Bioresource Technology 337 (2021) 125388

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Co-digestion of cheese whey with sewage sludge for caproic acid production: Role of microbiome and polyhydroxyalkanoates potential production

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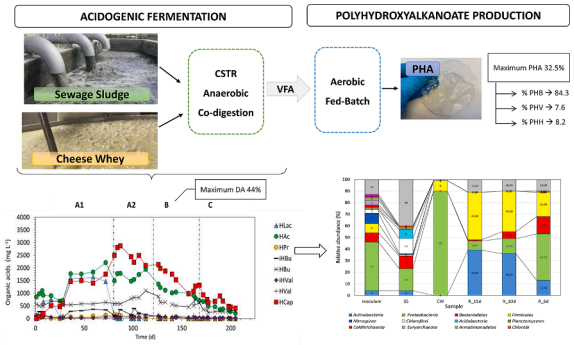
HIGHLIGHTS

- Caproic acid was produced in the co-digestion of cheese whey and sewage sludge.
- The optimal conditions for the process were 10 days HRT and 2 feeding cycles per day.
- Microbiome was enriched in organisms involved in the caproic acid production.
- The use of organic acids stream for polyhydroxyalkanoates production was verified.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Caproic acid
Cheese whey
Sewage sludge
Microbiome
Organic acids
Polyhydroxyalkanoates

ABSTRACT

The main aim of this work was to evaluate the efficiency of producing caproic acid and other volatile fatty acids using a co-digestion between cheese whey and sewage sludge in a continuous reactor. The effect of two different feeding regimes (one and two per day) and three hydraulic retention times (HRT) (15, 10 and 6 days) on the organic acids production were studied. The optimal conditions for the process were 10 days HRT, 2 feeding cycles per day, reaching a maximum degree of acidification of 44%. Under these conditions, the most abundant organic acid was caproic acid. The analysis of the microbial community dynamics in the reactor during the HRT changes revealed a microbiome enriched in organisms involved in caproic acid production. Additionally, the production of polyhydroxyalkanoates using the organic acids stream as feeding was verified in a fed-batch experiment obtaining a copolymer formed by hydroxybutyrate, hydroxyvalerate and hydroxyhexanoate.

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https://doi.org/10.1016/j.biortech.2021.125388
Received 31 March 2021; Received in revised form 3 June 2021; Accepted 4 June 2021
Available online 8 June 2021

1. Introduction

Over the last years, biorefineries have emerged as an alternative to petroleum-based refineries, where organic wastes can be used as raw materials to be transformed into a variety of bioproducts as for example biofuels, industrial biochemicals and biomaterials (Baleta et al., 2019; Mansouri et al., 2017). In this context, the European Commission (EC) presented the European Green Deal (European Commission, 2020a), a roadmap towards a climate-neutral circular economy. On March 2020, the EC adopted a New Circular Economy Action Plan (European Commission, 2020b) which was built on the work done in 2015, "Closing the loop – An EU action plan for the Circular Economy" which was focused on actions related to production, consumption, waste management, stimulating markets for secondary raw materials and water reuse. Consequently, the biorefinery sector is crucial within the circular economy.

In this framework, the carboxylate platform technology has been proposed as an opportunity to convert different organic wastes into valuable intermediates. The carboxylate platform is based on the use of anaerobic biological reactors with the ability to transform complex raw materials into short-chain carboxylic acids (SCCA) (commonly named volatile fatty acids, VFA) via acidogenesis. VFA are acetic, propionic, butyric and valeric acids, which have a lot of applicability for several uses in the biotechnological industry. Besides, through reverse β-oxidation, VFA can be elongated, yielding medium-chain carboxylic acids (MCCA), for instance caproic acid (HCap) (also called hexanoic acid). Following that process, acetic and butyric acids are extended with two carbon units through the conversion of acetyl-CoA into HCap (Agler et al., 2011). Although ethanol has been considered as the main electron donor in biologically-mediated processes of HCap formation, particularly for the microorganism *Clostridium kluyveri* (San-Valero et al., 2019), lactic acid has also been recently reported in the literature as electron donor in the HCap synthesis (Cavalcante et al., 2017). Nowadays, in industrial processes, HCap is mainly extracted using coconut or palm kernel oils as starting materials. The low fraction of these oils in starting materials (less than 1%) contributes to keep the HCap prices high in the market (0.55 € per kg), and restricts its applicability.

A significant use of VFA and MCCA is as carbon source for polyhydroxyalkanoates (PHA) production (Domingos et al., 2018). PHAs are a large family of biopolyesters with potential characteristics to be considered as substitutes for traditional petroleum-based plastics (Blunt et al., 2018). The production of PHA using mixed cultures and substrates from wastes can decrease the cost of production by 50% (Fauzi et al., 2019). Several studies have demonstrated the feasibility of the fermentation of different agroindustrial wastes, such as cheese whey or sugar cane molasses, and urban wastes as sewage sludge (SS) as single substrates for producing PHA using mixed microbial cultures (MMC) (Pakalapati et al., 2018). On the other hand, cheese whey (CW) is the most abundant subproduct in the dairy industry. The high content of organic matter in the CW is mostly biodegradable and composed mainly of lactose (80%) and organic acids (lactic and acetic acids) (Calero et al., 2018). In recent years, several studies have been published using CW as a feedstock to produce HCap via reverse β-oxidation. Most of these approaches used lactic acid formed during the fermentation of CW or supplemented lactic acid or ethanol as electron donors to stimulate the chain lengthening process (Chwialkowska et al., 2019; Kucek et al., 2016). Even though VFA chain elongation is a promising process, the sustainability of this technology can be enhanced by reducing the addition of external electron donors and chemicals for pH control (Chen et al., 2017). Therefore, the co-digestion of CW with other organic wastes such as, for example, sewage sludge (SS) could reduce the addition of chemicals to keep the pH in a suitable range in the chain elongation process. Besides, the possible use of the fermented effluent obtained for PHA production, would avoid the downstream process of organic acids separation and purification.

This research evaluates the effectiveness of producing an effluent rich in HCap and other VFA by using a co-digestion process between CW and SS in an anaerobic lab-scale reactor. The effect of two different feeding regimes and three different hydraulic retention times (HRT) on the organic acids production was investigated. Besides, the variations in the microbiome composition during the different conditions applied on the acidogenic reactor were explored via high-throughput 16S rRNA gene-based sequencing. Additionally, the production of PHA using the organic acids stream as feedstock was evaluated in a fed-batch experiment using a mixed microbial culture with PHA accumulation potential.

2. Material and methods

2.1. Inoculum and organic wastes

The CW was collected from a cheese factory (INNOLACT S.L) located in Galicia (Spain). The CW was kept at 4 °C; however, in order to reflect real conditions as when CW is stored at the factory, volumes of CW were moved to the reactor feed bottle every three days. Furthermore, the SS comes from a secondary container of a WWTP in Carral (A Coruña, Spain) and was also stored at 4 °C and thickened previous to its characterization. The SS was thermally pretreated in autoclave at 120 °C for 20 min and 2 atm (Selecta P. Autester Mod. 437-G). The inoculum was an anaerobic digester sludge from a brewery WWTP. The main characteristics of the inoculum were total solids concentration (TS) of 97.97 ± 2.26 g kg^{-1}, volatile solids concentration (VS) of 87.93 ± 1.82 g kg^{-1} and a percentage of VS/TS of 89.76 ± 0.43%.

