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OPTIMIZATION OF SHORT CHAIN VOLATILE FATTY ACIDS PRODUCTION FROM HOUSEHOLD FOOD WASTE FOR BIOREFINERY APPLICATIONS

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ABSTRACT

Household Food Wastes (HFW) are the most abundant organic wastes at urban level with a worldwide annual production of about 2 billion tons. This material can be the right feedstock for a carboxylate biorefinery platform. This work investigated the influence of different operational parameters (pH, temperature, Organic Loading Rate) on the Volatile Fatty Acids production and on their composition. It was demonstrated that, keeping constant the fermentation duration at 6 days, neutral pH, thermophilic temperature (55°C) and an organic loading rate of 11 gTVS per Liter per day maximized the VFA production with a yield of 0.38 gVFA per gTVS fed. Under these conditions, the main VFAs were butyric and caproic acids with percentages around 60 and 20%, respectively.3. The analysis of the mixed microbial community performed over the reactor operation showed the dominance of

members of *Firmicutes* phylum mainly affiliated to *Clostridium* and *Lactobacillus* genera. The process was mostly driven by *Clostridium* species in mesophilic runs characterized by stable VFAs production and highest yields whereas *Lactobacillus* was enriched under thermophilic operating conditions.

Keywords: Volatile Fatty Acids; Food wastes; Acidogenic fermentation; Microbial community structure; process optimization; Bioeconomy; Biorefinery

1. Introduction

Decarbonization of modern society passes through a change in our economic models (WEF, 2018): bioeconomy, where organic waste, by-products and residues rich in carbon can be used as feedstock to obtain high added value bio-based molecules, will be an important part of this new paradigm (EEA, 2018). These residual streams can be the new oil feeding the chemical sector, now depending on fossil oil and gas (JRC, 2019).

The worldwide production of solid wastes has reached the alarming level of 17 billion tons and it has been estimated to be 27 billion tons within 2050 as consequence of the human population growth (Laurent et al., 2013), with half of the world population living in cities, causing 70% of the total emissions of carbon dioxide (Angeli et al., 2018). With an annual production of approximately 2 billion metric tons on a dry matter basis, household food wastes (HFW) are the most abundant organic wastes (substrates) produced in the urban context (Battista et al., 2020). HFW can be defined as the sorted organic fraction of wastes collected at domestic level. The abundance and presence of these wastes, and the necessity to produce high added value molecules beside biogas and compost (European Commission, 2008) make HFW an optimal candidate as renewable carbon sources for feeding a multi-purpose and multi-product biorefinery (Battista et al., 2020).

In particular, HFW can be adopted as feeding materials for the production of volatile fatty acids (VFAs) within the so called “carboxylate platform” by hydrolysis and fermentation carried out by mixed microbial cultures (MMCs) (Monti et al., 2005). VFAs have several interesting applications, as they are considered biological precursors for different biofuels and high added value compounds, in particular bioplastics (Moretto et al., 2020). VFA are short-chain aliphatic mono-carboxylate compounds with two (acetic acid) to six (caproic acid) carbon atoms, deriving from acidogenic fermentation of organic wastes (Bhatia and Yang, 2017).

HFW include very heterogeneous types of fruit and vegetable, meat, fish and other carbohydrates wastes, which influence the choice of best operating parameters to increase the VFAs production. In particular, the variations of the Hydraulic Retention Time (HRT), the Organic Loading Rate (OLR), the pH and the temperature determine different VFA yields and compositions. In particular, the operational conditions, optimizing the VFA yield, seemed to be: slightly controlled pH (6.0–7.0), short HRT (3-7 days depending on the substrates complexity), thermophilic temperatures and high OLR (Strazzera et al., 2018, Battista et al. 2020). The combination of these parameters gives different VFA yields and requires to be optimized for complex and heterogenous substrates, such as HFW. The VFAs profile is also strongly influenced by HFWs chemical nature. Generally, lipids in HFWs are less prone to fermentation than carbohydrates and proteins because of their low solubility and slower biodegradation kinetics (Alibardi and Cossu, 2016). Furthermore, the hydrolysis of lipids produces glycerol and long chain fatty acids (LCFAs) (Alibardi and Cossu, 2016; Shen et al., 2017). On the contrary, carbohydrates are immediately available for glycolysis and fermentation into VFAs (Moretto et al., 2019; Valentino et al., 2019). In particular, glucose conversion privileges the formation of acetic acid, which is immediately used for the production of H_2 or for microbial growth by microorganisms, resulting in a low final concentration of the mentioned acid. More complex carbohydrates typically lead to a total VFA production where acetic acid, butyric and propionic acids as most abundant VFAs. Proteins are generally characterized by lower kinetics due to their tertiary and quaternary structures. Anyway, also in this case, acetic, propionic and butyric acids are the most

relevant products of protein fermentation, with a dominant presence of acetic acid, which usually accounts for 70% of the total VFAs produced (Strazzera et al., 2018; Yin et al. 2016).

The main aim of this work is the individuation of the best operational parameters to exploit the huge potential of HFW for the creation of a robust continuous process which can maximize the VFAs production by MMC's acidogenic fermentation. In particular, different OLR, pH ranges and mesophilic/thermophilic temperature conditions were tested for the conversion of HFW into VFAs in a lab-scale reactor working in a continuous configuration. At the best of the authors knowledge, the variations of the operational conditions were not previously performed in the same reactor, along an experimental campaign of almost 450 days using the same batch of food waste. This aspect also allowed the investigation of an element which is very important in an industrial full-scale process: the ability of the system to react at eventual working stresses or changes. Finally, the influence of the different operational parameters on microbial community dynamics and VFAs profile was evaluated.

