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Research article

From conventional to adapted microbiomes: Promoting high short-chain fatty acid yields and productivities from agricultural waste

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ABSTRACT

Microbial consortia play an essential role in anaerobic fermentation (AF) devoted to the production of short-chain fatty acids (SCFAs) from organic wastes. AF is usually performed by a conventional anaerobic microbiome (CM) sourced from anaerobic digestion reactors. During AF, the microbiome undergoes an adaptation period to the imposed operational conditions and substrate characteristics, leading to the bio-enrichment of certain microorganisms. This work compared the use of CM and a bio-enriched microbiome (BM) as inoculum for AF of agricultural wastes in continuous stirred tank reactors (CSTR) with hydraulic retention time (HRT) of 8 d. The novelty of this study lies in demonstrating that using a BM enhances the production rate of SCFAs when compared to CM. BM, composed of adapted microorganisms previously working at an HRT of 10 d, allowed the highest SCFAs productivity (1.97 g/L·d) and concentration (15.6 g/L). Bioconversion efficiencies achieved with BM and CM (60.1 % and 71.8 %, respectively) were among the highest reported in literature. Microbiome analysis revealed inoculum-driven changes in the microbial community. However, *Clostridium* and *Megasphaera*, which are involved in the hydrolysis and acidification steps of AF and are associated with acetic acid formation and chain elongation, predominated in all cases (up to 48 % of the microbial abundance within the total community). These results evidenced the feasibility of operating CSTRs at an HRT of 8 d with diverse inoculum sources to maintain exceptionally high SCFA productivity and bioconversion. The outcomes also highlighted the robustness of the microbial community, even under short HRT, providing a novel strategy for AF processes optimization.

1. Introduction

Sustainable development goals (SDG), established as part of the 2030 Agenda, include 17 key objectives. Among them, SDG7 (Affordable and Clean Energy), SDG12 (Responsible Consumption and Production) and SDG13 (Climate Action) can be addressed through the revalorization of organic wastes. Agricultural residues are a major source of organic waste, with 1.3 billion tons discarded every year (FAO, 2022), resulting in significant economic and environmental impacts. The organic matter in the residues can be transformed into energy and valuable products, contributing to a circular economy while reducing waste disposal. Out of the available organic waste conversion technologies, anaerobic fermentation (AF) has emerged as a reliable alternative for the production of short-chain fatty acids (SCFAs), which can be used as chemicals precursors in different industrial sectors (*e.g.*, biopolymers, biofuels, pharmaceuticals, etc.) (Lim et al., 2008; Muhorakeye et al., 2022; Tang et al., 2023).

AF is a shortened anaerobic digestion (AD) process where SCFAs are accumulated after hydrolysis, acidogenesis and acetogenesis steps, whereas the last step, methanogenesis, is inhibited. Given the diverse microbial population in anaerobic digesters, microbiomes for AF are normally collected from wastewater treatment plants (Greses et al., 2022; Wang et al., 2022; Zhou et al., 2024). These conventional microbiomes (CM) present a wide range of microorganisms responsible for hydrolyzing organic residues into simpler molecules (carbohydrates, proteins and lipids), as well as for producing intermediate metabolites,

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ultimately leading to biogas production. When targeting compounds other than biogas, not only the operational parameters but also the CM needs to be adapted to produce the desired final products. Thus, bio-enriched microbiomes (BM), composed of microbial communities previously adapted to the selected AF conditions, may serve as a valuable alternative to CM for achieving high SCFAs concentrations. Conventional approaches often rely on bioaugmentation with specific hydrolytic and acidogenic strains, that usually fails to ensure long-term stability. In contrast, BM could play a key role in enhancing overall process stability, increasing total SCFAs yields and improving bioconversion efficiencies, as microorganisms are already adapted to the process conditions. However, the impact of microbial populations composition on the AF process is still poorly understood.

Operational parameters such as the hydraulic retention time (HRT), temperature and pH as well as the composition of the residue play important roles on microbial community dynamics and consequently, in the process outcome. For example, carbohydrates-rich agricultural wastes are more easily degradable than protein-rich substrates at short HRT (<10 d) (Bi et al., 2019; De Groof et al., 2021). The short HRT suppresses methanogenesis while favoring the proliferation of SCFA producers (Aboudi et al., 2023; Lv et al., 2022). Additionally, short HRT enables the treatment of larger amounts of organic matter and reduces the investment costs associated with process operation. Nonetheless, an extremely short HRT may result in the washout of microorganisms from the system or in a reduced overall process performance (Kumar et al., 2016; Llamas et al., 2022).

While previous studies have assessed AF under different temperatures, pH, HRTs and OLRs, the use of BM for subsequent operation has not been systematically evaluated. Therefore, the novelty of this study lies in the use of a specialized BM, sourced from a previous AF reactor run at an HRT of 10 d, in a subsequent AF process operated at shorter HRTs (8 d). The use of BM could minimize the adaptation to the operational conditions, shorten operational times and promote high SCFAs yields and productivities through the development of an efficient microbiome. The combination of BM and short HRT could promote a resilient and metabolically efficient microbial community capable of achieving high efficiencies and productivities at higher turnover rates. Thus, the combined effect of short HRT (8 d) and microbial dynamics on AF performance using two different inoculum sources (CM and BM), is explored. To better understand how the different inocula affect process performance, microbial community analysis was conducted, providing valuable insights into microbial dynamics and their role in maintaining AF functionality and high product yields.

2. Materials and methods

2.1. Inoculum and substrate

Agricultural residues provided by the company Cajamar in Sta Maria del Águila-El Ejido, Almería (Spain) were composed of equal parts of melon, tomato, zucchini and cucumber (25 % w/w of each). The feedstock was blended for complete homogenization and stored at −21 °C to avoid self-fermentation. Waste mixture was characterized every time a new batch was prepared by determining total and volatile solids (TS and VS), total and soluble chemical oxygen demand (TCOD and SCOD) and ammonium (NH_4^+-N) (Table 1).

