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Short-chain fatty acids production from maize silage: impact of stepwise hydraulic retention time reduction on microbial adaptation and process output

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Abstract

The conversion of maize silage, byproduct of bioethanol production, into short-chain fatty acids (SCFAs) through anaerobic fermentation (AF) is a promising approach. Due to the diverse microbial metabolisms involved, precise control of operational parameters is crucial to maintain the stability of the AF and achieve maximum process outputs. This study investigates the impact of the stepwise hydraulic retention time (HRT) reduction on the stability of microbial communities and SCFAs production. To address this, a 40-L semi-pilot continuous stirred tank reactor was operated under different HRT conditions: 15, 12.5, 10, and 7.5 days. Results showed that the maximum bioconversion of 28% g COD_{SCFAs}/g COD_{in} (expressed as chemical oxygen demand [COD]) was reached at HRTs higher than 7.5 days, while the highest total SCFAs concentration of 14.3 g/L was observed at HRT of 15 days. SCFAs derived from maize silage mainly consisted of acetic, propionic, butyric, and valeric acids, maintaining a consistent profile across all evaluated HRTs. Canonical correspondence analysis results indicated synergistic and cross-feeding interactions among key microbial bacteria involved in AF such as unclassified Clostridiales, *Megasphaera*, Lachnospiraceae, Ruminococcaceae, and *Prevotella* for all tested HRTs. This finding indicated that these microorganisms were capable of maintaining their function and stability across different HRT conditions.

Keywords Short-chain fatty acids, Maize silage, Anaerobic fermentation, Canonical correspondence analysis, Microbial communities

Introduction

Bioethanol production has significantly increased in recent years due to its renewable nature and ability to reduce harmful vehicle emissions, including CO_2, CO, exhaust hydrocarbons, and fine particulate matter. Bioethanol production volumes reached 26 million m^3 in 2020 and 30 million m^3 in 2023 [1]. This growing tendency markedly rose the generation of maize silage. More specifically, 9 to 14 L of maize silage is generated to produce 1 L of ethanol [2]. Maize silage can be considered multipurpose waste, suitable for use as animal feed and as a substrate for bioenergy generation through anaerobic digestion process. However, the uncontrolled discharge

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of maize silage into soils, water bodies, or its direct application for crop irrigation possesses a risk of environmental contamination. This risk is associated with the high pollutant load of maize silage, which contains elevated levels of organic matter, nutrients, and chemicals such as potassium, nitrogen, phosphorus, and sulfate [2]. Yet, this biowaste is rich in lipids, non-fermentable carbohydrates, and proteins, holding great potential for further valorization. As a residue, maize silage treatment implies an important challenge for the bioethanol industry due to the large volumes produced and the complexity of the biowaste.

In line with the so-claimed circular economy, the high biodegradable organic matter in maize silage renders this biowaste an attractive feedstock for biotechnological valorization, especially through anaerobic bioprocesses in which all macromolecules can be used. In addition to biogas production, anaerobic bioprocesses have been lately used to increase the products portfolio that these technologies might offer. In this sense, aiming at substituting petrochemicals at some extent, anaerobic fermentation (AF), has been intensively investigated to produce several biocompounds, such as short-chain fatty acids (SCFAs) [2, 3].

SCFAs (i.e., acetic, propionic, butyric, valeric, and caproic acids) serve for the synthesis of chemicals like esters, ketones, alcohols, alkanes, and olefins, which are extensively utilized in the food, pharmaceutical, plastics, and textile industries [4]. Currently, SCFAs are mainly produced by oxidation or carboxylation of chemical precursors, including aldehydes and alkenes, and their production depends on nonrenewable petrochemical sources [5]. The emerging carboxylate platform, which focuses on converting biowastes into SCFAs as intermediate building blocks, plays a crucial role in waste biovalorization [6]. In the particular case of maize silage, SCFAs production will not only imply a sustainable method for producing these platform molecules but would also reduce waste disposal problems and increase the global economy of the bioethanol production process.

The production of SCFAs through AF involves three distinct stages: hydrolysis, acidogenesis, and acetogenesis. If methanogens are not effectively inhibited, they can further convert these acids into methane, transitioning the process into anaerobic digestion. The impact of operational parameters, including pH, hydraulic retention time (HRT), temperature, and type of inoculum, on the anaerobic digestion of maize silage for methane production has been extensively investigated. However, understanding of specific microbial communities thriving under varying HRT for SCFAs production remains limited, and it has been mainly assessed at lab scale. Therefore, this work investigates the selective pressure of stepwise reduction of HRT on the AF of maize silage in semi-pilot continuous stirred tank reactors (CSTRs). Specifically, HRT was decoupled from the organic loading rate (OLR) to precisely determine its influence on microbial community structure, bioconversion, and SCFAs production.

Material and methods

Anaerobic fermentation process

Feedstock

Corn grains were fermented to produce bioethanol at Vertex Bioenergy's facilities in Madrid, Spain. Following the fermentation and bioethanol distillation, the maize silage was used as a feedstock for AF. Maize silage was characterized in terms of total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), total solids (TS), volatile solids (VS), and ammoniacal nitrogen ($N–NH_4^+$). Results showed a tCOD of 855.0 ± 4.2 g/L (sCOD/tCOD (%) = 36.4 ± 2.2), TS of 904.6 ± 1.1 g/L (VS/TS = 93.0 ± 0.3% DW), and $N–NH_4^+$ of 1.1 ± 0.1 g N/L. Carbohydrates, proteins, and lipid contents ranged 27.6 ± 4.9% dry weight (DW), 25.4 ± 1.2% DW, and 40.6 ± 2.8% DW, respectively, while the ash content was 6.3 ± 0.2% DW. Notably, no ethanol was found in the maize silage.

