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Upscaling volatile fatty acids production: Demonstrating the reliability of anaerobic fermentation of food wastes from the lab towards industrial implementation

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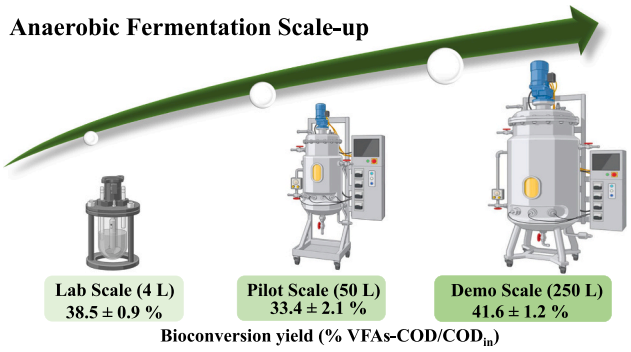
HIGHLIGHTS

- Upscale of Anaerobic auto-fermentation from laboratory to demonstration scale
- Butyric acid production prevailed in the metabolites pool at the three reactors sizes
- Endogenous microbiota was mainly represented by Lactobacillus
- Microbiota shifted into a specialized community for volatile fatty acids production
- The robustness and reliability of AF was demonstrated at different scales

GRAPHICAL ABSTRACT



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ABSTRACT

In recent years, the anaerobic fermentation (AF) of food waste (FW) has gained significant attention as a sustainable solution for waste valorization. However, the challenge of scaling up biotechnological processes for industrial applications remains a key barrier to commercialization. This investigation addressed this challenge by scaling up an auto-AF process from laboratory scale (4 L) to pilot (50 L) and demonstration scale in an industrial environment (250 L), using a lipid-rich FW (46.6 %, w/w) as the feedstock and endogenous microbiota as the inoculum. The applied operating conditions promoted the hydrolysis (>35 % volatile solids (VS) removal) and acidogenesis (>58 % of soluble chemical oxygen demand (sCOD) acidified) steps. As the reactor size for technology testing was increased, efficient mixing was crucial to ensure a proper homogenization of the fermentation broth. Lactic acid bacteria (LAB) prevailed in the endogenous microbiota, contributing to the enhanced

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hydrolysis and acidification efficiencies determined at all the scales. The minimal performance variations determined at different reactors' scales, along with the stability of the metabolite profiles, demonstrated the robustness and reliability of AF, opening the door to continue further industrialization.

1. Introduction

Volatile fatty acids (VFAs) are aliphatic monocarboxylic acids widely used as chemical building blocks in several industries (i.e. pharmaceutical, food and chemical) (Ramos-Suarez et al., 2021). Nowadays, the industrial production of these acids is made via chemical synthesis using petrochemical resources, which results in a significant carbon footprint (Ramos-Suarez et al., 2021; Riemenschneider, 2000). A promising and sustainable alternative to petrochemical synthesis is the production of VFAs via AF. AF is a shortened anaerobic digestion (AD) process where the final step (methanogenesis) is hampered. When the process is halted before its final step, intermediary metabolites such as VFAs, ethanol and lactic acid are accumulated (Llamas et al., 2023), rather than being consumed for biogas production purposes, as it takes place in conventional AD. To achieve this shortened version of AD, the methanogens are inhibited by applying physical and chemical pretreatments to the inoculum (Magdalena and González-Fernández, 2020) or by manipulating the operational parameters (Greses et al., 2021).

This biological production route allows to recover the carbon from a wide variety of biodegradable organic residues and converting them into valuable chemicals. These residues (e.g. food wastes (FW)) consist mainly of organic matter rich in carbohydrates, lipids, nitrogenous compounds, vitamins or even minerals, making them promising carbon sources for AF. The use of FW to produce VFAs perfectly aligns with the United Nations' sustainable development goals 9, 12 and 13, aiming at reducing residues generation and promoting the recycling of resources (United Nations, 2023).

To produce VFAs, the robustness of AF depends on a well-consolidated microbiota, as the synergy between microorganisms is fundamental for efficient hydrolysis and acidogenesis. Before industrial implementation, bioprocesses should be carefully assessed at increasing reactor volumes to test the reliability of the technology. However, developing an efficient scale-up by maintaining similar chemical outputs and process yields between scales is one of the major bottlenecks in the industrialization of biotechnological processes. During scale-up, the total fermentation volume increases, requiring the optimization of agitation configurations or spargers to ensure a proper homogenization of the fermentation broth that avoids mass transfer limitations. An inefficient mixing technology in large-scale AF reactors with high solids content can indeed not only lead to mass transfer limitations in systems but also hamper proper temperature or pH-control (Li et al., 2023). Additionally, during the upscale of the reactor, the shear force applied by the mixing technology can substantially increase (Lindmark et al., 2014). This can generate stress in the microorganisms and disrupt the interspecies reactions between the microbial communities, breaking their synergism. Scaling up biotechnological processes often introduces significant challenges, particularly in maintaining efficiency and stability across increasing volumes.

This work provides novel insights on the establishment of a stable AF of real FWs for VFAs production at large scale in an industrial environment, using the wastes' endogenous microbiota as inoculum. The study assessed the reproducibility of this process across different reactor scales to demonstrate the feasibility of AF industrialization. Moreover, the identification of the microbiome allowed tracking and studying the impact of larger fermentation volumes on the key bacteria involved in AF stability.

2. Materials and methods

2.1. Feedstock and inoculation of the reactor

The feedstock utilized was a blended mixture of depackaged FWs (DFW) collected from an industrial waste management plant (Ennezat, France) after their depackaging. The AFs were performed using the same batch of DFW as a feedstock. The chemical characterization of the DFW was performed by determining total and soluble chemical oxygen demand (tCOD and sCOD), total and volatile solids (TS and VS), pH, ammonium (NH_4^+-N), acetic acid (HAc), lactic acid (HLact), ethanol (EtOH), and the percentage of carbohydrates, lipids, proteins, and ash (Table 1).

The presence of HLact, HAc and EtOH in the feedstock indicates partial decomposition of the DFW by the endogenous microorganisms in the storage container. The AFs' reactors were directly filled with DFW. The endogenous microbiota (microorganisms present in FW) was the only bacteria used as microorganism to conduct the AF. Usually, to reduce the start-up period, AF is conducted by seeding the reactors with an inoculum collected from conventional anaerobic reactors (Greses et al., 2021). Organic residues, being non-sterile feedstocks, contain natural endogenous microbiota capable of degrading and consuming these wastes for growth. Their metabolic similarity with typical VFAs-producing consortia enabled the inoculation of an AF focused on VFAs accumulation with these endogenous microbes (Strazzera et al., 2021).

