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Biological caproic acid production in microbiome-based processes

PhD dissertation

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Preface

The presented work was performed at Poznan University of Technology, Faculty of Environmental Engineering and Energy, Water Supply and Bioeconomy Division from September 2015 to December 2020 under the supervision of Professor Piotr Oleśkowicz-Popiel. The research described in this dissertation was conducted and is part of two scientific projects:

- the project 'CaproBiome' funded by the Polish National Centre for Research and Development under the LIDER V programme for young scientists, contract no.: LIDER/013/261/L-5/13/NCBR/2014 (PI: Piotr Oleśkowicz-Popiel)

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The dissertation consists of two parts. The first one is an introduction that provides the context, main goals of the work, background on the open culture fermentations for the caproic acid production in microbiome-based processes and future outlook. The second part consists of the following publications, which are the basis of this dissertation:

Paper I: Duber, A., Jaroszynski, L., Zagrodnik, R., Chwialkowska, J., Juzwa, W., Ciesielski, S., Oleskowicz-Popiel, P., 2018. Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey, Green Chemistry 20, 3790–3803. https://doi.org/10.1039/C8GC01759j

Impact Factor 9.405, 45 points of MNiSW, individual input: 60%.

Anna Duber conceptualised and conducted the research, performed microbiological analyses, interpreted the results and wrote the manuscript.

Paper II: Duber, A., Zagrodnik, R., Chwialkowska, J., Juzwa, Oleskowicz-Popiel, P., 2020. Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation, Science of The Total Environment 728, 138814. https://doi.org/10.1016/j.scitotenv.2020.138814

Impact Factor 7.963, 200 points of MNiSW, individual input: 70%.

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Paper III: Duber, A., Zagrodnik, R., Gutowska N., Łężyk M., Oleskowicz-Popiel, P. 2022. Lactate and Ethanol Chain Elongation in the Presence of Lactose: Insight into Product Selectivity and Microbiome Composition. ACS Sustainable Chemistry & Engineering 10, 3407−3416

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Impact Factor 8.198, 140 points of MNiSW, individual input: 75%.

Anna Duber initiated and led the planning of the research, conceptualised and conducted the research, performed the chromatographic and microbiological analyses, interpreted the results and wrote the manuscript.

Scientific papers and patent application that are not part of the dissertation, but are relevant to the topic of the dissertation:

Paper IV: Jankowska, E., Duber, A., Chwialkowska, J., Stodolny, M., Oleskowicz-Popiel, P., 2018. Conversion of organic waste into volatile fatty acids – The influence of process operating parameters, Chemical Engineering Journal 345, 395–403.
https://doi.org/10.1016/j.cej.2018.03.180

Paper V: Chwialkowska, J., Duber, A., Zagrodnik, R., Walkiewicz, F., Lezyk, M., Oleskowicz-Popiel, P., 2019. Caproic acid production from acid whey via open culture fermentation – Evaluation of the role of electron donors and downstream processing, Bioresource Technology, 279, 74–83.
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Paper VI: Zagrodnik, R., Duber, A., Lezyk, M., Oleskowicz-Popiel, P., 2020. Enrichment Versus Bioaugmentation – Microbiological production of caproate from mixed carbon sources by mixed bacterial culture and *Clostridium kluyveri*, Environmental Science & Technology, 54, 9, 5864-5873.
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Paper VII: Brodowski, F., Duber, A., Zagrodnik, R., Oleskowicz-Popiel, P., 2020. Co-production of hydrogen and caproate for an effective bioprocessing of waste. Bioresource Technology, 318, 123895.
https://doi.org/10.1016/j.biortech.2020.123895

The patent application (European Patent Office): Oleśkowicz-Popiel P., Zagrodnik R., Duber A., Method for one-pot co-production of caproic acid and hydrogen, Date of filing: 15.07.2018, Application number: 18183574.5.
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Abstract

In a bioeconomy concept, the use of fossil resources for chemicals and energy production is replaced by renewable bioresources. The strategy enables to move from a linear 'take-make-use-dispose' model to a circular economy model which prioritises prevention, reuse, and recycling in the waste management cycle. Thus, in such an approach, waste becomes a resource and can be valorised sustainably for valuable biochemicals. A large fraction of waste is of organic origin. Organic waste can be converted through thermochemical and/or biological processing. The biological conversion relies on microbial processes, one of which is an open culture fermentation where complex organic feedstock undergoes a cascade of biochemical pathways driven by microorganisms (so-called reactor microbiome) for the final product formation. The main advantage of open culture fermentation is the possibility to carry out the process in unsterile conditions. Moreover, by proper control of operational conditions (temperature, pH, hydraulic retention time, etc.) it is possible to steer the bioprocess in a way to prevent a disperse of carbon flux into competing metabolic pathways and to achieve high production rates of preferred metabolite. One of the promising process directions is the so-called chain elongation process which enables an effective recovery of value-added biochemicals from a range of different organic waste streams. The key product is caproic acid, an attractive commercial chemical for industrial and agricultural applications.

This dissertation aimed to develop a biological caproic acid production process in a microbiome-based system from an organic waste feedstock, i.e. acid whey. The specific objectives of the work were focused on (i) investigation of operational parameters that influence the process performance, (ii) impact of the feed composition and particular substrates compounds on the caproic acid production process, (iii) identification of possible metabolic pathways, (iv) characterisation of microbial community structure, dynamics and activity, and (v) identification of key microbial groups responsible for caproic acid production. To achieve the goals, an experimental stand was designed and two long-term continuous bioprocesses of open culture fermentation of acid whey, as well as batch experiments using a synthetic medium, were conducted. For the process analysis, advanced analytical methods and state-of-the-art molecular biology tools were applied. The results of the research were published in three

independent articles, further referred to as Paper I, Paper II and Paper III, which are the basis of this dissertation.

Considering the process operating parameters the results showed that in continuous bioreactor processes the pH level of 5.5 was low enough to effectively inhibit methanogenesis – the main competitive metabolic pathway in anaerobic open culture fermentation. Furthermore, it was shown that gradually shortening HRT from 20 days to 2.5 days contributed to washing out most methanogens from the reactor, and improved the caproic acid production rate. The designed system enabled to produce the caproic acid where the highest average production rate was 134.3 ± 30.9 mmol C/L/day (0.11 g/L/h) with a median of 146.1 mmol C/L/day, and specificity between 58% and 83% (with a median of 79%). However, high caproic acid concentration in the bioreactor (up to 8.5 g/L) was a self-limiting factor due to product toxicity and caused the process inhibition, despite the availability of the substrates.

Caproic acid production based on the chain elongation relies on two crucial electron donors needed in the process, i.e. ethanol or lactate. In this study, the caproic acid formation depended mostly on lactate-based chain elongation, despite ethanol being produced in the reactor or its external delivery to the system with feed. However, some ethanol production in the reactor seemed important for the chain elongation process as it coincided with caproic acid production, and a decrease in ethanol concentration in the reactor coincided with the disturbance in caproic acid production. Dosing acid whey with an increasing ethanol loading rate led to ethanol oxidation with acetate and butyrate generation and hydrogenotrophic methane formation. Change of the feedstock composition (higher lactate and lactose content and cut off of external ethanol delivery) resulted in a sharp drop in the short-chain carboxylic acids formation and prompted a medium-chain carboxylic acids production with caproic acid as the main product. The batch trials demonstrated that the presence of additional substrate i.e. lactose may divert the carbon flux and impact the selectivity of the final product by providing additional intermediates for the biochemical reactions.

The analysis of the reactor microbiome showed that functional enrichment of a specific microbiome capable of forming caproic acid is possible through appropriate control of operating parameters, i.e. pH and HRT. Moreover, the results showed a strong

dependence of the biodiversity and structure of the microbial population on the feed composition. In the conducted processes, the dominance of the Coriobacteriaceae, Ruminokoccaceae (Ruminococcus, Oscillospira), Prevotellaceae and Veillonellaceae families, which most likely contributed to the elongation of the carbon chain, was demonstrated.

The presented dissertation showed that using an open culture fermentation enables the acid whey waste valorisation to caproic acid. The achieved results can contribute to the novel resource recovery strategies by biowastes utilisation using advanced biotechnological methods. Ultimately, it can lead to a reduction of our dependency on petrochemical resources and would pave the way for an innovative industrial-integrated biorefinery system.

Streszczenie

W koncepcji biogospodarki wykorzystywanie zasobów kopalnych do produkcji chemikaliów i energii jest zastępowane przez zasoby odnawialne. Zastosowanie ww. koncepcji umożliwia przejście od modelu gospodarki liniowej „weź, wyprodukuj, użyj, wyrzuć" do gospodarki o obiegu zamkniętym, w której priorytetem jest zapobieganie, ponowne użycie i recykling w cyklu gospodarowania odpadami. W takim podejściu odpady stają się więc zasobem, który może zostać poddany waloryzacji do wartościowych produktów. Dużą frakcję odpadów stanowią odpady organiczne. Odpady organiczne mogą zostać przetworzone za pomocą metod termochemicznych i/lub biologicznych. Konwersja biologiczna opiera się na procesach mikrobiologicznych takich jak fermentacja kultur otwartych, w której złożony surowiec organiczny przechodzi kaskadę szlaków biochemicznych napędzanych przez mikroorganizmy (tzw. mikrobiom reaktorowy) w celu wytworzenia produktu końcowego. Główną zaletą fermentacji kultur otwartych jest możliwość prowadzenia procesu w warunkach niesterylnych. Ponadto, poprzez odpowiednią kontrolę warunków operacyjnych (temperatura, pH, czas retencji hydraulicznej itp.) możliwe jest sterowanie bioprocesem w taki sposób, aby zapobiec rozpraszaniu strumienia węgla na konkurencyjne szlaki metaboliczne i osiągnąć wysokie tempo produkcji preferowanego metabolitu. Jednym z obiecujących kierunków prowadzenia procesu jest tak zwany proces wydłużania łańcucha, który umożliwia efektywne odzyskiwanie wartościowych substancji z szeregu różnych strumieni odpadów organicznych. Jednym z kluczowych produktów procesu jest kwas kapronowy, komercyjnie atrakcyjna substancja chemiczna do zastosowań przemysłowych i rolniczych.

Celem niniejszej pracy było opracowanie procesu biologicznego wytwarzania kwasu kapronowego za pomocą mikrobiomu z odpadów organicznych, tj. serwatki kwaśnej. Cele szczegółowe pracy koncentrowały się na (i) zbadaniu wpływu parametrów operacyjnych na przebieg procesu, (ii) wpływu składu substratu i poszczególnych związków substratu na produkcję kwasu kapronowego, (iii) identyfikacji możliwych szlaków metabolicznych, (iv) scharakteryzowaniu struktury, dynamiki i aktywności kultur mikroorganizmów oraz (v) identyfikacji kluczowych grup drobnoustrojów odpowiedzialnych za produkcję kwasu kapronowego. Dla osiągnięcia celów zostało zaprojektowane stanowisko badawcze i przeprowadzono dwa długoterminowe procesy

fermentacji kultur otwartych w trybie ciągłym z wykorzystaniem serwatki kwaśnej jako substratu, oraz procesy okresowe z użyciem pożywki syntetycznej. Do analizy przebiegu procesów zastosowano zaawansowane metody analityczne oraz najnowocześniejsze narzędzia biologii molekularnej. Wyniki badań zostały opublikowane w trzech niezależnych artykułach, zwanych dalej Publikacją I, Publikacją II i Publikacją III, które stanowią podstawę niniejszej rozprawy.

Rozważając parametry operacyjne procesu, wyniki wykazały, że w procesach bioreaktorowych ciągłych poziom pH 5.5 był wystarczająco niski, żeby skutecznie hamować metanogenezę – główny konkurencyjny szlak metaboliczny w fermentacji kultur otwartych. Ponadto wykazano, że stopniowe skracanie HRT z 20 do 2.5 dnia przyczyniło się do wypłukania większości metanogenów z reaktora i wyraźnie poprawiło tempo wytwarzania kwasu kapronowego. Zaprojektowany system umożliwił produkcję kwasu kapronowego gdzie najwyższa średnia szybkość produkcji wyniosła 134.3 ± 30.9 mmol C/L/dobę (0.11 g/L/h), mediana 146.1 mmol C/L/dzień, a specyficzność od 58% do 83% (mediana 79%). Niemniej jednak wysokie stężenie kwasu kapronowego w bioreaktorze (do 8.5 g/L) było czynnikiem limitującym ze względu na toksyczność produktu i powodowało zahamowanie procesu pomimo dostępności substratów.

Produkcja kwasu kapronowego w oparciu o wydłużanie łańcucha opiera się na dwóch kluczowych donorach elektronów potrzebnych w procesie, tj. etanolu lub mleczanie. W niniejszej pracy powstawanie kwasu kapronowego zależało głównie od wydłużenia łańcucha węglowego z mleczanu, pomimo wytwarzania etanolu w reaktorze lub jego zewnętrznego dostarczania do układu wraz z substratem. Niemniej jednak pewna produkcja etanolu w reaktorze wydawała się istotna dla procesu, ponieważ zbiegła się z produkcją kwasu kapronowego, a obniżone stężenie etanolu zbiegało się z zaburzeniem produkcji kwasu kapronowego. Dozowanie serwatki kwaśnej z rosnącą szybkością dozowania etanolu prowadziło do utleniania etanolu z wytworzeniem octanu i maślanu oraz wodorotroficznym tworzeniem się metanu. Zmiana składu substratu (większa zawartość mleczanów i laktozy oraz brak etanolu) spowodowała gwałtowny spadek tworzenia krótkołańcuchowych kwasów karboksylowych i przyspieszyła produkcję średniołańcuchowych kwasów karboksylowych z kwasem kapronowym jako głównym produktem. Procesy okresowe wykazały, że obecność laktozy, może zmienić kierunek

wykorzystania źródła węgla i wpłynąć na selektywność produktu końcowego poprzez dostarczenie dodatkowych związków pośrednich dla reakcji biochemicznych.

Analiza mikrobiomu reaktorowego wykazała, że funkcjonalne wzbogacenie specyficznego mikrobiomu zdolnego do tworzenia kwasu kapronowego jest możliwe poprzez odpowiednią kontrolę parametrów operacyjnych procesu, tj. pH i HRT. Co więcej, wyniki wykazały silną zależność bioróżnorodności i struktury populacji drobnoustrojów od składu substratu. W prowadzonych procesach wykazano dominację rodzin Coriobacteriaceae, Ruminokoccaceae (Ruminococcus, Oscillospira), Prevotellaceae i Veillonellaceae, które najprawdopodobniej przyczyniły się do wydłużania łańcucha węglowego.

Przedstawiona praca wykazała, że zastosowanie fermentacji kultur otwartych umożliwia waloryzację odpadu jakim jest serwatka kwaśna do kwasu kapronowego.

Osiągnięte wyniki mogą przyczynić się do opracowania nowatorskich strategii odzyskiwania zasobów poprzez utylizację bioodpadów przy użyciu zaawansowanych metod biotechnologicznych. Ostatecznie prowadzi to do zmniejszenia naszej zależności od zasobów petrochemicznych i toruje drogę dla stworzenia innowacyjnego, zintegrowanego systemu biorafinerii.

List of abbreviations

SCCAs – short-chain carboxylic acids

MCCAs – medium chain carboxylic acids

PHAs – polyhydroxyalkanoates

BOD – biological oxygen demand

COD – chemical oxygen demand

CE – chain elongation

ED – electron donor

16S rRNA – part of a small subunit of ribosomal ribonucleic acid

GC – gas chromatography

HPLC – high-performance liquid chromatography

UASB – up-flow anaerobic sludge blanket (reactor)

HRT – hydraulic retention time

TCD – thermal conductivity detector

FID – flame ionization detector

OCF – open culture fermentation

C6 – caproate (carboxylate with six carbons in the molecule)

Cx – straight-chain carboxylates with x carbons in the molecule

wv – working volume

BES – 2-bromoethanesulfonate

RBO – reversed β-oxidation

FAB – fatty acid biosynthesis

p*K*a – is the negative base -10 logarithm of the acid dissociation constant (K_a) of a solution ($pKa = -\log_{10}K_a$)

1 Background

1.1 Organic waste for bioeconomy

The rapid growth of the global population and the increasing energy demand calls for novel bioeconomy solutions for sustainable development of the future world. The expanding urbanisation leads to a depletion of natural resources as well as to the generation of a considerable amount of biowaste (Venkata Mohan et al., 2016). Nowadays economy still refers to a linear 'take-make-use-dispose' model and is based on the use of conventional energy resources such as fossil fuels for commodity chemicals and energy production. It brings negative consequences as the petrochemical refineries and fossil products gravely affect the natural environment, which results in pollution and damage, while the vast amount of organic waste is simply landfilled or introduced to the environment in another way. An emerging alternative is a bio-based economy concept. The early definition of the bioeconomy was proposed by the Organisation for Economic Co-operation and Development (OECD) and stated that "*A bio-based economy is defined as a concept that uses renewable bioresources, efficient bioprocesses and eco-industrial clusters to produce sustainable bioproducts, jobs and income"* (EOCD, 2004). It was later defined by the same institution as the process of "*transforming life science knowledge into new, sustainable, eco-efficient and competitive products*" (EOCD, 2009). In 2017 the European Commission released a review on the European Bioeconomy Strategy set in 2012. The described strategy defined the bioeconomy as *"the production of renewable biological resources and the conversion of these resources and waste streams into value-added products, such as food, feed, bio-based products as well as bio-energy*", with the aim "*to pave the way to a more innovative, resource-efficient and competitive society that reconciles food security with the sustainable use of biotic renewable resources for industrial purposes, while ensuring environmental protection*" (European Commission, 2017). Thus, organic waste is considered renewable bioresources for biochemicals and alternative energy production. Furthermore, integration of proper management of a particular waste and wastewater together with applying biotechnological processes and environmental engineering principles would be undeniably beneficial for both: waste valorisation and environmental protection.

Value-added resource recovery can be achieved in a biorefinery system (Scoma et al., 2016). The concept of a biorefinery refers to replacing fossil carbon products from traditional petrochemical refineries with carbon-based products (e.g., chemicals, liquid fuels, biomaterials and so forth) generated from biowaste using a variety of integrated technologies (Fava et al., 2015; Venkata Mohan et al., 2016). It would support the transition from the current linear economic model into the circular economy, which relies on 'closing the loop' by re-use and recycling materials and waste in various sectors of the economy with simultaneous minimal waste generation (Lago et al., 2019). This way, the circular economy model prioritises prevention, reuse, and recycling in the waste management cycle and turns waste into a resource (Scarlat and Dallemand, 2019). Biowastes will play an increasing role in the upcoming development of bioenergy.

Residual waste can be converted into a valorised product through thermochemical and/or biological processing (Fig. 1). Thermochemical conversion, e.g. pyrolysis (for bio-oil production) or gasification (where biowaste is gasified to syngas and then catalytically transformed into fuels), is suitable for dry biomass processing, while wet biomass is preferable for biological conversion, as water is required for biochemical processes to occur.

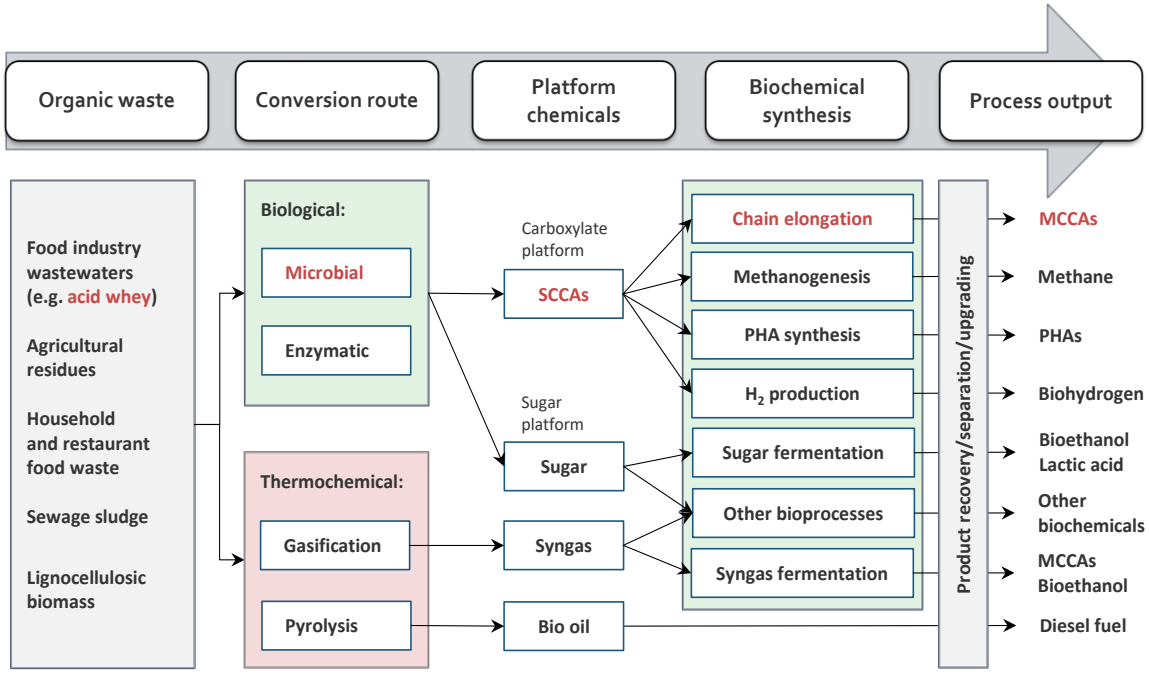

Figure 1. Graphical scheme of possible conversion routes of organic waste through platform chemicals. The font in red indicates the conversion of organic waste employed in this dissertation. SCCAs – short-chain carboxylic acids, MCCAs – medium-chain carboxylic acids, PHAs – polyhydroxyalkanoates.

1.2 Biological conversion of organic waste

Organic wastes (e.g. agricultural residues, food industry wastewaters, household and restaurant food waste, lignocellulosic biomass, sewage sludge) are renewable energy sources, that can be valorised through biological conversion into commodity chemicals and chemical building blocks (Tamis et al., 2015; Coma et al., 2017). The biological conversion of waste can be carried out using pure or open (mixed) cultures, also known as microbiomes. A disadvantage of pure culture, however, is that only a certain fraction of waste can be fermented (e.g. sugar fermentation to ethanol), and the process must be usually preceded by various pretreatment methods. The advantage of open cultures of microorganisms over pure culture lies in their economic (no sterilisation procedure is required) and the technical aspect (open cultures are functionally diverse and therefore more robust) (Regueira et al., 2020). The biological conversion that relies on microbiome-based processes may transform a broader range of waste where complex organic feedstock undergoes a cascade of biochemical pathways for the final product formation (Angenent et al., 2016). In such processes, the substrate type and efficient management and control of the operational parameters enable the creation of favourable environmental conditions for the proper microbiome development and desired product generation (Kleerebezem and van Loosdrecht, 2007; Hoelzle et al., 2014; Cavalcante et al., 2017).

For biological conversion two main chemicals platforms are described: sugar platform (where biomass is enzymatically hydrolysed into simple sugars for further fermentation, e.g. bioethanol), and carboxylate platform (where biomass is hydrolysed and further converted to carboxylates by microorganisms) (Fig. 1). The carboxylate platform is a mixture of short-chain carboxylic acids (SCCAs) in a dissociated form with at least one carbonyl group and includes mainly acetate, propionate, n-butyrate, and lactate. There are several known fermentation processes driven by microorganisms where different SCCAs can be obtained (Angenent et al., 2016). While SCCAs generated as such in a primary fermentation step have rather low economic value (Kleerebezem et al., 2015), they stand as an intermediate chemicals platform for higher value end-products generation (Agler et al., 2011). The preferred end-products may be formed in a secondary fermentation step or a separate (bio)process (Agler et al., 2011), and include but are not limited to polyhydroxyalkanoates for biodegradable bioplastics (Kleerebezem and van Loosdrecht,

2007), biohydrogen (Singhania et al., 2013; Yang and Wang, 2018), biogas (Bong et al., 2018) or medium-chain carboxylic acids (MCCAs) (Spirito et al., 2014).

1.3 Caproic acid

The presented work was focused on microbial caproic acid production based on bioeconomy principles. Caproic acid (hexanoic acid) belongs to MCCAs and is a straight-chain, saturated fatty acid with one carboxyl group (Angenent et al., 2016). The hydrophobic structure of the caproic acid molecule determines its solubility in water of only 10.19 g/L, which would enable its in situ recovery from fermentation broth through direct extraction into an organic phase (Agler et al., 2014). Caproic acid is an attractive commercial product for industrial and agricultural applications. It can be used as an antimicrobial, feed additive, plant growth promoter, or as a precursor for manufacturing products, including pharmaceuticals, cosmetic fragrances, lubricants, rubbers, paint additives and dyes, etc. (Angenent et al., 2016; Chen et al., 2017). Other studies proposed it as an intermediate precursor for liquid biodiesel and jet fuel production as part of a C6 fuel platform, due to its relatively high caloric value (Harvey and Meylemans, 2014; Andersen et al., 2015; Layton and Trinh, 2016). A required purity of caproic acid is dictated regarding the target application. Despite the broad range of possible applications, caproic acid is currently obtained in very low amounts. It is extracted from plant oils coming from food crops, such as butter, coconut or palm kernel oil, where its content is lower than 1% (Chen et al., 2017). An option for caproic acid extraction is its recovery through enzyme-mediated esterification of the abovementioned oils in an aromatic ester form (e.g. 2-phenethyl hexanoate), which can directly target the cosmetic market for flavour and fragrance formulations, but also in food products, such as cheese (Li et al., 2014; Sá et al., 2017). However, both methods are of low yield, seasonal dependant, and thus, generating skyrocketing prices, which is limiting regarding its market use. While chemical caproic acid production is also not well established, a promising alternative is its biotechnological production using microorganisms. To date, two different biotechnological companies associated with caproic acid production upraised. The first one is ChainCraft – Biobased Innovators, a start-up venture settled in Amsterdam, the Netherlands which converts organic waste streams into a mixture of fatty acids salts in liquid or powder form (with the highest concentration of sodium caproate)

at operational and semi-commercial demonstration scale, as well as purified specific fatty acids upon request including caproic acid ("ChainCraft – Biobased Innovators," n.d.). Another company is Capro-X, an agritech spin-off company from Cornell University in Ithaca, NY ("Capro-X Inc.," n.d.) that upgrades dairy waste streams of the American market into clean water and natural bio-oils.

To current knowledge, different biotechnological methods were studied for caproic acid production using complex microbial cultures (Agler et al., 2012a; Steinbusch et al., 2011; Weimer et al., 2015; Zhu et al., 2017). For the purpose of this work, acid whey was selected as an organic waste feedstock. Acid whey is a residual fraction produced in large volumes as a primary waste stream after crude cheese and Greek yoghurt manufacturing (Erickson, 2017; Xu et al., 2017). Due to remarkable consumer demand for those products, its worldwide production is systematically growing and reaches 165 million tonnes per year (Macwan et al., 2016). Furthermore, acid whey imposes high biological and chemical oxygen demand (BOD of 30,000–50,000 mg/L and COD of 60,000–80,000 mg/L, respectively) (Lievore et al., 2015; Macwan et al., 2016), thus significantly contributes to rising the environmental footprint when simply disposed of (Macwan et al., 2016), and the disposal costs run high. Even though it may be processed into whey powder or used for the production of protein concentrate (Nishanthi et al., 2017) and monosaccharides (Lindsay et al., 2018) owing to its high functional and nutritional properties, its utilisation is assessed to be only 75% in Europe and less than 50% in the rest of the world (Macwan et al., 2016). Moreover, several research was carried out on whey conversion into other valuable products, such as biogas or biohydrogen (Göblös et al., 2008; Azbar et al., 2009), but the proposed technologies are not well established yet. Based on the fact that the dairy industry is a sector of the economy where the greatest reduction potential can be achieved through sustainable acid whey management, the development of caproic acid production technology from acid whey would create an innovation opportunity for the dairy industry in the European market and established technology could be fitted into existing facilities.

2 Aim of the study

The main objective of the study was **to develop a biological caproic acid production process in a microbiome-based system from an organic waste feedstock**. For this purpose, two long-term continuous processes, as well as batch experiments were conducted. The continuous processes were performed in a single-phase reactor microbiome-based system using acid whey wastewater as a model substrate (**Paper I, Paper II**). Batch experiments were performed in serum bottles on a synthetic medium (**Paper III**).

The particular aims of the processes were:

- to demonstrate the feasibility of the long-term caproic acid production in a single-phase reactor microbiome-base system from a real organic waste feedstock – acid whey **(Paper I)**
- to discern and characterise the impact of crucial operating parameters (pH, temperature, hydraulic retention time, product toxicity) on the caproic acid production from acid whey for effective process control **(Paper I)**
- to investigate and explain the influence of change in feed composition on the chain elongation (CE) and the medium-chain carboxylic acids production, in particular, to investigate whether the presence of additional ethanol content could boost the process performance **(Paper II)**, and to investigate the influence of various concentration of lactose in presence of electron donors (ED) (lactate, ethanol or both) on process selectivity and conversion efficiency **(Paper III)**
- to identify possible metabolic pathways in the fermentation process **(Paper I, Paper II, Paper III)**
- to characterise the microbial community in terms of its structure, dynamics and activity along with the continuous processes **(Paper I, Paper II)** and to explore community variation and enrichment of functional groups responsible for specific product formation under applied substrates combination **(Paper III)**
- to identify key microbial groups responsible for caproic acid production **(Paper I, Paper II).**

3 Material and methods

Research were performed on a laboratory scale in the form of continuous reactor processes, as well as a series of batch processes via open culture fermentation. For the process control and samples analysis, advanced analytical methods and state-of-the-art molecular biology tools were applied. The graphical presentation of the employed methodology is presented in Fig. 2. Analytical methods performed at each stage of the study enabled evaluation of the substrate conversion and product formation in an open culture fermentation processes. Microbiological methods were based on high-throughput technologies, such as Illumina sequencing and bioinformatics, and flow cytometry combined with cell sorting. The first method employed amplicon metasequencing of 16S rRNA gene fragment that enabled microbiome characterisation including microbial structure and dynamics assessment and key microbial groups identification at different points of reactor processes operation. The second method enabled the evaluation of the microbiome activity at the single-cell level.

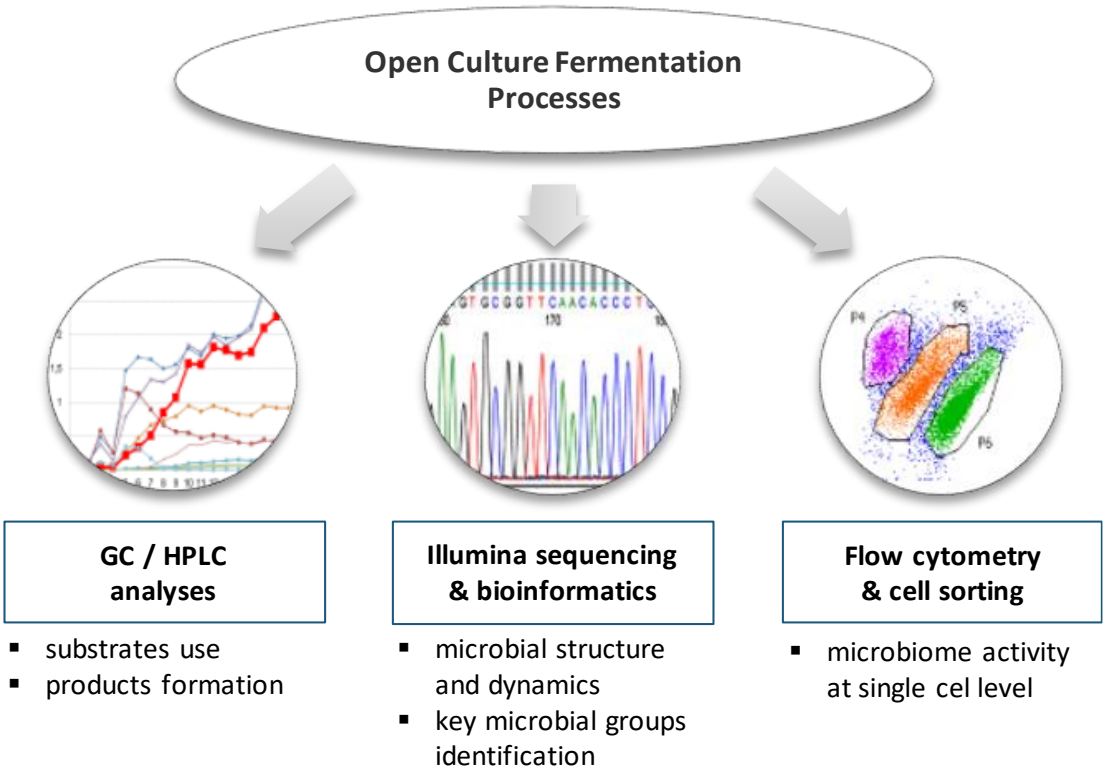

Figure 2. Graphical scheme of the methodology applied in the research.

3.1 Continuous processes configuration

For continuous processes for long-term caproic acid production, an experimental stand was designed. The workstation was set in a laboratory fume hood to prevent the release of hazardous or unpleasant fumes, vapours and gases (Fig. 3A), and consisted of the following components:

- Custom-designed up-flow anaerobic sludge blanket (UASB) reactor of 1L working volume cylindrical vessel covered with a water coat equipped with a circulating heater to maintain the set temperature, and 2.5L upper vessel for the surplus effluent collection, and recirculation tube (Fig. 3B)
- dedicated controller and set of pumps and tubes for pH control, feed supply, effluent pump out, and solid free wastewater recirculation from the up to the bottom – to keep the biomass within the reactor in suspension.
- fridge for feedstock storage
- effluent storage tank
- gasometer
- sampling ports for collection of biomass (coming out directly from the reactor vessel), liquid phase (set on a recirculation tube), and gases (set on a gas outflow tube, before the gasometer)

The proposed workstation configuration enabled to proceed with the fermentation processes under set pH and temperature conditions, and with hydraulic retention time (HRT) control. The description of each reactor set-up is presented in (**Paper I and Paper II**). In brief, in both cases, the UASB reactor was inoculated with sludge coming from the anaerobic digester of the municipal wastewater treatment plant (Koziegłowy, Poznan Area, Poland) and fed with acid whey delivered from Dobrzyca Dairy Industry, Poland. Despite the processes having been conducted in the same pH and temperature conditions, specifically, the mesophilic temperature of 30°C and pH of 5.5, the differences between both processes lay in the following operational conditions. In **Paper I** the process lasted **410 days** and was performed with gradually decreasing HRT, starting with an HRT of 20 days and ending at an HRT of 2.5 day. The feedstock supplied to the system was stored in a room temperature, thus its composition was fluctuating along the process and it was not controlled. In **Paper II** the process lasted **129 days** with gradually decreasing HRT, starting

with an HRT of 20 days and ending up at an HRT of 1.25 day. The feedstock came from two different batches that were precisely prepared for each operating period before use, i.e. pre-fermentation of acid whey until the concentration of ethanol reached the expected level was carried out and additionally external ethanol was added to the feedstock at a specific period of the fermentation process and in the last phase the feedstock did not contain ethanol (**Paper II, Table 1**). Then prepared feedstock was supplied to the reactor from the refrigerated reservoir (4°C), thus the loading rates of particular feedstock components were under control. Gaseous, liquid and biomass samples were collected regularly from dedicated sampling ports prior to analyses.

