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Consecutive lactate formation and chain elongation to reduce exogenous chemicals input in repeated-batch food waste fermentation

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ABSTRACT

The production of biochemicals from renewables through biorefinery processes is important to reduce the anthropogenic impact on the environment. Chain elongation processes based on microbiomes have been successfully developed to produce medium-chain fatty acids (MCFA) from organic waste streams. Yet, the sustainability of chain elongation can still be improved by reducing the use of electron donors and additional chemicals. This work aimed to couple lactate production and subsequent chain elongation to decrease chemicals input such as electron donors and hydroxide for pH control in repeated-batch food waste fermentation. Food waste with adjusted pH was used as substrate and fermentation proceeded without pH control. During fermentation, lactate was first formed through the homolactic pathway and then converted to fatty acids (FA), mainly n-butyrate and n-caproate. The highest n-caproate carbon selectivities (mmol C/mmol C_{FA}) and production rates were 38% and 4.2 g COD/L-d, respectively. Hydroxide input was reduced over time to a minimum of 0.47 mol OH^-/mol MCFA or 0.79 mol OH^-/kg COD_{FA}. Lactate was a key electron donor for chain elongation and its conversion was observed at pH as low as 4.3. The microbiome enriched in this work was dominated by *Lactobacillus* spp. and *Caproiciproducens* spp. The high abundance of *Caproiciproducens* spp. and their co-occurrence with *Lactobacillus* spp. suggest *Caproiciproducens* spp. used lactate as electron donor for chain elongation. This work shows the production of n-caproate from food waste with decreased use of hydroxide and no use of exogenous electron donors.

1. Introduction

Sustainable production of biochemicals is a key factor to reduce anthropogenic contributions to climate change and to develop a biobased economy (Cherubini, 2010). The biobased economy is grounded in biorefinery processes that allow effective conversion of renewable materials into a broad spectrum of chemicals, materials and fuels. Numerous microbial chain elongation processes exist that are able to elongate single- or short-chain molecules into longer-chain molecules. One recently developed microbial chain elongation process makes use of anaerobic microbiomes to convert organic wastes into medium-chain fatty acids (MCFA, i.e., fatty acids with 6–12 carbons), such as n-caproate (Steinbusch et al., 2011). MCFA are preferred products over short-chain fatty acids (SCFA, i.e., fatty acids with <6 carbons) due to their higher energy content and their increased hydrophobicity makes MCFA easier to separate from the fermentation broth. MCFA are versatile compounds with different applications e.g., feed additives, bioplastics, lubricants, aviation fuels. Although chain elongation is a promising technology, the sustainability of the process can be improved considerably by reducing the use of electron donors and additional chemicals for pH control (Chen et al., 2017). Thus, electron donors with a more sustainable nature are needed to improve the environmental performance of the chain elongation technology.

Chain elongating bacteria can use a variety of electron donors as energy and carbon sources. In ethanol-based chain elongation,

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ethanol is metabolized by *Clostridium kluyveri* into acetyl-CoA and NADH. Electrons in NADH are used to drive the reverse β-oxidation (RBO) pathway elongating available acetate (C2) to n-butyrate (n-C4) by the condensation of two acetyl-CoA (Angenent et al., 2016). When enough ethanol is available, n-butyrate is condensed with another acetyl-CoA in a second round and elongated to n-caproate (n-C6) (Angenent et al., 2016). In lactate-based chain elongation, pyruvate and NADH are products of lactate oxidation. Pyruvate is further oxidized to acetyl-CoA and CO_2 with electrons released in form of reduced ferredoxin (Spirito et al., 2014). The derived acetyl-CoA are used for n-butyrate and n-caproate formation through RBO (Zhu et al., 2017). In this paper, we denote n-caproate as undissociated n-caproic acid and dissociated n-caproate together, and we refer to each specific form when appropriate. Bacteria possessing the RBO pathway have been enriched in MCFA-producing microbiomes fed with ethanol, lactate and sugars (with ethanol as intermediary) (Ding et al., 2010; Kucek et al., 2016a; Steinbusch et al., 2011). Recently, a few processes have been developed to produce n-caproate from organic waste streams which either contain electron donors for chain elongation (Kucek et al., 2016b) or were acidified to produce electron donors such as lactate (Nzeteu et al., 2018; Xu et al., 2018).

Lactate is produced by lactic acid bacteria (LAB) mainly from carbohydrates (Gänzle and Follador, 2012). During lactate production, the release of protons (eq. (1)) causes acidification of the medium and eventually stops microbial activity due to metabolism disruption or toxicity of undissociated acids (Kashket, 1987). In contrast, a net proton consumption occurs in lactate-based chain elongation due to a decrease in the total acids molar concentration (eqs. (2) and (3)). Therefore, the proton consuming behaviour of lactate-based chain elongation could be used to compensate for the protons released during lactate production in the acidification phase. Besides, propionate production from lactate, an important competing pathway in lactate-based chain elongation (Kucek et al., 2016a), could be restricted when coupling these two processes since LAB and lactate elongators are able to grow at $pH \leq 5.0$ (Kashket, 1987; Weimer and Moen, 2013) which can limit the occurrence of propionate producing bacteria (Hettinga and Reinbold, 1972; Janssen, 1991).

$$C_6H_{12}O_6 \rightarrow 2C_3H_5O_3^- + 2H^+ \quad (1)$$

$$2C_3H_5O_3^- + H^+ \rightarrow C_4H_7O_2^- + 2H_2 + 2CO_2 \quad (2)$$

$$3C_3H_5O_3^- + 2H^+ \rightarrow C_6H_{11}O_2^- + 2H_2 + H_2O + 3CO_2 \quad (3)$$

Therefore, the aim of this study was to evaluate the effectivity of producing n-caproate from food waste through chain elongation with *in situ* produced lactate via repeated-batch operation which would result in 1) no need of exogenous electron donor addition and 2) reduction of chemicals addition for pH control. With repeated-batch operation, a concentrated food waste was consecutively added over 3 cycles which enabled to increase the n-caproate concentration over time. Food waste fermentation was performed without pH control. Changes in the microbiome composition were evaluated using 16S rRNA gene-based metagenomic data.