2.2. Laboratory scale reactor

A 6 L volume semi-continuous stirred anaerobic tank reactor was inoculated with 5.6 g VS L^{-1} of biomass. The feedstock was divided into 25% SS and 75% CW in terms of total chemical oxygen demand (COD). The selection of this feed mixing ratio was based on the results of the previous study of Iglesias-Iglesias et al. (2020), choosing the one that showed a synergistic effect on VFA production. The reactor stirring was set at 120 rpm and the temperature was 37 °C. pH was regulated through the addition of a NaOH solution (2 M). The different experimental phases are shown in Table 1. In the first and second operational periods (phases A1 and A2) the reactor was operated at 15 HRT and the effect of the number of feeding cycles per day was evaluated (one per day and two per day). Afterwards, the effect of three HRT (15, 10 and 6 days) was evaluated using two feeding cycles per day.

2.3. Fed-batch experiment

The suitability of the stream obtained during the acidogenic step for PHA production was tested in a fed-batch assay. The experiment was done in a 2 L reactor inoculated with an enriched biomass with PHA accumulation potential. The biomass enrichment was done in a 10 L sequencing batch reactor (SBR) under a feast-famine acclimation cycle, whose feedstock was provided by an acidogenic reactor fed only with CW (Lagoa-Costa et al., 2020). Information on the selection procedure and enrichment reactor is not detailed here, but is available elsewhere

Table 1
Comparison of the operational parameters applied during the different experimental phases.

Experimental phases	A1	A2	B	C
Substrate mixing ratio (% COD)	SS 25%: CW 75%			
Duration (d)	1–80	81–121	122–168	169–205
HRT (d)	15	15	10	6
T (°C)	37	37	37	37
pH	5.5	5.5	5.5	5.5
OLR (mg COD L^{-1} d^{-1})	1065–2420	1808–2442	1099–1511	979–1257
N° of feeding cycles/day	1	2	2	2

(Manuscript under submission).

Oxygen was provided by supplying compressed air at a flowrate of 1 vvm (vessel volume per minute) and the stirring speed was 150 rpm. Temperature was established at 30 °C, pH at 8. The fed-batch assay was carried out by adding successive pulses and without nutrients supplementation. A new pulse is added when the dissolved oxygen level increases indicating the total acid consumption. Once the fed-batch was completed, the extraction of the biopolymer was done as defined by Duque et al. (2014).

2.4. Analytical methods

Total and soluble COD (tCOD and sCOD), total and volatile suspended solids (TSS, VSS), TS and VS, as well as soluble ammonium and phosphorus concentrations were quantified according to Standard Methods (APHA-AWWA-WEF, 2017). The organic acids and lactose content were determined by HPLC using a Hewlett-Packard Serie 1100 equipped with Hi-Plex-H 300 × 7.7 mm column and two detectors, an ultraviolet and a refractive index detector, with detection wavelength at 210 nm. Sulphuric acid (0.05%) was used as mobile phase with a flowrate of 0.8 ml min^{-1} and the column temperature was kept at 30 °C.

2.5. Microbial community analysis

2.5.1. DNA sampling and extraction

The samples for microbial community analysis were taken by duplicate from the reactor at the end of each of the three HRT studied (operational phases A2, B and C). Inoculum, raw SS, and raw CW were also analyzed. The DNA was isolated through phenol/chloroform extraction technique (Alonso-Gutiérrez et al., 2009). Qubit and NanoDrop were used to determine the quantity and quality of DNA. The metagenomic analyses of V3 – V4 region of 16S rRNA gen were carried out by the company STABVIDA (Lisbon, Portugal). Illumina MiSeq platform was used for sequencing the genomic DNA. Library construction was performed using the Illumina 16S Metagenomic Sequencing Library preparation protocol (Amplicon et al., 2013). The DNA libraries were sequenced with MiSeq Reagent Kit v3 in the lllumina MiSeq platform, using 300 bp paired-end sequencing reads. Raw reads are available in the sequence read archive database (SRA) of NCBI under the BioProject PRJNA665375 with the accession numbers SAMN16253047, SAMN16253048, SAMN16253049, SAMN16253050, SAMN16253051 and SAMN16253052.

2.5.2. Sequencing data analysis

Raw sequencing data processing was performed with CLC Workbench software (V.8.0.2) with Microbial genomics module plug in (QIAGEN Bioinformatics, Germany). Standard quality filtering, OTUs (operative taxonomical units) clustering, taxonomical assignment (Greengenes v13_5 database) were carried out following the protocol described by Treu et al. (2018). Moreover, alpha and beta diversity were calculated. Through BLASTn platform (NCBI 16S ribosomal RNA database) the OTUs consensus sequences of the most abundant microbes were checked to verify the taxonomical assignment. Heatmaps and hierarchical clustering analyses were carried out taking into account the 50 most abundant OTUs (i.e. average relative abundance calculated on all the samples greater than 1%) and were completed using Multi experiment viewer software (MeV 4.9.0) (Saeed et al., 2006). Clustering was performed using Pearson correlation and complete linkage. For the purpose of comparing the microbial composition of the samples, a PCoA analysis based on weighted Unifrac pairwise distances matrices was performed following the protocol described by De Francisci et al. (2015).

2.6. Calculations

2.6.1. Degree of acidification

The Degree of Acidification (DA) was the parameter used to evaluate the acidogenic potential of the co-digestion, and it was defined as the sum of each individual organic acid produced in the effluent, expressed as COD equivalents, divided by the initial total COD added (tCOD initial).

$$DA(\%) = \sum Organic\ acids\_COD_{effluent} \Big/ tCOD_{initial} \quad (1)$$

2.6.2. Quality of the effluent

The Quality of the effluent was the parameter used to evaluate the percentage of the sCOD in the effluent represented by organic acids as COD.

$$Quality\ of\ the\ effluent(\%) = \sum Organic\ \ acids\_COD_{effluent} \Big/ sCOD_{effluent} \quad (2)$$

2.6.3. PHA and active biomass growth yield

The total PHA content, calculated as a percentage, was determined by dividing the PHA concentrations by the VSS concentrations. In the active biomass (X) calculation, the weight of the cellular PHA was subtracted from the VSS value, in addition to consider the ammonium concentration by considering that all ammonium was used only for growth. Storage ($Y_{PHA/S}$) and growth ($Y_{X/S}$) yields were calculated as the fraction of the formed product over the substrate (S) consumed. Specific substrate uptake rate ($-q_S$, Cmmol-S Cmmol-$X^{-1}h^{-1}$) and PHA storage rate (q_{PHA}, Cmmol-PHA Cmmol-$X^{-1}h^{-1}$) were determined from the linear regression of the experimental data of each concentration. X expressed in g L^{-1} was converted into Cmmol L^{-1} considering the generic chemical formula $C_5H_7NO_2$ (44.2 Cmmol g^{-1}).

