



Influence of different household Food Wastes Fractions on Volatile Fatty Acids production by anaerobic fermentation

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HIGHLIGHTS

- The influence of different Household Food Wastes fractions on VFA was investigated.
- Fractions rich in proteins and starch led to the highest VFA yield (12–15 g/L).
- Fractions rich in cellulose, fibers, and sugars showed low VFA production (<2 g/L).
- Metagenomic analysis of the different HFW fractions on VFAs production was conducted.
- The bacterial community compositions and VFAs profile was correlated.

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ABSTRACT

This research investigated for the first time the influence of the single fractions (proteins, lipids, starch, cellulose, fibers and sugars) composing Household Food Wastes on Volatile Fatty Acids (VFA). A production at different pH (uncontrolled, 5.5 and 7.0): both the amount and profile of VFA were investigated. It was found that fractions rich in proteins and starch led to the greatest VFA productions (12–15 g/L), especially at neutral pH condition. On the contrary, fractions rich in cellulose, fibers, and sugars showed a very low VFA production (<2 g/L). The chemical nature of HFW influenced the speciation of the microbial communities too. Lactobacillaceae family was highly represented in proteins-, starch-, fibers and sugars-rich substrates and Atopobiaceae, Eggerthellaceae, Acidaminococcaceae and Veillonellaceae displayed positive correlation to VFAs production. Instead, Comamonadaceae showed high relative abundance in lipids- and cellulose-rich fraction and was negatively correlated to the VFAs generation.

1. Introduction

A recent report from Food and Agriculture Organization (FAO) estimated a global food wastage around 1.3 billion tonnes per year (FAO, 2019). In the European Union, 88 million tonnes of food waste are generated annually; more than half of them (47 million tonnes) derives from household, food service and retail (European Commission, 2020). Food wastage is not only an ethically wrong practice but is also correlated to the deployment of resources. Consequently, the conversion of food waste into biofuels and biomaterials is becoming one of the main priorities of the new environmental policies promoted by the European Commission (Battista et al., 2020). In the last decades, food waste has been widely used for the mere biogas production by Anaerobic Digestion

(AD). In fact, food waste is considered a good substrate for AD, owing to higher methane production potential (434.0–593.6 L/kg volatile solid (VS)) than other organic wastes such as sewage sludge, lignocellulosic waste and livestock manure (Pramanik et al., 2019). Besides the biogas production, the scientific community started to be interested in the acidogenic fermentation of food waste for the production of Volatile Fatty Acids (VFAs), the so called carboxylate platform (Strazzera et al., 2018). VFA are very useful precursors in the chemical industry for the synthesis of esters, ketones, aldehydes, alcohols and alkane. Moreover, they are also adopted as substrates for polyhydroxyalkanoates (PHAs) production in pure and microbial mixed culture systems. In this context, the optimization of the operational parameters for the VFAs production from food waste is fundamental to maximize the process yields. Previous

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studies showed that a controlled pH in the neutral range (6.0–7.0), short HRT (lower than 10 days), thermophilic temperatures and an organic loading rate of about 10 kgVS/m³d, allow a high VFAs concentration between 10 and 30 gCOD/L (Strazzera et al., 2018; Fernández-Domínguez et al., 2020). But food wastes' composition has also a strong effect on the amount, and the quality of the produced VFAs.

According to previous researches, lipids, carbohydrates and proteins give different results in terms of fermentative products. Generally, lipids in food waste are less suitable for fermentative metabolism than carbohydrates and proteins as they present lower biodegradation kinetics (Alibardi and Cossu, 2016). Furthermore, hydrolysis of lipids produces glycerol and long-chain fatty acids (LCFAs). Although glycerol can be used as a fermentation substrate, LCFAs are able to adhere to cellular wall, affecting the transport of nutrients, and, consequentially, inhibiting the metabolism of anaerobic bacteria producing VFA (Alibardi & Cossu, 2016; Shen et al., 2017). On the contrary, carbohydrates are easily converted by microorganisms into glucose, which is immediately used for fermentation into VFAs through the glycolysis pathway (Shen et al., 2017). Yin et al. (2016) investigated the pure glucose fermentation in batch reactor, at mesophilic range, under pH 6, using granular activated sludge as inoculum. They obtained a maximal VFAs production of 38.2 gCOD/L, with butyric, propionic, acetic, and valeric acid accounting for 50, 30, 17, and 3% of the total VFAs amount, respectively. Another study was conducted at mesophilic temperature and uncontrolled pH fermentation using starch coming from fresh potatoes in a packed-bed reactor. The total VFAs production was of around 18 g/L, with acetate, butyrate and propionate representing about the 45, 28, and 14% of total, respectively. The same authors observed that, doubling the loading rate, VFAs production remained constant (17 g/L), but VFA profile changed with acetate representing about 70% of the total VFAs (Parawira et al., 2004). Alibardi and Cossu (2016) found the fermentation of fresh food rich in carbohydrates, was achieved at a concentration of butyric acid close to that of acetic acid, with a butyrate/acetate ratio of around 0.8.

Proteins are generally characterized by a lower biodegradability with respect to carbohydrates, due to their tertiary and quaternary structure, which make them less susceptible to degradation action (Battista and Bolzonella, 2018). For this reason, the hydrolysis of food waste rich in proteins is considered as a rate-limiting step during acidogenic fermentation (Shen et al., 2017; Yin et al., 2016). Also the VFA profile observed when fermenting food waste rich in proteins is particular. Acetic acid is the main VFA obtained from fermentation of peptone, as reported by Yin et al. (2016), accounting for the 70% of total VFAs produced, while butyrate and propionate representing around the 10 and 15%. Moreover, VFAs production seems also to be affected by origin of proteins. It was demonstrated that fermentation of animal or vegetal proteins lead to a different VFAs profiling. Vegetal proteins favored a not equal distribution of the acetic, propionic, butyric, and valeric acids (56.3:15.7:10.4:17.6), while their concentrations are similar when animal proteins' are fermented (Young and Pellett, 1994).