2. Materials and methods

2.1. Experimental set-up and procedure

Food waste was used as the only substrate in this study and consisted of outdated food remainders. Food waste was obtained from a recycling company (Rotie, the Netherlands) and preserved at −20 °C for long-term storage. This was the same original food waste used in an earlier study in which microbially acidified waste was used to develop an effective ethanol-based chain elongation process (Roghair et al., 2018). Prior to use, the waste was thawed in a 4 °C room and kept at this temperature during the experimental work. The waste had a total solids content (TS) of 200.2 ± 2.2 g TS/L, volatile solids content (VS) of 179.4 ± 1.1 g VS/L and total acidity of 15036 ± 152 mg $CaCO_3$/L (Table S1). Chemical oxygen demand (COD) for the total (COD_T) and soluble (COD_S) fractions of food waste were 276.6 ± 52.5 g COD_T/L and 125.2 ± 0.6 g COD_S/L. Total carbohydrates were estimated to be 83.53 ± 4.19 g/L (in glucose equivalents). Some organic acids and alcohols were present in the waste e.g., lactate (18.64 ± 1.34 g/L), ethanol (4.41 ± 0.04 g/L), acetate (4.87 ± 0.43 g/L), propionate (1.92 ± 1.45 g/L), butyrate (0.36 ± 0.06 g/L) and valerate (0.20 ± 0.02 g/L).

Fermentation was carried out in a stirred tank reactor (1L-RBF) with working volume of 1 L which was inoculated with a microbiome obtained from previous food waste fermentation. This was the same reactor used in earlier chain elongation studies (Roghair et al., 2016). For the start-up, the 1L-RBF reactor was fed with a mixture of mineral medium, vitamins and food waste to reach a food waste concentration of 10% v/v. The pH of the mixture was adjusted to an initial value of 6.0. Mineral medium and vitamins were added only at the beginning of the experiment with concentrations as reported by Roghair et al. (2016), except that $(NH_4)H_2PO_4$ was reduced to 1.8 g/L. During 1L-RBF operation, concentrated food waste was adjusted to pH 6.0 with 4 M KOH before addition to the reactor to have comparable pH in the waste fed and fermentation was allowed to proceed without pH control. Food waste was added on days 0 and 15 at concentrations of 10% v/v and on day 32 at 15% v/v. Nitrogen gas was bubbled for 15 min after each substrate addition to ensure anaerobic conditions. The reactor was connected to a gas flow meter (μFlow, Bioprocess Control) to quantify gas production and temperature was 35 °C.

Duplicate experiments were performed using 500 mL Schott bottles with working volume of 300 mL to systematically assess repeated-batch fermentation (0.3L-RBF). Procedure in 0.3L-RBF experiments was the same as in 1L-RBF except that food waste was added at constant concentration of 10% v/v once lactate was depleted (every 7−8 days). In the same way, mineral medium and vitamins, were added only at the beginning of the experiment together with yeast extract (1 g/L). For the 0.3L-RBF experiment, inoculum was taken from the 1-L reactor on day 19 and added at 3% v/v. The same procedure as in 0.3L-RBF was followed in duplicate single-batch experiments except that food waste was added only once. Single-batch experiments were performed to evaluate the products distribution of long-term hydrolysis and acidification of food waste. The 0.3L-RBF and single-batch experiments were placed in a shaker at 120 rpm and 35 °C. To evaluate the effect of higher temperature on hydrolysis/lactate formation, duplicate experiments were operated likewise to 0.3L-RBF but at a temperature of 50 °C until day 17. The same mesophilic inoculum was mixed with thermophilic anaerobic sludge from a recycling company (Heerenveen, the Netherlands) and added (3% v/v) as inoculum for the thermophilic experiments. On day 17, temperature was switched to 35 °C for subsequent chain elongation. A modified cap was used for the Schott bottles to allow liquid sampling and gas was collected via connected gas bags.

Liquid and gas samples were taken regularly to measure metabolites, pH and gas composition. The following parameters were used to evaluate the performance of the process and for comparison with literature: substrate hydrolysis, estimated as the increase in measured COD_S between the beginning and end of each cycle divided by the TS in the added food waste (g COD_S/kg TS_{added}); conversion efficiency, regarded as the amount of COD in a given

product with respect to either COD_T or COD_S measured at the end of the cycle (g $COD_{product}$/g COD); carbon specificity, equivalent to the product/fatty acids ratio, in mmol C/L; hydroxide input, calculated based on the production of both MCFA (mol OH^-/mol MCFA) and total fatty acids (FA) (mol OH^-/kg COD_{FA}).

2.2. Analytical methods

Gas headspace composition, fatty acids and alcohols were determined by gas chromatography methods (Roghair et al., 2018). Fatty acids and alcohols measured in the soluble fraction of samples were: straight-chain fatty acids (C2–C8), isobutyrate, b-valerate (both 2- and 3-methylbutanoic acids together), isocaproate (4-methyl-pentanoic acid) and straight-chain alcohols (C1–C6). L-lactate, succinate and formate were measured in the soluble fraction of samples by HPLC (Thermo Scientific Dionex UltiMate 3000, Thermo Fischer) equipped with a refractive index detector (Shodex RI-71, Separations) and an Alltech OA-1000 column (length 300 mm; ID 6.5 mm). The column oven was maintained at 60 °C and 1.25 mM sulphuric acid was used as mobile phase at a continuous flow of 0.6 mL/min. The injection volume was 20 μL. Chromatography data were analysed with Chromeleon software (version 6.80 SR13). COD_S measurements were performed at the end of each cycle with LCK514 kits (HACH GmbH, Germany) after sample centrifugation (10,000 rpm, 10 min) and dilution of the supernatant. Raw experimental data is available in the DANS-EASY database (https://doi.org/10.17026/dans-z44-2z4d).

2.3. Microbiome composition analysis

Samples for microbiome composition analysis were taken (Fig. S1) from the fermentation broth, centrifuged at 10,000 rpm for 10 min and stored at −20 °C for DNA extraction and sequencing. DNA was extracted from the pellets applying a PowerSoil DNA isolation kit, according to the manufacturer's instructions. The isolated DNA was used as template for amplifying the V3–V4 region of 16S rRNA via Illumina sequencing using the primer sets described by Takahashi et al. (2014). This allowed simultaneous amplification of bacterial and archaean 16S rRNA. The sequences were deposited in the ENA database under accession number PRJEB33791. DNA sequences were processed with the DADA2 pipeline to identify ASVs (amplicon sequence variants) (Callahan et al., 2016). ASVs are alleged to be independent of reference databases, reproducible in future data sets and reusable across studies (Callahan et al., 2017). The sequences were submitted to the SILVA database for taxonomic identification (Quast et al., 2013). The entire ASV table was normalized using the cumulative-sum scaling (CSS) method which helps to avoid biases generated with current sequencing technologies due to uneven sequencing depth (Paulson et al., 2013). A Bray–Curtis dissimilarity matrix was calculated and used to build Principal Coordinate Analyses and Constrained Principal Coordinate Analysis, both retrieved from Phyloseq and Vegan packages (McMurdie and Holmes, 2013; Oksanen et al., 2019). To compare the differences in taxonomic composition and to assess whether some bacterial taxa were differentially abundant, we conducted a statistical analysis in which we assessed separately the read counts at ASV level using the metagenome Seq package (Paulson et al., 2013). With the coefficients from the model, we applied moderated t-tests between accessions and the differences in the abundance of taxa between accessions were considered significant when adjusted P-values were lower than 0.05.