2. Materials and Methods

2.1 Substrates and inoculum characterization

HFW used in this study was appositely prepared to carry out the entire experimental work with the same substrate, thus reducing the external variability of real HFW. The chemical compositions of the different fresh food fractions considered to assemble the synthetic HFW, were taken from the United States Department of Agriculture database (https://www.usda.gov).

In particular, to produce this synthetic HFW, the most common fresh foods in the Mediterranean area and paper materials were mixed, according to the proportions reported in Figure 1, then they were cut and homogenized by means of a professional blender (Cutter Mixer K25 produced by DitoSama). The obtained synthetic HFW was stored at -20±1°C, until its usage.

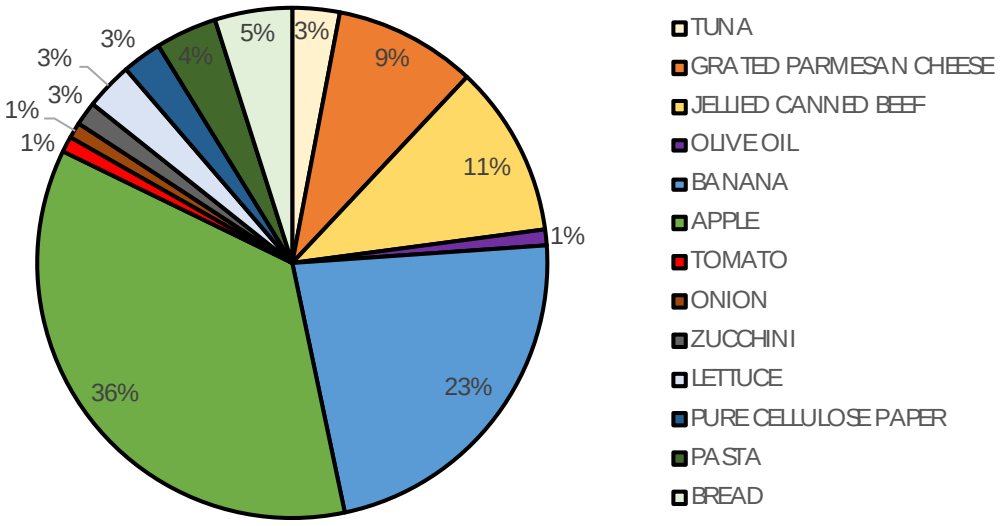


Figure 1. The proportion (% w/w) of the single fresh food and paper materials used to assemble the synthetic HFW.

The synthetic HFW was formulated considering the most typical carbohydrates, lipids, proteins and fibers concentrations' ranges of real HFWs collected in Countries of the Mediterranean Area, considering data from the scientific literature (Garcia et al., 2005; Matsakas et al., 2014; Alibardi and Cossu, 2016). With reference to data reported in Table 1, this material is clearly equivalent to typical real HFW with a solid content around 25% and a COD content of some 290 g/kg. Nitrogen and phosphorus were at 9.9 and 1.4 g/kg dry matter with a C/N value of 29. Proteins, carbohydrates, and lipids were all well represented with average values around 23.9%, 21.1%, and 15.6%, respectively.

Table 1. Physic-chemical characteristics and macromolecular composition of synthetic HFW

Parameter	Value	Parameter	Value (%TS)
pH	5.66 ± 0.18	**Proteins**	53.9 ± 1.0
TS (g/kg)	257.26 ± 13.92	**Carbohydrates**	21.1 ± 0.6
TVS (g/kg)	250.87 ± 13.03	**Lipids**	15.6 ± 1.0
COD* (g/kg)	292.42 ± 47.93	**Fibers**	31.6 ± 0.5
sCOD (g/L)	51.68 ± 8.82	**Cellulose**	10.7 ± 0.6
TKN* (g/kgTS)	9.90 ± 1.95	**Hemicellulose**	10.4 ± 1.0
TP* (g/kgTS)	1.45 ± 0.51	**Lignin**	10.5 ± 1.0
C/N	29	**Sugars**	30.2 ± 1.0

*on dry basis

The inoculum for the fermentation tests was represented by the endogenous microorganisms, already present in the food wastes. Along the fermentation, the most adapted microorganisms to the operational conditions of the different experimental runs survived and grew, influencing the VFA amount and profile.

2.2 The optimization of the VFA production from HFW

The study aimed at optimizing the operational parameters of the fermenter to maximize the VFAs production from acidogenic fermentation of the synthetic HFWs in a semi-continuous mode. The HRT was set up and kept constant at 6 days along the operation, as suggested by the good performances obtained by a previous research work conducted by Moretto et al. (2019), while OLR changed along the experimentation.

The effects of different OLR, pH and temperatures' ranges on the VFAs production were investigated along five experimental runs (Table 2). In particular, during the first run, acidogenic fermentation

was tested at uncontrolled pH, high OLR (22 gTS/Ld) and mesophilic temperature (37°C). Then the pH was set at 5.5 during the second run, keeping constant the OLR at 22gTS/Ld and the temperature at the mesophilic value of 37°C. These conditions did not allow for reaching steady state conditions of the system. Thus, the OLR was reduced at 11 gTS/Ld keeping constant the pH at 5.5 and the operative temperature (37°C), in the third experimental run. Then, the pH was shifted to neutral value in the fourth experimental run. Finally, temperature was increased to thermophilic temperature (T=55°C), while keeping constant the OLR and pH at 11 gTS/Ld and 7, respectively, during the last experimental run. The passage from one experimental run to the next one was conducted after the reaching of steady stable condition in term of VFAs concentration. In particular, the steady state of the reactor was considered reached when the VFAs concentration was stable at least for 3 HRTs, that means at least 18 days. With reference to the passage from run 4 to run 5, it is important to remark that the reactor's feeding was stopped for 45 days. This passage corresponded to the change from a mesophilic temperature (37°C) to a thermophilic one (55°C), which comported a deep change of the microbial communities too. In order to assure the complete acclimatation at the new operational condition to the thermophilic microorganisms without any other external interference, the feeding was interrupted for 45 days.