In this scenario, the aim of this work is the methodic investigation of the effect of the chemical nature of the main carbon fractions contained in food waste on the quantity and quality of VFAs production. Moreover, the corresponding microbial communities were studied. At the best of authors knowledge, this goal was pursued for the first time through the separated acidogenic fermentation of the different chemical fractions into the synthetic HFW. To limit the great variability of food waste, a synthetic Household Food Waste was formulated to simulate a real HFW typical of Mediterranean Countries. Then, all the ingredients, composing the synthetic HFW, were classified into five organic fractions according to the main carbon chemical nature: lipids, proteins, cellulose, starch, fibers and sugars which were separately fermented in different batch tests. The tests were conducted at different pH (uncontrolled, 5.5, and 7) and at mesophilic conditions (37°C), to determine their effect on VFA production and on the microbial communities' profile.

2. Material and methods

2.1. The synthetic HFW and its separation in five main fractions

HFW is a very heterogeneous waste whose composition depends from the municipality, the period of the year in which it is collected and the typical diet of the region. To simulate the chemical and physical characteristics of the real substrate, a standardized synthetic HFW was defined. In particular, the synthetic HFW was formulated considering the most typical carbohydrates, lipids, proteins, sugars, fibers and cellulose concentrations in the real HFWs collected in Mediterranean Area (Garcia et al., 2005; Matsakas et al., 2014; Alibardi and Cossu, 2016). The most common fresh foods in the Mediterranean area and paper materials were mixed, according to the proportions reported in Fig. 1, then they were cut and homogenized through a professional blender. The so obtained synthetic HFW was stored at $-20 \pm 1^\circ\text{C}$, until its usage.

Table 1 summarizes the physical-chemical characterization of the synthetic HFW and the TS concentration of the different carbon fractions included in the synthetic HFW.

The synthetic HFW was divided into five fractions, on the basis of its theoretical content in terms of carbohydrates, proteins, and lipids, starch, fibres and cellulose (USDA, 2016). The following fractions were created:

- *proteins-rich* ingredients: parmesan cheese, tuna in brine, and canned beef meat;
- *lipids-rich* ingredients: olive oil;
- *starch-rich* ingredients: bread and pasta;
- *cellulose-rich* ingredients: pure cellulose paper;
- *fibers and sugars-rich* ingredients: fruits and vegetables.

These fractions were fermented separately, according to the operative conditions illustrated in the following paragraph. The complete HFW mixture was also tested to simulate the acidogenic fermentation of a real HFW.

2.2. Description of the batch tests

The substrates of the five fractions from the synthetic HFW were finely milled and were fermented in 1 L batch reactors, with a working volume of 500 mL. As inoculum was employed an agricultural digestate from a full-scale anaerobic digester in Verona (Italy). The physical chemical characterization of the different fraction of the synthetic HFW and of the inoculum has been reported in Table 2.

A substrate: inoculum ratio (expressed as COD) of 7 was used for each test. In particular, 31.0 g proteins-rich; 2.8 g lipids-rich; 44.0 g sugars and fiber-rich; 7.8 g cellulose-rich; 13.0 g starch-rich; 17.9 g of whole HFW mixture were fed to the batch tests. The amount of the inoculum was constant for all the tests, at 12 g. Then, the final volume was adjusted to 500 mL with tap water. To verify the pH influence on the batch reactors, three different options (uncontrolled, 5.5 and 7) were chosen. The complete synthetic HFW was also fermented, following the same procedures, in order to evaluate the influence of the different fractions on the complete HFW. A blank test (control reactor) was also prepared filling the reactor of inoculum and water in the same amounts of the previous tests, to evaluate the endogenous VFA production. All the tests were located in thermostatic chamber, setting the working temperature to $37 \pm 2^\circ\text{C}$.

Biogas production was also monitored by a TG1PP gas meter (RITTER, Germany), while its composition was analysed by a portable biogas analyzer, the BIOGAS5000 (Geotech, United Kingdom).

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

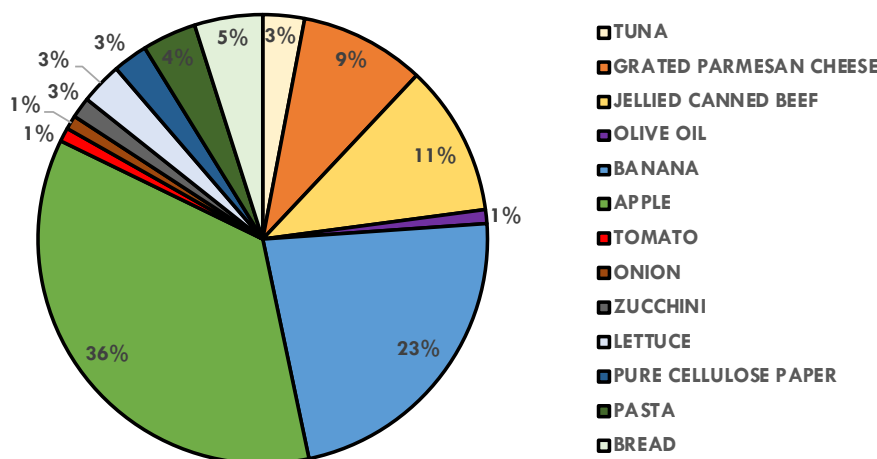


Fig. 1. The proportion (% w/w) of the single fresh food and paper materials used to assemble the synthetic HFW.

Table 1

Physical-chemical characteristics and macromolecular composition of synthetic HFW.

| Parameter | Value | Parameter | TS (%w/w) |
|-------------|----------------|---------------|------------|
| pH | 5.66 ± 0.18 | Proteins | 23.9 ± 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/kg) | 9.90 ± 1.95 | Hemicellulose | 10.4 ± 1.0 |
| TP* (g/kg) | 1.45 ± 0.51 | Lignin | 10.5 ± 1.0 |
| C/N | 29 | Sugars | 30.2 ± 1.0 |

* On dry basis.

Table 2

Chemical-physical characteristics of substrates and inoculum.

| Fraction | TS (g/kg) | TVS (g/kg) | COD (g/kg) | TKN (g/kg) |
|------------------|----------------|----------------|-----------------|--------------|
| proteins | 417.86 ± 27.18 | 348.35 ± 0.67 | 242.65 ± 11.34 | 31.08 ± 2.78 |
| starch | 634.93 ± 26.02 | 555.62 ± 0.78 | 581.83 ± 24.56 | 10.49 ± 2.10 |
| sugars and fiber | 134.03 ± 1.25 | 123.10 ± 0.54 | 167.29 ± 27.78 | 1.32 ± 0.08 |
| cellulose | 941.25 ± 1.76 | 938.87 ± 1.52 | 962.30 ± 19.11 | 1.69 ± 0.15 |
| lipids | n.d.* | n.d.* | 2678.59 ± 84.76 | 2.86 ± 0.58 |
| HFW | 290.51 ± 19.50 | 253.78 ± 15.12 | 352.72 ± 8.86 | 9.90 ± 0.32 |
| inoculum | 48.58 ± 3.55 | 43.23 ± 2.77 | 75.10 ± 12.45 | 3.14 ± 0.44 |

* Not determined.