2.2. Experimental setup

The AF of DFW was evaluated in continuously stirred tank reactors (CSTR) at three different scales, namely laboratory, pilot and demonstration-size reactors. Laboratory scale was evaluated by setting the AF in a 4 L reactor (university Clermont-Auvergne, Clermont Ferrand, France), with 2 L of working volume (R4). The AF under pilot scale was performed on a 50 L reactor (IMDEA Energy Institute, Móstoles, Spain), with a working volume of 30 L (R50). To avoid any disparity in the composition of the feedstocks utilized, part of the same batch of DFW was shipped to Spain in order to perform the experiment at the pilot-scale. The demonstration-scale was studied in a 250 L reactor (Biovalo, Methelec AD plant, Ennezat, France) with a working volume of 180 L (R250). The feedstock transportation between the experimental sites can be also considered as a real scenario by which wastes have to be sent to the treatment facility. Both R4 and R50 were conventional CSTRs utilizing a conventional blade propeller stirring system while in R250 a

Table 1
Chemical characterization of DFW in terms of mean ± standard deviation.

	DFW
TS (g/L)	180.2 ± 6.2
VS/TS (%)[a]	90.6 ± 0.2
tCOD (g/L)	275.1 ± 5.7
sCOD/tCOD (%)	34.0 ± 0.6
HAc (g/L)	5.32 ± 0.01
HLact (g/L)	27.1 ± 0.1
EtOH (g/L)	7.4 ± 0.1
NH_4^+-N (g/L)	6.2 ± 0.6
Carbohydrates (%)[a]	21.3 ± 1.0
Proteins (%)[a]	22.7 ± 2.3
Lipids (%)[a]	46.6 ± 2.9
Ash (%)[a]	9.4 ± 0.2

[a] Dry matter basis.

recirculation system was utilized to maintain a proper homogenization while decreasing the shear force associated. In R250, the re-circulation system consisted in an exhaust pipe located at the bottom of the reactor, connected to a hydraulic pump and ending at the top of the reactor. The fermentation broth was constantly being recirculated from the bottom to the top of the reactor with a flow-rate of 1 m^3/h. The differences between the mixing technologies were compared by calculating volumetric power input (VPi) of the mixing technologies. The VPi was calculated by dividing the power input (P) and the working volume (V). The power input using a mechanical impeller ($P_{Stirring}$) was calculated using eq. 1,

$$P_{Stirring}\,(W) = N \times \rho \times \mathrm{Np} \times \mathrm{ni} \quad (1)$$

where N represents the mixing speed in revolutions/s, the ρ is the density (kg/m^3), ni is the number of impellers and Np is the power number of the impellers (5.2 according to Qazizada. (2016)).

When utilizing the recirculation mixing technology, the power input ($P_{recirculation}$) was calculated following eq. 2:

$$P_{Recirculation}\,(W) = \Delta P \times Q \quad (2)$$

where ΔP represents the pressure loss (Pa) in the recirculation loop and the Q the flow rate in (m^3/s).

Except for the mixing technology utilized in the reactors, all the remaining operational conditions (temperature, pH, ORL and HRT) were the same across the different scales, allowing the direct comparison of the AFs between scales. The AFs at different scales were conducted at a pH of 6.0, mesophilic temperatures (35 °C), organic loading rate (OLR) of 9 g VS/Ld and a hydraulic retention time (HRT) of 8 days. The selection of these operational conditions related to pH and temperature was based on previous research (Cheah et al., 2019).

After filling the reactors for each scale experiment with the feedstock (DFW) and before feeding the reactor in a semicontinuous mode, the CSTRs were subjected to a 3-days period of incubation in batch mode, at the operational temperature and pH detailed above (35 °C and pH 6).

The AF in batch mode acted as a conditioning phase intended to promote the endogenous microbial populations able to thrive under these operational conditions and inherent to the substrate. After the incubation period, the CSTRs were fed daily for a duration of 24 days (the equivalent to 3 HRTs) in order to achieve stability (VFAs concentration and composition, solids, COD and NH_4^+-N concentration) in AF effluents. The same volume fed was also withdrawn from the reactors in a semicontinuous operation mode. Two times per week, the effluents were collected from each CSTR for monitoring the metabolite profile along the AFs. Once the AFs showed stability, samples from the steady-state period were selected to calculate the performance of the process in terms of bioconversion yields of organic matter into VFAs (eq. 3), conversion yield of the soluble fraction of the organic matter (sCOD) into VFAs (acidification yield (%), eq. 4) and the efficiency of the hydrolysis via the percentage of VS removal (eq. 5).

$$\text{Bioconversion yield}\,(\%) = \frac{\mathrm{COD_{VFAs\ eff}}}{\mathrm{tCOD_{in}}} \bullet 100 \quad (3)$$

$$\text{Acidification yield}\,(\%) = \frac{\mathrm{COD_{VFAs\ eff}}}{\mathrm{sCOD_{eff}}} \bullet 100 \quad (4)$$

$$\mathrm{VS_{removal}}\,(\%) = \frac{\mathrm{VS_{in}} - \mathrm{VS_{eff}}}{\mathrm{VS_{eff}}} \bullet 100 \quad (5)$$

Where the $tCOD_{in}$ (g/L) represents the total COD in the feedstocks while the $COD_{VFAs\ eff}$ represents the summation of HAc (gCOD/L), propionic acid (HPro) (gCOD/L), isobutyric acid (isoHBu) (gCOD/L), butyric acid (HBu) (gCOD/L), isovaleric acid (isoHVal) (gCOD/L), valeric acid (HVal) (gCOD/L) and caproic acid (HCa) (gCOD/L) in the effluents. The COD equivalents for each acid were 1.067, 1.513, 1.82, 2.039, and 2.207, respectively. The $sCOD_{eff}$ (g/L) represents the soluble COD analysed in the effluents of the AF. The VS_{in} (g/L) and VS_{eff} (g/L) represents the concentration of VS in the feedstock and in the effluents of the AF, respectively.