Table 2. Operational parameters during each experimental run

	T (°C)	**HRT (d)**	**OLR (gTS/Ld)**	**pH**
RUN 1	37 ± 1	6	22	Not controlled
RUN 2	37 ± 1	6	22	5.5
RUN 3	37 ± 1	6	11	5.5
RUN 4	37 ± 1	6	11	7
RUN 5	55 ± 1	6	11	7

Each test was carried out in a hermetically sealed laboratory scale CSTR, with a working volume of 4.2 L and a headspace of 0.8 L for a total volume of 5 L. The reactor was fed daily diluting the synthetic HFW with a proper volume of tap water (for a total amount of the feeding mixture of 700

mL) to reach the designed OLR, specific for each experimental run. pH was monitored twice a day and adjusted manually, according to the specific condition of the experimental run, using a NaOH solution (30% w/v).

At the same time, an equal amount of reaction medium was discharged from the reactor to keep constant the reactor volume and collected for the analysis. In particular, the feeding and discharging operations were performed manually through two different valves at the top and at bottoms of the reactor, respectively. The reactor was continuously stirred at 16.5 rpm, and volumetric biogas production was measured by a Milligascounter (RITTER, Germany). Temperature was controlled by water flowing through an external jacket.

2.3 Analytical methods

Total Solids (TS), Total Volatile Solids, Total Kjeldahl Nitrogen (TKN), Total Phosphorous (TP), Chemical Oxygen Demand (COD) and soluble COD (sCOD) were measured according to Standard Methods (APHA-AWWA-WPCF, 2005). pH was determined using a portable probe (Eutech pH 700). N-NH4+ concentrations were measured by an ion selective electrode (Orion 9512). VFAs concentration was determined by ion chromatography system (Dionex ICS 1100 with AS23 column). Lignocellulosic composition was determined in terms of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and crude fiber (CF) according to Van Soest and Wine (1967). Lipids analysis and crude protein were determined by the standard procedure of the Association of Official Analytical Chemists (AOAC, 1990). Sugars were determined by mass spectrometry (MS), previous sample filtration at 0.20 μm. Lactic acid was measured using a commercial kit (Megazyme, Bray, Ireland). Grams of VFAs and lactic acids are following reported as grams of COD.

2.4 Parameters for the evaluation of the process performances

Two main parameters were used for the evaluation of the performances of the tests. The first is represented by the specific VFAs yield, determined by the ratio between the daily VFAs production rate and the grams of TS fed per day, as follows:

$$Yield_{totVFAs} = \frac{gVFAs/d}{gTS\ fed/d} \quad \text{/Equation 1/}$$

The second parameter is the solubilization rate of the substrates, useful to reveal a potential inhibition of the process. It was calculated as the ratio between the variation of the soluble COD concentration in the reaction medium ($sCOD_t$) and the soluble COD concentration in the feeding mixture ($sCOD_0$), and the particulate COD (pCOD) daily fed, as follows:

$$Solubilization = \frac{sCOD_t - sCOD_{0\ (g/d)}}{pCOD\ (g/d)} \quad \text{/Equation 2/}$$

2.5 Microbial community structure: high-throughput 16 s rRNA gene sequencing

The analysis of the bacterial populations involved in the acidogenic mono-fermentation of HFW was performed by high-throughput 16 s rRNA gene sequencing. Two samples were analyzed for each run, one at the beginning of steady state and one at the end for run 1 (40.90 – 80.87 days), 3 (240.83 and 257.80 days), 4 (289.82 and 324.84 days), and 5 (419.08 and 440.12 days). Due to instability of the system, a sample taken at the first maximum (104.13 day) and at the first minimum (131.07) of total VFAs production were analysed within run 2. Genomic DNA was extracted using about 0.25 g of dry weight of each sample. The extraction was performed with PowerSoil DNA Isolation kit (MoBio, Italy), according to the manufacturer's instructions. DNA was eluted in 50 μL of sterile water and the concentration and purity were determined by NanoDrop (2000c) spectrophotometer (Thermo Scientific, USA). Aliquots were stored at −20 °C for a few days and then used for high-throughput 16 S rRNA gene sequencing. PCR reactions were set up in 25 μL volumes containing 12.5 ng of DNA, 0.5 μM primers V1-3 (27 F AGAGTTTGATCCTGGCTCAG and 534 R ATTACCGCGGCTGCTGG) and 1× Phusion High-Fidelity PCR Master Mix (Thermo Fisher

Scientific, Waltham, MA USA). All PCR reactions were run in duplicate and pooled afterwards. The amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). Library concentration was measured with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA USA). The purified sequencing libraries were mixed in equimolar concentrations and diluted to a final concentration of 4 nM. The samples were paired end sequenced (2x301bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. 10% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples. Sequences were processed using QIIME2 version 2018.2. To denoise paired-end sequences, dereplicate them and filter chimeras with the "consensus" method, DADA2 software package was employed (Callahan et al., 2016) to resolve amplicon sequence variants (ASVs) differing by as little as one nucleotide and Silva 132 database were used to assign the taxonomy (Kehrmann et al., 2017).