3. Results and discussion

3.1. Acidogenic reactor experiment

With the purpose of studying the co-digestion of SS with CW and to enrich the effluent with HCap and VFA, an anaerobic reactor was operated during 4 operational phases which are summarized in Table 1. During the first and second operational periods (phases A1 and A2), the reactor was operated at 15 days HRT and the effect of the number of feeding cycles per day was evaluated (one per day and two per day). Afterwards, the effect of three different HRT (15, 10 and 6 days) was also evaluated.

The first phase of the operation of the reactor (A1) started after the inoculation and it was operated for 80 days (Fig. 1). During this phase, the reactor was fed once per day (one cycle every 24 h) and the organic loading rate (OLR) applied varied in the range of 1065–2420 mg COD L^{-1} d^{-1}. In stage A1, the concentration of organic acids varied between 2580 and 10049 mg COD L^{-1}, depending on the OLR applied (Fig. 1a). In Fig. 1b it can be observed that from the beginning of the operation of the reactor, acetic and lactic acids were produced as major acids of the VFA profile, reaching their maximum concentrations between days 59 and 72, when the OLR was 2420 mg COD L^{-1} d^{-1}. Besides, HCap was also present from the beginning of the research and its concentration followed a continuous increase until the end of this phase achieving a maximum concentration of 1750 mg L^{-1} the last day. On the other hand, *iso*-butyric and butyric acids concentrations were 300 and 600 mg L^{-1}, respectively, meanwhile propionic, valeric and *iso*-valeric acids were present at concentrations below 100 mg L^{-1}. During this phase, when the OLR applied was the highest, it was observed that lactose was not present in the effluent, however there was accumulation of lactic acid which was not successfully consumed and transformed, suggesting that the microorganisms effortlessly fermented lactose but needed extra time

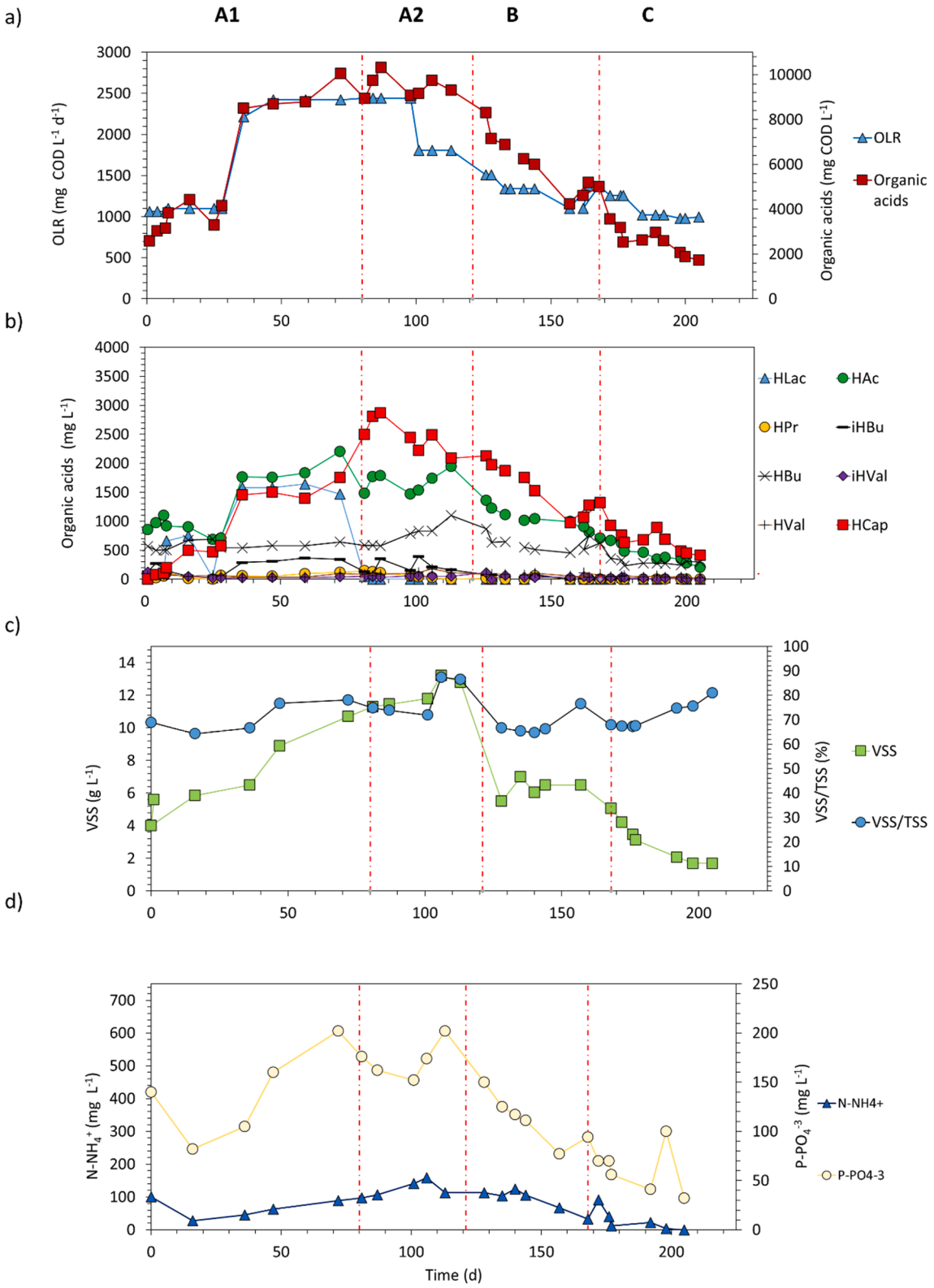

Fig. 1. Results during operational phases in terms of **(a)** OLR and organic acids production in COD terms, **(b)** organic acids profile **(c)** VSS concentration and **(d)** ammonium and phosphates concentration. **HLac:** lactic acid, **HAc:** acetic acid, **HPr:** propionic acid, **iHBu:** *iso*-butyric acid, **HBu:** butyric acid, **HVal:** valeric acid, **iHVal:** *iso*-valeric acid, **HCap:** caproic acid.

to convert lactic acid into other organic acids. The above results are in accordance with those of Duber et al. (2018), who reported that lactic acid accumulation in the reactor resulted in a reduction of HCap production.