2.4. Co-occurrence and identity networks

Network analyses were performed to assess the dynamics of the interactions in the microbiomes. Non-random co-occurrence analyses were performed using SparCC, a tool capable of estimating correlation values from compositional data (Friedman and Alm, 2012). Using Python, we calculated SparCC correlations between microbial taxa at ASV level based on the 16S rRNA extracted from the reads. Correlations with a magnitude >0.8 or < −0.8 and statistical significance ($P < 0.01$) were included in the network analyses. For the identity network, a multiple alignment sequences of approximately 400 bp was performed using Muscle v3.7 (Edgar, 2004) and used to calculate a pairwise distance with Clustal Omega - 1.2.3 (Sievers et al., 2011) for all detected 16S rRNA sequences and thresholds were settled at 0.9 and 0.95 for the clustering. Networks visualizations were constructed using Cytoscape (v. 3.7.1) (Shannon et al., 2003). More information on the DNA analysis and networks construction can be found in the Supplementary Data.

3. Results

3.1. Microbiome can be steered to produce n-caproate as the dominant product

Fermentation of complex substrates (e.g., food waste) may result in a mixture of products (fatty acids, alcohols, diols, methane) when using microbiomes. In this study, the main products were fatty acids with increasing overall length over time. The operation of the 1L-RBF was divided in three cycles. Hydrolysis, acidification and chain elongation were expected to occur in each cycle. No methane was observed during 1L-RBF operation (Fig. S2). During the first cycle, we could not detect any lactate formation from food waste. Instead, the lactate present in food waste was consumed within two days for SCFA production. Mainly acetate (C2) and n-butyrate (n-C4) accumulated (~2 g/L by day 15) and pH decreased from 6.0 to 4.7 (Fig. 1). Other fatty acids such as propionate (C3), n-valerate (n-C5) and n-caproate (n-C6) were detected at concentrations below 500 mg/L. After a second addition of food waste in cycle 2, lactate was again consumed within three days and a further increase of n-butyrate and n-caproate was observed. n-butyrate and n-caproate concentrations increased ~1.7 and ~3-times, respectively, whereas acetate and propionate concentrations were stable (Fig. 1) as well as pH which remained between 4.9 and 5.0.

In contrast to the first two cycles, lactate production was observed when increasing food waste concentration to 15% v/v in cycle 3. Lactate was detected at a maximum concentration of 4.5 g/L and was subsequently consumed within 5 days (Fig. 1). n-caproate was the main product during lactate consumption with n-caproate concentrations displaying a ~5-fold increase from 1.2 ± 0.03 (days 29 and 32) to 5.4 ± 0.01 g/L (days 39 and 41); which equals to 11.9 g $COD_{n\text{-}C6}$/L. The concentrations of SCFA increased to a lesser extent, ~1.3 times for acetate and ~1.6-times for n-butyrate. In cycle 3, n-heptanoate (n-C7) and n-caprylate (n-C8) were detected at a maximum concentration of 89 and 142 mg/L, respectively. During the period of lactate production, pH went down from 5.1 to 4.7 and increased back to ~5.3 when lactate was consumed. Overall, changes in pH and metabolites profile indicate that two separate phases occurred: an acidification phase towards lactate as the main product followed by a chain elongation phase where n-caproate production was favoured the most (Fig. S3). The synthesis of n-caproate in cycle 3 seemed to be linked to lactate as electron donor. Hydrolysis was estimated to be 20, 265 and 96.7 g COD_S/kg TS_{added} for cycles 1, 2 and 3, respectively. These values are comparable to those obtained by Bolaji and Dionisi (2017) who observed a maximum substrate degradation of ~283 g COD_S/kg TS_{added} when vegetables waste was fermented to a mixture of SCFA and MCFA. The highest n-caproate production rate of 1.89 g/L-d (4.2 g $COD_{n\text{-}C6}$/L-d) (days 34–36) was measured in cycle 3 while the overall n-

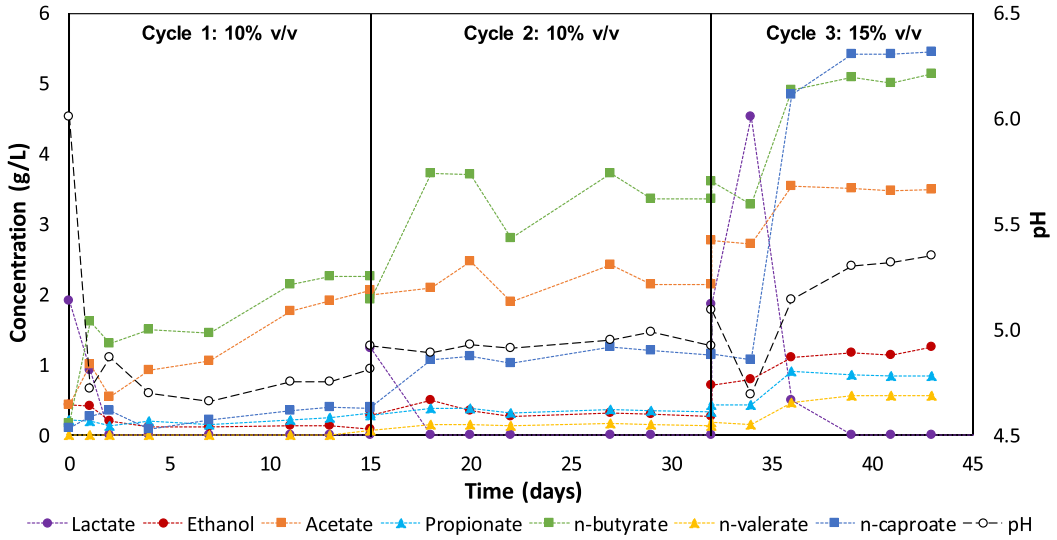

Fig. 1. Profile of metabolites and pH during 1L-RBF operation. Potential electron donors (circles); even-chain fatty acids (squares); odd-chain fatty acids (triangles). Solid vertical lines show the days of substrate addition.

caproate production rate in this cycle (including hydrolysis, acidification and chain elongation; days 32–39) was 0.61 g/L-d (1.35 g $COD_{n\text{-}C6}$/L-d) (Table S2).

