



Research Article

Conductive materials change the composition and activity of butyrate-degrading enrichment cultures with hydrogen as the main electron shuttle

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ABSTRACT

Conductive materials (CM) can improve methane production (MP) efficiency in many methanogenic systems. However, several types of CM exist, and there are uncertainties regarding whether they all improve MP efficiency to the same extent and modulate microbial communities in a similar way. To investigate that, different microbial enrichments with and without activated carbon (AC), magnetite (Mag), and zeolites (Zeo) (at 0.5 g/L) were developed. MP profiles and microbial composition changes were compared among enrichments. The behavior of all enrichments was different, although the initial inoculum sludge was the same. Lag phase duration was lower in AC enrichment, while the complete conversion of butyrate to methane was faster in Mag enrichment. *Syntrophomonas* was the most abundant bacterial genus in all enrichments, but changes in the methanogenic community were evident. Acetoclastic methanogens were more diverse in Mag enrichment, with microorganisms assigned to *Methanosarcina* and *Methanoxix* genera, but *Methanoxix* was the only acetoclastic methanogen in the other enrichments. On the other hand, different species of hydrogenotrophic methanogens prevailed in distinct enrichments. The metatranscriptomics results revealed that the dominant mechanism of interspecies electron transfer in the AC enrichment utilized hydrogen as the electron carrier, and no evidences of direct interspecies electron transfer (DIET) could be found. These results showed how different CM modulate microbial communities and affect MP efficiency through mechanisms that do not necessarily involve DIET or mediation via CM.

1. Introduction

Anaerobic digestion (AD) is an attractive engineering technology based on microbial activity to produce methane from various organic wastes (e.g., wastewater effluents, food waste). Conductive materials (CM) have shown a potential to accelerate methane production rates (MPR), the stability of the AD process, and biogas/methane yields (Chen et al., 2022; Martins et al., 2018). This subject deserves particular attention because improving methane production (MP) efficiency can have a significant impact on the environment and society. For instance, conductive minerals (e.g., magnetite (Mag), iron oxide minerals) or black carbon are present in natural environments (e.g., lake and river sediments), enhancing their methanogenic activity (Rotaru et al., 2018). The anthropogenic activity can also increase the accumulation of CM in sediments (e.g., via runoff from activities of the forestry industry or other industries nearby) (Rotaru et al., 2018). Since methanogene-

sis has a central role in the global carbon cycle, CM have the potential to increase atmospheric methane emissions, which is a potent greenhouse gas, and therefore, this is an issue of great concern (Stams et al., 2012; Stams and Plugge, 2009). On the other hand, boosting biogas production via CM in engineered bioreactors and recovering the methane (Castilho et al., 2022; Prussi et al., 2021) can be an excellent strategy for a fast, clean, and renewable energy transition (Alberici et al., 2022; Commission, 2022a, 2022b).

From a practical perspective, understanding the impact of CM on AD microbiology is crucial for identifying the most effective materials that enhance methanogenic efficiency. From a fundamental perspective, understanding whether the improvement of MP efficiency is related to changes in interspecies electron transfer (IET) mechanisms that can potentiate microbial interactions and, consequently, methane recovery from waste, is of significant scientific interest.

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Several reasons are suggested for the improved performance in the presence of materials, such as their role as support material for microbial growth, and as electron conduit between different species (Akram et al., 2024; Sun et al., 2023). Conductive material-mediated interspecies electron transfer (CIET) has been suggested as the potential mechanism responsible for the improvements in MP efficiency (Fu et al., 2018; Kato et al., 2012; Li et al., 2015; Xia et al., 2019; Zhang and Lu, 2016; Zhang et al., 2018), although some authors do not exclude the possibility that CIET may occur simultaneously with interspecies hydrogen transfer (IHT) and interspecies formate transfer (IFT) (Fu et al., 2018; Xia et al., 2019; Zhang et al., 2018). IHT/IFT are considered the typical electron transfer mechanisms occurring during the AD process as a means of dissipating the electrons generated from the degradation of organic matter (Martins et al., 2018; Rotaru et al., 2021). It involves the production and consumption of electron carriers (i.e., hydrogen and formate) by syntrophic bacteria and methanogenic archaea, respectively. A common example of a syntrophic relationship involving IHT/IFT is the *Syntrophomonas wolfei* with *Methanospirillum hungatei* (Schmidt et al., 2013; Sieber et al., 2015). *S. wolfei* can only degrade volatile fatty acids (VFA), like butyrate, in the presence of a suitable hydrogen/formate-utilizing partner, like *M. hungatei* (Schmidt et al., 2013; Sieber et al., 2015). The role of the methanogenic partner will be to maintain hydrogen/formate (the electron carriers produced during butyrate degradation) at low levels, allowing butyrate degradation to be thermodynamically favourable (Schmidt et al., 2013; Sieber et al., 2015, 2014). Another possible IET mechanism is the direct interspecies electron transfer (DIET) that relies on direct cell-to-cell electrical contacts by electrically conductive pili (e-pili) and/or c-type cytochromes (membrane-bound electron transport proteins), which are the main biological structures known to participate in this type of IET (Lovley, 2017; Rotaru et al., 2021). This mechanism is shuttle-free, meaning it does not involve the exchange of electron carriers, such as hydrogen or formate (Martins et al., 2018). In CIET mechanism, CM can function as electrical conduits, allowing the attachment of syntrophic partners on the surface of CM through which electron exchange occurs (Barua and Dhar, 2017; Rotaru et al., 2021). This external cell-to-cell electron transfer can enhance the syntrophic conversion of different organic compounds to methane, without the need for cells to form agglomerates or to be in direct contact as it occurs in DIET (Barua and Dhar, 2017; Rotaru et al., 2021). However, only a few studies effectively demonstrated that CIET occurred in the presence of activated carbon (AC), Mag, biochar, or carbon cloth, with these materials replacing the biological structures required for DIET (Chen et al., 2014a, 2014b; Liu et al., 2015, 2012; Lovley, 2017), or facilitating the IET between bacteria and methanogenic archaea (Chen et al., 2014a, 2014b; Liu et al., 2012; Rotaru et al., 2014a).

Previous microbial enrichment experiments in the presence of CM (e.g., Mag, carbon nanotubes (CNT), biochar, granular activated carbon (GAC)) and using anaerobic microbial communities from different natural sediments as inoculum have demonstrated the acceleration of MPR (data compiled and available at (Braga et al., 2024a)). However, there are still open questions regarding the effects of CM on microbial communities. For example, it remains unclear how microbial communities adapt and evolve when exposed to CM over extended periods. It is also worth knowing how different materials affect the taxonomic composition of microbial communities, once the majority of CM tend to improve methanogenic microbial activities, while simultaneously promoting the growth of some taxonomic groups. Another doubt remaining is whether IET mechanisms change in microbial communities adapted to CM. The majority of studies reported in the literature were performed with different inocula and carbon sources, preventing a direct comparison of the effects of different CM on the composition and activity of microbial communities.