Considering this fact, a new feeding strategy was proposed during phase A2. Meanwhile, during phase A1, the feeding was carried out once per day, during phase A2 the reactor was fed twice per day (every 12 h) keeping the OLR in a similar range to the A1 (1808–2442 mg COD L^{-1} d^{-}

^{1}), for the purpose to avoid a fast increase of lactic acid happening since the very beginning of the cycle. The concentration of organic acids reached was around 8934–10327 mg COD L^{-1} (Fig. 1a). The modification of the feeding regime drastically changed the organic acids profile, and HCap became the main product, followed by acetic and butyric acids, while lactic acid was completely utilized by the microbes (Fig. 1b). Interestingly, propionic acid continuously decreased during the entire duration of this phase until it was completely undetectable, confirming that the acrylate metabolic pathway was completely inhibited. These results are in line with those described by Kucek et al. (2016), which suggested the relationship between the inhibition of propionic acid formation and the absence of residual lactic acid in the effluent. During this phase, the highest HCap concentration was achieved (2871 mg L^{-1}), after 87 days of operation. Agler et al. (2012) proposed that at pH 5.5, which is similar to the pK_a of *n*-caproic acid (4.88), there is an accumulation of the undissociated form, which can have a toxic effect on the microbial cells and therefore on the chain elongation development. At this point the corresponding undissociated HCap concentration was around 5 mM. This concentration was less than the concentration previously hypothesized as inhibitory to the process (higher than 7 mM) (Cavalcante et al., 2017). Moreover, the presence of small quantities of *iso*-valeric and valeric acids, generated without the existence of propionic acid, was probably related to the fermentation of the amino acids belonging to the proteins present in the feed SS through the Strickland reaction (Iglesias-Iglesias et al., 2019). The VSS concentration and the OLR concomitantly increased, due to the VSS concentration of the feedstock and due to the growth of microorganisms which continued during phase A2, reaching a concentration around 13 g L^{-1} (Fig. 1c). Ammonium and phosphate concentrations are represented in Fig. 1d. Considering the increase of the HCap percentage in the organic acids profile, two feeding cycles per day were maintained throughout the rest of the experiment.

3.1.1. Effect of the HRT: Phases A2-B-C

At day 122, the HRT was decreased to 10 days and the operational phase B was started. Stage B was maintained until day 168 of operation, keeping the other operational parameters unchanged with respect to the previous stage (temperature 37 °C, pH 5.5 and two feeding cycles per day). When the OLR was between 1099 and 1511 mg COD L^{-1} d^{-1}, the organic acids concentration was in the range of 4237–8307 mg COD L^{-1} (Fig. 1a). By looking at the organic acids profile reported in Fig. 1b, it is evident that HCap was still the predominant one, followed by acetic and butyric acids. In addition, low amounts of valeric acid were observed. The decrease of the HRT did not affect the dominance of HCap in the acids profile, while propionic acid production continued to be inhibited. Starting from day 169, the HRT was changed to 6 days (phase C) until the end of the experiment. During stage C, the applied OLR varied between 979 and 1257 mg COD L^{-1} d^{-1}, and the organic acids reached values ranging between 1738 and 3559 mg COD L^{-1} (Fig. 1a). Regarding the acids profile, it remained very similar to phase B with HCap still remaining the most abundant one. Therefore, it can be concluded that the decreased HRT did not result in a reduction of the relative percentage of HCap in the organic acids profile. The results are in line with those obtained by Duber et al., (2018), where the production of HCap trough the fermentation of CW with mixed cultures had been investigated. In this study, the decrease of the HRT from 15 days to 10 and then to 6 did not affect to the dominance of HCap. According to the decrease of the HRT and the SRT, during this phase (6 days HRT) the VSS concentration decreased with respect to phase B (10 days HRT) (Fig. 1c). Besides, ammonium and phosphate concentrations were decreasing throughout this phase, as the OLR decreased (Fig. 1d).

It should be noted that during the entire duration of the experiment, which lasted 205 days, NaOH (2 M) consumption was lower than 2 L over the entire period, suggesting that the strategy of co-digestion between SS and CW can save important operational costs to carry out this process at industrial scale.

3.2. Comparison of the process efficiency among the different operational phases

The different parameters selected for the comparison of the process efficiency among the different operational stages applied in the acidogenic reactor are shown in Table 2. In phase A1, the average degree of acidification reached was 25% and the quality of the effluent was 75%. The HCap production rate was 0.16 g HCap_COD L^{-1} d^{-1}. In phase A2, with the change in the feeding regime, the DA increased up to a value of 30% and the quality of the effluent was80%. The HCap production rate increased up to 0.36 g HCap_COD L^{-1} d^{-1}. The change of the number of feeding cycles per day affected the percentage of lactic acid in the organic acids profile, which was completely consumed and transformed into caproic acid, which consequently doubled its percentage. However, the remaining percentages of acids were not affected.

Regarding the effect of the HRT on the reactor performance, it can be observed that decreasing its value from 15 to 10 days led to an increase in the DA from 30% to 44%, which was the maximum DA reached during the experiment. However, the subsequent decrease of the HRT from 10 to 6 days decreased the DA to 39%. Besides, the quality of the effluent was affected by the decrease of the HRT, dropping from 80% at 15 days HRT to 72% at 6 days HRT. The maximum effect on the HCap production rate was obtained by decreasing the HRT from 10 to 6 days, which resulted in a decreased value of g HCap_COD L^{-1} d^{-1} from a value of 0.34 to 0.24; despite this, it remained the most abundant acid of the SCCA profile (46%-45%).

3.3. Microbial community analysis

To figure out the effect of the HRT on the microbial community, a high throughput amplicon sequencing was performed on three samples collected from the reactor (R_15d, R_10d and R_6d). Samples were collected by duplicate at the end of the different operational phases: phase A2-15 days, phase B-10 days and phase C-6 days HRT, respectively. In addition, samples from the inoculum, the SS and the CW were also included in the sequencing. Due to the thermal pre-treatment of the SS at 120 °C for 20 min, it is highly probable that these microorganisms did not affect the microbial community dynamics of the reactor.

3.3.1. Microbial diversity in response to HRT changes in the reactor

Nearly 50% of the reads were taxonomically assigned (on average 39,000 per sample). This result reflects stringent criteria used for taxonomic classification. According to the rarefaction curves obtained the

Table 2
Results obtained in the different phases of the experiment including the DA, quality of the soluble effluent, caproic acid production rate and the percentage of each organic acid obtained in each one.

Experimental phases	A1	A2	B	C
HRT (d)	15	15	10	6
T (°C)	37	37	37	37
pH	5.5	5.5	5.5	5.5
OLR (mg COD L^{-1} d)	1065–2420	1808–2442	1099–1511	979–1257
DA (%)	25 ± 3	30 ± 5	44 ± 6	39 ± 7
Quality of soluble effluent (%)	75 ± 6	80 ± 8	74 ± 6	72 ± 4
Caproic acid production rate (g HCap_COD L^{-1} d)	0.16	0.36	0.34	0.24
HLac/Torganic acids (%)	23	0	0	0
HAc/Torganic acids (%)	31	31	30	29
HPr/Torganic acids (%)	2	1	0	0
iHBu + HBu/Torganic acids (%)	19	18	19	21
iHVal + HVal/Torganic acids (%)	3	3	3	4
HCap/Torganic acids (%)	22	46	46	45

sequencing depth was high enough to described the complexity of the microbiomes under investigation (Campanaro et al., 2018; Wu et al., 2021).