3 Results and discussion

3.1.1 Effects of different operational parameters on VFA production

The effects of different operational conditions are clearly reported in Figure 2 and Figure 3 which show the total VFAs production and the VFA profile from HFW fermentation along the different experimental runs, respectively. At the beginning, high OLR (22 gTS/Ld), mesophilic condition, and uncontrolled pH were tested. The high OLR immediately caused a quick accumulation of VFAs in the reaction medium, with the consequent decline of pH, which was not corrected, as scheduled for this experimental run (see Table 2). When the pH declined to very acidic values (3.6 ± 0.2), the microbial activity was inhibited, and the system reached a low performant steady state condition of VFA production rate (3.5 g/Ld). Acetic acid was almost the unique fermentation product (Figure 3) accounting for 90% of total VFAs produced. The predominance of acetic acid when VFA production is low, was already reported by a recent work (Luo et al. 2021). As consequence, a very low VFAs yield of 0.03 gVFA/gTVS fed was achieved along this first experimental run. Low VFA production

yields were also reported in previous studies carried out in similar conditions (Bolzonella et al., 2005; Kong et al., 2016). Although the low VFA yield, this first experimental run showed a lactic acid production of about 9.40 g/Ld. As discussed, high OLR led to the acidification of the systems, inhibiting the VFA production process. The acidification occurs more frequently with food waste, as this substrate is characterized by a high C/N ratio and very fast hydrolysis process (Kong et al., 2016; Bolzonella et al., 2018). Moreover, in line with the results obtained in this experimental run, Kong et al. (2016) detected acetic acid as the main VFA in the reaction medium. At low pH (pH < 4), VFAs are present in the medium under their undissociated form. This condition makes VFA more liposoluble and increases their ability to diffuse in the reaction medium and to penetrate in the bacterial cells. This causes a decrease of the intracellular pH, compromising the cellular activities of the microorganisms and, consequently, the capability of producing VFAs (Palmqvist and Hahn-Hagerdal, 2000). But the lactic acid producing microorganisms have a lower inhibition as effect of the lower pKa of the lactic acid (3.10). It means that at the pH condition of the first experimental run (pH 3.6), lactic acid is not still in the undissociated form and was not able to penetrate across the cellular membrane of the lactic acid producing microorganisms, inhibiting their biological activities (Gottardo et al., 2017). Another research work confirms that low pH has a strong inhibiting effect on the growth of most microbes in acidogenic fermentation, with the only exception of some acidic tolerant ones, such as the ones responsible of the lactic acid production (Luo el al., 2020). The inhibition was also confirmed by the COD conversion into VFA. Even if the solubilization rate was high, with observed values equivalent to 0.30 g soluble COD per gTVS fed to the system per day, only 5% of the influent COD was indeed bio-converted into VFAs.

A first strategy to avoid the acidification of the system, keeping the feeding at high OLR, considered a pH correction. High solids content, in fact, are preferable as they allow to operate with reduced reactor volumes and, consequently, capital and operational costs. Thus, a continuous correction of the pH at slightly acidic value (5.5) was operated, while OLR and temperature were kept constant. Although the test lasted for a long time (more than 100 days), these conditions did not permit to reach

a stable process and VFAs production rate followed a symmetric oscillation of concentration between maximum and minimal valued of 30 g/L and 4 g/L, respectively. This phenomenon was already described by other authors working on anaerobic digestion of food wastes, and it was ascribed to a detrimental effect of substrate overloading on system stability (Nagao et al., 2012; Cheng et al., 2016). Recently, Jia et al. (2019) investigated the hydrogen production from HFW at high OLR and observed that high OLR caused a pH dropping. The authors buffered the system to repristinate the initial pH value, but these continuous pH oscillations had a negative effect on the VFA production without the reaching of a steady state condition, as in the case of this work. Probably, this situation was the combined effect of the high OLR and the pH correction at 5.5: the high OLR led to a very quick VFA accumulation causing the inhibition of the VFA producing microorganisms, as already happened in the run 1. Contrary to run 1, in this second run the pH was corrected at 5.5, allowing to microorganisms to react against the inhibition. It favoured the VFA production's recovery, until the reaching of the VFA inhibition's level again.