Intending to address these questions, this study developed several enrichment cultures (EC) specialized in butyrate degradation in the presence of AC, Mag and zeolites (Zeo). Mag and AC have been previously reported as materials that can facilitate CIET (Martins et al.,

2018). Zeo was recently found to improve the methanogenic activity of *Methanobacterium formicicum* (Braga et al., 2024b). Butyrate was chosen as the carbon and energy source because it is a VFA typically converted to methane by syntrophic cultures, which exchange hydrogen or formate between butyrate-degrading bacteria and hydrogenotrophic methanogens (Schmidt et al., 2013; Stams and Plugge, 2009). Therefore, such EC are ideal to investigate if microbial communities' composition and electron transfer mechanisms change when in contact with different materials. The composition and activity of the microbial communities adapted to the three materials were compared, and genes expressed during butyrate conversion to methane in the presence of AC were identified. This allowed new insights into the impact of CM on IET mechanisms and microbial interactions in long-term enrichments.

2. Materials and methods

2.1. Development of butyrate-degrading microbial enrichments

The inoculum to start the butyrate-degrading EC was an anaerobic granular sludge collected from a brewery wastewater treatment plant (Super Bock, Porto, Portugal). Before inoculation, the sludge was incubated overnight (statically, at 37 °C) to deplete any residual substrate, and then it was disrupted by using an electric blender.

Not all enrichments were prepared in parallel. Butyrate-EC with and without materials were developed using the same inoculum but staggered in time, and thus differences on microbial composition and activity are expected (Appendix A Fig. S1). Enrichments with AC, Mag and without materials were performed simultaneously, using inoculum 1, while enrichments with Zeo (and another without materials) were set-up 12 months later, using inoculum 2. The cultures developed with AC and Mag were denominated AC-b1 and Mag-b1, respectively, and the corresponding EC without materials was denominated Bty-b1. Similarly, EC developed with Zeo was denominated Zeo-b2, and the correspondent EC without materials was Bty-b2. The cultures were continuously transferred (10 % V/V) until they reached similar performances in consecutive transfers. Then, each culture was characterized in triplicate regarding butyrate consumption, MP and microbial composition.

The commercial materials used in the incubations were AC from Norit (ROX 0.8), and Mag (Iron (II, III) oxide, Fe_3O_4) and Zeo (Molecular sieve, type 13X - beads, 4–8 mesh) from Sigma-Aldrich. These materials were chosen as representatives of each group of materials, i.e., carbon-based materials (AC), iron-based materials (Mag) and silicon-containing materials (Zeo). Their physicochemical characterization is given elsewhere (Braga et al., 2024b).

To initiate the EC, the anaerobic sludge (3 g/L of volatile solids) was distributed into bottles of 120 mL (50 mL of working volume) containing an anaerobic basal medium previously sterilized by autoclaving. The materials, i.e., AC, Mag and Zeo, were added at a concentration of 0.5 g/L. The bottles were sealed with rubber stoppers and aluminum caps, and the headspace was flushed with N_2/CO_2 (80 %:20 % V/V; 170 kPa). Vitamins' solution and bicarbonate containing sodium sulfide solution (0.8 mmol/L; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, Acros Organics) were added to the bottles to provide a saline bicarbonate-buffered mineral salt medium, supplemented with vitamins and sodium sulfide as reducing agent, as described elsewhere (Stams et al., 1993). Vitamins' solution and sodium sulfide were sterilized by filtration (0.22 μm) and under aseptic conditions. Butyrate (Sigma-Aldrich) stock solution was sterilized by autoclaving and added at a final concentration of 10 mmol/L. The batch incubations were performed without agitation at 37 °C and protected from light. After total butyrate conversion to methane, the cultures were transferred (10 % V/V) periodically to a fresh medium containing butyrate, and 0.5 g/L of each material (for enrichments with AC, Mag and Zeo), and MP from butyrate was followed over time.

When the cultures were well-adapted, i.e., the duration of incubations required for butyrate degradation became similar across successive transfers, they were characterized in terms of butyrate consumption, ac-

etate formation, MP, and microbial composition (**Appendix A Fig. S1**). The incubations were performed in triplicate.

2.2. Analytical methods

Gas samples were collected from the incubation bottles by using a Pressure-Lock syringe (SGE Syringe, Trajan Scientific Australia Pty Ltd., Australia) and were injected in a gas chromatograph-FID Chrompack 9000 (Shimadzu, Kyoto, Japan) with Carbowax 20 M column (2 m × 2 mm, 80–120 mesh), using the conditions described elsewhere (Duarte et al., 2020). According to Avogadro's Law, the CH₄ concentration from partial pressure in the headspace was converted to mmol/L in a liquid medium. Acetate and butyrate were analysed by high-performance liquid chromatography (Jasco, Tokyo, Japan) as described elsewhere (Duarte et al., 2020). The growth medium's conductivity, redox potential (ORP), and pH were measured at the beginning and end of the incubations. ORP was measured with a portable meter C533 (Consort, Turnhout, Belgium) equipped with an ORP electrode D 223 (VWR, Radnor, PA, USA). pH and electrical conductivity of the growth medium were measured with a Consort multi-parameter analyser C1020 and C3010, respectively.

2.3. Taxonomic characterization of microbial communities: RNA extraction and 16S rRNA gene amplicon sequencing

The taxonomic composition of the microbial EC was determined in duplicate by 16S rRNA gene sequencing. Aliquots of the inoculum sludge and adapted butyrate-degrading EC were collected and centrifuged (15 min, 15 °C, 7000 g). The pellet was resuspended in RNA later (Sigma-Aldrich, Germany) and frozen at −20 °C until RNA extraction. RNA was extracted using the FastRNA ProTM Soil-Direct Kit commercial kit (MP Biomedicals, USA), following the manufacturer's instructions. The isolated RNA was treated with DNase I (Thermo Scientific, USA) to eliminate the co-extracted DNA. cDNA was synthesized by using a SuperScriptTM IV Reverse Transcriptase (InvitrogenTM, Thermo Fisher Scientific) and a prokaryotic universal reverse primer (1492r) (Lane, 1991). cDNA was precipitated with ethanol and sent to the Research and Testing Laboratory (RTL, Texas), where cDNA amplification, Illumina libraries preparation, amplicon sequencing (Illumina MiSeq, Inc. San Diego, USA) and bioinformatics analysis was performed as described elsewhere (Salvador et al., 2019). The universal primer set 515F/806R (Caporaso et al., 2011), targeting the prokaryotic 16S rRNA gene, was used in the amplification step. FASTQ files were submitted to the European Nucleotide Archive (ENA), under the project accession number PRJEB77088.

2.4. Phase contrast microscopy

The EC were regularly observed under a contrast phase microscope CX41 RF (Olympus, Tokyo, Japan), and the images were captured using an Olympus digital camera (high-resolution SC50) combined with the software OLYMPUS cellSens Entry.

2.5. Metatranscriptomics analysis

2.5.1. RNA isolation and sequencing of transcripts

AC-adapted EC (20 % V/V of inoculum) was incubated in the same conditions described above but in bottles with a working volume of 300 mL and a headspace of 300 mL. Samples were taken during the initial phase of MP using the AC-b1 EC. Six biological replicates were performed. The content of each bottle was collected when approximately 3 mmol/L of methane was achieved. Cells were harvested by centrifugation (15 min, 10,000 ×g, 10 °C) and preserved in RNA later (Sigma-Aldrich), at −20 °C until RNA extraction. The RNA extraction was performed using the FastRNA ProTM Soil-Direct Kit commercial kit (MP

Biomedicals, USA) following the manufacturer's instructions. RNA samples were preserved at −80 °C and sent to the Centre Tecnològic de Catalunya (Eurecat), where DNase treatment, cDNA synthesis, library preparation and sequencing (NextSeq2000 sequencing system, Illumina) were performed as described by (Martins et al., 2021). FASTQ files were submitted to ENA under the project accession number PRJEB78461.