The diversity within the samples was measured as the Shannon index (Fig. 2a). In the inoculum, CW and SS samples, the Shannon index was quite different among them. SS presented the highest Shannon index (7.1) meanwhile the CW presented a highly enriched microbiome (Shannon index around 3.6). Regarding the reactor samples collected at different HRT, the Shannon index was around 4.7 for samples of 15 days and 10 days HRT, while the diversity of the microbial community increased, reaching the maximum level at 6 days HRT where the Shannon index was 5.5. The Shannon indexes presented in this study are higher than the results presented in similar studies using CW as single substrate for HCap production where the Shannon index was around 4 (Duber et al., 2020; Kucek et al., 2016). This fact is related with the addition of SS as part of the feedstock which directly influenced the diversity of the microbiome analyzes.

The PCoA plot is based on the first two principal coordinates (PCo1 and PCo2) and it explains 31% and 24% of overall of variance between the samples (Fig. 2b). According to the distance measured among samples in the PCoA, the results evidenced a similar bacterial community in the reactor with minor variations in the HRT microbiome between 15 days and 10 days and a higher variation respect to the 6 days HRT microbiome.

Fig. 3 reports the relative abundance of microorganisms based on the taxonomical classification of the microbial community at phylum level in all the samples (only OTUs with a relative abundance higher than 0.5% were considered). The inoculum was clearly dominated by the phylum *Proteobacteria* (42%) followed by the phyla *Firmicutes, Bacteroidetes* and *Nitrospirae* that have very similar relative abundances (8–9%). As previously reported these phyla frequently dominate samples collected from the anaerobic digestion systems (Campanaro et al.,

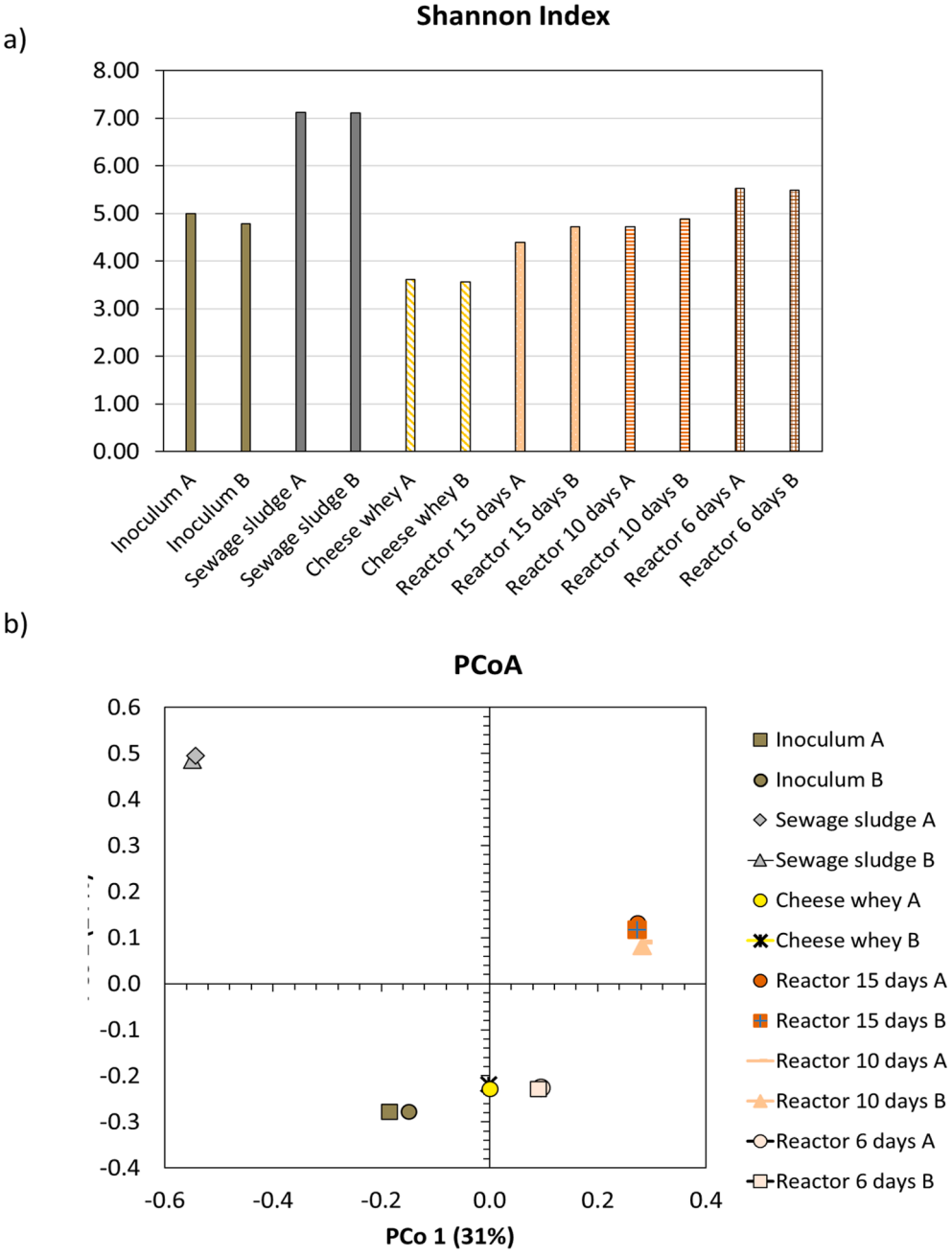

Fig. 2. **(a)** Shannon index and **(b)** principal component analysis (PCoA) of the samples.