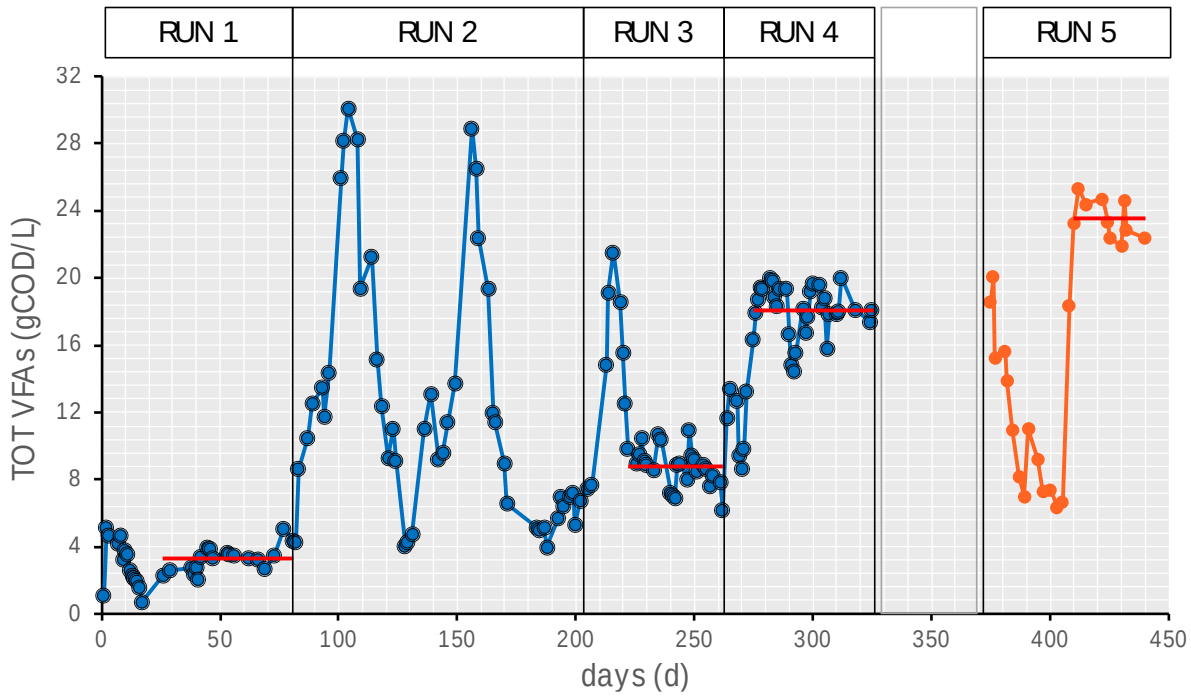


Figure 2. Total VFAs production trend. Blue curve is referred to mesophilic fermentation of HFW; orange curve is referred to thermophilic fermentation of HFW. Red lines show the average

productivity at each steady-state; grey rectangle show the period of time during which the reactor was not fed to avoid washing-out phenomena.

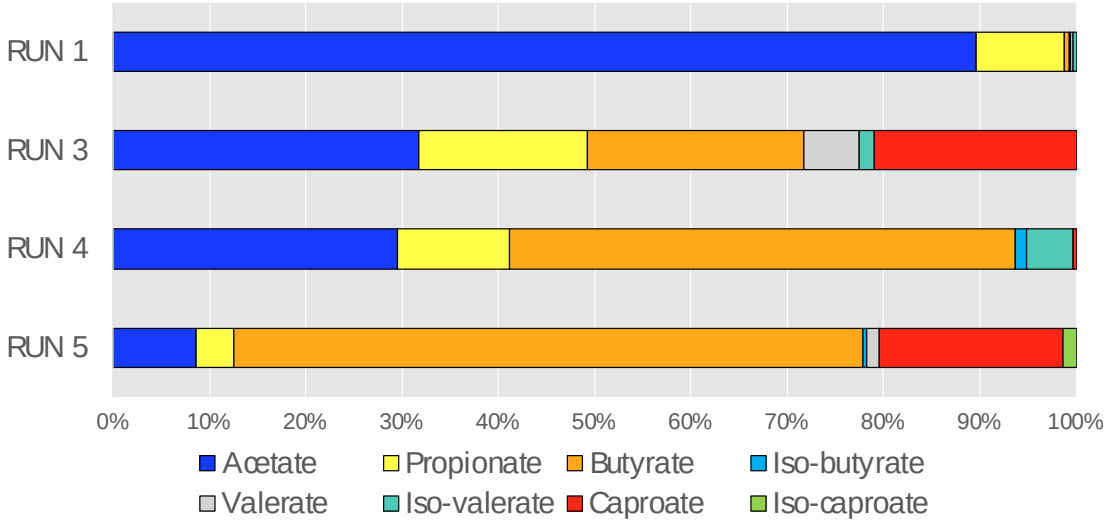


Figure 3. Average distribution pattern among VFAs for steady state at each experimental run. Run 2 is not included, due to the instability of the system.

Considering the controversial performances during Run 2 due to a too high organic matter content in the feed, the OLR was halved, from 22 to 11 gTS/Ld, in the third experimental run (Run 3). pH was kept constant at 5.5. This new OLR condition, allowed the achievement of a steady state condition after about 15 days of operation: a stable VFAs concentration around 8.5 g/L was observed, corresponding to a yield of 0.14 gVFA/gTVS fed. VFAs showed a wider distribution, as shown in Figure 3: acetic acid was still the main product, accounting for 32% of total, but also butyric, caproic, propionic, and valeric acids were produced, accounting for 23%, 21%, 17%, and 6 % of total VFAs, respectively. An average lactic acid concentration around 7 g/L was achieved. The positive effect of lower OLR was also discussed by Jiang et al. (2013), who explained that high substrates' concentration inhibited the microorganisms' metabolism. Moreover Battista et al. (2020) found that high solids concentration reduced also the efficiency of the mass and heat diffusions in the CSTR, causing a loss of the process performances.

During the fourth experimental run, the influence of neutral pH values was also investigated increasing pH from 5.5 to 7. As a consequence, the VFAs concentration increased to stable values

around 18 g/L, after 11 days of operation, corresponding to a yield of 0.29 gVFA/gTVS fed. This value is in line with the best yields reported in literature. A similar yield, 0.27 gVFA/gTS fed, was achieved by Zhang et al. (2016) in tests of acidogenic fermentation of kitchen waste under similar operating conditions. The improvement of acidogenic fermentation performance due to neutral pH condition was due to an increase of the hydrolytic rate. The optimal pH range of the VFA producing microorganisms is around the neutrality, which optimize their kinetics and their metabolisms (Detman et al., 2019). Along the third and fourth experimental runs, the average sCOD concentrations were 30 g/L during run 3 and 50 g/L during run 4, corresponding to solubilization rates of 0.27 and 0.59 g soluble COD per gram VS fed to the system, respectively. Also, the acidification efficiency increased reaching 30% and 38% of sCOD under the third and fourth experimental runs, respectively. Similar results were also obtained by Jiang et al. (2013) who studied the influence of different pH values on VFAs production from synthetic kitchen waste. They found out that a pH value between 6 and 7 brought to an increase of around 20% of the hydrolysis rate, achieving a sCOD concentration of 82 g/L. At lower uncontrolled pH conditions, the sCOD concentration decreased to 60 g/L. The high hydrolysis performances allowed for the increase of the following VFAs production which doubled the concentration. Also, other fermentation products showed increased concentrations compared to the run with uncontrolled pH (run 1).