2.3. Analytical methods and bioprocess monitoring

The TS, VS, ash and Total Kjeldahl Nitrogen (TKN) of the feedstocks were determined following the procedures described in Standard Methods (APHA et al., 2017). The feedstocks' content of COD (total and soluble) and NH_4^+-N were analysed with commercial kits ISO15705 and ISO000683 (Merck), respectively. The analysis of sCOD and NH_4^+-N required the utilization of the soluble fraction of the effluents. This fraction was obtained by centrifuging the effluents (5 min at 14000 rpm, Eppendorf 5424) and further filtration of the supernatants using filters of 0.45 μm pore size. The total proteins in the feedstock were estimated by multiplying the TKN by a protein factor of 6.25 (González-López et al., 2010) and the total carbohydrates were analysed by following the protocol of the phenol-sulphuric method (adapted from Dubois et al., 1956). The lipid percentage of the DFW was calculated by the difference of 100 % and the percentage of ashes, proteins and carbohydrates in dry matter basis.

The pH of the effluent was monitored by using an online pH meter and adjusted to 6 by adding 5 M NaOH. The AFs were monitored by analysing the reactors' liquids and gaseous effluents three times a week. The TS, VS, tCOD, sCOD and NH_4^+-N of the effluents were analysed following the methods described above in this section. The concentrations of VFAs, lactic acid (HLact) and ethanol (EtOH) were analysed by high-performance liquid chromatography (HPLC). In order to maintain the integrity of the equipment, the samples were firstly centrifuged (5 min at 14000 rpm, Eppendorf 5424) and the supernatant was then filtered by 0.22 μm pore size membranes before injecting them in the HPLC. The HPLC (1260 HPLC, Agilent) was coupled with a pre-column (Cation H Refill Cartridge Microguard column, Biorad), an ion exclusion column (Aminex HPX-87H 300 × 7.8 mm I.D., Biorad) and a refractive index detector. The mobile phase utilized was composed of H_2SO_4 at 5 mM. The HPLC was operated with a flow rate of 0.6 mL/min, an oven temperature of 44 °C and a detector temperature of 35 °C. The gaseous effluent of the reactor was collected from the headspace of the CSTRs and analysed via gas chromatography (GC). The equipment was coupled with a thermal conductivity detector (Claurus 580 GC, PerkinElmer) and 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). Helium was utilized as gas carrier with a flow rate of 30 mL/min. The temperatures set on the equipment were: the injector, 80 °C, the oven, 62 °C, and the detector, 200 °C.

2.4. Microbial community analysis

Microbial communities developed in the experiments at the different scales were determined by sequencing the 16S rRNA gene at the inoculum, after the incubation period, and at the steady state of AF. This analysis tracked the progression of the inocula from the initial feedstock to the steady state and facilitated microbiome comparisons across scales. The samples were collected from the effluent of the CSTRs in each period aforementioned in section 2.2 and stored in 1 mL aliquots at −80 °C prior to DNA extraction. To this end, DNA was extracted from 1 mL aliquots using the kit "FastDNA SPIN Kit for Soil" (MP Biomedicals, LCC), according to the manufacturers' procedure, and its quality was determined by using nanodrop (Omega Spectrostar BMC Labtech). The bacterial and archaea populations were identified by amplifying the hypervariable regions V3 and V4 of the 16S rRNA gene using the primers 341F (F–CCTACGGGNGGCWGCAG) and 805R (R –GACTACHVGGGTATCTAATCC). The samples were sequenced on MiSeq (Illumina) sequencer by FISABIO (Valencia, Spain) and the raw sequences were treated bioinformatically following the guidelines described elsewhere (Greses et al., 2017).

3. Results

3.1. Anaerobic fermentation performance efficiencies for VFAs production at different scales

The AF conducted at the laboratory scale (R4) presented a stable response to the incubation phase with the consumption of the primary metabolites from the DFW degradation and the accumulation of VFAs. By the end of the batch phase, the AF had already accumulated 26.0 ± 0.4 g/L of VFAs, evidencing a specialization of the microbiota into VFA-producing microorganisms. The AF under continuous mode presented a slight increase in the total VFAs concentrations to 26.6 ± 0.8 g/L (corresponding to a bioconversion yield of 38.5 ± 0.9 %). It should be highlighted that this high VFA production yields were obtained by utilizing a feedstock with high lipid content (46.6 ± 2.9 % w/w, Table 1). Although AF of lipid-rich feedstocks under continuous mode is barely studied, Sánchez et al. (2021) studied mesophilic batch AF of lipid-rich feedstocks (w/w) and under acidic pHs obtained a total VFAs yield of 25.3 %. Conventionally, high yields (>50 % of bioconversion yields) are attained with carbohydrate-rich substrates (Gonçalves et al., 2024a; Greses et al., 2022) while protein and lipid-rich substrates might present drawbacks and consequently, lower concentrations and yields. In the particular case of lipid-rich substrates, lipids are subsequently degraded into simple molecules (i.e. long-chain fatty acids-LCFAs). The adsorption of LCFAs (initial degradation product of lipids) into the microorganisms' cell walls is widely reported as the main toxicity factor when using lipid-rich substrates (Pereira et al., 2005). These molecules can adhere to the microorganisms' surface, creating a physical layer or solubilize into the lipid bilayer. In both cases, the excess of lipids hinders the transport of substrates and products hampering the microbial activity and limiting VFAs accumulation (Liu et al., 2017; Pereira et al., 2005). This phenomenon was also found by Liu et al. (2017) who tested the addition of oil until a concentration of 35 g/L and observed a decrease in the total VFAs from 41.1 g/L (in the control reactor) to 13.4 g/L (under 35 g/L of oil). Additionally, Law et al. (2023) found a VFAs bioconversion yield decrease from 39 % to 19 % concomitantly with the lipid content of feedstock (increasing from 20 % to 30 % w/w). The authors also observed a total acidification percentage 51 % under 30 % of lipids, which were remarkable lower than those found in the present study (Table 2). This fact could be related to the short HRT applied (4 days), which could reduce the hydrolytic potential of the system. Longer HRTs increase the reaction time of microorganisms promoting the hydrolytic and acidogenic steps (Gonçalves et al., 2024a). In the present study, the high acidification yield (65.9 ± 1.1 %, Table 2) confirmed the good performance of the acidogenic bacteria, demonstrating their ability to cope with substrates that exhibit remarkable high lipids content (46.6 % w/w, Table 1).