2.5.2. Bioinformatics analysis

Sequencing data was analysed using the MOSCA pipeline (version 1.6.1) (Sequeira et al., 2024). Briefly, FASTQ files were submitted to preprocessing with Trimmomatic for removal of adapters, low-quality regions (where the 10th percentile or the median of base quality fall below a Phred score of 10 or 25, respectively), low-quality reads (average Phred score <20) and short reads (lower than 50 bp). SortMeRNA was used to remove rRNA reads. Assembly of reads into contigs was performed with Trinity (Celaj et al., 2014). Gene calling was obtained by running FragGeneScan on the contigs, and annotation was performed both with UPIMAPI (Sequeira et al., 2022) (using the entire UniProt database as reference), and reCOGNizer (Sequeira et al., 2022) (using CDD, Protein Clusters, NCBIfam, TIGRFAM, Pfam, COG and SMART databases as references). Quantification of gene expression was obtained by aligning the reads to the assembled transcripts by using Bowtie2 (Langmead and Salzberg, 2012). Normalization of quantification values were obtained by dividing the read counts by the length of the contigs and by using the EdgeR package (using the trimmed mean of *M*-values method) (Robinson et al., 2010).

Special attention was given to the most expressed enzymes and to those involved in the following processes: degradation of fatty acids, electron transfer, and methanogenesis. The list of enzymes involved in each process were obtained based on information from the KEGG pathways and in the literature (Berghuis et al., 2019; Du et al., 2021; Enzmann et al., 2018; Fu et al., 2019; Kurth et al., 2020; Lie et al., 2012; Müller et al., 2010; Park et al., 2019; Schmidt et al., 2013; Sieber et al., 2015, 2010b; Sikora et al., 2018; Wang et al., 2020a; Xiao et al., 2020; Ziels et al., 2019).

3. Results

3.1. Characterization of butyrate-degrading enrichments

Microbial EC obtained by successive transfers (**Appendix A Fig. S1**) with and without different materials presented different performances regarding butyrate conversion to methane (**Fig. 1**).

The MP profiles of the EC showed transient acetate accumulation and, consequently, two different stages of MP could be distinguished, i.e., a first stage characterized by an initial methane production rate (IMPR) corresponding mainly to methane produced via the methanogenic hydrogenotrophic pathway, and a second stage characterized by the methane production rate (MPR) corresponding to the methane produced via the methanogenic acetoclastic pathway. The kinetic parameters, i.e., lag phase duration, IMPR, and MPR, were notably different under the various enrichment conditions. The Bty-b1 enrichment (**Fig. 1a**) showed a lag phase of approximately 2 days, while in the parallel EC containing CM (AC-b1 and Mag-b1, **Fig. 1b and c**, respectively) MP started earlier (after 1 day of incubation) (**Table 1**). Thus, IMPR was higher in AC-b1 and Mag-b1 EC than in Bty-b1, with Mag-b1 EC producing methane faster at the beginning of the incubation, when compared to the other EC. Main differences were also observed regarding acetate conversion to methane. For instance, the AC-b1 EC presented a low MPR (approximately 0.7 mmol/(L·day)), whereas acetate was converted to methane much faster by Bty-b1 and Mag-b1 EC (showing a MPR of around 1.9 mmol/(L·day) and 2.1 mmol/(L·day), respectively) (**Table 1**). Consequently, the total time of incubation required for the complete conversion of butyrate to methane was quite different among enrichments. The AC-b1 EC took around 31 days to completely convert

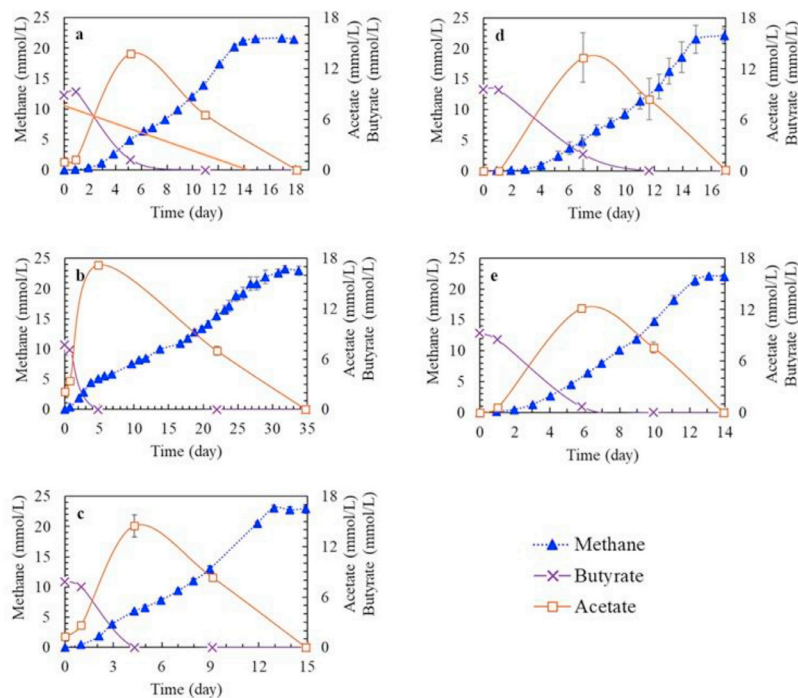


Fig. 1 – Cumulative methane production (MP) and butyrate and acetate consumption profiles of butyrate-degrading enrichment cultures (EC) developed: (a) without materials (Bty-b1), (b) with activated carbon (AC) (AC-b1), (c) with magnetite (Mag) (Mag-b1), (d) without materials (Bty-b2), and (e) with zeolites (Zeo) (Zeo-b2). The Bty-b1 was developed simultaneously with AC and Mag enrichments, while Bty-b2 was developed along with Zeo enrichment.

Table 1 – Characterization of butyrate-degrading enrichment cultures without and with materials in terms of the number of transfers performed, lag phase duration, initial methane production rate (IMPR), methane production rate (MPR), and the time (*t*) required for the total conversion of butyrate to methane.

Enrichment	Number of transfers	Lag phase (day)	IMPR ^a (mmol/(L·day))	R ²	MPR ^b (mmol/(L·day))	R ²	<i>t</i> (day)
Bty-b1	11	2	1.13 ± 0.11	0.91 ± 0.01	1.90 ± 0.18	0.98 ± 0.00	14
AC-b1	8	1	1.35 ± 0.03	0.99 ± 0.00	0.70 ± 0.05	0.97 ± 0.00	31
Mag-b1	11	1	1.72 ± 0.03	0.99 ± 0.00	2.12 ± 0.03	0.98 ± 0.00	13
Bty-b2	8	3	0.09 ± 0.04	0.90 ± 0.03	1.73 ± 0.18	0.98 ± 0.01	15
Zeo-b2	8	2	0.23 ± 0.04	0.99 ± 0.01	2.17 ± 0.07	0.98 ± 0.00	12

^a IMPR was calculated for the first slope of MP: Bty-b1 between days 1 and 5; AC-b1 between days 1 and 4; Mag-b1 between days 1 and 4; Bty-b2 between days 0 and 3; Zeo-b2 between days 0 and 2.