The waste mixture was diluted to maintain the desired HRT and organic loading rate (OLR) (Eq. (1)).

$$VS\ (g/L) = \frac{OLR \cdot (V_{reactor})}{\frac{V_{reactor}}{HRT}} \quad (1)$$

CM, collected from the wastewater treatment plant in Móstoles (Madrid, Arroyo de El Soto), was used as inoculum for AF in two continuous stirred tank reactor (CSTR) at HRT of 10 d (referred to as CM-HRT10) and at HRT of 8 d (CM-HRT8). When the CM-HRT10 system reached the steady state, the obtained bio-enriched microbiome (BM) was collected to be used as inoculum in subsequent experiments. CM and BM were characterized according to standard methods (APHA, 2012) and their composition is shown in Table 2.

Table 1
Agricultural waste characterization (mean and standard deviation (SD)).

	Agricultural waste
	Mean ± SD
pH	4.4 ± 0.05
TCOD (g/L)	84.7 ± 5.4
SCOD (%)	79.1 ± 1.4
TS (g/L)	66.3 ± 0.7
VS (%)	87.6 ± 2.0
NH_4^+-N (g/L)	0.3 ± < 0.1
Ash (% w/w)	9.8 ± 2.8
Lipids (% w/w)	7.8 ± 1.4
Proteins (% w/w)	12.9 ± 1.8
Carbohydrates (% w/w)	69.5 ± 4.5

The AF performance of both BM and CM was compared in CSTRs operated at HRT of 8 d (referred to as BM-HRT8 and CM-HRT8, respectively).

2.2. Reactor operation and culture conditions

AF was conducted in 3-L CSTR reactors at 25 °C, pH 5.8-6, and OLR of 3 g VS/L·d. Lower temperature, slightly acidic pH and relatively high OLR (compared to those typically applied in AD) were selected, as these conditions have been shown to inhibit methanogenic activity and to enhance SCFAs production (Greses et al., 2020). Temperature was controlled with a water bath (Julabo CD-0200F). pH was measured with a Crison Hach Lange probe and manually adjusted by adding NaOH (5 M).

All AF (CM-HRT10, CM-HRT8 and BM-HRT8) were run until the steady state was reached, which was equivalent to at least three HRTs and constant concentrations of TS, VS, NH_4^+-N and SCFAs in the effluents.

2.3. Analysis methods

All analyses were performed twice per week to assess process performance. TS and VS, TCOD and SCOD and NH_4^+-N were monitored following the Standard methods 2540B, 2540E, 5220D and 4500-NH_3F, respectively (APHA, 2012). Carbohydrates in the waste mixture were determined using the phenol-sulphuric method (Dubois et al., 1956), whereas Total Kjeldahl nitrogen (TKN) was calculated following the Standard methods 4500-N_{org} (APHA, 2012). Protein content was calculated by multiplying the TKN value by a conversion factor of 6.25 (López et al., 2010). The content of ash was determined from the TS and VS content, whereas lipids were calculated by subtracting the percentages of carbohydrates, proteins and ash from 100 %.

Gas production linked to methanogenesis was monitored daily by using a gas flow meter (Bioprocess Control, Sweden). Gas chromatography (GC) was used to evaluate the gas composition (H_2, CO_2 and CH_4) twice per week to verify whether the implemented operational

Table 2
Inoculum characterization.

	CM	BM
TS (g/L)	14.1 ± 2.3	31.7 ± 1.3
VS (%)	24.5 ± 1.3	47.5 ± 3.5
TCOD (g/L)	16.8 ± 4.6	37.2 ± 3.2
SCOD (%)	9.2 ± 0.6	65.7 ± 4.1
NH_4^+-N (g/L)	0.5 ± 0.3	0.2 ± 0.1
pH	7.7 ± 0.1	6.3 ± 0.1

conditions were suitable for low methane production. The GC was equipped with a thermal conductivity detector (Clarus 580 GC, PerkinElmer) and two columns (HSN6-60/80 Sulfinert P 7′ x 1/8″ O.D. and MS13X4-09SF2 40/60P 9'x 1/8″ O.D., PerkinElmer).

Metabolites production was measured using a high-performance liquid chromatography (HPLC) (1260 HPLC_RID, Agilent) equipped with a refractive index detector, a pre-column (Cation H Refill Cartridge Microguard column, Biorad) and an ion exclusion column (Aminex HPX-97-H 300 × 7.8 mm, I. D., Biorad). The mobile phase was 5 mM H_2SO_4 solution (VWR, international) with a flow rate of 0.6 mL/min 20 μL samples were injected for quantification.

Acidification, bioconversion, COD and VS removal were calculated according to Eqs. (2)–(5).

$$\%\ \text{Bioconversion} = \frac{\text{COD}_{\text{metabolites}}}{\text{TCOD}_{\text{in}}} \cdot 100 \quad (2)$$

$$\%\ \text{Acidification} = \frac{\text{COD}_{\text{metabolites}}}{\text{SCOD}_{\text{out}}} \cdot 100 \quad (3)$$

$$\%\ \text{COD}_{\text{removal}} = \frac{\text{TCOD}_{\text{in}} - \text{TCOD}_{\text{out}}}{\text{TCOD}_{\text{in}}} \cdot 100 \quad (4)$$

$$\%\ \text{VS}_{\text{removal}} = \frac{\text{VS}_{\text{influent}} - \text{VS}_{\text{effluent}}}{\text{VS}_{\text{influent}}} \cdot 100 \quad (5)$$

Bioconversion and acidification (Eqs. (2) and (3)) considered all the SCFAs (acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acids) in terms of COD. Acid concentration was thereby multiplied by its stoichiometric value, namely 1.066, 1.512, 1.816, 1.816, 2.037, 2.037 and 2.2, respectively.