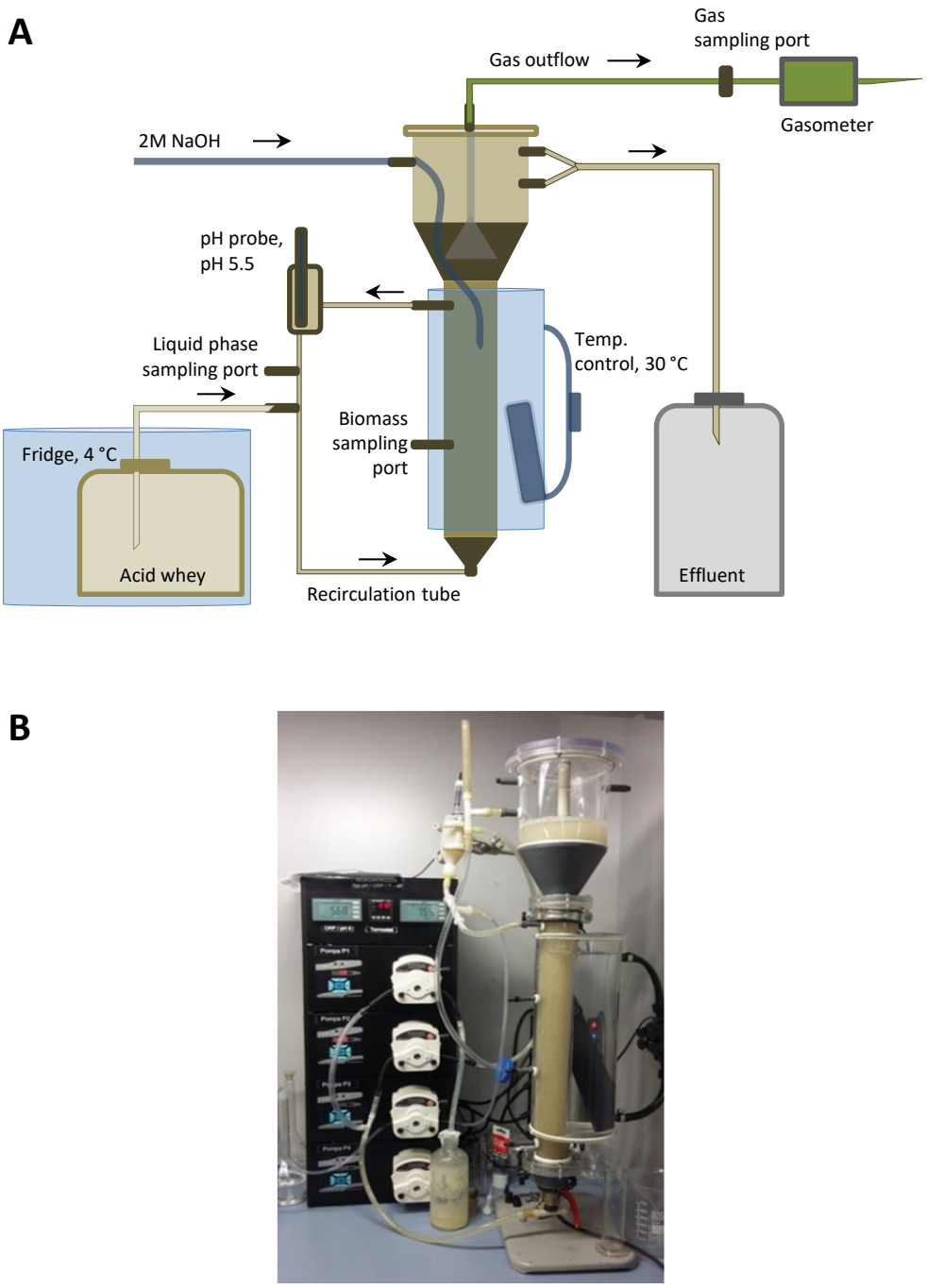

Figure 3. **A.** Scheme of the created research workstation and **B.** UASB reactor used in the research.

3.2 Batch processes configuration

The batch mode experiment was conducted in 120 mL serum bottles on a synthetic medium under strictly anaerobic conditions as described in **Paper III.** A scheme of serum bottle bioreactors for the batch mode fermentation processes and a picture of the bottles are presented in Fig. 4.

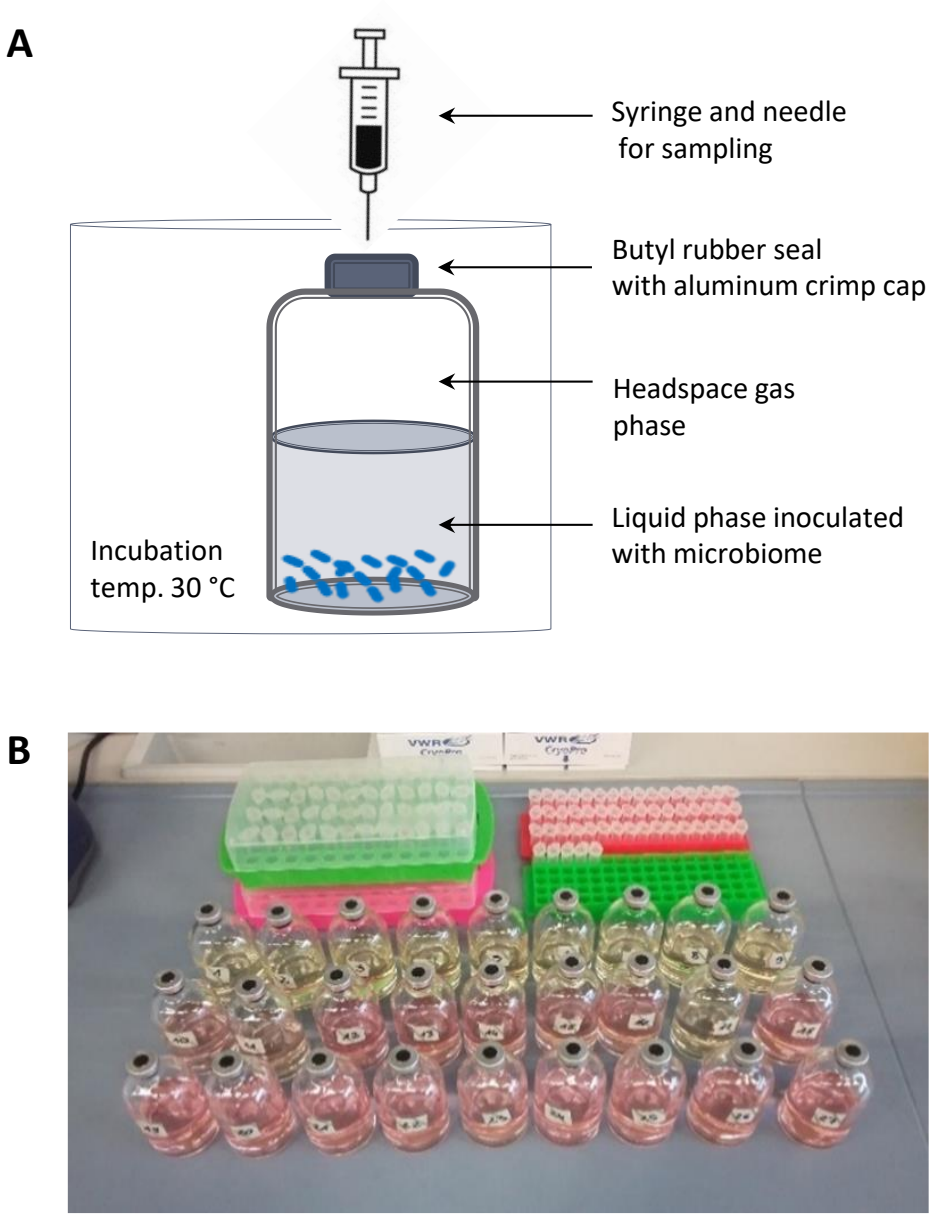

Figure 4. A. Scheme of serum bottle bioreactors for the batch mode fermentation processes and **B.** Bottles prepared for the batch mode experiment.

In short, nine batch trials of different carbon substrates composition (lactate, ethanol and lactose) were prepared in biological triplicates to study the content of lactose, a model carbon source (the main component of acid whey), in presence of lactate and/or ethanol on CE, product selectivity and conversion efficiency. The specific batch tests configuration and substrate composition are presented in **Paper III, Table 1**. Before fermentation, the bottles were filled with the DSMZ-52 synthetic medium prepared according to the DSMZ protocol (Leibniz Institute, Germany) with the modification of the composition of carbon sources used, closed tightly with butyl rubber septa and aluminum crimp caps, flushed with N_2 gas for 10 minutes to create anaerobic conditions, and autoclaved

for 20 minutes at 121 °C. Carbon substrates were prepared separately by autoclaving (ethanol, lactate) or filter sterilisation (lactose) and added to the bottles under sterile conditions. For bottles inoculation 1 mL aliquots of biomass from the UASB reactor (working on a stand-by in a laboratory) were washed with 1x PBS (Sigma), cell pellets were collected by gentle centrifugation (approximately 1500 rpm for 2 min), dissolved in previously sterilised medium and inoculated into the bottles. The total working volume of the bottles was 60 mL, and the initial pH was 7.5. Fermentations were performed in mesophilic conditions of 30°C without mixing and run for 22 days. Gaseous and liquid samples were systematically collected using syringe and needle under sterile conditions and analysed with GC and HPLC systems towards gaseous and liquid substrates and metabolites identification and quantification. Biomass samples were collected at the end of the process for microbial analyses.

3.3 Analytical methods

Qualitative and quantitative analysis using gas chromatography (GC).

Gaseous samples were analysed directly after collection for H_2, CO_2 and CH_4 using a gas chromatography system (Shimadzu 2014 GC System, Japan) equipped with a Porapak N column and thermal conductivity detector (TCD). For the analysis 0.2mL of the gaseous sample was collected regularly using a gas-tight syringe (SGE, Analytical Science) and injected manually. Nitrogen was used as the carrier gas at a flow rate of 15 mL/min and the temperature of the injector, column and detector were 110, 50 and 80 °C, respectively.

Liquid samples were analysed for short and medium-chain carboxylates (acetate, propionate, i-butyrate, butyrate, i-valerate, valerate, caproate, heptanoate, and caprylate) and alcohols (ethanol, propanol, i-propanol, butanol, i-butanol) using gas chromatography system (Shimadzu 2014 GC System, Japan) equipped with a flame ionization detector (FID) and a high-performance capillary column with a free fatty acid phase (Zebron ZB-FFAP, Phenomenex). Prior to analysis, samples were centrifuged for 10 minutes at 10 000 rpm, the supernatants were acidified using phosphoric acid and filtered with syringe filters (with a 0.45 µm pore size). In the methods of **Paper, I** and **Paper II** helium was provided at a flow rate of 7.38 mL/min. The initial oven temperature was 70 °C, maintained for 1 min, raised

to 240 °C at 10 °C/min, and finally held at 240 °C for 3 min. The temperature of the FID and the injection port were 250 and 200 °C, respectively, according to the standard protocol based on (Vasquez et al., 2014). In **Paper III** the method was modified as follows: helium was provided at a flow rate of 5.3 mL/min. The initial oven temperature was 70 °C, maintained for 3 min, raised to 185 °C at 10 °C/min, and finally held at 185 °C for 8 min. The temperature of the FID and the injection port were 250 and 250 °C, respectively.

Qualitative and quantitative analysis using high-performance liquid chromatography (HPLC). The concentration of substrates and other metabolites, such as lactose and lactate were determined and analysed according to the standard protocol based on (Sydney et al., 2014) using a high-performance liquid chromatography system (HPLC 20AT, Shimadzu, Japan) equipped with RezexTM ROAOrganic Acid H+ (8%) column and refractive index detector. Prior to analysis, samples were centrifuged for 10 minutes at 10 000 rpm, and the supernatants were filtered with syringe filters (with a 0.25 µm pore size). For elution 5 mM aqueous sulfuric acid was used at the flow rate of 0.6 mL/min at 63 °C.

3.4 Microbiome analyses

Biomass sampling and sequencing. For the analysis of the microbial populations, a biomass sample of the seed sludge was taken before each continuous process inoculation and samples from the UASB reactor and the feed were collected throughout the continuous processes regularly.

For **Paper I, Paper II** and **Paper III** genomic DNA was isolated using the commercial kit GeneMATRIX Soil DNA Purification Kit (Eurx®, Poland), according to the manufacturer's instructions. Genomic DNA extraction from sorted subpopulations was conducted according to (Koch et al., 2013) (**Paper I and Paper II**). The Illumina metagenomic sequencing analyses were performed based on the fragment of gene encoding 16S rRNA, according to Illumina protocol by an external commercial institution (Genomed, Warsaw, Poland). The libraries were prepared based on V3 and V4 regions of 16S rRNA gene fragment using Q5 Hot Start High-Fidelity 2X Master Mix, and the following primers set:

341F (5´- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG)

785R (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Bioinformatics.

In **Paper I** and **Paper II** α-diversity (OTU diversity) within every bioreactor sample and inoculum were calculated as observed OTUs, observed species, Shannon index (H'), Simpson index, Chao1 and Gini coefficient. In **Paper I and Paper III** a heatmap of OTU and ASV, respectively, that exceeded 1% relative abundance in at least one sample was created, additionally, in **Paper I** the OTUs on the heatmap were clustered based on the Bray–Curtis dissimilarity index. The β-diversity that compares bacterial communities between samples was calculated as the Principal Coordinate Analysis (PCoA) plot generated based on weighted UniFrac pairwise distance matrices (**Paper I** and **Paper II**), and as the NMDS plot based on Bray-Curtis dissimilarity, where the total abundances of less than 0.1% were removed from the analysis, stress = 0.1365 (**Paper III**).

Flow cytometry with cell sorting. The multi-parameter microbiome examination was performed using flow cytometry with cell sorter BD FACS Aria™ III (Becton Dickinson, USA) in cooperation with Poznan University of Life Sciences based on (Juzwa et al., 2016) (**Paper I and Paper II**). The multiparameter measurement enabled a quantitative and qualitative assessment of the microbial community at an individual cell level, considering the different metabolic activities of the distinguished subpopulations. A subpopulation of the highest metabolic activity was sorted for sequencing.

3.5 Calculations

Production rates **[mmolC/L/day or g/L/day]** of carboxylates were calculated for the working volume of the reactor in mmolC/L/day, and if necessary in g/L/day according to (Xu et al., 2017) (**Paper I and Paper II**).

Specificity **[% mol C]** understood as the product-to-carboxylate ratio in % mol C was calculated for caproate, SCCAs and/or MCCAs according to (Xu et al., 2017) (**Paper I and Paper II**).

Conversion efficiency **[% mol C]** was calculated as the final concentration of product(s) in mol/L C divided by net consumed mol/L C of substrates (lactose, lactate, ethanol) (**Paper II, Paper III**).

***Selectivity* [% mol e eq]** was calculated as the concentration of electron equivalents (e eq) in the generated product(s) (acetate, propionate, butyrate, valerate, caproate, H_2 and CO_2) divided by the net consumed electron equivalents in the substrates (Grootscholten et al., 2013b). The number of electrons for the following compounds are (mole electrons/mol compound): 8/acetate, 14/propionate, 20/butyrate, 26/valerate, 32/caproate, 12/ethanol, 12/lactate, 48/lactose, 2/H_2, 8/CH_4 (**Paper III**).

***Pearson correlation coefficient* (r)** was calculated to determine the correlation between the substrates load and the final concentration of produced carboxylates at $p < 0.05$ using Statistica v13 (**Paper I, and Paper III**).

Caproic acid is an undissociated form of the molecule while caproate is its dissociated form and the concentration of each of the forms in the fermentation broth is pH-dependent. Therefore, to simplify the nomenclature in the dissertation the caproate form will be used hence after, except if the use of the other form is necessary.

4 Open (mixed) culture fermentation

Open culture fermentation (OCF), also described in the literature as mixed culture fermentation, is a microbiome-driven process widely studied for nutrient and energy recovery from a range of different residual streams (Angenent et al., 2016; Arslan et al., 2016). Using reactor microbiomes is beneficial for several reasons. Microbiomes consist of mixed microbial communities represented by a multitude of metabolically interconnected and functionally organized species. It means that different subcommunities of the same microbiome have common metabolic pathways that interplay with each other in a synergistic way (Koch et al., 2014). It determines their resistance to some accidental process disturbances (Marshall et al., 2013), and in contrast to pure cultures fermentation, sterilisation procedures may be omitted (Angenent et al., 2016). Compared to aerobic bioprocesses, anaerobic fermentation is advantageous as it circumvents the addition of oxygen, which also reduces operating and capital costs considerably. Moreover, caproate production in OCF is an opportunity for the microbes to conserve energy under anaerobic conditions, thus little energy is lost in the process (Angenent et al., 2016). The ability to convert different waste components under non-sterile conditions and achieve high product yields makes the OCF processes economically attractive. Up to current knowledge, the same microbiome can be shaped toward desired purpose, usually done by alteration of operational conditions (Marshall et al., 2013). Taking into account that the success of the process outcome depends on many operational factors, including the source of inoculum, feedstock type, EDs, fermentation conditions (pH, temperature, hydraulic retention time), headspace composition, hydrogen partial pressure and so forth, the biggest challenge is to manage the process for an effective product formation.

Current investigations of caproate production are conducted using different reactor types, sources of inoculum (open cultures), substrates, and fermentation conditions to achieve the highest production rate (Jankowska et al., 2018; De Groof et al., 2019). Microbiome-based studies detailing reactor and batch processes that involved lactate for caproate production are listed in Table 1, which is an updated and extended version of **Paper I, Table 1**. The presented studies showed a wide range of caproate production rates (3.0 – 23.8 g/L/day) and maximum caproate concentration (3.1 – 23.4 g/L). The inoculum for the processes came from many different sources, usually, from anaerobic digester

sludge from WWTP (Duber et al., 2018, 2020; Zagrodnik et al., 2020; Brodowski et al., 2020), but also from thin stillage (Andersen et al., 2015; Sträuber et al., 2016), a mixture of pit mud and activated sludge (Wu et al., 2018), a mixture of several different sources (Contreras-Dávila et al., 2021), reactors already run in laboratories for MCCAs production (Kucek et al., 2016a; Khor et al., 2017; Candry et al., 2020; Contreras-Dávila et al., 2020; Brodowski et al., 2022; Duber et al., 2022) and so forth. In the presented processes various substrates were used for the fermentation starting with a synthetic medium containing lactate and other components required for CE (Zhu et al., 2015; Kucek et al., 2016a; Wu et al., 2019b; Liu et al., 2020; Candry et al., 2020; Contreras-Dávila et al., 2021; Brodowski et al., 2022; Duber et al., 2022), by wastewaters intrinsically containing lactate (Xu et al., 2017; Zhu et al., 2017; Duber et al., 2018; Wu et al., 2018; Duber et al., 2020), cafeteria food waste extract (Nzeteu et al., 2018; Contreras-Dávila et al., 2020; Crognale et al., 2021), percolate of a maize silage/maize silage (Sträuber et al., 2016; Lambrecht et al., 2019), thin stillage (Andersen et al., 2015; Carvajal-Arroyo et al., 2019), etc.

Considering reactor processes with acid whey, Xu et al. proposed to lead the OCF process in separate steps and at different temperatures, where lactose from acid whey was first completely acidified to lactate in one reactor, which then was used as a feedstock pumped to a fermentative reactor (Xu et al., 2017) to produce mainly caproate. However, in this dissertation (**Paper I**) it was demonstrated that both steps were achievable to occur simultaneously in a single-phase reactor bioprocess. **In Paper I the successful upgrading of lactose and lactate-rich wastewater (acid whey) to caproate was shown. The process was demonstrated in a UASB reactor using open culture microbiome. It was also demonstrated that in the applied system of acid whey fermentation neither methanogen inhibitor nor external ED was needed for long-term caproate generation.** The paper included a comprehensive description of the process run at each stage of the fermentation, process performance (production rates, metabolites concentrations and C6 product specificity) visualised in **Paper I**, **Fig**. **2**, and a detailed discussion of the obtained results.

Table 1. Microbiome-based studies detailing continuous and batch processes that involved lactate for caproate (C6) production.

Reactor type	**Inoculum**	**Carbon source**	**pH**	**temp. [°C]**	**HRT [day]**	**C6 prod. rate [g/L/day]**	**Max C6 conc. [g/L]/C6 product extraction**	**C6 Related microbes**	**Reference**
No data	Thin stillage	Thin stillage	5.4-5.7	35	3	0.5	NA/In-line electrolytic extraction	*Megasphera spp.*; *Lactobacillus spp*.	(Andersen et al., 2015)
Batch reactor (wv. 1 L)	Reactor microbiome fed with diluted 'Yellow water' (from Chinese Strong-Flavour Liquor fermentation) (containing lactate, ethanol, and glucose)	Synthetic medium + lactate	6.5	30	28	3.0	23.4	Ruminococcaceae (Clostridium cluster IV), Lactobacillaceae (*Lactobacillus*), Clostridiaceae	(Zhu et al., 2015)
Leach-bed fermentation reactor with percolation (wv. 4.4 L)	Digestate of maize silage	Percolate of the maize silage digestion (two-phase process)	4.2-5.7	37	-	-	3.1	Clostridiales (*Clostridium* cluster IV and Clostridiaceae)	(Sträuber et al., 2016)
Fed-batch anaerobic filter (wv. 0.55 L)	Reactor microbiome fed with ethanol-rich yeast fermentation beer	Synthetic medium + lactate and butyrate	5.0	34	1.9	3.1	NA/In-line extraction	*Acinetobacter spp.*; species of *Ruminococcus*, *Oscillospira;* genera from families: Veillonellaceae, Ruminococcaceae; Clostridiales	(Kucek et al., 2016a)

150 mL serum bottles with agitation; (wv. 50 mL)	A mixture of wastewaters + Ruminococcace strain CBP6 (*Clostridium* cluster IV)	Fermentation pit of a liquor brewing factory (containing lactate, acetate, butyrate, glucose, and ethanol) mixed with municipal wastewater	5.5	30	-	5.29	16.6	Ruminococcace strain CBP6 (*Clostridium* cluster IV)	(Zhu et al., 2017)
100 mL bioreactor; (wv. 50 mL)	Reactor microbiome fed with thin stillage and microbes native to the grass	Grass fermentation effluent containing lactate	5.5-6.2	32	2	23.8	4.1/ membrane electrolysis extraction	*Clostridium* cluster IV and *Lactobacillus*	(Khor et al., 2017)
Upflow anaerobic filter (wv.0.55 L)	Reactor microbiome fed with ethanol-rich yeast fermentation beer	Primarily fermented acid whey waste (temperature-phased system)	5.0	30	2.1	1.6	In-line extraction	unknown Bacteroidales and Clostridiales	(Xu et al., 2017)
6L Leach-bed reactors (wv. 3 L)	Anaerobic granular sludge from an anaerobic digester	Homogenized restaurant food waste (single phase)	7.0	37	7	3	10	*Clostridium sp.*	(Nzeteu et al., 2018)
UASB reactor (wv. 1 L)	Anaerobic digester sludge from WWTP	Acid whey wastewater (containing lactate and lactose)	5.5	30	2.5	7.4	10.5	Coriobacteriaceae, Ruminococcaceae (*Ruminococcus, Oscillospira*), Prevotellaceae	(Duber et al., 2018)
1 L serum bottles	A mixture of pit mud and activated sludge	Chinese liquor-making wastewater ethanol, lactate and SCCAs	6.5	35	-	-	5.6	Different Clostridia	(Wu et al., 2018)

1.5 L batch anaerobic reactors	Excess sludge and pit mud	synthetic medium with lactate, acetate and H2	7	35	-	-	21	*Clostridium spp*.	(Wu et al., 2019b)
1.6 L glass upflow reactor	Biomass from a CSTR fermenting solid-free stillage	Solid-free thin stillage	5.5	34	0.38	12.3	6.8	Ruminococcaceae, Coriobacteriaceae, Veillonellaceae (granular bed)	(Carvajal-Arroyo et al., 2019)
15 L continuous stirred-tank reactor (wv 12 L)	Acidogenic percolate and methanogenic digestate from a pilot-scale biogas plant	maize silage mixed with deionised water, trace element solution, and ammonium bicarbonate solution	5.5	38	4	-	6.1	*Bifidobacterium* and *Olsenella*	(Lambrecht et al., 2019)
1 L repeated batch stirred tank reactor (1L-RBF)	Reactor microbiome fermenting food waste	food waste (containing lactate, ethanol, and some organic acids)	6.0	35	-	1.89	5.5	*Lactobacillus spp., Caproiciproducens spp.*	(Contreras-Dávila et al., 2020)
120 mL serum bottles (wv 55 mL)	Heat-treated anaerobic digester sludge from WWTP	synthetic medium with lactose, acetate, ethanol, and lactate	6.8	37	-	-	9.1	Clostridia	(Zagrodnik et al., 2020)
UASB reactor (wv 1L)	Anaerobic digester sludge from WWTP	Fresh (raw) and prefermented acid whey wastewater (containing lactate, lactose, and/or ethanol)	5.5	30	1.25	4.5	8.5	Coriobacteriaceae, Veillonellaceae, Prevotellaceae	(Duber et al., 2020)
UASB reactor (wv 1L)	Anaerobic digester sludge from WWTP	Acid whey wastewater	5.5	30	2.5	2.7	8.6	-	(Brodowski et al., 2020)

Continuous stirred tank reactors (CSTR) with magnetic stirring (wv 0.9 L)	Pre-adapted lactic acid chain elongating culture enriched from a granular fermentation reactor converting a carbohydrate-rich stream to caproic acid	A synthetic medium with lactic acid	6	34	4	1.1	4.2	*Caproiciproducens*	(Candry et al., 2020)
Semi-continuous stirred tank reactor (wv 12 L)	Lab-scale CE reactor fed with lactate-rich corn silage	A synthetic medium with lactate and xylan	5.5	38	4	3.72	14.9	*Ruminiclostridium* 5 and *Pseudoramibacter*	(Liu et al., 2020)
Continuous reactor (wv 3 L)	Anaerobic inoculum from a digester of a WWTP	Cafeteria food waste extract	6	37	4	1.1	4.8	*Pseudoramibacter*	(Crognale et al., 2021)
Upflow anaerobic reactor (wv 3.2 L)	Several sources: ethanol-based CE and food waste fermentation, unfiltered Chinese liquor; rumen fluid; a full-scale lipid-degrading anaerobic digestor; and lab-scale acetate-oxidizing bioanode and biocathode producing n-butyrate	Feeding solution containing lactate, minerals, and nutrients	5.5	30	1	4.4	-	*Caproiciproducens*	(Contreras-Dávila et al., 2021)
Lambda Minifor fermenters (wv 0.8 L)	Sludge from (UASB) reactor producing caproate from acid whey in the CE process	A synthetic medium with lactate and acetate	5.5	30	5	1.4	6.9	Ruminococcaceae bacterium CPB6 and *Acinetobacter*	(Brodowski et al., 2022)
Batch in serum bottles (wv 60 mL)	Acidogenic reactor fed with acid whey, working on stand-by in laboratory	A synthetic medium with lactose and lactate	7.5 initial, not adjusted	30	-	-	1.8	Bacillaceae	(Duber et al., 2022)

NA – not analysed, wv - working volume, C6 – caproic acid

5 Process strategies and operational factors

Caproate production through open culture fermentation is dependent on several fermentation parameters. The major strategy that needed to be addressed for enhanced caproate production was to **avoid competing metabolic pathways** (methanogenesis, propionate-producing fermentative pathways such as acrylate pathway and others) (Wu et al., 2019a). The strategy relied on suppressing the methanogenesis to enable effective CE to caproate. Then the process operating conditions were set to drive the reaction towards CE and prevent any other competing metabolic pathway and dispersing lactate-carbon flow. In the works of this dissertation, OCF processes for caproate production were run in continuous and batch mode, and **vital parameters such as temperature, pH, and hydraulic retention time (HRT) were characterised** (**Paper I**). Other important aspect touched **the product toxicity resulting in process inhibition** (**Paper I**). Then **substrate impact** in terms of a feed composition, particularly the content of ethanol as an external electron donor (**Paper II**) and content of lactose (**Paper III**) on the process selectivity and the conversion efficiency were investigated.

5.1 Competing metabolic pathways

5.1.1 Methanogenic activity

Sludge coming from an anaerobic digester of a wastewater treatment plant is the most common inoculum for the OCF processes. However, it contains both, methanogenic Archaea and a plethora of microbes belonging to the Bacteria kingdom. The OCF is based on an anaerobic digestion process, where four simultaneous phases are distinguished: hydrolysis of complex organic waste, acidogenesis (formation of short-chain carboxylates, alcohols, carbon dioxide, hydrogen and other simple compounds), acetogenesis (conversion of longer carboxylates to acetate and hydrogen) and methanogenesis (Fig. 5), where methane is the main end-product (Kleerebezem et al., 2015; Arslan et al., 2016). To direct the process toward MCCAs generation, the methanogenesis must be suppressed to accumulate the shorter carboxylates as central intermediates and form a carboxylate platform. In this dissertation, due to anaerobic digester-derived microbial communities, the occurrence of methanogenic activity in OCF posed a threat, as the methanogens compete with the other

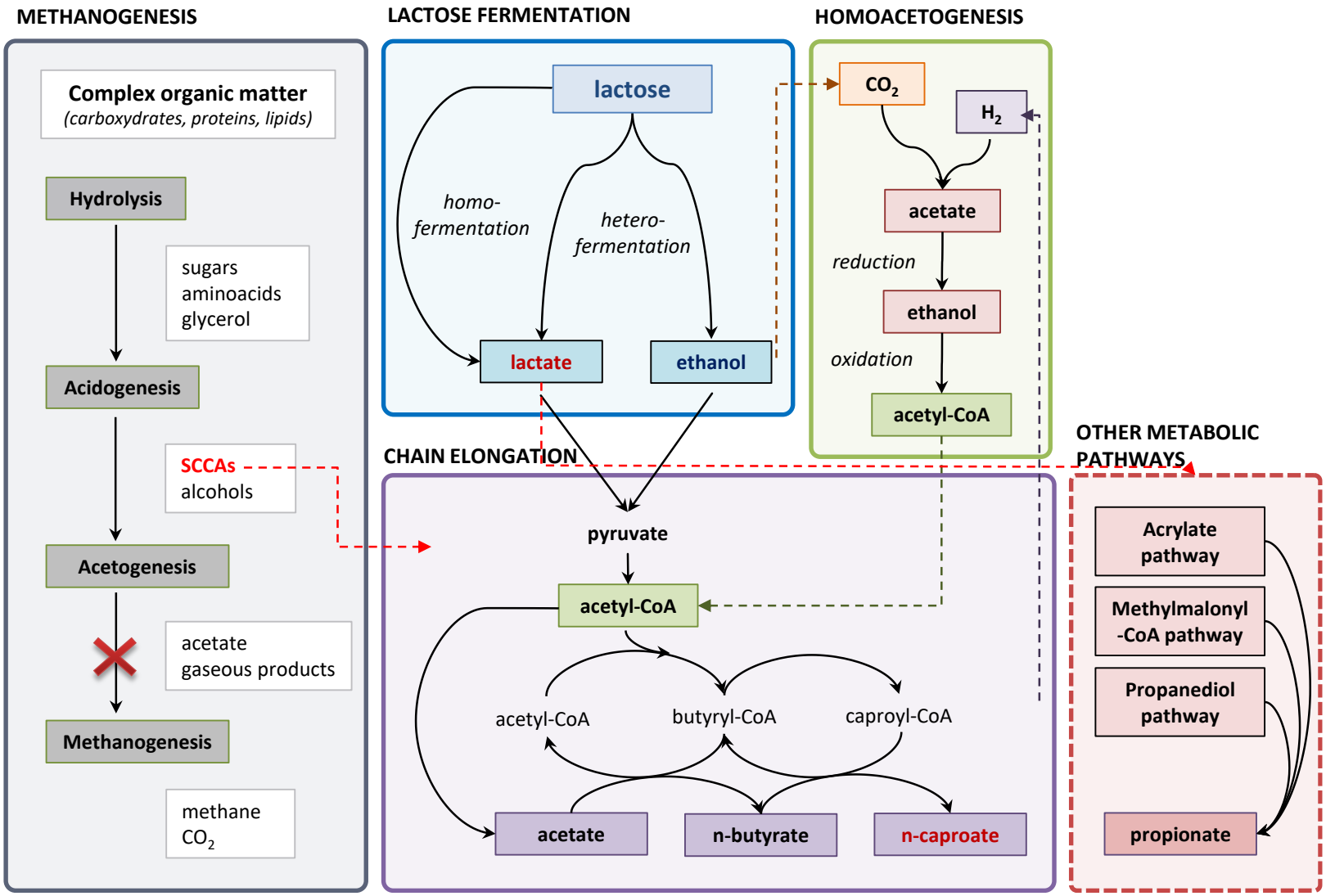

Figure 5. Scheme of possible metabolic pathways during the fermentation process based on (Duber et al., 2018, 2020).

microbes for substrates. Biological competition must be avoided as it decreases the process efficiency (Agler et al., 2012a). The microorganisms which should be primarily prevented, are acetoclastic methanogens, that consume acetate – the electron acceptor for carboxylate CE (**Paper II, Table 2**). Elimination of methanogens can be imposed by specific inhibition methods, e.g. sludge heat treatment prior to inoculation (Zagrodnik et al., 2020), chemical agent addition (2-bromoethanesulfonate, BES; iodoform, CHI_3) (Steinbusch et al., 2011), reduction of pH value (Agler et al., 2012a) or shortening the hydraulic retention time (Cavalcante et al., 2017). However, those methods are not perfect, for instance, thermal pretreatment or exposure to chemical suppression not only deactivates the Archaea but also eliminates a vast of bacterial species that may be needed for the CE process or for a syntrophic relationship with key caprogenic species (addition of BES was reported to act as a competing electron acceptor for sulphate-reducing bacteria and dehalogenating microorganisms) (Ye et al., 1999; Seedorf et al., 2008). According to the study of De Vrieze et al., optimum pH level for most methanogens is between 6.8 and 7.5 (De Vrieze et al., 2012). **In continuous bioreactor processes described in Paper I and Paper II the pH level of 5.5 was low enough to effectively inhibit methanogenesis where acetoclastic methanogens were completely outcompeted**. In batch trials (**Paper III**) the inoculum came from a long-term working UASB reactor, therefore the methanogenic Archaea were suppressed already, and the applied microbiome was established for organic acids production. Thus, applying that low pH was not necessary.

5.1.2 Chain elongation

Caprogenesis may occur through the carboxylic acid CE process based on the carboxylate platform. In CE (Fig. 5) the SCCAs (chain length from C2 to C5), which are primary metabolites of the OCF, are elongated in a secondary fermentation step into MCCAs (mainly with a chain length from C6 to C8). According to literature, the well-known CE pathway is a cyclic process called reversed β-oxidation (RBO) (Spirito et al., 2014), which was reported based on *Clostridium kluyveri* strain metabolism (Seedorf et al., 2008). For caproate generation, the process is comprised of two consecutive RBO cycles, that require energy-rich, reduced compounds (EDs) to form acetyl-CoA molecule for the carbon coupling to the carboxylic acid chain (Cavalcante et al., 2017). In the first cycle acetate

is elongated to butyrate, then in the second cycle butyrate is elongated to caproate, and if the CE proceed caproate may be elongated to caprylate on the same basis. The whole process is commenced by substrate oxidation to provide initial energy. The most studied EDs for the reaction are ethanol (Ge et al., 2015; Kucek et al., 2016c; Andersen et al., 2017) and lactate (Kucek et al., 2016c; Xu et al., 2017; Carvajal-Arroyo et al., 2019); however, other compounds that could be easily converted to acetyl-CoA, such as methanol (Chen et al., 2016) or galactitol (Kim et al., 2015) were also investigated. Reactions of even-numbered carboxylic acid CE from ethanol and lactate are presented in **Paper II, Table 2**. CE may also strike for odd-carbon numbered carboxylates (Grootscholten et al., 2013c); however, it is energetically less favourable. An alternative route described for CE is the fatty acid biosynthesis (FAB) pathway (Han et al., 2018). Compared to RBO, the FAB pathway is less efficient as it has more steps and consumes more energy. To current knowledge, it is unknown whether there are other complementary CE routes (Han et al., 2018).

The nutritive properties of acid whey make it a perfect feedstock for a microbiome-based caproate production. It contains high content of lactose, lactate, and some ethanol, which contribute to the CE process. In this dissertation, the primary fermentation step refers to lactose acidogenesis, where it undergoes a homo- or heterofermentative route, depending on the metabolising capabilities of specific microbes (**Paper I**). In the first case lactose is converted only to lactate, while in the second case lactate, ethanol and some acetate are generated. In both cases, the fermentation products serve as EDs for the CE process, as they can be easily converted by other microbial groups in the secondary fermentation step into pyruvate and then acetyl-CoA. The presentation of the proposed elaboration is precisely described in **Paper I** and **Paper II**.

