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Research Paper

# Long hydraulic retention time mediates stable volatile fatty acids production against slight pH oscillations

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ABSTRACT

The effect of operational conditions on the stability of acidogenic fermentation (AF) devoted to volatile fatty acids (VFAs) production still presents numerous gaps to achieve high yields and fully understand the responses of open microbiomes associated to this technology. To cope with that, this investigation was designed to assess the stability of VFAs production via AF of agro-food wastes at high hydraulic retention times (HRTs) (20 and 30 d) and pH oscillations (5.8–6.2). Similar bioconversion efficiencies (~50 %) were reached regardless of the HRT, revealing that HRT of 20 d can be considered as a threshold from which, no further improvement was achieved. The combination of long HRTs, 25 °C and acid pHs promoted a robust microbiome that resulted in a stable outcome against pH variations, being Clostridiales order identified as key player of AF stability. These conditions mediated a high selectivity in the VFAs production profile, with acetic and butyric acids, prevailing in the VFAs pool (~80 % of total VFAs) at HRT 20 d. The selection of appropriated conditions was shown to be critical to maximize the hydrolysis and acidogenesis of the substrate and attain a stable effluent against pH oscillations.

## 1. Introduction

As a consequence of the increasing population and social development, food demand is continuously rising around the world. According to the Food and Agriculture Organization (FAO, 2019), food production gives rise to 1.3 billion tonnes of residues per year, out of which 30 % corresponded to fruits and vegetables discarded in the early stage of the food supply chain (Ribeiro et al., 2018). Proper disposal or valorisation of food residues is of paramount importance to reduce their economic and environmental impact. In this framework, the European Commission has adopted a circular action plan to pave the way for a cleaner and more competitive Europe. This strategy encompasses not only the need of waste reduction but also the imperative of deriving value from these materials (European Commission., 2020).

Out of the potential valorisation routes that can be employed, anaerobic digestion (AD) is a sustainable bioprocess that can contribute to the circular economy approach to solve this agro-food waste (AFW) overproduction. Besides being environmentally friendly, this biological treatment mitigates the environmental impact linked to waste and yields an energetically valuable by-product—biogas. AD comprises four different steps, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis. As precursors of biogas, volatile fatty acids (VFAs) are produced in fermentative stages, namely the acidogenesis and acetogenesis steps. VFAs are chemical building blocks highly demanded by various industries, such as chemical, pharmaceutical, or food industries (Deshmukh and Manyar, 2021). Thus, acidogenic fermentation (AF) has emerged as an alternative approach to AD to valorize residues, where methanogenic archaea activity is hampered leading to VFAs accumulation. To inhibit methanogens, and thereby promoting VFAs accumulation, several strategies have been reported, including energy-demanding

*Abbreviations:* AD, Anaerobic digestion; AF, Anaerobic fermentation; AFW, Agro-food waste; CCA, Canonical correspondence analysis; COD, Chemical oxygen demand; CSTR, Continuous stirred tank reactor; EtOH, Ethanol; FW, Food waste; $H_2$, Hydrogen; HAc, Acetic acid; HBu, Butyric acid; HCa, Caproic acid; HLact, Lactic acid, HPro, Propionic acid; HRT, Hydraulic retention time; HVal, Valeric acid; IsoHBu, Isobutyric acid; IsoHVal, Isovaleric acid; OLR, Organic loading rate; OTUs, Operational taxonomic units; SCOD, Soluble chemical oxygen demand; TCOD, Total chemical oxygen demand; TKN, Total Kjeldahl nitrogen; TS, Total solids; VS, Volatile solids; VFAs, Volatile fatty acids.

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inoculum pretreatments or the addition of expensive chemicals (Magdalena and González-Fernández, 2020). However, the cost-efficiency trade-off of those strategies remains questionable. Alternatively, VFAs accumulation can also be attained by manipulating the operational parameters implemented in the AF to promote acidogenic bacteria activity and hamper methanogenic metabolisms (Magdalena and González-Fernández, 2020; Vázquez-Fernández et al., 2022). Although the effect of operational parameters on AD has been widely studied to maximize methane production, their correlation with VFAs production efficiency, profile and the microbiome operated in continuous mode remains unclear.

Process pH has been identified as a key regulator parameter in AF. However, controversy exists in reported results concerning the optimal pH, predominantly influenced by the characteristics of the residue and the targeted end-product. For instance, an optimal pH range of 6–8 was determined to anaerobically ferment proteins (Feng et al., 2009), while basic pHs (>8) were found beneficial for hydrolysing complex residues with high content of proteins or lipids (pH 10–11) (Battista et al., 2022). Recently, slightly acid pHs (5.5–6.5) were also identified as optimal to metabolise carbohydrate into VFAs (Lv et al., 2022; Tomás-Pejó et al., 2023). Prior studies identified slightly acidic pH conditions to be optimum for the acidification step based on their effectiveness for methanogenesis inhibition (Greses et al., 2022a; Zhang et al., 2020). Indeed, microbial metabolisms present in AF can shift according to minor pH variations in the optimum range, thereby resulting in different outputs (Greses et al., 2023). The oscillations are intricately tied to other operational conditions (such as HRT and temperature) established in the AF process, hampering the extrapolation of the results. In this regard, HRT plays a crucial role in process efficiency and stability.

Conventionally, short HRTs are imposed in AF to promote methanogens wash-out and promote VFAs accumulation (Battista et al., 2022). However, VFÁs yields may be compromised when utilizing complex residues as feedstock, requiring extended HRTs to facilitate efficient hydrolysis (Swiatkiewicz et al., 2021). Extended HRTs foster the development of a microbiome rich in biodiversity, enhancing the resilience of AF. More importantly, this also facilitates the proliferation of slow-growing microorganisms, including methanogenic archaea, which consume volatile fatty acids (VFAs) to produce biogas (Greses et al., 2017; Lv et al., 2022). Long HRT could be combined with an acid pH to maximize acidogenesis biodiversity but minimize methanogens activity. Consequently, extended HRTs were hypothesised to have the potential to circumvent these pH oscillations. In this context, the novelty of this investigation was to elucidate the HRT threshold to maximize the efficiency of AF of AFW at acid pH, assessing the robustness of the process against pH oscillations. The microbial community was analysed to link the prevailing microbiota and the bioprocess chemical outcome to identify the essential microorganisms responsible for the VFAs production stability.