COD removal (%) determined the organic matter in terms of COD transformed into CH_4 and H_2 (Eq. (4)). The VS removal (%) indicated the hydrolysis efficiency of the AF process (Eq. (5)). This implies the amount of organic matter transformed into simpler compounds (sugars, amino acids and fatty acids). The SCFAs productivity (Eq. (6)), together with the bioconversion efficiency (Eq. (2)) were calculated in the steady state period.

$$\%\ \text{SCFAs Productivity} = \frac{\text{SCFAs (g/L)}}{\text{HRT (d)}} \cdot 100 \quad (6)$$

Mineralization was also calculated at the steady state to determine the conversion of organic nitrogen into NH_4^+-N (Eq. (7)).

$$\%\ \text{Mineralization} = \frac{\left(\left(NH_4^+\text{-}N_{effluent}\right) - \left(NH_4^+\text{-}N_{in}\right)\right)}{\left(\text{TKN}_{\text{in}}\right) - \left(\text{NH}_4^+\text{-N}_{\text{in}}\right)} \cdot 100 \quad (7)$$

To assess the statistical significance of the results, a one-way ANOVA analysis was carried out with a 95 % confidence interval, considering significant difference at a p-value <0.05. Eight to ten time-point samples per reactor were used for statistical analysis, corresponding to repeated measurements under steady-state conditions. Prior to the analysis, data normality and homoscedasticity were evaluated using the Shapiro-Wilk and Levene's tests, respectively. No deviations from ANOVA assumptions were observed.

2.4. Microbial analysis

Microbial populations were analysed in samples collected from the inoculum and from steady state AF CSTR. Samples were kept at −80 °C until analysis. DNA was extracted using the FastDNA SPIN kit for Soil (MP Biomedicals, LCC) from 1-mL sample. The quality and concentration of the extracted DNA were assessed using a Nanodrop spectrophotometer (SPECTROstar Omega) (BMG Labtech, DE). Samples were sequenced at FISABIO (Valencia, Spain) on a MiSeq Sequencer (Illumina) using the 341F and 805R primers (F: CCTACGGGNGGCWGCAG and R: GACTACHVGGGTATCTAATCC). The primers target the hypervariable regions V3 and V4 of the 16S rRNA gene and were used to analyze both bacteria and archaea. The paired-end reads for each sample were combined using the software tool PEAR. Those with a quality score higher than 30 were selected using PRINSEQ and primer sequences were removed using Mothur. The remaining sequenced sharing >97 % similarity were grouped into operational taxonomic units (OTUs). Alpha diversity (Shannon index) were calculated using the Quantitative Insights Into Microbial Ecology (QIIME) software, version 1.9.1 to assess the evenness and richness of the samples. After sequencing, statistical analysis was carried out to correlate operational parameters with chemical and biological results using PAST4 (Hammer et al., 2001).

Canonical-correlation analysis (CCA) at both phylum and genus level were carried out to establish the relationship between the different fermentation conditions (HRT, inoculum) and the microbial communities developed. Microbiome dissimilarities were calculated following a Bray-Curtis distance metric through SIMPER analysis (Hammer et al., 2001).

3. Results and discussion

3.1. Development of a bio-enriched anaerobic microbiome for SCFAs production purposes

Previous studies aimed at enhancing organic matter conversion into SCFAs have often focused on inoculum enriching via bio-augmentation with hydrolytic and acidogenic bacteria (Atasoy and Cetecioglu, 2020; Chi et al., 2018; Reddy et al., 2018). Likewise, different inoculum sources (*e.g.*, wastewater, sewage sludge, co-digestion sludge and food waste digestate) have been thoroughly evaluated to promote AF (Qu et al., 2022; Wang et al., 2023; Yang et al., 2007). Yet, these approaches do not avoid the acclimation period that microorganisms undergo when they adapt to the imposed operational conditions. To reduce this adaptation stage, minimize the lag phase, and promote high SCFAs productivities, the production of a BM that had already been exposed to AF operational conditions was aimed in this study. This BM was thereby obtained from the steady stage of an AF reactor inoculated with CM and operated at HRT of 10 d (CM-HRT10), 25 °C, pH 6 and OLR of 3 g VS/L·d.

To confirm the microbiome selectivity and adaptation to the operational conditions, the microbial population in CM and BM were firstly assessed by comparing the alpha-diversity index (OTUs and Shannon index). When comparing CM and BM, a decrease in the richness and evenness of the microbial population was determined (Table 3). The number of OTUs, which is influenced by the physicochemical parameters of the substrate and the operational conditions, decreased from 670 in CM to 119 in BM. The Shannon index followed a similar trend, decreasing from 7.26 in CM to 3.45 in BM.

CM exhibited a typical phylum distribution of conventional AD inocula (Fig. 1), in which Bacteriodota, Firmicutes, Cloroflexi, Actinobacteria, Proteobacteria, Patescibacteria, Desulfobacteria and Cloacimonadales were the main phyla (Venkiteshwaran et al., 2016). By opposite, BM was dominated by Firmicutes, Actinobacteriota, Bacteroidetes and Proteobacteria (Fig. 1). Specifically, in BM, Firmicutes comprised 86 % of the total community while Actinobacteria only accounted for 6 %. These two phyla are responsible for hydrolyzing the complex organic matter into intermediate compounds (sugars and amino acids) that are further utilized for SCFAs-producing acidogenic

Table 3
Biodiversity and richness indexes in microbial populations of the inocula (CM and BM) and AF reactors at the steady state.