Experimental setup

The AF of maize silage was performed in semi-pilot CSTR with a working volume of 40 L, operated in a semi-continuous feeding mode (once daily) and at 25 °C. pH was monitored with a GLP 21 pH meter (Crison, Hach Lange) and adjusted to 5.5 by adding 5-M NaOH. The temperature and pH conditions were selected based on previous studies that optimized AF of food waste. Acidic conditions (pH 5.5–6.0), a low fermentation temperature (25 °C), and a high OLR (3 gVS/L·day) favored the development of a specialized microbial community associated with the reverse β-oxidation pathway. This metabolic process led to the predominant accumulation of valuable SCFAs as caproic and isobutyric acids [7]. The CSTR was mechanically stirred at 100 rpm with a Hei-TORQUE Expert 200 (Heidolph, Schwabach, Germany). The operational conditions including the flow rate (Q), the HRT, and the OLR are detailed in Table 1.

Semi-pilot CSTR with an HRT of 15 days and controlled temperature of 25 °C was inoculated with anaerobic sludge collected from the mesophilic anaerobic digester at the "El Soto" wastewater treatment plant in Móstoles, Madrid, Spain. The inoculum was characterized by 21.0 ± 1.1 g TS/L, 15.0 ± 0.5 g VS/L, 2.0 ± 0.2 g $N-NH_4$/L, and pH 7.0 ± 0.2.

The experimental setup aimed to support microbial adaptation through a stepwise decrease in HRT from 15

Table 1 Operational conditions of the CSTR devoted to SCFAs production from maize silage

Period	Q (L/day)	HRT (day)	OLR (g VS/L·day)
1	2.7	15	3
2	3.2	12.5	3
3	4.0	10	3
4	5.3	7.5	3

to 7.5 days. Reactor was transitioned to shorter HRTs after achieving steady-state conditions. For example, the inoculum for the 12.5-day HRT reactor was collected from the 15-day HRT operation, the inoculum for the 10-day HRT reactor was collected from the 12.5-day HRT operation, and so on.

Process performance assessment

The evolution of maize silage's AF was monitored by analyzing the effluents obtained from the CSTR twice a week. The steady state was considered to be reached when the experiment lasted at least three times the applied HRT and the effluent composition stabilized. After achieving stability, AF performance was evaluated based on bioconversion efficiency and acidified COD (using Eqs. 1 and 2, respectively):

$$\text{Bioconversion (\%)} = \left(\frac{COD_{\text{SCFAs}_{\text{out}}}}{\text{tCOD}_{\text{in}}}\right) \times 100 \quad (1)$$

$$\text{COD acidified (\%)} = \left(\frac{COD_{\text{SCFAs}_{\text{out}}}}{\text{sCOD}_{\text{out}}}\right) \times 100 \quad (2)$$

where $\text{COD}_{\text{SCFAsout}}$ represents the SCFAs measured as COD equivalents in the effluent (g COD/L); tCOD_{in} refers to the total organic matter fed into the reactors, measured as COD (g COD/L); and sCOD_{out} denotes the soluble organic matter in the reactor's effluents (g COD/L). The COD equivalents for SCFAs were calculated using the following stoichiometric conversions: 1.07 for acetic acid (HAc), 1.51 for propionic acid (HPro), 1.82 for isobutyric acid (isoHBu), 1.82 for butyric acid (HBu), 2.04 for isovaleric acid (isoHVal), 2.04 for valeric acid (HVal), and 2.21 for caproic acid (HCa).

The COD removal (%) was determined to the percentage of organic matter removed as biogas (Eq. 3):

$$\text{COD removal (\%)} = \left(\frac{tCOD_{out} - tCOD_{in}}{tCOD_{in}}\right) \times 100 \quad (3)$$

where tCOD_{out} refers to the total organic matter content in the reactor's effluents (g COD/L) and tCOD_{in} represents the total organic matter fed to reactors (g COD/L).

Analytical techniques

Carbohydrates were quantified using the phenol–sulfuric acid method [8]. Total organic nitrogen was measured using the Kjeldahl method, following standard protocols [9], and protein content was determined by multiplying the total organic nitrogen by a conversion factor of 6.25 [10]. Additionally, APHA (2023) methods were employed to determine total solids (TS), volatile solids (VS), ash, ammonium (N-NH_4^+), sCOD, and tCOD [9]. Lipid content was estimated by subtracting the ash, protein, and carbohydrate contents from the TS. To measure soluble compounds (N-NH_4^+ and sCOD), the collected samples were first centrifuged at 18,440 RCF for 5 min (Centrifuge 5424 R — Eppendorf) and then filtered through a 0.45-µm pore filter.