## 2. Materials and methods

### 2.1. Feedstock and inoculum used for VFAs production

A mixture of AFWs composed of cucumber, tomato, eggplant, and melon was provided by an agricultural producing company (Estación Experimental Cajamar, Almería, Spain). This mixture has been selected based on the waste production data (amount and season-dependency) of the producer, identifying these AFWs as potential feedstock for AF. The AFWs was blended using a kitchen blender and further characterized (Table 1). The characterization comprises the pH, total and volatile solids (TS and VS, respectively), total and soluble chemical oxygen demand (TCOD and SCOD, respectively), organic acids, the percentage of carbohydrates, lipids, proteins, and ash, and ammonium ($NH_4^+$-N). To avoid autofermentative processes, the thoroughly mixed residue was stored at −20 °C until use.

The inoculum used for the AF process was anaerobic sludge collected from a conventional mesophilic anaerobic digester of a wastewater treatment plant (El Soto, Mostoles, Spain). The anaerobic inoculum presented TS of 7.5 ± 0.2 g/L, VS of 4.5 ± 0.1 g/L, TCOD of 9.7 ± 0.1 g/L, SCOD of 1.6 ± 0.0 g/L and $NH_4^+$-N of 0.5 ± 0.0 g N/L.

**Table 1**
AFW composition presented as mean ± standard deviation.

| | AFW |
|---|---|
| pH | 4.1 ± 0.2 |
| TCOD (g/L) | 101.1 ± 6.7 |
| SCOD/TCOD (%) | 76.2 ± 6.8 |
| TS (g/L) | 72.7 ± 1.2 |
| VS/TS (% w/w) | 89.9 ± 1.8 |
| $NH_4^+$-N (g/L) | 0.01 ± 0.00 |
| Carbohydrates (% w/w) | 63.3 ± 5.4 |
| Proteins (% w/w) | 13.5 ± 1.8 |
| Lipids (% w/w) | 13.1 ± 5.3 |
| Ash (% w/w) | 10.1 ± 1.8 |
| HAc (g/L) | 2.1 ± 0.2 |
| HLact (g/L) | 12.4 ± 0.3 |
| EtOH (g/L) | 4.5 ± 0.1 |

### 2.2. Experimental setup

AF of AFWs was performed in continuous stirred tank reactors (CSTRs) with 1 L of working volume and 0.5 L of headspace. The headspace was connected to a water trap to measure the biogas production by water displacement. The agitation in the reactor was accomplished by magnetic stirring (Agimatic-S, J.P. Selecta, S.A., Spain) and the temperature was set at 25 °C in a controlled temperature room. The reactors were filled with the selected inoculum and fed once a day over the entire experimental period. To induce VFAs accumulation, an organic loading rate (OLR) of 3 g VS/Ld was established. It is important to highlight that temperature (25 °C) and OLR (3 g VS/Ld) values were selected in accordance to Greses et al., (2022a), who concluded that these conditions promoted VFAs accumulation when using carbohydrate-rich feedstock. To elucidate the effect of the HRT on AF performance, HRTs of 20 d and 30 d were evaluated in two CSTRs operated in parallel under similar pH (5.8). These HRTs were selected because those values have been widely recommended to maximize complex residues hydrolysis in conventional AD process (Cremonez et al., 2021). Once the optimal HRT was selected, the effect of a minor pH variation (from 5.8 to 6.2) was studied as minor pH fluctuations have been identified to be able to alter AF performance, particularly when carbohydrate-rich residues are employed as feedstock (Greses et al., 2023).

All reactors were operated for three HRTs whereby the process reached the steady state (VFAs, TS, VS, $NH_4^+$-N concentration in the effluents and biogas production and composition exhibited stability). The AF performance was evaluated by calculating the bioconversion yield of organic matter into VFAs, the acidification percentage of the SCOD and the hydrolysis efficiency as VS removal, according to the equations (1), (2) and (3):

$$\%Bioconversion = \frac{COD_{VFAs,effluent}}{TCOD_{influent}} \hat{A}\cdot 100 \quad (1)$$

$$\% \text{ Acidification } = \text{Acidification} = \frac{\text{COD}_{\text{VFAs,effluent}}}{\text{SCOD}_{\text{effluent}}} \hat{A}\cdot 100 \quad (2)$$

$$\%VS_{removal} = \frac{(VS_{influent} - VS_{effluent})}{VS_{influent}} \hat{A}\cdot 100 \quad (3)$$

at the equations, $COD_{VFAs,effluent}$ represented the sum of concentrations of acetic acid (HAc), propionic acid (HPro), isobutyric acid (isoHBu), butyric acid (HBu), isovaleric acid (isoHVal), valeric acid (HVal) and caproic acid (HCa) in the CSTRs effluents in terms of g COD/L. The COD

equivalents for each VFA are 1.067, 1.513, 1.82, 2.039 and 2.207 for HAc, HPro, HBu, HVal and HCa, respectively. The $TCOD_{influent}$ represents the total COD (g COD/L) of the AFW fed to the reactors and the $SCOD_{effluent}$ (g COD/L) represents the soluble fraction of the COD analyzed in the CSTRs effluents. The $VS_{influent}$ was the VS concentration of AFWs fed to the reactors while the $VS_{effluent}$ was the VS content concentration in the CSTRs effluents.

### 2.3. Analytical methods and process monitoring

In order to characterize the feedstock, TS, VS, ash and Total Kjeldahl nitrogen (TKN) were calculated according to Standard Methods procedures (APHA, 2017). $NH_4^+$-N, and TCOD and SCOD were analyzed by using commercial kits (ISO 000,683 and ISO 15705, respectively, Merck). For analyse $NH_4^+$-N and SCOD, the soluble fraction of the samples was obtained by centrifuging and subsequently filtering the supernatant through 0.45 μm filters. The carbohydrate content of the AFWs was determined by using the phenol–sulfuric acid method (Dubois et al., 1956) and the protein content by using a TKN-protein conversion factor of 6.25. Thereafter, the lipid content (in percentage) was calculated by the difference between 100 and the percentage of proteins, carbohydrates, and ash. The pH was daily measured using a pH meter (GLP 21, Crison, Hach Lange) and adjusted to the desired value by adding NaOH (5 M).