Process efficiency was evaluated in terms of COD and carbon conversion. In 1L-RBF, between 25 and 34% of the COD_T added as food waste was identified in the measured metabolites (Fig. S4) which equals to 50–80% of COD_S. Conversion efficiency of COD_S into n-caproate ($COD_{n\text{-}C6}/COD_S$) was 29.7 ± 0.5% (days 39–41) in cycle 3, equivalent to a 5-fold increase during the experiment (Fig. S5). The same increasing trend was observed for n-valerate (~6.8-fold increase) although it remained at low proportions. The highest COD_T conversion efficiency towards n-caproate ($COD_{n\text{-}C6}/COD_T$) was 12.4% in cycle 3. Carbon specificity (product/fatty acids ratio) for n-caproate reached a maximum of 38.2 ± 0.1% in cycle 3 (Table S2). In a comparable continuous one-stage reactor, Xu et al. (2018) obtained a n-caproate carbon specificity of 23% using solely yogurt-waste as substrate. These authors also observed equivalent carbon specificities for propionate, which has been reported as a product of the competitive acrylate pathway in lactate-based chain elongation with in-line extraction (Kucek et al., 2016a). Here nevertheless, propionate was always a minor metabolite and propionate carbon specificities remained at 4–5% probably due to the low pH levels which can limit the growth of propionate producing bacteria (Hettinga and Reinbold, 1972).

Fermentation proceeded without pH control but food waste pH was adjusted before being added to the reactor. Hydroxide used for pH adjustment equals to 0.77–4.35 mol OH-/mol MCFA and hydroxide input showed a decreasing trend after every cycle (Table S2). These values are 0.8- to 2.3-times the amount of hydroxide used by Roghair et al. (2018) in a chain elongation reactor fed with the same original food waste (previously acidified at pH 5.5) and ethanol.

3.2. Subsequent acidification and chain elongation regulate pH dynamics while repeated food waste addition enhances n-caproate selectivity

Results of 1L-RBF suggest that lactate was an important electron donor for fatty acids chain elongation. Therefore, food waste fermentation to lactate and fatty acids was systematically studied in the duplicate 0.3L-RBF experiments. In these experiments, lactate was produced within 1–2 days causing the pH to drop and was subsequently consumed for FA formation (Fig. 2a). The corresponding increase in pH was observed during lactate consumption. This same behaviour in lactate and pH profiles was observed after each addition of food waste. In cycle 2 and 3, n-butyrate, n-valerate and n-caproate were the main fatty acids formed during lactate consumption, all being products of the RBO pathway. Thus, reproducible acidification and chain elongation phases could be distinguished. In batch and repeated-batch food waste fermentation, initial environments rich in nutrients and easily fermentable substrate would promote lactate formation as a readily produced ATP-yielding electron sink. After lactate production, the medium is acidified and only a few specialists (e.g., *M. elsdenii*) can take up lactate at low pH levels to produce FA (Counotte and Prins, 1981). Hydrolysis was estimated to be 212.5 ± 30 g COD_S/kg TS_{added} in cycle 1 but high deviations were observed in the following cycles and it is, therefore, not clear whether hydrolysis occurred in cycles 2 and 3 (data not shown). During the 0.3L-RBF experiments, n-caproate concentrations increased after every cycle and n-caproate was the second main metabolite produced after n-butyrate. Methane was not detected in any of the cycles in 0.3L-RBF (Fig. S6). The highest n-caproate concentrations and production rates in 0.3L-RBF were observed in cycle 3 (Fig. 2a, Table S2), being 3.4 ± 0.20 g/L and 0.3 ± 0.11 g/L-d, respectively.

Similarly to the 0.3L-RBF experiments, lactate was also produced in single-batch fermentation and was further converted to SCFA with the respective changes in pH (Fig. 2b). Metabolites profile and final pH at the moment of lactate depletion (day 8) were similar in 0.3L-RBF and single-batch experiments. After lactate depletion, however, the single-batch experiment showed a fairly stable pH (~4.9) and product spectrum. n-caproate concentration remained <1 g/L after more than 40 days of reaction time with acetate and n-butyrate produced at similar concentrations. Thus, chain elongation is promoted by both the addition of substrate and the availability of electron acceptors. Addition of substrate results in more lactate which in the presence of electron acceptors (i.e., C2, n-C4) can be more efficiently used for MCFA production. Experiments with pure cultures have shown that the lactate needed to produce one molecule of n-caproate was reduced in 11% when acetate was