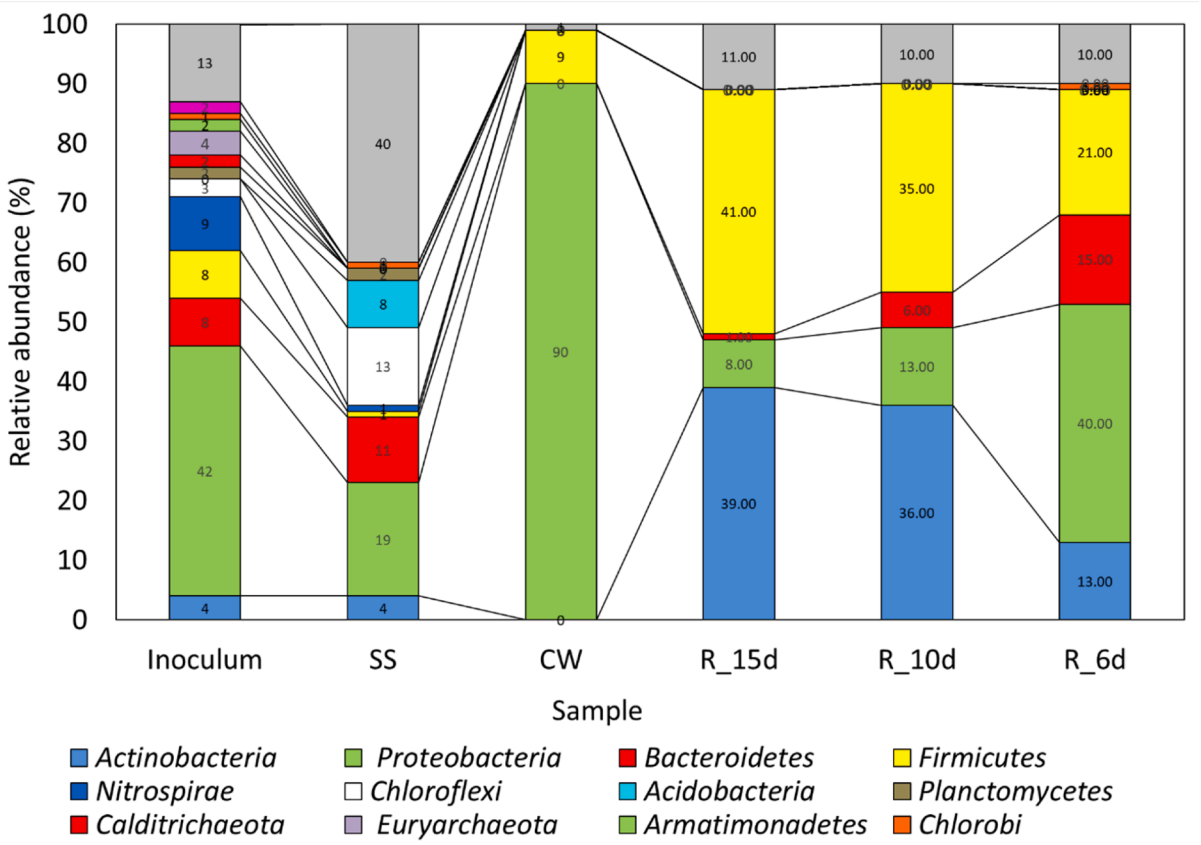

Fig. 3. Relative abundance shown as percentage of microorganisms from taxonomical classification of the microbial community in the samples determined at phylum level (greater than 0.05 OTUs of relative abundance).

2020). Other two quite abundant phyla are *Calditrichaeota* and *Actinobacteria,* identified respectively at 2 and 4% relative abundance. It has been demonstrated that these bacteria are able to discharge different enzymes in anaerobic granular sludge reactors (Doloman et al., 2019). In the case of the SS used as feedstock, the most abundant phylum was *Proteobacteria* with a relative abundance of 19%, followed by *Cloroflexi, Bacteroidetes,* and *Acidobacteria*; meanwhile the CW microbiome was clearly dominated by *Proteobacteria* representing more than 90% of the OTUs. On the other hand, the general trend of the three samples of the acidogenic reactor performance confirmed that the predominant phyla in the fermentation of the organic matter are *Actinobacteria, Firmicutes, Proteobacteria* and *Bacteroidetes*. These results are in agree with earlier research which reported their ability to perform the anaerobic digestion using a range of different substrates (Guo et al., 2015). Nevertheless, it can be observed that the relative abundance of the phyla in the different operational phases of the reactor changed drastically from the samples R_15d and R_10d to the sample R_6d. The samples R_15d and R_10d were clearly dominated by the phyla *Firmicutes* and *Actinobacteria* having similar abundances and representing more than 80% of the OTUs. However, the microbiome in R_6d was dominated *Proteobacteria* followed by *Bacteroidetes*. These results showed that the decrease on the HRT led to a decrease of the phyla *Actinobacteria* and *Firmicutes*, and this trend was even more important comparing the samples collected in reactors operated at 10 and 6 days HRT. These four phyla have been widely reported in studies about the VFA production using CW or SS as separate substrates (Iglesias-Iglesias et al., 2019; Lagoa-Costa et al., 2020). Moreover, as was expected, the phylum *Euryarchaeotata* which includes the most important methanogens of the anaerobic digestion system, completely disappeared in the reactor confirming a successful acidogenic microbiome enrichment.

In order to have a clear representation of the modifications occurring during the different stages of the reactor performance, a heat-map was elaborated taking into account the 50 OTUs with the highest relative abundance in all the samples, upper than 1% in at least one sample (Fig. 4). Analyzing the microbial community dynamics inside the reactor, seven main OTUs clusters were observed that showed different behavior and were influenced in different ways by changes in HRT.

OTUs present in "cluster 1" have an inverse correlation with HRT and increased their relative abundance at the decrease of HRT (from R_15d to R_10d and from R_10d to R_6d). This cluster was composed by OTUs assigned to *Cloacibacterium normanense , Pseudomonas sihuiensis* and *Acinetobacter johnsonii*, which were present in the raw substrates used as feedstock and therefore, their increment can be related with the loss of part of the microbial community enriched at 15 days HRT and with the increment of the microbial community added with each feeding. However, some of the OTUs of this cluster were not observed in the raw SS as for example *Nguyenibacter vanlangensis* and *Komagataeibacter intermedius,* which presence was previously reported in WWTP (Lin et al., 2016; Vu et al., 2013); this finding suggests that bacteria present in the SS used in the feedstock varied in each lot of SS used. On the other hand, some anaerobic bacteria were enriched with the decrease of the HRT in the reactor. For example, *Bacteroides luti*, which underwent a twofold increase at every HRT decrement, reaching more than a 1% relative abundance in R_6d. This species, previously described as a sugar-fermenting bacteria, has been also identified in an anaerobic reactor performing SS treatment (Hatamoto et al., 2014). With the two-step decrease in the HRT, an OTU tentatively assigned to the specie *Faecalibacterium prausnitzii* increased; interestingly this species was recently reported as butyrate producer involved in degradation of carbohydrates and proteins (Fu et al., 2019).

The second cluster includes the OTUs remaining unchanged during the shift from R_15d to R_10d and increasing in relative abundance more