Similarly, the inoculum was characterized in terms of TCOD, SCOD, TS, VS and $NH_4^+$-N, following the procedures described above.

The processes were monitored by analysing the pH, TCOD, SCOD, TS, VS and $NH_4^+$-N twice a week in the effluent of AF using the methods described above. The metabolites (VFAs, lactic acid (HLac) and ethanol (EtOH) were analyzed by high performance liquid chromatography (1260 HPLC, Agilent). The HPLC was 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 300x7.8 mm, I. D., Biorad). The mobile phase, flow rate and temperature of the HPLC's oven and detector were set following the description provided by Greses et al. (2023). Samples were filtered by 0.22 μm before being injected in the HPLC. The biogas composition was characterized by analysing 0.1 mL of biogas twice a week through a gas chromatograph (GC) equipped with a thermal conductivity detector (Claurus 580 GC, PerkinElmer). The GC used helium as gas carrier (30 mL/min) through two coupled packed columns (HSN6-60/80 Sulfinert P 7′ × 1/8″ O.D. and MS13X4-09SF2 40/60P 9′ × 1/8″ O.D., PerkinElmer) with the temperature of the oven, injector and detector at 62, 80 and 200 °C, respectively.

### 2.4. Microbial community analysis

The effect of the different HRTs and pHs on the microbial community was evaluated by analyzing the 16S rRNA gene in the inoculum and samples collected from each AF when the steady state was reached. DNA was extracted using the kit "FastDNA SPIN Kit for Soil" (MP Biomedicals, LCC), following the manufacturer's procedure. The amount and the quality of the DNA were analyzed through nanodrop (Omega Spectrostar BMC Labtech). The hypervariable regions V3 and V4 of the 16S rRNA gene were amplified using the primers 341F (F – CCTACGGGNGGCWGCAG) and 805R (R – GACTACHVGGGTATCTAATCC) to target bacterial and archaeal communities. These samples were further sequenced on MiSeq (Illumina). The resulting sequences were bio-informatically processed according to the procedure described by Greses et al. (2017), including the biodiversity index calculation (Shannon and operational taxonomic units [OTUs] at 97 % identity). PAST (Hammer et al., 2001) was used to perform a canonical correspondence analysis (CCA), which allowed establishing a correlation between metabolites production and microbial community in each condition. Similarity percentage analysis (SIMPER) was performed also using PAST (Hammer et al., 2001).

## 3. Results and discussion

### 3.1. Effect of HRT on VFAs production

#### 3.1.1. Bioconversion yields and process efficiencies in terms of hydrolysis and acidification

The effect of HRT was tested in semi-continuously operated CSTRs working at 20 and 30 d with a controlled pH of 5.8. Fig. 1 represents the VFAs profile and the bioconversion yield attained for both CSTRs. After reaching the steady state, VFAs achieved a concentration of 29.6 ± 2.1 g/L (47.0 ± 2.1 g COD/L) and 30.1 ± 1.0 g/L (44.8 ± 3.0 g COD/L) for HRT-20 d and HRT-30 d, respectively, corresponding to a bioconversion of 49.2 ± 2.0 % and 51.2 ± 2.7 % (Fig. 1, Table 2).

No significant differences were found between both HRTs in terms of bioconversion efficiency, evidencing the benefit of working at long HRTs for effluent concentration stability. It should be highlighted that these results represented high values (VFAs production ~ 30 g/L and bioconversion yields ~ 50 %) when compared with previous studies assessing the carbohydrate-rich feedstock bioconversion info VFAs via AF. Lim et al. (2008) found a lower bioconversion (0.36–0.39 g VFAs/g $VS_{in}$) than the present investigation (approx. 0.5 g VFAs/g $VS_{in}$) when AF of carbohydrate-rich food wastes (FWs) was performed at similar pH, but at 35 °C and HRT of 12 d. Likewise, Greses et al. (2020) reported a bioconversion efficiency of 40 % when a carbohydrate-rich residue was anaerobically fermented under similar conditions (25 °C, OLR 3 g VS/Ld and pH 5.6) and at an HRT of 12.5 d. These results indicated that an extended HRT could have a more positive effect on complex organic matter hydrolysis and acidification than the use of high operational temperature or its combination with acid pHs. The importance of long HRTs to enhance the hydrolysis and promote VFAs production via AF of AFW was confirmed by Bolaji and Dionisi (2017), who reported a VFAs concentration increase from 9.1 g COD/L to 19 g COD/L when increasing the HRT from 10 to 30 days. However, the use of 35 °C as fermentation temperature also promoted methanogenesis, which led to subsequent VFAs consumption and lower VFAs production yields (25 % bioconversion).

The hydrolytic efficiency was determined by using the VS removal. As it can be seen in Table 2, VS removal was 53.8 % and 49.8 % for HRT of 20 d and 30 d, respectively., The attained results were pretty similar to those obtained by Bolaji and Dionisi (2017) at long HRT and 35 °C (49 % $VS_{removal}$). The low bioconversion yield (25 %) in terms of VFAs accumulation, indicated that the conditions set by Bolaji and Dionisi (2017) were suitable to get a good hydrolysis but not favourable to boost VFAs production. By opposite, the long HRTs implemented herein supported a high hydrolysis efficiency, and its combination with low temperatures and pH, led to an efficient methanogenesis inhibition. This fact was proven by the high HAc accumulation in the CSTR (methane precursor) and the negligible methane production. It can be thus stated that combining long HRTs with low process temperatures (25 °C) and pH supported higher yields by promoting organic matter hydrolysis without compromising VFAs accumulation. As a matter of fact, the straightforward effect of low temperature on metabolic kinetics could also support the hydrolysis efficiency stability since bioreactivity has been determined to be more rapidly fluctuant at high process temperature (Jiang et al., 2013). The application of lower temperatures would also decrease the energetic costs associated with the reactor's heating, increasing the overall profits when scaling up VFAs production.

