Fig. 6. Principal coordinate analysis (PCoA) showing PCos 1 and 2 of the microbial composition of samples. Ordination was calculated from the weighted unifrac distance matrix. The two first components were chosen to represent the differences between the three experiments (BES, CO and Na) and the control samples analysed in the 16SrRNA gene amplicon sequencing approach. Explained variance of each component is shown in percentage in each axis.

Thermoclostridium genus had a high relative abundance in all the samples (Fig. 5). One of the OTUs belonging to this genus, was tentatively assigned to *Thermoclostridium caenicola* according to the 97.7 % identity determined from the BLAST search. This OTU had a relative abundance in CO samples around 3-fold higher comparing with the other conditions. This cellulolytic thermophilic microorganism was recently reclassified as a novel genus (Zhang et al., 2018). The same happens with another group belonging to *Clostridiales* sp. (OTU16) that had 7-fold higher abundance in CO samples comparing to the control. Since the highest propionic acid accumulation was observed in CO samples (see Fig. 1), these microorganisms may be involved in propionate formation, as corroborated by CCA analysis (see supplementary materials). The propionate production can trigger the proliferation of syntrophic propionate oxidizing species such as those belonging to the W5 taxon (Dyksma and Gallert, 2019). An OTU associated to the W5 taxon was indeed identified at higher abundance in CO samples compared with the control (28-fold enrichment).

Despite there is not a big difference between the microbial community in the ETH2120 experiment and the other samples, there are some taxa that are worthy of mentioning. For instance, *Syntrophomonas* sp. relative abundance was higher in ETH2120, especially when compared with CO experiment (11-fold higher). *Syntrophomonas* is a hydrogen-producing acetogenic bacterium able to metabolize C_3 acids such as propionate. *Mesotoga* sp. was also 40-fold more abundant in ETH2120 experiment in comparison to control. *Mesotoga* is a SAOB that has been recently reported as an auxotroph for different amino acids (Du et al., 2022). *Proteiniphilum* sp. also had a higher abundance in ETH2120 experiment (around 4-fold compared with the control). *Proteiniphilum* is a proteolytic bacterium able to convert peptone, arginine, yeast extract and glycine to NH_3 and acetate (Chen and Dong, 2005).

PCoA evidenced that the community is clearly different in the case of BES experiment comparing with the other samples (Fig. 6). There is a difference in the three more representative microorganisms: in comparison with the control, the group *Aminivibrio* sp. was more abundant both in BES samples, and also in the other two conditions (ETH2120 and CO). On the other hand, the other two most abundant microorganisms (*Aminipila* sp. and *Rikenecellaceae* sp.) were less abundant in BES samples. A lower relative abundance of *Rikenecellaceae* sp. in BES samples could be expected since this group is able to ferment amino acids in syntrophic association with hydrogenotrophic methanogens, which are

being inhibited by BES.

Despite BES treatment resulted in a strong CH_4 production inhibition, there were no differences in the relative abundance of the most abundant archaeal methanogens (see supplementary materials). As mentioned in Section 3.1., methanogens could be present in an inactive state. The genomic analysis carried out in the present study cannot determine the activity of microorganisms, so, even archaea were present, it does not mean that these microorganisms were active. A further transcriptomic approach (RNA analysis) should be applied to know the activity of the microorganisms. De Vrieze et al. (2018) investigated the archaeal and bacterial community of 48 full-scale anaerobic digestion plants on DNA (total community) and RNA (active community) and observed a clear difference in active (RNA) and total (DNA) community profiles. Moreover, a significantly higher diversity on DNA compared with RNA level for archaea was reported. The only difference observed in methanogens comparison between experiments was in genus *Methanobrevibacter*, which was 54-fold higher in BES samples compared to the control (see supplementary material), even CH_4 has being produced in the control and not in the BES samples. This is in accordance with a previous study (Webster et al., 2016) that revealed a high activity of hydrogenotrophic methanogens when BES was applied at 0.5 and 10 mM. In that study, *Methanobrevibacter* represented the highest fraction of the active archaeal community when the inhibitor was applied.