(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, acid detergent fiber, acid detergent lignin, and crude fiber according to Van Soest and Wine (1967). Lipids and crude protein analysis were determined by the standard procedure of the Association of Official Analytical Chemists, (1990). Sugars were determined by mass spectrometry, before the 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. Finally, biogas composition was analyzed by a portable biogas analyzer (BIOGAS 5000 Geotech, UK).

2.4. VFA evaluation performances

VFA performances of the batch tests were expressed as acidogenic conversion or VFA yield. This latter is expressed as the total VFA production (COD equivalents) referred to the COD amount, fed at the beginning of the tests (Cerdan et al., 2021).

$$VFA \text{ yield } (\%, w/w) = 100 * (gCOD_{VFA} / gCOD_{fed}) \quad (1)$$

2.5. Microbial community structure: High-throughput 16S rRNA gene sequencing

The characterization of the bacterial communities was performed on samples collected at the end of each run by means of a high-throughput 16S rRNA gene sequencing analysis. Total DNA was obtained from 0.25 g of dry weight with the FastDNATM SPIN Kit for Soil (MP Biomedicals) as described by Andreoli et al. (2016), sampled from each run. DNA concentration and purity were determined by NanoDrop (2000c) spectrophotometer (Thermo Scientific, USA). Aliquots were stored at -20°C for a few days and then sent for high-throughput 16S rRNA gene sequencing to Eurofins Genomics (Germany). V1-3 variable regions were used as target for procedure to bacterial 16S rRNA amplicon sequencing. Ten ng of extracted DNA was used as template and the PCR reaction (25 µL) contained dNTPs (400 nM of each), MgSO₄ (1.5 mM), Platinum® Taq DNA polymerase HF (2 mU), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and barcoded library adaptors (400 nM) containing V1-3 specific primers: 27 F AGAGTTT-GATCCTGGCTCAG and 534 R ATTACCGCGGCTGCTGG. 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 Quant-iT™ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a TapeStation 2200, using D1K ScreenTapes (Agilent, USA).

The purified sequencing libraries were mixed in equimolar concentrations and diluted to a final concentration of 4 nM. The samples were paired and sequenced (2 × 301 bp) 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. 20% Phix control library was spiked in to overcome low complexity issue often observed with amplicon samples. Bacteria V1-3 forward reads were trimmed for quality using Trimmomatic v. 0.32, with the settings SLIDINGWINDOW:5:3 and MINLEN:275 and clipped to a length of 275 bp. The cut down forward reads were dereplicated and formatted for use in the UPARSE workflow. The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090

-usearch_global command with -id 0.97. Taxonomy was assigned using the RDP classifier according to the sequence similarity with a 97% identity threshold as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME using the SILVA database.

PAST software (version 4.03) was adopted to measure: (i) the Shannon–Wiener and Simpson bacterial diversity indices, (ii) clustering analysis based on Neighbour-Joining algorithm and (iii) heatmap

representation of Pearson's correlation matrix (P-value cut-off of 0.05) (Hammer et al., 2001).