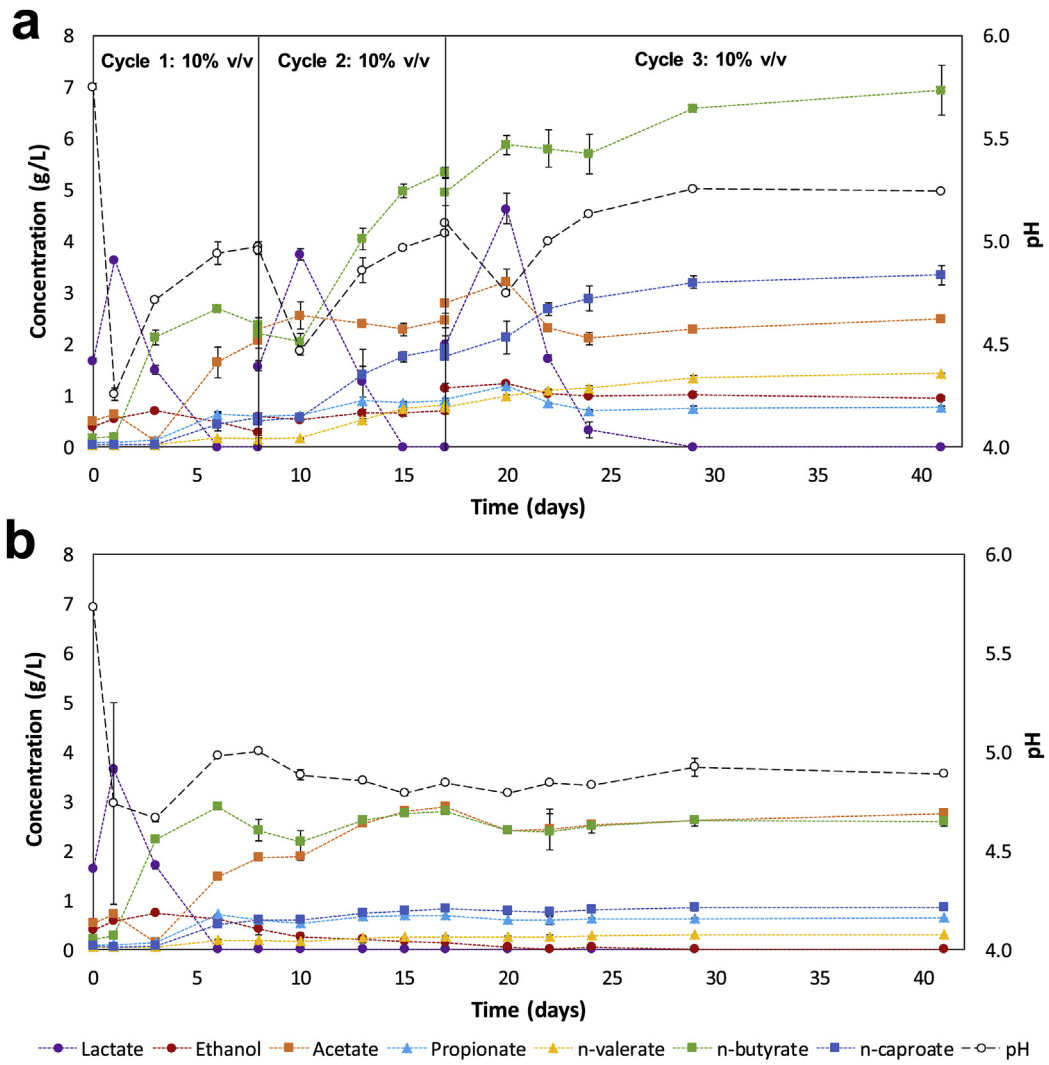

Fig. 2. Metabolites produced during food waste fermentation in (a) 0.3L-RBF and (b) single-batch experiments. Potential electron donors (circles); even-chain fatty acids (squares); odd-chain fatty acids (triangles). Duplicates values were averaged and error bars depict actual values with respect to the mean.

supplemented or in 37% when n-butyrate was added to the medium (Zhu et al., 2017). In our experiments, electron acceptors were always available and they might have substantially contributed to n-caproate formation. Whether one-time addition of 30% v/v food waste (the total amount added after 3 cycles) would allow the occurrence or enhancement of acidification and chain elongation was not assessed. In cycle 3 of the 0.3L-RBF, acidification and chain elongation occurred simultaneously and lactate, SCFA and n-caproate were produced during the acidification phase (Fig. S3). Also, part of the products formed during chain elongation in cycle 3 could not be accounted for since probably they sorbed onto the organic phase (i.e., solids or oily phase of food waste).

Thermophilic conditions did not improve lactate production after two additions of food waste compared to 0.3L-RBF experiments (Fig. S7). The lactate produced accumulated over time (~8.5 g/L) and was not metabolized to fatty acids at 50 °C. However, lactate conversion to n-butyrate and traces of n-caproate was observed (after ~24 days) when temperature was switched to 35 °C suggesting a similar behaviour as in 0.3L-RBF experiments.

Regarding process efficiency, similar tendencies to previous experiments were observed. Measured metabolites in 0.3L-RBF comprised 36–42% of COD_T (Fig. S4). COD_S conversion efficiency and carbon specificity showed an increasing trend for n-caproate with values between 8-18% and 9–22%, respectively (Fig. S8). The branched-chain SCFA isobutyrate (i-C4) and isovalerate (b-C5) were detected at maximum concentrations of 350 and 500 mg/L, respectively, together with other minor metabolites i.e., succinate, formate, butanol, propanol, hexanol, methanol, n-heptanoate and pentanol (Fig. S9). Among them, succinate (up to 500 mg/L) and formate (up to 1.1 g/L) were the most dominant and were metabolized during fermentation. However, all minor metabolites together never reached concentrations higher than 2.5 g COD/L. In the case of 0.3L-RBF, the hydroxide input ranged from 0.47 to 1.47 mol OH^-/mol MCFA (Table S2). The decreasing trend in hydroxide input together with the increasing trend in pH after every cycle suggest that besides hydroxide being used more efficiently after every cycle due to higher MCFA selectivity, buffer capacity also accumulated and contributed to neutralize acids.

3.3. *Lactobacillus and Caproiciproducens species dominated the microbiome composition*

Despite the complex composition of the food waste, only a few microorganisms were enriched during fermentation experiments in both 1L-RBF and 0.3L-RBF. Two genera were persistently dominant in all the samples: *Caproiciproducens* and *Lactobacillus* which

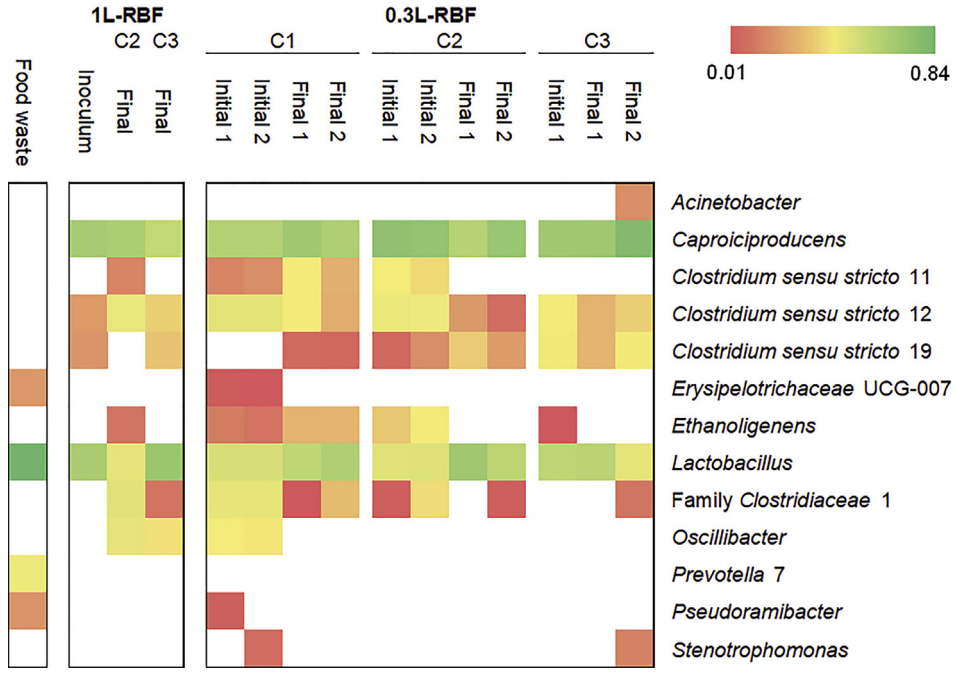

Fig. 3. Heat map with relative abundance for effective ASVs (after CSS normalization) at genus level unless specified otherwise. 0.3L-RBF duplicate reactors are designated with numbers 1 and 2. Samples were taken at the beginning (Initial) and end (Final) of cycles 1 (C1), 2 (C2) and 3 (C3). No DNA could be extracted from the beginning of cycle 3 in 0.3L-RBF reactor 2.