	Conventional microbiome (CM)	BM (resulting from CM-HRT10)	BM - HRT8	CM – HRT8
OTUs	670	119	278	177
Shannon Index	7.26	3.45	4.40	4.23

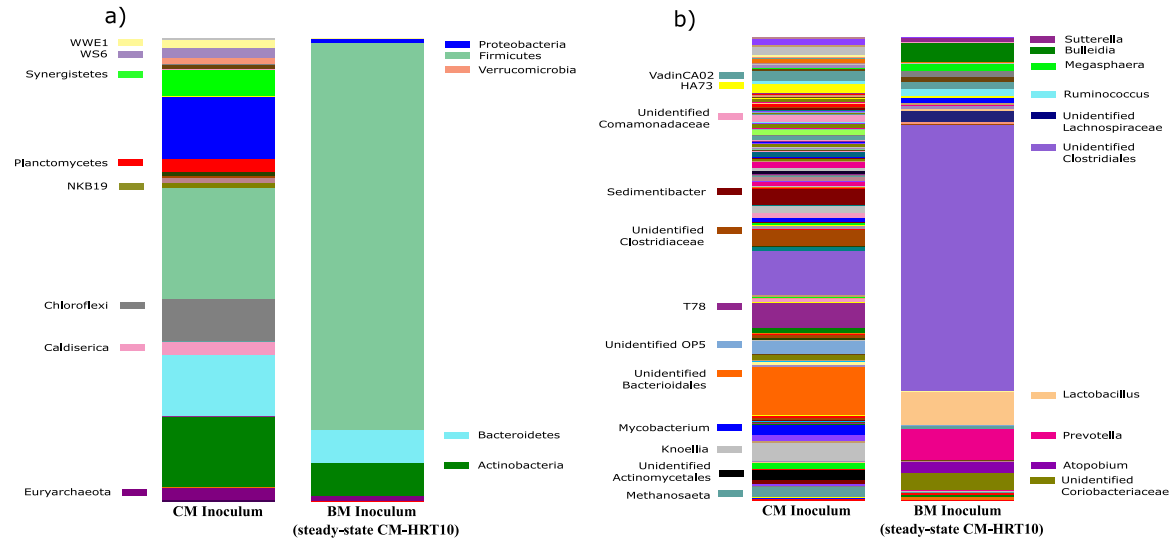

Fig. 1. Microbial population relative abundance at phylum (a) and genus (b) levels in the inoculum (CM) and CM-HRT10 at steady state (namely, BM). Taxonomic groups with relative abundance lower than 1 % were not included in the legend.

bacteria within the same phylum (Cheng et al., 2023; Liu et al., 2020). At genus level, *Clostridium* was predominant in BM, accounting for 79 % of the total. The presence of these hydrolytic and acidogenic microorganisms agreed with the chemical output attained in the effluents.

The developed microbial population in reactor CM-HRT10 (*i.e.*, the BM) maximized organic matter degradation into SCFAs, reaching a concentration of 15.9 ± 0.7 g SCFAs/L. VS removal and acidification efficiency were 36.2 ± 3.8 % and 99 ± 3.1 %, respectively. As expected, VS removal was lower than that observed in AF fed with similar feedstock (around 50 %) but different HRT (Gonçalves et al., 2024). Short HRTs have been reported to result in a decreased hydrolysis efficiency (Gonçalves et al., 2024; Malinowsky et al., 2021; Sillero et al., 2022). In CM-HRT10 prevailing acids were acetic and butyric acids, and the bioconversion efficiency attained was 57.5 ± 3.5 %. These values are significantly higher than that reported by other authors that employed agricultural wastes as feedstocks operating CSTRs at HRTs 12 d and 20 d (49 % and 39 %, respectively) (Bolaji and Dionisi. (2017); Gonçalves et al. (2024)). Besides the slight differences in feedstock composition, the higher bioconversion efficiencies in this study may result from the shorter HRT. While a short HRT could lead to greater substrate availability, contributing to a more efficient system, it may also reduce the residence time of microorganisms with the substrate. In this study, however, the high degradability of carbohydrate feedstocks ensured that even at short HRTs, the feedstock was sufficiently accessible to sustain high bioconversion rates. In this sense, the robustness of the microbial community together with the low HRT provided high process stability. Therefore, feedstock variability driven by seasonality is not expected to be a limiting factor if the macromolecular composition, specifically the carbohydrates content, remains stable as also seen in other studies with different carbohydrate-rich feedstocks (Gonçalves et al., 2024; Greses et al., 2020).

Once confirmed that the microbial population of CM-HRT10 was efficient in terms of SCFAs production, the microbiome was used as inoculum (BM) to operate CSTR at an even shorter HRT of 8 d.

3.2. *Influence of the inoculum source on microbial community dynamics, SCFA productivity, and bioconversion efficiency*

BM was used as inoculum in an AF process operated at HRT 8 d (referred to as BM-HRT8). To determine the advantages of using an adapted inoculum, the performance of the BM was compared with that of CM under the same AF operational conditions (CM-HRT8). As observed in Table 4, 41.8 ± 3.4 % and 44.6 ± 1.5 % of hydrolysis efficiency (*i.e.*, VS removal %) were obtained with BM-HRT8 and CM-HRT8, respectively. The solubilization of organic matter (SCOD) followed a similar trend, being 73.1 ± 3.2 % in case of BM-HRT8 and 77.7 ± 2.4 % for CM-HRT8. Therefore, no significant differences were found in VS removal and SCOD between reactors. It could be hypothesized that, since the BM inoculum already exhibited high SCOD (65.7 ± 4.1 %, Table 2) and substantial presence of hydrolytic bacteria (Fig. 1), the degraded organic matter was available for the sequential reactors (BM-HRT8). The higher SCOD could have triggered a lower development of hydrolytic bacteria. However, no significant differences were seen in terms of VS removal showcasing no limitation of the hydrolytic activity of microorganisms within BM-HRT8.

Table 4
Process outputs for CM-HRT10, BM-HRT8 and CM-HRT8 during the steady state. # Indicates statistically significant differences from CM-HRT10. * Indicated statistically significant differences between BM-HRT8 and CM-HRT8.† Indicated that all reactors are statistically different between them. ($p < 0.05$).