5.1.3 Other metabolic pathways

Instead of direct lactate oxidation to acetate and CO_2 and then product incorporation into the CE process, lactate may be converted into propionate in alternative fermentative pathways such as propanediol pathways, methylmalonyl-CoA pathway or acrylate pathway (Fig. 5) (Gonzalez-Garcia et al., 2017). Acrylate pathway was observed under abundant lactate conditions of 16 gCOD/L/day lactate (that corresponded to 506 mmol C/L/day and lactate concentration of 657 mM C, HRT 1.3 day), where lactylyl-CoA, acrylyl-CoA,

and then propionyl-CoA were formed as intermediates (Kucek et al., 2016a) leading to a propionate production and possibly odd-carbon numbered CE. Kucek et al. (2016a) indicated that the acrylate pathway is a competing route of lactate utilisation, which once induced can be difficult to direct back to the RBO route and even-carbon CE. Authors also suggested that the acrylate pathway may be constrained by keeping residual lactate concentration near zero and applying pH as low as 5.0, once, at that pH, lactose homofermentative bacteria and lactate-based chain elongators can grow, and the propionate producing bacteria are limited (Contreras-Dávila et al., 2020). However, the microbial growth may be suppressed at that low pH in bioreactor systems, where the obtained products are not being extracted online, due to inhibition by emerging undissociated forms of carboxylates (Wu et al., 2018). In the continuous processes of this dissertation, the propionate production was avoided, even though the maximum lactate loading rate was as high as 380 mmol C/L/day at stage III (HRT 2.5 day) (**Paper I**) and 555 mmol C/L/day in period IV (HRT 1.25 day) (**Paper II**). However, propionate production was noticed in **Paper III** batch group I with lactate (R1), and lactate with lactose (R2). Even though the maximum initial concentration of lactate was only 120 mM C (R1), the process could have been channelled to propionate formation due to the high pH value in the reactor (around 7). In the work of (Lambrecht et al., 2019) lactate conversion route to propionate was reported after the pH increase to 7.0.

5.2 Fermentation parameters

Temperature, pH and EDs. Process temperature and pH are operational factors that directly impact the growth and activity of the microbial population which drive the biochemical reactions. The OCF process temperature for MCCAs production is usually set within the mesophilic range of 30°C – 38°C, and the pH is usually mildly acidic to neutral (4.2-7.5) as those conditions may be beneficial for the development of lactate-utilizing and caproate-producing bacteria (Table 1). pH in the range between 4.2 – 6 was proven to inhibit methanogens that are active in neutral and mildly alkaline conditions. At pH higher than 6-6.5 chemical inhibitors or other methods are needed to avoid methanogenic activity (Agler et al., 2014).

The biochemical reactions are also dependent on the available EDs for RBO reaction (lactate, ethanol, sugars) coming from the feedstock. Ethanol and lactate are the most

studied EDs for CE. While ethanol chain elongators prefer neutral pH, lactate chain elongators perform the reaction of CE in lightly acidic conditions (i.e. pH 5.5) (Kucek et al., 2016c). For example, in works where acid whey rich in lactate was supplied as feedstock, the temperature of 30°C and pH of 5.0 were applied, which was favourable for microorganisms delivered with the substrate (Xu et al., 2017). In other work where lactate CE to caproate was observed, the temperature of 30°C and pH of 5.5 were set (Zhu et al., 2017). On the other hand, in works where CE was based on ethanol utilisation, the pH close to neutral was favoured (Steinbusch et al., 2011; Agler et al., 2012a).

Based on collected literature sources, a temperature of 30°C and pH of 5.5 were chosen for continuous fermentation processes (**Paper I**, **Paper II)**. The same temperature conditions but a higher initial pH value (7.5) was used in the batch processes, and the pH was not adjusted along the fermentations (**Paper III**). Higher initial pH is a common practice when fast pH drop is expected in the process, for instance in the work of (Yin et al., 2017) where batch trials were initiated at a pH of 7.5. Thus, in **Paper III** the initial pH was set at 7.5 as lactose is easily fermentable sugar and its conversion led to fast acidification of the reactors and pH drop to a level more convenient for CE. If the initial pH was lower, the pH would drop too low and lead to process inhibition.

Hydraulic retention time (HRT). Other important parameter that influences the CE and process performance is HRT. HRT is defined as the ratio between the reactor volume and the influent flow rate and represents the average residence time that the microbial biomass and feed compounds remain in the reactor. The choice of adequate HRT is impacted by the complexity of the feed components. While complex substrates require higher HRT to be decomposed, the HRT for feedstock rich in easily fermentable components such as acid whey can be considerably shortened from several to a few days. The literature data show that the MCCAs productivity can be improved by working at low HRT when solids retention time is decoupled from HRT and the biomass is retained in the reactor, such as in UASB reactors or granular sludge reactors (Grootscholten et al., 2013d; Carvajal-Arroyo et al., 2019; Wu et al., 2020; De Groof et al., 2020). In the work of Roghair et al. when CE was run from acidified food waste and ethanol the maximum caproate production rate of 5.5 g/L/d at an average concentration of 23.4 g/L was obtained for HRT of 4 days, while when working on HRT 1 day the caproate production rate

was 5.6 g/L/d at an average concentration of 7.1 g/L (Roghair et al., 2018b). Furthermore, in HRT shorter than 4 days an in-line product extraction might need to be applied due to high product concentrations in the reactor resulting in possible product toxicity (Agler et al., 2012a; Andersen et al., 2015; Kucek et al., 2016c; Xu et al., 2017). **In Paper I it was shown that gradually shortening the HRT from 20 to 2.5 days improved the caproate production rate.** This parameter is also associated with the specific growth rates of methane- and caproate-producing microorganisms, as it should be adequate for the growth of desirable microorganisms. **In Paper I and Paper II it was shown that decreasing HRT contributed to washing out most methanogens from the UASB reactor and enhanced caproate production.**

5.3 Product toxicity

CE might be limited by a toxic effect of an undissociated form of short and medium chain carboxylic acids. Undissociated acids release pressure on the microbial cells, which restrict the bioproduction, and the antimicrobial properties increase with the chain length (Desbois and Smith, 2010; Coma et al., 2016). At the temperature of 30°C, the p*K*a value of short and medium carboxylates is between 4.8-4.9 (4.88 for caproic acid), therefore at pH 5.5 the carboxylates are present in vast part in an undissociated form (Agler et al., 2012a; Grootscholten et al., 2013a). Ge et al. reported the upper toxicity limit for undissociated species of caproic acid of 7.5 mM (45 mM C or 0.87 g/L) at a pH of 5.0 when ethanol-rich wastewater was used as feedstock (Ge et al., 2015). In **Paper I** undissociated caproic acid presented an inhibitory effect at a concentration above 17.2 mM (103.2 mM C or 2 g/L) and in **Paper II** it was above 12.4 mM (74.38 mM C or 1.44 g/L), which are much higher concentrations than in the aforementioned work. The discrepancies between these studies can be explained by complex interactions between actual substrate composition, environmental conditions and other factors that may affect the condition of a specific reactor microbiome, its susceptibility and adapted preventing mechanisms (Agler et al., 2012b). Andersen et al. showed in their work that medium-chain fatty acids toxicity had a substantial impact on the microbial community which determined a decline in the overall production (Andersen et al., 2017). On the other hand, in the work of Roghair et al. authors proved that open cultures can adapt to high caproate concentrations to perform CE (Roghair et al., 2018a), which was achieved in **Paper I**. Thus, the effects

of different concentrations of undissociated carboxylates are not universal for a specific reactor microbiome. To alleviate the mechanism of product inhibition, decreasing its concentration in the bioreactor is required (Coma et al., 2016). Overcoming the toxicity issue, while achieving high caproate production rates, can be achieved by setting a favourable pH for organic acids dissociation (close to neutral), working at high dilution rates (short HRT), and/or in-line product removal/extraction (Gildemyn et al., 2017). **In Paper I gradually reduced HRT (from 20 days to 2.5 day) let to eliminate the caproate accumulation to some extent. Similarly, in Paper II, decreasing HRT from 2.5 day to 1.25 say enabled circumventing the butyrate toxicity. However, such a short HRT was too short for the microbes to assimilate nutrients and grow. In Paper II low caproate yield in Period IV (HRT 1.25 day) was attributed to high caproate concentration (up to 8.5 g/L) that was self-limiting and caused the process inhibition, despite the availability of the substrates.** Thus, in the works of this dissertation, the main limitation was the lack of robust technology for in situ product (caproate) recovery, thus higher production rates were not achievable.

5.4 Substrate composition

Processes conducted with real waste feedstock may be difficult to manage due to different compositions of batches and an inevitable changing of the feedstock composition during storage. It may naturally happen with the storage of acid whey wastewater in outside tanks at the dairy factory where the temperature is not controlled and biological processes occur naturally. Thus, achieving stable biological production of a specific metabolite at a high production rate and selectivity may be troublesome. In **Paper I** raw acid whey containing mostly lactose and lactate was used for a fermentation process. To feed the reactor, portions of acid whey were transferred from a fridge to a room temperature tank, with the purpose to reflect realistic conditions that occur while storage at the dairy factory. Investigations in **Paper II** went one step further, where the temperature of the feedstock to feed the reactor was controlled at 4°C and the impact of particular acid whey composition on the process outcome was evaluated. The study considered OCF of acid whey that, besides lactate, contained external ethanol as ED for CE within a certain period. For that purpose, the acid whey feedstock was prefermented in the laboratory

prior to feeding the reactor and a defined portion of ethanol was externally added during a specific period of the fermentation process. In **Paper III** the influence of various concentrations of lactose in presence of EDs (ethanol, lactate or both at equal molar concentration) on CE was investigated.

In the continuous bioprocesses (Paper I and Paper II) the results showed that caproate formation depended mostly on lactate-based CE, despite ethanol being produced in the reactor (Paper I) or externally delivered to the system with feed (Paper II). However, some ethanol production in the reactor seemed important for the CE process (as it coincided with caproate production) and its decrease coincided with the process disturbance (**Paper I**). In **Paper II** results showed a strong dependency of the produced metabolites share on the content of the substrates (lactate, ethanol, and lactose) in the feed. **Dosing acid whey with an increasing ethanol loading rate led to ethanol oxidation with SCCAs generation and hydrogenotrophic methane formation. Change of the feedstock composition (higher lactate and lactose content and delivery of external ethanol cut off) resulted in a sharp drop in the SCCAs formation and prompted MCCAs production with caproate as the main product.** The MCCAs production rate has grown from 0.7 ± 0 to 4.12 ± 1 g/L/day (38.3 ± 5 to 212.6 ± 60 mmol C/L/day) and reached a specificity of 48 ± 18% mol C. Short and medium-chain carboxylates production rates, specificity and conversion efficiency during selected periods of continuous bioprocesses of acid whey fermentation is presented in Table 2. Taking into account that higher MCCAs performances were generated without external ethanol delivery, **the conclusion was that in a one-step process it was essential to keep low ethanol concentration in the reactor (it was about 1-2 g/L) and abundant lactate and lactose content in feed (up to 20 g/L and 30 g/L, respectively) for caproate production**.

Table 2. Short and medium chain carboxylates production rates, specificity and conversion efficiency during selected periods of continuous bioprocesses of acid whey fermentation.

	1st continuous process	2nd continuous process		
	Acid whey fermentation (no ethanol addition)	**Start-up period**	**Feed with ethanol addition**	**Feed without ethanol addition**
Days of the process [d]	351 - 410	15 - 27	71 - 84	85 -129
HRT [d]	2.5	10	2.5	1.25
SCCA production rate [g/L/day]	1.4 ± 0.8	0.8 ± 0.1	11.2 ± 0.8	7.6 ± 7.0
SCCA specificity [% mol C]	31 ± 14	88 ± 2	92 ± 1	52 ± 18
SCCA conversion efficiency [% mol C]	10	17.9	51.0	24.4
MCCA production rate [g/L/day]	2.4 ± 0.6	0.1 ± 0.0	38.3 ± 5.0	4.2 ± 1.2
MCCA Specificity [% mol C]	69 ± 14	12 ± 2	8 ± 1	48 ± 18
MCCA conversion efficiency [% mol C]	20	2.5	4.5	16.3
Caproate production rate [g/L/day]	2.32 ± 0.59	0.1 ± 0.0	0.7 ± 0.1	4.1 ± 1.2
Caproate specificity [% mol C]	68 ± 14	9 ± 1	8 ± 1	47 ± 18
Caproate conversion efficiency [% mol C]	-	1.8	4.4	16.0

Fermentation processes performed in batch mode confirmed the dependency of products formation on the substrate composition (**Paper III**). In **Paper III** it was **demonstrated that the presence of additional substrate i.e. lactose may divert the carbon flux and impact the selectivity of final products by providing additional intermediates for the biochemical reactions in the OCF process.** For instance, when only lactate was used as an ED and different lactose content was applied, the process went in two different directions depending on the initial lactose concentration. When there was no lactose in the batch mostly acetate and propionate were formed as the end products, but the higher the lactose content the higher the caproate production was observed. In the study, the highest selectivity and conversion efficiency to caproate (based on % mol C) were 65% and 53 %, respectively, and it was observed in the trial with the use of lactate and the highest lactose content. In trials with lactose and ethanol, there was no caproate production, while in fermentations with lactose and both EDs the highest caproate selectivity and conversion efficiency reached only 8% and 7%, respectively. **Overall, the results provided that the choice of metabolic pathway and diverting the process outcome depends not only on the presence of EDs but also on other available compounds such as lactose and pH, which is especially important in designing waste and wastewater valorisation bioprocesses.**

6 Reactor microbiome characterisation

Open (mixed) cultures are widely studied in terms of their structure, dynamics, and function to develop engineered systems for biotechnological process application. Carboxylate metabolism is strongly related to the preponderance of various *Clostridia* (Han et al., 2018), where *Clostridium kluyveri* is the model organism capable to produce caproate from acetate and ethanol as sole carbon sources (Seedorf et al., 2008). However, the pH 5.5 applied in **Paper I** and **Paper II** was not favourable for *C. kluyveri* growth as presented in (Andersen et al., 2017), and the strain was not detected in both mentioned studies despite ethanol and acetate availability (**Paper II**). Thus, it was concluded that other species were performing CE. In papers of this dissertation the reactor microbiomes were characterised in terms of microbial composition along the processes (**Paper I, Fig. 4 and Fig. S2; Paper II, Fig. 4**) or final microbial composition of batch trials (**Paper III, Fig. 4**); alpha biodiversity, shown as Shannon diversity index (H´) and Gini index (**Paper I, Table S3; Paper II, Fig. 3A**); beta biodiversity demonstrated as the Principal Coordinate Analysis (PCoA) (**Paper I, Fig S3; Paper II Fig. 3B**); and cellular metabolic activity (flow cytometry analysis combined with cells sorting) (**Paper I, Fig. 5; Paper II, Fig. S3**). Moreover, a non-metric multidimensional scaling plot (NMDS) of Bray-Curtis dissimilarity between microbial communities in samples of batch trials was analysed. In **Paper I** the study showed that **functional enrichment of the specific microbiome for caproate formation was feasible by proper control of operational parameters, namely pH and HRT.** Furthermore, the results of analyses in **Paper II and Paper III** showed a **strong dependency of the structural biodiversity and composition of the microbial population on the substrate composition**.

The studies of **Paper I** and **Paper II** indicated a predominance of Coriobacteriaceae, Ruminococcaceae (Ruminococcus, Oscillospira), Prevotellaceae and Veillonellaceae families, which likely participated in the metabolic progression of CE in lactate-chain elongating reactor microbiomes. The only known caproate producer was aligned to *Caproiciproducens* spp., which reached a relative OTU abundance between 3 and 7% (**Paper II**). Even though the data analysis of sequences in **Paper I** did not reveal any specific caproate producer, the sequences were reanalysed and described 3 years after using an updated database by (Candry and Ganigué, 2021), and the study showed that

Caproiciproducens was also present in **Paper I,** but the species (sequences) were different from those in other studies on lactate-based CE (Carvajal-Arroyo et al., 2019; Contreras-Dávila et al., 2020). In **Paper III** batch group I, where lactose and lactate were used as substrates up to 70% of the total microbial community was assigned to the Bacilli class, (where up to 38% belonged to the Bacillaceae family), the other bacteria belonged to Clostridia, Gammaproteobacteria and Bacteroides. The simultaneous presence of Bacilli and Clostridia was previously noted in the work of Lambrecht et al., where complex biomass was fermented (Lambrecht et al., 2019). The members of *Caproiciproducens* spp. were present in this batch group but at a very low level of about 1%, which made it unlikely to be responsible for caproate production. When lactose with lactate and ethanol were used as substrates the microbial community structure was different suggesting that the presence of ethanol influenced the enrichment of other microbial groups, where the majority of sequences were aligned to Clostridia-like members. Anaerobic bacteria from the Clostridial cluster such as *Clostridiaceae*, *Ruminococcaceae* or *Oscillospiraceae* occurred often in ethanol-related CE processes (Agler et al., 2012a; Kucek et al., 2016b). In terms of methanogenesis, accidental methane formation in Paper I and Paper II was associated with *Methanobrevibacter*-related phylotypes, that are hydrogenotrophic Archaea, and therefore posed no direct threat to the CE process. In the batch trials (**Paper III**) pre-adapted consortia from the UASB reactor were used to shorten acclimation times and avoid methane production.

Results of α-diversity analyses that show the structural biodiversity of a reactor microbiome in every sample revealed that the biodiversity of the microbial population (showed as the Shannon index) in the bioreactor fed with acid whey (**Paper I**) was a bit higher than in other similar studies (Kucek et al., 2016a, 2016c; Xu et al., 2017); however, it indicated a varied but stable microbial community during the process. In the same bioprocess, the Gini Coefficient was close to 1 which implied a functionally specialised microbiome for caproate production. In **Paper II**, the Shannon biodiversity was decreasing along the process and it was lower than in **Paper I** and other studies, while the Gini Coefficient was growing over time reaching the value of 0.99 at the end of the process (likewise to **Paper I**, where it was 0.98 and other work, where it was 0.94 (Xu et al., 2017)). β-diversity presented as PCoA plot (**Paper I, Paper II**) and as NMDS plot (**Paper III**) depicted differences

in microbial community between samples of the bioreactor and inoculum of each bioprocess. The results showed a strong dependency of the microbial biodiversity on the composition of the substrates, i.e. there was a clear temporal variation between samples of different operating periods (**Paper II**) or in different batch groups (**Paper III**) due to a different feed composition.

A fundamental issue in running the UASB reactor is to separate the microbial biomass from the liquid phase and retain it in the bioreactor in the form of granules. Stable granular sludge is attributed to slow-growing microbes, thus the growth rate and growth yield play a key role in fermentative granules formation (Carvajal-Arroyo et al., 2019). Up to date diverse granular sludges were developed and proven their advantages in anaerobic wastewater treatment, e.g. for nitrogen removal. However, there are only a few studies on biomass granulation devoted to volatile fatty acids production (Tamis et al., 2015), lactic acid production (Kim et al., 2016), and only two studies reported on MCCAs production (Roghair et al., 2016; Carvajal-Arroyo et al., 2019). Under operational conditions applied in **Paper I** and **Paper II** there was a lack of microbial granulation, but rather aggregated flocks were formed and the remaining cells were floating in the liquid phase as presented in Fig. 6. In 2016 Roghair et al. developed an ethanol-based CE bioprocess with granular sludges formation using a synthetic medium containing propionate, ethanol and CO_2 for MCCA production (namely caproate and heptanoate) (Roghair et al., 2016). A year after the **Paper I** had appeared Carvajal-Arroyo et al. reported a microbial granules formation at pH 5.5 in a bioprocess treating the solid-free supernatant of thin stillage (that contained 1.1 g/L of acetate, 3.9 g/L of lactate, 0.4 g/L of ethanol, 6.0 g/L of glycerol, and total carbohydrates expressed as 20.5 g glucose/L) (Carvajal-Arroyo et al., 2019). In mentioned bioprocess the CE was lactate-dependant and caproate production reached sustained rates of 12.3 g/L/d, which was 4 times higher than in **Paper I**, and the concentration of caproate in the reactor of 6.8 g/L. The fermentative granulation in mentioned work was possible due to an up-flow velocity >14 m/h that caused high shear stress, which is a requirement for the formation of the granules. In the study of this dissertation, the up-flow velocity within the reactor was as low as 1.24 m/h, which explains the lack of microbial granulation.

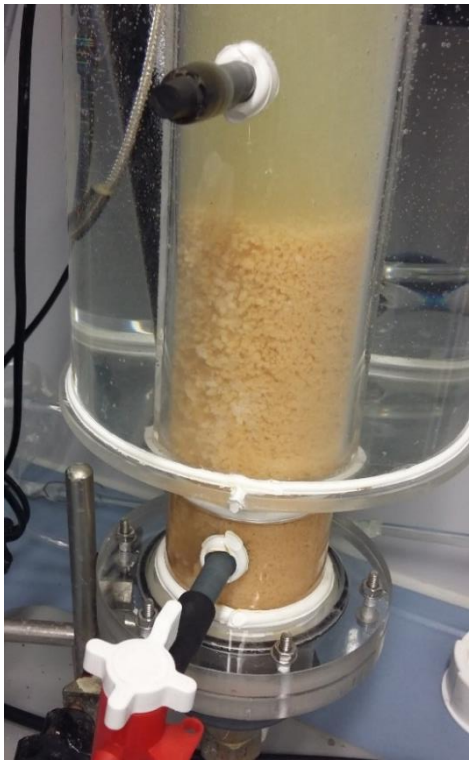

Figure 6. Flocks and aggregates formed during the continuous fermentation processes.

Even though the biomass was not forming granules, the fermentative activity within the formed flocks and cells floating in the liquid phase were analysed at different process stages using flow cytometry analysis combined with cell sorting and subsequent Illumina sequencing. In **Paper I** the analysis allowed to investigate active, mid-active and non-active microbial groups (**Paper I, Fig. 5**), whereas in **Paper II** when the CE to caproate was noted, the most metabolically active microbial group was sorted out and sequenced(**Paper II, Fig. S3**). The results showed that the structure of the microbial community was only slightly different than the total microbial community and was represented by Coriobacteriaceae, Prevotellaceae and Clostridiaceae families (relative abundance of 70%, 20%, 5%, relatively) (**Paper II, Fig. 4**). The results are contrary to the other study where Ruminococcaceae (Clostridia) was the dominating family, while Coriobacteriaceae were found rather in suspended biomass (Carvajal-Arroyo et al., 2019).

Results of the flow cytometry measurement coupled with cell sorting and subsequent metagenomic sequencing provided a comprehensive knowledge of the active microbial subgroups that were directly involved in the CE process and caproate production. **Combining of aforementioned methods put a new light on the analysis of different subgroups of the microbiome and opened a new path for its deep characterisation.**

7 Conclusions

The research in this doctoral dissertation were conducted using a custom-designed bioreactor as well as in a batch route, and the applied analyses were performed using high-throughput, state-of-the-art technology to ensure high-quality results. The presented work enabled to draw the following conclusions:

- valorisation of lactose and lactate-rich wastewater (acid whey) to caproic acid in a continuous mode through a novel biotechnological process with the use of an open culture fermentation is feasible**;**
- it was shown that both, lactose fermentation and CE were achievable to occur simultaneously in a single-phase reactor bioprocess;
- in the applied system of acid whey fermentation neither methanogen inhibitor nor external ED were needed for long-term caproate production;
- for continuous bioreactor processes the pH level of 5.5 was low enough to effectively inhibit methanogenesis where acetoclastic methanogens were completely outcompeted;
- it was shown that decreasing HRT contributed to washing out most methanogens from the UASB reactor;
- the results showed an acceleration of caproate formation with decreasing HRT, precisely, gradually shortening the HRT from 20 to 2.5 day improved caproate production rate and let to eliminate the caproate accumulation;
- HRT of 1.25 day enabled to circumvent the butyrate toxicity, however, it was too short for the microbes to assimilate nutrients and grow;
- the designed system enabled to produce the caproate where its highest average production rate was 134.3 ± 30.9 mmol C/L/day (0.11 g/L/h) and it lasted for 37 days (with a median of 146.1 mmol C/L/day), and specificity between 58% and 83% (with a median of 79%);
- high caproate concentration (up to 8.5 g/L) was self-limiting and caused the process inhibition, despite the availability of the substrates;

- considering possible metabolic pathways in the acid whey fermentation the caproate formation depended on a lactate-based CE, not an ethanol-based CE, despite ethanol being produced in the reactor or externally delivered to the system with feed. Nevertheless, some ethanol production in the reactor was important for the CE process as it coincided with the caproate production, and its decrease coincided with the process disturbance;
- increasing ethanol loading rate led to ethanol oxidation along with a SCCAs generation and a hydrogenotrophic methane formation;
- higher lactate and lactose content and a lack of external ethanol delivery resulted in a sharp drop in the SCCAs formation and prompted the MCCAs production with the caproate as the main product;
- in a one-step process (single phase) it was essential to keep low ethanol concentration in the reactor (it was about 1-2 g/L) and abundant lactate and lactose content in feed (up to 20 g/L and 30 g/L, respectively) for an efficient caproate production;
- it was demonstrated that not only availability of EDs in feed impact the choice of the metabolic pathway but the presence of an additional substrate i.e. lactose, and the pH may divert the carbon flux in biochemical processes and impact the selectivity of final products by providing additional intermediates for the biochemical reactions, which is especially important in designing waste and wastewater valorisation bioprocesses;
- a functional enrichment of the specific microbiome for a caproate formation was feasible by proper control of operational parameters, namely pH and HRT;
- the structural biodiversity and composition of the microbial population were strongly impacted by the substrate composition;
- the continuous studies indicated a predominance of Coriobacteriaceae, Ruminococcaceae (Ruminococcus, Oscillospira), Prevotellaceae and Veillonellaceae families, which likely participated in the biochemical processes leading to a caproate production;
- in the continuous bioprocesses run on acid whey the only known caproate producer was aligned to *Caproiciproducens* spp.;

- combining a flow cytometry measurement with a cell sorting and a subsequent metagenomic sequencing provided a comprehensive knowledge of the active microbial subgroups that were directly involved in the CE process and a caproate production. The proposed approach put a new light on the analysis of different subgroups of the microbiome and opened a new path for its deep characterisation.

8 Final remarks and future outlook

The carboxylic acid market size was worth more than USD 13 billion in 2015 and it is increasing with an estimated gain of 5% (Kiran and Soumalya, 2016). At present, the caproic acid market size is limited, but its potential is significant. However, one of the key issue in upscaling the process is to limit the addition of an external ED, which negatively impact the economic and environmental indicators (Chen et al., 2017). Two promising solutions for upgrading acid whey wastewater to caproic acid have been recently developed omitting the need for external ED, i.e. one-step process presented in **Paper I** of this dissertation and a two-step process via lactic acid fermentation (Xu et al., 2017). In the first case, the advantage is lower investment and operating costs due to a single bioreactor. However, the latter achieves better substrate conversion and, henceforth, higher income. The other limitation regards product extraction. The initial techno-economic analysis presented in (Chwialkowska et al., 2019) indicated that it would be feasible to produce caproic acid under the current market price if effective downstream processing was established. Thus, an effort to integrate the production process with advanced techniques of the product extraction is necessary to prevent product toxicity, and enable caproic acid production at market prices. The downstream processing of caproate separation and recovery remains still a challenge due to the very complex composition of the fermentation broth. Laboratory scale investigations provided a few promising methods, such as adsorption, membrane processes, solvent extraction, pertraction, but a mature cost-effective technology is still missing (Crognale et al., 2021).

The presented dissertation enabled to characterise the crucial parameters for the OCF process control for caproic acid production from acid whey. Benefits that lower general operating expenditures and can be attributed to the processes presented in this dissertation are the possibility of using non-sterile conditions, no need for oxygen and any external substrates (such as ethanol) addition, and no need for any expensive heat shock or chemical agent addition for methanogenesis inhibition. Furthermore, the results of this work provide guidelines to create industrial biorefineries with a novel organic waste recycling technology, that perfectly fits into the bioeconomy concept.

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Paper I

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Exploiting the real wastewater potential for resource recovery – *n*-caproate production from acid whey†

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In light of an increasing demand for energy and sustainable development, bioenergy production is a must. A carboxylate platform-based bioprocess now enables the conversion of organic waste into a valuable bioproduct *via* open culture fermentation due to a complex microbial activity. Caproic acid is one of the promising products that can be further processed into liquid biofuels. In this study we have shown stable, long-term production of *n*-caproate from acid whey by shaping the reactor microbiome in a UASB bioreactor. The reactor was operated at pH 5.5 with gradually decreasing hydraulic retention time. The results showed acceleration of *n*-caproate formation and the highest average *n*-caproate production rate that lasted for 37 days was 134.3 ± 30.9 mmolC L^{-1} d^{-1} (0.11 g L^{-1} h^{-1}) (with a median of 146.1 mmolC L^{-1} d^{-1}) and specificity between 58 and 83% (with a median of 79%). Microbiological studies showed the prevalence of fermentative microorganisms from the families Coriobacteriaceae, Ruminococcaceae and Prevotellaceae that are the likely agents for lactose and lactate to *n*-caproate conversion. A flow cytometry based evaluation of the reactor microbiome demonstrated the inherent heterogeneity of microbial subpopulations in terms of microbial activity at different points of fermentation.

Introduction

Intense economic development and urbanization result in an inevitable increase in energy demand, which is also linked to the depletion of conventional energy sources such as crude oil, natural gas and other fossil fuels. The above trend focuses the world's attention on the search for novel biotechnological solutions that enable efficient and sustainable development. The continuous increase in organic and biomass waste brings a great deal of energy potential and it can make a substantial contribution to waste management and supply of so-called green energy. Recently, waste streams have been regarded as renewable bioresources that can be valorised to value-added commodities, such as liquid biofuels, bioplastics and other biochemicals.

Acid whey is a byproduct of the dairy industry; it is estimated that its global production reaches 180–190 million tonnes. Sweet whey, a by-product from hard cheese production, is turned into valuable products such as protein powder. Acid whey, on the other hand, a by-product from the manufacture of Greek yogurt, cottage cheese, and the like, constitutes a serious environmental problem and typically the dairy industry is obliged to pay for it to be taken away.[1] The consumption of milk products associated with acid whey by-production has skyrocketed in recent years. According to Erickson, only in the US, the total amount of acid whey produced was 2 million tons in 2015 and its worldwide volume is expected to rise significantly in the coming years.[1]

A carboxylate platform is one of the biorefinery platforms where carbon and energy from organic feedstock, usually agricultural or industrial waste biomass, can be recovered through open culture fermentation (OCF). In OCF, similarly to anaerobic digestion, the raw feedstock is first homogenised by hydrolytic bacteria. In the subsequent step the obtained intermediates undergo primary fermentation into economically low-value compounds, mostly short-chain carboxylic acids (SCCAs) that range from two to five carbons including acetate, propionate, *n*-butyrate, *n*-valerate and lactate.[2] In the same fermenta-

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tion process, they can be either used as precursors in the secondary fermentation step or directly extracted from the fermentation broth and then converted into industrially useful biochemicals in downstream processing. In the secondary fermentation step the direction of interest is elongation of SCCAs to medium-chain carboxylic acids (MCCAs) ranging from six to nine carbons.

Caproic acid, a carboxylate of six carbons, is one of the MCCAs that have gained more attention recently. Caproic acid has a wide range of applications such as a feed additive, an antimicrobial agent or a plant growth promoter as well as a precursor to various commodities including fragrances, paint additives and pharmaceuticals.[3] It is also used as a synthetic lubricant and refrigeration lubricant in the production of metal working fluids to enhance the rust resistance, cutting capabilities and grinding capabilities of fluids.[4] At high concentrations it is corrosive and has an unpleasant odour; in contact it may severely irritate the skin, eyes and mucous membranes. Currently, caproic acid is produced by extraction from coconut or palm kernel oils. Its low content in these oils (less than 1%) drives the price of caproic acid up and limits its applications. Its synthetic production is not well established.

A shift towards bio-based products is observed with the aim of decreasing carbon footprinting and reducing the dominant dependence on petrochemicals.[4] Caproic acid, due to its high caloric value and slightly hydrophobic properties, is a suitable intermediate for biofuel and biochemical production. Consequently it could be further processed into liquid biodiesel and jet fuels.[5] Layton and Trinh indicated that isobutyl hexanoate could be a suitable drop-in additive in the A-1 jet fuel.[6] However, with the current market prices ($0.67 per kg based on IATA Fuel Price Analysis – http://www.iata.org – accessed 02/02/2018), further process optimization and intensification along with economically effective downstream processing and the search for low cost feedstock would be critical to enter this market. Recently, Urban *et al.* reported that the MCCAs, including caproic acid, gained through microbial and electrochemical conversions can be suitable for use as drop-in fuels without any further downstream processing.[7] The biggest threat to commercial reality of open microbial processes is a low product concentration and therefore difficult downstream processing and product recovery. The acid whey to caproic acid value chain, based on a carboxylate platform, could be integrated either directly with a milk and cheese factory or it could be incorporated into a lignocellulosic biorefinery where effluents from sugar fermentation are mixed with acid whey and directed to OCF.

Biological formation of *n*-caproate is driven by microbial consortia and can be conducted through the elongation process called reversed β-oxidation (RBO). The reaction needs an electron donor, such as ethanol, lactate or other molecules that can be transformed into acetyl-CoA with energy conversion through pyruvate. The electron donor can be delivered separately or produced within the process itself. In the reaction an acetyl-CoA molecule is added to a carboxylate resulting in cyclic two carbon (C2) chain elongation (*e.g.* acetate to *n*-butyrate to *n*-caproate).[8]

Using reactor microbiomes for biological production of biochemicals such as caproic acid is advantageous as open cultures are resistant to possible disturbances that may occur during the process and, in contrast to using pure culture, no sterilization procedures are required prior to the fermentation.[8,9] To date, various strategies for caproic acid production have been investigated including batch, fed-batch or continuous reactor processes of open or pure culture fermentation and using synthetic media or real waste stream as a substrate. In each case different electron donors/carbon sources have been used for the chain elongation process, including ethanol,[10,11] ethanol and acetate,[12,13] methanol,[14] sugars,[15] municipal solid waste,[16] cellulosic biomass,[17] yeast-fermentation beer,[18] thin stillage,[5,19] wine lees,[20] grass,[21] acid whey,[22] restaurant food waste.[23] However, most of the studies demonstrated the dependence on addition of external ethanol for efficient chain elongation. Thus, one of the possible secondary fermentation pathways to produce *n*-caproate may occur through the coupling of ethanol oxidation and *n*-butyrate reduction (ethanol + *n*-butyrate → *n*-caproate).

Recently, in addition to ethanol, lactate has been proposed as an interesting electron donor alternative for *n*-caproate formation,[21,22,24–27] although it was typically related with odd-numbered carbon chain elongation *via* a competitive acrylate pathway.[28] Previous studies related to lactate utilisation for *n*-caproate production are presented in Table 1.

In the work of Zhu *et al.*, lactate was used as the sole carbon and energy source for *n*-caproate production in batch tests.[24] The reactor was inoculated with a portion of diluted 'yellow water' coming from Chinese Strong-Flavour Liquor fermentation and run at a controlled pH of 6.0–6.5. The maximum *n*-caproate concentration reached 23.4 g L^{-1} at an average production rate of 1.08 g L^{-1} day^{-1}. The major microbial population developed along the process belonged to *Clostridium* cluster IV (79.1%) followed by *Lactobacillus* and *Clostridium*. In the following paper,[26] Zhu *et al.* demonstrated high *n*-caproate production from lactate using the newly isolated bacterium CPB6 belonging to *Clostridium* cluster IV (Ruminococcaceae). The process was performed in serum bottles using a lactate-containing real waste stream (fermentation pit of a liquor brewing factory) mixed with municipal wastewater and bioaugmented with CPB6 strain. In the process 16.6 g L^{-1} of *n*-caproate was produced with a maximum production rate of 5.29 g L^{-1} day^{-1}. In another work, Kucek *et al.*[25] have demonstrated for the first time the conversion of lactate to *n*-caproate in a continuously fed microbiome system with in-line product extraction. In the study, an upflow anaerobic filter reactor was fed synthetic medium containing L-lactate and *n*-butyrate as a carbon source. The work demonstrated that lowering the pH from 5.5 to 5.0 as well as an increase in the lactate loading rate led to an increase in *n*-caproate production, reaching a maximum *n*-caproate production rate of 6.9 g COD L^{-1} day^{-1} (3.1 g L^{-1} day^{-1}). It was also found that maintaining a low residual lactate concentration in the bioreactor broth played an essential role in directing the reaction towards the formation of *n*-caproate instead of propionate. During the period of high *n*-caproate production rate,

Table 1 Microbiome-based fermentation processes for *n*-caproate production from lactate

Reactor type	Substrate	pH	Temp. [°C]	Max. C6 prod. rate [g L^{-1} day^{-1}]	Max. C6 conc. [g L^{-1}]	Ref.
No data	Thin stillage	5.4–5.7	35	—	In-line extraction	5
Batch reactor (wv. 1 L)	Synthetic medium + lactate	6.5	30	3.0	23.4	24
Modified CSTR (wv. 5.5 L)	Percolate of the maize silage digestion (two-phase process)	4.2–5.7	37	—	3.1	28
Fed-batch anaerobic filter (wv. 0.55 L)	Synthetic medium + lactate and butyrate	5.0	34	3.1	In-line extraction	25
150 mL serum bottles with agitation (wv. 50 mL)	Fermentation pit of a liquor brewing factory (containing lactate, acetate, butyrate, glucose and ethanol) mixed with municipal wastewater	5.5	30	5.3	16.6	26
100 mL bioreactor (wv. 50 mL)	Grass fermentation effluent containing lactate	5.5–6.2	32	23.8 (electricity assisted)	4.1	21
Upflow anaerobic filter (wv. 0.55 L)	Primarily fermented acid whey waste (temperature-phased system)	5.0	30	1.6	In-line extraction	22
6 L leach-bed reactors (wv. 3 L)	Homogenized restaurant food waste (single-phase)	7	37	3	10	23
UASB reactor (wv. 1 L)	Acid whey wastewater (single-phase)	5.5	30	3.2	10.45	This study

wv. – working volume.