To assess the conversion of SCFAs into biogas, daily gas production was measured using a µFlow unit (Bioprocess Control, Sweden), and its composition was analyzed (CH_4, CO_2, and H_2) twice a week with a gas chromatograph equipped with a thermal conductivity detector (Clarus 580 GC, PerkinElmer, USA). SCFAs were measured by high-performance liquid chromatography (HPLC) using an Agilent 1260 HPLC equipped with a refractive index detector. The HPLC setup included a pre-column (Cation H Refill Cartridge Micro-Guard, Bio-Rad) and an ion exclusion column (Aminex HPX-87H 300 × 7.8 mm ID, Bio-Rad). Details of the used chromatographic methods are provided in previous studies [11, 12].

Microbial analyses

To evaluate the effect of HRT on microbial communities, 16S rRNA analysis was performed on the inoculum and samples taken from period 1, period 2, period 3, and period 4 (Table 1) when reactors reached the steady state. Samples were stored at − 20 °C until analysis. DNA extraction was carried out from 1-mL sample using FastDNA SPIN Kit for Soil (MP Biomedicals, LLC), with DNA quantity and purity verified via spectrophotometry (SPECTROstar Omega e BMG Labtech). The V3–V4 regions of the 16S rRNA gene for bacteria and archaea were amplified using primers 341 F and 805 R. Amplicon sequencing was conducted with Illumina MiSeq (FISABIO, Spain). Microbial identification was achieved by processing the sequenced raw data with bioinformatics tools as described by Greses and co-authors (2021) [6]. Alpha diversity, measured in terms of operational taxonomic units (OTUs) at 97% sequence identity, and the Shannon index were obtained using the QIIME 1.9.1 software package [13]. Canonical correspondence analysis (CCA) was performed using PAST [14], enabling the establishment of a correlation between SCFAs

production and the microbial community developed at each HRT tested. The sequence data from the samples analyzed in this study were uploaded to the Sequence Read Archive (SRA) under the accession numbers SRR25436197 to SRR25436200, as part of BioProject PRJNA907204.

Results and discussion

Anaerobic fermentation of maize silage for SCFAs production

Table 2 displays the variations in the most critical process parameters for each HRTs tested. At 15-day and 12.5-day HRT, the VS content remained constant at an average of 25 g/L, while at 10-day and 7.5-day HRT the VS content averaged 20 g/L, demonstrating efficient disintegration of particulate organic matter when HRTs were below 12.5 days. Furthermore, during the acidogenesis stage, the stepwise variation in HRT supported a strong acidogenic activity, as indicated by acidification percentages, reaching a maximum value of 95.2 ± 12.2% $COD_{SCFAs,out}$/$sCOD_{out}$ for 15-day HRT. At the lowest HRT (7.5 days), the acidification percentages fall to 82.4 ± 5.9% $COD_{SCFAs,out}$/$sCOD_{out}$. Xu and co-authors (2023) stated that the acidification percentage decreased as HRT was reduced, likely due to the shorter reaction time available for hydrolysis and acidogenesis [15]. In any case, these values were notably higher than those reported in the available literature. For instance, the acidification percentage ranged between 61–69% $COD_{SCFAs,out}$/$sCOD_{out}$ [6] for AF of agroindustrial waste, 57–70% $COD_{SCFAs,out}$/$sCOD_{out}$ for AF of microalgae biomass [11], and 39–77% $COD_{SCFAs,out}$/$sCOD_{out}$ for AF of lipid-enriched food waste [15].

During the AF of maize silage, a lipid-rich biowaste (≅41% DW, "Feedstock" section), tSCFA concentration increased linearly when increasing HRT, reaching a maximum concentration of 14.3 g/L (equivalent to 21.8 g $COD_{SCFA,out}$/L) at 15-day HRT. Results obtained herein were similar than that obtained for batch AF of synthetic substrate (lipids = 50%, carbohydrates = 25%, proteins = 25%) with comparable macromolecular composition to maize silage and an initial tCOD of 79.9 g/L [16]. In addition, Law and co-authors (2023) reported lower SCFA concentrations (4.9 g COD_{SCFA}/L) at 4-day HRT in a continuous reactor operated at OLR of 6.5 g COD/L·day and fed with lipid-enriched food waste (lipids content = 30%) [15].

The findings suggest that at *HRTs* < 10 days, no further enhancements in bioconversion were achieved. The bioconversion was maintained at 28% g COD_{SCFAs}/g $tCOD_{in}$ at HRT of 15 days, 12.5 days, and 10 days, while for 7.5-day HRT the bioconversion decreased to 21.7% g COD_{SCFAs}/g $tCOD_{in}$. Law and co-authors (2023) described a similar bioconversion of 17% g COD_{SCFAs}/g $tCOD_{in}$ for AF of food waste supplemented with 13% lipids at 4-day HRT in CSTR [15]. These authors also observed a decrease in bioconversion from approximately 39 to 19% g COD_{SCFAs}/g $tCOD_{in}$ when lipid content increased from 20 to 30% at the same HRT [15]. Thus, feedstock with high lipid content, as in maize silage (≅41% DW), may reduce bioconversion, and careful balance between lipid content and process HRT is crucial for achieving optimal AF.