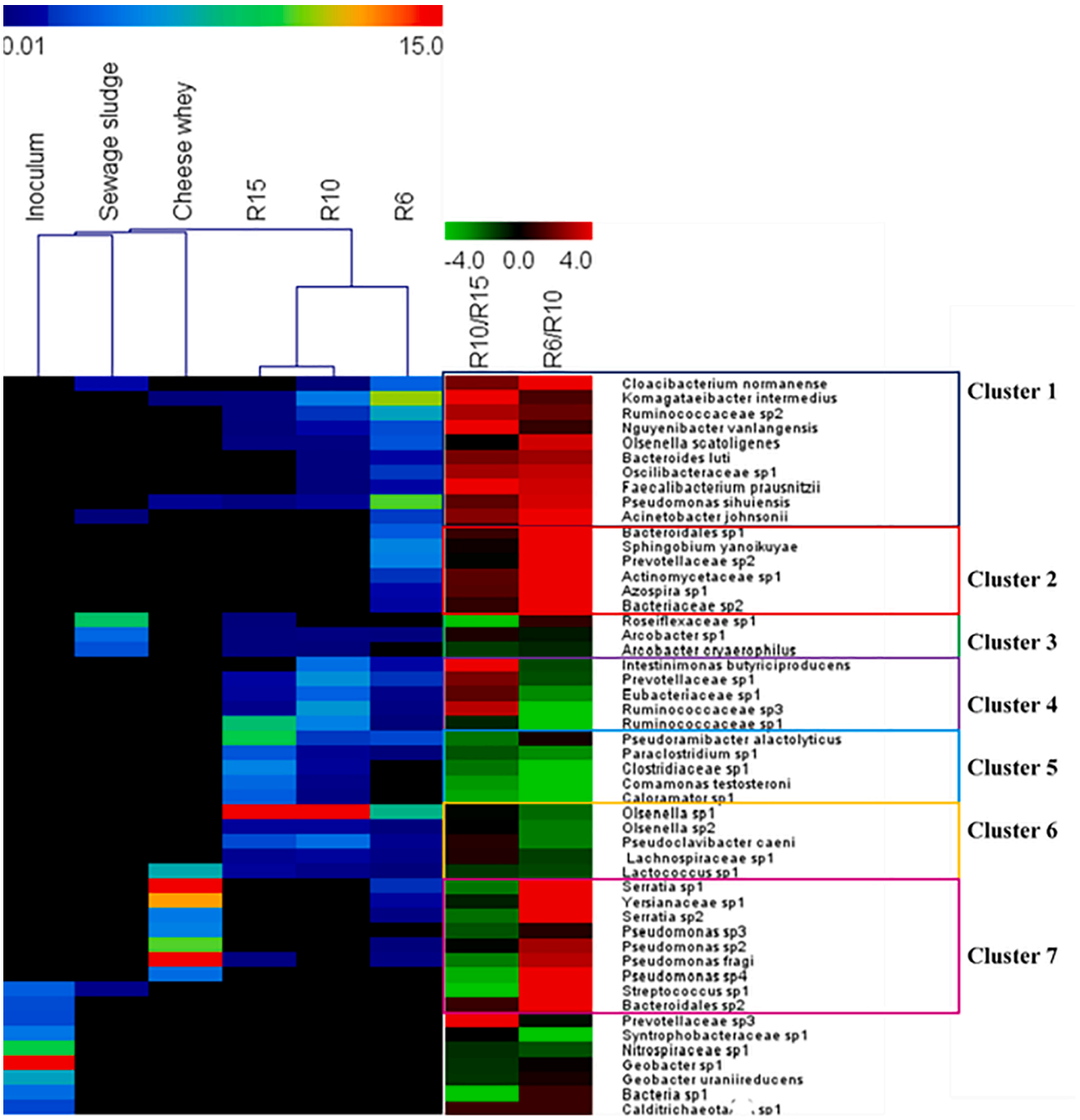

Fig. 4. Heatmap of the average relative abundance of 50 OTUs most numerous in all the samples. At the top of each graph are the color scales. On the left side, the most abundant microorganisms are identified in red and the least abundant in black and blue. On the right side of the graph, the part colored red reflects the increase in relative abundance at fold change, the decrease in relative abundance is indicated in green. If there is no fold change, it is indicated in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

than two folds at R_6d. These OTUs were assigned to microorganisms typically present in the SS (*Actinomycetaceae* sp.*1, Azospira* sp.*1* and *Sphingobium yanoikuyae)* but they were absent in the SS the OTUs assigned for the SS used as feedstock. The third cluster includes the OTUs having high abundance in the SS such as *Roseiflexaceae* sp.*1, Arcobacter* sp.*1* and *Arcobacter cryaerophilus*; these OTUs experienced a decrease in relative abundance during the reactor operation.

The microorganisms related to the VFA and HCap production were englobed in the cluster of microorganisms that increased, not changed or decreased from R_15d and R_10 d, but all of them decreased from R_10d to R_ 6d (clusters 4, 6 and 5, respectively).

The "cluster 4", includes OTUs increased from R_15d to R_10d, such as for example *Intestinimonas butyriciproducens. Prevotellaceae* sp. *, Eubacteriaceae* sp. *, Ruminococcaceae* sp. *1 and Ruminococcaceae* sp. *3*. Members of the family *Ruminococcaceae* have been reported as one of the dominant bacteria in the studies describing the production of caproic acid using lactic acid as electron donor (Contreras-Dávila et al., 2020; Duber et al., 2020). Moreover, members of the *Eubacteriaceae* family were also confirmed as MCCA producers from lactic acid (Scarborough et al., 2018).

On the other hand, *Intestinimonas butyriciproducens* has been related with the butyrate production from aminoacids and it is linked with the previous mentioned *Faecalibacterium prausnitzii* another butyrogenic species being both of them within *Clostridium* cluster IV (Bui et al.,

2016). In the previous studies describing the production of SCCA and MMCA from CW fermentation, these two microorganisms were not identified, showing that SS addition (a substrate enriched in proteins) strongly influenced the presence of these bacteria and their role in VFA production and in the biosynthesis of intermediates involved in the subsequent chain elongation.

The "cluster 5" includes OTUs decreasing in abundance along with the reduction of the HRT. Among these OTUs, *Pseudoramibacter alactolyticus* was present at relative abundance of nearly 9% in R_15d and it decreased to a relative abundance of nearly 2% at R_10d and R_6d. This specie has been previously reported as a SCCA and MMCA producer (Scarborough et al., 2019). Other OTUs assigned to this cluster are related to *Paraclostridium sp,* which has been reported as organic acids produces from soluble sugars (Silva Rabelo et al., 2020*)* and to *Clostridiaceae sp*. and *Calormator* sp.*,* which are able to ferment sugars and aminoacids for VFA production (Iglesias-Iglesias et al., 2019; Rubiano-Labrador et al., 2013).

In "cluster 6", OTUs were assigned to genera *Olsenella* and *Lactococcus,* among others. *Olsenella* sp.*1* was the most abundant OTU reported in samples R_15d and R_10d. These results are consistent with those of Duber et al. (2018), which reported *Olsenella* species as key organisms during caproate formation from acid whey fermentation; this was attributed to their ability to convert the lactose into lactate and acetate as the main products. *Lactococcus* sp.*1*, a well-known lactic acid bacterium, which was continuously imported in the reactor during feedstock addition, was identified during all the experiment. In addition, OTUs assigned to the genus *Caproiciproducens* were observed at a relative abundance higher than 1% only at R_10d; this genus was previously reported for its ability in converting lactate into n-caproate at pH 5.5.

The results obtained in this experiment confirmed the crucial role of the microorganisms associated to *Prevotellaceae, Olsenella, Ruminococacceae* and *Pseudoaramibacter* in HCap production through the co-digestion of CW and SS. All those organisms decreased their relative abundance when an HRT of 6 days was applied to the reactor, confirming the negative effect observed in the HCap production rate. Also, it was demonstrated that the use of SS as feedstock enriched the OTUs potentially involved in HCap production and this was confirmed by the presence of genera as *Faecalobacterium, Calormator and Intestimimonas* having proteolytic activity.