Regarding the absence of VFAs yield changes when HRT was increased from 20 to 30 d (Fig. 1, Table 2), it is important to highlight that increasing HRT is only advantageous until a certain threshold above which, further increases do not result in any improvement. This fact has been previously observed by Scoma et al. (2013) who compared the effect of different HRTs in the AF of olive mill wastewaters. Whereas an HRT increase from 1 to 5 d evidenced a bioconversion improvement from 10 to 36 %, a further HRT increase to 7 d did not mediate any enhancement in the AF efficiencies. These results suggested that a

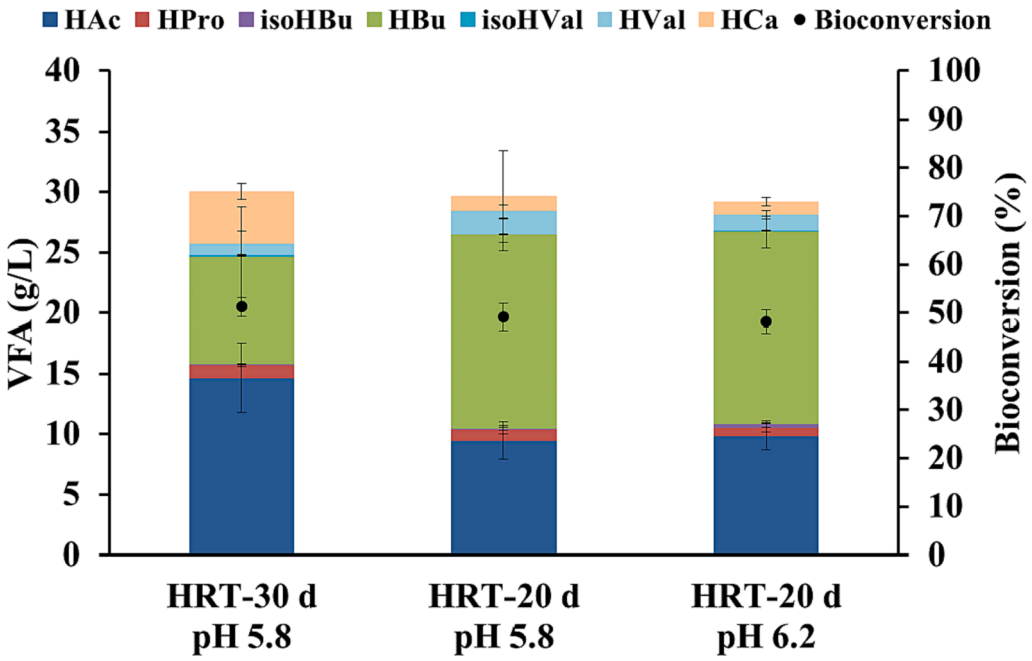


**Fig. 1.** VFAs profiles, concentration and bioconversion yields calculated for the steady state of each AF.

**Table 2**
AF effluents composition measured at the steady state of the bioprocess (mean ± standard deviation).

| | HRT 30 dpH 5.8 | HRT 20 dpH 5.8 | HRT 20 dpH 6.2 |
|---|---|---|---|
| TCOD (g/L) | 67.1 ± 3.2 | 66.1 ± 2.3 | 62.7 ± 2.4 |
| SCOD (%) | 78.9 ± 5.8 | 74.9 ± 3.1 | 76.1 ± 3.9 |
| TS (g/L) | 62.3 ± 2.5 | 52.8 ± 3.2 | 53.6 ± 3.1 |
| VS (%) | 60.7 ± 2.2 | 60.8 ± 2.0 | 58.2 ± 1.0 |
| $NH_4^+$-N (g N/L) | 0.4 ± 0.0 | 0.4 ± 0.1 | 0.3 ± 0.1 |
| Total VFAs (g VFAs/L) | 30.1 ± 1.0 | 29.6 ± 2.1 | 29.2 ± 1.7 |
| Total VFAs (g COD/L) | 44.8 ± 3.0 | 47.0 ± 2.1 | 45.9 ± 2.3 |
| HAc (% w/w) | 48.9 ± 5.7 | 31.9 ± 8.9 | 33.7 ± 2.4 |
| HPro (% w/w) | 3.4 ± 1.0 | 3.0 ± 0.3 | 2.3 ± 0.9 |
| isoHBu (% w/w) | 0.4 ± 0.3 | 0.3 ± 0.1 | 0.9 ± 0.5 |
| HBu (% w/w) | 29.5 ± 2.6 | 54.1 ± 7.9 | 54.5 ± 2.8 |
| isoHVal (% w/w) | 0.3 ± 0.1 | 0.1 ± 0.1 | 0.3 ± 0.0 |
| HVal (% w/w) | 3.1 ± 0.8 | 6.5 ± 1.8 | 4.5 ± 0.5 |
| HCa (% w/w) | 14.4 ± 5.4 | 4.1 ± 1.0 | 3.8 ± 0.7 |
| Bioconversion (%) | 51.2 ± 2.7 | 49.2 ± 2.0 | 48.1 ± 2.4 |
| Acidification (%) | 85.6 ± 4.1 | 95.8 ± 5.2 | 92.7 ± 2.4 |
| $VS_{removal}$ (%) | 53.8 ± 2.0 | 49.8 ± 0.6 | 51.0 ± 1.6 |
| VFAs/TS (gCOD/$gTS_{in}$) | 0.50 ± 0.02 | 0.68 ± 0.03 | 0.67 ± 0.03 |
| VFAs/VS (gCOD/$gVS_{in}$) | 0.56 ± 0.02 | 0.77 ± 0.03 | 0.75 ± 0.04 |

threshold was reached in the present investigation and 20 d was a long enough HRT for attaining high VFAs production via AF of AFW under slightly acidic pHs.