The bioconversion reached in the present study agrees with values observed for AF of maize silage (17% g COD_{SCFAs}/g $tCOD_{in}$ [17]) performed in a 5-L CSTR fed in a semicontinuous mode. However, while promising, current values are below the levels documented for continuous anaerobic reactors fed with food waste supplemented with 30% lipid content and operated at 4-day HRT (51% g COD_{SCFAs}/g $tCOD_{in}$) [15]. This variation could be linked to the physicochemical properties of food waste (sourced from supermarkets and restaurants) and the composition of lipids added (derived from waste cooking oils).

Table 2 Main process parameters measured during the steady-state period in the AF of maize silage under stepwise HRT decrease (mean ± standard deviation)

Chemical parameters	15-day HRT	12.5-day HRT	10-day HRT	7.5-day HRT
pH	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1
$tCOD_{out}$ (g/L)	44.7 ± 1.6	53.9 ± 5.6	54.9 ± 3.1	43.8 ± 6.8
$sCOD_{out}$/$tCOD_{out}$ (%)	44.3 ± 3.1	36.4 ± 2.6	30.1 ± 1.2	22.0 ± 1.9
TS (g/L)	34.1 ± 5.7	31.1 ± 3.8	27.2 ± 1.3	26.5 ± 1.3
VS (g/L)	25.9 ± 3.8	24.3 ± 3.1	20.2 ± 1.5	19.9 ± 1.7
N-NH_4^+ (g N/L)	675.8 ± 63.4	439.2 ± 11.2	312.7 ± 15.6	291.6 ± 7.9
tSCFAs (g/L)	14.3 ± 0.6	10.6 ± 0.4	9.6 ± 0.6	5.9 ± 0.1
% acidification ($COD_{SCFAs,out}$/$sCOD_{out}$)	95.2 ± 12.2	87.2 ± 10.0	90.5 ± 3.3	82.4 ± 5.9
COD removal (%)	27.9 ± 2.2	13.06 ± 4.3	7.9 ± 3.4	3.4 ± 0.8

Time courses for sCOD, COD removal (%), TS, VS, and VS removal (%) during AF at HRTs of 15, 12.5, 10, and 7.5 days are available in Fig. 1S

Besides, the rates of biodegradation and hydrolysis vary among lipids, proteins, and carbohydrates, with lipids exhibiting the lowest rate, especially for complex substrates that require longer hydrolysis times under the acidic conditions [18].

Lipid degradation is often considered the rate-limiting step in the anaerobic treatment [19]. In AF, these lipids are hydrolyzed to glycerol and long-chain fatty acids and further transformed into acetate and H_2 via β-oxidation. AF of lipid-rich waste often fails due to the inhibition caused by lipid hydrolysis products (*e.g.*, long-chain fatty acids (LCFAs)). Due to their adsorption onto the microorganism's surface, the transport/protective function might be hampered [20]. Given the inherent complexity of lipid degradation, the bioconversion achieved in this study is relevant. The findings revealed that the bioconversion remained unaffected by the gradual reduction in HRT from 15 to 10 days, highlighting the robustness of the AF of maize silage.

In the present study, adjusting reactor operating parameters (*e.g.*, $HRT \leq 15$ days and pH = 5.5) as strategy to inhibit the growth of methanogens has proven inadequate in fully controlling their activity during the AF of maize silage (ca. 32-mL CH_4/g CODin; CH_4: 14%). HRT and acidic pH alone did not exert sufficient selective pressure to eliminate methanogens. However, these microorganisms, responsible for SCFAs consumption and methane production, were reasonably controlled. As a result, a relevant SCFAs production was sustained, and COD removal reached 27.9% at 15-day HRT. Regarding the SCFAs profile resulting from each HRT assessed (Fig. 1), the SCFAs composition was dominated by acetic (~31% w/w), propionic (22–27% w/w), and butyric acids (~19% w/w) followed by minor valeric acid concentration (15–19% w/w), while caproic acid presence increased as HRT decreased, ranging from 1.6 to 9.6% w/w. Variations in the relative distribution of SCFAs have been previously reported during reductions in HRT [11]. In conventional anaerobic digestion processes for biogas production at elevated HRTs, acetoclastic methanogenic archaea rapidly utilize acetic acid. Conversely, in systems designed for SCFAs production at reduced HRTs, the persistence of acetic acid is regarded as evidence of successful AF, indicating the reduction of acetoclastic archaea activity and the absence of acetic acid consumption [18].

Acidic pH conditions tend to favor the accumulation of acetic, propionic, and butyric acids [18, 21]. In our study, the predominance of these acids was possibly due to the influence of process pH (=5.5) on the SCFA profile and macromolecular composition. Cavinato and co-authors (2017) reported similar findings for continuous AF of