3.4. PHA production experiment

The suitability of the effluent from the acidogenic step for PHA production was evaluated in a fed-batch assay. The effluent was collected in the operational phase B. The feeding solution was an organic acids rich stream composed of 27.9% acetic, 1.4% *iso*-butyric, 17.9% butyric, 1.0% *iso*-valeric, 2.6% valeric acids, and 49.1% HCap. The fed-batch was planned to introduce a carbon concentration of 30 Cmmol L^{-1} in each pulse. As shown in Fig. 5a, the acids consumption occurs simultaneously with the PHA accumulation. The slow substrate

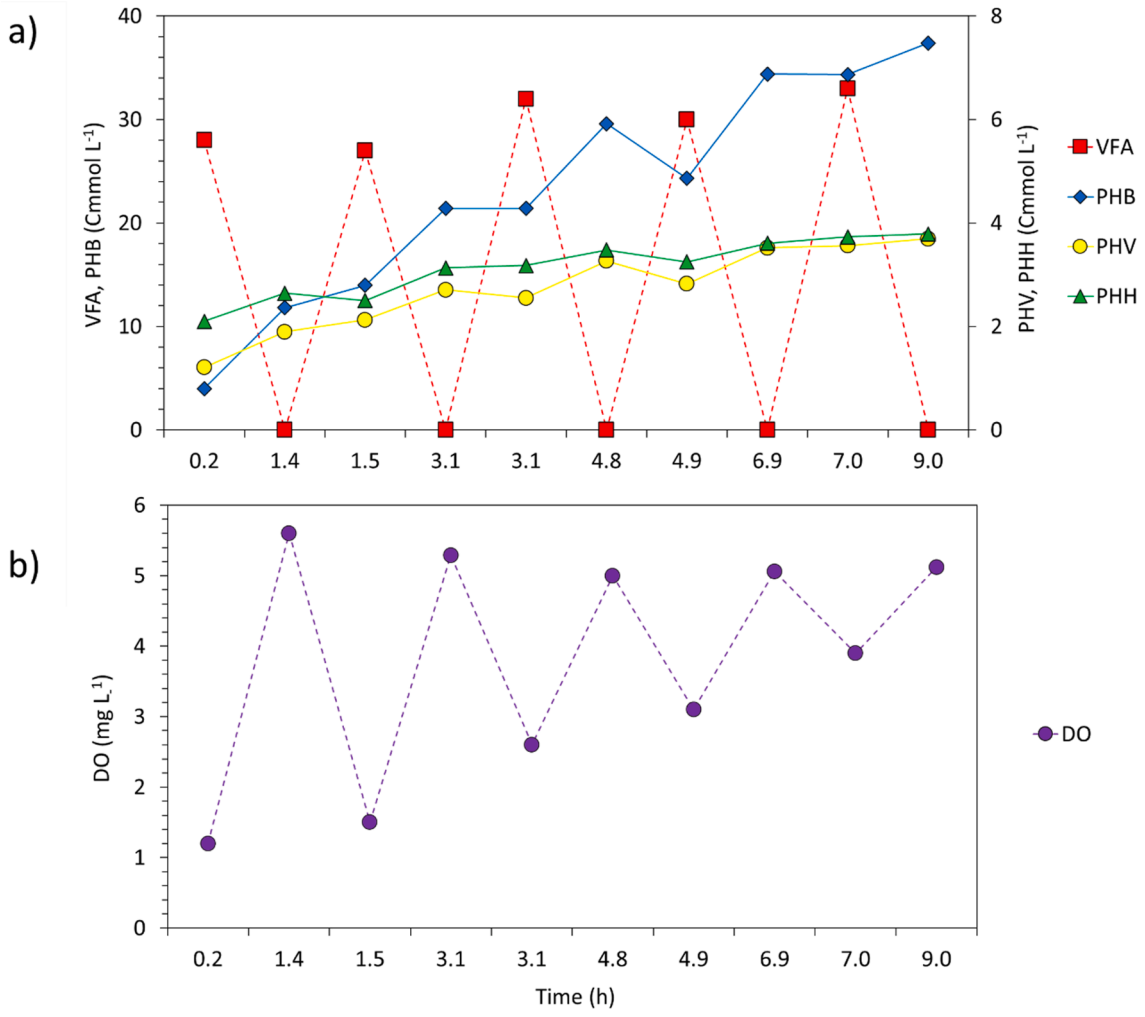

Fig. 5. (a) Volatile fatty acids (VFA), hydroxybutyrate (HB), hydroxyvalerate (HV) and hydroxyhexanoate (HH) concentrations profile during the fed-batch assay. (b) Changes in dissolved oxygen (DO) concentration during the fed-batch experiment.

consumption and no decrease of the dissolved oxygen (Fig. 5b) indicated that the sixth pulse was the last, consequently 150 Cmmol L^{-1} of organic acids were consumed. According to this, the YPHA/S was 0.25 Cmmol Cmmol^{-1} assuming that the whole carbon source consumed was used for PHA synthesis. The substrate uptake rate (-q_S) and PHA storage rate (q_{PHA}) were 0.189 and 0.065 Cmmol Cmmol$^{-1}h^{-1}$, respectively, decreasing pulse by pulse. The maximum PHA content reached was 32.5%, being affected by the nitrogen present in the feedstock. In each pulse, above 0.3 Nmmol entered in the reactor, increasing the biomass growth instead of accumulating PHA (Lagoa-Costa et al., 2017). The composition of the polymer was mainly formed by hydroxybutyrate (HB) (84.2%), and a minor part distributed between hydroxyvalerate (HV) (7.6%) and hydroxyhexanoate (HH) (8.2%). The literature focusing on the production of HH from MMC, with a non-synthetic substrate, is still scarce. Most authors report the production of HH from pure cultures and from a variety of carbon sources (Bhatia et al., 2019; Shi et al., 2020). Other authors reported the use of CW as a substrate to produce medium chain acids (Domingos et al., 2018). However, when those authors used the organic acid stream for subsequent PHA production, the biopolymer produced was not composed of HH. It was reported that PHA with higher molecular weights have better mechanical properties which contributes to their better applicability (Tan et al., 2020), and that the bonding of short and medium-chain-length monomers results in improved flexibility and softness compared to homopolymers (Nanni and Messori, 2020). Therefore, the use of the stream obtained in the acidogenic step of this study would be valuable for potential PHA production.

4. Conclusions

This study evaluated the effectiveness of producing HCap and other VFA using a co-digestion between CW and SS in a lab-scale continuous stirred reactor. The maximum degree of acidification was 44% and it was reached when the reactor was operated at 10 days HRT and with two feeding cycles per day. Under these conditions, HCap was the most abundant acid of the profile and the microbial community was dominated by *Olsenella* spp. and *Ruminococcaceae* spp. Additionally, the use of the effluent obtained for the production of PHA was verified with the accumulation of a copolymer mainly formed by HB, HV and HH.

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.

Acknowledgements

This study received funding from the European FEDER framework program CTQ2017-88292-R and from the Spanish Ministry of Economy and Competitiveness (MINECO), also by Xunta de Galicia under the grant agreement for Competitive Reference Research Groups (GRC) (ED431C 2017/66). The authors would like to thank the companies EMALCSA S.A., and INNOLACT S.L. for their contribution.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125388.

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