The threshold can be also confirmed by assessing the acidification yield. Similar to the hydrolysis efficiency, proper reaction times are required for acidogenesis. When targeting at VFAs production, acidification yields should be maximized. In this regard, the values attained for the acidification yield were similar at both HRTs (85.6 % at 30 d of HRT vs 95.8 % at 20 d of HRT). Since similar VFAs and SCOD were attained in both HRTs (Table 2), the slight variation observed in the acidification yield was mainly attributed to the COD equivalence of the VFAs profile. This result corroborated that an HRT longer than 20 d did not support any improvement in an AF.

### 3.1.2. VFA profile distribution

Regardless of the HRT applied, HAc and HBu were the most abundant VFAs. In the reactor set with HRT of 20 d, HAc and HBu accounted for 86 % (w/w) of the total VFAs produced and solely, HBu content surpassed 50 % of the total VFAs concentration (Table 2), which indicated the presence of a particularly selective production. For the AF at HRT of 30 d, HAc and HBu represented above 70 % of the total VFAs.

The VFAs distribution profile is commonly reported to be highly influenced by the operational parameters and the feedstock composition. The predominance of HAc and HBu is associated with the AF of high carbohydrate-content AFWs (Strazzera et al., 2018). In correlation, the profile described above is advantageous for further industrial implementation. According to Ramos-Suarez et al. (2021), downstream processes for separation and purification of carboxylates require high VFAs concentrations and selectivity to achieve significant yields. The selectivity in the VFA profile distribution with over 70 % being composed only of two VFAs would simplify the separation and purification methodologies.

The increase of HCa at the longest HRT (30 d) might be linked to the microbiome biodiversity enrichment. Although similar bioconversions were attained, a long HRT entails the prevalence of slow-growing microorganisms that could alter metabolisms. More specifically, Greses et al. (2021) found that long HRT at slightly acid pH promoted microorganisms involved in carbon chain elongation, which could justify the HCa production at the expenses of a lower HBu concentration.

HVal and HCa were obtained at both retention times. Whereas HVal was higher at HRT-20 days (6.5 %) than at HRT-30 days (3.1 %), HCa production increased from 4.1 % to 14.4 % when increasing the HRT. The low HVal production agreed with the macromolecular composition of the feedstock. It has been reported that HVal is commonly found in AF of substrates rich in proteins under acidic conditions (Llamas et al., 2022). The AFWs was composed of 13 % of proteins (Table 1), and thereby the VFA profile was poor in odd carbon chain carboxylates. Overall, the results obtained herein agreed with those reported by Bolaji and Dionisi (2017) and De Groof et al. (2021) who concluded that longer chain VFAs were produced concomitantly with increasing HRT.

## 3.2. Effect of slight pH oscillations on VFAs production

### 3.2.1. Bioconversion yields and process efficiencies in terms of hydrolysis and acidification

Since process efficiency was not improved at an HRT longer than 20

d, this value was selected to study the effect of minor pH variations on AF performance. In this manner, two pHs (5.8 and 6.2) were tested while maintaining the HRT at 20 d. The results showed similar VFAs concentration of 29.6 ± 2.1 g/L (47.0 ± 2.1 g COD/L) at pH 5.8 and 29.2 ± 1.7 g/L (45.9 ± 2.3 g COD/L) at pH 6.2. These VFAs productions corresponded to a bioconversion yield of 49.2 ± 2.0 % for pH 5.8 and 48.1 ± 2.4 % for pH 6.2. The pHs applied herein were comprised in the optimal range for promoting acidogenesis, leading to remarkably high VFAs production and bioconversion yield. No significant differences between the assays conducted at different pH were attained, indicating that AF of AFW at an HRT of 20 d promoted a robust microbiome that was able to maintain a high VFAs production efficiency against pH oscillations (±0.4). This agrees with the fact that long HRTs are reported to confer microbial robustness and stability while CSTRs operated at short HRTs are prone to instabilities (Gómez-Quiroga et al., 2022). Similar to the trend observed herein, Greses et al. (2020) found that slight pH oscillations (from 5.6 to 6.0) in the AF of vegetable wastes did not provoke significant variations in bioconversion yields. These results confirmed that the use of long HRTs (20 d) reduced the AF instabilities, which would decrease the control complexity and the related costs.

The robustness of the AF process can be also observed in the hydrolysis efficiency. It should be stressed that the hydrolytic step is highly influenced by pH. Nevertheless, as it can be seen in Table 2, the AF conducted at different pHs mediated similar results. $VS_{removal}$ of 49.8 ± 0.6 % and 51.0 ± 1.6 % were determined in the AFs conducted at pH-5.8 and pH-6.2, respectively. These high hydrolysis efficiencies were in agreement with Ma et al. (2019) who tested pH values in the range of 4 to 11 in AF of FWs and concluded that the hydrolytic activity was maximized at acidic pH. These results did not only demonstrate the AF stability but also the high hydrolytic efficiency of the process, which avoided the use of costly pretreatments to enhance the organic matter availability.

Regarding the acidogenesis stage, the different pHs applied also supported a high acidogenic microbial activity since the acidification percentage was 95 % and 92 % at pH-5.8 and pH-6.2, respectively (Table 2). Those values were comparably higher than the ones available in the literature. Kumar and Mohan (2018) compared the effect of a wide range of pH (4, 6, 7 and 10) when subjecting AFWs to AF. These authors achieved maximum acidification at a pH of 6.0 (54 %), followed by pHs 10, 7, and 4 (obtaining acidification efficiencies of 43.5 %, 30.2 %, and 26 %, respectively). Likewise, Greses et al. (2021) reported an acidification yield of 61.1 % and 69.2 % when fermenting melon wastes (pH of 5.8) and watermelon wastes (pH of 5.6), respectively. Although those authors showed a slight variation in the acidification efficiency with the pH, it is important to highlight that AFs were performed with different residues (melon and watermelon residues), hindering the comparison between their processes. Nevertheless, the percentage of SCOD acidified herein was significantly higher due to the proper combination of operational conditions since not only the pH but also the HRT (20 d) and the temperature (25 °C) were selected to maximize organic matter conversion into VFAs. It could be thus confirmed that the acidification efficiency was optimum at acidic pH while the decrease in pH from 6.2 to 5.8 did not show any advantage or disadvantages when it came to the hydrolysis or acidogenesis of AFWs. Based on that, it could be more industrially beneficial to work at pH of 5.8 than at 6.2 since high AF efficiencies will be maintained while decreasing the expenses related to the need of chemicals for pH control. Moreover, those results support the fact that those conditions allowed the microbial system to cope with pH oscillations (±0.4) without exhibiting a performance decrease.