Limnochordia sp. showed a relative abundance of 3.4 % in the control, while its abundance increased in BES samples (4.9 %) and had lower values in ETH210 and CO samples (3.3 % and 2.9 % respectively). This bacterium is able to grow consuming acetate and its relative abundance is correlated with H_2 concentration (as shown in CCA analysis) in the reactor (Treu et al., 2018). This is in accordance with the present work: as described in Section 3.1., H_2 concentration in BES samples was higher comparing with the rest of experiments since this gas was not completely consumed due to archaea activity inhibition.

Other differences were observed in the BES experiment in some groups belonging to order Clostridiales. Specifically, *Desulfohalotomaculum* sp. and *Herbinix* sp. were higher in BES (6-fold and 4-fold higher than the control, respectively), while *Tissierellaceae* sp. was 6-fold lower, comparing to the control.

While most of the microorganisms were found in all samples tested (even with different relative abundances), two groups of microorganisms belonging to the Firmicutes phylum were only found in the control samples. One of them was the family Lachnospiraceae and the other one was *Sedimentibacter* sp. with relative abundances of 0.9 % and of 0.5 %, respectively. In addition, another group of Synergistaceae family was found in a higher abundance in control samples. It was 4-fold higher than ETH2120, 3-fold higher than CO and 20-fold higher than BES experiment so this microorganism could be affected by the three compounds added to the samples. This group matched the genus *Cloacibacillus* sp., according to 90.8 % similarity obtained from the BLASTn search against NCBI database and was isolated from the mucosal lining of a pig cecum (Looft et al., 2013). Nevertheless, the sequence identity is lower compared to the accepted taxonomic threshold for genus assignment (greater than94.5 %) (Yarza et al., 2014) and thus, assignment to *Cloacibacillus* genus is not well supported.

Although in the present work hydrogenotrophic methanogenesis is supposed to be the fundamental pathway in H_2/CO_2 biomethanation, methyl respiration seems to be the key for CH_4 production. Additionally, other metabolic routes as homoacetogenesis are taking place in the complex culture used in the present study working in batch mode. According to the obtained results, CO_2 biomethanation was not observed in the present work: due to the presence of that main pathway for CH_4 production, H_2 was quickly consumed while CO_2 was not consumed in all experiments, so, the compounds used for trying to improve CO_2 biomethanation (ETH2120 and CO) would not be useful, since methane is not being upgraded and CO_2 is not being consumed.

4. Conclusions

Effects of ETH2120, CO, and BES on homoacetogenesis and methanogenesis to enhance CH_4 production were evaluated. BES had an inhibitory effect on methanogenesis (no CH_4 was produced even at 10 mM BES concentration). 20 kPa of CO seemed to have a negative effect on homoacetogenesis, but a positive effect on methanogenesis. Although 60 μM ETH2120 decreased acetate production, no significant increase in CH_4 production was observed. However, when these compounds were applied to a complex environmental sample, their expected effects on specific metabolic pathways were not observed. Using continuous reactors will help to fully understand the effects of these inhibitors.

E-supplementary data of this work can be found in online version of the paper.

CRediT authorship contribution statement

Rebecca Serna-García: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Panagiotis Tsapekos:** Conceptualization, Methodology, Writing – review & editing. **Laura Treu:** Formal analysis, Software. **Alberto Bouzas:** Supervision, Writing – review & editing. **Aurora Seco:** Supervision, Writing – review & editing. **Stefano Campanaro:** Supervision, Writing – review & editing, Software. **Irini Angelidaki:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research work was supported by the Science and Innovation Spanish Ministry (Projects CTM2014-54980-C2-1-R / C2-2-R) and the European Regional Development Fund (ERDF), which are gratefully acknowledged. The Science and Innovation Spanish Ministry have also supported this study via pre-doctoral FPI fellowship to the first author (BES-2015-071884, Project CTM2014-54980-C2-1-R). This study was supported by the EU project CRONUS Capture and Reuse of biogenic gases for Negative-emission- sustainable biofUelS. Project number: 101084405.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128922.

