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KEYWORDS

agro-industrial waste – valorization – carboxylate platform – immobilized cells –chain elongation – volatile fatty acids – packed bed bioreactor

ABSTRACT

Volatile fatty acids (VFAs) were produced using cheese whey as feedstock. A mixed culture packed bed bioreactor was set-up to digest anaerobically under acidogenic condition a water solution of a cheese whey powder. Batch tests pointed out that the whole VFAs production process occurred via two sequential phases: (a) conversion of lactose into lactic acid; (b) conversion of lactic acid into a mixture of VFAs. Furthermore, the same tests showed that the ceramic material Vukopor S10 can be used as an effective support for cell immobilization in anaerobic fermentation processes.

The effect of the hydraulic retention time (HRT) and organic loading rate (OLR) were then studied in a bench-top bioreactor operated continuously. By a HRT of 6 days, OLR of 25 $g_{lactose}.L^{-1}.d^{-1}$ and pH 5.8-6, 16 $g.L^{-1}$ of total VFAs were produced, with a yield higher than 75% ($Cmol_{VFAs}.Cmol_{lactose}^{-1}$). Characterization with Illumina-based sequencing suggested that high VFAs productivities were obtained when microbial community structures developed in the biofilm reactor were highly enriched in few genera.

INTRODUCTION

Volatile fatty acids (VFAs) are chemically defined as short-chain length (C_2 to C_6) carboxylic acids, namely: acetic, propionic, butyric, valeric and hexanoic ones[1]. They are potential precursors for the obtainment of a variety of compounds such as ketones, aldehydes, esters, alcohols and alkanes[2]. Many important compounds - including traditional and new products - could be obtained through a route involving their utilization[3] either directly (e.g.: as reactants) or

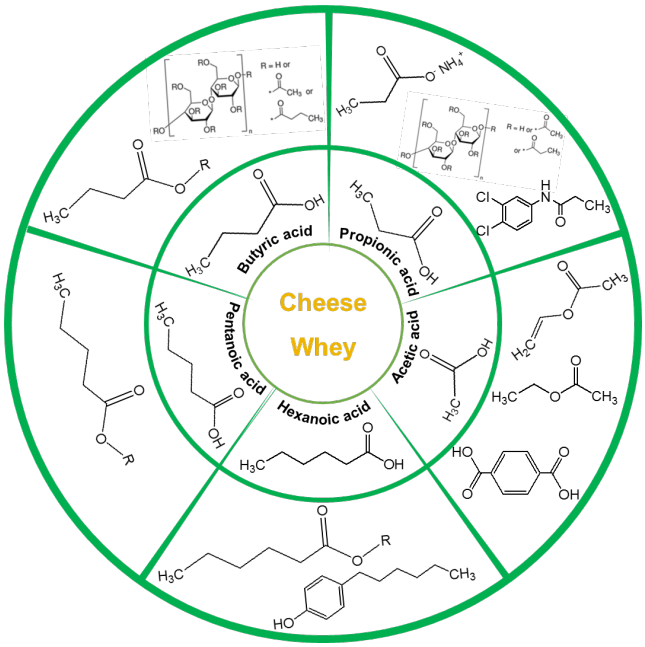

Figure 1: VFAs related products

indirectly (e.g.: as solvents) (**Figure *1***). As a result, VFAs are considered molecules of interest, some of them even as bulk products for the chemical industry.

At the present time VFAs are produced by means of petrochemical processes[4], but globalised concern about sustainability of production processes has prompted the development of alternative green technologies for replacing petrochemical-based ones. The main target of this approach is to achieve economically-feasible and environmentally-friendly processes by making use of renewable resources as feedstock. This could lead to the development of new industrial production routes, e.g., polyhydroxyalkanoates and single esters.

Anaerobic digestion represents the core biotechnology for the transformation of renewable resources into VFAs. This is an oxygen-free bioprocess fed with organic substrates (e.g., agro-industrial wastes), in which a consortium of microorganisms first transforms complex molecules into simple intermediate products -such as VFAs- and finally mineralizes carbon into its most oxidized (CO_2) and reduced (CH_4) form[5]. When methane production is inhibited (e.g., by setting low pH and/or high organic loading rates) VFAs tend to accumulate, accentuating the

methanogenesis repression[6]. This process is called acidogenic fermentation. Also pure cultures of acidogenic strains can be employed for the production of organic acids, achieving high yields, remarkable reproducibility and final product titers (50-180 $g.L^{-1}$)[7]. Nevertheless, this approach is typically expensive due to the request of high quality substrates and reactor sterilization costs. Alternatively, mixed microbial consortia can make profitable use of undefined leftover material occurring in agro-industrial wastes[8] without requiring sterilization. *Scoma et al*.[9] recently discussed some potential valorization platforms for high-impact agro-industrial residues of the Mediterranean area, which include olive mill wastewater, tomato pomace, grape pomace and cheese whey among others. *Adom et al*.[10] reported valorization strategies of dry-grind ethanol, which could supply the succinic acid and histidine industrial demands. Both these studies focus on the fact that many agro-industrial wastes are mainly composed of carbohydrates, proteins and lipids, which represent *per se* valuable compounds for many biotechnological processes. However, with few exceptions (e.g.: biodiesel glycerol, molasses and oils) where the substrate concentration is comparable to those of prepared feeding solutions, the organic content in these residues would not allow developing conventional pure culture fermentations with high productivities and titers. Nevertheless, they hold high chemical oxygen demands (COD; $30 < COD < 100\ gO_2.L^{-1}$), a strong acidic potential, possible anti-microbial activities and, therefore, slow biodegradability. As crops are subjected to seasonality, leftovers are generated within 2-4 month periods, and their massive discharge prior to any treatment may lead to eutrophication of water bodies and/or reduced fertility of cultivable soils. Anaerobic fermentation carried out by mixed microbial consortia (AFMC) can play a strategic role in changing waste treatment into a biotechnological valorization of carbohydrates, proteins and lipids hold by these residues, in order to generate added-value molecules such as VFAs.