CSTR	CM-HRT10	BM-HRT8	CM-HRT8
SCFAs (g/L)	15.9 ± 0.7$^{\#}$	15.6 ± 0.6*	14.6 ± 0.3*$^{\#}$
SCFAs (g COD/L)	25.1 ± 1.8	23.8 ± 1.9	24.5 ± 0.6
Acidification (%)	99.0 ± 3.5	96.2 ± 3.8	94.7 ± 3.5
Bioconversion (%)	57.5 ± 3.5$^{\#}$	60.1 ± 4.1*	71.8 ± 3.1*$^{\#}$
VS removal (%)	36.2 ± 3.8	41.2 ± 3.6	44.8 ± 1.5
SCOD/TCOD (%)	56.3 ± 6.5$^{\#}$	72.4 ± 4.2$^{\#}$	77.1 ± 2.4$^{\#}$
COD removal (%)	25.6 ± 7.5†	16.4 ± 2.3†	3.0 ± 1.9†
Productivity (g/L·d)	1.59 ± 0.1†	1.97 ± 0.1†	1.80 ± 0.1†

Bioconversion efficiencies were 60.1 ± 4.1 % and 71.8 ± 3.1 % in BM-HRT8 and CM-HRT8, respectively (Table 4). This higher bioconversion efficiency in CM-HRT8 could be attributed to the increased accumulation of valeric and caproic acids (Fig. 2). Due to their stoichiometry (COD/g C equivalents), these two acids contributed with a higher COD to the bioconversion efficiency, despite the lower SCFAs concentration. These values represent some of the highest efficiencies reported in the literature and may be related to the high biodegradability of the substrate and the complete inhibition of the methanogenesis in CM-HRT8 under the selected conditions. Complete methanogenic inhibition led to a low COD removal (3.0 ± 1.9 %, Table 4). COD removal is closely linked to the methanogenic activity, as the conversion of the organic matter into CH_4 represents a primary pathway in anaerobic systems. By inhibiting methanogenesis (the intended target of the implemented operational conditions), the carbon

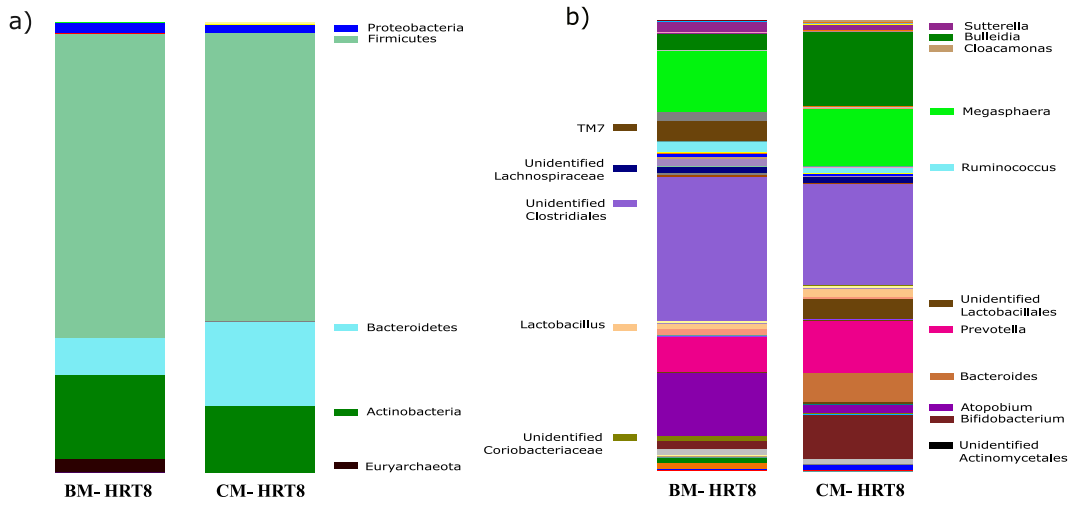

Fig. 2. SCFAs profile and bioconversion yields calculated at steady state of each reactor.

in the feedstock could be directed towards SCFAs accumulation, resulting in a slightly higher SCFA-COD concentrations for CM-HRT8 (24.5 ± 0.6 gCOD/L). By contrast, archaea were still detected in BM-HRT8 despite the HRT decrease, probably as result of the interactions stablished among microorganisms that allowed their maintenance within the system (Fig. 3), yielding an average of 50 mL CH_4/g VS_{in} (33.8 mL CH_4/gCOD_{in}) at the steady state (16.4 ± 2.3 % COD removal). The presence of archaea in BM-HRT8 could be due to their occurrence in the BM, as the inoculum was adapted to a longer HRT (*i.e.*, 10 d). Under these conditions, methanogenic microorganisms were not completely restricted and could adapt to the fermentation conditions, still remaining when the BM in CM-HRT10 was used as inoculum for BM-HRT8. In contrast, when using CM directly at an HRT 8 d, methanogens were unable to adapt. Indeed, the high bioconversion efficiencies can also be explained by the developed microbial community, especially Firmicutes, involved in organic matter degradation. Firmicutes was the most abundant phylum at the steady state of BM-HRT8 and CM-HRT8, accounting for 60 % and 66 % of the total population, respectively (Fig. 3). Firmicutes, especially *Clostridium, Prevotella* and *Ruminococcus* genera, have been reported as hydrolytic bacteria capable of secreting hydrolytic enzymes such as cellulases and proteases (Cayetano et al., 2021; Awasthi et al., 2022; Zhang et al., 2023). In both reactors, BM-HRT8 and CM-HRT8, Clostridia was the predominant genus (35 % and 27 %, respectively Fig. 2b), leading to high SCFAs production and bioconversion efficiencies. Bacteria within this genus are widely known for exhibiting efficient hydrolytic and acidogenic activities (Atasoy and Cetecioglu, 2020).