#### 3.2.2. Vfas profile composition

The VFAs profile attained in the reactors were mostly composed of HAc and HBu, with these two carboxylic acids accounting for 86 % and 88.2 % (w/w) of the VFAs pool at pH-5.8 and pH-6.2, respectively (Table 2). As mentioned above in Section 3.1.2., the selected conditions enabled a high selectivity of carboxylates in the effluent. No significant differences were found in the VFAs profile within the slight pH changes applied, confirming the AF robustness and stability. With regard to the prevalence of VFAs with an even number of carbons, the results were in accordance with literature indicating that AF of carbohydrate-rich AFWs under slightly acidic pH results in a VFAs profile composed mainly of HAc and HBu (Valentino et al., 2018). The abundance of these carboxylates suggested a production from carbohydrate degradation through a butyrate-type fermentation (Greses et al., 2022b). At this point, it should be stressed that HAc is the VFA with the highest market volume within the industry (Deshmukh and Manyar, 2021). Likewise, HBu has a wide range of applications and its production through biotechnological pathways provides a sustainable alternative to it petrochemical production (Dwidar et al., 2012).

### 3.3. Microbial community analysis

Microbial community characterization was performed by collecting samples from the CSTRs when the process exhibited stability. The inoculum was also analysed to evaluate microbial changes against the operational parameters implemented in the CSTR aiming at producing VFAs. The inoculum exhibited high biodiversity (Table 3). This feature is characteristic of microbiomes collected in anaerobic digesters (Sundberg et al., 2013). High biodiversity has been related to high resistance to stressful environmental conditions, given that a wider range of metabolisms can co-exist (Wang et al., 2017). This analysis revealed a considerable loss of biodiversity from the inoculum to the samples collected from the CSTRs, resulting in a notable OTUs richness decrease (Table 3). Nevertheless, considering that Shannon index also accounts for both richness and evenness, this index revealed a less pronounced biodiversity decrease. These results, along with the high AF efficiencies, suggested that the microbiome suffered a specialization for VFAs production.

Among the studied operational conditions, HRT showed a higher effect on evenness (HRT 30 d, 4.214) than pH variation, which could justify the slightly different VFAs profile observed in the reactors.

In accordance with the biodiversity, the microbial profile in the samples also changed significantly from the inoculum to the CSTRs (Fig. 2). The heterogeneous phyla identified in the inoculum were mainly composed by Firmicutes (16.6 % relative abundance), Actinobacteria (15.2 %), Bacteroidetes (14.9 %), Proteobacteria (14.8 %), Chloroflexi (14.4 %), WWE1 (2.1 %) and Euryarchaeota (3.1 %). This microbiome profile has been previously found in well balanced AD processes devoted to biogas production (Campanaro et al., 2018; Sundberg et al., 2013). For instance, the dominance of T78 as main genus of Chloroflexi (Fig. 2) is a common microbial feature in AD since the growth of these bacteria requires long HRTs, mesophilic conditions, and neutral pH values (Greses et al., 2017). Protein-degraders, such as WWE1 and Proteobacteria, are also normally present in anaerobic inocula collected from WWTP since this microbiome is adapted to degrade a protein-rich organic matter (sewage sludge) (Llamas et al., 2022). Moreover, the presence of *Methanosaeta* as main genus in Euryarchaeota phylum has been also described as the most common archaea in conventional AD since biogas production at mesophilic conditions is normally performed by these strictly acetoclastic methanogens (Zhang et al., 2023).

The correlation of these microorganisms with conventional AD can be confirmed based on their disappearance when the CSTRs were

**Table 3**
Biodiversity indexes from inoculum and all the AFs at steady state.

| Sample | Observed OTUs | Shannon index |
|---|---|---|
| Inoculum | 1038 | 7.190 |
| HRT 20 d, pH 6.2 | 230 | 3.927 |
| HRT 20 d, pH 5.8 | 197 | 4.001 |
| HRT 30 d, pH 5.8 | 210 | 4.214 |