Acinetobacter ssp. were the most abundant species (62.9%) in the reactor. Recently, Xu *et al.* have demonstrated for the first time medium-chain carboxylic acid production from an acid whey waste *via* lactic acid in a temperature-phased system without external electron donor addition.[22] The process was performed in two separate bioreactors under different temperature conditions where acid whey was first converted into lactic acid in one reactor and then it was funnelled to the chain elongation reactor. The approach let them create specialised microbiomes in each reactor and reach a maximum caproic acid production rate of 81 mmolC L^{-1} day^{-1} (1.6 g L^{-1} day^{-1}) at a specificity of 48.2% (based on product-to-carboxylic acid production ratio in % mol C).[22]

The main objective of our study was to perform long-term production of *n*-caproate from acid whey (AW) in a single-phase reactor microbiome-based system. For this purpose we fed the upflow anaerobic sludge blanket reactor (UASB) with AW for the operating period of over a year. AW is an effluent of the dairy industry from quark production that inherently contains lactose, lactate, minerals, peptides, vitamins and other minor components,[29,30] and thus can be used as a complex feedstock for OCF. To inhibit methanogenic activity and promote the conversion of lactate and lactose to *n*-caproate, we evaluated the process under the regime of low pH (5.5) to prevent methanogenesis[11] and under decreasing hydraulic retention time (HRT) to promote MCCA production. The other aim was to characterize the microbial community in terms of its structure, dynamics and activity along the process and identify the key *n*-caproate producers.

Experimental

Continuous process configuration

Inoculum and substrate. The sludge from an anaerobic digester was used as a seed for the process (Poznan Wastewater Treatment Plant, Poland). AW, the dairy industry wastewater after a traditional quark production (Dobrzyca Diary Industry, OSM Kowalew–Dobrzyca, Poland), was used as a feedstock. After the quark production, AW is stored in closed stainless steel tanks outside the factory until utilisation; therefore it could partly undergo a fermentation process when it is stored. The AW delivered to the laboratory was stored at 4 °C. However, to feed the reactor, portions of AW were transferred to the room temperature tank once a week and thereafter more frequently with each HRT decrease to reflect realistic conditions that occur when AW is stored at the factory. Thus, the used feedstock was partly fermented, first at the factory and then in the laboratory; still, the average lactose-to-lactate molar ratio fed to the reactor was 0.78 based on mmol C mmol C^{-1}. The characteristics of both, inoculum and the delivered feedstock, are presented in Table S1.† Prior to inoculation, 900 mL of sludge was first stored at 30 °C for 48 hours for degasification and then diluted with reject waters to reach an initial total soluble solid (TS) concentration of about 5 g L^{-1}. Next, 50 mL of AW was added and then the reactor was operated in a continuous mode.

Reactor system. The experiment was carried out in a UASB reactor with a dedicated controller (Fig. 1). The reactor consisted of a 1 L working volume cylindrical Plexiglass® vessel of 6 cm inner diameter and a 2.5 L upper part to collect the surplus effluent. The excess effluent was discarded into the separate reservoir through the overflow line attached to the top of the tank. The produced gases were collected in an inverted funnel in the upper part of the reactor and discharged. The sludge bed was in suspension by the recirculation of solid-free wastewater from the upper part of the reactor to the bottom. The temperature inside the reactor was maintained at 30 °C by circulating heated water through a water jacket. The pH probe (Elmetron EPP-1, Elmetron, Poland) was set at the recirculation tube. The pH in the reactor was automatically adjusted to 5.5 with 2 M NaOH using a corresponding peristaltic pump and it

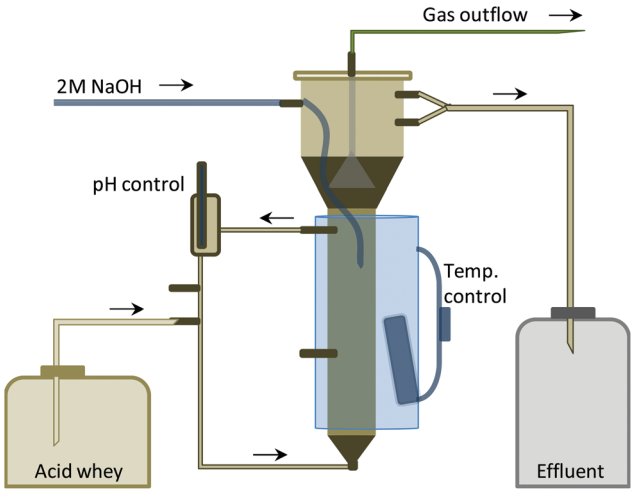

Fig. 1 Scheme of the upflow anaerobic sludge blanket reactor configuration.

was calibrated once a week. AW was fed to the reactor at a continuous flow rate *via* an inlet in the recirculation tube. The feed flow rate was determined volumetrically. The effluent sampling port was mounted at the recirculation tube and the biomass sampling port was placed in the middle of the reactor vessel.

Operational strategy. The reactor was operated for 410 days with decreasing HRT, where three subsequent operating stages were distinguished along the process. Stage I, which was the start-up stage, lasted from day 0 to 177. During that period the HRT was maintained at 20 days to let the development of an *n*-caproate producing microbiome. The stage lasted until stable *n*-caproate production was observed. In every next step the HRT was shortened by half to promote and maximize the *n*-caproate production. In stage II, which lasted from day 178 to 350 (HRT = 10 days from day 178 to 230; HRT = 5 days from day 231 to 350), product toxicity was evaluated, which enabled determination of the inhibitory threshold of the undissociated caproic acid concentration in the fermentation broth. Stage III (HRT = 2.5 days from day 351 to 410) was to examine the microbiome's capability to overcome the toxicity effect of undissociated caproic acid.

The production rates were calculated for the working volume of the reactor (mmolC L^{-1} day^{-1}) and product specificity was calculated as the product-to-carboxylic acids production ratio (in % mol C) according to Xu *et al.*[22] The operating and performance parameters for each stage are presented in Table S2.† The concentration of the undissociated form of caproic acid was calculated based on the daily noted pH value.

Analytical methods

GC and HPLC analyses. Broth samples were collected from the bioreactor recycle line daily or every other day. Organic acid and alcohol concentrations were monitored by gas chromatography (GC) using a Shimadzu 2014 GC System (Shimadzu, Japan) based on the method described by Vasquez *et al.*[31] Prior to the analysis, samples were filtered with 0.45 μm syringe filters and acidified with H_3PO_4. A flame ionization detector (FID) and a high-performance capillary column with a free fatty acid phase (Zebron ZB-FFAP, Phenomenex, USA) of 30 m × 0.53 mm coated with 1 μm film thickness was used. As the carrier gas, helium was supplied at a flow rate of 7.38 mL min^{-1}. The initial oven temperature was 70 °C, maintained for 1 min, increased to 240 °C at 10 °C min^{-1}, and finally held at 240 °C for 3 min. The temperatures of the FID and the injection port were 250 and 200 °C, respectively. The concentrations of lactate and lactose were determined by high performance liquid chromatography (HPLC 20AT, Shimadzu, Japan). The HPLC instrument was equipped with a Rezex™ ROA-Organic Acid H^+ (8%) column (Phenomenex, USA), and a refractive index detector (RID-20A). 5 mM aqueous sulphuric acid was used for elution at a flow rate of 0.6 mL min^{-1} at 63 °C.[32]

Microbial analysis

Biomass sample collection and DNA extraction. Biomass samples from days 36, 77, 119, 155, 175, 195, 217, 230, 260, 292 and 336 of the fermentation process as well as inoculum and AW were chosen for analysis. Aliquots of the reactor samples were washed in PBS buffer and the obtained pellets were stored at −20 °C. Total genomic DNA was extracted from 150 mg of each sample using a mechanical method based on the protocol of Ciesielski *et al.* with some modifications as described below.[33] For extraction approx. 300 mg of glass beads (∅ 0.25–0.6; Carl Roth, Germany) and 1 mL of extraction buffer (100 mM Tris base, 100 mM Na_2EDTA, 1.5 M NaCl, pH 8) were added to the samples and then shaken for 20 min at 5000 rpm in a bead beating device (Uniequip, Germany). Next, 200 μL of 10% SDS solution was added and the samples were incubated for 30 minutes in 65 °C for further disintegration. After incubation the samples were centrifuged for 10 min at 8000 rpm and the supernatant was placed on silica washing columns (A&A Biotechnology, Poland) and washed twice with 70% ethanol solution (A&A Biotechnology, Poland). DNA attached to the filter was eluted with 50 μL of sterile, DNase free water and stored at −20 °C.

Illumina sequencing and bioinformatics data processing. To characterise the microbial population, metagenomic sequencing of V3–V4 hypervariable region of 16S rRNA gene was performed with an Illumina Miseq v2 Reagent kit by GENOMED (Warsaw, Poland) using 2 × 250 bp paired-end reads and primers 341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 785R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).[34] For the PCR reaction a Q5 Hot Start High Fidelity 2× Master Mix was used according to the manufacturer's protocol. Miseq Reporter (MSR) v 2.6 was used for demultiplexing and raw fastq files generation. The fastq files were quality filtered and trimmed at a minimum quality score threshold of 20 and a minimal length of 30 with cutadapt program (v1.9.1).[35] Further data processing was carried out using Quantitative Insights Into Microbial Ecology QIIME (v1.9).[36] The paired-end reads were joined using fastq-joint algorithm with a min.

overlap of 10 and then clustered into an operational taxonomic unit (OTU) based on 97% pairwise identity using the default uclust algorithm.[37] Representative sequences from each OTU were aligned to the Greengenes v13_8 database.[38] Chimeras were removed from the reference set using ChimeraSlayer.[39] OTU diversity within each sample (alpha diversity) was calculated as Shannon index (H') and Gini coefficient (Tabe S2†). For heatmaps those OTU that exceeded 1% relative abundance in at least one sample were clustered based on the Bray–Curtis dissimilarity index (Fig. S2†). To compare bacterial communities between samples (beta diversity) the Principal Coordinate Analysis (PCoA) plot was generated based on weighted UniFrac pairwise distance matrices (Fig. S3†). Additionally, Pearson correlation coefficient (r) was calculated to determine the correlation between the microbial members and the produced fatty acids.

Flow cytometry analysis. An evaluation of the redox potential of microbial cells from bioreactor samples was performed using a BacLight™ Redox Sensor™ Green Vitality kit (Life Technologies, Carlsbad, CA, USA). 1 mL of a fresh liquid bioreactor phase (effluent) and 0.5 mL of a suspended biomass phase (flocks) were sampled and filtered using a nylon net 20 μm syringe filter (assembled with a Swinnex filter holder (25 mm) – both from Merck Millipore, Darmstadt, Germany). Sample filtration was employed to prevent the flow arrest in case samples include particles capable of blocking the light of the sample line or nozzle and to avoid binding of florescent dyes to non-cellular particles, which could lead to a reduced labeling efficiency. Filtered samples were washed with PBS and centrifuged for 4 min at 3800 rpm. The obtained pellet was resuspended in 1 mL of PBS. Samples were analysed using a BD FACS Aria™III (Becton Dickinson, San Jose, CA, USA) flow cytometer (cell sorter). FACS flow solution (Becton Dickinson, USA) was used as the sheath fluid. The configuration of the flow cytometer was as follows: 70 μm nozzle and 70 psi (0.483 MPa) sheath fluid pressure. The cells were characterized by two non-fluorescent parameters: forward scatter (FSC) and side scatter (SSC), as well as two fluorescent parameters: green fluorescence (FITC) from RedoxSensor™ Green reagent collected using a 530/30 band pass filter and red fluorescence (PE-Texas Red) from propidium iodide (PI) reagent collected using a 616/23 band pass filter. For the excitation of both fluorescent reagents a 488 nm blue laser was employed. Each sample was analysed in triplicate.

The flow cytometry analyses were performed using logarithmic gains and specific detector settings (10 000 events were recorded per analysis). Data were acquired on a four-decade logarithmic scale as area signals (FSC-A, SSC-A, FITC-A and PE-Texas Red-A) and analysed using FACS DIVA software (Becton Dickinson, USA). The threshold was set on both: the FSC and FITC (RedoxSensor™ Green reagent) signals. The threshold setting procedure was performed according to Cyplik *et al.* and Juzwa *et al.*[40,41] The populations were defined by gating in the dot plots of green fluorescence (FITC) *versus* red fluorescence (PE-Texas Red). The calculation of the cellular redox potential was performed using the medians of green fluorescence (FITC-A) signals of gated populations defined on a bivariate dot plot (FITC-A *vs.* PE-Texas Red-A).[40,41] Sub-populations P4, P5 and P6 (distinct and demonstrating low CV (Coefficient of Variation) values in the measured parameter) of the analysed cells were selected based on differences in the level of metabolic activity measured as cellular redox potential (CRP). To each sub-population the arbitrary selected CRP ranges were attributed (Table S4†).

Results and discussion

Decreasing HRT enhanced the *n*-caproate production rate

The operating and performance parameters for each stage of the fermentation process including average production rates and specificities (presented as product-to-carboxylic acids ratio in % mol C) are presented in Table S2.†

Stage I. At the beginning of the process (Fig. 2) SCCAs were produced with acetate as the main product, reaching 12.2 mmolC L^{-1} day^{-1} on day 54. However, *n*-caproate appeared from the very beginning and its concentration in the fermentation broth was slowly increasing. After 55 days of the trial, acetate started to decrease in favour of growing *n*-caproate production. Interestingly, from day 55, increasing ethanol accumulation was noticed (up to 88.7 mM C) and then its concentration in the fermentation broth was fluctuating between 42 and 62 mM C. From day 105 on, the concentration of ethanol, butyrate and *n*-caproate started to decrease from 82.1, 166.8 and 397.9 mM C to 5.6, 23.5 and 177.9 mM C on day 134, respectively. This indicated fermentation process disturbance, possibly due to pH fluctuations on the previous days. The pH adjustment was reflected on the subsequent days in lactate accumulation followed by parallel *n*-caproate and ethanol formation. Thus, stable pH conditions were crucial for the recuperation process. It is worth noting that lactose was not accumulated during these days, suggesting that the *n*-caproate production was dependent not only on dosed lactate, but also on lactose fermentation. For stage I the maximum *n*-caproate production rate was 22.9 mmolC L^{-1} day^{-1} (day 173) with the *n*-caproate to carboxylic acid ratio ranging between 72 and 79% for over 22 days (155–177). When the *n*-caproate concentration remained stable for over 10 days we decreased the HRT to evaluate its effect on the acceleration of *n*-caproate production.

Stage II (days 178 to 350). Initially, a decrease of HRT to 10 days caused the decrease in *n*-caproate production rate to 19.2 mmolC L^{-1} day^{-1} on day 190 and lactate accumulation up to 403.9 mM C on day 194 (Fig. 2). However, in the subsequent days the SCCA concentration started to increase with *n*-butyrate concentration exceeding 410 mM C on day 218 and the *n*-caproate production rate increased from 19.2 to 49.6 mmolC L^{-1} day^{-1} on day 225 and the *n*-caproate concentration was 495.8 mM C. Even though its specificity was only 54%, its undissociated form accounted for 123.3 mM C (Fig. S1†). Moreover, on those days the total concentration of all produced carboxylates reached 918.7 mM C, which as a

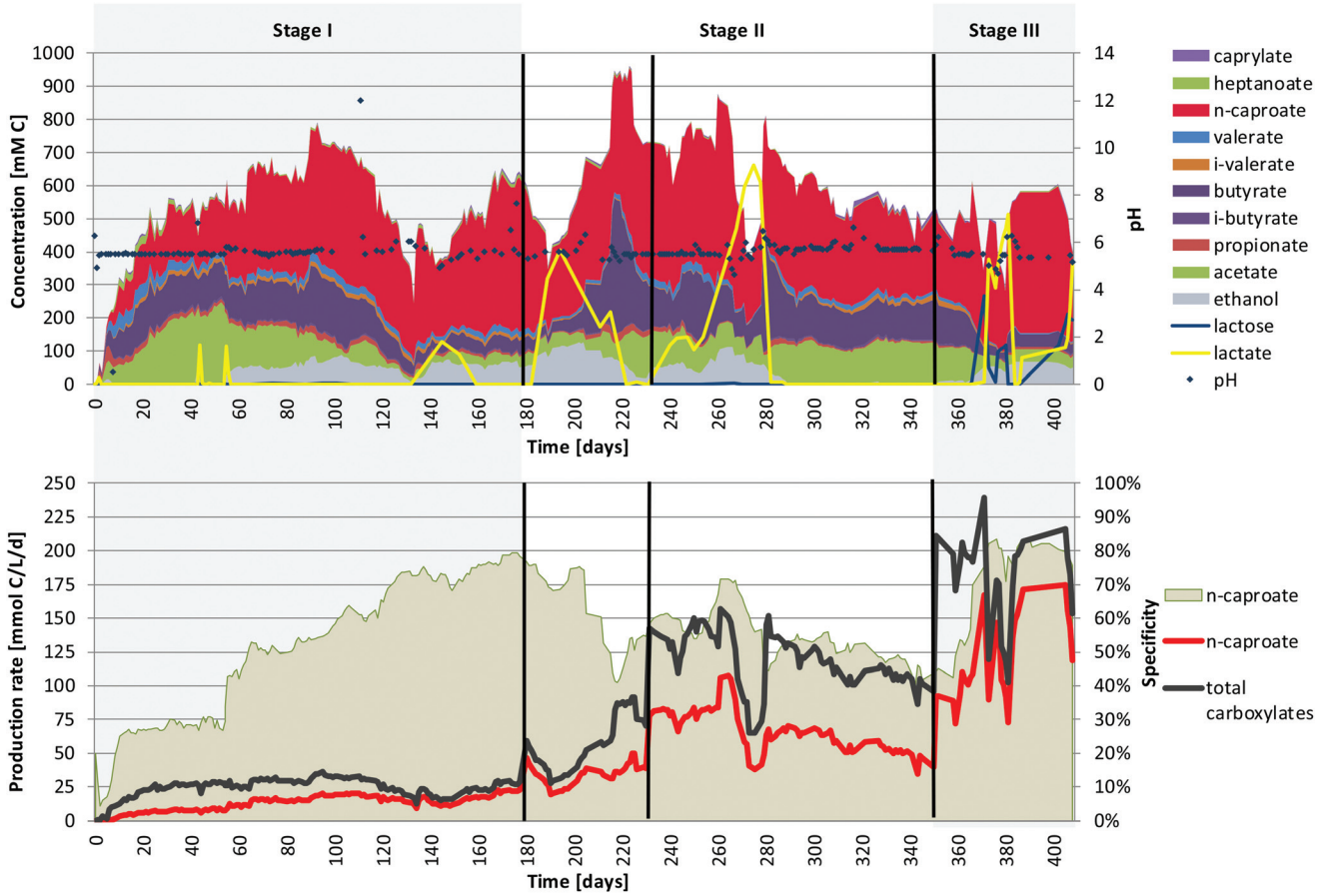

Fig. 2 The concentration profile of carboxylates, ethanol and lactate along the fermentation of acid whey (top) and *n*-caproate and total carboxylate production rate and *n*-caproate specificity (bottom).

consequence might cause microbial toxicity. The effect was reflected in a temporal decrease in the *n*-caproate production rate during days 226–230 and lactate accumulation on the following days. However, a further decrease of HRT to 5 days enhanced the *n*-caproate production rate to 108.0 mmolC L^{-1} day^{-1}, resulting in an *n*-caproate concentration of 540.1 mM C (10.45 g L^{-1}) and a specificity of 72% on day 264, which was the highest concentration of *n*-caproate in the fermentation broth measured in this study. However, the result was also reflected in a high concentration of undissociated caproic acid in the fermentation broth, which again had a toxic effect on the microbial cells. Agler *et al.*[11] suggested that at pH 5.5 the accumulated undissociated *n*-caproic acid (close to its pK_a of 4.88 at 30 °C) has an inhibitory effect on the microbial cells and thus on the carboxylate chain elongation process. In the work of Ge *et al.*[18] the authors found out the product toxicity of undissociated *n*-caproic acid at a concentration of 7.5 mM when the pH was decreased to 5.0. In this study the process inhibition was observed when the undissociated caproic acid concentration in the effluent was above 17.2 mM (Fig. S1†). This result is more than two times higher than that previously reported, suggesting that the developed microbiome was composed of highly acid-resistant species. Consequently, the chain elongation activity was inhibited, which triggered the lactate accumulation up to 660.6 mM C on day 275 and shifted the process outcome from *n*-caproate, which dropped to around 315 mM C, to rapid accumulation of *n*-butyrate up to 367.2 mM C on day 280. On subsequent days the concentration of butyrate and *n*-caproate kept decreasing and stabilized at the concentrations of around 109 and 287 mM C, respectively. From day 295 on, we have observed a stable carboxylate production rate for over 50 days although *n*-caproate concentration was decreasing slightly with time. During that period neither ethanol nor lactate accumulation was observed. The highest *n*-caproate production rate during that stage was 65.52 ± 15.76 mmolC L^{-1} day^{-1} at an *n*-caproate-to-carboxylic acid ratio of 55 ± 7% and lasted for 119 days (HRT = 5 days).

Stage III (days 351 to 410) was the process recovery period. To overcome the effect of the product toxicity, the HRT was shortened to 2.5 day that resulted in a substantial increase in *n*-caproate production rate (Fig. 2). The highest average *n*-caproate production rate during that stage and in this study lasted for 37 days (during days 371–408) and was 134.3 ± 30.9 mmolC L^{-1} day^{-1} (0.11 g L^{-1} h^{-1}) with a median of 146.1 mmolC L^{-1} day^{-1} and specificity between 58 and 83%

(with a median of 79%). During that period (day 387) we experienced the reactor leakage, yet it was repaired and did not disturb the overall fermentation outcome. The product toxicity was a limiting agent of the process performance; however, it could be omitted by in-line product recovery. Moreover, the in-line product extraction can enhance the product generation.[11] Different technologies for the recovery of MCCAs from the fermentation broth were proposed, *i.e.* biphasic extraction,[42] pertraction,[43] liquid–liquid extraction,[44] liquid–liquid extraction combined with Kolbe electrolysis.[7]

Promising *n*-caproate production from real waste feedstock with a single-phase reactor microbiome

This is the second study of long-term, continuous *n*-caproate production from acid whey waste without ethanol or other exogenous electron donor addition, where lactate was identified as the electron donor for *n*-caproate production. In the first study the caproic acid was produced in two temperature-phased bioreactors, where acid whey came from Greek-yogurt production and contained lactose, other milk sugars and lactic acid.[22] In the study the lactose-to-lactate molar ratio (based on mol C mol C^{-1}) was 5.3. In this work we used acid whey (AW) waste from quark production, which inherently contained lactate and lactose at a lactose-to-lactate molar ratio of 0.78 based on mol C mol C^{-1}. When the content of lactose is higher, like in the first study, a two-phase system may be needed, while in this study a much lower lactose-to-lactate ratio enabled production of *n*-caproate in a single bioreactor system. This was mostly possible due to the fact that the acid whey delivered from the factory was partly fermented (see the Experimental section) and separating the fermentation processes in the two bioreactors was not necessary in this case. During 410 days of the process the *n*-caproate production rate was increasing with each HRT shortening and the average maximum production rate (Stage III, days 371–408) accounted for 134.3 ± 30.9 mmolC L^{-1} day^{-1} (0.11 g L^{-1} h^{-1}) with a maximum *n*-caproate specificity of 83% (Fig. 2). Moreover, we succeeded in shaping the reactor microbiome for stable *n*-caproate production.

To our knowledge, reproducible *n*-caproate production using a reactor microbiome from real waste, where lactate was the electron donor for carboxylic fatty acid chain elongation, was reported only in a few other papers.[5,21,23,28,45] In other works based on microbiome biotechnology a platform run on real waste feedstock *n*-caproate was mainly generated using endogenous or exogenous ethanol.[8] In the work of Ge *et al.*[18] ethanol-rich yeast fermentation beer was fed to the reactor for *n*-caproate production in a long-term operating period with an average *n*-caproate production rate of 3.4 ± 0.4 g L^{-1} day^{-1}. In more recent work, Kucek *et al.*[20] converted wine lees (containing inherent ethanol) to *n*-caproate and *n*-caprylate achieving a total MCFA production rate of 3.9 g COD L^{-1} day^{-1} at equal specificities of 36% each. In both studies continuous in-line extraction was applied. However, the highest *n*-caproate production rate of over 55 g L^{-1} day^{-1} (>121 g COD L^{-1} day^{-1}) was reported in a continuous process using synthetic ethanol and acetate for MCFA production.[46]

Although in many previous studies ethanol delivery was proved necessary to produce *n*-caproate, Chen *et al.* pointed out that its external supply is a dominant cause of environmental impact over the life cycle and it should be reduced.[3] Here, we confirmed that ethanol does not have to be delivered to the system as it was produced within the bioreactor itself; additionally, lactate was identified as the main electron donor for *n*-caproate formation. Moreover, as the feedstock composition is one of the important factors shaping the process outcome, it has to be a sufficient source of substrates for the chain elongation process. Choosing AW, a lactate and lactose-rich feedstock, we moved on to the next stage in the development of caproic acid production technology in a bioreactor where additional dosage of external ethanol becomes redundant.

n-Caproate production was lactate-dependent despite ethanol production within the reactor

The reactor was fed with lactic acid and lactose, where most of the carbon was introduced as the carbon in lactic acid (see the Experimental). Moreover, lactose can be fermented mostly to lactic acid, which suggested that lactate was not only delivered to the reactor with feed but also produced within the reactor. Lactate accumulation was observed after each HRT shortening, so a higher organic loading rate, which was reflected in a delayed reactor microbiome response to the introduced change. It was negatively correlated with *n*-caproate production indicating that the microbiome easily fermented lactose, but needed more time to adjust to the higher lactate concentration. The predominance of lactate with simultaneous ethanol production in the reactor indicated that the chain elongation process was performed primarily by lactate utilisation and to a lesser extent through ethanol. The ethanol depletion occurred only twice, first during stage I and then during stage II, in both cases as a result of process inhibition. Thus, we concluded that *n*-caproate production was carried out mostly through lactate utilisation, despite the ethanol production within the reactor.

Even though the amount of carbon delivered with lactose was lower than the carbon coming with lactate, its fermentation also played an important role. Lactose was utilized directly after the addition during most of the process regardless of the HRT. Its accumulation was observed only at the end of stage III likely due to reactor overloading and the toxicity effect of undissociated caproic acid on the microbial cells.

Lactose is a disaccharide that can be fermented by both yeast, among others *Kluyveromyces lactis* and *K. marxianus*,[47] and bacteria, mostly lactic acid bacteria (LAB);[48,49] however, yeast cells were not identified in this study. Metabolic pathways of lactose metabolism depend on the enzymatic apparatus of the microorganism and environmental conditions. Therefore, regarding a considerable diversity between strains in OCF a plenitude of metabolic streams occur simultaneously, resulting in a range of different products (Fig. 3). In the

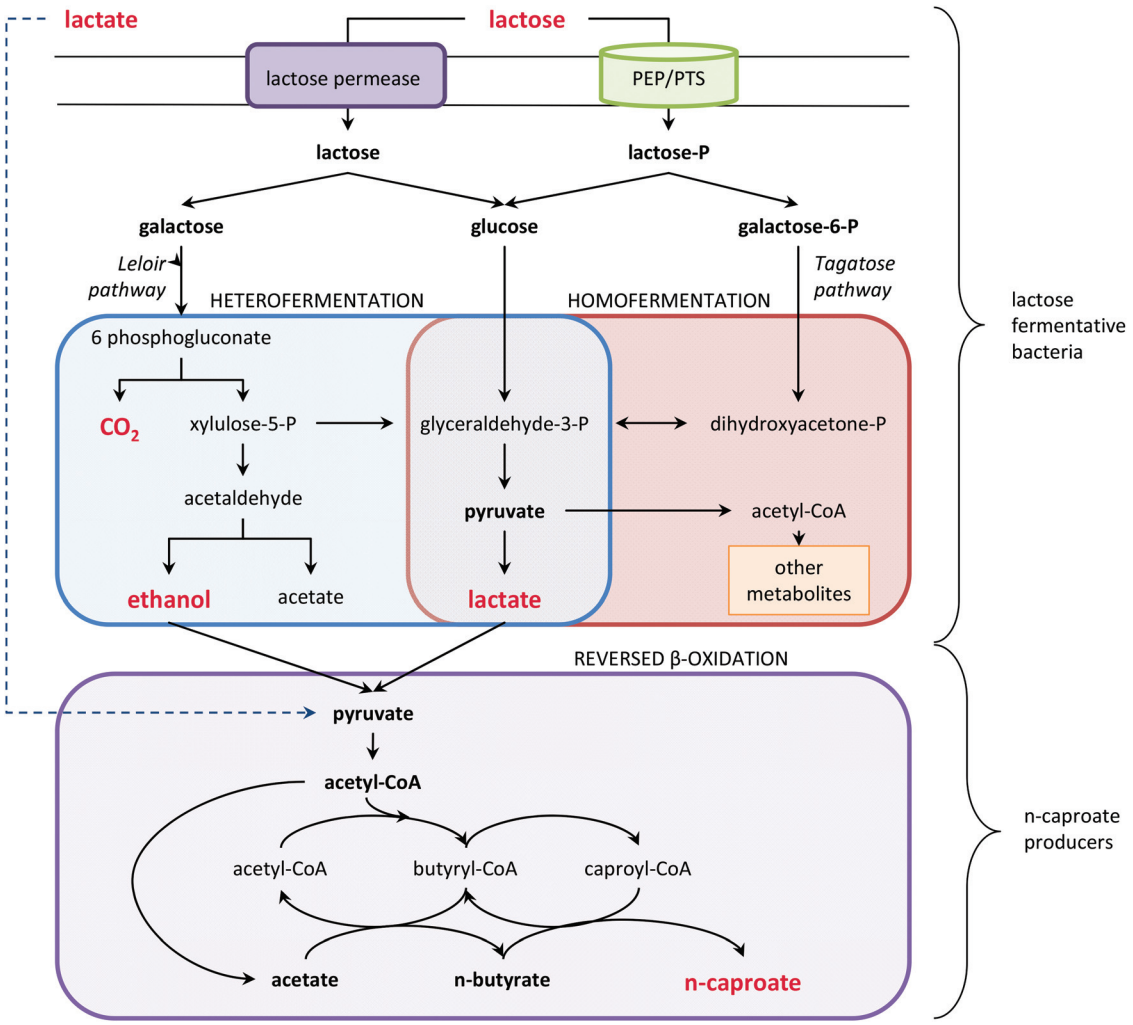

Fig. 3 Lactate utilisation and lactose metabolism combined with reversed β-oxidation for *n*-caproate production in the open culture fermentation process. The diagram shows possible metabolic pathways of lactose fermentation and lactate utilisation for *n*-caproate production through reversed β-oxidation. The elaboration is proposed based on previous models.[26,27,49–53]

process, lactose is first transported into the microbial cells across the inner membrane which can occur through a phosphotransferase system (PTS) associated with energy production through substrate phosphorylation so that lactose enters the cells as lactose-phosphate.[49,50] The other mechanism of lactose uptake involves a lactose permease system.[49,50] In general, there are two major routes for further lactose metabolism. Within the microbial cells, lactose or lactose-phosphate is hydrolysed to glucose and galactose or glucose and galactose-6-phosphate, respectively.[49,50] In the homofermentative pathway each mole of glucose generates 2 moles of lactate and 2 moles of ATP *via* pyruvate. Alternatively, in heterofermentation each mole of glucose yields one mole each of lactate, CO_2, ethanol or acetate and one mole of ATP. Galactose and galactose-6-phosphate enter the glycolysis *via* the Leloir pathway or the tagatose-6-phosphate pathway, respectively, resulting in pyruvate and then lactate formation.[48–50] However, in fermentation processes where other substrates such as organic acids are involved, the ratio of end products differs from the theoretical value.[49] Moreover, depending on the microbial metabolic capabilities and in response to changes in environmental conditions, pyruvate can be converted through acetyl-CoA branches not only into lactic acid but also ethanol, acetate, butyrate or other metabolites.[24,51] The metabolites can serve as intermediates and can be further consumed by other microbiome members.[51] Thus, the lactose metabolism provides the intermediates to the RBO pathway for the fatty acid chain elongation process with lactate as the key intermediate that can be metabolised in secondary fermentation reactions to VFAs and MCFAs.

Acidic pH and decreasing the HRT led to methanogenesis inhibition and functional enrichment of the microbiome

Due to anaerobic digester-derived microbial communities, the occurrence of methanogenic activity in OCF poses a threat, as the digestate is a source of not only the acidogenic bacteria

but also the methanogenic archaea, especially acetoclastic methanogens that compete with the microbes for substrates.[54] Elimination of methanogens from the microbiome can be imposed by specific inhibition methods, such as digestate boiling or addition of inhibiting chemical agents (such as 2-bromoethanesulfonate).[10,52] In this study we confirmed successful methanogen inhibition coupled with *n*-caproate production by applying acidic conditions of pH 5.5 and decreasing the HRT without addition of any external inhibitor as was first demonstrated by Agler *et al.*[11] Some pH fluctuations happened during the course of the trial due to the recirculation tube clogging. However, the short disturbances did not have any considerable influence on the fermentation process until the pH dropped below 5. Such a low pH value favours the undissociated fatty acid form that caused the toxicity effect on microbial cells. However, under acidic environmental conditions of 5.5, carboxylates occur only partly in an undissociated form that causes a selective pressure favourable to acid-tolerant species, which as a consequence enabled us to outcompete sensitive microbes and inhibit methanogens. At the same time, applying an extended period of 20 days HRT and its further decrease provided advantageous conditions to shape the microbiome towards *n*-caproate production and accelerate the process efficiency.

Microbial analysis revealed the dominance of Coriobacteriaceae, Ruminococcaceae and Prevotellaceae family members

Reactor microbiome structure and dynamics were evaluated based on 11 bioreactor samples and one sample each of the inoculum and feedstock. Among all the analysed samples OTU assignment resulted in 2568 OTUs from high quality sequence reads. The sequence number per sample ranged from 67 678 to 99 910 with a median of 82 601. At the family level there were 36 OTUs that exceeded 1% relative abundance in at least one bioreactor sample.

The structural biodiversity of the bioreactor microbiome was calculated as the Shannon index (H') that takes into account not only the number of OTU (richness) but also their relative abundance. For the reactor samples, H' was 4.3 on average, which is higher than the results presented in similar studies with reactor microbiomes, where it was approximately 3.6,[25] 3.7 [20] and 4.15.[22] This indicates that the reactor microbiome was quite diverse but also stable, as the index did not vary much along the process (Table S3†). The Gini index is a measure of the evenness of the microbial community (values range from 0 to 1) and reflects its functional organisation. The higher the value the more uneven the community is (only a few OTU dominate) and therefore the more functionally organised the microbiome.[11,55] The Gini index was very high (0.98) over the operating period, similarly to the work of Xu *et al.* (0.94),[22] proving that the reactor microbiome was highly specialised for *n*-caproate production.