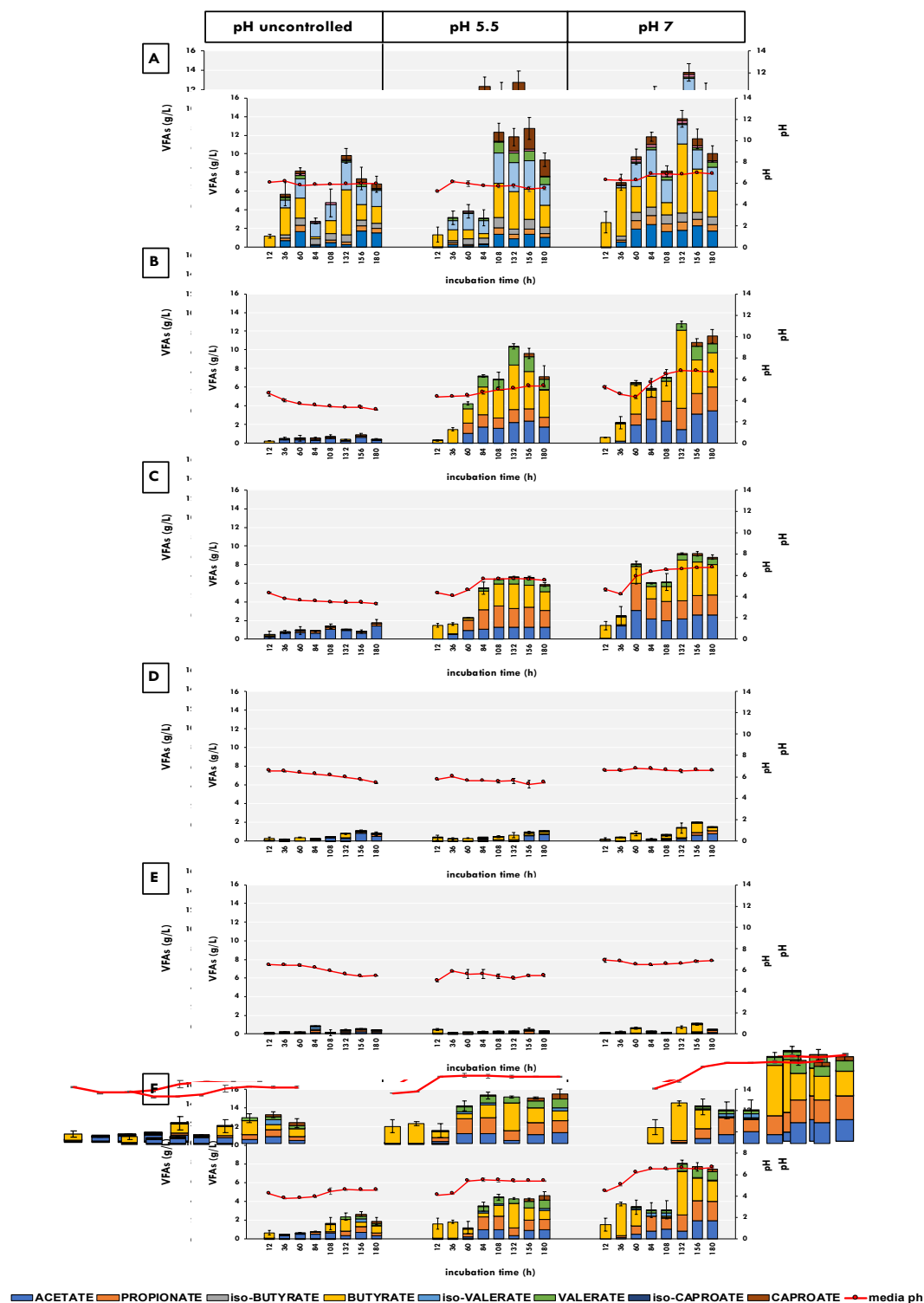


Fig. 2. Fermentation performances from each substrate. a) protein-rich; b) starch-rich; c) fibers and sugars; d) cellulose-rich; e) lipid-rich fraction; f) HFW mix. Red curves are referred to the average pH value in the reactors at time of sampling. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussions

3.1. Effect of C-source chemical nature (food waste fraction) on VFAs production

Fig. 2 shows the fermentation performances achieved for each HFW fraction test in terms of VFAs total amount and profile. The pH values of the tests are reported too.

The total concentration of VFAs obtained from protein-rich fraction (Fig. 2A) shows that the highest production was achieved when pH was adjusted to 7 with a total VFA concentration of 13.77 ± 0.89 gCOD/L after 130 h of incubation. The performances were slightly lower when the reactor was set at a pH of 5.5 and uncontrolled pH with a VFA production of 12 and 10 gCOD/L after 130 h, respectively. This is a typical result and it is generally associated with the capability of protonated VFA (low pH) to pass the membrane cell of bacteria then determining a cytoplasmatic unbalance (Strazzera et al., 2018). It was noted that pH setting influenced also the VFA profile: a pH of 7 led butyrate to be the main fermentation product (53% of total VFAs), followed by *iso*-valeric, acetic, *iso*-butyric, propionic, *iso*-caproic, caproic, and valeric acids accounted for 14.97, 13.17, 6.95, 6.51, 2.03, 1.35, 1.31% of total VFAs, respectively. The butyrate concentration decreased at 25.61% when pH was set at 5.5 in favor of caproate, *iso*-valerate, and valerate which represented the 17.10, 23.74, and 8.57% of total VFAs.

These results are consistent with previous research works. Liu et al. (2012) observed a higher valeric acid production from proteinaceous substrate at slight acid pH than at neutral one. It can be explained considering that proteins-rich fraction was the only substrate capable to lead a production of branched VFAs, in particular *iso*-valeric acid. In fact, branched-chain amino acids, such as valine and leucine, present in high concentration in protein substrates, are converted to the branched-chain VFAs (*iso*-butyric and *iso*-valeric acid) through oxidative deamination (Apajalahti et al., 2019).

It is also important to emphasize that tests carried out using substrates rich in proteins generally show pH at a constant level of 5.5, even when pH is not controlled. This phenomenon is due to the abundance of nitrogen compounds derived from their degradation.

Regarding the tests on fractions rich in starch material, the best fermentation performance was achieved when the pH was set at neutrality, with a maximum VFAs production of 12.77 gCOD/L, after 130 h of fermentation. The butyric acid was the main fermentation product, representing 65.85% of total VFAs, while propionic, acetic, and valeric acid accounted for 11.77, 17.37, 5.01% of total VFAs, respectively. Similarly to the previous case, the VFAs productivity decreased for pH 5.5 (10.38 gCOD/L) after 130 h. Butyric acid remained the main product but its concentration was lower (45.48% of total VFAs) with a consequent increasing of the acetate and valerate concentrations, representing the 21.53 and 18.57% of total VFAs, respectively. Instead, the percentage of propionic acid remained almost the same. Finally, when the tests were conducted at uncontrolled condition, pH quickly dropped at an acidic value (pH 3.14), negatively affecting the VFAs production which was only around 1 gCOD/L. These results emphasize the key role of pH on acidogenic fermentation. When a strong acidification took place, VFAs are under undissociated form (H-Acid), becoming more liposoluble. At this form, VFA can diffuse across the plasma membrane. Once they are in the cytosol, dissociation occurs, due to the neutral intracellular pH, causing the decrease of the cytosolic pH, with a subsequent growth-inhibiting effect on microorganisms (Palmqvist and Hahn-Hägerdal, 2000). In particular, Awasthi et al. (2018) observed that the activity of α -amylase, the main hydrolytic enzyme for starch solubilization, is strongly affected by pH. They studied the degradation process under a pH range from 4 to 10, observing that a neutral pH had a favorable effect both on proliferation of microbial strains producers of α -amylase and on the hydrolytic activity of the enzyme itself. On the contrary, a gradual adjustment of pH both toward acid and alkaline conditions had a negative effect on α -amylase production.

Tests on food waste fractions characterized by high levels of sugars and fibers (Fig. 2C) had a very similar behavior of starch rich fractions: when it was not controlled, pH quickly dropped down to a value of about 3.50. Even in this case, the acidification of medium negatively affected VFAs production, with the highest value of 1.74 ± 0.34 g/L at the end of fermentation. Acetic acid as main fermentation product, accounting for more than 80% of total VFAs. An increase of pH toward 5.5 allowed to increase VFAs production until a value of 6.51 ± 0.14 gCOD/L, after 108 h of incubation, remaining more or less constant for the remaining fermentation time. Butyric acid was the main fermentation product, accounting for 39.82% of total VFAs, while propionic, acetic, and valeric acid represented the 30.56, 18.97, and 10.57% of total VFAs produced, respectively. An adjustment of pH toward neutrality allowed to increase total VFAs production by 30%, reaching a maximum production of about 9 gCOD/L after 132 h of incubation. Regarding the VFAs profile, butyric, acetic, propionic and valeric acid accounted for 47.50, 23.81, 21.36, and 6.32% of total VFAs produced, respectively. This distribution is in line with typical distributions among fermentation products achieved by acidogenic fermentation process carried out by Microbial Mixed Cultures from simple sugars, such as glucose (Yin et al., 2016).