References

Agneessens, L.M., Ottosen, L.D.M., Andersen, M., Berg Olesen, C., Feilberg, A., Kofoed, M.V.W., 2018. Parameters affecting acetate concentrations during in-situ biological hydrogen methanation. Bioresour. Technol. 258, 33–40. https://doi.org/10.1016/j.biortech.2018.02.102.

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J.L., Guwy, A.J., Kalyuzhnyi, S., Jenicek, P., van Lier, J.B., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. Water Sci. Technol. 59 (5), 927–934. https://doi.org/10.2166/wst.2009.040.

Asimakopoulos, K., Grimalt-Alemany, A., Lundholm-Høffner, Gavala, H.N., Skiadas, I.V., 2021. Carbon sequestration through syngas biomethanation coupled with H_2 supply for a clean production of natural gas grade biomethane. Waste Biomass Valor. 12, 6005–6019. doi: 10.1007/s12649-021-01393-2.

Bassani, I., Kougias, P.G., Treu, L., Angelidaki, I., 2015. Biogas upgrading via hydrogenotrophic methanogenesis in two-stage continuous stirred tank reactors at

mesophilic and thermophilic conditions. Environ. Sci. Technol. 49, 12585–12593. https://doi.org/10.1021/acs.est.5b03451.
Bertsch, J., Müller, V., Lovell, C.R., 2015. CO metabolism in the acetogen Acetobacterium woodii. Appl. Environ. Microbiol. 81 (17), 5949–5956.
Borrel, G., O'Toole, P.W., Harris, H.M., Peyret, P., Brugère, J.F., Gribaldo, S., 2013. Phylogenomic data support a seventh order of Methylotrophic methanogens and provide insights into the evolution of Methanogenesis. Genome Biol Evol. 5 (10), 1769–1780. https://doi.org/10.1093/gbe/evt128.
Boudry, G., Le Huërou-Luron, I., Michel, C., 2016. Chapter 15 - Dietary Protein and Colonic Microbiota: Molecular Aspects, Ed(s): Dominique Dardevet. The Molecular Nutrition of Amino Acids and Proteins. Academic Press, pp. 207–220.
Buan, N.R., 2018. Methanogens: pushing the boundaries of biology. Emerg. Top. Life Sci. 2 (4), 620–646. https://doi.org/10.1042/ETLS20180031.
Chen, S., Dong, X., 2005. Proteiniphilum acetatigenes gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. Int. J. Syst. Evol. Microbiol. 55, 2257–2261. https://doi.org/10.1099/ijs.0.63807-0.
Cordova, S.S., Gustafsson, M., Eklund, M., Svensson, N., 2022. Potential for the valorization of carbon dioxide from biogas production in Sweden. J. Clean. Prod. 370, 133498 https://doi.org/10.1016/j.jclepro.2022.133498.
De Vrieze, J., Pinto, A.J., Sloan, W.T., Ijaz, U.Z., 2018. The active microbial community more accurately reflects the anaerobic digestion process: 16S rRNA (gene) sequencing as a predictive tool. Microbiome 6, 63. https://doi.org/10.1186/s40168-018-0449-9.
Du, J., Yin, Q., Zhou, X., Guo, Q., Wu, G., 2022. Distribution of extracellular amino acids and their potential functions in microbial cross-feeding in anaerobic digestion systems. Bioresour. Technol. 360, 127535 https://doi.org/10.1016/j.biortech.2022.127535.
Dyksma, S., Gallert, C., 2019. *Candidatus Syntrophosphaera thermopropionivorans*: a novel player in syntrophic propionate oxidation during anaerobic digestion. Environ. Microbiol. Rep. 11 (4), 558–570. https://doi.org/10.1111/1758-2229.12759.
European Commission, 2022. REPowerEU: Joint European Action for More Affordable, Secure and Sustainable Energy. 8.3.2022 COM (2022) 108 Final; European Commission: Brussels, Belgium.