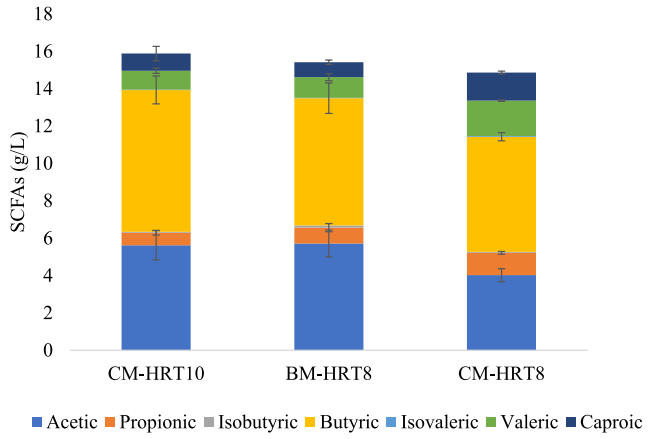

Fig. 3. Relative microbial abundance of the Bacterial and Archaea communities at phylum (a) and genus (b) levels during the steady state of AF performed with BM and CM. Relative abundance lower than 1 % were not represented in the figure.

To assess how the inoculum source influenced SCFAs production and bioconversion, microbial populations at steady state of BM-HRT8 and CM-HRT8 were evaluated using the alpha diversity index. The OTUs increased from 119 in BM (Table 3) to 278 in BM-HRT8 (Table 3), highlighting a microbiome enrichment when decreasing the HRT. This could be explained by the more frequent feeding at shorter HRT which enriches the microbial community with the endogenous microbiota present in the feedstock. It has been shown that this type of feedstocks presents lactic acid bacteria (*Leuconostoc*, *Lactobacillus* and *Weissella* genus) and bacteria from the Bacillales order, with high hydrolytic activity, that can lead to self-fermentative processes (Gonçalves et al., 2025; Tang et al., 2016, 2023). OTUs richness for CM-HRT8, decreased from 670 to 177 when compared to the CM (Table 3), and it was also lower than that of BM-HRT8 (278). Regarding the Shannon index, it increased from 3.45 in BM to 4.4 for BM-HRT8 (Table 3). However, microbial evenness was very similar when comparing BM-HRT8 and CM-HRT8 (4.23), despite the lower microorganism diversity (OTUs) in the latter.

The abundance of Bacteroidetes, that are hydrolytic bacteria capable of degrading proteins and carbohydrates through hydrolytic enzymes (*e.g.*, proteases and lipases), was 10 % in reactor BM-HRT8 and 20 % with CM-HRT8, in detriment of Actinobacteria (Fig. 3). Megasphaera abundance (Fig. 2b) reached 13 % and 15 % in BM-HRT8 and CM-HRT8, respectively. This genus is mainly associated to the acidification step, being responsible for chain elongation, through the reverse β-oxidation pathway (Kang et al., 2022; Zhang et al., 2023). Accordingly, higher concentrations of valeric and caproic acid were observed mainly in CM-HRT8 (Figs. 3 and 4) when compared to BM-HRT8.

When comparing microbial populations in CM-HRT10 and BM-HRT8, a shift from Firmicutes to Actinobacteria was observed (Figs. 1a and 2a). Specifically, at genus level, *Clostridium* was replaced by *Megasphaera, Prevotella* and *Atopobium* (Figs. 1b and 2b). Species of these three genera have an average doubling time of 40 - 180 min, which might vary depending on the selected fermentation conditions, leading to its predominance at shorter HRTs (Franke and Deppenmeier, 2018; Maki and Looft, 2018; Nagaraja and Taylor, 1987; Weimer and Moen, 2013). *Atopobium,* in addition to being involved in carbohydrates degradation into lactic, acetic acid, is also responsible for butyric acid production (Lv et al., 2022; Qin et al., 2021). Also, a slight increase was observed for *Bifidobacterium*, that releases carbohydrate-degrading enzymes for acetic and lactic acid production (Detman et al., 2021; Pokusaeva et al., 2011). These last genera are usually found together

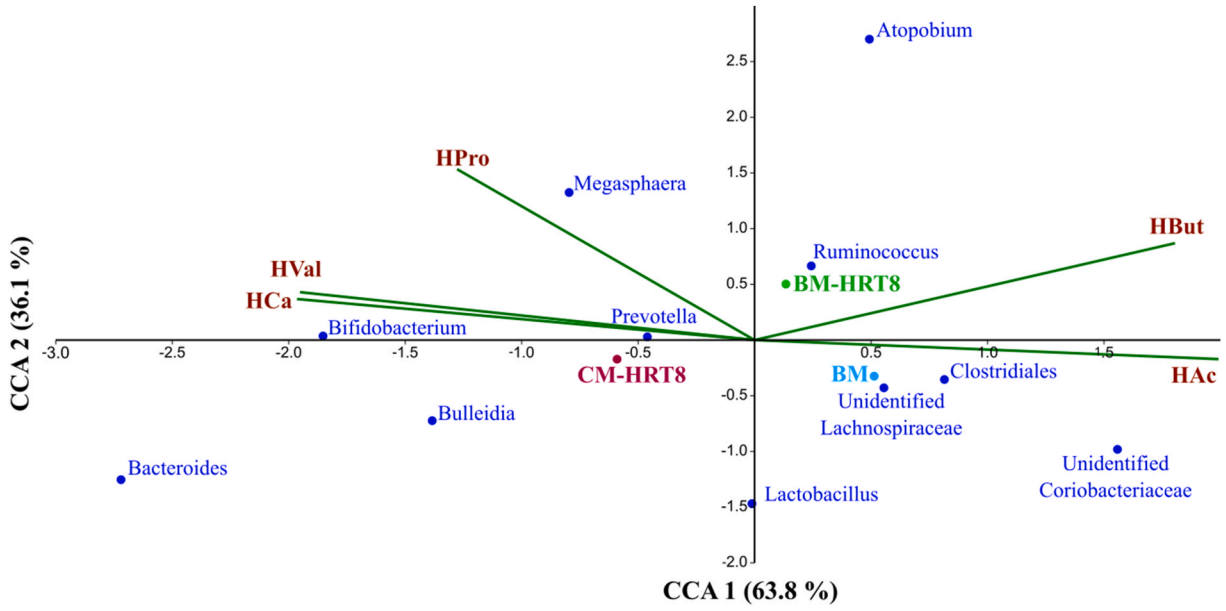

Fig. 4. Canonical correspondence analysis (CCA) ordination triplot for the steady state of the CSTRs at genus level (b), between BM (CM-HRT10) and BM-HRT8 and CM-HRT8. The inocula are not represented in the plot. Species are shown as blue dots, whereas SCFAs, and process parameters are represented as green arrows.

working synergistically (Gonçalves et al., 2024; Gulhane et al., 2017).