Lipids, being the most energetically dense compared to proteins and carbohydrates, have been reported to be the slowest and hardest component to degrade (Law et al., 2023). Nevertheless, the solubilization of the feedstock was remarkably efficient. The soluble fraction of the organic matter in the reactor (sCOD %) at the steady state of R4 almost doubled (60.0 ± 5.6 %) its value when compared with the feedstocks' (34.0 ± 0.6 %) composition (Tables 1 and 2). Additionally, the percentage of VS removal (34.7 ± 3.1 %), which represents the efficiency of the hydrolysis, was noteworthy in comparison with previous studies. Law et al. (2023) found that the increase in lipid content decreased the AFs' hydrolysis efficiencies from 27 % (at 13 % w/w of lipids) to 0 % (at 30 % w/w of lipids). The solubilization percentage and $VS_{removal}$ obtained herein confirmed a successful hydrolysis step during the AF. The efficient hydrolysis increased the content of available carbon (sCOD) for bacterial consumption, which consequently boosted the acidogenesis step and led to high acidification and bioconversion percentages. Indeed, the process efficiencies attained herein were remarkably high when compared with studies utilizing carbohydrate-rich feedstocks, which are easier to degrade and consume by microorganisms. For instance, Greses et al. (2021) attained a maximum bioconversion yield of 46.3 % with a corresponding solubilization percentage of 68.2 % when carbohydrate-rich FWs (80.4 % w/w) was used as feedstocks for AF. Similarly, Gonçalves et al. (2024a) performed the AF of agro-FWs (63.3 % w/w of carbohydrate), reaching bioconversion yields and hydrolysis efficiencies of 48.1 % and 51 %, respectively. These investigations evidenced that, despite the utilization of more easily degradable feedstocks (carbohydrate-rich FW) than lipid-rich feedstock, the hydrolysis and bioconversion efficiencies were in the range of those achieved herein. This result can be related to the microbiota used as inoculum. Conventionally, AF have been inoculated with anaerobic sludge from conventional AD reactors due to the presence of a specialized microbiota capable of converting organic matter into VFAs. Nevertheless, by utilizing the endogenous microbiota from the feedstock as inoculum (auto-AF), the microbiome was naturally adapted to the composition of the raw materials (i.e. high lipid content) (Table 1). The incubation phase (in batch mode) allowed the proliferation of specific bacteria of interest, with high hydrolytic and acidogenic activities and resistant to the high lipid content of the feedstock. Taking advantage of naturally growing bacteria should thus not be disregarded as optimum degrading activities might already come along with the substrate.

It should be remarked that the methanogenesis step was successfully inhibited during the whole experimental time since methane production was not detected in the gaseous phase, which was confirmed by the low COD removal obtained (7.0 ± 1.7 %, Table 2) in R4. This inhibition was reached by the conditions imposed in the reactor and most probably by a rapid accumulation of VFAs which inhibited the growth of archaea. Moreover, the microbial populations usually found in spoiled FW are usually deprived of methanogenic archaea (Lee et al., 2013; Wu et al., 2018). These features are relevant when it comes to AF, as it contributes to the successful inhibition of the methanogenesis step.

When the AF was upscaled to the pilot scale (R50), the overall fermentation performances slightly decreased. The total VFAs accumulation by the end of the incubation phase was 20.4 g/L of VFAs and slightly increased to 21.0 ± 1.4 g/L (36.6 ± 2.8 g COD/L) in the steady-state period, with a bioconversion efficiency yield of 33.4 ± 2.1 %. Concomitantly with the slight decrease in the bioconversion efficiencies, the percentage of sCOD acidified into VFAs (acidification %) also decreased compared with R4. Even though the increase in the AF scale caused a reduction in the VFAs production efficiency, the results attained herein were in line with previous investigations where AF was performed under a similar scale. Garcia-Aguirre et al. (2019) investigated the scaling up of co-AF of sewage sludge and the organic fraction of municipal solid waste in an 80 L reactor, achieving a maximum VFAs average concentration of 19.4 g COD/L (43.9 % of the sCOD acidified into VFAs) when conducting the AF at pH of 6.0. Similarly, during the reactor upscale from 5 L to 50 L of a two-stage AD of fruit pulp wastes,

Table 2
Chemical characterization of effluents evaluated at the steady-state of each operation in terms of mean ± standard deviation.

	Laboratory Scale (R4)	Pilot Scale (R50)	Demonstration Scale (R250)
pH	6.0 ± 0.1	6.0 ± 0.2	6.0 ± 0.1
tCOD (g/L)	119.7 ± 9.2	109.8 ± 3.1	103.7 ± 4.0
sCOD/tCOD (%)	60.0 ± 5.6	59.5 ± 3.5	63.8 ± 2.7
TS (g/L)	54.0 ± 3.2	58.0 ± 4.4	53.0 ± 2.6
VS/TS (%)	91.1 ± 4.7	71.9 ± 0.6	61.0 ± 3.0
NH_4^+-N (g N/L)	2.02 ± 0.03	1.83 ± 0.04	2.48 ± 0.04
Total VFAs (g VFAs/L)	26.6 ± 0.8	21.0 ± 1.4	30.3 ± 0.9
Bioconversion (%)	38.5 ± 0.9	33.4 ± 2.1	41.6 ± 1.2
Acidification (%)	65.9 ± 1.1	58.8 ± 4.0	73.8 ± 1.4
$VS_{removal}$ (%)	34.7 ± 3.1	44.2 ± 1.6	53.6 ± 2.6
$COD_{removal}$ (%)	7.0 ± 1.7	4.2 ± 0.3	9.9 ± 1.1

Carvalheira et al. (2018) observed 20 percentual points decrease in the degree of acidification taking place in the first stage. The authors considered the inefficient homogenization of the fermentation broth as the major drawback, affecting the mass transfer in the AF. The poor homogenization was a consequence of the scale increase and the use of organic matter in suspension causing a disturbance in the overall performance. Mixing intensity requires a special attention since high-intensity mixing strategy can increase the shear stress in the microorganisms and can break the microbial syntrophic interactions (Lindmark et al., 2014). It should be also highlighted that upon high-intensity mixing, substrates such as the ones employed here (high lipid (46.6 ± 2.9 %) and protein (22.7 ± 2.3 %) content) increase the possibility of foam formation. In R50, the high-speed mixing (180 rpm) applied led to the production of foam in the CSTR, which could have affected the homogeneity of the mixture in the reactor and the access to the substrate for microorganisms. Additionally, the stress induced from the high mixing could have broken the interspecies interactions, partially limiting the acidogenesis step of AF. Consequently, 4.2 ± 0.1 and 3.7 ± 0.3 g/L of HLact and EtOH were accumulated, respectively, corroborating the stress provoked in the reactor.