^b MPR was calculated for the second slope of MP: Bty-b1 between days 6 and 13; AC-b1 between days 5 and 29; Mag-b1 between days 5 and 13; Bty-b2 between days 4 and 14; Zeo-b2 between days 3 and 12.

butyrate to methane, while Mag-b1 and Bty-b1 needed less than half of the time (i.e., 13 days and 14 days, respectively).

Slight differences in MP profiles were obtained in the two enrichments developed without CM, i.e., Bty-b1 and Bty-b2 (Fig. 1a and d), which were developed with the same inoculum sludge but staggered in time (1 year of gap). Nevertheless, the two enrichments showed similar methanogenic activities (Table 1). By comparing Bty-b2 with Zeo-b2, it is noticeable that the lag phase duration was lower and methane production rates (IMPR and MPR) were higher in the enrichment containing Zeo (Fig. 1d and e; Table 1).

The presence of different materials altered the ORP values at the beginning of the assays. ORP values were higher with Mag (-254 ± 18 mV) relative to Bty-b1 EC (-332 ± 6 mV), and with Zeo (-336 ± 18 mV) relative to Bty-b2 (-273 ± 18 mV) (Appendix A Table S1). At the end of incubations, the ORP values were similar for all conditions, except for Mag incubations, which presented higher ORP values. The pH values were also very close in the different conditions, between 7.3 and 7.4, at the beginning or end of incubations (Appendix A Table S1), except for the growth with Zeo that had a higher pH value at the beginning and end of the incubations (approximately 7.5). This is in line with the fact that Zeo presents a basic character since its pH_{pzc} is around 11.6 (Braga et al., 2024b), and, as a result, the presence of Zeo may slightly increase the pH of the growth medium. On the other hand, the values of conductivity were not significantly different among the conditions

for the enrichments developed from inoculum 1, except for the value of conductivity measured at the end of the growth medium in Mag-b1 incubation, which was the highest value recorded ((7.35 ± 0.06) mS/cm) (Appendix A Table S1). In the incubations obtained from inoculum 2, the conductivity of the growth medium was not significantly affected by the presence of Zeo.

3.2. Microbial diversity of butyrate-degrading enrichments

Microscopic observations of the EC revealed major differences, but also some common morphotypes (Fig. 2). *Methanothrix*-like cells (filamentous rods) were present in all EC. In AC-enrichments, *Syntrophomonas*-like cells (slightly curved rods) were very abundant (and were also detected in the other EC), as were irregular cocci cells (Fig. 2b). On the other hand, *Methanosarcina*-like cells (cocci) were only detected in Mag-enrichments (Fig. 2c).

To gain further insights into the structure of microbial communities degrading the same substrate with and without CM, we compared the taxonomic composition of all EC. Appendix A Table S2 shows the complete taxonomic identification and relative abundances, while Table 2 presents the taxonomic information only at a relative abundance higher than 2.00 %. The microbial communities changed significantly from the inoculum sludge to the enrichments specialized in butyrate degradation (Table 2, Appendix A Table S2). The inoculum sludge was rich in mi-

Table 2 – Taxonomic characterization at the genus level (at relative abundance ≥ 2.00 %) of anaerobic inocula sludge and well-adapted enrichments, with and without materials, based on 16S rRNA gene amplicon sequencing. The relative abundance of each taxon (given in percentage) is the average and standard deviation for (at least) duplicate samples.

Kingdom	Phylum	Class	Order	Family	Genus	Inoc-b1	Bty-b1	AC-b1	Mag-b1	Inoc-b2	Bty-b2	Zeo-b2
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobacterium</i>	9.00 $\pm$ 3.26	nd	< 0.50	< 0.50	3.71 $\pm$ 2.31	< 0.50	nd
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanofollis</i>	nd	< 0.50	< 0.50	< 0.50	nd	2.42 $\pm$ 1.36	7.82 $\pm$ 0.79
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Unclassified	nd	12.95 $\pm$ 2.82	29.22 $\pm$ 16.75	28.32 $\pm$ 2.57	nd	nd	nd
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanolinea</i>	9.07 $\pm$ 0.63	< 0.50	< 0.50	< 0.50	2.70 $\pm$ 2.18	< 0.50	nd
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanospirillum</i>	< 0.5	25.88 $\pm$ 0.89	23.64 $\pm$ 2.77	16.53 $\pm$ 0.68	nd	25.47 $\pm$ 1.27	20.87 $\pm$ 0.03
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanobacterium</i>	20.00 $\pm$ 0.96	37.27 $\pm$ 2.01	14.88 $\pm$ 2.95	25.72 $\pm$ 0.51	4.13 $\pm$ 5.60	37.19 $\pm$ 16.64	46.21 $\pm$ 2.93
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Methanosarcina</i>	nd	< 0.50	nd	8.21 $\pm$ 10	nd	< 0.50	nd
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	<i>Anaerophaga</i>	< 0.50	2.19 $\pm$ 0.14	3.16 $\pm$ 2.73	1.36 $\pm$ 0.32	< 0.50	1.55 $\pm$ 0.72	< 0.50
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	<i>Syntrophomonas</i>	nd	15.62 $\pm$ 0.08	26.18 $\pm$ 12.40	12.43 $\pm$ 0.49	nd	15.43 $\pm$ 7.44	10.91 $\pm$ 1.84
Bacteria	Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	< 0.50	< 0.50	< 0.50	nd	0.61 $\pm$ 0.50	< 0.50	6.99 $\pm$ 2.27
Bacteria	Firmicutes	Clostridia	Clostridiales	Unclassified	Unclassified	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	3.61 $\pm$ 3.93	< 0.50
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacteriales	Desulfobacteriaceae	<i>Desulfobacterium</i>	< 0.50	< 0.50	< 0.50	< 0.50	1.52 $\pm$ 1.09	< 0.50	< 0.50
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacteriales	Desulfobacteriaceae	<i>Geobacter</i>	14.42 $\pm$ 0.89	< 0.50	< 0.50	< 0.50	27.32 $\pm$ 1.59	< 0.50	nd
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacteriales	Desulfobacteriaceae	Unclassified	< 0.50	nd	< 0.50	nd	6.49 $\pm$ 6.80	< 0.50	nd
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacteriales	Syntrophobacteraceae	<i>Syntrophobacter</i>	15.87 $\pm$ 0.84	nd	nd	nd	26.32 $\pm$ 9.57	< 0.50	< 0.50
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacteriales	Unclassified	Unclassified	0.62 $\pm$ 0.09	nd	< 0.50	< 0.50	2.75 $\pm$ 0.74	< 0.50	< 0.50
Bacteria	Proteobacteria	Unclassified	Unclassified	Unclassified	Unclassified	4.19 $\pm$ 0.75	< 0.50	< 0.50	nd	0.54 $\pm$ 0.34	< 0.50	nd
Bacteria	Synergistetes	Synergistia	Synergistales	Unclassified	Unclassified	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50	0.72 $\pm$ 0.02	2.39 $\pm$ 0.40
Bacteria	Unclassified	Unclassified	Unclassified	Unclassified	Unclassified	18.64 $\pm$ 2.73	1.68 $\pm$ 0.43	< 0.50	4.65 $\pm$ 0.61	14.15 $\pm$ 3.05	11.10 $\pm$ 2.04	1.17 $\pm$ 0.60

^a nd: not detected; Inoc-b1: Inoculum 1; Inoc-b2: Inoculum 2.

croorganisms assigned to *Bacteria* (i.e., around 61 % and 89 % in inocula 1 and 2, respectively) and, after the enrichment process, methanogens (belonging to the *Archaea* domain) became more abundant, reaching percentages ranging from 66 % to 79 % in the EC.