Regarding the VFA profile, the concentrations of acetate and propionate were similar along the third and fourth phases (Figure 3), while caproate and valerate disappeared during the passage from the third to the fourth experimental run. On the other hand, butyrate became the main component of VFAs with a concentration around 50% of the total VFAs. In addition, a strong decrease of the lactic acid production was observed along the fourth run (concentrations decreased to 1.09 g/L), in line with the results reported by Kim et al. (2009), Gottardo et al. (2017) and Al Dhabi et al. (2020). According to the results reported in these research works, the lactic acid production has optimized for pH values in the range 5.0-5.5. In particular, Al Dhabi et al. (2020), who optimized lactic acid production from

cofermentation of food wastes and municipal sludge, achieved the maximum lactic acid production after 48 h at pH 5.5, while it decreased at higher pH ranges (6.0–7.0).

In the last experimental run, the working temperature was increased to 55°C, while the other parameters remained constant (Table 2). Passing from mesophilic to thermophilic conditions determined the concentration decline of the mesophilic microorganisms, and a consequent growing of the thermophilic microbial communities. To avoid any disturbance to microorganisms during this transient condition, the reactor feeding was stopped for 45 days. A stable total VFAs concentration around 23.5 g/L was achieved after 30 days of operation. Butyric acid was the main product, accounting for around 65% of total VFAs, while caproic, acetic, and propionic acids constituted only the 19%, 9%, and 4% of total VFAs, respectively. Furthermore, a yield of 0.38 gVFA/gTVS fed was achieved. The good performances of the system were probably due to the acidogenic fermentation, rather than a higher hydrolytic activity, since a solubilization rate of 0.63 g sCOD per g VS was achieved during run 5, comparable to the one observed during run 4 (0.59 g sCOD per g VS). A higher conversion of soluble COD into VFAs was also observed: 45% versus 38% observed during run 4. The importance of the temperature choice in the organic matter hydrolysis and, consequently, on its conversion into VFA has been recently confirmed by Soomro et al. (2020), who remarked the positive effect of higher temperatures on the performances of acidogenic fermentation of Organic Fraction of Municipal Solid Wastes (OFMSW). Finally, an increase of lactic acid concentration was observed compared to the previous run: lactic acid passed from 1.09 g/L to 8.37 g/L, along the fourth and fifth experimental run, respectively.

As discussed above, the variations of the operational conditions were conducted along the same experimental campaign to observe the system's kinetics in the reaching of a new steady-state conditions. Except for run 2, when a new steady state was not reached, it was demonstrated that the OLR and pH changes (runs 3 and 4) were followed by a lag phase period in the range of 15-25 days. In a full-scale prospective, this time is economically more advantageous than the discharge of the reactor and the starting of a new process at different operational conditions. The lag phase was longer

(around 40 days, without considering the period when reactor was not fed) after the change of the temperature (from mesophilic to thermophilic conditions). With the temperature shift, as better explained below, was highlighted a switch in the microbial population inhabiting the reactor. This rearrangement was probably the cause of a longer lag phase previously described.

Regarding the biogas production, it increased in correspondence with the run characterized by better VFA yields, reaching an average value of about 1.19 L/Ld along run 5. Hydrogen was the main component, accounting for the 55% *v/v* of the total produced biogas. Methane was absent in the first four experimental runs, appearing just in the last one, even if its concentration was lower than 1% *v/v*. It is a further demonstration the reactor was set at optimal operational conditions for VFA production, without the risk to shift towards methanogenic phase.

3.2 Microbial community structure and dynamics

The main bacterial genera found over the operation are reported in Figure 4. As expected, the lowest microbial diversity was observed in Run 1 where selective operating conditions were adopted (high OLR and not controlled pH). Indeed, despite the complex composition of the food waste, only lactobacilli populations were enriched during run 1 (up to 99% of total reads were affiliated to *Lactobacillus* genus). Remarkably, *Lactobacillum gasseri* (ASV1 in Table 1 Supplementary information), an obligate homofermentative bacterium typically retrieved in the gastrointestinal tracts of humans and animals, was found highly abundant in both samples taken during stable VFAs production in Run 1 (82.1% and 42.2% in 1A and 1B samples respectively). This finding is in line with the average yield of 0.07g of lactic acid per gram of TS fed obtained during run 1, twice the value of VFAs concentration. The higher lactic acid yield may be explained by the known acid tolerance of lactobacilli that are also specialized lactic acid producers (Corcoran et al., 2005). Nonetheless, a certain production of VFAs was ensured by heterofermentative *Lactobacillus* spp. in the reactor such as *L. pontis, L. vaginalis, L. buchneri, and L. farraginis* (Filya et al., 2003; Endo and