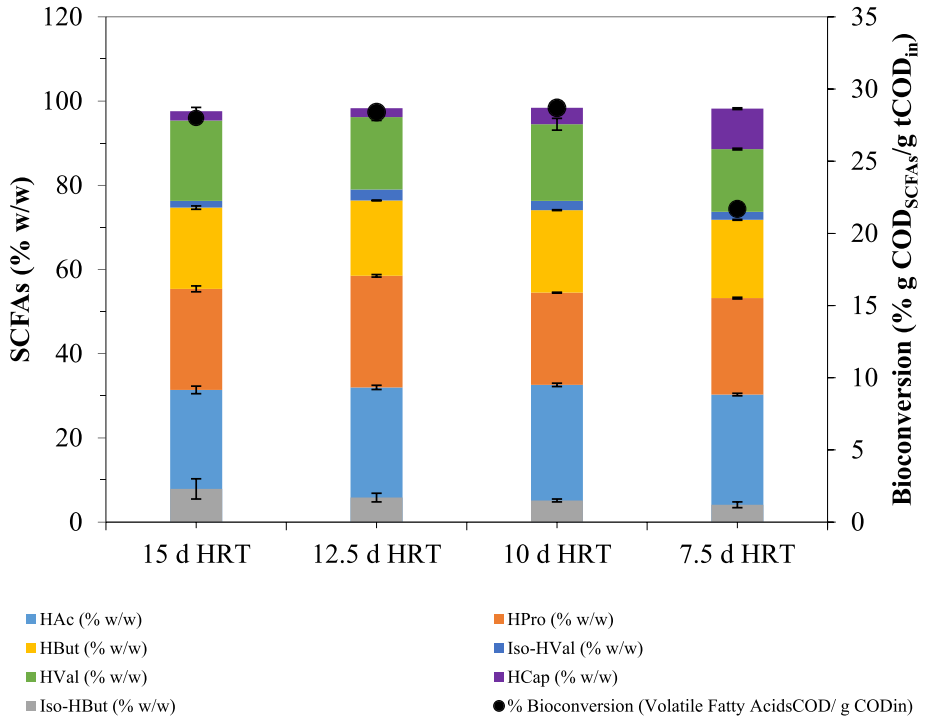

Fig. 1 SCFAs profile and corresponding SCFAs concentration (g COD/L) reached in AF of maize silage during the steady state of 15-day HRT, 12.5-day HRT, 10-day HRT, and 7.5-day HRT

maize silage ($tCOD = 996$ g/kg; $TS = 333$ g/kg), operated at 6-day HRT and an acidic pH of 5.6. Under these conditions, acetic (22%), butyric (21%), and propionic acids (13%) were identified as the predominant SCFAs [17].

In this study on AF of maize silage, the absence of ethanol and lactate suggested that sugars might serve as the primary electron donors, providing the reducing power needed for microbial chain elongation to synthesize caproic acid through reverse beta-oxidation [22]. This observation aligns with previous findings, such as those by Lambrecht and co-authors (2019), which reported caproate levels up to 6.12 g/L in a CSTR fed with maize silage and operated at pH of 5.5 and HRT of 4 days, using lactate as electron donor [23]. This highlights the potential for achieving relatively high caproic acid production under similar conditions, even with sugars as electron donors.

Microbial ecology and its correlation with abundance and reactors performance

Taxonomic analysis was conducted to assess how the stepwise decrease of HRT affected relative abundance of microbial communities and reactors performance. As it can be seen in Table 3, the inoculum demonstrated the greatest biodiversity, reflected by the highest number of observed OTUs (1333). The Shannon index (7347), which considers sample richness and evenness, also confirmed a comparable trend. Furthermore, a clear reduction in biodiversity was noted when comparing the anaerobic inoculum with the microbiome obtained in each HRTs tested. This shift indicated a specialization of the microbial community towards metabolic pathways associated with SCFA synthesis under the imposed conditions. Among the HRTs tested, the OTUs remained relatively stable. This trend was also reflected in the Shannon index, indicating a more specialized and homogeneous microbial population during each period (Table 3). These findings were consistent with the literature, as higher biodiversity is typically observed at longer HRTs and mild conditions devoted to biogas production, such as mesophilic temperatures and neutral pHs [6].

The phylum profile in the inoculum consisted of a wide variety of microorganisms attributed to the origin of the sludge (Fig. 2). The inoculum was obtained from a conventional anaerobic digester, typically operated at HRTs from 20 to 30 days to stabilize the sewage sludge. In the inoculum, the five main phyla in terms of relative abundance were Firmicutes (21.87%), Actinobacteria (21.72%), Proteobacteria (12.53%), Bacteroidetes (10.01%), and Chloroflexi (9.41%). Firmicutes and Bacteroidetes have been identified as the predominant bacterial phyla in most anaerobic digesters [24]. These phyla contribute to both the hydrolysis and fermentation process, facilitating the conversion of complex substrates into simpler molecules during anaerobic treatment [25]. While Chloroflexi growth in stable anaerobic digestion systems designed for biogas production, often operated at long HRT (> 20 d) [26]. Additionally, Euryarchaeota, a phylum that includes diverse methanogens capable of methane production as a metabolic byproduct, was detected at low abundance (1.9%). At the genus level, the inoculum exhibited a highly diverse microbial community, with the most dominant groups including the family unclassified Intrasporangiaceae (5.82%), unclassified Bacteroidales (6.32%), and the order Clostridiales (5.43%).

As the HRT was reduced, the microbial population became predominantly composed of bacteria from the Firmicutes (65.62–76.18%), Bacteroidetes (15.99–23.75%), and Actinobacteria (4.51–7.40%). These phyla are typically observed in anaerobic reactors designed for SCFAs production [6, 27]. For all tested HRTs, the Firmicutes population showed a 3- to 3.5-fold increase relative to the inoculum. This is consistent with the role of Firmicutes which have been identified as key fermentative bacteria that contribute to hydrolysis, acidogenesis, and acetogenesis in anaerobic metabolic pathways [11, 27]. In addition, some studies confirmed that Firmicutes can contribute significantly to lipid degradation and the synthesis of SCFAs [28, 29]. Bacteroidetes has been recognized as degrading complex organic matter, including cellulose and protein [27], while Actinobacteria are essential for degrading complex carbohydrates and proteins and are involved in acetic acid synthesis during the AF process. Furthermore, Actinobacteria produce an extensive array of extracellular enzymes, notably efficient lipases, which are actively involved in breaking down a wide range of lipids [30]. These findings suggest that Firmicutes and Actinobacteria might involve in the lipid hydrolysis during the AF of maize silage.