Cheese whey (CW) is a common residue worldwide resulting from cheese production. As a rule of thumb, the production of 1 kg of cheese requires 10 kg of milk, 9 kg of which are set aside as CW by-product[11]. This means 191.7 M tons of CW generated per year worldwide according to cheese productivities reported by FAO (21.3 M tons.year^{-1})[12]. CW is composed by lactose (44-52 g.L^{-1}), proteins (6-10 g.L^{-1}, primarily β-lactoglobulin) and lipids (4-5 g.L^{-1})[13,14], has a high COD (50-102 gO_2.L^{-1}), and contains some hardly biodegradable proteins which require CW to be treated before being discharged[11]. Several studies about CW treatment were reported[11,15], including its valorization through the production of: H_2 [20,23,25], micronutrient fertilizers[15], VFAs[5,19,27,29,31] or biogas production using immobilized cells[16]. Regarding this last, cell immobilization has several advantages as compared to suspended cultures since: (i)- it prevents microbes washout in continuous process by separating dilution rate and hydraulic retention time (HRT), (ii)- it allows to work at higher cell density, (iii)- it confers an advantage for the microbial consortium against adverse conditions[13], and (iv)- it helps downstream processes as cells are retained within the support material and separated from the liquid.

Due to the many potential applications of VFAs the present work aimed at studying their production through AFMC. A packed bed bioreactor (PBBR) was proposed as the culture system owing to the advantages it represents for industrial application, due to the fact that: (a)- cell retention systems allow to operate at small volumes[27] while still processing large amounts of non-easily biodegradable wastes such as CW, and (b)- cell retention attained by immobilization is cheaper than by membrane bioreactors (MBR), which typically requires much energy for cell recycling[19]. The ceramic material Vukopor was chosen as immobilization support due to its low cost and good performances in the AFMC of olive mill wastewater[17,18]. The operational parameters HRT and lactose feed concentration were tested during the continuous process and

the outcomes in terms of total VFAs, VFAs mixture profile, productivities and microbial community structure were studied. This allowed to analyze how microbial community structures link to VFAs productivity and mixture profile[32,33].

Microcosm-scale experiments were first carried out to test CW bioconversion into VFAs and verify the possibility of employing the immobilization material. Afterwards, experiments at bench scale with PBBR were performed in order to evaluate the culture system performance during continuous operations.

MATERIAL AND METHODS

Substrate and inoculum. All tests were carried out using a liquid stream composed of water and cheese whey powder (CWP), courtesy of Lactogal–Produtos Alimentares S.A. (Porto, Portugal). The CWP was dried at the factory for animal feeding, composition reported by *Duque et al*[19]. Unless otherwise stated, CWP solutions were prepared by dissolving 20 g of powder in 1-L of distilled water (15 $g.L^{-1}$ of lactose)[19]; no other mineral or organic supplement was added.

The inoculum was a fully acclimated anaerobic acidogenic consortium obtained from a membrane bioreactor fed with the same CWP solution to generate VFAs at high yields[19].

Experiment at microcosms scale. Small-scale batch experiments were carried out in quadruplicate with both freely suspended and immobilized cells, in order to study lactose bioconversion and to verify the possibility of using the proposed ceramic support. Microcosms were prepared in 100-mL Pyrex bottles with a working volume of 50 mL (55 for immobilized condition); inoculated at 10% v/v in 45 mL of CWP solution. 12 g of Vukopor S10 (VK) (Lanik, Boskovice, CZ) porous cubes (12 x 12 x 9 mm^3) were used for cell immobilization[20]. Three preliminary sequential batches were carried out for the immobilized condition in order to allow

biofilm formation. Each of them lasted until all organic acid concentrations reached their *plateau* (16-14 days). Afterwards, liquid suspensions were centrifuged (8000 rpm, 6 ºC, 10 min), pellets re-suspended in 50 mL fresh CWP solution, and poured within the original corresponding bottles to start the consecutive batch. The former procedure was carried out maintaining anaerobic condition (nitrogen flux), and the packing material was not washed nor new inoculum was added between runs. The final experimental batch run was started just by replacing the liquid with fresh CWP solution. Incubation conditions were 37°C, pH 6 and 150 rpm, as reported previously by the inoculum donor[19]. Biogas and other metabolites production monitoring, and pH control were carried out following the same procedure reported elsewhere[21]. VK samples were withdrawn from microcosms at 0, 5 and 12 days of the last incubation for microbial community characterization and PCR-DGGE analysis carried out according to *Scoma et al.*[22]. Briefly, metagenomic DNA was extracted from liquid samples with the UltraClean Soil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) by using approximately 250 mg of pellet. PCR-DGGE analysis of the bacterial and archaeal communities was performed after amplification of the 16S rRNA genes with the primer pairs GC-357f/907r and GC-344f/915r, respectively, as described elsewhere[23].

Experiments with bench scale PBBR. An anaerobic acidogenic PBBR was set-up, as described by *Monti et al.*[24], for continuous process tests with immobilized cells. The PBBR consisted of a 1 L-glass column (5 cm of diameter and 40 cm high) filled with 120 ± 1 g of VK (PBBR-VK); ***Abstract figure*** and ***SI Figure S1***. A serpentine silicon tubing (S) connected to a thermostat was used to maintain the temperature at 37 ± 2 ºC. Peristaltic pumps (Masterflex) were used to feed CWP solution (P1), and recycle and mix the liquid phase (P2). The bioreactor effluent (transferred by overload) and gas were collected in a bottle (outlet), which was hydraulically

connected to a “Mariotte system”. The bioreactor had a working volume of 0.8 L (0.74 L liquid) and were inoculated with 20% v/v. It was operated initially under batch conditions for biofilm development (13-7 days) as in the experiment at the microcosm scale. Next to this, continuous operation start-up was done at a HRT of 6 days, high enough to guarantee an easy system adaption to changing from discontinuous to continuous operation. After 49 days, a third pump (P3) was implemented to continuously supply 10 M NaOH (flux manually tuned), which sensibly reduced pH variations (***SI Figure S3,*** HRT6_15).