364 Okada, 2007; Ibrahim, 2016) that represented 5.1% and 44% of total reads in sample 1A and 1B,
365 respectively.
366 The analysis of the sample "2 max", taken at the first maximum of total VFA production (day 104)
367 and "sample 2 min", taken at the first minimum of total VFA production (day 131) showed the
368 dominance of *Lactobacillus* species. In the first case, most of the sequences were attributed to the
369 genera *Lactobacillus* (68.7%) and *Caproiciproducens* (29.5%) whereas sample "2 min" was almost
370 entirely composed by *Lactobacillus* species. Sample 2 max was mainly composed by the
371 heterofermentative *L. rhamnosus* with the dominance of *L. rhamnosus* ASV4 (32.5% of total reads)
372 whereas *Lactobacillus* ASV3 was highly abundant (84.1%) in sample 2min (Table S1 supplementary
373 information).
374 *Caproiciproducens* ASV2 was found only in sample "2 max" (Table S1 supplementary information).
375 Members of the genus *Caproiciproducens* are strictly anaerobic bacteria able to produce caproic acid
376 along with acetic and butyric acids (Kim et al., 2015; Bengelsdorf et al., 2019). Some
377 *Caproiciproducens* species are capable of making a lactate-based chain elongation. The pyruvate
378 produced during lactate oxidation is further oxidized to acetyl-CoA and CO_2 with electrons released
379 in form of reduced ferredoxin. The derived acetyl-CoA is used for butyrate and caproate formation
380 through a reverse β-oxidation pathway (Spirito et al., 2014).
381

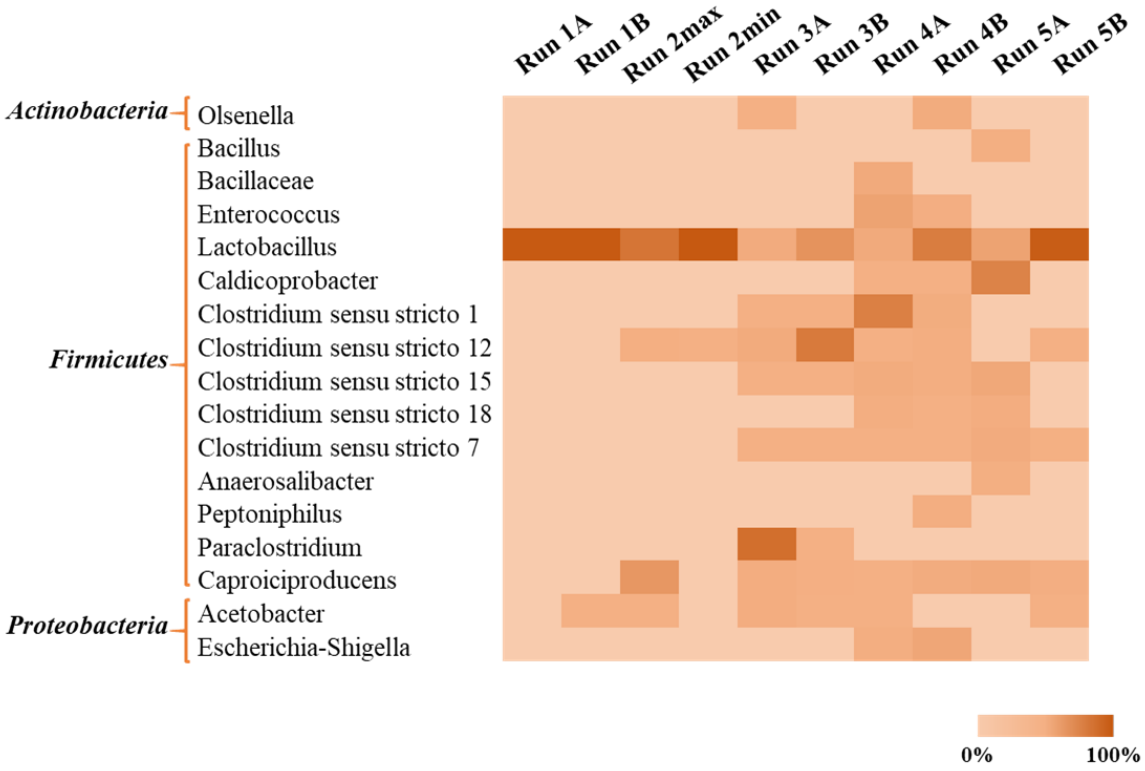
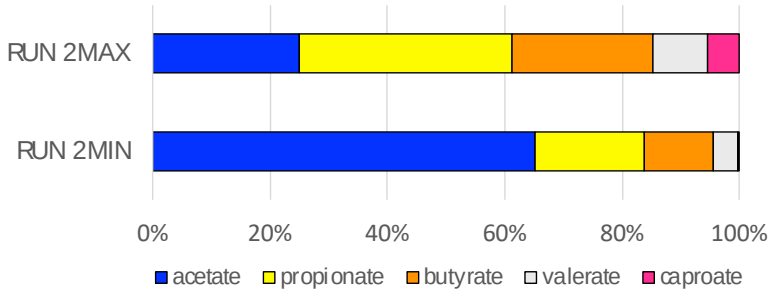


Figure 4. Frequency heat-map of microbial communities at taxonomical genera levels (only taxonomy group ≥ 2% of abundance in at least one sample are shown). The colour intensity in each cell shows the relative abundance.

This pathway allows the conversion of the available acetate (C2) to butyrate (C4) by the condensation of two acetyl-CoA, and, if other molecules of acetyl-CoA are available, further elongation to caproic acid (C6) (Kucek et al., 2016). Figure 5 shows the punctual distribution among VFAs produced at time of sampling during run 2. As it can be noticed, when *Caproiciproducens* ASV2 was present in the reactor along with *Lactobacillus rhamnosus* ASV4 during the first maximum of total VFA production, a greater amount of butyric and caproic acids and a lower amount of acetic acid were produced, probably as consequence of a chain elongation process mediated by *Caproiciproducens*, using lactic acid produced by lactobacilli as electrons donor (de Leeuw et al., 2019).