At genus level, for each tested HRTs, the microbial profile resulted in a marked dominance of bacterial communities predominantly from Firmicutes phylum, mainly unclassified Clostridiales, *Megasphaera*, and *Bulleidia*. Concurrently, the phylum Bacteroidota was represented mainly by the genus *Prevotella*, maintaining a stable presence across all HRT conditions. In addition, bacteria from the unclassified Clostridiales, within the phylum

Table 3 Biodiversity indexes calculated for the microbial community of inoculum, 15-day HRT, 12.5-day HRT, 10-day HRT, and 7.5-day HRT

Biodiversity index	Inoculum	15-day HRT	12.5-day HRT	10-day HRT	7.5-day HRT
OTUs	1333	564	627	601	589
Shannon	7347	4793	5287	5287	5237

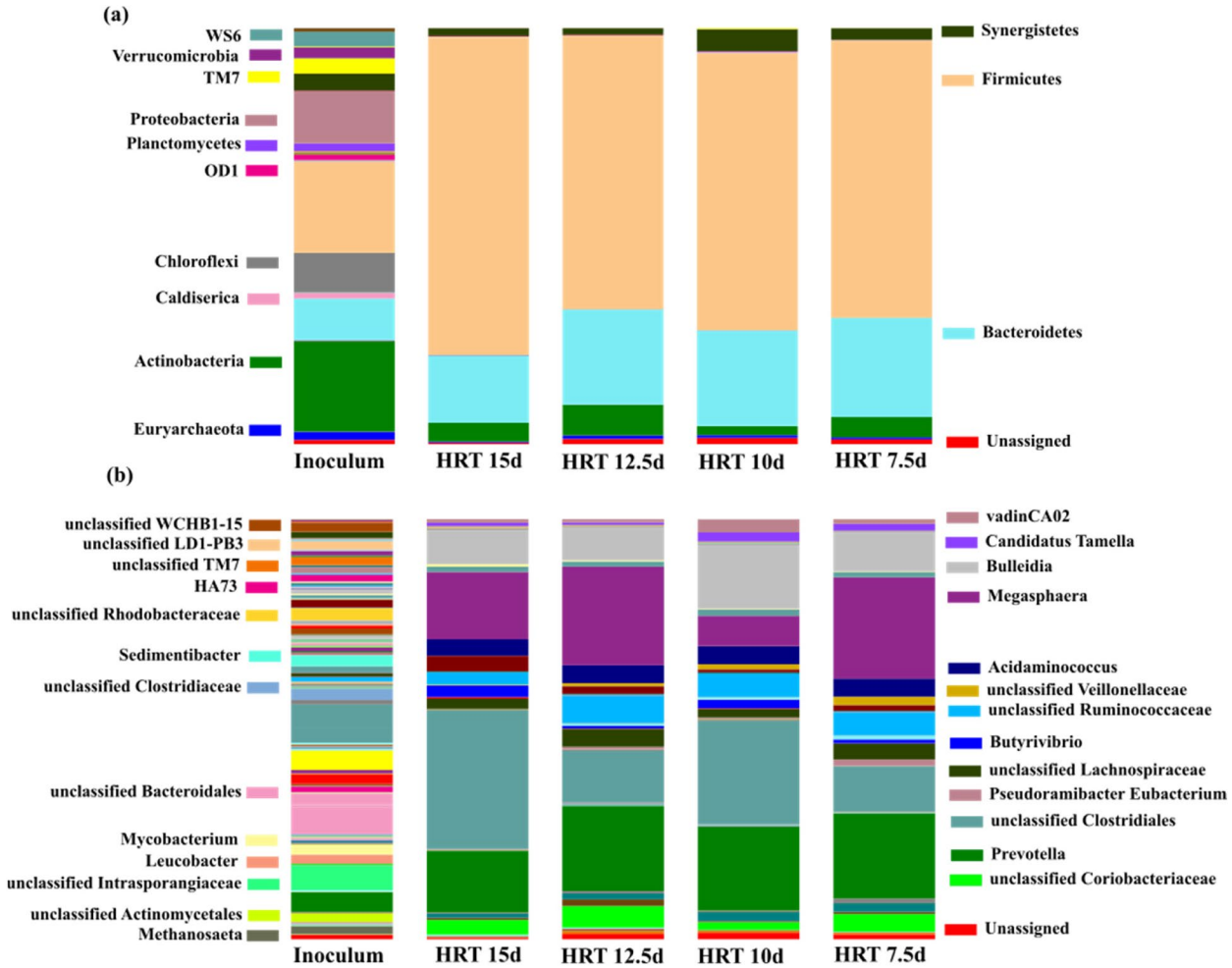

Fig. 2 Relative abundance of bacteria and archaea at **a** phylum and **b** genus level identified in the inoculum and each hydraulic retention time studied. Plot legend only includes microorganisms with relative abundance > 1%