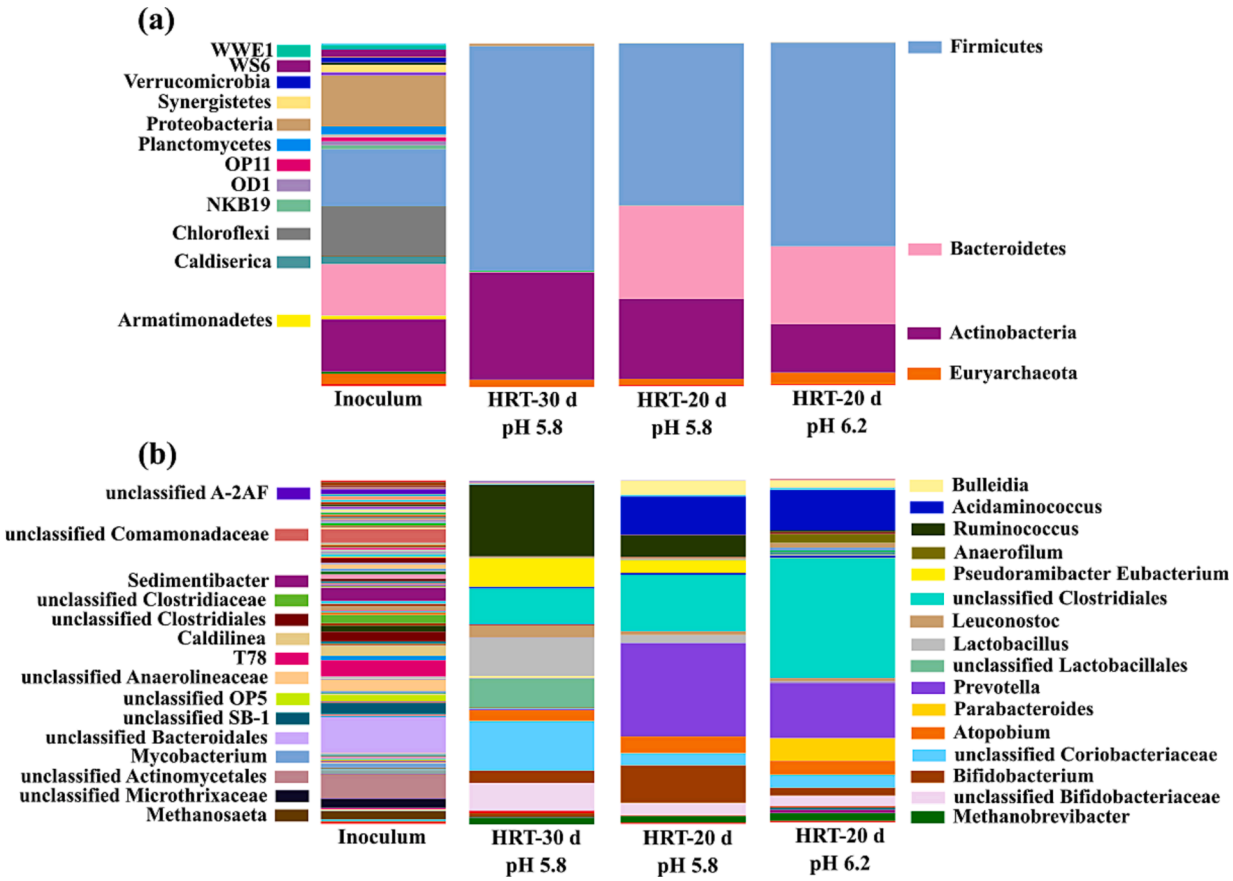


**Fig. 2.** Relative abundance of Bacteria and Archaea at (a) phylum and (b) genus levels (only microorganisms with a relative abundance > 1 % were included in the legend).

subjected to fermentative conditions (pH 5.8–6.2 and 25 °C) and employing a carbohydrate-rich substrate (63.3 % w/w). Fig. 2 shows that Firmicutes became the most abundant phylum in all the CSTRs, accounting for 59.3 %, 47.1 %, and 65.4 % for HRT-20 d and pH-6.2, HRT-20 d and pH-5.8, and HRT-30 d and pH-5.8, respectively. Bacteria belonging to Firmicutes phylum have been determined as key microorganisms in AF since they play a relevant role in the hydrolysis and acidogenesis (Greses et al., 2022a; Llamas et al., 2022). Beyond Firmicutes, Bacteroidetes and Actinobacteria phyla were also identified at HRT of 20 d, while the AF conducted at 30 d of HRT provoked a notable Firmicutes and Actinobacteria overgrowth at the expense of Bacteroidetes decrease. Members of both, Bacteroidetes and Actinobacteria, exhibit cellulolytic activities to degrade complex carbohydrates into HAc and HBu, thereby being active players in hydrolysis and acidification (Llamas et al., 2021). Nevertheless, previous studies showed an Actinobacteria dominance at long HRTs (Greses et al., 2021) and Bacteroidetes enrichment at short ones (Iglesias-Iglesias et al., 2019), justifying Bacteroidetes phylum decrease at an HRT of 30 d. The abundance of these cellulolytic bacteria in the AFs was likely related to the high carbohydrate content of AFWs. Moreover, the cellulolytic metabolisms of those phyla supported the high hydrolysis efficiency attained when the reactors were operated at both HRTs of 20 and 30 d.

Although the phylum composition of 20 d HRT at both pH (5.8 and 6.2) was similar, some changes were observed at genus level. SIMPER analysis revealed only 34.5 % of dissimilarity between pH 6.2 and 5.8 (HRT 20 d), out of which 82.5 % was mainly explained by bacteria belonging to Clostridiales order, *Prevotella*, *Bifidobacterium*, *Parabacteroides*, *Ruminococcus* and *Pseudoramibacter Eubacterium* (Table S1).

Genera identified in Clostridiales order are cellulolytic bacteria involved in sugars fermentation into HAc and HBu (Wang and Yin, 2022). This metabolism was in agreement with the CCA, which evidenced a statistical correlation of Clostridiales with the high production of HBu (Fig. 3). Similarly, *Prevotella*, well-known for its capability to metabolize sugars into HAc, HBu, and $H_2$ (Greses et al., 2021; Swiatkiewicz et al., 2021), was one of the main contributors of the VFAs profile resulting from the CSTRs subjected to pH oscillations. The metabolic similarity between Clostridiales and *Prevotella* could justify the consistent HAc and HBu profile found at both pHs, considering that these microorganisms dominated the bacterial profile variance.

The abundance increase of *Bifidobacterium* at pH 5.8 (HRT 20 d) was also correlated to HAc and HBu (Fig. 3). This bacterium prevails at low pH and it is known for transforming HAc and lactic acid into HBu (Detman et al., 2021). Thus, these bacteria are synergically found with HAc- and HLact-producers such as *Prevotella* and *Atopobium*, respectively (Detman et al., 2021; Gulhane et al., 2017). *Parabacteroides* are saccharolytic bacteria belonging to *Bacteroidetes* and have HAc and succinate as fermentation end products (Zhang et al., 2016). Although these bacteria can grow under a pH range between 5.5 and 8.0 (Benabdelkader et al., 2020; Tan et al., 2012), their optimum condition is close to neutral pH, explaining their presence only in the reactor operated at pH 6.2.

*Ruminococcus* and *Pseudoramibacter Eubacterium* have been also described as HBu producers (Greses et al., 2021). The similar VFAs profile resulting from both studied pHs suggested that these bacteria exhibited a metabolic redundancy, contributing to the high accumulation of HBu at both pHs. This fact confirmed the robustness of the microbiome against pH oscillation in the range of 5.8–6.2, conferring stability to AF to maintain VFAs yield and profile.