Grimalt-Alemany, A., Lezyk, M., Asimakopoulos, K., Skiadas, I.V., Gavala, H.N., 2020. Cryopreservation and fast recovery of enriched syngas-converting microbial communities. Water Res. 177, 115747 https://doi.org/10.1016/j.watres.2020.115747.
Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4, 9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.
Hashim, H., Zubir, M., Kamyab, H., Zahran, M., 2022. Decarbonisation of the industrial sector through greenhouse gas mitigation, offset, and emission trading schemes. Chem. Eng. Trans. 97. https://www.cetjournal.it/index.php/cet/article/view/CET2297086.
Honda, T., Fujita, T., Tonouchi, A., 2013. Aminivibrio pyruvatiphilus gen. nov., sp. nov., an anaerobic, amino-acid-degrading bacterium from soil of a Japanese rice field. Int. J. Syst. Evol. Microbiol. 63, 3679–3686. https://doi.org/10.1099/ijs.0.052225-0.
Kottenhahn, P., Schuchmann, K., Müller, V., 2018. Efficient whole cell biocatalyst for formate-based hydrogen production. Biotechnol. Biofuels 11, 93. https://doi.org/10.1186/s13068-018-1082-3.
Kougias, P.G., Campanaro, S., Treu, L., Zhu, X., Angelidaki, I., 2017. A novel archaeal species belonging to Methanoculleus genus identified via de-novo assembly and metagenomic binning process in biogas reactors. Anaerobe 46, 23–32. https://doi.org/10.1016/j.anaerobe.2017.02.009.
Li, Y., Leahy, S.C., Jeyanathan, J., Henderson, G., Cox, F., Altermann, E., Kelly, W.J., Lambie, S.C., Janssen, P.H., Rakonjac, J., Attwood, G.T., 2016. The complete genome sequence of the methanogenic archaeon ISO4-H5 provides insights into the methylotrophic lifestyle of a ruminal representative of the *Methanomassiliicoccales*. Stand. Genomic Sci. 11 (1), 59. https://doi.org/10.1186/s40793-016-0183-5.
Liu, H., Wang, J., Wang, A., Chen, J., 2011. Chemical inhibitors of methanogenesis and putative applications. Appl. Microbiol. Biotechnol. 89, 1333–1340. https://doi.org/10.1007/s00253-010-3066-5.
Lloyd, K.G., Schreiber, L., Petersen, D.G., Kjeldsen, K.U., Lever, M.A., Steen, A.D., Stepanauskas, R., Richter, M., Kleindienst, S., Lenk, S., Schramm, A., Jørgensen, B.B., 2013. Predominant archaea in marine sediments degrade detrital proteins. Nature 496 (7444), 215–218.
Logroño, W., Nikolausz, M., Harms, H., Kleinsteuber, S., 2022. Physiological effects of 2-bromoethanesulfonate on hydrogenotrophic pure and mixed cultures. Microorganisms 10 (2), 355. https://doi.org/10.3390/microorganisms10020355.
Loh, H.Q., Hervé, V., Brune, A., 2021. Metabolic potential for reductive acetogenesis and a novel energy-converting [NiFe] hydrogenase in bathyarchaeia from termite guts – A genome-centric Analysis. Front. Microbiol. 11, 635786. doi: 10.3389/fmicb.2020.635786.
Looft, T., Levine, U.Y., Stanton, T.B., 2013. Cloacibacillus porcorum sp. nov., a mucin-degrading bacterium from the swine intestinal tract and emended description of the genus Cloacibacillus. Int. J. Syst. Evol. Microbiol. 63, 1960–1966. https://doi.org/10.1099/ijs.0.044719-0.
Mayer, A., Schädler, T., Trunz, S., Stelzer, T., Weuster-Botz, D., 2018. Carbon monoxide conversion with Clostridium aceticum. Biotechnol. Bioeng. 115 (11), 2740–2750. https://doi.org/10.1002/bit.26808.
Navarro, S.S., Cimpoia, R., Bruant, G., Guiot, S.R., 2016. Biomethanation of syngas using anaerobic sludge: Shift in the catabolic routes with the CO partial pressure increase. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.01188.