Contrary to the slight decrease in VFAs production yields, the hydrolysis efficiencies (percentage of VS removal) increased with the scale increase, reaching 44.2 ± 1.6 % (Table 2). The resistance of the hydrolysis step to the stress conditions provoked by the intense mixing indicated that hydrolytic bacteria were more robust than acidogenic bacteria. NH_4^+-N concentration was stable regardless of the reactor volume evaluated. This meant that the nitrogen mineralization, linked to the degradation of proteins, was stable as well. Thus, the hydrolysis increase was rather associated with the enhancement of the degradation of carbohydrates or lipids. Furthermore, these results also suggested that the increase of the viscosity in the fermentation broth could have reduced the microorganisms' access to the solubilized organic matter, thereby encouraging hydrolytic reactions to obtain a consumable organic source.

When the scale of the AF was further increased to the demonstration scale (R250), the highest fermentation yields and concentrations of VFAs were achieved. After the incubation period (batch mode), the concentration of VFAs was 26.6 g/L. When feeding the reactors in semicontinuous mode, the concentration of VFAs increased gradually to 30.3 ± 0.9 g/L (bioconversion yield of 41.6 ± 1.2 %, Table 2). These results in R250 were promising as they were slightly higher than the R4, indicating a replicable process under larger scales. Additionally, the yields attained were remarkably high in comparison with previous studies operating fermenters at demonstration scale. Castro-Fernandez et al. (2024) optimized the scale-up of AF of FWs from laboratory to demonstration scale (1.5 m^3 reactor). At the demonstration scale, the authors obtained a VFA production of 37.6 g COD/L (vs 48.9 ± 1.4 g COD/L obtained herein), which corresponded to a bioconversion of 37.9 %, which were comparable to the ones attained at R250. Valentino et al. (2019) obtained a maximum concentration of 19 g COD/L (25 % of bioconversion yield) in a 380 L AF using the organic fraction of the municipal solid waste as feedstock at mesophilic conditions. The authors demonstrated the promotion of the acidogenic bacteria activity by obtaining an acidification efficiency of 75 %. These results agreed with the ones attained herein, corroborating that AF of FWs in large scales can promote high acidification yields (73.8 % sCOD acidified into VFAs, Table 2) at similar operational conditions such as mesophilic temperatures, acidic pHs and longer HRTs (15 days). As in the other CSTRs, CH_4 was negligible confirming that the methanogenesis was successfully inhibited also at this scale.

Additionally, at R250, the mixing technology was readjusted to a recirculation strategy, which was less intense than R50, preventing foam production and maintaining the synergism between species. At this point, it should be highlighted that R4 and R50 employed mechanical mixing. The intensity of the mixing technologies between the different scales was compared by the Volumetric Power Input (P/V), which presents the power necessary (W) to mix the reactor per litre of working volume. The mechanical steering applied in the CSTR 50 L at 180 rpm required 5.6 W/L to fully homogenize the reactor, while the recirculation technology employed in CSTR 250 L only utilizes 0.54 W/L. Remarkably, the hydrolysis efficiency was even higher when the process was scaled up to 250 L, pointing out to the promotion of the microbial activity. Additionally, the low foam formation in the reactor led to the decrease in the viscosity, increasing the mass transfer reactions. VS removal of 53.6 ± 2.6 % and a solubilization percentage of 63.8 % were obtained (Table 2). The high hydrolysis and sCOD concentration, coupled with the high bioconversion yields and acidification percentages, corroborated the high acidogenic activity of the endogenous microbiota and the robustness and reliability of the process towards the scale-up.

The utilization of recirculation strategy vs mechanical stirring was advantageous in this particular case, allowing to homogenize the reactor while applying low power inputs, which directly reduces the operation costs. However, it should be considered that the success of a recirculation mixing technology is highly dependent on the physicochemical characteristics of the feedstock, where feedstocks with very high viscosity and big size particles can generate clogging of the recirculation system. On the other hand, mechanical stirring is more versatile for all types of feedstocks. Additionally, the stirring technology can be optimized by varying the shape and the number of the impellers, increasing their stirring efficiency. Generally speaking, the mixing technology in fermentation reactors is widely discussed in the literature and it should be selected considering the conditions of the AF.

3.2. Chemical distribution of metabolites obtained at the different reactors' scales

The chemical distribution of the AF in R4 was marked by the presence of even chain VFAs. At the steady state of the AF, HBu was the prevailing VFA in the fermentation broth (10.1 ± 0.6 g/L), followed by HAc (6.0 ± 0.3 g/L) (Fig. 1). The content of these acids, together with the remaining even chain VFAs (HCa and isoHBu) made up a total VFAs concentration of 19.4 g/L (73.2 % w/w of the total VFAs pool). The composition of the VFAs pool was mostly related to the combination of the applied operational conditions together with the composition of the feedstock. AF of FWs under slightly acidic pHs, combined with mesophilic temperatures, tend to promote HAc and HBu production (Cheah et al., 2019; Strazzera et al., 2021). The high lipid content of the feedstocks was also responsible for the VFA profile. It has been reported that upon hydrolysis, lipids are degraded into glycerol and the main metabolite produced from its fermentation is pyruvate (Liu et al., 2017). This metabolite is the pivotal control of the acidogenesis and can be converted into a wide range of metabolites such as, HAc, HBu, HPro or EtOH (Zhou et al., 2018). In R4, 3.4 ± 0.3 g/L of HPro was accumulated in the reactor. The production of HPro is commonly associated with protein content in the feedstocks and its content can increase proportionally with the concentration of proteins (Regueira et al., 2020). Likewise, HAc and HBu, the lipid content of the feedstock can affect the production of HPro. Following the pyruvate metabolism, HPro is (Gonzalez-Garcia et al., 2017) also one of the main products. The high content of HLact in the feedstock was also responsible for the metabolite distribution in the AF since this metabolite is a primary fermentation product commonly used for VFAs production (Greses et al., 2022). On one hand, HLact can be oxidized by the lactate dehydrogenase into pyruvate which is further converted into several compounds (Detman et al., 2019). On the other hand, following the pyruvate metabolism, HLact is consumed for the production of HPro.