Thereafter, AFMC experimental tests were carried out using the same CWP concentration as in the microcosms (15 $g_{lactose}.L^{-1}$) at HRT 6 and 4 days (hereafter referred to as HRT6_15 and HRT4_15, respectively). A third condition tested CWP at 25 $g_{lactose}.L^{-1}$ and 6 days HRT (hereafter referred to as HRT6_25). Liquid samples from PBBRs were taken every 2-3 days to analyse the metabolites concentrations. While sampling, the pH was manually controlled. Steady state condition was considered achieved when variations of total acids concentration and yield were lower than 20%. After at least 6 residence times PBBRs were sampled by taking out a VK sample. Cells were detached (VK sample, 20 mL ethanol, 25 ºC, 150 rpm for 3 hours), harvested (10000 rpm, 6 ºC for 10 min) and stocked (-20 ºC) for the microbial community analysis by high throughput sequencing using the Illumina platform[25].

Molecular analysis with Illumina. The total DNA was extracted from the pellets with 1 mL lysis buffer (pH 8.0) and 200 mg glass beads (0.11 mm, Sartorius) in a FastPrep®-96 instrument (MP Biomedicals, Santa Ana, USA) by bead-beating two times for 40 s (1600 rpm). The beads were removed by centrifugation (5 min, max speed), the DNA extracted from the supernatant with phenol-chloroform and precipitated with 1 volume ice-cold isopropyl alcohol and 1:10 volume 3 M sodium acetate (1 h; -20°C). Isopropyl alcohol was removed by centrifugation (30

min, maximum speed) and the DNA pellet dried and resuspended in 30 µL 1x TE buffer (10 mM Tris, 1 mM EDTA). Following the extraction procedure, the DNA samples were stored at -20°C prior to further analysis. The quality of the DNA samples was assessed by 1% (w/v) agarose (Life technologiesTM, Madrid, Spain) gel-electrophoresis and the DNA quantified by a fluorescence assay (QuantiFluor® dsDNA kit; Promega, Madison, USA) and Glomax®-Multi+ system (Promega, Madison, USA). Samples were then normalised to contain 1 ng DNA.μL^{-1} and sent to LGC Genomics (Germany) for library preparation and sequencing on an Illumina Miseq platform. Further details on the Illumina platform procedure, on amplicon sequencing processing, sequences classification and collector curves are provided in the ***SI DIMAI, Figure S2***. Search for type strains in highly productive PBBRs was carried out to check whether known bacteria were eventually enriched in the present study. Results are reported in ***SI Table S3***, and made use of the database RDP (rdp.cme.msu.edu), searching for isolated type strains with a sequence size ≥1200. A statistical analysis between high and low VFA-productive PBBRs was performed by using the results obtained during steady-state production phases (n= 10 to 15). The analysis stood on a 95% confidence interval (95% CI) calculated with a Student *t*-test with a two-sided distribution. The statistical significance was assessed using a nonparametric test (Mann–Whitney test) which considered a two-sided distribution with 95% CI.

Analytical procedures. The liquid samples were centrifuged (14000 rpm,25 ºC, 10 min), the supernatant separated, and diluted within distilled water (for HPLC) or oxalic acid (for GC) and finally filtered (0.45 µm cellulose acetate) prior to analysis. The lactose and lactic acid concentrations were determined by HPLC-IR (Agilent Technologies, Milano-Italy) according to what previously reported[26]. The VFAs concentrations were determined by GC-FID analysis (Agilent Technologies, Milano, Italy) as described previously[21]. The standard VFAs mixture

(Supelco) was purchased from Sigma–Aldrich (Milano, Italy). The biogas production volume was measured by using a graduated glass syringe in the microcosms experiments while in PBBR a "Mariotte" system was connected to the bioreactor outlet (***Abstract figure***). The biogas composition was measured as described previously[21]: microcosms were directly connected to the μGC (model 3000, Agilent technologies, Milano, Italy) while for PBBR the biogas composition was determined by collecting biogas samples in a 10 mL vial previously flushed with nitrogen gas. The COD of the feeding and effluent solutions, both soluble (SCOD) and total (TCOD), were measured with a commercial kit (AQUALYTIC Vario MR).

Calculations. The total VFAs yield in C-mol basis ($Y_{VFAs;C\text{-}mol}$) was calculated both by dividing the total C-moles of VFAs by the C-moles of lactose occurring in the CWP solution and in terms of COD[27] ($Y_{VFAs;COD}$); the total VFAs concentrations were calculated as COD equivalent by oxidation stoichiometry of the single acids (COD_{TVFAs}). Therefore the yields were calculated as follows $Y_{VFAs;COD} = \frac{COD_{TVFAs}}{SCOD_{Feeding} - (SCOD_{Exit} - COD_{TVFAs})}$

RESULTS AND DISCUSSION

Experiment at microcosms scale. The results of the AFMC of CW by freely suspended and immobilized cells are presented in **Figure *2 A,B*** and **Figure *2 C,D***, respectively. The first difference recorded was that the support implementation shortened the microbial conversion of lactose into VFAs from 19 to 12 days; this probably due to higher cell concentration in the