Fig. 2D and 2E describe the VFAs productions from cellulose-rich and lipids-rich fractions' tests. In both cases a very low VFAs production was observed under every pH value tested. In particular, along cellulose-rich fraction hydrolysis was probably the limiting step of the dark fermentation. Instead, the low yields from lipids-rich fraction's tests, can be explained by the high presence of LCFA, known to damage to cell membranes, to reduce nutrients transport and decrease cell permeability with a consequent fermentation inhibition. The absence of acidogenic fermentation processes is further confirmed by the behavior of pH, which remained more or less constant at the initial value, during the whole process.

Finally, Fig. 2F describes the fermentation performances obtained from the whole mixture representing the synthetic HFW. The highest VFAs production was achieved under neutral pH, with a maximum VFA production of 8.02 ± 0.42 gCOD/L after 130 h of incubation. The main observed product was butyric acid with a concentration of 58% of total VFAs, followed by propionic, acetic, and valeric acid which were 21.18, 10.37, and 9.87%, respectively. When pH was set at 5.5, the maximal VFA productivity decreased at 4.5 gCOD/L. This value was achieved after 108 h since the start of the test, remaining almost constant until the end of the fermentation. At the end of experiment, a wider distribution of fermentation products was observed than at pH 7. Acetate, propionate, and butyrate, in fact, were present in percentages around 20%, while caproate and *iso*-valerate represented 10.20 and 4.54% of total VFAs, respectively. As for the previous cases, uncontrolled pH gave the worst performance: a maximum VFAs production of 2.64 ± 0.25 gCOD/L was achieved after 156 h of incubation, with a distribution among fermentation products very similar to the one observed under pH 5.5. Even in this case, pH showed its crucial role in determining the fermentation performance. Without pH control, the system went quickly to very acidic values (3.76) which inhibited the fermentation. But, contrary to what was observed during tests on sugars rich fractions, pH started to increase till 4.4 as consequence of the protein degradation into ammonia. This conclusion is supported by values of N-NH_4^+ measured in each reactor at the end of experiment. As expected, the highest amount of N-NH_4^+ was found in the reactor inoculated with protein-rich fraction. In this case, a value of about 800 mg/L of N-NH_4^+ was measured at the end of experiment in all the reactors set up. On the contrary, tests having high concentration of starch and carbohydrates were characterized by very low ammonia concentration (<10 mg/L), which explains the absence of buffering capability of the system and the consequent pH drop. It demonstrated that an equilibrium between starch, sugars and proteins contents is fundamental to achieve higher VFA yields.

Table 3 summarizes the VFA yields and the specific biogas productions from the batch tests.

Table 3

VFA yields and biogas production of the batch tests.

| | VFA yield (%), pH 7 | VFA yield (%), pH 5.5 | VFA yield (%), pH NC | Biogas, pH 7 (L/ Kg _{COD}) | Biogas, pH 5.5 (L/ Kg _{COD}) | Biogas, pH NC (L/ Kg _{COD}) |
|------------------|------------------------------|--------------------------------|-------------------------------|--|---|--|
| Proteins | 81.74% | 71.23% | 59.36% | 30.00 | 50.00 | 45.00 |
| Starch | 75.80% | 59.95% | 50.45% | 80.00 | 45.00 | 8.50 |
| fiber/ sugars | 53.42% | 38.64% | 10.33% | 70.00 | 40.00 | 18.00 |
| cellulose | 11.87% | 5.94% | 5.31% | 4.00 | 3.00 | 3.00 |
| Lipids | 8.90% | 5.02% | 4.41% | 3.00 | 2.00 | 1.00 |
| HFW | 47.61% | 26.71% | 16.62% | 35.00 | 29.00 | 42.00 |

Regarding the specific biogas production, protein, starch and fibers/sugars rich fractions achieved the higher values in the range of 40–80 L_{biogas}/kg_{COD}. The highest biogas yields were achieved by starch-rich and sugars and fiber-rich fractions, when pH was adjusted to 7 (Table 3), with correspondent biogas productions of 80 and 70 L/kg_{COD}, respectively. Hydrogen and carbon dioxide constituted the 48.5 and 50.0% v/v, respectively, of the biogas produced. High protein rich fraction had lower biogas production (Table 3), with a hydrogen of 53% v/v, the same of the ones from starch-rich and sugars and fiber-rich fractions. Other carbon fractions had very low biogas production (Table 3), with a high carbon dioxide content higher than the 90% v/v. These very low biogas values, indicate that organic matter was essentially converted in VFA or remained in the mixture. In particular, protein and starch rich fractions led to high VFA conversion of the organic matter, especially at neutral conditions (more than 80 and 75%, respectively). On the contrary, the other fractions had lower VFA yields as effect of the acidification of the system (fibers/sugars rich fraction) or the recalcitrant nature of the substrate (cellulose rich fraction). Consequently, the final conversion of the whole HFW is strongly depending on its composition, with an intermediary VFA yield value of about 45% at neutral pH.

Very different VFA yields in the range 25–85% (w/w) are also present in the scientific literature, confirming the strong influence of the various fractions' amounts on the whole HFW (Atasoy et al., 2019; Cerdan et al., 2021). More data, such as the VFA compositions of the different carbon fraction at each considered pH, the sCOD and ammonia concentrations, are available as the [Supplementary material](#).

3.2. Bacterial community composition

Different fermentation conditions such as pH and temperature affected both the VFAs production and the composition of bacterial communities (Zhang et al., 2020). On the other hand, at the best of author's knowledge, this is the first study where a standardized synthetic HFW was modified in terms of carbon composition and the impact on VFAs production was further monitored through the acidogenic fermentation. Hence, the bacterial community structure in each batch test was monitored through 16S rRNA gene sequencing.

The bacterial diversity analysis was carried out by using Shannon and Simpson indices (Table 4).