The AF under these conditions also promoted the accumulation of longer-chain VFAs. The total concentrations of HVal and HCa in the metabolites pool were 3.7 ± 0.1 g/L and 2.7 ± 0.4 g/L, respectively. Their production could have resulted from the degradation of long chain fatty acids by the β-oxidation pathway could have degraded these

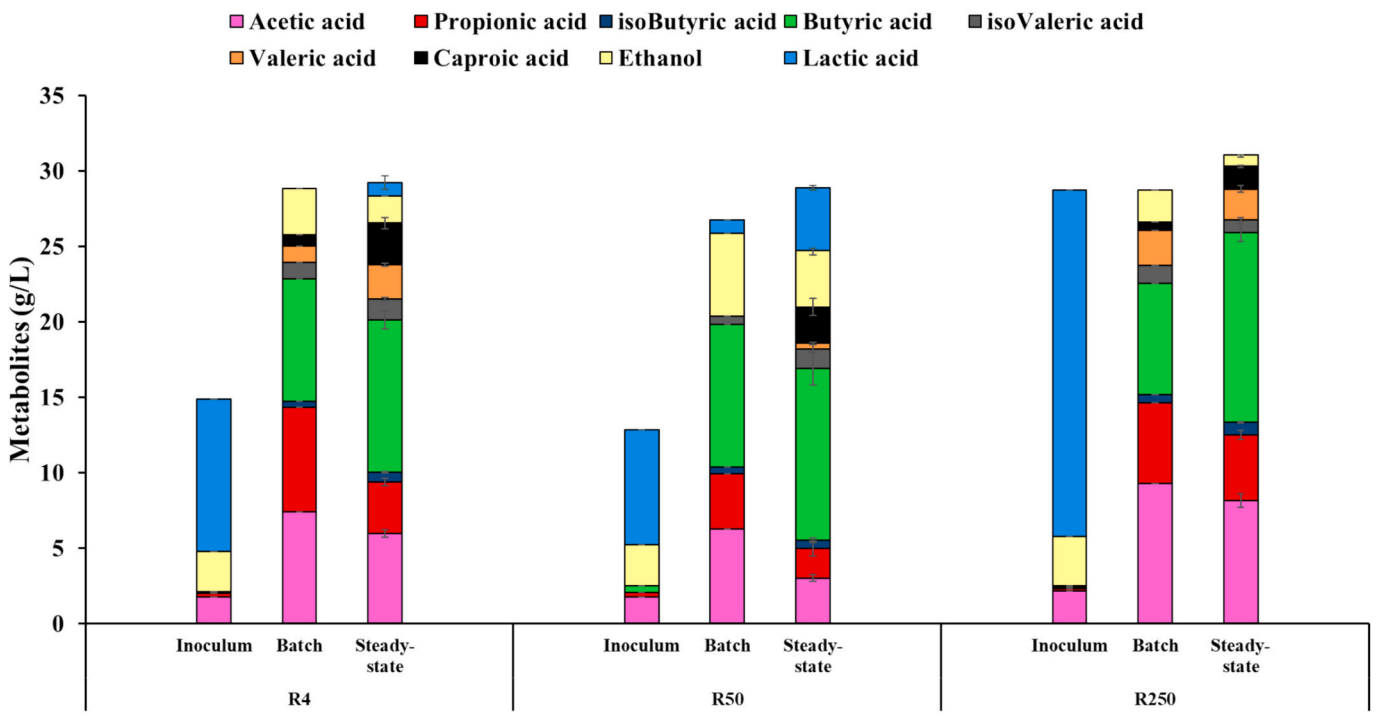

Fig. 1. Metabolite profiles and concentration determined for each stage of the AFs, at the three different reactor scales.

compounds after the hydrolysis of the lipids present in the feedstock (Jimenez-Diaz et al., 2017).

When the scale increased to R50, the total abundance of even chain VFAs increased to 82.5 % (w/w) with regard to the total VFAs pool. Remarkably, the concentration of HBu increased to 11.4 ± 1.1 g/L at the expense of HAc decrease to 3.0 ± 0.2 g/L, as well as the odd chain VFAs. These variations in the VFAs were the major differences observed in comparison with R4. Additionally, as aforementioned, it could be observed a slight accumulation of HLact in the steady-state operation. The intense mixing technology might generate shear stress in the microorganisms, resulting the in accumulation of a VFAs precursor. The accumulation of AFs' intermediates, such as HLact, was already identified as a process damage indicator in partially inhibited fermentations (Gonçalves et al., 2024b). Simultaneously, the HCa concentration was also noticeable, accumulating a total of 2.4 ± 0.6 g/L which was equivalent to 12.4 ± 1.1 % (w/w) of the total VFAs pool, the highest obtained under all the scales. The partial inhibition of the acidogenesis and the promotion of the hydrolysis step during the upscale agreed with the pathway proposed above where the accumulation of longer VFAs could have been correlated with the degradation of the LCFA. The content of lipids from the feedstock under an efficient hydrolysis step could lead to a consecutive degradation of those compounds into LCFA and further into smaller VFAs such as HCa and HBu (Jimenez-Diaz et al., 2017). With this regard, it is crucial to control the homogenization during the scale-up of AF to avoid the application of extreme stress to the microorganisms (Lindmark et al., 2014). The stress applied can influence not only the fermentation yields but also the final metabolite distribution.

Regarding the R250, increasing the reactor volume resulted in a higher concentration of HBu, remaining the predominant acid with 12.6 ± 0.6 g/L (41.4 ± 1.4 % w/w). In R250, HLact was completely consumed during the incubation period in batch mode. Simultaneously, HBu and HCa production slightly decreased with the promotion of HAc. The decrease of the longer chain VFAs, suggested that the depletion of HLact was not only associated with its consumption as a precursor for VFAs production but also with the inhibition of HLact bacteria.

Regardless of the slight changes in the VFAs distribution profile, the variations were not significant as the major variation was approximately 12 % of HAc from R50 to R250. The small differences observed in the metabolite profiles, together with the previously mentioned bioconversion increase at R250, indicated a successful upscaling of this carboxylate production technology. Furthermore, the acids profile was highly selective as the percentage of HAc and HBu together accounted for over 70 % of the total pool at the demonstration scale. This feature is also particularly relevant given that the simplicity and effectivity of downstream processes for further purification of the VFAs rise with the increase of the total acids' concentration and the selectivity of its profile.

3.3. Microbial populations characterization

When studying the behaviour of the AF at different scales, some slight variations were observed in AF performance efficiencies and chemical outputs. To understand these differences, studying the microbial populations provides key information.