In the archaeal community, *Methanobacterium* genus was dominant (around 49 %) in Bty-b1 EC, followed by *Methanospirillum* (approximately 34 %) and an unclassified genus within Methanomicrobiaceae (around 17 %). In the presence of Mag, the methanogenic community became more diverse with the detection of two acetoclastic methanogens (around 33 % of *Methanobacterium* and 10 % of *Methanosarcina*, within the methanogenic community) and two hydrogenotrophic methanogens (uncultured Methanomicrobiaceae and *Methanospirillum* in percentages of approximately 36 % and 21 %, respectively, relative to the archaeal community). While the Mag-enrichment presented a highly diverse methanogenic community, the enrichment with AC showed a lower diversity and a poor acetoclastic community, as *Methanobacterium*, the only acetoclastic methanogen detected, was present in a relatively low percentage when compared with the other enrichments (approximately 22 % of the archaeal community). These results are in line with the long periods necessary for complete acetate conversion to methane in AC-enrichment (Fig. 1b).

In the second set of enrichments (Bty-b2 and Zeo-b2), *Methanobacterium* was the most abundant genus within the archaeal community (around 57 % in Bty-b2 and 61 % in Zeo-b2), followed by *Methanospirillum* (about 39 % in Bty-b2 and 28 % in Zeo-b2) and *Methanofollis* (approximately 4 % in Bty-b2, and 10 % in Zeo-b2).

In all EC, *Syntrophomonas* species (butyrate-oxidizers) were the most abundant bacteria, representing over 11 % of the total microbial community and over 44 % of the bacterial community, although it was not detected in the inocula sludge (Table 2). For example, in AC-b1 EC, this genus was detected in 26.18 % ($\pm$ 12.40 %) (Table 2), representing around 82 % of the bacterial community, confirming the prevalence of these microorganisms as observed by microscopic observations (Fig. 2b). On the other hand, although the genera *Geobacter* (electroactive bacteria, generally butyrate and acetate oxidizers (Koch and Harnisch, 2016; Lovley et al., 2011)) and *Syntrophobacter* (typical propionate-oxidizing bacteria (Zhang et al., 2023)) were in relatively high abundance in the inocula sludge, they were not detected in the microbial EC, neither with or without CM (Table 2). These results indicate a major role of *Syntrophomonas* species in butyrate degradation in all EC, suggesting that the rate of butyrate conversion to methane was dependent on their syntrophic interactions with methanogens and on the activities of both methanogens and bacteria inside the cultures. Other members of bacterial community were present in percentages lower than 1 % (Appendix A Table S2). The microorganisms present in abundances higher than 1 % include the ones assigned to *Anaerophaga* (strictly anaerobic fermentative bacteria (Tao et al., 2020)) in Bty-b1, AC-b1, Mag-b1 and Bty-b2, a microorganism belonging to the phylum Firmicutes in Bty-b2, and *Desulfomicrobium* (sulfate-reducers (El Houari et al., 2020)) in inoculum 2 (Table 2, Appendix A Table S2). In Zeo-b2, bacteria assigned to *Candidatus Cloacimonas* (acetogenic bacteria (Lendormi et al., 2022)) and *Sulfuricurvum* (sulfide-oxidizing bacteria (Bagheri Novair et al., 2024)) were enriched but were not detected in Bty-b2 (Table 2).

3.3. Metatranscriptomics analysis of a butyrate-degrading enrichment culture with AC

Since only MP via the hydrogenotrophic pathway was accelerated in the AC-b1, the gene expression was investigated to obtain further information on the IET mechanisms and the microorganisms that were actively functioning within the microbial community.

The metatranscriptomics analysis of the butyrate-degrading enrichment developed with AC, provided interesting insights on the role of specific microorganisms. Appendix A Table S3 shows the list of all genes expressed with the corresponding taxonomic assignment, while

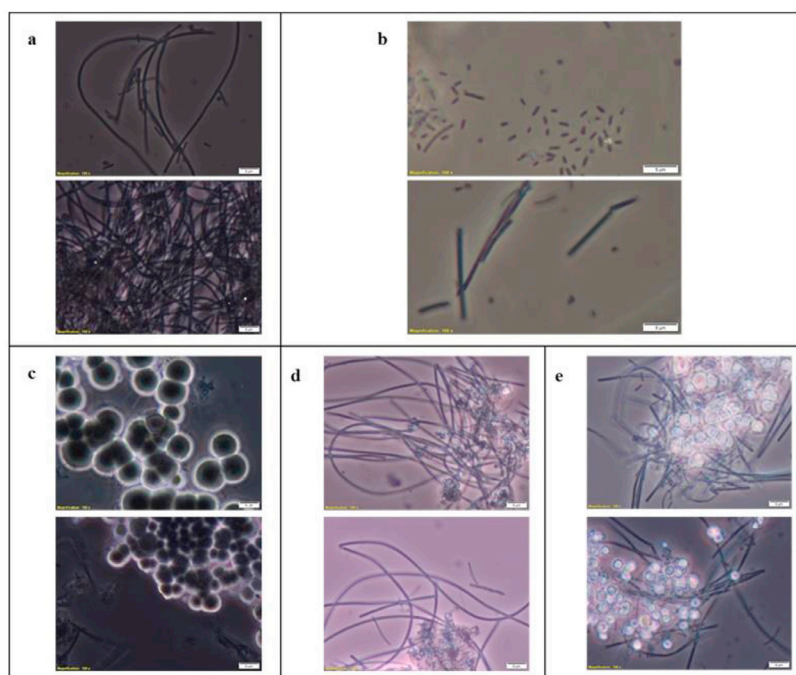


Fig. 2 – Microscopic observations during the development of the butyrate-degrading EC: (a) *Methanotherix*-like cells in Bty-b1, (b) irregular cocci cells and *Syntrophomonas*-like cells in AC-b1, (c) *Methanosarcina*-like cells in Mag-b1, (d) *Methanotherix*-like cells in Bty-b2, and (e) *Methanotherix*-like cells in Zeo-b2. The images were captured with a magnification of 100 ×, corresponding to a scale of 5 μm.

Tables 3 and 4 summarize only the genes expressed which code for enzymes involved in methanogenesis and β -oxidation pathways, respectively. The results revealed that 59 % of the genes were expressed by methanogenic microorganisms belonging to Archaea, and 36 % by members of Bacteria. Among methanogens (Table 3), hydrogenotrophic microorganisms belonging to Methanomicrobiales represented over 55 % of gene expression, with *Methanofollis* (35 % of the total expression) and *Methanospirillum* (21 % of the total expression) as the most active methanogens. Acetoclastic methanogens assigned to Methanosarcinales represented 32 % of total gene expression, with *Methanotherix* as the most active methanogen converting acetate to methane (22 % of the total expression). Because butyrate is degraded via the β -oxidation pathway, we searched for the microorganisms that were expressing the enzymes involved in β -oxidation. We found that around 94 % of the genes for β -oxidation were assigned to a Syntrophomonadaceae bacterium (Table 4). Similarly, we searched for the microorganisms expressing genes coding for proteins involved in methanogenesis and could identify which groups of methanogens were more active in the enrichment (Table 3). With the information collected about the key players for butyrate degradation and MP, it was possible to suggest the most probable syntrophic interactions occurring in the butyrate-degrading enrichment developed with AC (Fig. 3). All proteins involved in syntrophic butyrate degradation via hydrogen and formate could be detected through metatranscriptomics and the majority of reads were assigned to a microorganism from the Syntrophomonadaceae family, and therefore this microbe is likely the most active in butyrate degradation (Table 4). Regarding MP, *Methanotherix* was undoubtedly the responsible for MP via acetate, and the most active hydrogenotrophic methanogen was *Methanofollis*, although *Methanospirillum* also showed hydrogenotrophic activity (Table 3).