Nayak, D.D., Marx, C.J., 2014. Methylamine utilization via the N-methylglutamate pathway in Methylobacterium extorquens PA1 involves a novel flow of carbon through C1 assimilation and dissimilation pathways. J. Bacteriol. 196 (23), 4130–4139.
Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: statistical analysis of taxonomic and functional profiles. Bioinformatics 30, 3123–3124. https://doi.org/10.1016/j.jhazmat.2020.124595.
Schmidt, S., Biegel, E., Müller, V., 2009. The ins and outs of Na+ bioenergetics in Acetobacterium woodii. Biochim. Biophys. Acta 1787, 691–696. https://doi.org/10.1126/science.1244758.
Schöne, C., Rother, M., 2018. Methanogenesis from Carbon Monoxide. In: Stams, A., Sousa, D. (eds) Biogenesis of Hydrocarbons, Handbook of Hydrocarbon and Lipid Microbiology. Springer, Cham. doi: 10.1007/978-3-319-53114-4_4-1.
Schuchmann, K., Müller, V., 2013. Direct and reversible hydrogenation of CO2 to formate by a bacterial carbon dioxide reductase. Science 342, 1382–1385. https://doi.org/10.1126/science.1244758.
Tasaki, K., 2021. Chemical-free recovery of protein from cow manure digestate solid and antioxidant activity of recovered protein. Environ. Challenges. 4, 100132 https://doi.org/10.1016/j.envc.2021.100132.
Treu, L., de Kougias, P.G., Diego-Díaz, B., Campanaro, S., Bassani, I., Fernández-Rodríguez, J., Angelidaki, I., 2018. Two-year microbial adaptation during hydrogen-mediated biogas upgrading process in a serial reactor configuration. Bioresour. Technol. 264, 140–147. https://doi.org/10.1016/j.biortech.2018.05.070.
Ueki, A., Goto, K., Kaku, N., Ueki, K., 2018. Aminipila butyrica gen. nov., sp. nov., a strictly anaerobic, arginine-decomposing bacterium isolated from a methanogenic reactor of cattle waste. Int. J. Syst. Evol. Microbiol. 68, 443–448. https://doi.org/10.1099/ijsem.0.002534.
Voelklein, M.A., Rusmanis, D., Murphy, J.D., 2019. Biological methanation: Strategies for in situ and ex-situ upgrading in anaerobic digestion. Appl. Energy 235, 1061–1071. https://doi.org/10.1016/j.apenergy.2018.11.006.
Wang, S., Huang, H., Kahnt, J., Mueller, A.P., Köpke, M., Thauer, R.K., 2013. NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in *Clostridium autoethanogenum* grown on CO. J. Bacteriol. 195, 4373–4386. https://doi.org/10.1128/JB.00678-13.
Webster, T.M., Smith, A.L., Reddy, R.R., Pinto, A.J., Hayes, K.F., Raskin, L., 2016. Anaerobic microbial community response to methanogenic inhibitors 2-bromoethanesulfonate and propynoic acid. MicrobiologyOpen. 5 (4), 537–550. https://doi.org/10.1002/mbo3.349.
Wu, L., Wang, R., 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol. Rev. 257 (4), 585–630. https://doi.org/10.1124/pr.57.4.3.
Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Karl-Heinz, S., Whitman, W.B., Euzéby, J., Aman, R., Roselló-Móra, R., 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat. Rev. Microbiol. 12, 635–645. https://doi.org/10.1038/nrmicro3330.
Zhang, X., Tu, B., Dai, L.R., Lawson, P.A., Zheng, Z-Z., Liu, L-Y., Deng, Y., Zhang, H., Cheng, L., Petroclostridium xylanilyticum gen. nov., sp. nov., a xylan-degrading bacterium isolated from an oilfield, and reclassification of clostridial cluster III members into four novel genera in a new Hungateiclostridiaceae fam. Nov. Int. J. Syst. Evol. Microbiol. 68 (10), 3197-3211. doi: 10.1099/ijsem.0.002966.