The results evidenced that, except for the protein-rich runs, uncontrolled pH displayed the lower bacterial richness among the same carbon source experiments. Both protein- and starch-rich reactors at pH 7, which accumulated the highest total VFA yield, showed different bacterial richness (Shannon index: 2.321 vs. 1.451). Moreover, although diversity indices showed higher microbiological diversity in cellulose-rich fraction (Shannon >2.287) in comparison to lipids-rich fraction (Shannon <1.831), very low VFAs production was observed from both these fractions at all pH values tested. It has been reported that higher microbial diversity was able to ferment different fermentation substrates, leading to a higher total VFAs yield (Wu et al., 2016; Zhang et al., 2020). On the other hand, the data here achieved suggest that the results

Table 4

. Statistical indices of microbial diversity derived from analysis of bacterial communities at phylum level. Note: The larger the Simpson and Shannon indices are, the higher the diversity is.

| | Simpson_Index | Shannon_index |
|---------------------------|---------------|---------------|
| HFW-Mix 5.5 | 0.81 | 2.07 |
| HFW-Mix 7 | 0.88 | 2.28 |
| HFW-Mix n.c | 0.73 | 1.51 |
| Lipids-rich 5.5 | 0.8 | 1.84 |
| Lipids-rich 7 | 0.77 | 1.73 |
| Lipids-rich n.c | 0.6 | 1.18 |
| Starch-rich 5.5 | 0.71 | 1.56 |
| Starch-rich 7 | 0.84 | 2.32 |
| Starch-rich n.c | 0.35 | 0.54 |
| Protein-rich 5.5 | 0.81 | 1.94 |
| Protein-rich 7 | 0.56 | 1.45 |
| Protein-rich n.c | 0.75 | 1.76 |
| Cellulose-rich 5.5 | 0.87 | 2.32 |
| Cellulose-rich 7 | 0.87 | 2.29 |
| Cellulose-rich n.c | 0.86 | 2.29 |
| Sugars and fiber-rich 5.5 | 0.74 | 1.57 |
| Sugars and fiber-rich 7 | 0.87 | 2.25 |
| Sugars and fiber-rich n.c | 0.49 | 0.69 |
| Inoculum | 0.74 | 1.89 |

of VFAs accumulation is scarcely correlated to the bacterial richness.

Therefore, the bacterial community compositions were analyses (Fig. 3).

The inoculum was dominated by Firmicutes (with relative abundances of 68.1%), wherein Lactobacillaceae represented the main bacterial family (46%). Firmicutes was the main phylum observed even in the HFW-mix samples (55.9–65.5%), in which Lactobacillaceae (10.8–38%) followed by Veillonellaceae (6–13%) were the main bacterial families. Moreover, a high percentage of Acetobacteraceae (6.5–29.6%) belonging to Proteobacteria phylum was found. The major bacterial phyla in starch-rich fermentation were Firmicutes (38.2–77%) and Proteobacteria (14.1–30.7%) in which Lactobacillaceae (6.3–77%) and Acetobacteraceae (13.4–26.6%) were the main families respectively. Moreover, it is important to underline a 46% of Prevotellaceae (mostly represented by *Prevotella* genus) at pH 5.5. The bacterial community in protein-rich fermentation revealed high abundance of Firmicutes (36.5–79.6%), Proteobacteria (14.3–50.7%) and Actinobacteria (6–8.3%). Here, Lactobacillaceae (18–56.1%), Enterobacteriaceae (5.5–38.7%) and Atopobiaceae (2.5–7.5%; represented mostly by *Olse-nella* genus) were the main families. Sugars- and fiber-rich fermentation runs were dominated by Proteobacteria (27.7–50.7%), Firmicutes (23.3–47%) where Acetobacteraceae (25.8–50%) and Lactobacillaceae (5.4–40.1%) were the main bacterial families, respectively. As regards cellulose-rich fermentation, Proteobacteria (34.6–63.2%), Firmicutes (22.1–30.6%) and Bacteroidetes (5.5–42.5%) were the main phyla. Here, an abundance of Desulfovibrionaceae family (3.9–25.6%) was revealed. Among the different carbon source trials, lipids-rich fermentation evidenced the lower abundance of Firmicutes (2.2–7.4%) and the higher percentage of Proteobacteria (67.3–95.8%). Among this latter phylum, Comamonadaceae (11.8–43.3%) and Rhodocyclaceae (11.3–39.8%) resulted the most represented families.

Firmicutes, the dominant bacterial phyla here identified, played an essential role in metabolizing organic compounds and producing the VFAs. For instance, Zhang et al. (2020) reported that the high relative abundance of Firmicutes through acidogenic fermentation and it was suggested that this family can positively affected the VFA production (Atasoy et al., 2019).

The heatmap (Fig. 4) reported that Comamonadaceae was negatively correlated to the VFAs accumulation: indeed this family was highly represented in both lipids- and cellulose-rich fractions.

Members of *Comamonas* sp. genus are obligate or facultative anaerobic bacteria and did not exhibit production of acid from multiple carbon substrates (Tamaoka et al., 1987; Zhang et al., 2013). Instead,