Nevertheless, CCA showed a strong correlation between HCa accumulation and *Ruminococcus* and *Pseudoramibacter Eubacterium* genera

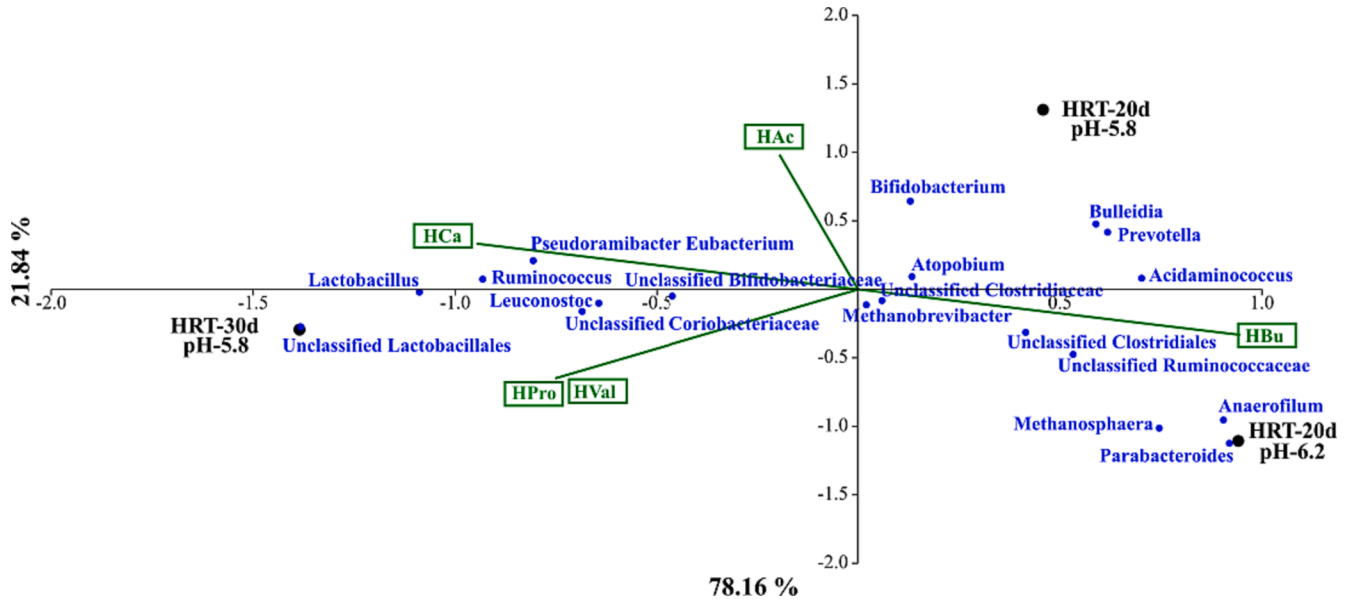


**Fig. 3.** Canonical correspondence analysis ordination triplot performed for the samples retrieved during the steady-state of the AF performed at pH 5.8 and HRT of 20 d (R1), pH 5.8 and HRT of 30 d (R2) and pH 6.2 and HRT of 20 d (R3).

(Fig. 3). *Ruminococcus* enrichment is usually present when reactors are operated at long HRTs (Greses et al., 2021), which could explain the high relative abundance of this genus at HRT of 30 d. Moreover, the relationship with HCa suggested that the carbon chain elongation metabolisms might take place in this CSTR. This metabolism requires the presence of an electron donor (HLact or EtOH) to elongate the carbon chain from HAc or HBu. As it can be seen in Fig. 2, 30 d of HRT also promoted the growth of HLact-producers, such as *Lactobacillus* (11.1 %), other members of Lactobacillales (8.4 %) and *Leuconostoc* (3.5 %), indicating a bacterial synergism. As a matter of fact, the growth of these bacteria (electron-producers and carbon-chain-elongators) at an HRT of 30 d provoked a microbial community dissimilarity of 57.9 % between both studied HRTs (Table 1S). This high dissimilarity agreed with the variety of metabolisms present in the reactor.

The displacement of *Methanosaeta* determined in the inoculum by *Methanobrevibacter* determined in the microbiomes also evidenced that the conditions were not suitable for biogas production purposes but optimum to hamper the activity of common archaea. *Methanobrevibacter* is a strictly hydrogenotrophic archaea less sensitive to harsh conditions, such as low pH or HRT (Bi et al., 2020). Although hydrogen-producers were also identified, *Methanobrevibacter* metabolisms was likely limited by the low process temperature (25 °C). This result confirmed that the temperature is a key factor to limit the metabolic activity of resistant archaea when VFAs were targeted as end-product. The washout out of the sensitive acetoclastic methanogens present in the inoculum by applying harsh operational parameters led to a successful methanogenic inhibition that supported VFAs accumulation.

## 4. Conclusions

The present investigation revealed that the proper selection of operational conditions in AF of AFWs developed a robust microbiome that was able to cope with pH oscillations. The use of long HRTs was critical to enable a high organic matter hydrolysis, being more relevant than conducting AF at higher process temperatures. Moreover, the high stability of AF at long HRTs was confirmed by the similar VFAs production yields and profile attained against pH oscillation (5.8–6.2). The combination of long HRTs with acid pH and 25 °C promoted acidogenesis, resulting in high VFAs accumulation (~30 g VFAs/L) and high bioconversion efficiencies (~50 %) in all CSTRs. The VFAs profile was slightly altered when the HRT was increased from 20 to 30 d. HRT of 20 d promoted a microbiome enriched in bacteria belonging to Clostridiales order, which was correlated to HAc and HBu production with high specificity. By contrast, an HRT-30 d promoted a synergy between *Ruminococcus* bacteria and HLact-producers that boosted HCa accumulation via carbon chain elongation. This study evidenced AF as feasible, robust and efficient technology to valorize AFWs into added-value metabolites with high industrial interest.

## CRediT authorship contribution statement

**M.J. Gonçalves:** Writing – original draft, Investigation, Data curation. **C. González-Fernández:** Conceptualization, Data curation, Writing – review & editing, Supervision, Funding acquisition. **S. Greses:** Writing – review & editing, Supervision, 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2024.01.012.

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