During the course of the trial the reactor microbiome was enriched only by the applied operational conditions, which was reflected in shifts in the reactor microbial community composition, especially at the beginning of stage I (Fig. 4). To shape the microbiome for *n*-caproate production a prolonged start-up stage was necessary. Along the process, phylotypes related to the families Coriobacteriaceae, Ruminococcaceae and Prevotellaceae were found to be dominant and accounted for 37.4% (±7.2), 20.5% (±8.4) and 17.9% (±8,5) of the mean

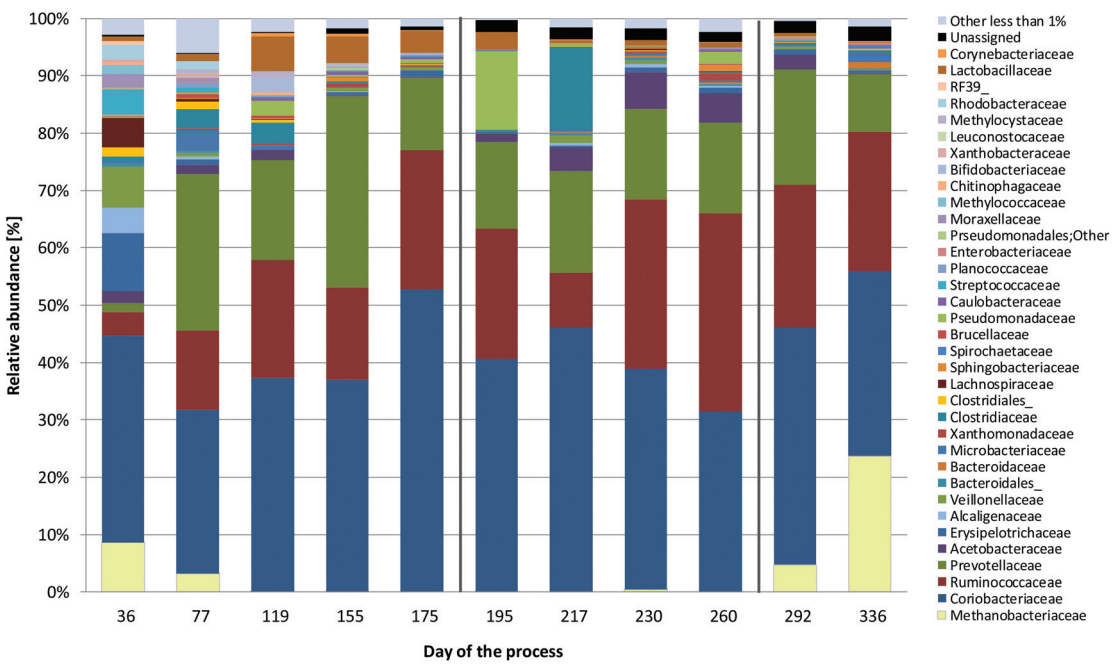

Fig. 4 Microbial composition of bioreactor samples at family level. Only families that exceeded 1% relative abundance in at least one bioreactor sample are presented.

relative abundance of the microbial society, respectively, making them the likely agents for *n*-caproate formation. Members of Coriobacteriaceae were enriched from the very beginning and became the key organisms found in this study. Although Coriobacteriaceae are generally reported to be asaccharolytic,[56] most of the sequences were aligned with high similarity to *Olsenella* species that are fermentative bacteria able to grow on lactose and convert it to lactate, acetate or formate as the main products.[57] Among the Ruminococcaceae family the community was dominated by *Ruminococcus* followed by *Oscillospira* that were found in reactor microbiomes associated with *n*-caproate production in previous studies.[20,25,58,59] Recently, Ruminococcaceae bacterium CPB6 has been isolated from a microbiome of efficient *n*-caproate production from lactate and a hypothetic pathway for *n*-caproate production was proposed based on its complete genome sequence analysis.[27] Moreover, the members of the Ruminococcaceae family, which belongs to *Clostridium* cluster IV, were found to be dominant bacteria of the microbiome synthesizing *n*-caproate from lactate.[23,24] Microorganisms belonging to Prevotellaceae were another highly abundant family that has been reported to be positive for β-galactosidase activity.[60]

Generally, from day 77 on, high similarity in community profile was observed with only a few exceptions. Pseudomonadaceae had higher relative abundance when the ethanol concentration was increasing and exceeded 2 g L^{-1} (r = 0.66, p = 0.020 on days 195, 260 and 413). Clostridiaceae (*Clostridium*) appearance coincided the following butyrate increase (r = 0.85, p = 0.000, on days 77, 119 and 217). In both cases, Coriobacteriaceae and Ruminococcaceae decreased noticeably during those stages (except for the start-up stage when the microbiome has not been already shaped).

LAB were added to the system continuously *via* substrate feeding. Although the AW microbial community was composed mostly of Streptococcaceae family represented by *Lactococcus* and Lactobacillaceae family dominated by *Lactobacillus* (89.4% and 5.8% relative abundance, respectively), only the latter were able to survive in the reactor. Lactobacillaceae members were present during all of the fermentation process; however, they decreased drastically during stage II to comprise less than 1% and appeared again during stage III (samples from days 217 to 336), which was in line with process recovery. Quite a low relative abundance of LAB during stage II confirmed that microbiome members other than LAB were metabolising lactose and lactate.

Members of the genus *Megasphaera* are rumen bacteria that were previously reported as lactate to *n*-caproate fermentative bacteria.[61,62] *Megasphaera elsdenii* is a well known species able to grow and convert lactate into *n*-caproate at a pH of 5.5 and lower.[63] Under the applied environmental conditions, we expected the microbe to dominate the microbiome and contribute to caproic acid production. Interestingly, in this study, microorganisms belonging to *Megasphaera* genus were only present at the beginning of the process, accounting for 5.57% relative abundance (on day 36), and were quickly diminished to comprise less than 1%. This is in line with the finding of Kucek *et al.*, where *M. elsdenii* was also absent in the reactor microbiome despite continuous lactate delivery and similar pH conditions.[25] The absence of *M. elsdenii* was also reported in the work of Nzeteu *et al.*, where lactate was involved in *n*-caproate production.[23]

The Archaea kingdom represented by *Methanobrevibacter* (Methanobacteriaceae family) were present at the beginning of the process and were successfully inhibited due to a low pH of 5.5. Interestingly, they appeared again at the end of stage II reaching over 23% relative abundance when the production process was inhibited. However, *Methanobrevibacter*-related phylotypes are hydrogenotrophic; thus they did not pose a threat of substrate competition with the bacterial community.[54]

Flow cytometry analysis as an early indicator of fermentation process disturbance

Even though we were successful in achieving a high *n*-caproate production rate and high specificity we were not able to form granules under the applied conditions, which is a fundamental issue in UASB reactor operation. However, using a UASB reactor enabled one to retain and separate the biomass (flocks) from the liquid phase. Flow cytometry measurement provides a fingerprint of microbial morphology and activity and thus allowed one to gain an insight into the complex microbial community and distinguish between microbiome compositions caused by varying operational conditions (*e.g.* change of HRT).[64,65] As we did not manage to obtain granules, an analysis was performed to characterise the reactor microbiome in terms of microbial activity within the formed flocks and cells floating in the liquid phase at different process stages.

The individual and representative subpopulations exhibiting low CV (Coefficient of Variation) values in the measured parameters were discriminated. Sub-populations P4, P5 and P6 demonstrating differences in calculated cellular redox potential values (CRP) were designated as non-active, mid-active and active groups, respectively. The possible cell agglomerates, doubles or other cellular particles were assigned "other". The analysis revealed the fermentation-stage dependent distribution of non-active, mid-active and active sub-populations (Fig. 5A). Examples of histograms showing cell distribution from the stages of shaping the reactor microbiome (day 121) and stable *n*-caproate production rate (day 237) for both, flocks and liquid phase, are presented in Fig. 5B.

In flock samples, a sub-population of active cells (P6) was dominant with the exception of samples from day 121, which demonstrated the prevalence of mid-active cells (26.4% *vs.* 50.3% of P6 *vs.* P5 cells, respectively). These results go along with quite a low carboxylic acid production rate during that period (Stage I), confirming that the process needed more time to form functionally active flocks. Moreover, active cells were also dominant in all the tested effluent samples showing the following percentages: 50.3, 63.7, 70.8, 73.6, 86.9 and 62.2 after 121, 155, 175, 230, 237 and 292 days, respectively. This suggested that although the substrates were easily available for

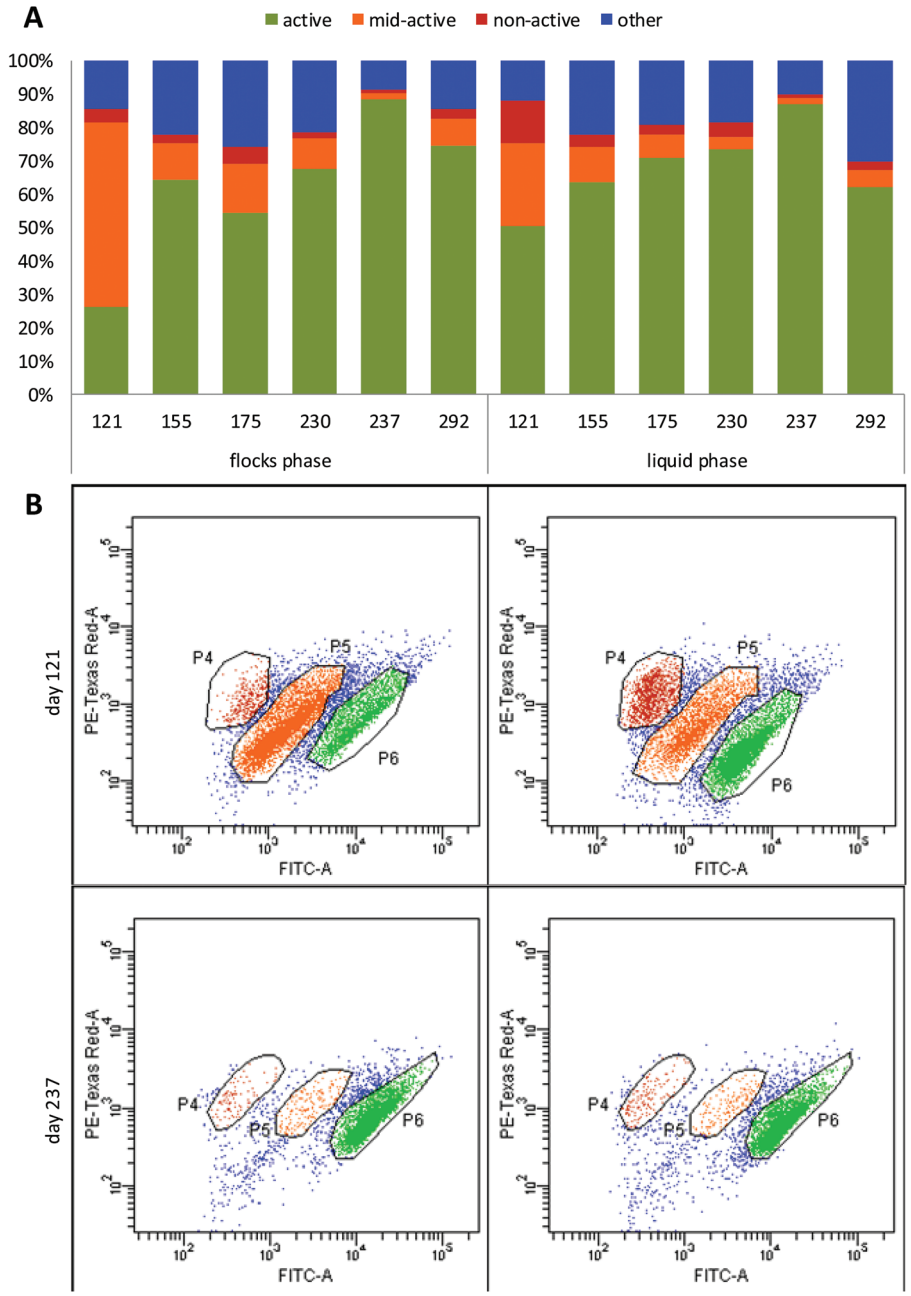

Fig. 5 Flow cytometric analysis of cellular integrity and metabolic activity of microbial cells from flocks and liquid phase of the UASB reactor. A. The non-active, mid-active and active bacterial sub-populations (P4, P5 and P6, respectively) were discriminated. The possible cell agglomerates, doublets or other cellular particles were assigned "other". The analysis demonstrated differences in the percentage of microbial cells from each sub-population at different points of the fermentation process (121, 155, 175, 230, 237 and 292 days) as presented in the bar chart. B. The sub-populations P4, P5 and P6 were defined on bivariate dot plots displaying fluorescence intensity signals from FITC *vs.* PE-Texas Red detectors, corresponding to cellular redox potential *vs.* viability measurements. Examples of FITC/PE-Texas Red dot plots (from days 121 and 237) for flock phase and liquid phase are shown in diagrams.

floating cells in the liquid phase, they equally reached more complex structures such as flocks and cell agglomerates.

Cytometric monitoring of microbial community dynamics along production processes can be used to estimate the stability of microbiome-based processes like biogas production by the analysis of segregated cell abundance changes.[66] Here, the flow cytometry-based evaluation of cellular metabolic activity within the reactor microbiome demonstrated the inherent heterogeneity of microbial populations within bioreactor flocks and liquid phases, which could be an early and fast indicator of bioreactor disturbance. However, more reproducible studies are required to confirm that hypothesis.

Conclusions

In this study we demonstrated successful long-term caproic acid production in a UASB reactor from acid whey (with lactate as the electron donor) using a microbiome. We confirmed that stable caproic acid production from AW was feasible without external ethanol addition where lactate was the main electron donor for carboxylic fatty acid chain elongation. We also showed that it was possible to shape the reactor microbiome in a single bioreactor for *n*-caproate production. The system demonstrated the capability of an average *n*-caproate production rate of 134.3 ± 30.9 mmolC L^{-1} day^{-1} (0.11 g L^{-1} h^{-1}) for 37 days with a max. *n*-caproate-to-carboxylic acids ratio of 83%. Having the advantage of the HRT control it was possible to achieve a proper microbial community composition, even though some bacteria required longer time for acclimation or growth, especially at the beginning of the process. At the same time, by modulating the feed rate, desired product concentrations can be achieved inside the reactor. The microbiome diverged from both digestate and the AW-derived Lactobacillaceae family and was functionally enriched along the process to produce *n*-caproate. Thus, we succeeded in shaping the reactor microbiome to produce *n*-caproate from acid whey in a UASB reactor system. However, the process could become self-limiting due to the inhibitory effect of a high final product concentration that was toxic for the microbial community. Thus, in-line product extraction would be advised for process maintenance.

Data availability

Sequences were submitted to EBI with accession number EBI: ERP106617.

Conflicts of interest

There are no conflicts to declare.

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Electronic Supplementary Information (ESI)

Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey

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Table S1. The characteristics of seed sludge and feedstock after delivery.

Parameter	Seed sludge	Acid whey wastewater
pH	7.48 ± 0.30	4.70 ± 0.16
Total solids (TS) [g/L]	30.86 ± 3.85	58.15 ± 1.58
Volatile solids (VS) [g/L]	19.046 ± 3.69	50.99 ± 1.14
Total chemical oxygen demand (TCOD) [g/L]	50.86 ± 0.21	71.92 ± 0.21
Soluble chemical oxygen demand (SCOD) [g/L]	1.54 ± 0.04	70.71 ± 0.12
VFA [g/L]	0.12 ± 0.04	0.75 ± 0.13
VFA [mM C]	0.01 ± 0.00	27.73 ± 6.15
Lactic acid [g/L]	-	25.16 ± 1.94
Lactic acid [mM C]	-	837.93 ± 64.77
Lactose [g/L]	-	18.63 ± 1.87
Lactose [mM C]	-	653.05 ± 65.50
Ethanol [g/L]	-	0.78 ± 0.38
Ethanol [mM C]	-	33.91 ± 16.71

Table S2. The operating and performance parameters for each phase of the fermentation process.
Specificity was calculated as product-to-carboxylates production ratio in % mol C. The table contain average production rates for each HRT of each phase.

		Stage I	Stage II		Stage III
HRT [d]		20	10	5	2.5
Days of the process [d]		0 - 177	178 - 230	231 - 350	351 - 410
SCCA (C2, C3, C4, C5 without lactic acid)	**Production rate [mmol C/L/d]**	11.25 ± 5.21	22.23 ± 15.60	52.55 ± 11.53	56.67 ± 31.48
	SCCA specificity [% mol C]	47 ± 20	35 ± 13	44 ± 7	31 ± 14
Caproic acid (C6)	**Production rate [mmol C/L/d]**	12.65 ± 5.67	34.03 ± 7.72	65.52 ± 15.76	119.95 ± 30.68
	n-caproate specificity [% mol C]	50 ± 20	63 ± 12	55 ± 7	70 ± 14
MCCA (C6, C7, C8)	**Production rate [mmol C/L/d]**	13.16 ± 5.60	35.03 ± 7.72	66.72 ± 15.38	122.40 ± 30.21
	MCCA specificity [% mol C]	53 ± 20	65 ± 13	56 ± 7	69 ± 14

Table S3. The microbial population biodiversity.

Seqs/ Sample	observed_ otus Ave,	observed_ otus Err,	observed _species Ave,	Observed _species Err,	Shannon Ave,	Shannon Err,	chao1 Ave,	chao1 Err,	Gini _index Ave,	Gini _index Err,
10	6.264	1.498	6.264	1.498	2.331	0.545	14.24	10.455	0.895	0.03
8269	354.05	122.585	354.05	122.585	4.263	1.237	546.08	148.966	0.981	0.017
16528	463.364	141.717	463.364	141.717	4.289	1.24	649.46	167.224	0.981	0.016
24787	532.221	151.283	532.221	151.283	4.299	1.245	709.485	173.731	0.981	0.016
33046	581.743	158.461	581.743	158.461	4.305	1.245	757.595	183.112	0.981	0.016
41305	620.114	164.489	620.114	164.489	4.307	1.246	792.933	189.09	0.981	0.016
49564	651.893	168.682	651.893	168.682	4.31	1.246	820.388	189.146	0.981	0.016
57823	678.357	171.628	678.357	171.628	4.312	1.246	844.205	192.206	0.98	0.016
66082	700.964	175.13	700.964	175.13	4.314	1.247	866.28	197.39	0.98	0.016
74341	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
82600	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan

Table S4. The flow cytometry measurement of non-active, mid-active and active microbial subpopulations.

			non-active			mid-active			active		
Day	Sample name	Sample type	P4 #Events	P4 %Parent	P4 FITC-A Median	P5 #Events	P5 %Parent	P5 FITC-A Median	P6 #Events	P6 %Parent	P6 FITC-A Median
121	1	effluent	1178	12.9	410	2295	25.1	1267	4607	50.5	4580
	1_001		1161	12.8	409	2226	24.5	1425	4581	50.4	4823
	1_002		1136	12.5	394	2275	25.1	1478	4551	50.1	4836
	2	flocks	361	4.4	585	4529	55.6	1415	2055	25.2	10302
	2_001		302	3.9	572	4290	54.7	1550	2112	26.9	10481
	2_002		290	3.7	588	4299	55.2	1603	2117	27.2	10154
155	C	effluent	347	3.9	493	899	10.1	995	6087	68.1	9182
	C_001		321	3.6	439	899	10.1	1373	5918	66.6	8379
	C_002		316	3.4	411	1022	11.1	1495	5183	56.5	9241
	O	flocks	229	2.9	468	839	10.5	995	5152	64.3	9968
	O_001		207	2.6	469	896	11.1	1034	5137	63.9	10155
	O_002		232	2.8	428	935	11.1	1022	5436	64.7	10397
175	C	effluent	167	2.1	461	563	7	1133	5906	73.5	17160
	C_001		220	2.7	501	625	7.8	1201	5688	70.9	16950
	C_002		282	3.2	515	614	6.9	1233	6080	67.9	16613
	O	flocks	427	5.4	825	1185	14.8	2245	4330	54.3	19842
	O_001		391	4.9	797	1156	14.5	2495	4386	54.9	20778
	O_002		362	4.6	751	1224	15.4	2643	4266	53.7	20516
230	C	effluent	418	4.6	667	324	3.6	3254	6596	72.7	21591
	C_001		384	4.2	650	330	3.6	3851	6744	74.4	20826
	C_002		396	4.4	605	340	3.8	4185	6672	73.8	20055
	O	flocks	173	2	895	724	8.4	3065	5932	68.9	24137
	O_001		155	1.8	866	795	9.3	3285	5795	67.5	24334
	O_002		189	2.2	878	780	9	3159	5810	67	24091
237	C	effluent	128	1.5	350	167	1.9	3303	7673	87.6	11752
	C_001		135	1.5	358	143	1.6	3195	7595	86.7	11672
	C_002		106	1.2	368	153	1.7	3057	7579	86.5	11686
	C_003		93	1.1	340	162	1.9	3054	7509	86.9	11644
	O	flocks	89	1	370	177	2.1	2777	7606	88.2	13212
	O_001		96	1.1	353	170	1.9	2860	7785	88.9	13354
	O_002		100	1.2	378	184	2.1	2988	7672	88.3	13328
	O_003		85	1	363	174	2	3261	7660	88.4	13165
292	C	effluent	290	3.4	271	612	7.3	423	5785	68.8	5165
	C_001		295	3.5	266	655	7.7	432	5792	68	4997
	C_002		128	1.7	401	259	3.4	604	4318	56.8	8933
	C_003		157	2.1	325	300	4	537	4366	58.2	8063
	C_004		130	1.7	405	217	2.8	689	4530	57.6	8504
	C_005		181	2.5	267	400	5.5	453	4655	63.9	6630
	O	flocks	217	2.7	262	618	7.7	366	5962	73.8	6411
	O_001		280	3.3	279	715	8.3	359	6422	74.8	6008
	O_002		251	2.9	265	710	8.3	368	6460	75.2	5692
	O_003		256	2.9	272	734	8.4	371	6517	74.4	5908

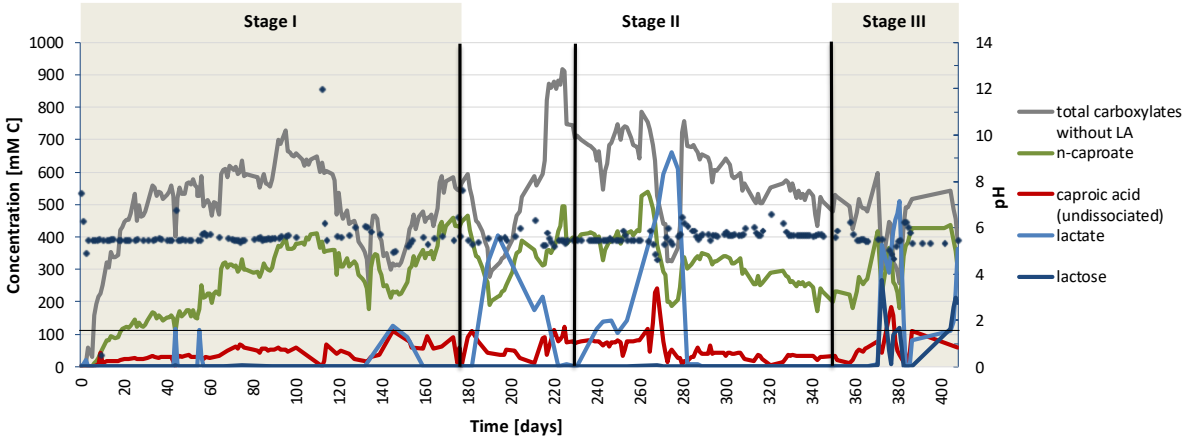
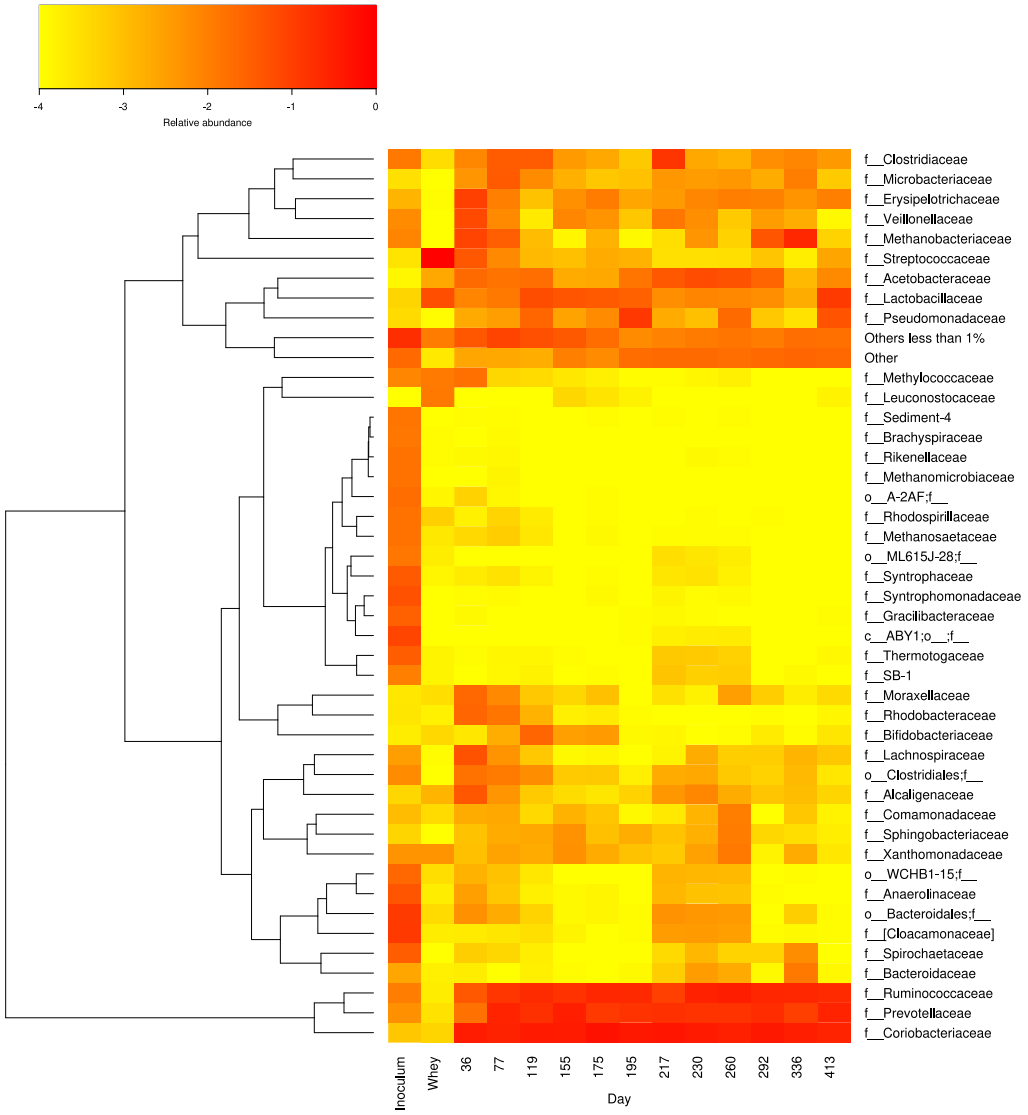


Fig. S1 Concentration of total carboxylates, n-caproate (dissociated form), caproic acid (undissociated form) lactate and lactose as well as pH along the fermentation process.

Fig. S2 Heatmap of relative abundance at family level. The relative abundance were calculated as log for better visualisation and clustered based on the Bray-Curtis dissimilarity index.



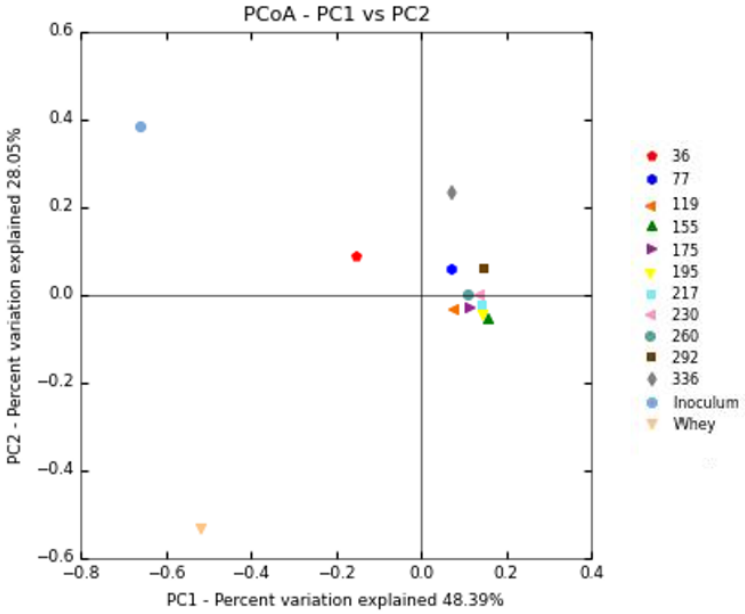


Fig. S3 PCoA analysis of the microbial society of inoculum, feedstock and samples taken throughout bioreactor operation. The PCoA analysis was performed based on the weighted UniFrac algorithm. The plot is based on the first two principal coordinates (PC1 and PC2) which explain 48.39% and 28.05% of overall phylogenetic variation, respectively.

PCoA analysis was used to determine the dissimilarity between the community of the inoculum, feedstock and samples taken throughout bioreactor operation based on the weighted UniFrac algorithm. Weighted UniFrac is a measure of the pairwise dissimilarity in community composition which takes into account the relative abundance of different members of the microbial community. The distances are visualised based on the first two principal coordinates (PC) that collectively explained 76.44% of overall phylogenetic variations between samples (PC1 explains 28% of the overall phylogenetic variation, while PC2 explains 48.4%). Inoculum and ACW samples were distanced from each other and from the reactor samples significantly. Sample from day 36 (beginning of the process) diverged from inoculum and feedstock, however it was slightly separated from the other reactor samples on the PCoA plot. Samples from days 77 to 292 were grouped together. Sample from day 336 was separated along the PC2 making it closer to the inoculum as at that time methanogens appeared in the reactor. The results showed similar bacterial community in the reactor microbiome along the process with small spacial and temporal variations indicating a quite stable microbial population.

Poznań, dnia 09.08.2018

OŚWIADCZENIE WSPÓŁAUTORÓW

Oświadcza się, że w pracy:

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Paper II

Duber, A., Zagrodnik, R., Chwialkowska, J., Juzwa, Oleskowicz-Popiel, P., 2020. *Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation*, Sci. Total Environ. 728, 138814.

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Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation

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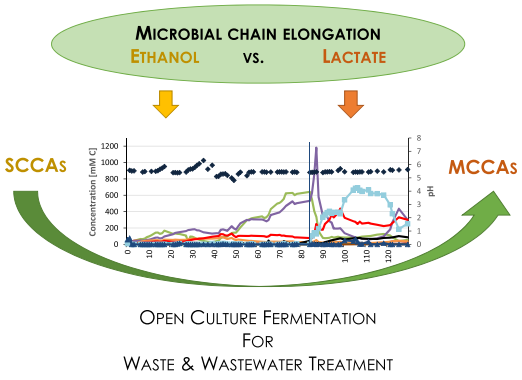
HIGHLIGHTS

- The feed composition strongly influenced the chain elongation process.
- Low ethanol content and abundant lactate were essential to produce caproate.
- Overdosing with ethanol directed the process towards ethanol oxidation.
- Removal of ethanol resulted in sharp SCCAs drop and growth of MCCAs production.
- Maximum caproate production rate reached 4.1 g/L/day at HRT of 1.25 day.

GRAPHICAL ABSTRACT



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ABSTRACT

The objective of this study was to investigate the effect of substrate composition on chain elongation pathways and on shaping reactor microbiome during open culture fermentation (OCF). The process was performed in a continuous mode in an upflow anaerobic sludge blanket (UASB) reactor fed with either fresh acid whey (AW) or AW at controlled stage of prefermentation (with controlled content of electron donors). Dosing AW with an increasing ethanol loading rate led to ethanol oxidation and short chain carboxylic acids (SCCAs) generation. Change of the feedstock composition (higher lactate and lactose content and ethanol cut off) shifted the process outcome towards medium chain carboxylic acids (MCCAs) production, with caproate as the main product. The MCCAs production rate has grown from 0.7 ± 0 to 4.12 ± 1 g/L/day (38.3 ± 5 to 212.6 ± 60 mmol C/L/day) and reached specificity of 48 ± 18% mol C. The differentiation between microbiome samples confirmed the reactor microbiome shaped according to the feed composition. The only known caproic acid producers were represented by *Caproiciproducens* ssp., that reached a relative OTU abundance between 3 and 7%. The developed method enables to substitute the use of fossil resources with products from the OCF of waste and wastewater. Thus, it contributes to reduce the carbon footprint and enhance the sustainability of the chemical industry.

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1. Introduction

Different organic waste feedstocks can be exploited via open culture fermentation (OCF) for a production of valuable MCCAs through complex microbial bioprocesses in order to minimize the negative impact of waste and limit the use of fossil resources. Open culture biotechnological processes relay on process operational conditions, such as pH, organic loading rate (OLR), hydraulic retention time (HRT), and a complex reactor microbiome activity. The reactor microbiome consists of mixed microbial consortia that are naturally delivered to the system with the feedstock. It can also proceed from a natural or enriched inoculum (Angenent et al., 2016). As the process can be controlled by setting specific operational conditions, the microbiome is also shaped for specific product(s) generation (Cabrera et al., 2019). However, when working with a real waste feedstock containing a mixture of substrates, numerous competing and intertwining biochemical pathways occur simultaneously. Therefore, not only the process operational conditions, but also delivered substrates, produced intermediates and products have an impact on the microbial succession, and thus on the following production (Jankowska et al., 2018). In a specific microbiome, different microbial groups express different metabolic potential for specific substrate(s) utilization and conversion linked to the thermodynamic feasibility of particular reactions. This way, at fixed operational process parameters, the feedstock composition may play a crucial role in shaping the microbiome and the process performance.

MCCAs are carboxylic acids with six to twelve carbon atoms, that can be produced biologically in OCF from variety of organic waste feedstocks (Angenent et al., 2016). MCCAs are valuable as they can be further upgraded to jet biofuels or other biochemicals (Layton and Trinh, 2016). It can be formed through chain elongation from short chain carboxylic acids (SCCAs), that range from two to five carbon atoms and include acetate, propionate, butyrate, valerate and lactate (Agler et al., 2011). Several pathways for microbial chain elongation are described (Spirito et al., 2014). In the most known pathway SCCAs, products of primary fermentation (hydrolysis and acidification) (Yuan et al., 2019), are elongated to MCCAs in secondary fermentation through the reversed β-oxidation (RBO) in the presence of electron donor (Agler et al., 2011). Even though many electron donors were investigated for that purpose, ethanol and lactate are considered the most suitable for chain elongation (Wu et al., 2018). The OCF processes for MCCAs formation were carried out either on a real wastewater with high ethanol concentration (ethanol occurs naturally in the feedstock) (Agler et al., 2012a; Ge et al., 2015; Kucek et al., 2016c; Andersen et al., 2017), or on real waste streams containing lactate (Zhu et al., 2015; Kucek et al., 2016a; Xu et al., 2018; Duber et al., 2018; Carvajal-Arroyo et al., 2019). Few research was conducted with a waste feedstock that intrinsically contains combinations of the mentioned electron donors, i.e. Chinese liquor-making wastewater (Wu et al., 2018).

A wastewater that may contain both, lactate and ethanol is acid whey (AW). It is rich in lactate, but also lactose and other components, such as peptides, vitamins. Therefore, it can be used as a valuable feedstock for microbiome-based processes (Carvalho et al., 2013) and can serve as a substrate (with intrinsic co-electron donors) for understanding the principles of chain elongation process. Depending on different factors, such as a source of milk (cow, sheep, bovine, etc.), an animals breed, feed, health, the stage of lactation and a cheese production process, AW may vary in a chemical composition (Chwialkowska et al., 2019), particularly lactose, lactate and ethanol content. It may change during the storage at the production site, due to the storage conditions and a natural presence of lactic acid bacteria (LAB). In general, LAB may spontaneously ferment lactose in two major routes: (i) through homofermentation to lactate or (ii) through heterofermentation to lactate, CO_2 and ethanol or acetate (Kandler, 1983), thus providing electron donors for chain elongation.