The selected operational conditions (pH 6, 25 °C, and HRT of 8 d) facilitated high organic matter conversion into SCFAs in all cases, resulting in high acidification and indicating strong microbial activity during the acidogenic phase, regardless of the inoculum source. In this sense, the SCFA concentration in BM-HRT8 (15.6 ± 0.6 g/L) was significantly higher to that achieved in CM-HRT8 (14.6 ± 0.3 g/L) (Table 4). Due to the higher presence of valeric and caproic acids at CM-HRT8 (Fig. 2), which present a higher COD equivalence, non-significant differences were observed in SCFAs concentration in terms of COD when comparing BM-HRT8 (23.8 ± 1.9 g COD/L) and CM-HRT8 (24.5 ± 0.6 g COD/L). Although the similar COD concentration in both fermentations is driven by the stoichiometric weighting of these acids, CM-HRT8 showed a higher bioconversion. According to Eq. (2), the difference therefore lies on the organic matter fed in the reactor (COD_{in}). Despite aiming for the same COD_{in}, this value was slightly lower in CM-HRT8 (27.2 vs 29 g COD_{in}), which reflects a more efficient substrate utilization for this reactor, rather than an effect of the stoichiometric weighting.

Proteobacteria and Actinobacteria (Bacteroidetes) abundance decreased in all CSTR when compared to CM due to the imposed fermentation conditions (Figs. 1 and 3). These two phyla, mainly present in AD systems at both short and long HRT, are proteolytic bacteria responsible for protein degradation (Cayetano et al., 2021; Liu et al., 2014; Luo et al., 2020; Lv et al., 2022). Besides being involved in the hydrolysis step, Proteobacteria phylum can also be responsible, albeit to a lesser extent, for acetic acid production (Atasoy et al., 2019). Proteobacteria was also absent in AF of agricultural wastes at HRT 20 d, 25 °C and pH 6 (Gonçalves et al., 2024). Nevertheless, although the presence of Proteobacteria can be beneficial for protein degradation, certain genera within alpha, beta and gammaproteobacteria may be detrimental for SCFAs accumulation as they can utilize propionate, acetate and butyrate as carbon source for their metabolic activities (Ariesyady et al., 2007; Gulhane et al., 2017; Luo et al., 2020).

Nitrogen mineralization percentage, that represents protein degradation into ammonium, reached 11.2 ± 1.0 % and 22.0 ± 2.0 %, in BM-HRT8 and CM-HRT8, respectively. Lower mineralization efficiencies in BM-HRT8 could be attributed to the use of BM, which mainly contained Firmicutes, primarily involved in carbohydrate degradation, rather than other protein-degrading microorganisms. These facts, together with the macromolecular feedstock composition, rich in carbohydrates (Table 1), led to a low NH_4^+-N concentration. The low abundance of Proteobacteria also agreed with the low protein mineralization.

In terms of productivity, BM-HRT8 and CM-HRT8 reached 1.97 ± 0.13 g/L·d and 1.80 ± 0.1 g/L·d of SCFAs, higher than that achieved in CM-HRT10 (1.59 ± 0.12 g/L·d). These results followed a similar trend to those results showed by Simonetti et al. (2023), where productivity increased inversely to the HRT. Productivity increase at lower HRT could be attributed to the increased flow rate, and to the reduced conversion of SCFAs into CH_4 or H_2. The SCFAs productivity values for BM-HRT8 and CM-HRT8 were also higher than those reported in other studies utilizing agricultural and food wastes as feedstocks. Gonçalves et al. (2024) reported 1.48 g/L·d, at HRT 20 d and same OLR and pH, whereas Lim et al. (2008), achieved a productivity of 1.70 g/L·d at HRT of 8 d and OLR of 5 g TS/L·d. The high SCFAs production rates, particularly in case of BM-HRT8, indicated an optimized AF process at short HRT, reducing the microorganism's adaptation period and maximizing agricultural waste conversion. The observed robustness of the microbial community may facilitate the implementation of the proposed strategy at pilot and full scale. Scale-up could be achieved by transferring an adapted microbiome from an existing AF reactor or by generating a short-term adapted microbiome at smaller scale to be used as inoculum. This, combined with the selected operational parameters (pH, OLR and low HRT (8 d)), could enhance the processing capacity of waste treatment plants by increasing the amount of waste treated within the existing infrastructure. Alternatively, this strategy could enable reduced reactor volumes for a given treatment capacity, thereby improving process efficiency and potentially lowering capital and operational costs.

3.3. *Microbial community statistical analysis and their effect on the SCFAs profile*

The SCFAs profile distribution achieved at the steady state for all reactors is shown in Fig. 2. The population developed in CM-HRT10 was very robust and gave rise to similar metabolites distribution when used as inoculum in BM-HRT8. However, higher concentrations of long-chain SCFAs were observed in CM-HRT8 with valeric and caproic acids showing the most significant differences (Fig. 2). Butyric acid accounted for 46.4, 43.6 and 41.4 % for CM-HRT10, BM-HRT8 and CM-HRT8,

respectively and it was the predominant SCFAs in all cases, followed by acetic acid. The acid profile was consistent with previous reactors using carbohydrate-rich feedstocks (Gonçalves et al., 2024; Greses et al., 2020).