After identifying the microbiotas present in the different experiments, the phylum distribution in the inoculum was mostly dominated by Firmicutes (Fig. 2). In the I4 and I250, Firmicutes comprehended over 85 % of the microbiome while in the I50 comprehended only 57.0 % of the total abundance. The remaining phyla observed in the inoculum of the reactors were mostly Proteobacteria, Actinobacteria and Bacteroidetes. The variations observed in the inoculum could have been related to the exposure of the DFW to aerated conditions while simultaneous defrosting of the feedstocks during transportation. It should be mentioned that the feedstock was transported from Clermont Ferrand (France) to Móstoles (Spain) in order to perform the experimentation at the pilot plant scale. These conditions promoted the growth of facultative anaerobic bacteria (Enterobacteriaceae, *Enterobacter* and *Pseudomonas*) and aerobic bacteria (from the order Streptophyta), commonly found in the surface of FWs (Lu et al., 2022; Wu et al., 2018). Meanwhile, the feedstocks kept in France were conserved in a mixed contained before freezing which could have inhibited these microorganisms' growth and degraded their DNA, preventing its identification.

During the incubation phase and at the steady state of the AFs, the phylum distribution of the microbiomes remained similar, with

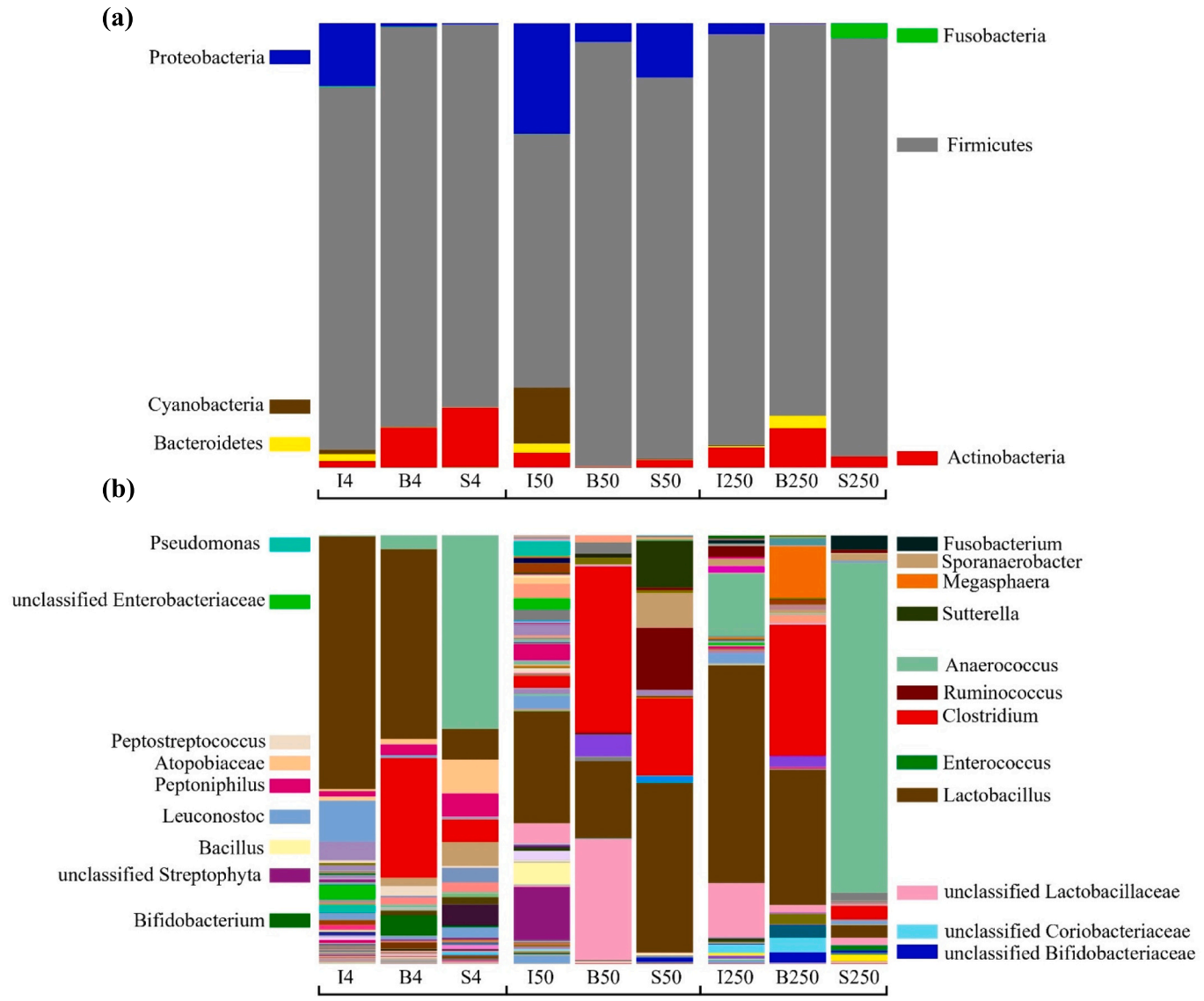

Fig. 2. Relative abundance of bacteria and archaea identified throughout the experiment at (a) phylum and (b) genus level. The labels I, B and S indicate that the samples were collected from the inoculum, at the end of the incubation phase and from the steady state, respectively. Microorganisms with relative abundance lower than 1 % have been excluded from the plot legend.

Firmicutes dominating the populations. Firmicutes is a wide and versatile phylum commonly found in anaerobic mixed cultures able to degrade organic matter and ferment it into organic acids (i.e. VFAs or HLact) (Greses et al., 2022; Greses et al., 2021). Additionally, Firmicutes and Proteobacteria have been reported as endogenous microbiota of processed FW, where a high hydrolytic and acidogenic capacity was detected (Kim et al., 2021).