AW is a byproduct form crude cheese manufacturing industry produced in large volumes (Erickson, 2017). It causes a real problem for the dairy industry and for wastewater treatment plants due to the high biological oxygen demand (BOD) (in the range 30,000–60,000 mg/L) owing ~90% to the high lactose content (Kissalita et al., 1989). Thus, the issue of AW disposal/utilization is actually a question of lactose disposal/utilization. Nowadays, when the AW/lactose disposal is not a desirable action due to the high BOD, there are a number of technological developments in the conversion of AW to other products, such as methane (Göblös et al., 2008) or biohydrogen (Azbar et al., 2009). However, the obtained products are of a low energy density compared to MCCAs. Utilization of AW wastewater in order to produce MCCAs offers a new use for this waste stream (Xu et al., 2018; Duber et al., 2018).

The objective of this study was to effectively recover carbon from waste, where AW was chosen as a model substrate. The effect of the substrate composition (different lactose/lactate/ethanol content) on chain elongation in long-term OCF was investigated. As AW can be fermented to lactate and/or ethanol, another hypothesis was, whether the presence of both electron donors could boost the chain elongation and MCCAs performance. To identify and better understand the ethanol role, the chain elongation was studied in a continuous mode by supplying feed with an increasing ethanol loading rate as well as feed without any content of ethanol. The response of the microbial structure to the change of the feedstock composition was investigated at each process stage. Results of this work provide better understanding of the chain elongation in response to the various composition of real waste feedstock, that intrinsically contain more than one electron donor.

2. Materials and methods

2.1. Process configuration

Sludge was obtained from an anaerobic digester of a municipal wastewater treatment plant (Poznan, Poland) and used as inoculum. The sludge was first left for degasification for 48 h at 30 °C and next it was diluted with reject waters to obtain an initial total soluble solids concentration of 5 g/L. Two different AW batches (coming from first and second delivery, henceforth called acid whey 1 and acid whey 2, respectively) delivered from the same company (Dobrzyca Dairy Industry, Poland) were used as a feedstock. The acid whey 1 was taken from a crude cheese production line and acid whey 2 was one week old AW taken from an outdoor storage tank. Upon delivery, both feedstocks were stored at 4 °C. The characteristics of inoculum and both delivered feedstocks are presented in Table S1. Prior feeding, each feedstock was prepared as described below and then pumped into the reactor from a refrigerated reservoir (4 °C).

The trial was carried out in an UASB type reactor as described before (Duber et al., 2018) with working volume of 1 L and 2.5 L upper part to collect the surplus effluent (Fig. S1). Recirculation assured that the sludge was in suspension. The pH in the reactor was measured at the recirculation (Elmetron EPP-1, Elmetron, Poland) and was maintained at pH of 5.5 by the addition of 2 M NaOH. The temperature was kept at 30 °C. The biomass, liquid and gas samples were collected as indicated on Fig. S1. The gas production was quantified using a volumetric gas flow meter (Ritter, Germany).

The process was divided into four operating periods (*Period I–IV*) based on the ethanol content in the feedstock and lactose-to-ethanol molar ratio (based on mol C/mol C). The ethanol concentrations in the influent were below its toxicity level and in a range in which chain elongation to MCCAs is known to occur (Grootscholten et al., 2013a). For every period we used either raw acid whey 1 from fresh crude cheese production, prefermented acid whey 1 containing ethanol or acid whey 2 as described below. Lactose-to-lactate molar ratio were in range 1–5 mol C/mol C during all operating periods (Table 1). Concentration of ethanol, lactate, lactose and acetate in the feedstock dosed to the reactor as well as lactose-to-ethanol and lactose-to-lactate molar ratio (based on mol C/mol C) during each operating period are

presented in Table 1. Additionally, the HRT was shortened by half in the middle of operating *Periods I–III* to accelerate the MCCAs production process as described below and in (Table S2).

During *Period I*, which was the startup period (days 0–27; HRT = 20 days from day 0 to 14; HRT = 10 from day 15 to 27), the reactor was fed with raw acid whey 1. During *Period II* (days 28–56; HRT = 10 from day 28 to 42; HRT = 5 days from day 43 to 56) we used acid whey 1 that was first prefermented. The AW prefermentation was carried out in room temperature in the unsealed storage tank through a spontaneous activity of lactic acid bacteria, that are naturally present in AW. The process lasted as long as ethanol concentration reached around 10 g/L (434 mol C/L or 217 mol/L) and then it was kept in the fridge (4 °C). During *Period III* (days 57–84; HRT = 5 days from day 57 to 70; HRT = 2.5 day from day 71 to 84) external ethanol was added to prefermented acid whey 1 to reach a concentration of 20 g/L (868 mol C/L or 434 mol/L). In *Period IV* (days 85–129; HRT = 1.25) acid whey 1 was replaced with acid whey 2, which did not contain ethanol. The operating and performance parameters for each HRT of each operating period of the fermentation process are presented in Table S2.

The substrates loading rate (Eq. (S1)) and carboxylates production rates (Eq. (S2)) were calculated for working volume of the reactor in [mmol C/L/day] and [g/L/day]. Product specificity (Eq. (S3)) was calculated as product-to-carboxylic acids (without lactic acid) production ratio (in % mol C) according to (Xu et al., 2018). Conversion efficiency [% mol C] (Eq. (S4)) was shown as carbon balance based on the loading rates and production rates of measured carbon substrates (acetate, ethanol, lactic acid, lactose) and products (SCCAs, MCCAs, CO_2 and CH_4).

2.2. Analytical methods

Methane, carbon dioxide and hydrogen content were measured using gas chromatograph equipped with TCD detector (Shimadzu 2014 GC System, Japan). Nitrogen was used as the carrier gas at a flow rate of 15 mL/min with the injector temperature of 110 °C, column of 50 °C and detector of 80 °C. Weekly collected feedstock and daily collected reactor liquid samples were analysed for organic acids, alcohols and lactose. Volatile organic acids and alcohols concentration were monitored with gas chromatography system equipped with FID detector (Shimadzu 2014 GC System, Japan) according to a method described in Duber et al. (2018). Concentrations of lactic acid and lactose were determined with high performance liquid chromatography system (HPLC 20AT, Shimadzu, Japan) as presented before (Duber et al., 2018). The total solids (TS), volatile solids (VS), total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were measured as described by Jankowska et al. (2018).

2.3. Microbiome analysis

Biomass samples for microbiome analysis were collected from the UASB reactor at seven time points (days 7, 14, 35, 56, 78, 107 and 125) and the seed sludge (inoculum). Samples were centrifuged at 13,000 rpm for 4 min, supernatant was discarded and obtained biomass pellets were stored in −20 °C until the process was finished and representative samples for microbial analysis were chosen based on all obtained results. Additionally microbial subpopulations from sample 107 showing difference in metabolic activity (further called 107_P4, 107_P5, 107_P6) were sorted and harvested for the flow cytometry analysis. Genomic DNA from sludge and reactor samples was extracted as described before (Duber et al., 2018) using GeneMATRIX Soil DNA Purification Kit (Eurx®, Poland), according to the manufacturer's instructions. Genomic DNA from sorted subpopulations was extracted using Chelex method (Chelex 100 Resin, Bio Rad Laboratories) according to a protocol described by Koch et al. (2013). Eluted DNA was stored at −20 °C prior analysis. Microbiome analysis was performed as previously described in Duber et al. (2018).

The flow cytometry analysis was performed for sample from day 107 according to a method described previously (Duber et al., 2018). In brief, microbial cells (1 mL of bioreactor sample) were filtered (20 μm nylon net syringe filter, Merck Millipore), washed with PBS, resuspended in 1 mL of PBS and stained with BacLight™ Redox Sensor™ Green Vitality Kit (Life Technologies, Carlsbad, CA, USA). Prepared sample was analysed using BD FACS Aria™III flow cytometer (cell sorter) (Becton Dickinson, San Jose, CA, USA) and acquired data was analysed with FACS DIVA software (Becton Dickinson, USA) as described in detail in (Duber et al., 2018). Subpopulation of active cells 107_P6 showing the highest level of the metabolic activity among all subpopulations, that was measured as cellular redox potential (CRP), was sorted to separate tube into sheath buffer, centrifuged at 13,000 rpm for 4 min, supernatant was discarded and pelleted cells were stored in −80 °C prior DNA isolation.

3. Results and discussion

3.1. Increasing ethanol loading rate promoted production of SCCAs

Substrates loading rates and carboxylates and gases production rates at each operating period are presented in Fig. 1. In a start-up phase (*Period I*) raw acid whey 1 with high lactose content of 42.3 ± 0.2 g/L or 1483 ± 7 mM C (lactose-to-ethanol molar ratio 62.3 mol C/mol C) (Table 1.) was used as a feed. During that phase carboxylates of even and odd carbon numbers from C2 to C6 were produced in a total concentration below 12 g/L (460 mM C). MCCAs concentration did not exceed 1.2 g/L (65 mM C). From day 28th on (*Period II*), prefermented feedstock containing much lower lactose content (11.4 ± 0.5 g/L or 399 ± 19 mM C) and ethanol at concentration of 11.2 ± 0.9 g/L (484 ± 40 mM C) (lactose-to-ethanol molar ratio of 0.8 mol C/mol C) was supplied to the reactor (Fig. 1A; Table S2). The role of ethanol was to provide reducing equivalents for the chain elongation process. However, the change in the substrate composition directed the process mostly to acetate and butyrate production (Fig. 1B) and slightly triggered the chain elongation to valerate and caproate (*Period II*, HRT = 5 day). MCCAs concentration reached 2.9 g/L (145 mM C). In *Period III* further increase of ethanol in the feedstock to 21.5 ± 1.0 g/L (934 ± 43 mM C or 467 ± 22 mol/L) and shortening the HRT to 2.5 days

Table 1
Concentration of feedstocks components fed to the reactor as well as lactose-to-ethanol and lactose-to-lactate molar ratio (based on mol C/mol C) during each operating period.

	Period I	Period II	Period III	Period IV
Ethanol [g/L]	0.55 ± 0.67	11.15 ± 0.93	21.51 ± 0.99	0.42 ± 0.04
Lactate [g/L]	8.84 ± 0.06	12.18 ± 0.05	10.91 ± 0.35	19.40 ± 0.70
Lactose [g/L]	42.31 ± 0.20	11.38 ± 0.53	20.34 ± 0.20	26.85 ± 0.97
Acetate [g/L]	0.71 ± 0.09	1.75 ± 0.34	1.63 ± 0.33	1.59 ± 0.06
Ethanol [mM C]	23.8 ± 2.9	484.1 ± 40.3	933.9 ± 43.0	18.4 ± 1.5
Lactate [mM C]	294.3 ± 2.0	405.7 ± 1.7	363.4 ± 11.8	644.3 ± 22.3
Lactose [mM C]	1483 ± 7	398.9 ± 19	713.2 ± 7	946.9 ± 35
Acetate [mM C]	23.6 ± 2.9	58.4 ± 11.2	54.2 ± 11.0	52.9 ± 2.0
Lactose-to-ethanol molar ratio [mol C/mol C]	62.3	0.8	0.8	51.6
Lactose-to-lactate molar ratio [mol C/mol C]	5.0	1.0	2.0	1.5

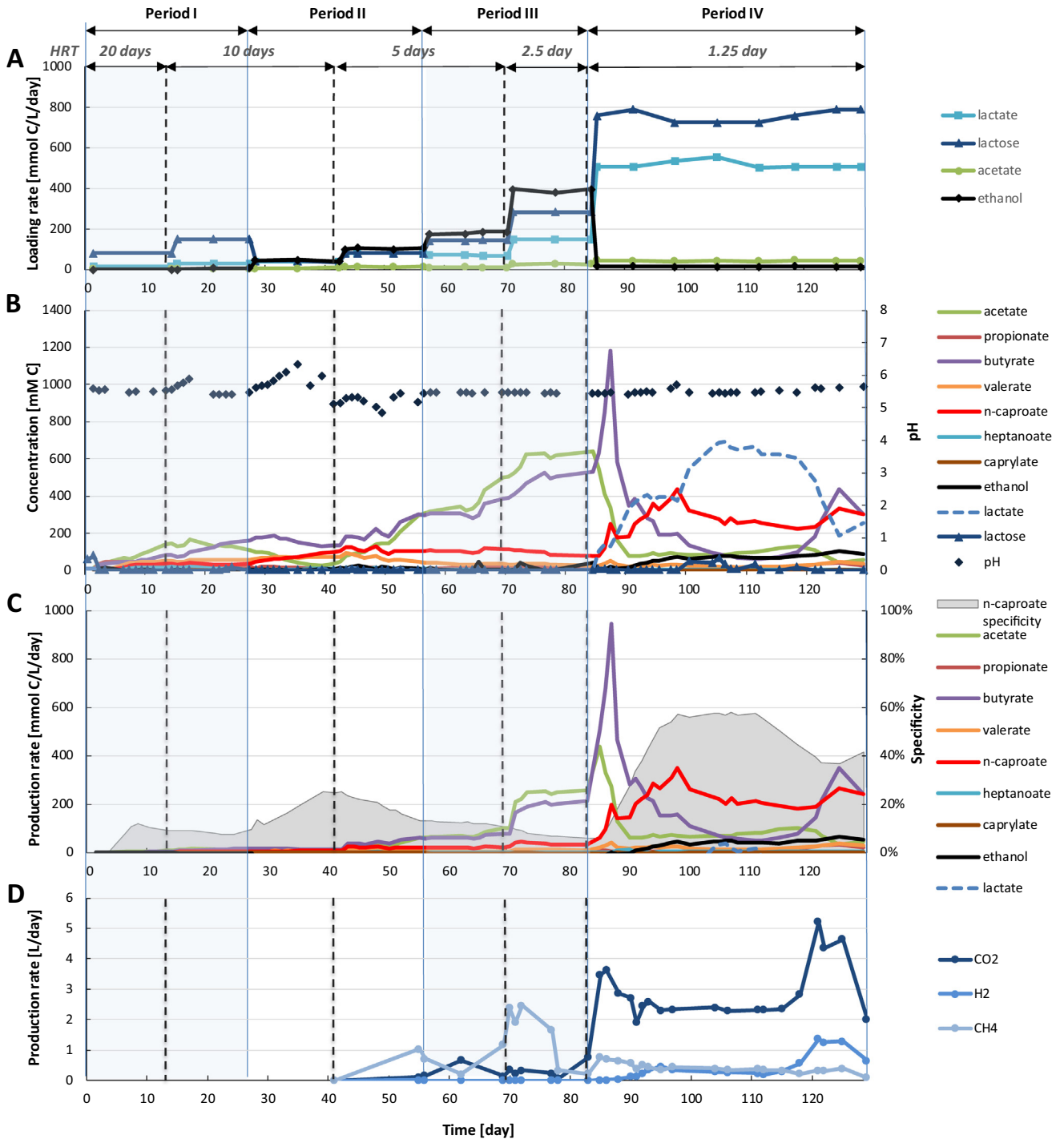

Fig. 1. Substrates loading rates, products concentration and carboxylates and gases production rates at each operating period. A Loading rate of feedstock components (ethanol, lactate and lactose) to the UASB reactor. B Carboxylates, lactose and ethanol concentration as well as pH in the UASB reactor during the fermentation process. C Production rate of carboxylates and ethanol as well as caproate specificity [%]. D Production rate of measured gases is presented as the volume of gases produced in the reactor per day. Vertical dotted lines indicate the change of HRT.

boosted acetate and butyrate production to 6.4 ± 0.5 and 4.3 ± 0.4 g/L/day (214 ± 16 and 193 ± 17 mmol C/L/day) respectively (Fig. 1C). At the same time caproate was produced at quite low but stable level of 0.7 ± 0.1 g/L/day (38 ± 4 mmol C/L/day) and it was accompanied with CH_4 formation (Fig. 1D). Other MCCAs were produced at inconsiderable level.

In works of Grootscholten et al. (2013a, 2013b) and Lonkar et al. (2016) authors showed that increasing ethanol loading rate led to higher MCCAs production rates, mostly caproate and heptanoate. However, in contrary to our study, Grootscholten et al. (2013a, 2013b) used a higher pH value and a shorter HRT. Therefore, they saw that an increasing ethanol loading rate worked positively on the caproate production rate. It is worth noting, that in our study during *Periods I–III* all delivered substrates were completely utilized. Despite its concentration in the feed, the ethanol added with AW during *Periods II–III* contributed mostly to ethanol oxidation to acetate and then to chain elongation to butyrate (and only partly to caproate). In the anaerobic oxidation of ethanol, it is oxidised to acetate and H_2 (Table 2, Eq. (1)) (Layton and Trinh,

2016). If the reaction is coupled with hydrogenotrophic methanogenesis, produced hydrogen is immediately used (Table 2, Eq. (2)) (Roghair et al., 2018) and the overall pathway is called syntrophic ethanol oxidation (Table 2, Eq. (3)) (Agler et al., 2014). This process explains the high concentration of acetate (up to 19 g/L or 633 mM C), which was coupled with the methane production up to 2.5 L/day (Fig. 1D). Taking into account that, generally, the chain elongation of carboxylates of even carbon numbers (Table 2, Eqs. (4)–(6)) is initialised with the ethanol oxidation, excess of ethanol could be elongated with acetate (in form of acetylo-CoA) to butyrate (Table 2, Eq. (4)) (Spirito et al., 2014; Angenent et al., 2016) and it would explain the ethanol consumption with a simultaneous acetate, butyrate and methane production during *Periods II–III*. Although the substrates needed for the RBO process were supplied to the system, the chain elongation might not proceeded further, due to the insufficient lactate availability (the lactate concentration in the feed was much lower than the ethanol content during *Period III*). Moreover, the high concentrations of produced butyrate would produce the inhibition on further chain elongation to caproate. Thus, the microbiome was redirected mainly to the syntrophic ethanol oxidation process resulting in a low MCCAs yield.

3.2. Ethanol cut off shifted the reactor outcome from SCCAs to MCCAs production

In *Period IV* HRT shortening to 1.25 day, as well as the change of the feedstock from prefermented AW containing ethanol to AW without ethanol and with a higher content of lactate (high lactose-to-ethanol molar ratio and a relatively low lactose-to-lactate molar ratio based on [mol C/mol C]) (Table 1.) resulted in a number of changes.

First, acetate and butyrate reached the highest concentration during the whole process of 19.3 g/L (642 mM C) on day 84 and 26.1 g/L (1183 mM C) on day 87, respectively (Fig. 1B). In the next days the concentration of both carboxylates dropped sharply and a metabolic switch from SCCAs to MCCAs formation (mostly caproate) was noticed (Fig. 1B). The caproate production rate increased in that operating period and from day 91 until the end of the process caproate was produced at a stable level of 4.5 ± 0.8 g/L/day (233 ± 42 mmol C/L/day) (Fig. 1C) with the highest concentration of 8.5 g/L (437 mM C) on day 98. Reaching higher concentrations of caproate could not be achieved mostly due to a product inhibition, as the undissociated species of caproic acid may cause an inhibition effect on microbial cells. At pH 5.5 the undissociated form of caproic acid is high (pKa 4.88). Ge et al. (2015) indicated its toxic limit of 7.5 mM. The concentration of undissociated caproic acid in *Period IV* was above the mentioned limit (Fig. S2). Thus, the reactor microbiome controlled itself by not producing more caproate and kept the inhibition down, despite the substrates availability. A continuous product extraction would address this limitation (Ge et al., 2015; Kucek et al., 2016c).

Due to the product inhibition and a three times higher lactate loading rate, lactate was not consumed completely and accumulated in the reactor (Fig. 1B). Moreover, during days 104–111 the lactate concentration in the UASB reactor was higher than its concentration in the feed as a result of in situ conversion of lactose to lactate (Fig. 2). Only after the 115th day, the microbiome adapted to the applied conditions and started to utilize lactate (Fig. 1B, C). Thus, lactate concentration in the reactor decreased from 18.7 g/L (on day 115) to 5.6 g/L (on day 125) (from 622 to 187 mM C, respectively). Consequently, the butyrate concentration increased from 1.6 to 9.6 g/L (from 74 to 435 mM C) and the caproate concentration increased from 4.7 to 6.4 g/L (from 240 to 331 mM C). Taking into account that the ethanol concentration was stable, lactose and lactate were the main sources of reducing equivalents for the production of caproate. The reaction was accompanied with a rapid increase in CO_2 and H_2 production rates as presented in (Fig. 1D) and (Table 2, Eqs. (7)–(9)) (Cavalcante et al., 2017).

It is worth noting that after ethanol cut off, it started to be produced in the reactor with growing tendency until day 100. Then, ethanol production rate remained stable at the average of 1.1 ± 0.2 g/L/day (46.5 ± 8 mmol C/L/day). It is in line with results of our previous work, where we showed that ethanol was produced itself during the AW fermentation process as one of the product of lactose conversion (Fig. 2) and the reaction coincided with the caproate production (Duber et al., 2018).

Generally, the CO_2 and H_2 production rates increased from 0.3 ± 0.2 L/day CO_2 and 0.0 L/day H_2 (in *Period III*), to 2.8 ± 1.0 L/day CO_2 and 0.4 ± 0.4 L/day H_2 (in *Period IV*) (Fig. 1D). At conditions of shorter HRT and higher OLR, excess electrons were utilized for hydrogen production. A shorter HRT is unfavourable for methanogens, which could utilize produced hydrogen. As a result a higher hydrogen production was observed. Hydrogen and carbon dioxide can be converted to acetate through homoacetogenesis (Table 2, Eq. (10)), the acetate reduced to ethanol with hydrogen, and then follows the chain elongation (Fig. 2). In work of Nzeteu et al. (2018) high hydrogen partial pressure was essential to reach high caproate selectivity. Authors also suggested that

Table 2
Thermodynamic information of Gibbs free energy ($\Delta G'^{\circ}_{r}$) for syntrophic ethanol oxidation and carboxylic acid chain elongation through ethanol or lactate for standard conditions (25 °C, pH 7.0) (Spirito et al., 2014; Cavalcante et al., 2017; Roghair et al., 2018).

Eq.	Process	Stoichiometries	ΔG^{0}_{r} (kJ/mol)	Coupled reactions
Syntrophic ethanol oxidation				
(1)	Anaerobic ethanol oxidation	Ethanol + H_2O → $acetate^-$ + H^+ + $2H_2$	10.1	×1
(2)	Hydrogenotrophic methanogenesis	$2H_2$ + $0.5CO_2$ → $0.5CH_4$ + H_2O	−32.7	×2
(3)	Overall syntrophic ethanol oxidation	Ethanol + $0.5CO_2$ → $acetate^-$ + H^+ + $0.5CH_4$	−55.4	
Carboxylic acid chain elongation from ethanol				
(1)	Anaerobic ethanol oxidation	Ethanol + H_2O → $acetate^-$ + H^+ + $2H_2$	10.1	×1
(4)	Chain elongation to butyrate	Ethanol + $acetate^-$ → $butyrate^-$ + H_2O	−38.7	×5
(5)	Chain elongation to caproate	Ethanol + $butyrate^-$ → $caproate^-$ + H_2O	−38.8	×5
(6)	Chain elongation to caprylate	Ethanol + $caproate^-$ → $caprylate^-$ + H_2O	−38.8	×5
	Overall chain elongation to caproate from ethanol	12 ethanol + 3 $acetate^-$ → 5 $caproate^-$ + $4H_2$ + $8H_2O$	−30.6	
	Overall chain elongation to caprylate from ethanol	18 ethanol + 2 $acetate^-$ → 5 $caprylate^-$ + $6H_2$ + $12H_2O$	−30.6	
Carboxylic acid chain elongation from lactate				
(7)	Lactate oxidation	$Lactate^-$ + H_2O → $acetate^-$ + $2H_2$ + CO_2	−8.79	×5
(8)	Chain elongation to butyrate	$Lactate^-$ + $acetate^-$ → $butyrate^-$ + CO_2 + H_2O	−57.5	×5
(9)	Chain elongation to caproate	$Lactate^-$ + $butyrate^-$ → $caproate^-$ + CO_2 + H_2O	−57.6	×5
	Overall chain elongation to caproate from lactate	15 $lactate^-$ → 5 $caproate^-$ + $10H_2$ + $10CO_2$ + $5H_2O$	−41.3	
Other				
(10)	Homoacetogenesis	$4H_2$ + $2CO_2$ → $acetate^-$ + H^+ + $2H_2O$	−95.0	
(11)	Acetoclastic methanogenesis	$Acetate^-$ → CH_4 + CO_2	−36.3	
(12)	Lactic acid oxidation	$Lactate^-$ + $3H_2O$ → $3CO_2$ + $6H_2$	85.61	Not plausible

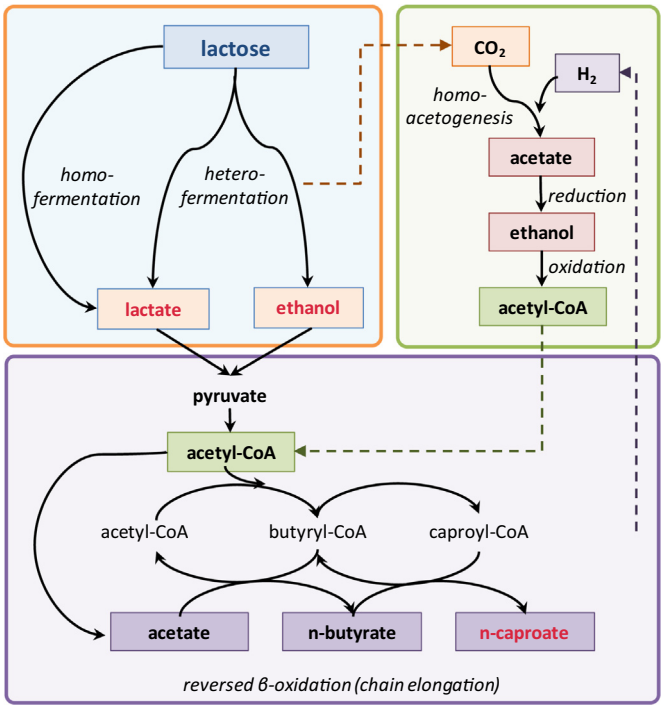

Fig. 2. Simplified scheme of the possible metabolic pathways of acid whey (lactose) utilization for MCCAs production, based on Duber et al. (2018) and Wu et al. (2018).

providing additional source of reducing power in the form of H_2 could facilitate the process of the chain elongation of lactate to caproate (Nzeteu et al., 2018). Moreover, competing processes such as an anaerobic oxidation of MCCAs and an excessive ethanol oxidation are thermodynamically inhibited at the hydrogen partial pressure above 0.03 atm (Angenent et al., 2016; Wu et al., 2019). In our study at the beginning of *Period IV* the hydrogen partial pressure was 0.1 atm and it grew to about 0.2 atm at the end of the process, creating favourable conditions for the chain elongation of lactate to caproate.

In *Period IV* the substrates conversion efficiency to SCCAs dropped from around 51.1% mol C (in *Period III*) to 24.4% mol C (in *Period IV*) (Table S2). At the same time the substrates conversion efficiency to MCCAs increased from about 5% mol C in previous period, to 16.3% mol C in *Period IV*, where caproate comprised 16.0% mol C (Table S2). The switch in the production outcome could have occurred due to a higher lactate loading, that may have caused an increase in the total energy available for the chain elongation pathway, and therefore, it contributed to the activation of more energetically favourable pathways. Even though the carboxylic acid chain elongation to caproate is thermodynamically plausible through both, ethanol and lactate (Chwialkowska et al., 2019), the latter option is energetically more favourable as the overall energy gain is higher (Table 2). Grootscholten et al. (2013c) proved that for the MCCAs formation from ethanol, butyrate production was essential. However, Zhu et al. (2017) shown that the caproate production from butyrate and lactate was more favourable compared to its formation from butyrate and ethanol. Taking into account that a higher production of MCCAs were generated without ethanol dosage, we concluded, that it was essential to keep high lactate availability and keep the product concentration below the toxicity limit to avoid the product inhibition and effectively produce MCCAs. Thus, the production of caproate and other MCCAs from AW depended mostly on lactate as an electron donor and eventually partially on ethanol coming from lactose conversion within the reactor. However, with a product extraction or without the product inhibition, both ethanol and lactate would be used for the chain elongation. These results can be applied in designing sustainable treatment of AW wastewater or other waste that typically undergo an anaerobic treatment to produce i.e. methane (Valenti et al., 2020), thus, increasing the value of the final product and supply valuable products for the society.

3.3. Feed composition clearly impacted the structural biodiversity and microbial composition of the reactor microbiome

To evaluate the reactor microbiome structure and dynamics, 8 bioreactor samples (7 samples taken directly from the bioreactor and one sample of sorted cells) and the inoculum were sequenced and analysed. The number of sequences per sample were from 47,661 to 91,559 with a median of 75,442. In all of the analysed samples there were 2040 OTUs assigned from high quality sequence reads. 29 OTUs exceeded 1% relative abundance at the family level in at least one bioreactor sample.

The structural biodiversity throughout the process was shown as Shannon diversity index (H') and Gini index (Fig. 3A). Based on the Shannon index, the biodiversity of microbial population was decreasing over time. At the same time the unevenness of the reactor microbiome shown as Gini index was growing along the operating periods (Fig. 3A). When AW with ethanol was dosed (*Period II* and *III*) H' was between 4.9 and 6.7 and Gini index was between 0.93 and 0.97, while in *Period IV* (feed without ethanol and with high lactate content) H' decreased considerably (values between 2.8 and 3.3) and Gini index was higher (0.99). In other studies with reactor microbiomes, where lactate was the electron donor, the H' were higher than in this study i.e. 3.6 (Kucek et al., 2016b), 3.7 (Kucek et al., 2016a), 4.2 (Xu et al., 2018), 4.3 (Duber et al., 2018). The high value of the Gini index in *Period IV* implied high functional organisation of the reactor microbiome, which is likewise to our previous work, where we had a Gini index of 0.98 (Duber et al., 2018) or to other work, where it was 0.94 (Xu et al., 2018). The observed tendency to produce SCCAs and MCCAs has shown strong dependency on the substrates composition in dosed AW. It was also confirmed by the results of β diversity analysis that showed a clear temporal variation between samples, that were grouped into three areas on the PCoA plot: (i) inoculum and samples 7 and 14 taken from the beginning of the process (*Period I*), (ii) samples from *Period II* and *III* when AW with ethanol was loaded (days 35, 56, 78) and lactose-to-ethanol molar ratio was low, and (iii) samples from *Period IV* when feed without ethanol and high lactose and lactate content was loaded (days 107, 107-P6, 125) (Fig. 3B). The differentiation between samples confirmed shaping the reactor microbiome according to the feed composition.

In the start-up phase (*Period I*, samples 7 and 14) the reactor microbiome was very diverse (Fig. 4). When AW with ethanol was introduced (*Period II* and *III*, samples 35–78) the microbiome started to shape. From that time on, members of Streptococcaceae family coming from the feedstock disappeared and the microbiome was dominated by increasing number of Coriobacteriaceae family (from 11% on day 35 to 35% on day 78) and Clostridia class that accounted for 28–31% of all assigned OTUs that were represented mostly by Veillonellaceae, Ruminococcaceae and other families. Although Clostridia were more or less diversified in *Period II* and *III*, the vast majority of the Veillonellaceae family have been identified as *Megasphaera* genus (11–20% of all assigned OTUs). *Megasphaera* sp. (e.g. *M. elsdenii*) is well known to grow on glucose or lactate with volatile fatty acids as end products (Weimer and Moen, 2013). However, *Megasphaera* sp. was scarce or not present in the next operating period, where lactate was the main electron donor suggesting, that it did not contribute to the MCCAs synthesis from lactate and it is in line with other studies (Zhu et al., 2015; Kucek et al., 2016a; Duber et al., 2018). The *Caproiciproducens* ssp., known to be caproate-producing bacteria that involve lactate as electron donor (Contreras-Dávila et al., 2020), displayed relative abundances of 3% (on day 56 and 78). Many other Clostridia species are known to be homoacetogens able to produce acetate from H_2 and CO_2 (Table 2, Eq. (10)) or acetogens, that produce acetylo-CoA (Guo et al., 2015) necessary for RBO reactions in chain elongation. It would explain high acetate and butyrate production during

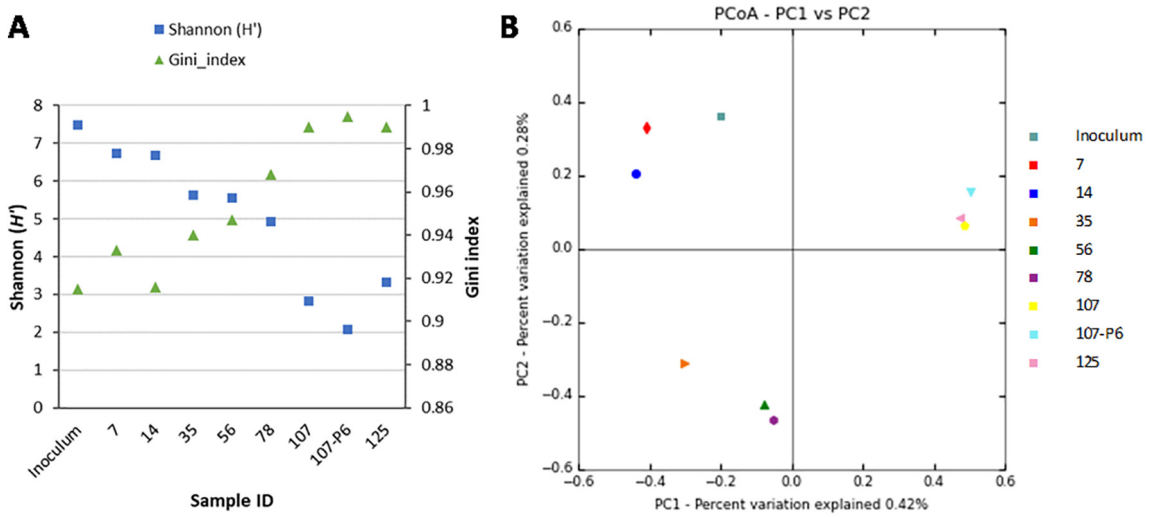

Fig. 3. Analysis of the microbiome: (A) α diversity of inoculum and microbial population along the process shown as Shannon diversity index (H′) and Gini index. (B) β diversity of inoculum and bioreactor samples shown as the PCoA analysis performed based on the weighted UniFrac algorithm. The distances are shown based on the first two principal coordinates (PC).

Periods II and *III*. *Clostridium kluyveri* being the model species producing MCCAs from ethanol and acetate (Weimer and Stevenson, 2012) was not identified in this study, despite both substrates availability. It might have been the result of a low pH (5.5), in which the undissociated forms of MCCAs (mostly caproic acid) caused an inhibitory effect on that microorganism (Candry et al., 2018) and plausibly hampered its growth.

Methanogenic Archaea represented by Methanobacteriaceae family (mostly *Methanobrevibacter*) were present in the bioreactor along the process. However, its highest relative abundance was noticed when ethanol loading rate was the highest in this study (14% and 12% on day 56 and 78, respectively), which corresponded with the highest methane production. Methanobacteriaceae are hydrogenotrophic methanogens, that reduce carbon dioxide with hydrogen to methane (Agler et al., 2014) (Table 2, Eq. (2)). Therefore, they possibly contributed to the overall syntrophic ethanol oxidation.