4. Discussion

This study evaluated the long-term effect of CM on the composition and performance of methanogenic communities. When CM are added to anaerobic bioreactors, they tend to improve waste conversion to methane. It is usually an immediate effect that is reflected in the reduction of the lag phase duration and consequently in the time required for complete waste conversion to methane (Castilho et al., 2022;

Chen et al., 2022; Liu et al., 2023). However, if the presence of CM affects the composition and performance of microbial cultures during an enrichment process, and the activity of well-adapted cultures, was still unknown. In this work, we adapted an anaerobic sludge to butyrate conversion to methane, with and without CM. We end up with 5 well-adapted EC, 2 developed without CM, and 3 developed with CM (AC, Mag and Zeo). Generally, acetoclastic methanogens (*Methanotherix* and *Methanosarcina*) were less abundant than hydrogenotrophic methanogens (Table 2), which explains why acetate transiently accumulated, and acetate conversion to methane took several days in all enrichments (Fig. 1). The poor enrichment of acetoclastic methanogens was more evident in the AC-b1, which took approximately 30 days to completely convert the acetate formed from butyrate degradation to methane. Indeed, the AC-b1 EC was the one with lower percentage of *Methanotherix*, the acetoclastic methanogen that was prevalent in these EC (Table 2). The less abundant acetoclastic community in AC-b1 explains the delay in MP (Fig. 1b). The efficiency of AC-b1 was much lower than the efficiency of the EC developed without CM (Bty-b1), where total butyrate degradation was much faster (approximately 14 days) (Fig. 1a). Note that *Methanotherix* was highly abundant in Bty-b1 culture (about 37 % of the total microbial community; Table 2). Nevertheless, in the AC-enrichment, interspecies interactions between butyrate-degrading bacteria and hydrogenotrophic methanogens were strengthened since the IMPR was higher than in Bty-b1 (Table 1). The microbial enrichment presenting the highest IMPR was Mag-b1 (Table 1). Mag-b1 EC was more efficient, although the time needed for complete butyrate conversion to methane by Mag-b1 and Bty-b1 was not that different (13 days and 14 days, respectively) (Fig. 1c and a, respectively; Table 1). Interestingly, *Methanosarcina* was only enriched in Mag-b1 EC (Table 2). This genus is known to perform the reduction of Fe(III) (present in Mag), as demonstrated previously (Fu et al., 2019; Wang et al., 2020b), and many studies report that the acetoclastic activity is improved by Mag (Fu et al., 2019; Wang et al., 2020b; Wu et al., 2020), which was also observed in this work.

Remarkably, the enrichment Zeo-b2 presented the highest MPR and was the fastest converting butyrate to methane (12 days; Fig. 1; Table 1). The effect of Zeo in methanogenesis has been poorly explored, but we recently showed its potential to accelerate the methanogenic activity (Braga et al., 2024b). The most efficient EC showed a common trend,

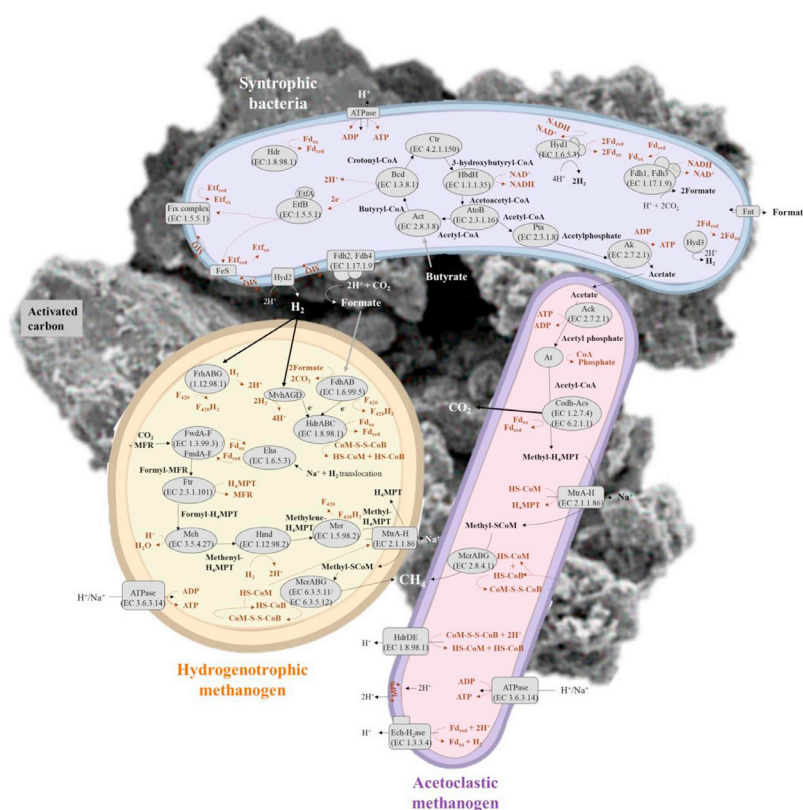
Table 3 – Expression of enzymes involved in methanogenesis via hydrogenotrophic and acetoclastic methanogenic pathways in the microbial EC developed with activated carbon (AC), with respective taxonomic assignment.