SIMPER analysis based on the Bray-Courtis distance matrix was carried out to elucidate which variables contribute the most to the dissimilarities among reactors. A 35 % dissimilarity was found between CM-HRT10 (*i.e.*, BM) and BM-HRT8. *Clostridium* explained 34 % of the dissimilarities, followed by *Megasphaera* and *Atopobium* (16 % and 15 % of the dissimilarity percentage). The high contribution percentage of these genera to dissimilarities in both cases was attributed to its decrease in relative abundance after the HRT decrease from 10 to 8 d. When comparing BM-HRT8 and CM-HRT8, a 40 % dissimilarity was found. *Bulleidia, Atopobium, Clostridium* and *Bifidobacterium* were the main contributors to the dissimilarity (56.4 %), out of which *Bulleidia* explained (17.4 %), *Atopobium* (15.8 %), *Clostridium* (12.5 %) and *Bifidobacterium* (10.7 %). The dissimilarity between BM-HRT8 and CM-HRT8 was the result of the different inoculum sources.

To stablish the connection between the genera community, the environmental conditions and the produced SCFAs, a CCA was applied. The distance between the three samples (Fig. 4), evidenced the difference in microbial abundances resulting from the selected operational conditions and the different inocula. The high presence of acetic acid could be attributed to Firmicutes, specially *Clostridium* (Fig. 4). This genus is capable of producing acetic acid through the Wood–Ljungdahl pathway, while producing acetyl-CoA for sequential chain elongation through reverse β-oxidation (Dong et al., 2023; Huang et al., 2023). Besides being one of the main factors explaining the dissimilarities between reactors, no differences in terms of acetic and butyric acids concentrations were seen between CM-HRT10 and BM-HRT8 (Figs. 3 and 4).

Clostridiales, which prevailed in CM-HRT10 (Fig. 1), are associated with acetic acid production (Fig. 4) but are also capable of oxidizing this acid into longer-chain acids such as butyric and caproic acids. In both BM-HRT8 and CM-HRT8, Clostridium dominance decreased but still remained the predominant genus (Fig. 3). In BM-HRT8, *Lactobacillales, Bulleidia* and *Megasphaera* presence increased in detriment of the *Clostridium* population (Fig. 3). SIMPER analysis attributed these genera, together with *Bifidobacterium*, as the main contributors to the dissimilarities between reactors. Consistently, the CCA also evidenced their association with the different SCFAs concentrations across reactors. This confirmed the results obtained with the SIMPER analysis, and suggested that the different microbial communities were linked not only to changes in abundance but also to the different metabolic outputs. The CCA also revealed a correlation between Coriobacteriaceae and the production of acetic and butyric acids (Fig. 4).

Valeric acid increased from 7.3 % in BM-HRT8 to 12.7 % in CM-HRT8. Equally, caproic proportion increased from 5.2 % in BM-HRT8 to 10.1 % in CM-HRT8. Fig. 4 confirmed the relation between *Bifidobacterium, Bacteroides* and *Lactobacillus,* producers of chain elongation precursors (Contreras-Dávila et al., 2020; Ulčar et al., 2023), with both valeric and caproic acids production. Although present in all reactors, valeric and caproic acids exhibited the highest concentrations (1.9 g/L and 1.5 g/L, respectively) in CM-HRT8, with *Bulleidia*, a fermentative bacterium, becoming the dominant genus after *Clostridium*. These results aligned with the fact that this genus is associated with glucose fermentation and protein degradation for acetic and caproic acid production (Greses et al., 2020; Gulhane et al., 2017). The presence of lactic acid producers (*Lactobacillus* and *Bifidubacterium* genera) also contributed to the higher valeric and caproic acid concentration in CM-HRT8, as lactic acid can serve as an electron donor for chain elongators through reverse β-oxidation (Candry et al., 2020; Gazzola et al., 2022). Although lactic acid was not accumulated, the presence of *Lactobacillus* and *Bifidobacterium* suggested that both genera were degrading carbohydrates into lactic acid (Okoye et al., 2022; Zhang et al., 2023). The sustained performance and dominance of fermentative bacteria (e.g. *Clostridium, Megasphaera, Bifidobacterium*) indicated that long-term microbial stability can be inferred under stable operational conditions. Previous reports also suggest that microbial communities can exhibit high resilience despite environmental and macromolecular changes (Gonçalves et al., 2025). In summary, the higher concentrations of valeric and caproic acids in CM-HRT8, resulting from the developed microbial community and their metabolic dynamics, contributed to the higher bioconversion achieved under this condition, despite the lower SCFAs concentration.

Overall, the statistical analysis elucidated the correlations between the microbial community, the different HRTs and inocula source responsible for producing specific SCFAs profiles. These CCA results revealed the microbiome in BM and CM responsible for the different SCFAs. In this sense, although CM-HRT8 exhibited a less varied microbiome in terms of OTUs (Table 3), its microbiome presented diverse microbial activities responsible for the predominant accumulation of acetic and butyric acids that were the prevailing acids in both CM-HRT8 and BM-HRT8.

4. Conclusions

The attaining of a bio-enriched microbiome enhanced SCFAs production rate due to an efficient microbial adaptation. This inoculum promoted agricultural waste revalorization, maximizing SCFAs yields at short HRT. At HRT 8 d, the use of CM at the selected conditions promoted one of the highest bioconversion efficiencies (71.8 %) reported in literature. This inoculum also promoted lactic acid producing bacteria for sequential chain-elongation, maximizing bioconversion efficiencies. Despite reactors dissimilarities, the microbiome was capable of efficiently hydrolyze and reach high acidification efficiencies at low HRTs, showcasing process robustness. The inocula exerted a greater effect on the microbial community and on the SCFAs profile than the HRT decrease. *Clostridium, Bulleidia,* and *Megasphaera* involved in carbohydrate fermentation prevailed in all reactors, shaping the SCFAs profile by producing mainly butyric and acetic acids.

CRediT authorship contribution statement

Marta de Vicente: Writing – original draft, Investigation, Data curation. **Elia Tomás-Pejó:** Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Cristina González-Fernández:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

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.

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Data availability

Data will be made available on request.

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