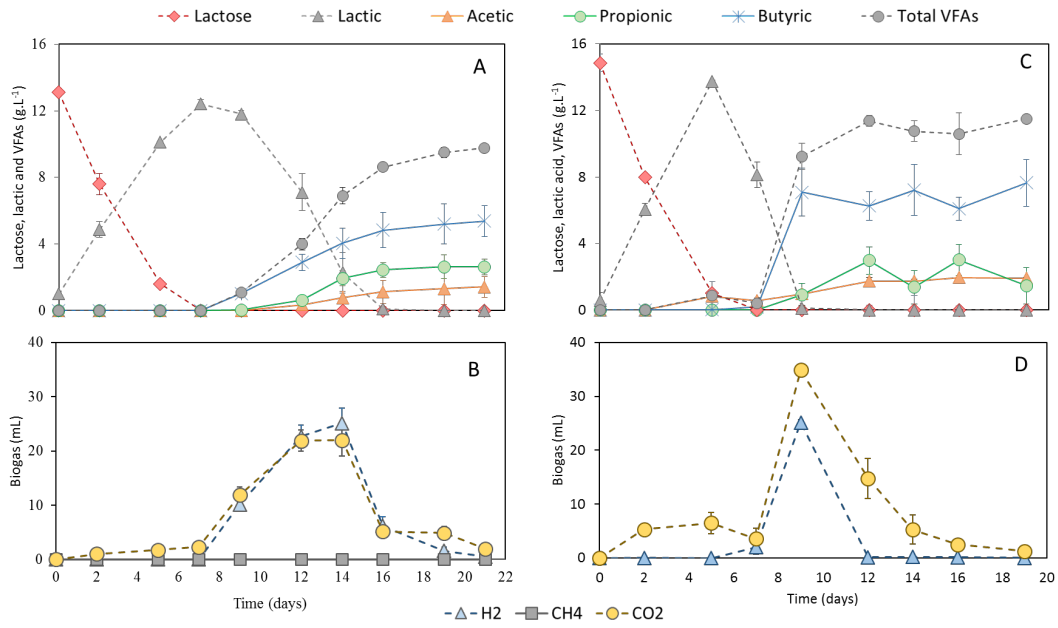

Figure 2: AFMC of CW at microcosm scale. Lactose, lactic acid, VFAs concentrations and biogas production trends for free suspended (A and B) and immobilized (C and D) cells conditions.

immobilized condition. Two distinct phases were detected in both conditions. Initially, lactose was converted into lactic acid (primary fermentation), whose concentration peaked at day 7 and 5 in freely suspended and immobilized cells, respectively. Afterwards, lactic acid was transformed into VFAs (secondary fermentation), being butyric, propionic and acetic acids the prominent ones. The VFAs accumulation was accompanied by a prompt increase in the production of a biogas rich in H_2 and CO_2 (**Figure *2 B,D***). No trace of CH_4 was detected even when the VFAs accumulation stopped. The H_2 maximum production was detected at day 12 and 9 in suspended and immobilized cells, respectively, finally accounting for 34 ± 1 and 36 ± 1 NmL H_2 . g^{-1} of lactose, respectively. As soon as all lactic acid was consumed the H_2 production dropped and the VFAs accumulation stopped (**Figure *2 A,B***). These results suggest that lactic acid was the major carbon source used for VFAs production during the secondary fermentation.

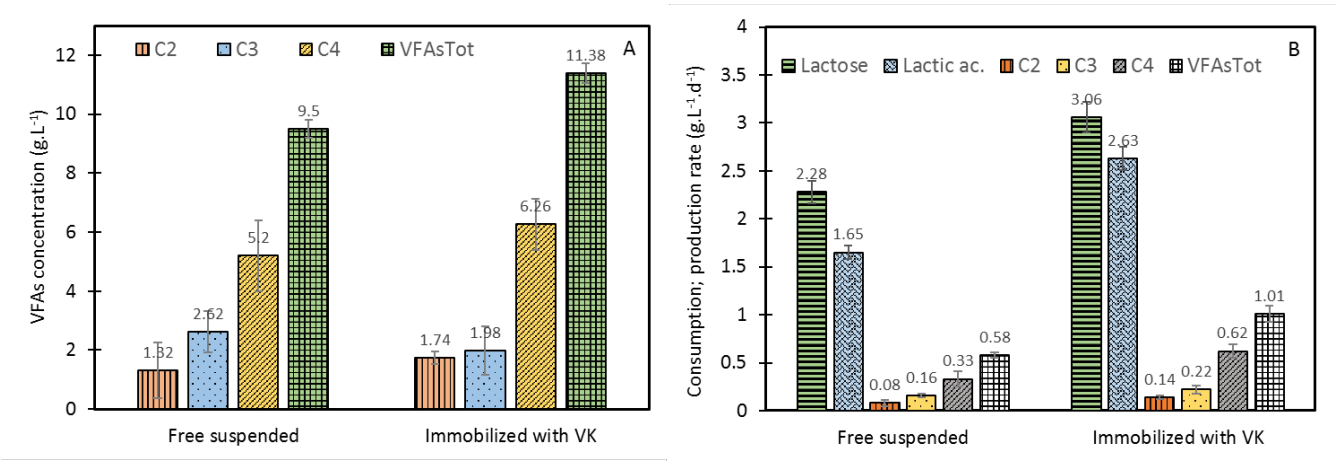

Figure 3: Microcosm experiment comparison between free suspended cells and immobilized cells. (A) Final VFAs concentration and (B) lactose consumption and acids productivities.