Abbreviation	Taxonomic assignment:		<i>Methanofollis</i>		<i>Methanospirillum</i>		<i>Methanothrix</i>		Other archaea	
	Protein name	EC number	Number of reads ^a	Percentage of reads ^b	Number of reads ^a	Percentage of reads ^b	Number of reads ^a	Percentage of reads ^b	Number of reads ^a	Percentage of reads ^b
Acs	Acetyl-coenzyme A synthetase	6.2.1.1	76,972	6 %	6856	< 1 %	1013,677	78 %	206,201	16 %
Codh	Carbon-monoxide dehydrogenase	1.2.7.4	589,274	46 %	78,237	6 %	600,642	47 %	75	< 1 %
MtrA-H	Tetrahydromethanopterin S-methyl-transferase	2.1.1.86	722,734	71 %	88,595	9 %	122,540	12 %	80,299	8 %
McrABG	Methyl-coenzyme M reductase	2.8.4.1	1783,123	29 %	1070,471	18 %	2759,415	46 %	443,096	7 %
HdrDE	Heterodisulfide reductase	1.8.98.1	2676,349	77 %	466,526	13 %	14,517	< 1 %	317,818	9 %
Ech-H ₂ ase	Hydrogenase	1.3.3.4	4074,412	58 %	363,711	5 %	1878,301	27 %	665,056	10 %
ATPase	V-type ATPase	3.6.3.14	1577,060	51 %	220,050	7 %	645,881	21 %	665,852	21 %
Eha	Energy-converting hydrogenase A	1.6.5.3	1170,414	80 %	8050	< 1 %	140,708	10 %	144,857	10 %
FwdA-F/FmdA-F	Formylmethanofuran dehydrogenase	1.3.99.3	1059,029	84 %	7309	< 1 %	20,574	2 %	171,112	14 %
MvhAGD	F ₄₂₀ -nonreducing hydrogenase		18,083	100 %	0	0 %	0	0 %	0	0 %
FdhAB	Formate dehydrogenase	1.6.99.5	587,915	91 %	45,340	7 %	3336	< 1 %	11,186	2 %
HdrABC	Soluble heterodisulfide reductase	1.8.98.1	914,692	71 %	107,837	8 %	3235	< 1 %	268,478	21 %
Ftr	Formylmethanofurantetrahydromethanopterin formyl-transferase	2.3.1.101	21,798	56 %	6068	16 %	0	0 %	10,941	28 %
FrhABG	F ₄₂₀ -reducing hydrogenase	1.12.98.1	723,928	89 %	87,690	11 %	0	0 %	0	0 %
Hmd	H ₂ -dependent methylenetetrahydromethanopterin dehydrogenase	1.12.98.2	0	0 %	144	100 %	0	0 %	0	0 %
Mer	5,10-methylenetetrahydromethanopterin reductase	1.5.98.2	526,228	85 %	2481	< 1 %	0	0 %	89,638	15 %
CoM	Methyl coenzyme M reductase	2.8.4.1	1758,333	29 %	1069,143	18 %	2766,329	46 %	440,182	7 %
Fd	Ferredoxin		2872,137	69 %	587,172	14 %	331,251	8 %	354,849	9 %

^a Total read counts by the enzyme.^b The percentages were obtained by dividing the number of reads by the total number of reads for the specific enzyme. The color gradient emphasizes the highest number of reads obtained for each taxon considered.
EC number – enzyme commission number.

Table 4 – Expression of enzymes involved β -oxidation of butyrate and hydrogen production in the microbial enrichment developed with AC, with respective taxonomic assignment.

Abbreviation	Taxonomic assignment:		Syntrophomonadaceae		Other bacteria	
	Protein/ enzymatic complex name	EC number	Number of reads ^a	Percentage of reads ^b	Number of reads ^a	Percentage of reads ^b
Act	Acetyl-CoA coenzyme A-transferase	2.8.3.8	4909,313	99.90 %	5341	< 1.0 %
Bcd	Butyryl-CoA dehydrogenase	1.3.8.1	6026,733	99.40 %	38,495	< 1.0 %
Ctr	Crotonyl-CoA hydratase	4.2.1.150	5541,852	99.70 %	19,163	< 1.0 %
HbdH	3-hydroxybutyryl-CoA dehydrogenase	1.1.1.35	2391,505	98.40 %	39,375	1.60 %
AtoB	Acetyl-CoA acetyltransferase	2.3.1.16	24,360,477	99.20 %	208,522	< 1.0 %
Pta	Phosphate acetyltransferase	2.3.1.8	351,756	100.00 %	41	< 1.0 %
Ak	Acetate kinase	2.7.2.1	583,771	99.40 %	3797	< 1.0 %
Hyd1	Electron confurcating hydrogenase	1.6.5.3	2835,871	93.20 %	207,816	6.80 %
Hyd2	Membrane-bound [FeFe]-hydrogenase		372,596	99.40 %	2118	< 1.0 %
Hyd3	Ferredoxin dependent hydrogenase		944,232	93.30 %	67,477	6.70 %
Fdh1	Formate dehydrogenase 1	1.17.1.9	3025,558	93.60 %	207,767	6.40 %
Fdh2	Formate dehydrogenase 2 (membrane-bound, quinone reducing)	1.17.1.9	2549,764	88.10 %	343,477	11.90 %
Fdh3	Formate dehydrogenase 3	1.17.1.9	3041,124	93.40 %	213,336	6.60 %
Fdh4	Formate dehydrogenase 4 (membrane-bound, quinone reducing)	1.17.1.9	2544,129	79.40 %	661,611	20.60 %
Fix complex: Etf	Electron transfer flavoprotein alpha subunit		596,128	99.70 %	1545	< 1.0 %
Fix complex: Fd	4Fe-4S ferredoxin-type domain-containing protein		2679	87.30 %	389	12.70 %
Fix complex	FAD_binding_3 domain-containing protein	1.5.5.1	14,009	98.70 %	191	1.40 %
FeS oxidoreductase: EtfB	Electron transfer flavoprotein subunit beta		285,500	99.80 %	533	< 1.0 %
FeS oxidoreductase: EtfA	Electron transfer flavoprotein subunit alpha		310,628	99.70 %	1012	< 1.0 %
FeS oxidoreductase	EtfAB:quinone oxidoreductase		712,785	99.00 %	7248	1.00 %
Hdr	Heterodisulfide reductase	1.8.98.1	67,765	73.10 %	24,953	26.90 %
ATPase	ATP synthase		12,908,876	99.40 %	84,285	< 1.0 %
Nitrite transporter	Nitrite transporter		187,688	100.00 %	0	0.00 %

^a Total read counts by enzyme or group of enzymes.^b The percentages were obtained by dividing the number of reads by the total number of reads for the specific enzyme. The color gradient emphasizes the highest number of reads obtained for each taxon considered. EC number – enzyme commission number.**Fig. 3** – Scheme of the syntrophic butyrate degradation occurring in AC-enrichment, where most of the β -oxidation enzymes were assigned to Syntrophomonadaceae spp., and genes involved in the MP via the hydrogenotrophic pathway were mainly assigned to the genera *Methanofollis* and *Methanospirillum*, while MP via the acetoclastic pathway was attributed to the activity of *Methanotrix*. Supporting references in addition to KEGG database for the representation of β -oxidation and methanogenic pathways: (Enzmann et al., 2018; Kurth et al., 2020; Lie et al., 2012; Sieber et al., 2010b; Stams et al., 2012; Stams and Plugge, 2009; Ziels et al., 2019). The abbreviations used for the enzymes are as follows: CoA - coenzyme A; EC number – enzyme commission number; CoB - coenzyme B; CoM - coenzyme M; Fd - ferredoxin; Fd_{ox} - oxidized ferredoxin; Fd_{red} - reduced ferredoxin (=F₄₂₀H₂); ATPase - ATP synthase. Syntrophic butyrate metabolism: Act - acetyl-CoA acetyltransferase; Bcd - butyryl-CoA dehydrogenase; Ctr - crotonyl-CoA hydratase; HbdH - 3-hydroxybutyryl-CoA dehydrogenase; AtoB - acetyl-CoA C-acetyltransferase; Pta - phosphate acetyltransferase; Ak - acetate kinase; Hyd1 - electron confurcating hydrogenase; Hyd2 - membrane-bound [FeFe]-hydrogenase; Hyd3 - ferredoxin dependent hydrogenase; Fdh1, Fdh3 - formate dehydrogenases; Fdh2, Fdh4 - formate dehydrogenase (membrane bound, quinone reducing); Hdr - Heterodisulfide reductase; Fnt - formate transporter; MQ - menaquinone; EtfAB - Electron transfer flavoproteins A and B; FeS - complex membrane bound FeS-oxidoreductase; Fix complex - electron transfer flavoprotein: associated ferredoxin. Hydrogenotrophic metabolism: Mtr - tetrahydromethanopterin S-methyl-transferase; Mcr - methyl-coenzyme M reductase; Hdr - soluble heterodisulfide reductase; Fdh - formate dehydrogenase; Mvh - F₄₂₀-non-reducing hydrogenase; Frh - coenzyme F₄₂₀-reducing hydrogenase; Fwd - formyl-methanofuran dehydrogenase; Eha - energy-converting hydrogenase A; MFR - methanofuran; Ftr -Formyl-methanofurantetrahydromethanopterin formyl-transferase; H₄MPT – tetrahydromethanopterin; Mch - Methenyl-tetrahydromethanopterin cyclohydrolase; Hmd - H₂-dependent methylenetetrahydromethanopterin dehydrogenase; Mer - 5,10- methylenetetrahydromethanopterin reductase. Acetoclastic metabolism: Ack - Acetate kinase; At - phosphate acetyltransferase; Acs - acetyl-CoA decarbonylase/synthetase; Codh - carbon-monoxide dehydrogenase; Codh-Acs - carbon monoxide dehydrogenase/acetyl-CoA decarbonylase/synthase complex; MtrA-H - tetrahydromethanopterin S-methyltransferase; McrABG - methyl-coenzyme M reductase; HdrDE - membrane-bound heterodisulfide reductase; Ech - energy-converting hydrogenase; MPH – methanophenazine.