After ethanol cut off (*Period IV*, samples 107 and 125) the reactor microbiome was clearly diminished in the Veillonellaceae family and enriched in Coriobacteriaceae, Prevotellaceae and Ruminococcaceae families that represented between 49–59%, 20–24% and 9–11% of all families, respectively. Coriobacteriaceae, the most abundant microbial group at that time, were present in all samples of the OCF process,

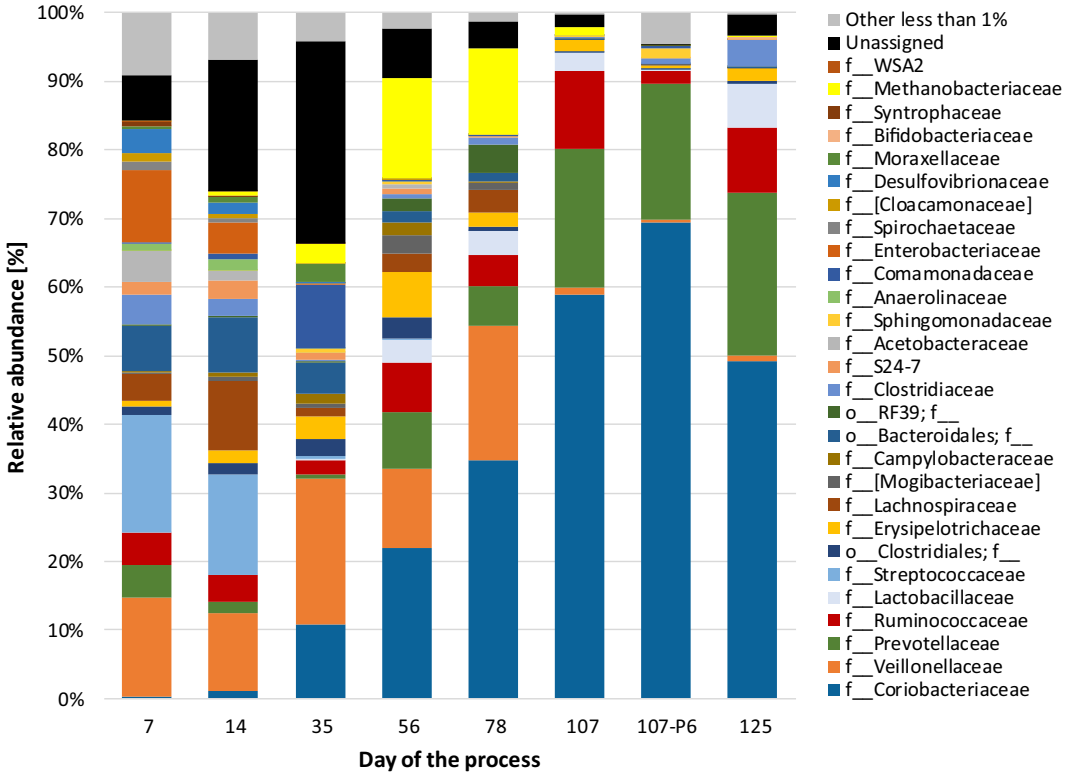

Fig. 4. Microbial composition of bioreactor samples at family level. Only families that exceeded 1% relative abundance in at least one bioreactor sample are presented. f_ refers to a family level and o_ refers to an order level.

suggesting that it was likely to contribute to both, SCCAs and MCCAs synthesis, regardless the concentration of ethanol, lactose and lactate. Some of its species were previously reported to be fermentative bacteria converting lactose to lactate, acetate or formate (Li et al., 2015). Coriobacteriaceae were also observed the most abundant family in caproate production from AW in our previous study (Duber et al., 2018). Therefore, it can be concluded that the Coriobacteriaceae members may contribute greatly but indirectly to the chain elongation process. Other bacterial group belonged to Prevotellaceae family that was likely involved in the conversion of lactose as they have been reported to have the β-galactosidase activity (Morotomi et al., 2009). Accordingly, it may play an important role in the use of lactose and lactate for MCCAs generation. The *Caproiciproducens* spp., reached a relative OTU abundance of 7 and 5% (on day 107 and 125, respectively). The Ruminococcaceae family have been found in several other studies to be involved in butyrate (Agler et al., 2012b) and/or caproate production from ethanol (Kenealy et al., 1995; Kucek et al., 2016c) or lactate (Kucek et al., 2016a, 2016b; Zhu et al., 2017; Contreras-Dávila et al., 2020). Nevertheless, the 16S rRNA analysis let to determine the relative microbial composition of a sample, but cannot assign the function to the identified species, which is a major limitation of the method (Cabezas et al., 2015).

In UASB reactors the formation of granular biomass is expected at some point of the operation. However, the sludge morphology was not changing much during the process and it remained in a form of suspended flocks over the operating periods. The main reason that could explain the lack of granules was a low upflow velocity within the reactor (1.24 m/h), as high recirculation flow rate is a requirement to achieve the microbial granulation (Carvajal-Arroyo et al., 2019). For comparison, in other work where granules were formed, the upflow velocity was fixed at 14.1 m/h (Carvajal-Arroyo et al., 2019). Even though the biomass was not aggregating into granules, the fermentative activity of present microbial species may demonstrate different values. Flow cytometry analysis combined with cell sorting and subsequent Illumina sequencing was performed to investigate, which microbial groups were the most metabolically active, when the carboxylic acid chain elongation to MCCAs was observed (*Period IV*, sample day 107). The analysis distinguished subpopulations of non-active (5.6%), mid-active (7.4%) and active microbial cells (64.7%) (Fig. S3). The remained 22.3% were designated as possible cell doublets, agglomerates or other cellular particles. The metabolically active subpopulation (107-P6) differed slightly from the total population of day 107 in terms of the microbial composition (Fig. 4). The vast majority of active cells were assigned to Coriobacteriaceae (70%) and Prevotellaceae (20%) families. The other represented groups were various Clostridia (5%), Alphaproteobacteria (3%) and other cells. Interestingly, the fraction of Ruminococcaceae family was much lower here than in the non-sorted sample from the same day (1.8 and 11.2% in sample 107-P6 and 107, respectively). In other study authors suggested Ruminococcaceae to be the main MCCAs producers, as it was the main family found in the chain elongating granules, while the Coriobacteriaceae family was present rather in a suspended biomass (Carvajal-Arroyo et al., 2019). Thus, our results suggested that Coriobacteriaceae and Prevotellaceae families must have played a crucial role in providing the intermediates from lactose for chain elongation process. *Caproiciproducens* were assigned to as few as 0.7% of all OTUs, hence the indication of main MCCAs producers remained unclear. For that reason more studies with mentioned microbial groups are needed to confirm the hypothesis.

4. Conclusions

In this study either fresh AW or AW at controlled stage of prefermentation (with controlled content of electron donors) was used as a feed to investigate the chain elongation and MCCAs production performance. The feed composition strongly influenced the chain elongation process. When AW with both electron donors (lactate and ethanol) was pumped to the reactor (*Period II* and *III*) the process was directed to ethanol oxidation and hydrogenotrophic methane formation, while the chain elongation proceeded only to a SCCAs production (regardless the ethanol content in the feed). A change of the feedstock into lactate-rich AW (higher lactate content and ethanol cut off, therefore high lactose-to-ethanol C molar ratios) resulted in a sharp drop in the SCCAs formation and shifted the reaction outcome towards MCCAs generation, with caproate as the main product. Taking into account that higher MCCAs performances were generated without ethanol dosage, we concluded that in one step process, it was essential to keep low ethanol content and abundant lactate and/or lactose to produce caproic acid. Nevertheless, to avoid the product inhibition and keep high MCCAs yield, the product extraction is needed. The study confirmed that for a sustainable AW wastewater recycling and carbon recovery, any prefermentation should be directed towards lactate whereas ethanol formation should be avoided.

Data availability

Sequences were submitted to EMBL-EBI with accession number ERP114146 and data analysed by MGnify (Mitchell et al., 2018) is available under accession number MGYS00003939.

CRediT authorship contribution statement

Anna Duber: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Roman Zagrodnik:** Conceptualization, Investigation, Formal analysis, Writing - review & editing. **Joanna Chwialkowska:** Formal analysis. **Wojciech Juzwa:** Formal analysis, Writing - review & editing. **Piotr Oleskowicz-Popiel:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138814.

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Supplementary material

Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation

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Equations

Substrates loading rate [mmol C/L/day]:

$$\frac{(Cs \times Ni) \times Q}{V} \qquad \textbf{(Eq. S1)}$$

Where:

Cs = concentration of a specific substrate in the influent, mM

Ni = number of carbons in a specific substrate

Q = effluent flow rate, L/day

V = volume of reactor, L

Carboxylates production rates [mmol C/L/day]:

$$\frac{(Cp \times Ni) \times Q}{V} \qquad \textbf{(Eq. S2)}$$

Where:

Cs = concentration of a specific product (carboxylate) in the reactor, mM

Ni = number of carbons in a specific product

Q = effluent flow rate, L/day

V = volume of the reactor, L

Product-to-carboxylates specificity [% mol C]

$$\frac{\gamma_p}{\sum_{i=1}^{n} \gamma_i} \times 100\% \qquad \textbf{(Eq. S3)}$$

γ_p = production rate of a specific product, mmol C/L/day

γ_i = production rate of all detected carboxylic acids (without lactic acid), mmol C/L/day

Substrate-into-product conversion efficiency (% mol C):

$$\frac{\gamma_p}{\sum_{i=1}^{n} C_i \times Q \times N_i / V} \times 100\% \qquad \textbf{(Eq. S4)}$$

Where:

γ_p = production rate of a specific product, mmol C/L/day

C_i = concentration of a specific substrate, mM

Q = effluent flow rate, L/day

N_i = number of carbons in a specific substrate

V = volume of the reactor, L

Tables

Table S1. The characteristics of seed sludge and the feedstocks after delivery.

Parameter	Seed sludge	Acid whey I (AW_I)	Acid whey II (AW_II)
pH	7.9 ± 0.3	4.5 ± 0.2	4.6 ± 0.2
Total solids (TS) [g/L]	34.9 ± 4.5	58.9 ± 0.1	58.2 ± 0.2
Volatile solids (VS) [g/L]	20.6 ± 2.7	51.4 ± 0.1	50.7 ± 0.2
Total chemical oxygen demand (TCOD) [g/L]	42.4 ± 6.4	67.8 ± 1.2	63.5 ± 0.5
Soluble chemical oxygen demand (SCOD) [g/L]	1.45 ± 0.15	53.4 ± 0.3	54.2 ± 0.4
VFA [g/L]	0.12 ± 0.04	0.9 ± 0.2	1.8 ± 0.1
VFA [mM C]	0.01 ± 0.00	35.0 ± 1.2	62.5 ± 2.0
Lactic acid [g/L]	-	8.8 ± 0.1	19.4 ± 0.7
Lactic acid [mM C]	-	294 ± 2	644 ± 22
Lactose [g/L]	-	42.3 ± 0.2	27.0 ± 1.0
Lactose [mM C]	-	1483 ± 7	947 ± 35
Ethanol [g/L]	-	0.0 ± 0.0	0.4 ± 0.0
Ethanol [mM C]	-	0.0 ± 0.0	18.4 ± 1.5

Table S2. The operating and performance parameters for each HRT of each period of the fermentation process. Specificity was calculated as product-to-all carboxylates (without lactic acid) production ratio in % mol C. Conversion efficiency [% mol C] is shown as carbon balance including the loading rates and production rates of measured carbon substrates (ethanol, lactic acid, lactose) and products (SCCA, MCCA, CH_4 and CO_2). Amount of CO_2 and CH_4 in mol C needed for carbon balance were calculated for the condition of 1 atm (101325 Pa) and 21°C (294.15 K).

		Period I		Period II		Period III		Period IV
HRT [d]		20	10	10	5	5	2.5	1.25
Flow rate [L/day]		0.05	0.1	0.1	0.2	0.2	0.4	0.8
Days of the process [day]		0 - 14	15 - 27	28 - 42	43 - 56	57 - 70	71 - 84	85 -129
Ethanol loading rate [g/L/day]		0.0 ± 0.0	0.1 ± 0.1	1.0 ± 0.1	2.4 ± 0.1	4.2 ± 0.1	8.9 ± 0.2	0.3 ± 0.1
Lactic acid loading rate [g/L/day]		0.5 ± 0.0	0.9 ± 0.0	1.2 ± 0.0	2.4 ± 0.0	2.2 ± 0.1	4.5 ± 0.0	15.5 ± 0.5
Lactose loading rate [g/L/day]		2.3 ± 0.0	4.2 ± 0.0	1.1 ± 0.1	2.3 ± 0.0	4.1 ± 0.0	8.1 ± 0.0	21.6 ± 0.8
Acetate loading rate [g/L/day]		0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.8 ± 0.1	1.3 ± 0.1
CO_2	**Production rate [L/day]**	ND	ND	ND	0.1 ± 0.0	0.3 ± 0.2	0.2 ± 0.1	2.8 ± 1.0
	Production rate [mmol C/L/day]	ND	ND	ND	4.38 ± 0.0	13.1 ± 9.5	9.9 ± 4.4	117.2 ± 41.9
	Conversion efficiency [% mol C]	ND	ND	ND	1.6	2.7	1.2	8.8
CH_4	**Production rate [L/day]**	ND	ND	ND	1.0 ± 0.0	0.7 ± 0.4	1.8 ± 0.8	0.4 ± 0.2
	Production rate [mmol C/L/day]	ND	ND	ND	42.4 ± 0.0	28.7 ± 15.4	72.4 ± 31.9	17.4 ± 6.1
	Conversion efficiency [% mol C]	ND	ND	ND	15.3	7.9	8.5	1.3
SCCA (C2, C3, C4, C5)	**Production rate [g/L/day]**	0.2 ± 0.1	0.8 ± 0.1	0.6 ± 0.2	2.0 ± 0.6	3.7 ± 0.6	11.2 ± 0.8	7.6 ± 7.0
	Production rate [mmol C/L/day]	9.9 ± 4.9	33.1 ± 2.8	28.1 ± 5.5	83.4 ± 21.2	144.7 ± 22.9	432.2 ± 30.4	324.0 ± 289.3
	SCCA Specificity [% mol C]	88 ± 8	88 ± 2	76 ± 8	76 ± 5	87 ± 1	92 ± 1	52 ± 18
	Conversion efficiency [% mol C]	10.2	17.9	21.1	30.2	35.8	51.0	24.4
Caproic acid (C6)	**Production rate [g/L/day]**	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	4.1 ± 1.2
	Production rate [mmol C/L/day]	1.2 ± 0.8	3.3 ± 0.4	7.3 ± 1.8	21.9 ± 2.4	21.0 ± 1.6	37.7 ± 4.4	212.5 ± 59.4
	n-caproate Specificity [% mol C]	8 ± 5	9 ± 1	21 ± 7	21 ± 4	13 ± 1	8 ± 1	47 ± 18
	Conversion efficiency [% mol C]	1.2	1.8	5.6	7.9	5.2	4.4	16.0
MCCA (C6, C7, C8)	**Production rate [g/L/day]**	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.0	0.7 ± 0.1	4.2 ± 1.2
	Production rate [mmol C/L/day]	1.7 ± 1.1	4.6 ± 0.8	8.2 ± 2.1	24.6 ± 2.9	29.1 ± 1.6	38.3 ± 5.0	216.6 ± 59.8
	MCCA specificity [% mol C]	12 ± 8	12 ± 2	24 ± 8	24 ± 5	13 ± 1	8 ± 1	48 ± 18
	Conversion efficiency [% mol C]	1.8	2.5	6.3	8.9	5.4	4.5	16.3

Table S3. The microbial population biodiversity.

Seqs/ Sample	observed _otus Ave.	observed _otus Err.	observed _species Ave.	observed _species Err.	shannon Ave.	shannon Err.	simpson Ave.	simpson Err.	simpson_ e Ave.	simpson_ e Err.	chao1 Ave.	chao1 Err.	gini_index Ave.	gini_index Err.
10.0	6.856	2.122	6.878	1.925	2.481	0.660	0.762	0.133	0.806	0.106	19.599	12.505	0.862	0.049
7553.0	409.056	201.783	409.056	202.344	4.964	1.768	0.850	0.150	0.040	0.028	576.469	279.006	0.963	0.029
15096.0	512.378	253.161	512.656	249.733	4.994	1.783	0.850	0.150	0.033	0.026	647.775	313.707	0.963	0.028
22639.0	572.256	281.184	571.456	280.715	5.003	1.785	0.850	0.150	0.030	0.026	701.557	337.717	0.963	0.028
30182.0	612.489	301.293	609.967	299.029	5.012	1.789	0.850	0.150	0.029	0.026	737.541	352.550	0.962	0.029
37725.0	642.122	313.879	642.578	314.131	5.013	1.792	0.850	0.151	0.028	0.025	764.907	362.950	0.962	0.029
45268.0	665.189	324.806	664.989	324.591	5.015	1.791	0.850	0.150	0.027	0.025	785.782	372.214	0.962	0.029
52811.0	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
60354.0	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
67897.0	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan
75440.0	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan	nan

Figures.

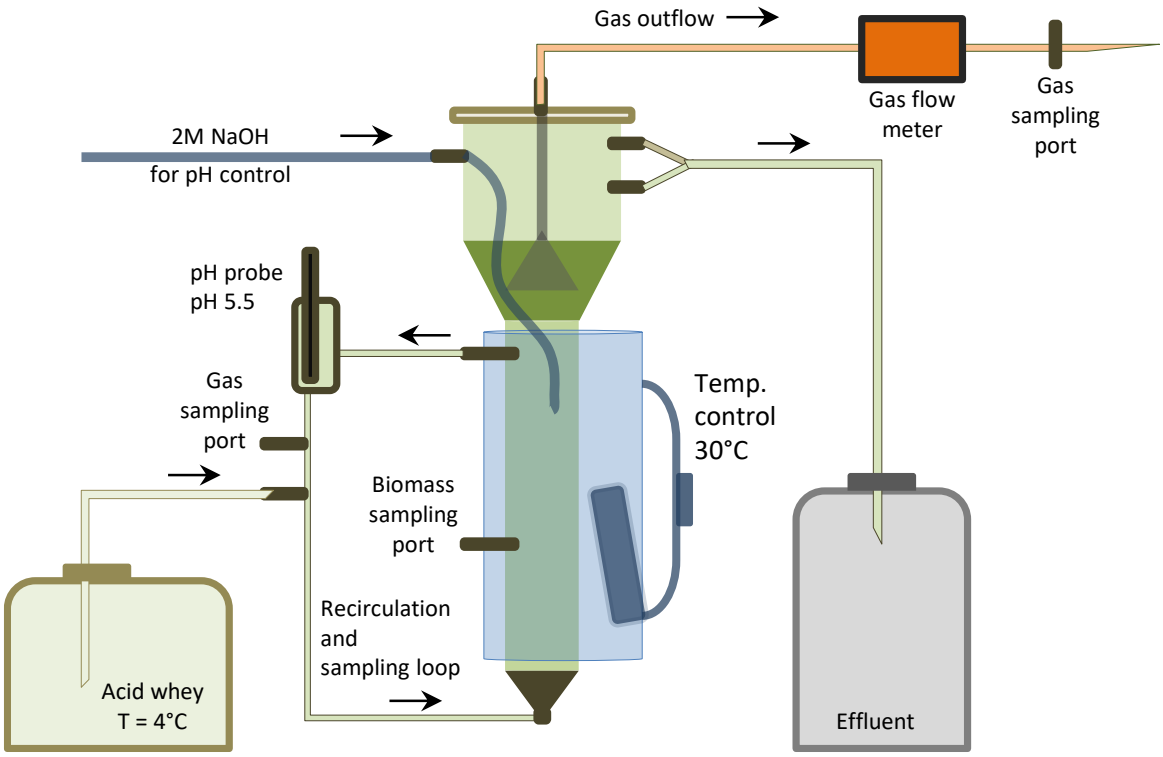

Fig. S1. Scheme of the reactor configuration, adapted from (Duber et al., 2018).

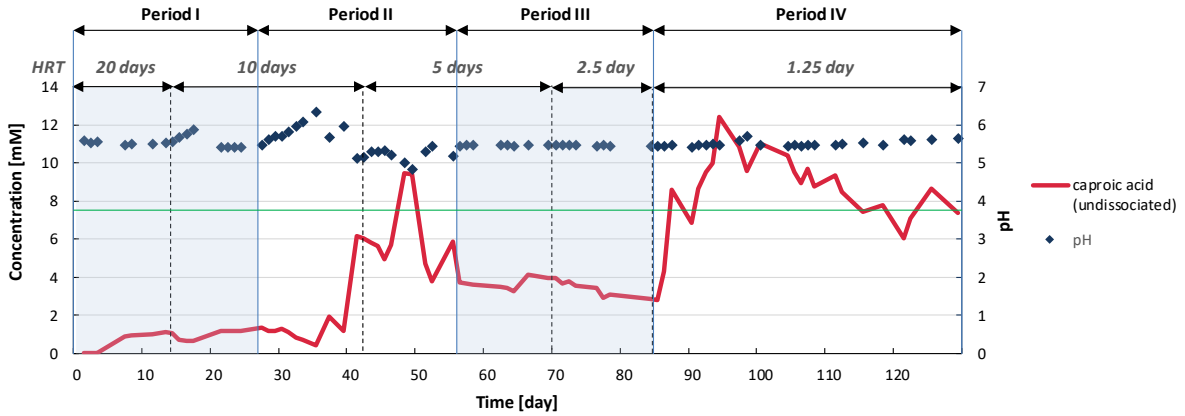

Fig. S2. Concentration of undissociated form of caproic acid [mM] and pH along the fermentation process. Horizontal green line indicates the toxic limit (7.5 mM) of the undissociated form of caproic acid found in (Ge et al., 2015).

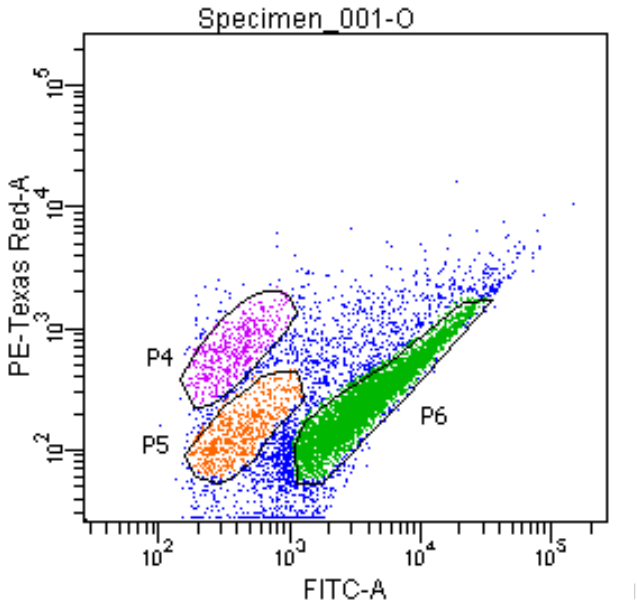

Fig. S3. The flow cytometry measurement of the microbiome from day 107. The histogram shows the distribution of non-active (P4), mid-active (P5) and active (P6) microbial subpopulations measured based on differences in calculated cellular redox potential values (CRP).

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Paper III

Duber, A., Zagrodnik, R., Gutowska N., Łężyk M., Oleskowicz-Popiel, P. 2022. *Lactate and Ethanol Chain Elongation in the Presence of Lactose: Insight into Product Selectivity and Microbiome Composition*. ACS Sustain. Chem. Eng. 10, 3407−3416

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Research Article

Lactate and Ethanol Chain Elongation in the Presence of Lactose: Insight into Product Selectivity and Microbiome Composition

Anna Duber,* Roman Zagrodnik, Natalia Gutowska, Mateusz Łężyk, and Piotr Oleskowicz-Popiel*

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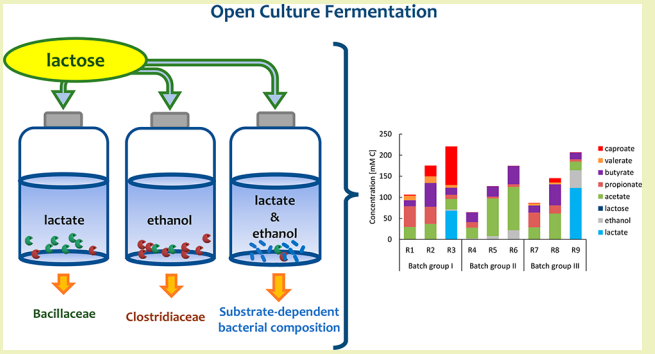
ABSTRACT: Organic waste streams rich in carbohydrates are costly to treat; however, they can be valorized to commodity chemicals such as medium-chain carboxylic acids (MCCAs), for example, caproic acid. Simple carbohydrates are easily fermentable, providing different intermediates for competing bacterial groups, which may lead to product diversification. For that reason, it is essential to understand the impact of sugar fermentation on chain elongation (CE) in the presence of key electron donors to control the biochemical pathways for MCCAs production. The research provides an insight into the impact of co-fermentation of lactose with lactate and ethanol as electron donors on CE and process selectivity in open culture fermentation. Co-fermentation of lactose with ethanol led mostly to acetogenesis and development of the *Clostridium* genus, while co-fermentation of lactose with solely lactate or with both electron donors activated the propionate production and diversified the process outcome to mix short- and medium-chain carboxylates. The highest caproate production efficiency and selectivity were achieved (53 and 65%, respectively) when lactose in the presence of lactate were used; CE improved with a higher initial lactose load. Interestingly, the microbiome was highly enriched in members of the Bacillaceae family, which was not reported before.



KEYWORDS: *chain elongation, caproic acid, microbiome, open culture fermentation*

INTRODUCTION

Organic wastes are alternative and renewable resources that could reduce our dependency on fossil resources for energy and chemicals' production. Thus, organic waste valorization is an appealing approach within a biobased economy and a sustainable development where waste can become a resource. However, designing microbial ecology-based bioprocesses for biological conversion of a real organic waste stream is still hindered by operation challenges, mostly by pH[1] and feedstock complexity.[2]

Whey wastewaters coming from cheese and Greek yoghurt production stand as a problematic worldwide issue for wastewater treatment plants in terms of their utilization due to a high biological oxygen demand (BOD), as described broadly in other studies.[3,4] However, when such a waste stream is considered a resource for producing chemicals, a high BOD is beneficial as it reflects the high content of fermentable carbon in the feedstock. In this case, it is caused mostly by abundant lactose content, a disaccharide easily fermentable to other molecules, such as lactate and ethanol, which can be used as precursors for biochemical production. However, batch-varied composition of whey feedstock, specifically carbohydrate (lactose) content, may affect significantly the biochemical pathways, diversify the product spectrum, and cause variable product selectivity.

Sustainable and rational upgrading of organic waste to commodity chemicals is possible through novel biotechnological processes run by microorganisms, such as open culture fermentation (OCF). OCF has its roots in anaerobic digestion (AD). The difference lies in the last part of the process: short-chain carboxylic acids accumulated during acido- and acetogenesis are elongated in the chain elongation (CE) process to more economically attractive medium chain carboxylic acids (MCCAs) instead of being oxidized by syntrophic proton-reducing bacteria during methanogenesis.[5] The produced MCCAs are straight-chain carboxylates that contain 6 to 8 C atoms in the molecule, which can be used in various industrial branches or serve as precursors for commodity chemicals or biofuel production. For instance, caproic acid, which gained much attention in the last years, is proposed for animal

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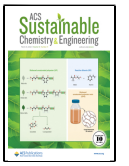

nutrition as a feed additive, an antimicrobial agent, a corrosion inhibitor, feedstock for flavors and fragrances, plasticizers, and in fuel production.[6,7]

The CE process requires an electron donor (ED) (e.g. lactate, ethanol, and sugars) that can be converted to an acetyl-CoA molecule, which is directly coupled to the carboxylate via reverse β-oxidation (RBO).[5,8] Thus, in CE, the carboxylate is elongated by cyclically added two carbon atoms (C2 → C4 → C6 → C8 or C3 → C5 → C7 for even- or odd-numbered carbon chain, respectively). Ethanol or lactate CE have been broadly studied separately, in co-fermentation on synthetic media and as a real waste component, in various configurations with other chemicals needed for CE, such as acetate or butyrate.[4,9−12] While the ethanol and lactate CE seem well described, little is known about the effect of co-fermentation of sugars in the presence of EDs on metabolic pathways. In the work of Xu et al., the authors proposed a temperature-phased process where lactose-rich wastewater was first fermented in one bioreactor to lactate, and then the effluent was directed to an MCCA-producing bioreactor, which simplified the control of the CE process.[13] In the one-pot bioprocess, where both fermentation steps occur simultaneously, as described for instance in the work of Duber et al.,[14] there might be a dispersion of the carbon flux to different products if other carbon sources occur simultaneously in the feedstock, which in consequence may lead to a low selectivity of a targeted product and entail difficulties in the process control. In our other work of acid whey fermentation (where lactose, lactate, and ethanol occurred simultaneously), the impact of the feed composition and specifically additional ethanol content on CE was investigated; however, the impact of lactose fermentation within the process was not fully explained.[4] In this regard, the question is if the lactose content in the presence of other EDs affects the direction of fermentative routes and product selectivity in OCF processes.

In this study, a set of OCF experiments was conducted to investigate the influence of various concentrations of lactose in the presence of EDs (ethanol, lactate, or both at an equal molar concentration) on the CE performance with a particular eye put on the selectivity of the formed carboxylates and conversion efficiency. Furthermore, the structure of the inoculum and the enriched microbial community of representative samples were compared and characterized to explore the community variation and enrichment of functional microbial groups.

MATERIALS AND METHODS

Fermentation Processes. *Inoculum and Media.* The inoculum was derived from a long-operated acidogenic reactor fed with acid whey, working as a stand-by in a laboratory, and producing mainly lactate, acetate, propionate, butyrate, and ethanol (15.2, 10.5, 1.2, 4.5, and 2 g/L, respectively) on the day of the inoculum harvesting. Methane production was inhibited by a low pH (5.5) inside the bioreactor, and thus, no additional inhibition of methanogens before inoculation was required. 1 mL aliquots from the reactor were centrifuged and washed with 1× phosphate buffer saline solution (Sigma) to prevent the transfer of nutrients from the reactor and then suspended in 1 mL of synthetic DSM-52 medium and inoculated into the bottles (1.6% v/v). The medium for the fermentations was prepared according to the DSMZ protocol (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH); the composition was reported in our previous study,[15] with the modification of the carbon sources used as presented in Table 1. Electron acceptors (such as acetate) were not added into the medium as they could be produced through lactose fermentation or anaerobic ethanol/lactate oxidation.[4]

Table 1. Experimental Design of Different Substrates' Compositions

		substrates' compositions in mM (mM C)		
batch group	trial	lactose	lactate	ethanol
I	R1	-	40 (120)	-
	R2	4.5 (54)	40 (120)	-
	R3	9.0 (108)	40 (120)	-
II	R4	-	-	40 (80)
	R5	4.5 (54)	-	40 (80)
	R6	9.0 (108)	-	40 (80)
III	R7	-	20 (60)	20 (40)
	R8	4.5 (54)	20 (60)	20 (40)
	R9	9.0 (108)	20 (60)	20 (40)

Batch Experiment Configuration. Batch mode experiment was conducted in biological triplicates in 120 mL serum bottles under strict anaerobic conditions. A total of 27 batch trials and blank controls with different substrates' compositions were prepared and conducted for 22 days (Table 1). The working volume was 60 mL. The medium was dispensed to the serum bottles, which were next sealed with butyl rubber septa and aluminum crimp caps. The headspace of the bottles was flushed with nitrogen gas for 10 min. The bottles were autoclaved for 20 min at 121 °C. All substrates were prepared separately, autoclaved (ethanol, lactic acid) or filter-sterilized (lactose) and added to the bottles prior to inoculation under sterile conditions. The initial pH was set at 7.5 and was not adjusted along the processes.[16] Bottles were incubated in 30 °C. During first 3 days of the process, samples were collected every 12 h, and later, samples were taken every 3 to 5 days.

Analytical Methods. *Gaseous Samples.* The headspace gas composition was analyzed for carbon dioxide, hydrogen, and methane using gas chromatography using a Porapak N column and a thermal conductivity detector (Shimadzu 2014 GC System, Japan). The flow rate of the carrier gas (nitrogen) was set at 15 mL/min. The injector, column, and detector temperatures were 110, 50, and 80 °C, respectively. For every analysis, 0.2 mL of the gaseous sample was collected using a gas-tight syringe (SGE, Analytical Science) and injected manually on the apparatus. All gas volumes were reported at 1 atm and 273.15 K.

Liquid Samples. Liquid samples were collected using a regular sterile syringe with needle. Organic acids (acetate, propionate, *i*-butyrate, butyrate, *i*-valerate, valerate, caproate, heptanoate, and caprylate) and alcohol (ethanol, propanol, *i*-propanol, butanol, and *i*-butanol) concentrations were monitored using a gas chromatography system (Shimadzu 2014 GC System, Japan) equipped with a flame ionization detector (FID) and a high-performance capillary column with a free fatty acid phase (Zebron ZB-FFAP, Phenomenex). Prior to analysis, samples were acidified using phosphoric acid and filtered with syringe filters (with a 0.45 μm pore size). Helium was provided at a flow rate of 5.3 mL/min. The initial oven temperature was 70 °C, maintained for 3 min, raised to 185 °C at 10 °C/min, and finally held at 185 °C for 8 min. The temperature of the FID and the injection port were both 250 °C.[14] Lactate and lactose concentrations were determined using a high-performance liquid chromatography system (HPLC 20AT, Shimadzu, Japan) equipped with a Rezex ROA organic acid H+ (8%) column and refractive index detector. For elution, 5 mM aqueous sulfuric acid was used at the flow rate of 0.6 mL/min at 63 °C.

Microbial Community Analysis. At the end (22nd day) of batch fermentations, biomass samples were collected by centrifugation and stored frozen at −20 °C until processing. Total metagenomic DNA was isolated from the inoculum and batch samples (two out of three biological replicates) using a GeneMATRIX Soil DNA Purification Kit (Eurx, Poland) according to manufacturer's recommendations.

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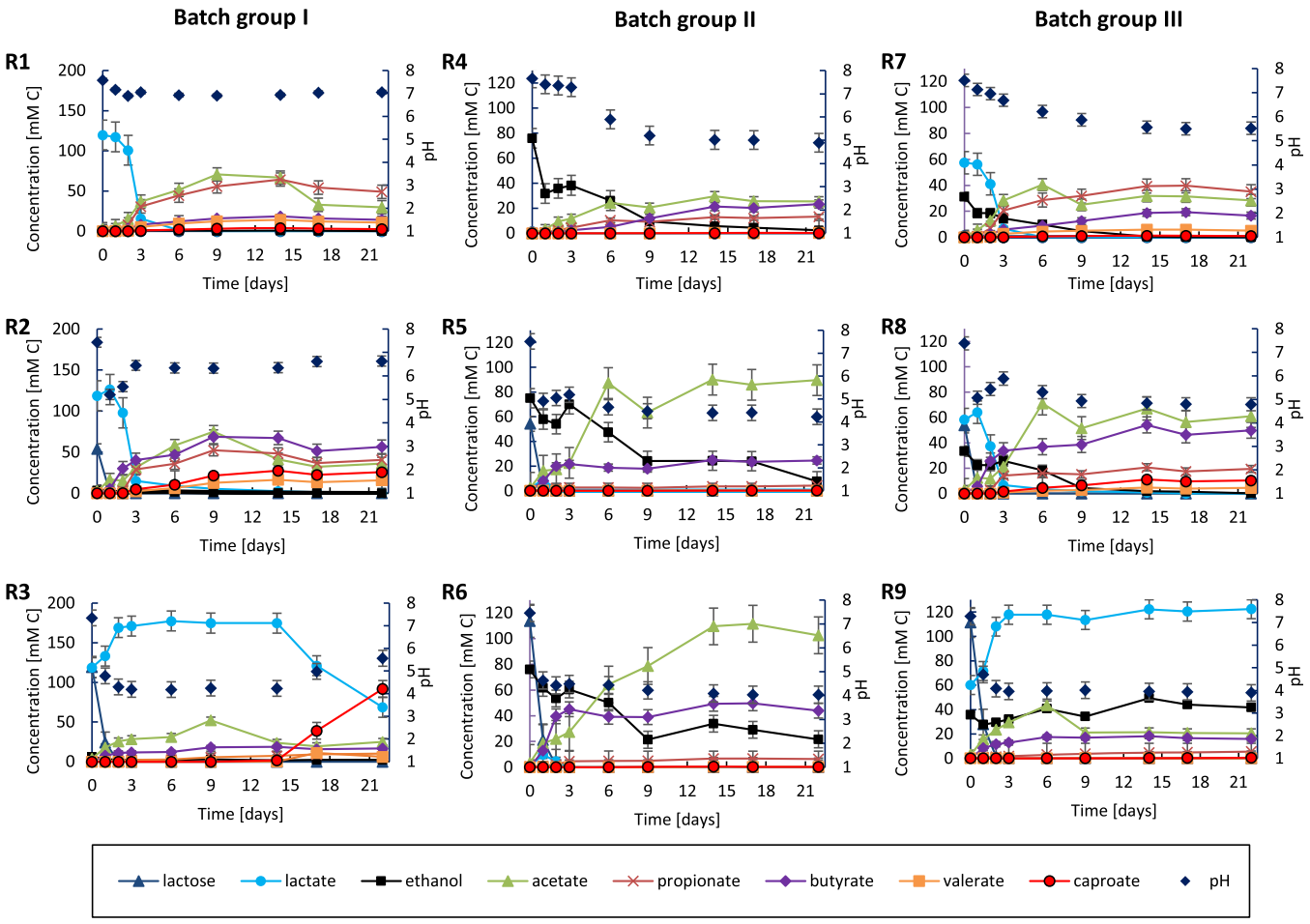

Figure 1. Substrates and carboxylates concentrations as well as pH values in the conducted processes (average of three biological replicates). Metabolites with a concentration below 4.4 mM C are not shown.