Provided that butyric acid was the most prominent VFA, its production through lactate oxidation may have been the leading pathway at these conditions[2]. The alternative pathway entails lactate reduction to propionate, which also yields some acetate. The present work does not allow singling out precisely the leading pathway, the molecular balance between lactate oxidation and reduction cannot be entirely justified by the obtained results. However, the higher butyric rather than acetic and propionic acid concentrations coupled with the high H_2 production rates are in agreement with findings obtained by *Davila-Vazquez et al*[28] for a condition with similar VFAs yield (0.75 g_{VFAs} . $g_{lactose}^{-1}$) and 4 times more H_2.

The total and individual VFAs concentrations were similar between the freely suspended and immobilized cell conditions (**Figure *3 A***), as well as the yields: 0.90±0.03 and 0.95±0.01 $Cmol_{lactic\ acid}.Cmol_{lactose}^{-1}$, and 0.89±0.01 and 0.86±0.05 $Cmol_{VFAs}.Cmol_{lactose}^{-1}$, both respectively. These lactic acid production yields are comparable to those obtained under batch conditions with pure cultures and freely suspended cells (*Lb. casei* NRRL B-441, L*b. bulgaricus ATCC 8001, PTCC 1332*)[34], but higher than those obtained with co-cultures (*Lb. helveticus & K. marxianu, Lb. bulgaricus & K. marxianus*)[35] and immobilized-cells (*L. bulgaricus*)[31]. These

comparisons confirm that lactic acid was produced from lactose and that the other minor fractions (i.e. proteins and lipids) did not contribute to lactic acid formation; otherwise the yields would have been significantly higher.

Concerning the total VFAs productivities, the values attained in both tests were higher (***Figure 3 B***) than those recorded by *Bengtsson et al*[27] for the AFMC of CW under batch conditions (0.32 g VFAs . $L^{-1}.d^{-1}$). Comparing lactose consumption and acids production rates, they were 1.3-1.9 times higher for immobilized cells (**Figure *3 B***), this showing that the proposed ceramic support can represent an effective cell immobilization material. Nonetheless, the lactic acid productivity obtained in this work was lower than what previously reported[31,34,35], likely due to low cells concentration (still requiring biofilm formation) and to the fact that no additional supplement was provided, such as manganese which is a component of the lactate dehydrogenase[11]; both reasons can positively impact on specific rates of CW anaerobic fermentation.

Experiments with bench scale PBBR. Immobilized cells microcosm experiments indicated that lactic acid and total VFAs productivities were 2.8 and 4.6 $g.L^{-1}.d^{-1}$, thus requiring a hypothetical HRT of about 5 days. Therefore, the impact of HRT on continuous AFMC of CW in PBBRs was tested at HRT 6 and 4.

The results obtained for condition HRT6_15 are shown in **Figure *4 A*** and **Table *1***. From day 0 to 49 the VFAs concentration and yield varied concomitantly with the pH (Figure S3, HRT6_15). From day 49 the pH was better controlled, this allowed to achieve the steady state after 11 days (from day 60). From there on, butyric, acetic and propionic acids were equally produced between 2.6 to 3.2 $g.L^{-1}$. Neither lactose nor lactic acid were detected all along the experiment.

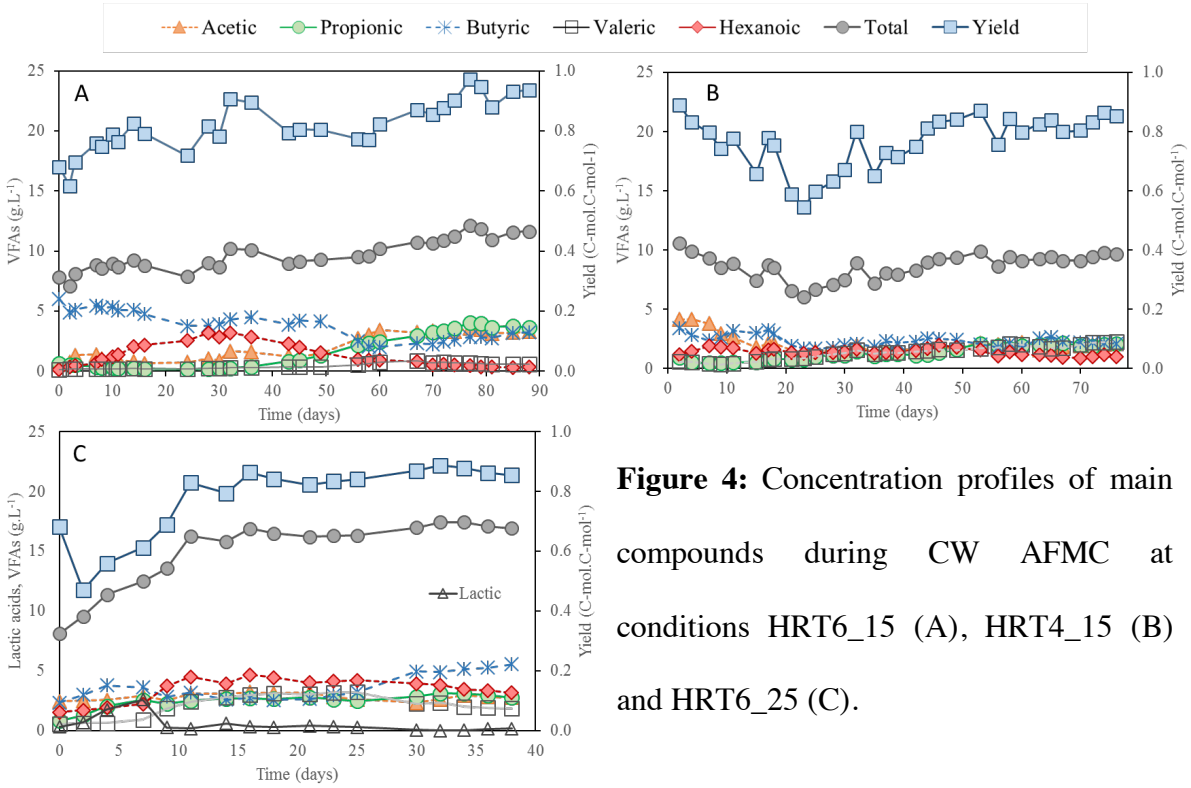

Figure 4: Concentration profiles of main compounds during CW AFMC at conditions HRT6_15 (A), HRT4_15 (B) and HRT6_25 (C).