which seems to rely on a good balance between hydrogenotrophic and acetoclastic communities (Table 2).

Methanogenic activity is not the sole determinant for achieving an efficient digestion process. In the particular case of VFA degradation, syntrophic relationships are essential. Butyrate is first converted to acetate and hydrogen or formate by bacteria capable of performing β -oxidation. If electron carriers (i.e., hydrogen or formate) accumulate, butyrate cannot be further degraded due to thermodynamic reasons (Sieber et al., 2012). Therefore, hydrogenotrophic organisms consuming hydrogen and formate are key players in VFA conversion to methane. In alternative, other mechanisms can possibly take place in methanogenic environments, like DIET or CIET. DIET was proven to occur between bacteria (*Geobacter*) and acetoclastic methanogens (*Methanosarcina* or *Methanoxanthus*) (Rotaru et al., 2014b, 2014a), while CIET was demonstrated between *Geobacter* and *Methanosarcina* genera in the presence of GAC (Liu et al., 2012; Rotaru et al., 2014a), carbon cloth (Chen et al., 2014a) or biochar (Chen et al., 2014b). On the other hand, other reports suggested the occurrence of CIET in anaerobic systems but without confirmation (Fu et al., 2018; Zhang et al., 2018). In this work, we found that *Syntrophomonas* species were dominant in all microbial enrichments (Table 2). *Syntrophomonas* are known as fatty acid-degrading microorganisms so their enrichment was not surprising (Sieber et al., 2010a; Stams et al., 2012; Stams and Plugge, 2009; Ziels et al., 2019). On the other hand, although *Syntrophobacter* and *Geobacter* were highly abundant in the inocula, none were enriched with butyrate as substrate, either with or without materials. Because the majority of *Syntrophobacter* species consume propionate (Stams et al., 2012) (although at least one species was reported as a butyrate oxidizer (Li et al., 2012)), it was not surprising that *Syntrophobacter* did not prevail in butyrate-degrading enrichments. On the contrary, the majority of *Geobacter* species consume butyrate and/or acetate (Lovley et al., 2011), and many studies reported their enrichment in the presence of several materials (Kato et al., 2012; Li et al., 2015; Rotaru et al., 2018; Yuan et al., 2018), but in this work (Table 2) and others (Fu et al., 2019, 2018; Wang et al., 2020a; Xia et al., 2019; Zhang and Lu, 2016; Zhang et al., 2018), this did not happen. Furthermore, gene expression analysis clearly showed that butyrate degradation via β -oxidation was performed almost exclusively by *Syntrophomonadaceae* species (Table 4). Deep inspection of metatranscriptomics data revealed that, although some enzymes involved in the β -oxidation pathway were expressed by other taxonomic groups, none of those expressed the complete pathway (data not shown). Therefore, our data strongly suggest that relevant syntrophic relationships occur between *Syntrophomonas* species and methanogens (Fig. 3). All proteins known to participate in butyrate degradation were expressed by *Syntrophomonadaceae* species, including several hydrogenases and formate dehydrogenases. Proteins involved in MP were expressed by *Methanofollis*, *Methanospirillum* and *Methanoxanthus* genera (Tables 3 and 4). The results indicate that, in the AC enrichment, both hydrogen and formate were exchanged between syntrophic bacteria and hydrogenotrophic methanogens. This does not exclude the occurrence of DIET, however genes coding for *e*-pili could not be detected, and genes coding for *c*-type cytochromes showed residual expression (Appendix A Table S3, sheet “bacterial *c*-type cyt”), which are genes considered essential for extracellular electron transfer (Van Steendam et al., 2019). Therefore, if DIET/CIET occurs in this enrichment, it should be a less relevant mechanism.

The reason why the taxonomic assignment obtained by 16S rRNA sequencing and metatranscriptomics do not completely match, may be the nature of the databases used to annotate that data. For 16S rRNA reads, a well curated and complete taxonomic database was used, ensuring a reliable taxonomic classification at least until the genus level. On the other hand, metatranscriptomics annotation was done against the UniProt database, which contains a limited number of complete genomes available. For instance, only 3 complete genomes of *Syntrophomonas* species (*S. wolfei*, *Syntrophomonas zehnderi* and *Syntrophomonadaceae bacterium*) have been sequenced so far, and probably the *Syntrophomonas*

species present in the EC does not have its genome sequenced to date. On the other hand, the most abundant methanogens (by 16S rRNA sequencing) belonged to an unclassified genus within *Methanomicrobiaceae* family, which probably corresponds to the *Methanofollis* genus found as highly active by the metatranscriptomics analysis. In fact, during microscopy inspection of the AC-enrichment, many *Methanofollis*-like cells (Garcia et al., 2006) could be observed, which present a typical irregular cocci cell shape (Fig. 2b). For the reasons just explained, the genes of the microorganisms were assigned to the genes of the close relatives available in the reference databases.

5. Conclusions

Different materials favoured the growth of different methanogens but had a minor impact on the bacterial community (dominated by *Syntrophomonas*). This resulted in faster or slower butyrate conversion to methane, depending on the composition of the microbial communities, and the possible syntrophic interactions. The acceleration of methane production via the hydrogenotrophic pathway in the culture containing AC is linked to the exchange of electrons via hydrogen and formate, as it was shown by gene expression analysis. These conclusions are dependent on the type of carbon and energy source available. Further studies should be conducted with other substrates (e.g., more complex wastes) to get further insights into the effects of materials on microbial communities' modulation and gene expression in AD process.

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.

CRediT authorship contribution statement

Cátia S.N. Braga: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **João C. Sequeira:** Writing – review & editing, Software, Methodology, Formal analysis, Data curation. **M. Salomé Duarte:** Writing – review & editing, Methodology, Investigation. **Gilberto Martins:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition. **Inês A.C. Pereira:** Writing – review & editing, Supervision. **Luciana Pereira:** Writing – review & editing, Supervision. **M. Madalena Alves:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Andreia F. Salvador:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

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Appendix A Supplementary data

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jes.2025.05.015.

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