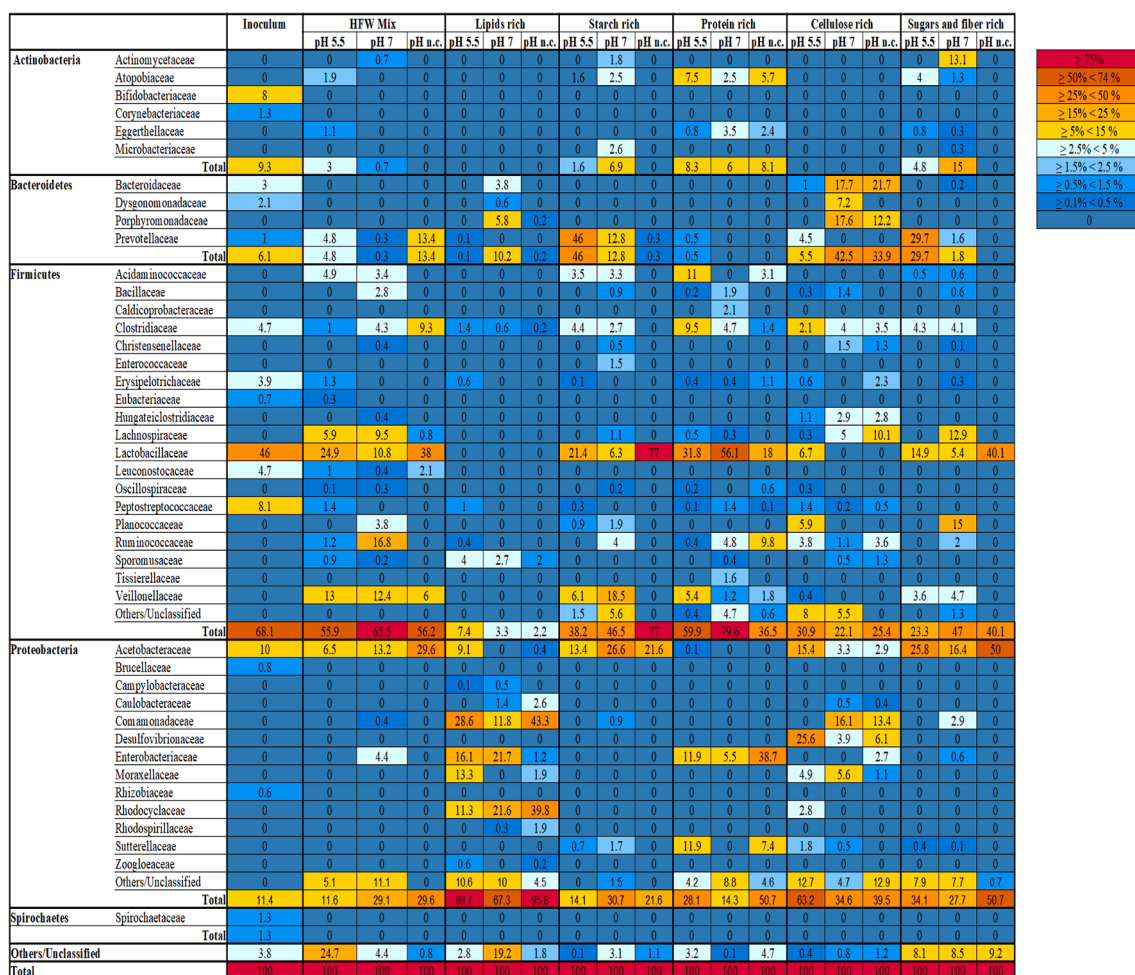


Fig. 3. Bacterial community composition at phylum and family levels revealed in the inoculum, HFW-mix and HFW enriched by lipids, starch, protein, cellulose and sugar and fiber fractions at the end of acidogenic fermentation. Each fraction was tested at pH 5.5, 7 and uncontrolled (n.c.). All taxonomic units with <0.1% of reads and unclassified OTUs are stated as category “Other/Unclassified”.

species belonging to this genus are able to convert oil palm and VFAs to PHA/PHB (Zakaria et al., 2010a, 2010b; Kumar et al., 2019). Eventually, high levels of cellulase were also observed in different *Comamonas* strains (Zheng et al., 2014; Mohammadpour et al., 2020). The heatmap showed that this genus was positively correlated to Rhodocyclaceae, which is an abundant family in lipids-rich samples. It was already reported that the most abundant genus *Azospira* sp. was negatively correlated with VFA accumulation (Cai et al., 2020). On the contrary, the heatmaps evidenced that *Comamonas* was negatively correlated to the Lactobacillaceae, which was, in fact, highly represented in mix-, starch-, protein- and sugar and fiber-rich samples. Indeed, here is reported that Lactobacillaceae showed a positive correlation with the increasing of percentage of acetic acid in the mix of VFAs. Bacteria of this family are aerotolerant or facultative anaerobic, with a variable ability to ferment carbohydrates to lactate and other by-products, such as acetate – in agreement with the results here reported – ethanol, CO₂, formate and succinate (Hammes and Vogel, 1995; Hammes and Hertel, 2009). The members of this family are commonly found in habitats where carbohydrate-containing substrates are available such as food: dairy products, grain products, meat and fish products, fruits and others (Hammes and Hertel, 2009). Moreover, Lactobacillaceae are often reported as dominant in previously VFAs fermentation studies (Xin et al., 2018; Zhang et al., 2020). In addition, Atopobiaceae family (mostly represented by *Olsenella* genus), which displayed positive correlation with VFAs production can anaerobically even produce lactic acid, acetic acid and other different VFAs (Olsen et al., 1991). Both *Olsenella* and

Lactobacillus were already abundantly found during anaerobic fermentation of food waste (Feng et al., 2018). Therefore, it was proposed that both lactic and acetic acid can be used as ideal substrates for anaerobic digestion (Gu et al., 2018) and rapidly converted to other VFAs by several microorganisms. Furthermore, the results showed a positive correlation between Veillonellaceae (represented mostly by *Megasphaera* genera) and VFAs production. Species belonging to *Megasphaera* sp. can utilize both glucose and lactic acid and/or acetic acid to produce different VFAs (Weimer and Moen, 2013; Feng et al., 2018). Thus, Prevotellaceae displayed positive correlation to propionic acid. Studies found higher abundance of the genera *Prevotella* after the lactate accumulation during acidogenic fermentation (Wu et al., 2015; Feng et al., 2018). *Prevotella* is able to grow at pH as low as 5.0–5.5 utilizing glucose to produce organic acids such as formic acid, acetate, fumaric acid succinic acid (Takahashi, 2003). Moreover, members of this genus can ferment starch producing acetate, butyrate, propionate, and valerate (Rodríguez, 2003; Chiquette et al., 2008). *Acetobacter* genus, belonging to Acetobacteraceae family which displayed positive correlation to the increasing of percentage of acetic acid, can indeed produce acetic acid using glucose during fermentation (Chen and Wang, 2016). *Acidaminococcus* can synthesize different VFAs from different ammonia acids. Indeed, the result so far achieved evidenced that *Acidaminococcus* showed positive correlation to valeric and caproic acids production, and protein-rich fraction (pH 5.5) showed the most abundant percentage of this family (11%). Both *Acetobacter* and *Acidaminococcus* were already identified during fermentation of food waste