The HRT was then shortened to 4 days (HRT4_15, **Figure *4 B*, Table *1***). The steady state was achieved after 44 days. Yield and total VFAs concentration remained almost constant for the following 32 days (equivalent to 8 residence times). The single VFAs concentrations were more equally distributed and an increase in productivity was attained as compared to HRT6_15. However, the total VFAs concentration was reduced (**Table *1***).

To enhance both productivity and VFAs concentration, a last test was performed where the CWP concentration was increased to 25 $g_{lactose}.L^{-1}$ (equivalent to 4.2 $g_{lactose}$. $L^{-1}.d^{-1}$) and the HRT was set back to 6 days (HRT6_25). High VFAs concentration facilitates the application of these processed effluents as renewable feedstock, as in the case of PHAs production employing either mixed or pure cultures[19,26]. Steady state VFAs production was achieved within 10 days (**Figure *4 C***) and yielded the highest total VFAs concentration of the present study (16.65 $g.L^{-1}$, **Table *1***). This value is higher than the ones reported by *Duque et al*. (7.05 $g.L^{-1}$) and *Bengtsson et al*. (3.08

$g.L^{-1}$)[27], but lower than that attained by *Davila-Vazquez et al*. (25.62 $g.L^{-1}$, with 46 $g.L^{-1}$ of lactose and extra supplemented nutrients)[28]. However, in the latter study the yield in VFAs per fed lactose was lower (0.56 g VFAs.$g_{lactose}^{-1}$). This indicates that an improvement of VFAs concentration may be attained also in the present PBBR system by further increasing lactose concentration in the feed, although its conversion efficiency might be affected.

The consumption of proteins and lipids (the minor fraction -25%- in CWP) could not be clear determined on the basis of COD balances ($[SCOD_{IN}\text{-}COD_{IN,lactose}] - [SCOD_{OUT} - COD_{OUT,VFAs}]$) due to high uncertainty caused by error propagation (Table S2). Nonetheless, it can be said that at least 10-20% of that fraction was consumed in all tested conditions, probably for cell growth and acids production[36]. Regarding a potential inhibition from ammonia arising from protein digest, this was not considered because: it was not observed in previous MBR fermentation test using the same CWP (on the contrary, lack of ammonia was reported)[19] and because it should have been noticed when increasing the CWP concentration for condition HRT6_25. Comparable Y_{VFAs} were obtained with respect to other VFAs production processes carried out under continuous operation mode. In the present study the Y_{VFAs} values ranged between 0.79-0.90 $g_{COD}.g_{SCOD}$, while *Duque et al.*[19] obtained 0.65–0.74 $g_{COD}.g_{SCOD}^{-1}$ with a membrane bioreactor, also comparable with what reported by *Bengtsson et al.*[27] (0.75–0.87 $g_{COD}.g_{SCOD}^{-1}$ at pH 6 and HRT 0.3–2.1 d). Y_{VFAs} obtained in the present work are higher with respect to those obtained in continuous fermentation processes using paper, wood or olive mill effluents as renewable feedstock[1].

Nonetheless, the present PBBR system needs to further improve its performance, especially in terms of immobilized biomass per reactor volume, since the obtained productivities were below those reported previously for the same CWP (7.05 $g.L^{-1}.d^{-1}$ [19]) and even more far away from an

expected industrial productivity of 106 $g.L^{-1}.d^{-1}$ [28], which however was obtained by employing CW supplemented with salts. On the other hand, the proposed system allowed to obtain hexanoic acid, which could represent a higher added value product and potentially easier to be separated[6]. In this respect, the remarkable relative increase of the hexanoic acid concentration with respect to the other VFAs in the HRT6_25 test suggests that higher lactose concentrations may yield interesting results also in terms of VFAs profile. Similarly, pH control may be considered to govern CW bioconversion pathways and steer microbial processes towards a different range of fermentation products. This was observed during the PBBR start up (HRT6_15, days 0 to 49, **Figure *4 A***) although it was not a variable studied in the present work. Indeed, the reduction on pH variations to less acid values caused an increase of the relative abundance of propionic acid and, at a lower extent, valeric acid, as opposed to butyric acid. This is consistent with the results obtained by *Bengtsson et al.*, who reported that acetic and butyric acids were mainly produced at pH 5-5.5, whereas a pH shift to a value of about 6 yielded propionic and valeric acids in a significant amount[27]. Propionic acid continued to be produced even at HRT4_15 (**Figure *4 B***), suggesting that pH has more influence than HRT.

Table 1: Main results describing the PPBR performances at steady state: acids concentrations (acetic,C2; propionic, C3; butyric, C4; valeric, C5; hexanoic, C6; and total acids VFAs), productivities (P_{Cx}), COD of the feeding and effluent solutions (COD_{Feed} and COD_{out}^{Liq}) and total acids yield ($Y_{VFAs/Lactose}$).