together showed a relative abundance of 57–93% (Fig. 3). *Caproiciproducens* spp. were the most dominant (32–72% relative abundance) followed by *Lactobacillus* spp. (12–56% relative abundance). *Clostridium* spp. were also persistent during the experiments although at lower relative abundances (≤15%). In total, 22 ASVs belonging to *Caproiciproducens*, 12 ASVs belonging to *Lactobacillus* and 13 ASVs belonging to *Clostridium* were identified (Fig. S10). *Lactobacillus* sp. ASV1 was the most abundant *Lactobacillus* (≤95%) (Fig. 4) and was closely related to *L. johnsonii* (100% identity), *L. gasseri* (99.5% identity) and *L. taiwanensis* (99.5% identity). These *Lactobacillus* species are obligately homolactic LAB (Vandamme et al., 1996) and their high abundance support that lactate formation occurred through homolactic fermentation. It is worth mentioning that even though obligately heterolactic LAB were the most abundant (~95% of *Lactobacillus* spp.) in the food waste (ASV18; 100% identity to *L. sanfransiscensis*), homolactic bacteria (present in the inoculum) were enriched in the experiments. This could be a result of the specific conditions of the experiments or due to inactivation of indigenous LAB during food waste storage. Since LAB are abundant in food waste (Wu et al., 2018), heterolactic and homolactic metabolisms might compete when fresh food waste is used as substrate.

3.4. Enriched *Caproiciproducens* spp. co-occurred with *Lactobacillus* spp.

The microbiomes developed in this study tended to be more alike as fermentation proceeded. Similarities between 1L-RBF and 0.3L-RBF microbiomes, shown by a reduced beta-diversity, increased at the end compared to the beginning of the experiments (Fig. S11a). Preliminary analysis of the 0.3L-RBF experiments showed major changes in the microbiomes during fermentation. The microbiomes clustered into two groups (Fig. S11b) referred to as: SCFA-producing microbiomes (end of cycle 1/beginning of cycle 2; group 1) and MCFA-producing microbiomes (from end of cycle 2 onwards; group 2). Differential abundance analysis revealed that many *Caproiciproducens* spp. including *Caproiciproducens* spp. ASV4 and ASV7 were enriched in MCFA-producing microbiomes whereas *Clostridium* spp. and *Ethanoligenens* spp. were enriched in SCFA-producing microbiomes (Fig. S12). This suggests that chain elongating microorganisms require longer time or specific conditions (e.g., higher pH or electron acceptors concentration) to grow. Furthermore, co-occurrence network analysis (Fig. S13) showed a positive interaction between ASV1, belonging to *Lactobacillus* spp., and ASVs 2, 3, 4 and 5, which were the most abundant ASVs belonging to *Caproiciproducens* spp. (Fig. 4).

Lactobacillus sp. ASV1 was a key LAB in the microbiome, closely related to microorganisms capable of using starch, oligosaccharides, disaccharides and monosaccharides as substrate for lactate production (Gänzle and Follador, 2012). The produced lactate would then be utilized by lactate elongators to produce MCFA. However, microorganisms commonly reported to use lactate for MCFA formation such as *Ruminococcaceae* bacterium CPB6 or species belonging to the *Megasphaera* genus were not found in this study. Instead, species of the genus *Caproiciproducens* are likely responsible of MCFA production in our experiments. ASVs belonging to the *Caproiciproducens* genus showed 91–95% similarity to *Caproiciproducens galactitolivorans*. *Caproiciproducens* is a recently defined genus with *Caproiciproducens galactitolivorans* strain BS-1 as the only species described so far. *Caproiciproducens galactitolivorans* is reported to utilize hexoses, pentoses, disaccharides and glycerol to produce acetate, n-butyrate, n-caproate and hydrogen (Kim et al., 2015). Therefore, sugars were most likely used for MCFA production. However, the remarkable dominance of *Caproiciproducens* in our experiments suggests that organisms belonging to this genus used lactate to produce n-caproate. At the moment of this research, there was no report on the capability of *Caproiciproducens* spp. to use lactate for MCFA production. The low similarity between *Caproiciproducens galactitolivorans* and the *Caproiciproducens*-related ASVs found here suggests that new species of *Caproiciproducens* were enriched in our experiments. The capability of *Caproiciproducens* organisms to metabolize both