In all the fermentations, the dominance of Firmicutes occurred with the decrease of Proteobacteria and, specifically at R50, with the disappearance of the phylum Cyanobacteria from the microbial communities. This specific decrease was marked by the disappearance of the aforementioned facultative anaerobic bacteria and aerobic bacteria. When considering the microbes' distribution at the genus level, their profile changed during the experiment. In all the experiments, the inoculum was dominated by LAB. *Lactobacillus* was the dominant genus in all the different scales while *Leuconostoc* and Lactobacillaceae were observed in lower abundances. Microorganisms belonging to Enterobacteriaceae, *Enterobacter*, and *Pseudomonas* were determined as well in all the inoculum microbiomes. All these microorganisms are LAB commonly predominant in the microbiotas of spoiled food and food residues (Wu et al., 2018). These bacteria belonging to Firmicutes are members of the order Lactobacillales and share in their metabolism the ability to metabolize several sugars and proteins to produce HLact as the main product via the Embden–Meyerhof–Parnas or the pentose phosphate pathways (Iyer et al., 2010; Salvetti et al., 2013). Additionally, some of these microorganisms, such as *Lactobacillus* can simultaneously perform homolactic and heterolactic fermentation. Heterolactic fermentation simultaneously produces HAc and EtOH as side products during HLact accumulation, which supports their production in the inoculum stages in all the different scales (Castillo Martinez et al., 2013).

In the incubation period of the fermentations (batch phase), the relative abundance of LAB slightly decreased and was dominated by *Lactobacillus* and Lactobacillaceae in all the different scales. LAB were previously reported to have high lipolytic and proteolytic activity (Abedi and Hashemi, 2020; Thierry et al., 2017). The remaining distribution of the microbiomes presented a high relative abundance of *Clostridium* and Clostridiaceae. The versatile *Clostridium* metabolism has been reported to degrade and ferment complex organic compounds into VFAs, consuming HLact to produce HBu and HAc (Detman et al., 2019; Gonçalves et al., 2024a). Based on that fact, the presence of these genera

agreed with the feedstock composition (high lipid and protein content) supporting the high hydrolysis and acidogenic activities. While LAB was responsible for HLact, HAc and EtOH production via heterolactic fermentation, *Clostridium* was associated with HAc, HPro and HBu production. The low content of HLact in the presence of LAB suggested that *Clostridium* consumed all the HLact produced as an electron donor in HBu fermentation. The incubation phase at the demonstration scale also promoted the proliferation of *Megasphaera* (11.8 %). This genus worked together with the rest of the microbial population for the accumulation of HPro and HVal in the reactor as they can ferment glucose, lactose and HLact into HPro, HBu and HVal (Gonçalves et al., 2024b). The synergetic correlation between the microorganisms allowed the production of the longer VFAs in the metabolites profile.

Opposite to the similar microbial population detected in the inoculum and batch period at all reactor scales, the microbial communities evolved differently at the steady state. In the S4, the microbial distribution was dominated by *Anaerococcus* (45 %). This genus was previously reported to hydrolyze and ferment complex carbohydrates and proteins into VFAs, having as main products HBu, HPro and HLact (Khor et al., 2017; Yang et al., 2021). Additionally, the remaining composition of the microbiota was composed of the genera *Clostridium* (5.0 %), *Lactobacillus* (7.1 %), *Sporanaerobacter* (5.7 %), *Peptoniphilus* (5.4 %), and the family Atopobiaceae (7.9 %). The combination of the hydrolysis and acidogenic capabilities of the genera mentioned (Wu et al., 2016; Yin et al., 2023) together with the fermentative capabilities from *Anaerococcus* contributed to the high yields observed (Khor et al., 2017; Yang et al., 2021). The metabolites distribution agreed with the metabolisms of the microbial populations identified since the dominating microorganisms during the steady state presented HAc and HLact as main fermentation products.

At S50, the most relevant genera identified in the populations were *Lactobacillus* (39.6 %), *Clostridium* (18.0 %) and *Ruminococcus* (14.5 %). Both *Clostridium* and *Ruminococcus* were associated with HBu production, being detected in the populations of AFs where this acid dominated the acids profile. (Gonçalves et al., 2024a).

The microbiota characterized at S250 was highly specialized, being dominated by *Anaerococcus* (77.1 %). The remaining fraction of the microbiota was composed of *Lactobacillus* (4.8 %), and *Clostridium* (3.7 %). The hydrolytic and acidogenic abilities of these microorganisms were aforementioned above in this section and their metabolisms combined were responsible for the highest yields and the metabolite distribution obtained herein. Additionally, the high hydrolysis yields in this lipid-rich feedstock suggested a high lipolytic activity from the dominant genus *Anaerococcus*. Indeed, the only fully sequenced genome of a specie from the genus *Anaerococcus*, the *Anaerococcus prevotii* DSM 20548 (GCA_000024105.1), presented the genes of the family of proteins GDSL-type esterase/lipase, one of the most important families of lipolytic enzymes (Ding et al., 2023). This fact suggested that these bacteria might present high lipolytic capabilities.

The utilization of the endogenous microbiota from the FWs as inoculum for the AF was shown to be an interesting approach to ensure a previous adaptation of the microorganisms to the complex organic residues. While LAB had a great presence in all the reactors during the inoculum and batch phase, at all the tested scales the microbiota specialized in VFAs accumulation. The differences in the mixing technologies under all the scales led to distinct shear forces applied to the microorganisms as well as the homogenization was distinct, affecting the mass transfer of the microbes. In this sense, the microbial populations changed based on the robustness of the bacteria to face the different conditions. St-Pierre and Wright (2014) showed several variations in the microbiotas of different anaerobic digesters from different scales and configurations, treating the same residue. Nevertheless, the differences observed herein were compensated by the similarities in their metabolisms. By sharing similar metabolic pathways different combinations of microorganisms allowed a replicable scale-up of FWs' AF.

4. Conclusions

The present investigation showed the reliability and robustness of anaerobic fermentation of food wastes where the endogenous microbiota of the feedstock has proven to be an efficient inoculum for this process. Despite some small variances in the product outcome, the applied operational conditions promoted butyric acid production in the metabolites pool at the three reactor sizes (4, 50 and 250 L). Lactic acid bacteria, such as *Lactobacillus*, were present at all scales and demonstrated to be an important microorganism in acidogenesis of food wastes. Likewise, *Anaerococcus* was proven to be a key microorganism in the anaerobic fermentation of lipid-rich food wastes, presenting high capabilities of fermenting complex organic matter into VFAs.

CRediT authorship contribution statement

Manuel João Afecto Gonçalves: Writing – original draft, Investigation, Data curation. **Silvia Greses:** Writing – review & editing, Formal analysis, Data curation. **Omar Kanine:** Investigation. **Jean-Sébastien Guez:** Writing – review & editing, Formal analysis. **Pierre Fontanille:** Writing – review & editing, Funding acquisition, Formal analysis. **Christophe Vial:** Writing – review & editing, Funding acquisition, Conceptualization. **Cristina González-Fernández:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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