	HRT6_15 [a]	**HRT4_15** [b]	**HRT 6_25** [c]
C2 ($g.L^{-1}$)	3.32 ± 0.13	1.89 ± 0.19	2.90 ± 0.28
C3 ($g.L^{-1}$)	3.45 ± 0.48	1.85 ± 0.29	2.77 ± 0.22
C4 ($g.L^{-1}$)	2.64 ± 0.40	2.23 ± 0.26	3.65 ± 1.15

C5 ($g.L^{-1}$)	0.68 ± 0.10	1.87 ± 0.25	2.66 ± 0.49
C6 ($g.L^{-1}$)	0.51 ± 0.21	1.27 ± 0.37	4.03 ± 0.41
VFAs ($g.L^{-1}$)	11.18 ± 0.61	9.27 ± 0.35	16.65 ± 0.53
COD_{Feed}	21.43 ± 1.81		40.28 ± 1.44
COD_{out}^{Mix}	18.62 ± 1.01	19.27 ± 0.88	35.32 ± 3.54
COD_{out}^{Liq}	18.32 ± 0.77	17.59 ± 1.00	31.23 ± 4.68
$Y_{VFAs;COD}$ [d]	0.85 ± 0.08	0.81 ± 0.09	0.80 ± 0.13
P_{C2} ($g.L^{-1}.d^{-1}$)	0.55 ± 0.02	0.47 ± 0.05	0.48 ± 0.05
P_{C3} ($g.L^{-1}.d^{-1}$)	0.58 ± 0.08	0.46 ± 0.07	0.46 ± 0.04
P_{C4} ($g.L^{-1}.d^{-1}$)	0.44 ± 0.07	0.56 ± 0.07	0.61 ±0.19
P_{C5} ($g.L^{-1}.d^{-1}$)	0.11 ± 0.02	0.47 ± 0.06	0.44 ± 0.08
P_{C6} ($g.L^{-1}.d^{-1}$)	0.09 ± 0.04	0.32 ± 0.09	0.67 ± 0.07
P_{VFAs} ($g.L^{-1}.d^{-1}$)	1.86 ± 0.10	2.32 ± 0.01	2.78 ± 0.09
$Y_{VFAs/Lactose}$ [e]	0.90 ± 0.05	0.82 ± 0.03	0.85 ± 0.03

Unless otherwise stated, the reported results are the average of the replicates with standard deviation represented by ±. Average values for days: [a] 60-88, [b] 44-76 and [c] 11-38; [e] ($Cmol.Cmol^{-1}$). [d] Deviation calculated by error propagation.

Microbial community. All operating conditions shared a number of dominant genera (**Figure *5***). The potential relation in between the production of specific VFAs and the process microbial community structures was investigated (***SI Table S4***). When comparing the tested operating conditions in terms of the highest *vs*. the lowest butyric and hexanoic acid productivities (HRT6_25 *vs.* HRT6_15, gVFAs L^{-1} d^{-1}, ***SI Table S4***), the high productivity of these VFAs was associated with a loss of richness and evenness (***SI Table S4***), i.e., when microbial communities were highly enriched in few genera. The microbial community that mediated high butyric and hexanoic acids production (HRT6_25, **Table *1***) was dominated by the genus *Lactobacillus* (OTU0001), which was identified as the type strain *L. delbrueckii* (***SI Table S3***). This strain was