Firmicutes, has been pointed out to be key for producing extracellular enzymes (such as cellulases, lipases, and proteases) which are involved in acetogenesis and syntrophic acetate oxidation by breaking down acetate into CO_2 and H_2. Members of this genus utilize an oxidative metabolic pathway to convert alcohols into acetic acid and other SCFAs [24]. Additionally, these bacteria are associated with butyric acid as a primary end product, which might explain the relative elevated butyric acid levels observed during AF of maize silage [24]. *Bulleidia* species are fermentative bacteria known to metabolize a variety of sugars and proteins, producing acetic and caproic acid as byproducts [12]. The genus *Megasphaera* is known for its ability to ferment sugar, resulting in the production of propionic, butyric, and valeric acids [31].

Prevotella abundance exhibited no significant variations (~ 20%) at HRTs less than 15 days, showing that this genus maintained its presence consistently during the AF of maize silage. *Prevotella* has been identified in batch anaerobic treatment of maize silage using a mix of cattle rumen and cattle manure as inoculum. Furthermore, it has been reported that *Prevotella* has the ability to hydrolyze pectin, xylan, and cellulose and break down a range of polysaccharides, generating acetate, succinate, and propionate, the latter being a crucial substrate for gluconeogenesis [32–34]. This metabolic pathway is essential for glucose synthesis under low-carbohydrate conditions, utilizing precursors such as amino acids, pyruvate, lactate, acetate, fatty acids, and glycerol [35]. Since the maize silage in this study predominantly contained high lipids content (40.6 ± 2.8% DW) and a moderate protein concentration (25.4 ± 1.2% DW), it is plausible that gluconeogenesis pathway was active during AF of maize silage.

Acidaminococcus was consistently detected across all tested HRTs with an average abundance of 4%. This genus is known for producing various SCFAs from amino acids

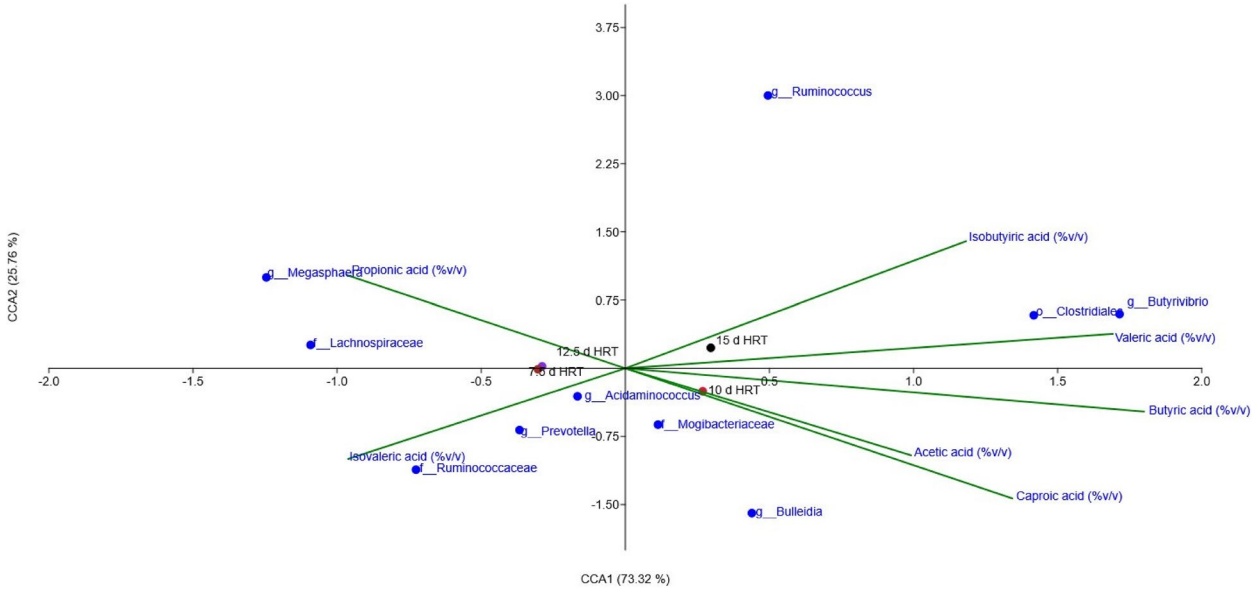

Fig. 3 Canonical correspondence analysis (CCA) ordination triplot performed for the samples retrieved during the steady state of the AF of maize silage. The figure displays the relationships among CSTRs (period 1- to 15-day HRT, black circle; period 2- to 12.5-day HRT, blue circle; period 3- to 10-day HRT, orange circle; period 4- to 7.5-day HRT, red circle), species (blue circles), and SCFAs (green arrows)

and was positively associated with the production of valeric and caproic acids [36].