In line with this finding, at the first minimum production of total VFA, apart from the extremely lower VFAs production rate, acetic acid was the main fermentation product mirrored by the establishment of lactic acid bacteria and the decline of *Caproiciproducens*. The correlation among lactobacilli and *Caproiciproducens* spp. was observed by Contreras-Dávila et al. (2020) during an

399 experiment on caproic acid production by reverse β-oxidation pathway using food waste as feedstock.
400 They observed that the process took place by two separate phases: an acidification phase towards
401 lactate as main product, followed by a chain elongation phase. The oscillation between these two
402 phases required an increase of OLR, as in the case of the second run.
403



404

405 Figure 5. Pattern of distribution at time of sampling during the maximum and minimum of total VFAs
406 production
407
408

409 During the operation at lower OLR (Run 3. 4 and 5), the production of more heterogeneous VFAs
410 mixtures is mirrored by the higher microbial diversity associated to known fermentative bacterial
411 genera (Figure. 4). The analysis of Run 3 revealed the establishment of a variety of putative
412 fermenters mostly belonging to *Paraclostridium*, *Clostridium sensu stricto* 12, *Lactobacillus* and
413 *Caproiciproducens*. *Paraclostridium* ASV5 (38% in sample 3A), *Clostridium sensu stricto 12* ASV6
414 (55.5% in sample 3B) and *Lactobacillus* ASV3 (Table S1) represented the main taxa even though
415 marked fluctuations of their relative abundance were observed over the operation. Nearly all members
416 of the taxon *Clostridium sensu stricto* form butyrate as main fermentation product, but also other
417 compounds, including various organic acids and alcohols, are produced. Members of *Clostridium*
418 *sensu stricto* have a wide physiological versatility and this may explain the greater distribution among
419 VFAs achieved during this experimental run. Consistently with the occurrence of lactobacilli, the
420 lactic acid production rate reached a stable value of 7.43 g/L.

421 In Run 4, the increase of pH toward neutrality led to an initial enrichment of bacteria belonging to
422 the class *Clostridia*. Consistent with the VFA distribution pattern observed in this run (Figure 3),

Clostridium butyricum ASV7, known butyric acid producing microorganism (Wiegel, 2015), dominated in sample 5A (46.8%; Table S1) together with *Lactobacillus* ASV3 found at lower abundance (6.4%). At the time of sampling, the lactic acid production rate was about 1.79 g/Ld, one of the lowest measured during the whole experimental campaign. This low production can be partially explained either by the relative low abundance of *Lactobacillus* spp., and, likely by a metabolic cross-feeding with lactate-utilizing, butyrate-producing bacteria. It is known that some bacteria, such as *C. butyricum*, are able to convert lactate and acetate to butyrate (Detman et al., 2019).

Lactobacillus ASV3 and ASV8 were the main taxa retrieved in sample 4B (31.7% and 16.5% respectively). This result can explain the heterogeneous distribution of fermentation products obtained within this run, which was however characterized by butyric acid as main fermentation product. Other conversion pathways of lactic acid to other products, such as caproic acid, most likely took place in the system, as revealed by the occurrence of *Caproiciproduces* spp., and this may explain the observed low lactate production rate.

Members of *Caldicoprobacter* genus represented one of the main microbial components under thermophilic operating conditions (52.4% in sample 5A). The peculiarity of species belonging to this genus is the capability of growing on xylan, and degrading hemicellulose (Yokoyama et al., 2020). *Lactobacillus* species were also enriched during operation (up to 95.8%) in line the observed increase of lactic acid concentration compared to previous mesophilic run.

4. Conclusions

The optimization of the VFA production from HFW in continuous mode was investigated in the present research work. It was demonstrated that different pH, temperature and OLR values influenced the VFA productivity and VFA profile. The conditions which allowed the best VFA yield were: OLR around 11 gVS/Ld, pH above 7 and thermophilic temperature. Under these conditions, the VFA yield was of 0.38 gVFA/gTVS, corresponding to a solubilization rate of 0.63 sCOD/gTVS. The VFA productivity of almost 0.40 gVFA/gTS is one of the highest present in the scientific literature. A very

recent work (Jon Jones et al., 2021) achieved a similar result of 0.35gVFA/gVS, but after a combination of filtration and electrodialysis on food wastes.

In view of a practical application of the process, it is fundamental to remark that, after the optimization of the VFA production, the next challenge is their recovery and purification, that means the finding of economically and efficient techniques for their separation from the fermentation broth and then, for their concentration till 70-100 g/L.

Moreover, other interesting observations for the economy of the process can be done from the discussion of the lag phase, which is around of 15-25 days when OLR and pH were gradually modified, while the passage from mesophilic to thermophilic temperature required longer time. These considerations must be considered in an eventual scale-up of the process.

It was also demonstrated that different operational parameters influence the VFA profile and the microbial community structure. Regarding the VFA composition, acetic acid was the main product when the process was not optimized and the VFA productivity was low. Instead, under the optimal conditions, butyric acid was the predominant VFA (more than the 60% of the total acids) followed by a significant amount (almost the 20%) by caproic acid. It demonstrated that the system can be adapted if the production of specific VFA is desired through the variation of the operational conditions.

As for microbiological aspects, the runs with the best performances during HFW mesophilic treatment showed the enrichment of bacteria belonging to the class *Clostridia*, while *Lactobacillus* species dominated under acidic conditions or when the process was performed at high temperature.

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