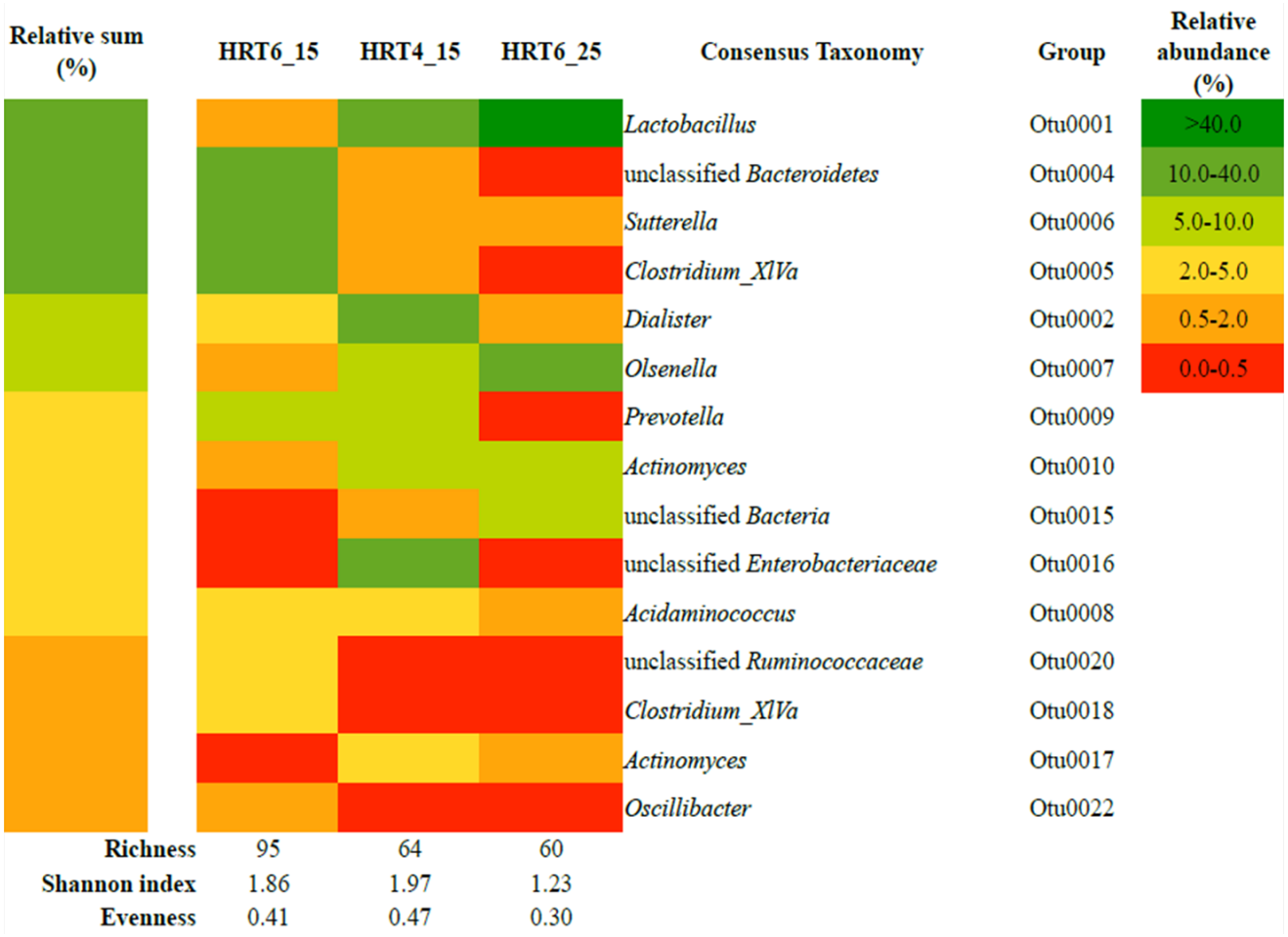

Figure 5: Predominant OTU representatives and their relative abundance in PBBR converting CW into VFAs.

the most abundant in all reactors (***SI File S1***) and was predominant in the CW feed (~98% of the sequences; ***SI Table S3***, ***SI File S1***). Other highly enriched genera were *Olsenella* (OTU0007), *Actinomyces* (OTU0010) and an *unclassified Bacteria* (OTU0015). All of them were not detected in the feed and inoculum (except *Olsenella* whose relative abundance was almost negligible; ***SI Table S3***; ***SI File S1***) and were poorly associated with known type strains (***SI Table S3***). When not related to type strains, they showed strong similarities with, respectively, *O. uli*, *A. hyovaginalis* and *Clostridium colinum* (≥0.99, ***SI File S1***). This result is consistent with data from microcosms experiments loaded with the supporting material, where *Lactobacillus* was the

predominant genera in the first 5 days of incubation (***SI Figure S4*** and ***SI Table S1***), that is during lactose to lactic acid conversion (**Figure *2*** C), while the second phase associated with VFAs production (**Figure *2*** C) was mostly enriched in *Clostridium* (***SI Figure S4*** and ***SI Table S1***).

The highest propionic and valeric acids producing PBBRs were also compared to the lowest producing ones, but no clear association in terms of richness or evenness was noted (***SI Table S4***). However, propionic acid productivity, which was the highest in HRT6_15 and comparable in HRT6_25 and HRT4_15 (0.46 $g.L^{-1}.d^{-1}$, **Table *1***), was associated with a reduction to 0.5-2.0% of the genera *Lactobacillus*, *Actinomyces* (both OTU0010 and OTU0017) and *Olsenella* (**Figure *5***), in concomitance with an increase in an *unclassified Bacteroidetes* (OTU0004), *Sutterella* (OTU0006), *Clostridium XIVa* (both OTU0005 and OTU0018), an *unclassified Ruminococcaceae* (OTU0020) and *Oscillibacter* (OTU0022) (**Figure *5***). Hence, all bacteria belonging to the class of *Clostridia* with the exception of the *unclassified Bacteria* (OTU0015) were associated with an increase in propionic acid productivity.

In conclusion, the PBBR turned out to be a robust culture system for the bioconversion of CW organic matter into VFAs. The process was monitored from both chemical and microbiological point of views. High bioconversion yields (>80%) and VFAs concentrations (up to 16 $g.L^{-1}$) were obtained by operating under different loading conditions and without nutrient supplement. The results obtained indicate that the process performance may be improved by increasing the OLR parameter ($4 < HRT \leq 6$ days; $CWP \geq 20$ $g.L^{-1}$), which in turn will impact on the microbial community structures by reducing the number of microbial representatives (mainly *Lactobacillus* and *Olsenella*).

ASSOCIATED CONTENT

Supporting Information.

Bench scale PBBR photo (**Figure S1**); Additional details on molecular analyses with Illumina (**DIMAI** and **Figure S2**); pH trends in the bench-scale PBBR experiments (**Figure S3**); Results obtained from DGGE analyses (**Figure S4** and **Table S1**); Results obtained for the microbial characterization with high throughput sequencing analysis (**Table S2**); Statistical analyses of hypotheses related with richness and evenness (**Table S3**).

A table showing sequences and identification of each phylotype from each sample (**File S1**).

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Notes

Any additional relevant notes should be placed here.

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ABBREVIATIONS

AFMC, anaerobic fermentation using mixed culture, VFAs, volatile fatty acids; HRT, hydraulic retention time; OLR, organic loading rate; AAD, anaerobic acidogenic digestion; COD, chemical oxygen demand; CW, cheese whey; PBBR, packed bed bioreactor; CWP, cheese whey powder; VK, vukopor; HPLC, high performance liquid chromatography; GC, gas chromatography; SCOD, soluble chemical oxygen demand; TCOD, total chemical oxygen demand; MBR, membrane bioreactor; OUT, operational taxonomic unit.

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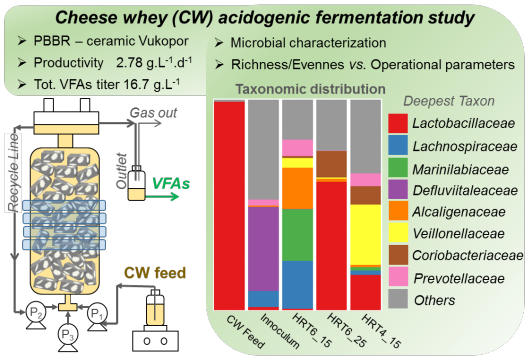

Carboxylic acids productivities, yields and compositions were related with different operational conditions and the resulted microbial consortium structures.