For all HRTs tested, the main changes in abundance, during the AF, were related to the genera *Bulleidia*, *Megasphaera*, and the unclassified Clostridiales. *Bulleidia* and *Megasphaera* showed variations in abundance from 7.82–15.29% and 7.21–24.12%, respectively. Given maize silage used here contain a moderate concentration of carbohydrate (27.6 ± 4.9% DW) and the absence of detectable concentrations of lactate or ethanol, these genus are presumed to play an essential role in AF, as it demonstrates a remarkable capacity to metabolize various sugars and employ them as electron donors [37]. Additionally, the abundance of unclassified Clostridiales ranged from 10.70 to 32.94%. These bacteria are known to efficiently ferment carbohydrates, generating acetic acid and butyric acid as byproducts [6].

Comparison of the hydrolytic and acidogenic communities during the different operational stages and initial inoculum demonstrated that the operational conditions and the maize silage composition shaped the anaerobic microbiome. This resulted in a community dominated by unclassified Clostridiales, *Bulleidia*, and *Megasphaera*, specialized in degrading lipid-rich substrates and producing SCFAs.

Canonical correspondence analysis (CCA) of the relationship between hydraulic retention time with microbial community and acids production

As illustrated in Fig. 3, the triplot reveals that the ordination axes together account for 99.08% of the total variation in the bacterial community, suggesting that the tested HRTs, pH, and temperature played a key role in influencing microbial community dynamics respect to inoculum.

The CCA plot showed that HRT of 15 days favored the production of valeric and isobutyric acids. This may be attributed to the longer HRTs, together with an acidic pH, promoting the formation of valeric acid [21, 38]. In the case of 10-day HRT, the proximity of *Acidaminococcus* to the plot's origin indicated that it was only marginally influenced by the changes in HRT. This genus was consistently in all reactors (~ 4%) and is likely involved in the degradation of monosaccharides present in the maize silage, and SCFAs, including acetic and butyric acids [39].

CCA showed a strong correlation between Ruminococcaceae and *Prevotella*. Ruminococcaceae, particularly the *Ruminococcus* genus, exhibit strong hydrolytic capabilities for degrading complex carbohydrates such as cellulose and hemicellulose [6], while *Prevotella* specializes in the breakdown of carbohydrates, proteins, and lipids [29, 40]. This conveys that Ruminococcaceae

may focus on decomposing fibers such as cellulose, releasing oligosaccharides and simple sugars that *Prevotella* subsequently ferments. These results highlight the complementary role of these microorganisms as fiber degraders and carbohydrate fermenters. Furthermore, significant correlation was observed between the family Lachnospiraceae and *Megasphaera*. Lachnospiraceae are a family of anaerobic, fermentative, and chemoorganotrophic bacteria recognized for their effective hydrolytic activities [41]. This family produces enzymes such as xylanase, pectin methylesterase, pectate lyase, and various glycosidases, which enable the breakdown of complex carbohydrates into simpler sugars, supporting subsequent fermentation processes. Additionally, Lachnospiraceae synthesize isobutyrate and isovalerate [41]. Meanwhile *Megasphaera* are known for their role in the fermentation of lactate and other substrates into SCFAs, including butyrate [42]. The anaerobic interaction between Lachnospiraceae and *Megasphaera* might be characterized by metabolic interdependence, where Lachnospiraceae degrade complex carbohydrates, generating simpler metabolites, which is used as substrates for *Megasphaera*.

The CCA clearly demonstrated that the HRTs had an important impact on microbial community dynamics and SCFA profile accounting for nearly 100% of the total observed variation. The results also highlight potential synergistic and cross-feeding interactions among key genera involved in AF, including unclassified Clostridiales, *Megasphaera*, Lachnospiraceae, Ruminococcaceae, and *Prevotella*. These interactions likely play a crucial role in supporting efficient fermentation processes.

Conclusion

This study showed that the HRTs tested (7.5–15 days) promoted microbial specialization of hydrolytic and acidogenic microorganisms, maximizing SCFAs production from maize silage. The findings suggest that *HRTs* > 7.5 days represent a critical point, after which no further enhancements in bioconversion occur (~ 28% g COD_{SCFAs}/g $tCOD_{in}$). The AF reached a relatively high SCFAs concentration (14.3 g/L) with acetic, propionic, butyric, and valeric acids as the dominant byproducts, together accounting for up to 76% of the total SCFA concentration. CCA results showed that key microbial groups such as unclassified Clostridiales, *Megasphaera*, Lachnospiraceae, Ruminococcaceae, and *Prevotella* are crucial for maintaining process stability by efficiently degrading a lipid-rich waste as maize silage.

Supplementary Information

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Additional file 1: Fig. 1S. Time courses for sCOD, COD removal (%), TS, VS, and VS removal (%) achieved in AF of maize silage in CSTRs operated at 15-d HRT (a, b), 12.5-d HRT (c, d), 10-d HRT (e, f), and 7.5-d HRT (g, h).

Authors' contributions

MLL performed the anaerobic fermentation experiments and revised the final version of the manuscript, JV analyzed and interpreted all the data regarding the microbial populations and anaerobic fermentation outputs, and was the major contributor in writing the manuscript. SG was in charge of the DNA extraction, sample preparation for microbial population analysis and revised the final version of the manuscript, ETP collaborated in the manuscript writing, funding acquisition and revision of the final version of the manuscript, CGF was in charge of the conceptualization of the work, funding acquisition and revision of the final version of the manuscript. All authors read and approved the final manuscript.

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Data availability

Persistent identifiers (e.g. DOI or accession number) for the data will be added when available.

Declarations

Competing interests

The authors declare no competing interests.

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