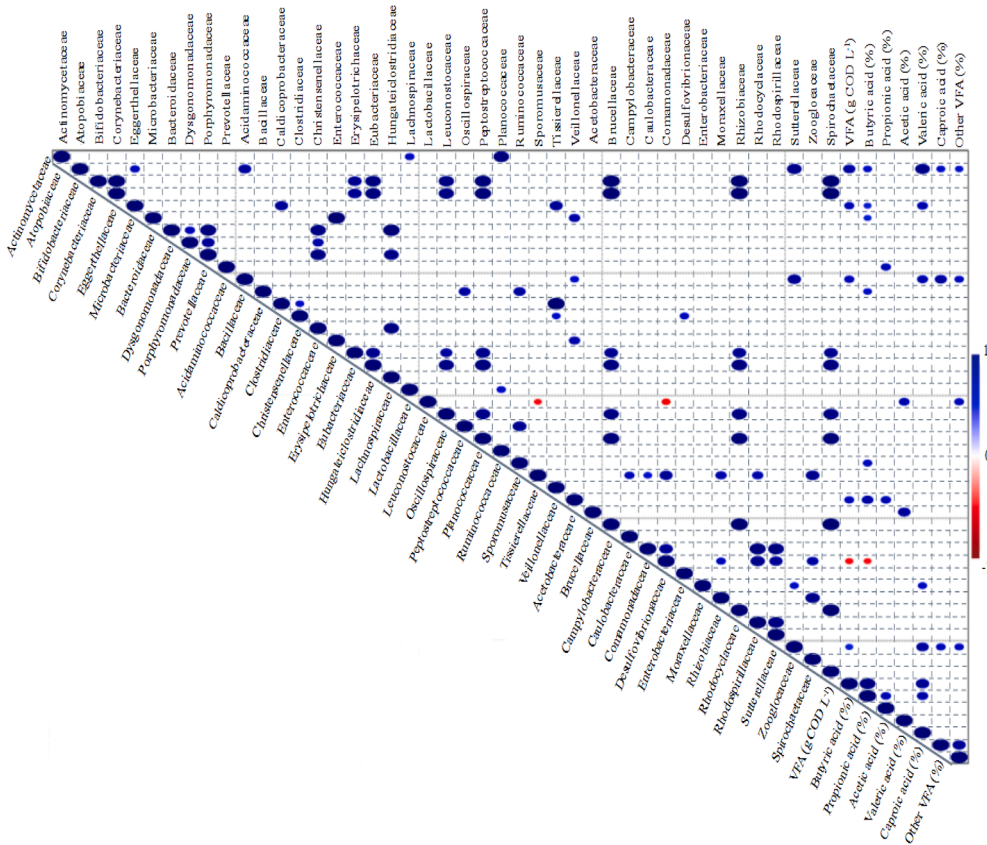


Fig. 4. Heatmap representation of Pearson's correlation matrix of the dominant bacterial families and VFA products: relative abundance (%) and total VFAs (g COD L⁻¹). P-value cut-off of 0.05. Strong correlations are indicated by large ovals, whereas weak correlations are indicated by small ovals. Color denotes the nature of the correlation: 1 (dark blue) indicating perfect positive correlation; -1 (dark red) indicating perfect negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

process (Feng et al., 2018). Eventually, although Eggerthellaceae family was here found directly correlated to VFAs production, only scarce information have been reported so far in literature regarding their role in anaerobic fermentation (Bertin et al., 2010).

The neighbor joining clustering analysis (Fig. 5) evidenced that all the lipids- and cellulose- rich runs were placed in the same cluster. Therefore, these carbon substrates drove towards a similar bacterial speciation.

This result is consistent with the very low VFAs production observed in both of these fractions. Thus, starch-rich fractions and sugar and fiber-rich samples both fermented pH 5.5 and 7 were located in an additional cluster. This observation confirms the effect of the carbon substrate and pH on the bacterial speciation. It was already reported that the relative abundance of bacterial families may greatly change at different pH (Zhang et al., 2020). For instance, the data so far achieved showed that the relative abundance of Lactobacillaceae and Prevotellaceae

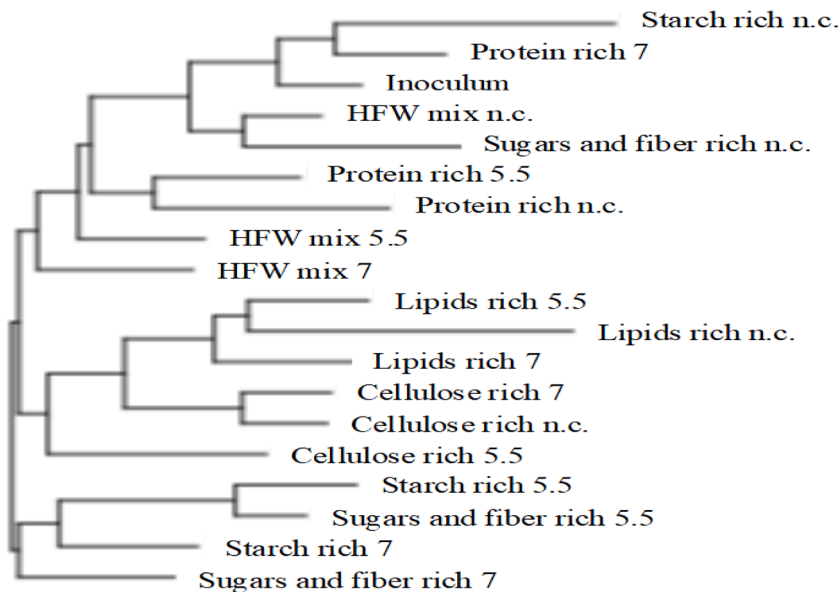


Fig. 5. Clustering analysis of based on Neighbour-Joining algorithm of the inoculum, HFW-mix and HFM enriched by lipids, starch, protein, cellulose and sugar and fiber fractions at pH 5.5, 7 and uncontrolled (n.c.).

drastically increased with decreasing of pH from 7 to 5.5. It has been reported that lactobacilli can survive at extremely low pH conditions and produce lactic acid until pH 4 (Itoh et al., 2012). Recent studies showed that lactate is one of the major product in the fermentation of food waste, with increasing levels at decreasing pH (Gu et al., 2018; Zhang et al., 2020;), suggesting that lactobacilli, due to its greater tolerance to low pH, may plays a key role in this process (Tang et al., 2017). Similarly, *Provetella* was the dominant genus during long-term continuous anaerobic fermentation of food waste at pH ~6 producing a wide variety of VFAs (Feng et al., 2018).

4. Conclusions

The influence of the chemical nature of different HFW fractions on the VFA production was investigated. Fractions rich in proteins and starch achieved the highest VFA concentrations (12–15 g/L) at neutral pH. Butyric, *iso*-valeric and propionic acids were the more abundant acids. It was observed that the chemical nature of HFW impacted also on the relative abundance and speciation of the main bacterial classes involved in the VFA production. Bacterial phyla such as Comamonadaceae (lipids- and cellulose-rich fractions) was negatively correlated to the VFAs production. Instead, Lactobacillaceae family was one of the most abundant family displayed positive correlation to VFAs production.

CRedit authorship contribution statement

Giuseppe Strazzera: . Federico Battista: . Marco Andreolli: . Miriam Menini: . David Bolzonella: Conceptualization, Supervision. Silvia Lampis: Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2021.125289>.

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