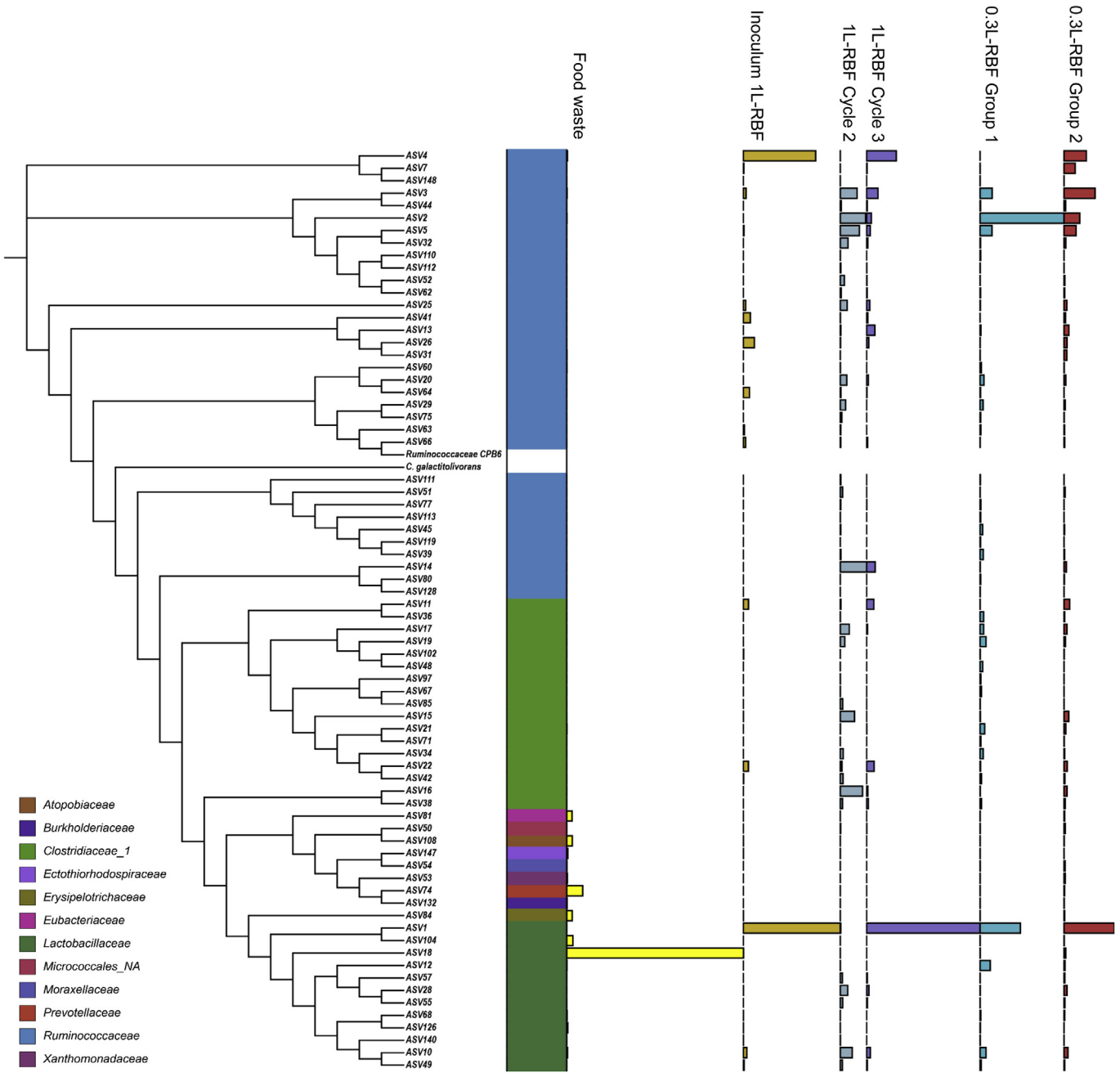

Fig. 4. Phylogenetic tree and relative abundance of bacterial taxa at ASV level for food waste, 1L-RBF and 0.3L-RBF microbiomes.

lactate and sugars could be foreseen, similarly to lactate elongators being capable of metabolizing sugars (Wang et al., 2018; Weimer and Moen, 2013). Hence, it is reasonable to hypothesize that *Caproiciproducens* spp. found in this study used lactate and sugars for chain elongation (Fig. S14).

4. Discussion

4.1. n-caproate production performance

In this study, n-caproate production was linked to lactate as electron donor, which is revealed by the increase in n-caproate concentrations and the increase in pH during lactate consumption. Lactate was produced through the homolactic pathway which is an efficient way to improve the production of MCFA (Scarborough et al., 2018). During acidification, lactate was the main product formed (>79% of acidification products, in COD) (Fig. S3) and negligible CO_2 release was accordingly observed (Fig. S2 and Fig. S6). Since various electron donors and acceptors could have been produced/consumed to different extents, it is difficult to quantify the effectivity of lactate conversion through the RBO pathway. Relatively small amounts of ethanol were detected that may also contribute as electron donor together with sugars, amino acids, D-galactitol, propanol and electron acceptors, such as succinate, that could be present in the system (Angenent et al., 2016). Hydrogen was present in the reactors and glycerol could be present in the food waste, both of which can act as indirect electron donors in chain elongation (Leng et al., 2017; Zhang et al., 2013). Besides,

degradation of lipids and proteins present in the food waste could also result in MCFA formation. Apart from lactate, other compounds (electron donors/acceptors) contributed to n-butyrate and n-caproate formation (Fig. S3).

Coupling acidification and chain elongation helped to reduce hydroxide input for pH control. However, buffer capacity (Fig. S15) also played an important role in determining the degree of acidification of the medium. Actual pH measured in 0.3L-RBF was higher than the expected levels based on the total acids concentration, making evident that the alkalinity of the system was important to keep the pH levels high enough to prevent microbial inhibition (Fig. S16). The initial buffer capacity helped to avoid extreme acidic conditions together with fatty acids and probably other buffer agents (NH_4^+, HS^-) that were formed during fermentation. It is also evident from Fig. S16 that the stoichiometry of lactate production and consumption partially describes the downward/upward pH trends caused by protons production/consumption and that the fluctuations in pH were attenuated by the buffer capacity of the system. Although hydroxide was added to the reactors with the neutralized food waste, the main changes in pH seemed to be due to acids production or destruction rather than substrate addition. However, buffer capacity accumulated in the reactor due to substrate addition and FA formation, which explains the increasing trend in pH by the end of the experiments. Even though the eventual accumulation of buffer capacity might be detrimental for the long-term operation, this effect could be reduced by tuning operational parameters such as substrate concentration and volume replacement in sequencing-batch or fed-batch reactors. A trade-off between buffer capacity accumulation, conversion efficiencies and rates could then be achieved.

Even though optimization was out of the scope of this study, our results are comparable to other chain elongation processes using carbon-rich wastes (Table 1). Overall, the n-caproate production rates observed in our study (0.3–4.2 g COD/L-d) are in agreement with other works that used solely organic waste streams. The highest n-caproate production rate in this study is 2.7- to 6.4-times higher than other studies working with solid organic wastes (Grootscholten et al., 2013; Reddy et al., 2018) and similar to the maximum obtained by Xu et al. (2018). Comparable n-caproate carbon specificities to this work (23–38%) have been reported from fresh food waste fermentation in batch leach-bed reactors (38%) (Nzeteu et al., 2018). Promising results have been obtained from liquid waste streams as well such as 45% n-caproate conversion efficiencies (Xu et al., 2018) from yogurt-processing wastewater and 79% n-caproate carbon specificities from acid whey (Duber et al., 2018). In a one-stage reactor equipped with in-line extraction, Xu et al. (2018) observed a lower n-caproate carbon specificity compared to our work (23% vs 38%). To improve the carbon specificity, the authors implemented a thermophilic acidification stage to produce lactate prior to the lactate-based chain elongation stage. The implementation of this two-stage system resulted in a doubled carbon specificity (48%) towards n-caproate and reached 66% for MCFA. Unfortunately, data on the amount of chemicals used to control pH in the two separate reactors are not available.

A previous work from our group focused on the efficient use of ethanol and hydroxide in a continuous chain elongation reactor fed with acidified food waste and ethanol. The efficient use of ethanol resulted in a higher MCFA conversion efficiency, MCFA carbon specificity and a reduced hydroxide input for pH control (0.92 mol OH^-/mol MCFA in the chain elongation stage and 5.5 mol OH^-/kg COD-FA for the overall process) (Roghair et al., 2018). Under these conditions, around 24% of the n-caproate produced was derived

Table 1
n-caproate production and hydroxide input in chain elongation processes from waste streams.

Substrate	Process	pH control	n-C6 concentration	Maximum n-C6 production rate	n-C6 conversion efficiency[a]	n-C6 carbon specificity	Hydroxide input		Reference
			[g/L]	[g COD/L-d]	[%]	[%]	[mol OH^-/mol MCFA]	[mol OH^-/kg COD-FA]	
Food waste	Repeated-batch[b]	Substrate pH adjusted	3.21	0.62	18	23	0.47	0.79	This study
Food waste	Repeated-batch[c]	Substrate pH adjusted	5.52	4.20	30	38	0.77	0.99	This study
OFMSW and ethanol	Batch	No	2.70	1.55	N.A.	N.A.	N.A.	N.A.	Grootscholten et al. (2013)
Food waste	Batch	Medium pH adjusted	10	6.61	N.A.	38[d]	N.A.	N.A.	Nzeteu et al. (2018)
Vegetable and salad waste	Fed-batch	No	1.80	N.A.	N.A.	22[d]	N.A.	N.A.	Bolaji and Dionisi (2017)
	Batch	Buffer addition	3.08	N.A.	N.A.	23[d]	N.A.	N.A.	
Wine lees	Continuous	Yes	<0.5[e]	4.30	N.A.	36	N.A.	N.A.	Kucek et al. (2016b)
Acidified food waste and ethanol	Continuous	Yes	23.40	12.16	59	76	0.92	5.50	Roghair et al. (2018)
	Continuous	Yes	7.10	12.38	13	46	1.93	N.A.	
Yogurt industry waste stream	Continuous single-stage	Yes	<0.5[e]	N.A.	N.A.	23	N.A.	N.A.	Xu et al. (2018)
	Continuous two-stage	Yes	<0.5[e]	3.71	45	48	N.A.	N.A.	
Food waste	Batch	No	8.10	0.66	N.A.	N.A.	N.A.	N.A.	Reddy et al. (2018)
Acid whey wastewater	Continuous	Yes	10.45	7.07	N.A.	79	N.A.	N.A.	Duber et al. (2018)
Thin stillage	Continuous	Yes	6.80	27.20	33	44	N.A.	N.A.	Carvajal-Arroyo et al. (2019)

N.A. = not available.
[a] Based on COD_S.
[b] Data from 0.3L-RBF, cycle 3.
[c] Data from 1L-RBF, cycle 3.
[d] $COD_{n\text{-}C6}/COD_{products}$.
[e] In-line extraction was applied.

from the acidified food waste (COD from acetate and n-butyrate consumed). In the present study, n-caproate came entirely from food waste and hydroxide addition was lowered by a 2-times (0.47 mol OH^-/mol MCFA) and 7-times (0.79 mol OH^-/kg COD-FA) factor (Table 1). These benefits were attained by changing from a two-stage ethanol-based chain elongation to a one-stage lactate-based chain elongation process. However, n-caproate concentrations and productivities were much lower in the present study. Improved n-caproate productivities from food waste could be achieved by using a fed-batch reactor with higher substrate concentration and shorter cycles. In this case, residual amounts of electron acceptors should be maintained to promote higher MCFA selectivities. Substrate conversion was limited by hydrolysis which can be improved by using leach-bed reactors (Nzeteu et al., 2018) or *in situ* extraction (Grootscholten et al., 2013).

4.2. Outlook and process boundaries

4.2.1. Relevance of pH for balanced acidification and chain elongation

When no pH control is intended, the balance between substrate supplementation, alkalinity, reactor operation and the microbiome resilience will determine the conditions to avoid bioprocess instability due to outranged pH.

Although, LAB show hydrolytic activity at pH as low as 4.0, glycolysis is not functional under these conditions and lactate production would be inhibited (Kashket, 1987; Ohkouchi and Inoue, 2006). Hence, pH >4.0 is essential to allow lactate formation. However, such acidic conditions could be inhibitory for lactate utilizers. Growth of *M. elsdenii* has been shown to be substantially affected at pH 4.65 (Weimer and Moen, 2013) and a recent work showed that lactate elongation to n-butyrate occurred only at pH $\geq$4.5 (Sträuber et al., 2018). In the present study, lactate utilization for n-butyrate production occurred at a lower pH (4.0–4.3) (0.3L-RBF, day 1; thermophilic experiment, day 41). Moreover, the highest n-caproate production rate in our study was detected at an initial pH of 4.7 (1L-RBF, day 34) even though pH <5.0 was reported to completely inhibit n-caproate production in a similar process (Nzeteu et al., 2018). It is worth mentioning that the undissociated n-caproic acid concentration (0.65 g/L) at this day was below the toxic limit (0.87 g/L) proposed to inhibit n-caproate production in ethanol-based chain elongation (Ge et al., 2015). Nevertheless, undissociated n-caproic acid concentration on day 36, when n-caproate formation was still observed, reached 1.72 g/L. These observations show that the microbiome developed in our experiments could function at more acidic environments than previous reports and in the presence of higher concentrations of the toxic undissociated n-caproic acid. The low pH and the presence of undissociated n-butyric ($\leq$35 mM) and n-caproic ($\leq$20 mM) acids could have prevented the acrylate pathway from being dominant despite the temporary high lactate concentrations. Propionate could still be formed at low pH levels when in-line extraction is applied and lactate accumulation is observed (Kucek et al., 2016a). Although it seems that lactate elongators can stand higher undissociated n-caproic acid concentrations than ethanol elongators or propionate producers, more research is needed to systematically determine the toxic limit of undissociated n-caproic acid for the three microbial groups.

4.2.2. Maximum hydroxide use for n-caproate production from carbohydrates

Reduced chemical use for pH control can help to increase the life cycle performance of the technology (Chen et al., 2017). At present, however, few studies report on either base or acid needed in their processes. In our study, homolactic fermentation (eq. (1)) and lactate-based chain elongation (eq. (2), eq. (3)) were the main identified processes. Considering glucose conversion to lactate through homolactic fermentation and lactate-based chain elongation to n-caproate, a maximum of 2/3 of the protons released can be self-neutralized by coupling these two processes. To keep a constant pH, base addition or intrinsic alkalinity from the substrate would still be needed. This equals to a maximum hydroxide need of 1 mol OH^-/mol n-C6 or 3.9 mol OH^-/kg COD-C6. In this study, n-caproate was almost the only MCFA produced and the values for hydroxide addition were always below the theoretical maximum (except for 1L-RBF cycles 1 and 2 were lactate was not produced, Table S2) even though SCFA were always produced together with n-caproate. Hydroxide input was improved as pH started to increase over cycles due to increased n-caproate selectivity and accumulation of buffer capacity. Clearly, the production of SCFA increases the base need and the opposite is true for longer MCFA production (e.g., n-caprylate).

5. Conclusions

Repeated-batch food waste fermentation without exogenous electron donor was steered to n-caproate as the dominant product. Lactate production and subsequent chain elongation from food waste regulated pH dynamics and buffer capacity of the substrate helped to avoid inhibitory acidic conditions and large pH fluctuations. These phenomena together lead to reduced hydroxide input for n-caproate production. Lactate was attributed as key electron donor for chain elongation and its uptake for n-caproate formation was observed at conditions suggested as inhibitory in the literature. *Lactobacillus* spp. and *Caproiciproducens* spp. dominated and co-occurred in the microbiome which shows the involvement of *Caproiciproducens* spp. in the use of lactate as electron donor.

Declaration of competing interest

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

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Appendix A. Supplementary data

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