The obtained DNA samples were submitted to GENOMED SA (Warsaw, Poland) for libraries' preparation according to the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, Part # 15044223, Rev. B) and sequencing using an Illumina MiSeq instrument (300 bp paired-end sequencing, MiSeq v3). Bioinformatic analysis of the resulting paired reads was carried out using the QIIME 2 software[17] and the Phyloseq R package.[18] The DADA2 package with standard parameters was used to extract unique sequences of biological origin, that is, amplicon sequence variant (ASV), ensuring the classification of the readings at the species level.[19] The taxonomy was assigned based on the reference sequences of the Silva v138 database using a hybrid vsearch/sklearn classifier.[20,21] Raw sequences obtained in this study were submitted to the NCBI-SRA database and are available under BioProject accession number PRJNA808231.

Calculations. Selectivity was calculated as the concentration of electron equivalents (e equiv) in the generated product(s) (acetate, propionate, butyrate, valerate, caproate, H_2, and CO_2) divided by the net consumed electron equivalents in the substrates.[22] The number of electrons for the following compounds are (mole electrons/mol compound): 8/acetate, 14/propionate, 20/butyrate, 26/valerate, 32/caproate, 12/ethanol, 12/lactate, 48/lactose, 2/H_2, and 8/CH_4. Conversion efficiency [%] was calculated as the final concentration of product(s) in mol/L C divided by net consumed mol/L C of substrates (lactose, lactate, and ethanol).

The Pearson correlation coefficient (r) was calculated to determine the correlation between the substrates' load and the final concentration of produced carboxylates at $p < 0.05$ using Statistica 13.3.

RESULTS AND DISCUSSION

Increased Initial Lactose Load in the Presence of Lactate Led to Chain Elongation to Caproate. In lactate CE, the reaction is preceded by lactate oxidation, where lactate is converted to acetate and CO_2 as the side product, and the reaction is favored in mildly acidic pH (5−5.5).[11] However, under slightly higher pH conditions (>6), lactate may undergo a competing fermentative pathway such as the propanediol pathway, the methylmalonyl-CoA pathway, or the acrylate pathway (AP) that may lead to propionate production,[23] for example, AP was specifically described in*Megasphaera elsdenii* where lactyl-CoA, acrylyl-CoA, and then propionyl-CoA are formed as intermediates to finally produce propionate and other odd-chain products.[11,24]

In the batch group I, where lactate was an ED, the process went in two different directions depending on the presence of lactose. In the trials R1 and R2, most of the lactate was depleted after the first 3 days of the process (Figure 1). In the trial without lactose where lactate was the only carbon source (R1), lactate was expected to be converted to caproate as it occurred in the study of Zhu et al., where lactate at an initial concentration of 30 g/L (333 mM) was used and pH maintained between 6 and 6.5.[25] However, in R1, the pH sustained around 7 (Figure 1. R1) and lactate was converted mainly into acetate and propionate (at selectivities of 22 and 47%, respectively, based on % mol e equiv) (Table 2). It is in line with the work of Candry et al. where the authors reported

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Table 2. Product Selectivity and Conversion Efficiency during the Batch Trials

batch group	I			II			III		
trial	R1	R2	R3	R4	R5	R6	R7	R8	R9
Selectivity [% mol e equiv]									
acetate	22	19	11	**20**	**52**	**49**	**27**	**36**	**38**
propionate	**47**	27	6	12	3	3	**39**	14	14
butyrate	15	**40**	11	**23**	**19**	**27**	20	**38**	**43**
valerate	12	12	4	0	0	0	6	4	0
caproate	3	19	**65**	0	0	0	1	**8**	1
H_2	0	0	2	0	0	0	0	0	0
CH_4	0	0	0	0	0	0	0	0	0
Conversion Efficiency [% mol C]									
acetate	22	19	12	**31**	**67**	**57**	**32**	**40**	**40**
propionate	**41**	23	6	16	3	3	**39**	13	13
butyrate	12	**32**	10	**28**	**19**	**25**	19	**34**	**37**
valerate	9	9	4	0	0	0	6	3	0
caproate	2	15	**53**	0	0	0	1	7	1
CO_2	11	0	15	0	0	0	0	0	0
CH_4	0	0	0	0	0	0	0	0	0

that at pH 6.0 and higher, propionate producers outcompeted chain elongators for lactate usage, while pH below 6.0 stimulated lactate chain elongators.[1] Lambrecht et al. observed higher propionate production after the pH increase to 7.0; however, it was still processed to medium odd-chain carboxylates.[26] Similarly, in trial R2 where the pH sustained at around 6.0 along the process, lactate chain elongators were inhibited and lactose fermentation resulted in a mixture of even- and odd-numbered carboxylates (Figure 1) and low MCCAs selectivity (Table 2). In trial (R3) where abundant initial lactose concentration (9 mM) as an additional carbon source was investigated, lactose was totally converted after the first 2 days of the process, producing mostly lactate and noticeable amounts of acetate. Regarding the pH in the trial R3, the short-chain carboxylic acids (SCCAs) abundance led to a drop of the pH below 4.5 already on day 2, which effectively inhibited the propionate producers. Simultaneously, high CO_2 (0.31 L/L_{medium}) and H_2 (0.27 L/L_{medium}) production was recorded (Figure 2). In trials with lactose as an additional carbon source (R2 and R3), hydrogen was formed in much larger amounts compared to the R1 trial because the metabolic pathways of sugar processing are the main source of hydrogen production (Figure 2).[27] In the batch R3, the overproduction of hydrogen from lactose inhibited acetate and propionate fermentation and forced CE to happen. Only after 14 days of the process, a gradual decline in lactate concentration was noted in favor of caproate generation (Figure 1) along with an improved H_2 and CO_2 release, which also suggested micro-

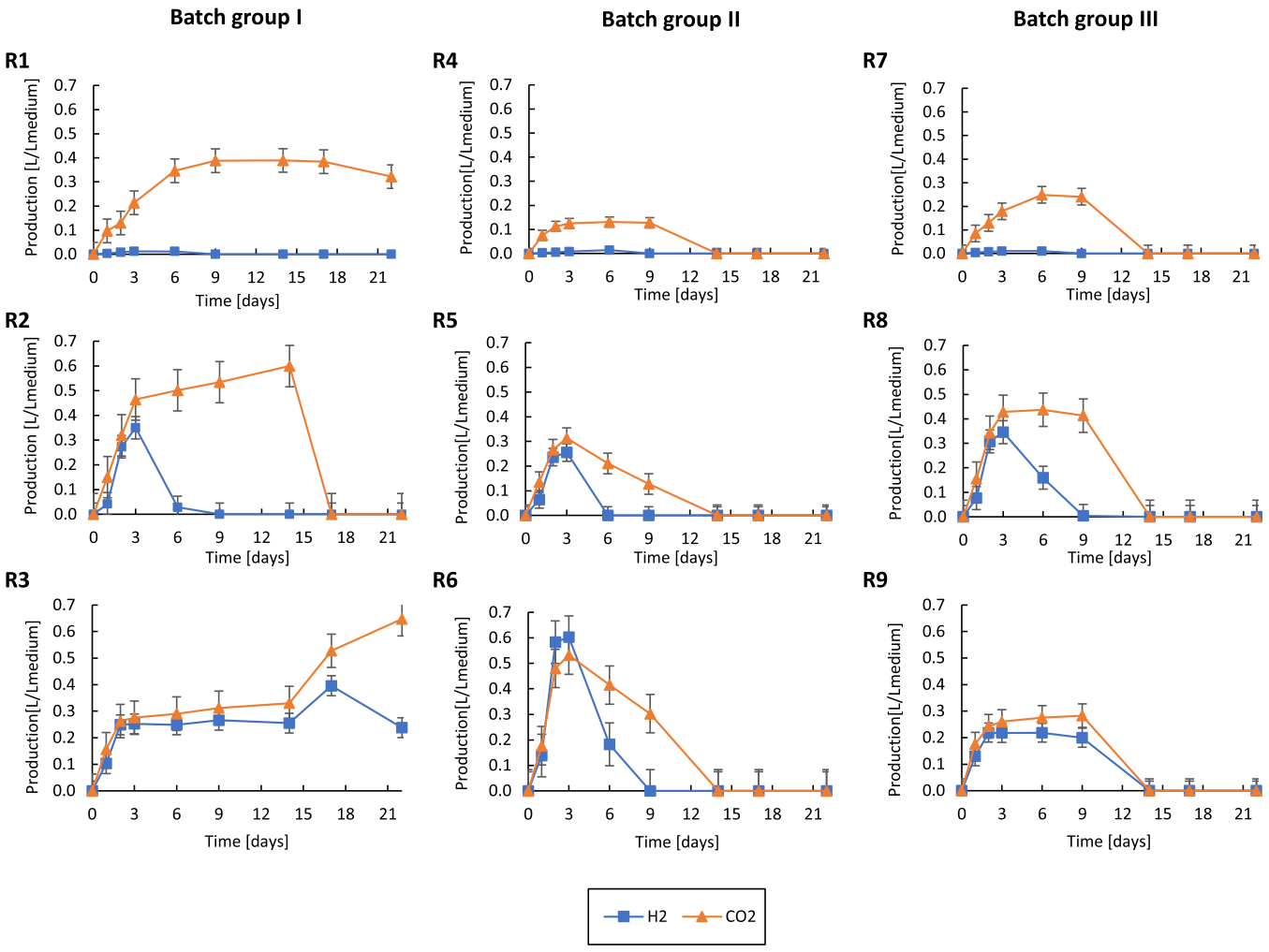

Figure 2. Gas production in the conducted processes (average of three biological replicates).

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biome adjustment to acidic conditions and driving the typical route of lactate oxidation and following lactate-based CE.[8,28] Co-production of even-numbered carboxylates and H_2 was previously described in the work of Brodowski et al., where lactose at the initial concentration of 135 mmol C/L (11.25 mM) together with lactate and acetate at different molar ratios were used as substrates for CE, and the initial pH was 5.5.[29] Moreover, in the work of Wu et al., the authors suggested that the coexistence of H_2 and CO_2 coming from ethanol and lactate conversion contributed to the MCCAs production.[30] In the batch group I, the initial lactose load was positively correlated with a caproate production ($r = 0.95$) and negatively correlated with a propionate and *iso*-valerate production ($r = -0.91$ and $r = -0.94$, respectively). The comparison of final substrates and products' concentrations is shown in Figure 3. It

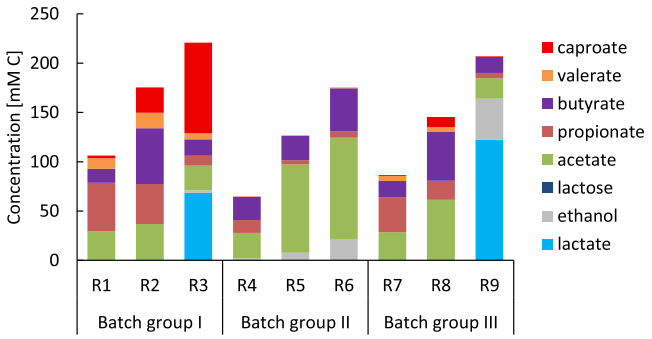

Figure 3. Comparison of the final substrates and products concentration in mM C in each batch group (average of three biological replicates).

is worth noting that in this batch group, the selectivity for butyrate was higher in the batch R2, (15, 40, and 11% for trials R1, R2, and R3, respectively) and further CE to caproate and its selectivities improved with a higher initial lactose load and were 3, 19, and 65% for trials R1, R2, and R3, respectively (Table 2). In the R3 trial, the maximum caproate concentration reached 92 mmol C/L (1.8 g/L) at a conversion efficiency of 53% and a selectivity as high as 65%, and seemingly the process of lactate CE to caproate would proceed if it was not ceased on day 22 (Figure 1).

Presence of Ethanol Directed the Lactose Conversion to Acetate and Butyrate. To date, several studies have been performed to investigate ethanol CE for MCCA production, usually in combination with acetate or CO_2.[10,22,31−35] In the batch group II, lactose with ethanol as the primary ED for CE were investigated (Table 1). In the batch trial where solely ethanol was used (R4), most of ethanol was consumed during the first days of the process (Figure 1) and a mixture of different SCCAs (except lactate) and CO_2 were generated as the end products (Figures 2 and 3). Formation of acetate during the anaerobic ethanol oxidation decreased the pH to 4.8. In R5 and R6 where lactose was used as an additional carbon and energy source, sugar was the first choice for microorganisms rather than alcohol, which delayed ethanol consumption (it was decreasing slowly along the fermentation). Lactose was consumed during the first or the second day (in R5 and R6 trials, respectively), which caused rapid acidification of the environment, mostly with acetate (at selectivities of 52 and 49% and conversion efficiencies of 67 and 57% in the R5 and R6 trials, respectively) and butyrate (Table 2). It is worth noting that a higher lactose concentration led to a higher H_2 and CO_2 production (Figure 2). Fermentation of sugars to acetate and butyrate is characteristic for the processes carried out by different Clostridia,[36] which was further confirmed by the composition of microorganisms in the batch group II. Decrease of the pH to around 4−4.5 hindered ethanol chain elongating communities, and the metabolism was directed only toward anaerobic ethanol oxidation to acetate, and homoacetogenesis of produced gases to acetate (Figure 2) regardless of the initial lactose concentration. CE from ethanol and acetate was described in several other studies; however, the reaction required a high ethanol/acetate substrate ratio.[16,37,38] Recently, Candry et al. reported that caproate production from ethanol and acetate is mostly affected by pH with higher concentrations of caproate obtained at pH 7 rather than 5.5.[39] Thus, in this batch group, the concentration of carboxylates with a longer chain was scarce. Interestingly, in the batch R6, a lactate formation from lactose was not observed, while in corresponding trials with lactate (R3), all added lactose was fermented and lactate-based CE proceeded to caproate despite the low pH. The results indicated that microbes capable for lactate-based CE may work at a lower pH (starting from pH 4.5 as noted in batch R3) than the ethanol-utilizing bacteria.

Lactate Utilization Outcompeted Ethanol Consumption. Trials of batch group III were conducted using two EDs (lactate and ethanol) simultaneously at the same initial millimolar concentration and different initial lactose concentrations (Table 1). In the batch group III, it was observed that the higher the concentration of lactose, the lower the pH, which resulted in lower final propionate and valerate concentrations ($r = -0.99$ and $r = -0.92$, respectively) compared with corresponding trials run with lactate as an ED. In the batch trial with lactate and ethanol without lactose (R7), propionate was the main end product at a process selectivity of 39% and a conversion efficiency of 39% (Table 2) as a result of lactate conversion. Other products were acetate, butyrate, and some valerate. When lactose was an additional carbon source (R8), the share of individual acids changed toward even-numbered carboxylates, where acetate and butyrate were the dominating products (36 and 38% selectivity, respectively), while propionate and valerate formation was much lower (14 and 4% selectivity) (Table 2). Also, in R8, self-maintenance of pH (that was slightly below 5) was favorable to proceed CE toward caproate; however, the process selectivity for caproate was only 8%. What is important is that when both lactate and ethanol were used together as substrates (R7 and R8), lactate was utilized during the first 3−6 days, while ethanol consumption lasted throughout the trial (22 days). In the work of Zhu et al., the authors noted that during fermentation of yellow water where both EDs and sugar occurred at concentrations of 20 g/L of lactate, 10 g/L of ethanol, and 4 g/L of glucose (numbers taken from the graph) at pH 6−6.5 and temp of 30 °C, the ethanol was accumulated as in this study but a high concentration of caproate was noted, which was due to the unique bacterial culture.[25] Given that the inoculum was already acclimatized for acid whey conversion, the microbial community consisted mostly of lactose- and lactate-utilizing bacteria. Therefore, it would explain why ethanol was not directly used, but the concentration of lactate might have been too low to force the reaction toward CE and caproate production. When a higher concentration of lactose was used (R9), all the lactose was fermented during the first 2 days mostly into lactate (Figure 1). Lactate, acetate, and butyrate

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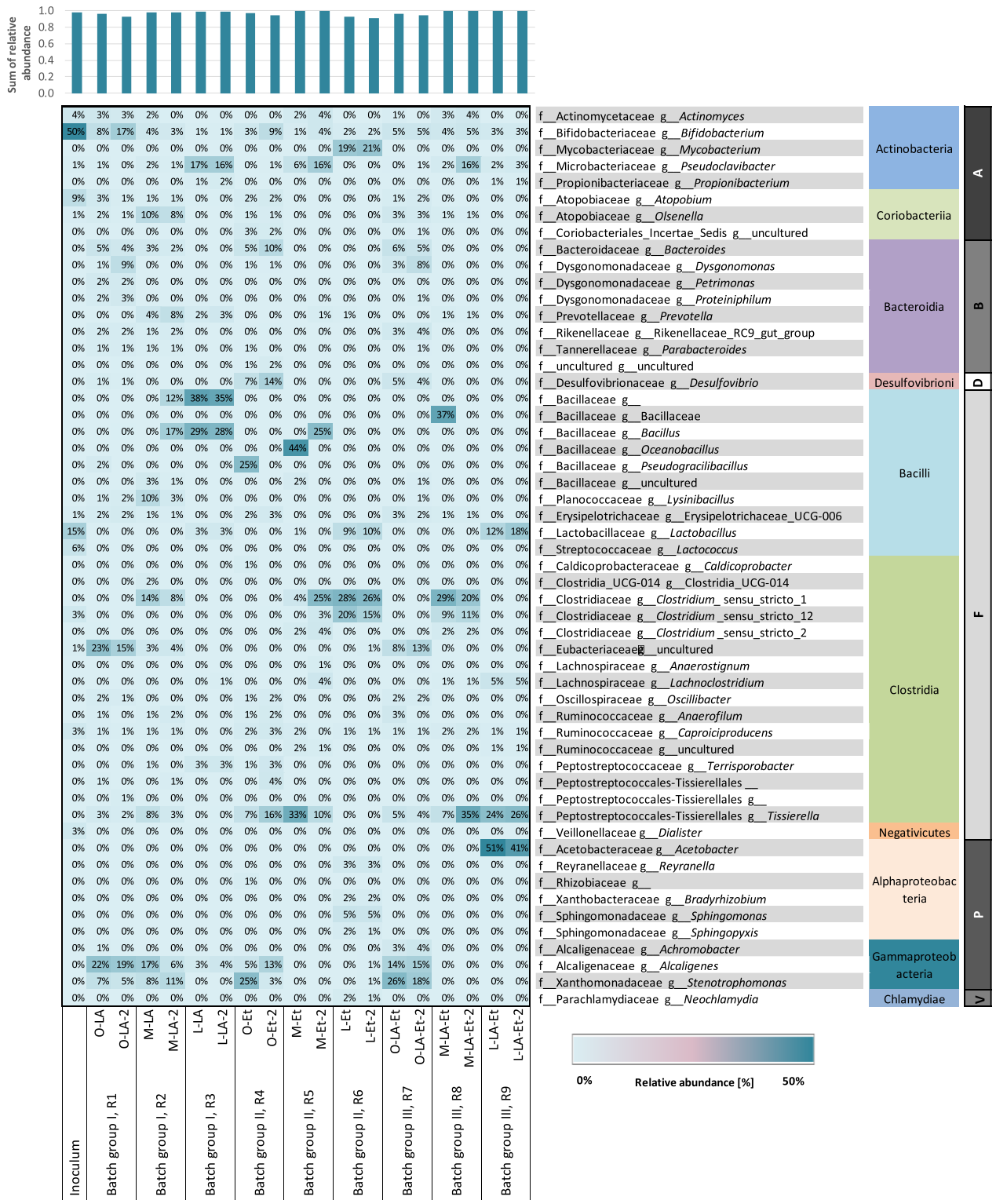

Figure 4. Distribution of microorganisms shown as a heatmap of ASVs with relative abundances >1% in the inoculum or in one or more batch samples. A bar plot of the sum of abundances of displayed assignments is shown above the heatmap. The class (colored boxes) and phylum (bold) of ASVs are shown to the right of the heatmap. Abbreviations: family (f), genus (g); Actinobacteriota (A), Bacteroidota (B), Desulfobacterota (D), Firmicutes (F), Proteobacteria (P), Verrucomicrobiota (V); abbreviations in names of samples: no lactose (O), 4.5 mM of lactose (M), 9 mM of lactose (L), lactic acid (LA), and ethanol (Et).

production caused the pH to drop below 4.0, which inhibited possible lactate CE, and the process did not run to produce medium chain carboxylates, as it happens in the batch group I trial R3. A low pH value also explains no ethanol utilization during the trial, as ethanol CE has been reported to occur in higher pH values.[22] Nevertheless, another explanation of CE to caproate in R3 but not in R9 might arise fromthe difference in the structure of reactor microbiomes and hydrogen partial

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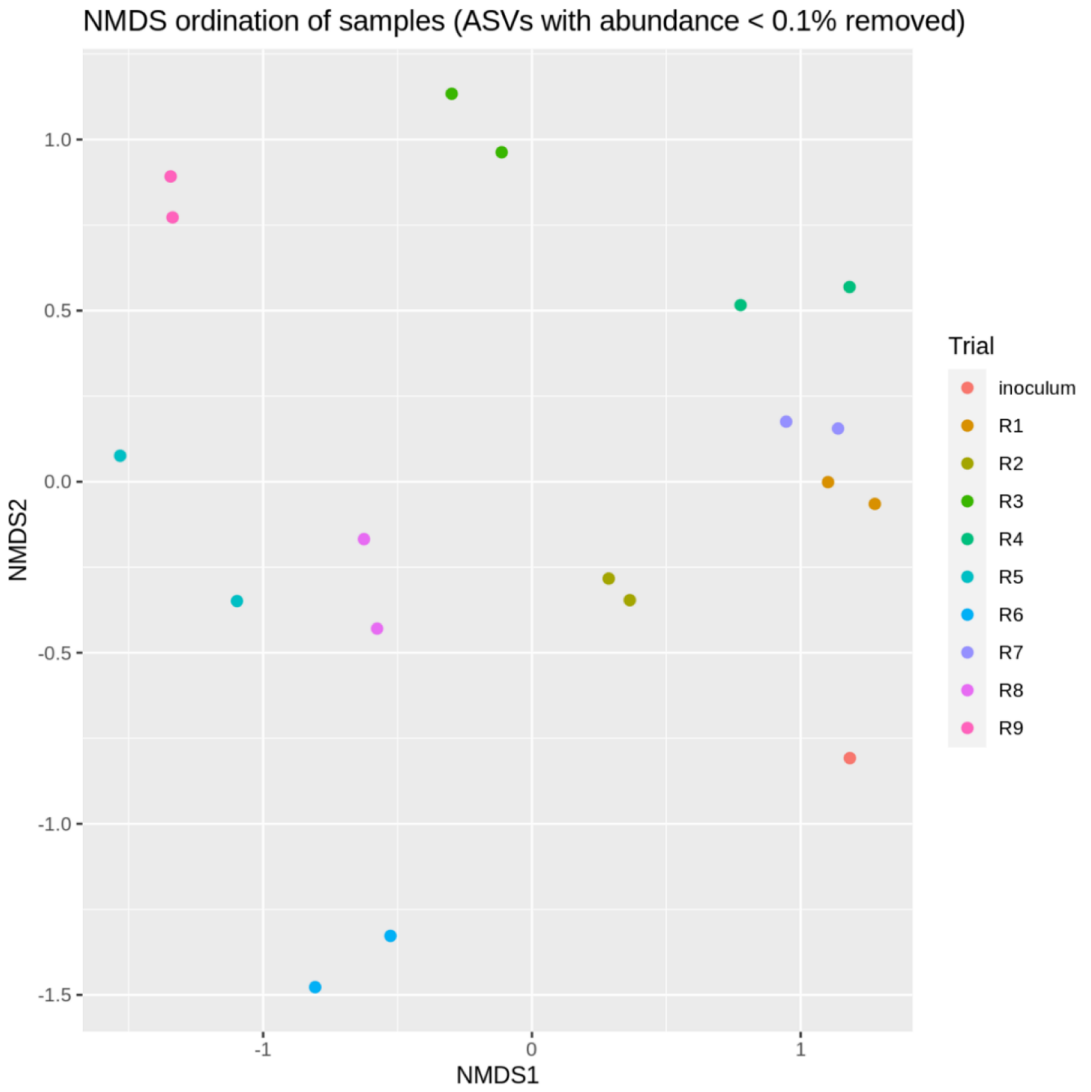

Figure 5. NMDS plot of Bray–Curtis dissimilarity between microbial communities in samples analyzed in this study. ASVs with the total abundance of less than 0.1% removed from the analysis. Stress = 0.1365.

pressure (P_{H_2}) in those trials. González-Cabaleiro et al. reported that MCCAs can be produced from SCCAs by increasing the P_{H_2}.[40] In other works, the authors suggested that the P_{H_2} above 0.03 atm is thermodynamically crucial to avoid excessive ethanol oxidation and anaerobic oxidation of MCCAs but must be lower than 0.1 atm to let ethanol oxidation into acetyl-CoA formation.[6,41] In trial R9, the hydrogen partial pressure was 0.13 atm on day 9 and then it was consumed, while in R3, it was 0.17 atm on the same day and kept on this level in the next days. Thus, there might be a proper range of the P_{H_2} that would guarantee a CE proceeding.[12]

Microbial Community Enriched Depending on the Substrates Used. Inoculum and batch samples (two out of three biological replicates) were subjected to amplicon sequencing of the 16S rRNA gene fragment for microbiological analysis. A total of 1 145 875 reads were obtained (41 801–78 096 reads per sample). Results showed that each microbiome strongly diversified (Figure 4) depending on the substrates used. However, in some duplicates, the structure of microorganisms differed slightly in a way that in one duplicate, some bacterial groups appeared, but they were not present in the other duplicates (R2, R4, R5, and R8). Despite some differences obtained in the microbiological composition of these samples, no significant changes were observed within the other analyzed process parameters, that is, the metabolite concentration within triplicates were comparable (low standard deviation values). When ordination of between-samples Bray–Curtis dissimilarity was plotted using non-metric multidimensional scaling (NMDS) (Figure 5), in all but one case (R5), sample replicates grouped much closer than due to the effect of substrate combinations. In none of the samples were methanogenic Archaea assigned to any ASV, which is in line with the fact that no methane was detected in any of the batch trials.

In the batch group I, where lactate and lactose were used, the Bacilli class dominated the microbiome, and its relative abundance was higher with a higher initial lactose dose. In R3, Bacilli included up to 66–70% of the total 16S rRNA sequences, among which 35–38% was assigned only to the family level (Bacillaceae); another 27–29% was assigned to *Bacillusuncultured bacterium* and the remaining 3% belonged to *Lactobacillus* species. It could be assumed that these bacteria simply ferment lactose; however, they were scarce or none in

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other batch samples. Actinobacteria that constituted up to 20% of total relative abundance in this batch group were represented mostly by*Pseudoclavibacter caeni*, a species able to produce acids from lactose and to assimilate acetate but not lactate.[42] However, it is a strict aerobic species, which indicate some possible oxygen contamination in that batch trial. Other numerous bacterial groups that were rather disappearing with higher lactose dose were Clostridia (mostly*Terrisporobacter glycolicus*, unassigned Lachnospiraceae and Ruminococcaceae), Gammaproteobacteria (*Alcaligenes faecalis*), and Bacteroidia, which constituted between 23−33, 25−30, and 13−21% of total relative abundance of reads in R1, respectively, and they were below 6, 4, and 3%, respectively, when abundant lactose was used (R3). In other studies regarding lactate CE through RBO, the microbial community was related to different families of Clostridia, such as*Caproiciproducens*,[1,4,43] Oscillospiraceae[44] (also described as Ruminococcaceae, i.e., *Ruminococcaceae* bacterium CPB6[45]), or Lachnospiraceae.[26] The presence of Bacilli along with*Clostridium*-like members was previously reported in work with a complex biomass conversion.[46] The only known caproate producer that occurred in this batch group was*Caproiciproducens*, but its total relative abundance was at most 1%, which make it unlikely to be responsible for all caproate production. Up to the authors' knowledge, none of the known species of mentioned bacteria was previously described in literature to be directly related with CE process or caproate production, which opens a new way for screening and isolation of novel caproate producers within these bacterial groups.

In the batch group II, phylogenetic analysis showed that a majority of reads were mapped to Clostridia and Actinobacteria classes that were up to 49 and 23% of total relative abundance of reads in R6, respectively. Gammaproteobacteria vanished in favor of various Alphaproteobacteria when a higher initial lactose dose was applied, and it accounted for 17% of all the microbiome members in R6. Clostridia were represented by species of*Clostridium butyricum* (15−18% of total read abundance) known to utilize lactose from whey to produce butyrate, acetate, and lactate,[47] and*Clostridium autoethanogenum* (13−15% of total read abundance) previously found to be able to utilize CO_2 and H_2 and to produce ethanol and acetate as the end products.[48] Thus, the results explain the presence of acetate, butyrate, and the residue ethanol in the batch group II (Figure 1) as well as the gases' consumption after day 3 (Figure 2). The microbial community of the batch group III were different for R7, R8, and R9 depending on the composition of the substrates used and the pH resulting from their fermentation in the first days of the process. In batch R7 without lactose, the microbiome was very diverse and into different Gammaproteobacteria (38−43%), Clostridia (23%), and Bacteroidia (13−19%). When lactose was an additional carbon source (R8), the microbiome was dominated by Clostridia (up to 72% of all community) represented by*C. autoethanogenum*,*C. butyricum*, and other unassigned Clostridia species. Interestingly, Bacilli occurred in batch groups I and II when lactose was used (viz., R2, R3, R5, and R6), but in each case, the class was represented by different species of the Bacillaceae family. The results suggest that the Bacillaceae family plays a significant role in lactose fermentation. However, in batch R9 where abundant lactose was fed, the Bacilli was represented by *Lactobacillaceae* (12−18% of total read relative abundance, mostly*Lactobacillus harbinensis*) rather than Bacillaceae. Lactobacillaceae are lactic acid bacteria that ferment lactose to lactate and that might have led to a rapid pH drop below 4. Apparently, in developed conditions, no lactate chain elongator was able to grow and produce caproate. Contrarily, the majority of the microbiome was*Acetobacter pasteurianus* (41−51%). The species has been known as a strict aerobic, ethanol-tolerant acetic acid bacteria, able to oxidize ethanol to acetate, which indicates some oxygen contamination and explains the acetate accumulation in the reactors.[49] About 31−33% of total relative abundance constituted for the Clostridia class, most of which belonged to*Tissierella* genus. *Tissierella* was found in AD systems, and the function of this bacteria might be volatile fatty acids, lactate, and CO_2 production.[50,51] Overall, the obtained results of the microbial community composition in R9 explain the rapid process acidification and no CE.

CONCLUSIONS

This work demonstrated that the presence of an additional substrate such as lactose, may divert the carbon flux and impact the selectivity of the final products by providing additional intermediates for the biochemical reactions in the OCF process. It was shown that additional sugar in the system strongly influenced the microbial community structure and triggered different microbial groups to dominate the reactor. The obtained results also confirmed the importance of stable pH during the process. Nevertheless, this work demonstrated which groups of microorganisms may develop depending on the substrates used at the actual pH, and it can be the basis for the appropriate selection of the feed composition and pH conditions for CE in continuous processes. Thus, the results provided that the choice of the metabolic pathway and diverting the process outcome depend not only on the presence of EDs but also on other available compounds, which is especially important in designing waste and wastewater valorization bioprocesses.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c05869.

Carbon balance of batch processes and electron equivalents' balance of batch processes (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting Information

Lactate and ethanol chain elongation in presence of lactose: insight into product selectivity and the microbiome composition

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4 Pages, 2 Tables

Content

Table S1. Carbon balance of batch processes. All values are expressed in [mM C], except for carbon recovery [%]. Final (F) and initial (I) concentrations as well as concentration difference (D) are presented (negative values indicate an overall consumption of the component in the trial).

Trial	R1			R2			R3			R4			R5			R6			R7			R8			R9		
	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D
lactose	1	0	-1	54	0	-54	119	0	-119	1	0	-1	54	0	-54	114	0	-114	1	0	-1	54	0	-54	111	0	-111
lactate	120	0	-120	119	0	-118	119	68	-50	1	0	-1	1	0	-1	1	0	-1	57	0	-57	58	0	-58	60	122	62
ethanol	0	0	0	3	0	-3	6	2	-3	76	2	-74	75	8	-67	76	22	-54	31	0	-31	33	0	-33	36	42	6
propanol	2	0	-2	2	0	-2	2	1	-2	2	2	0	1	1	0	1	2	1	1	0	0	0	0	0	0	0	0
butanol	0	0	0	0	0	0	0	0	0	0	2	2	0	2	2	0	11	11	0	0	0	0	0	0	0	0	0
acetate	3	30	27	3	36	34	4	25	21	0	23	23	2	85	82	3	99	96	0	28	28	3	61	58	3	21	17
propionate	0	49	49	0	41	41	0	10	10	0	12	12	0	4	4	1	6	6	0	35	35	0	19	19	0	5	5
i-butyrate	0	1	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0
butyrate	0	14	14	0	57	57	0	17	17	0	21	21	0	23	23	0	42	42	0	17	17	0	50	50	0	16	16
i-valerate	0	3	3	0	2	2	0	0	0	0	2	2	0	0	0	0	1	1	0	2	2	0	1	1	0	1	1
valerate	0	11	11	0	16	16	0	6	6	0	0	0	0	0	0	0	0	0	0	5	5	0	4	4	0	0	0
i-caproate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
caproate	0	3	3	0	26	26	0	92	92	0	0	0	0	0	0	0	0	0	0	1	1	0	10	10	0	0	0
heptanoate	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
caprylate	1	0	-1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	-1	0	0	0	0	0	0	0	0	0
CO_2[a]	0	13	13	0	0	0	0	27	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total mM C	125	124	-2	181	180	-1	250	249	-1	79	65	-14	134	124	-9	196	184	-12	90	90	0	149	148	0	210	208	-2
Carbon recovery [%]	98.8			98.9			99.7			82.3			92.5			93.9			99.9			99.7			99.0		

[a] - CO2 in the liquid phase was ignored

Table S2. Electron equivalents balance of batch processes. All values are expressed in [mmol/L e^-], except for electron recovery. Initial (I) and final (F) concentrations as well as concentration difference (D) are presented (negative values indicate an overall consumption of the component). All the involved compounds and their electrons are (mol electrons/mol compound): 12/lactate, 48/lactose,12/ethanol, 18/propanol, 24/butanol, 8/acetate, 14/propionate, 20/i-butyrate, 20/butyrate, 26/i-valerate, 26/valerate, 32/i-caproate, 32/caproate, 38/heptanoate, 44/caprylate, 2/H_2 (negative values indicate an overall consumption of the component in the trial).

Trial	R1			R2			R3			R4			R5			R6			R7			R8			R9		
	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D	I	F	D
lactose	4	0	-4	216	0	-216	478	0	-478	4	0	-4	217	0	-217	454	0	-454	4	0	-4	216	0	-216	446	0	-446
lactate	479	0	-479	475	2	-473	475	219	-255	2	0	-2	4	0	-4	3	0	-3	230	0	-230	234	0	-234	240	465	225
ethanol	0	0	0	20	0	-20	36	15	-21	455	12	-443	450	44	-406	456	126	-331	187	0	-187	201	1	-200	215	250	35
propanol	10	0	-10	14	0	-14	14	3	-11	9	11	2	5	8	3	4	12	8	3	1	-2	0	0	0	0	3	3
butanol	0	0	0	0	0	0	0	0	0	0	13	13	0	15	15	0	66	66	0	0	0	0	2	2	0	0	0
acetate	10	118	108	11	145	135	15	100	85	0	92	92	10	339	329	13	396	383	0	114	114	11	244	233	13	82	70
propionate	0	229	229	0	190	190	0	46	46	0	55	55	0	19	19	3	29	26	0	164	164	0	91	91	0	26	26
i-butyrate	0	6	6	0	6	6	0	0	0	0	3	3	0	1	1	0	2	2	0	5	5	0	3	3	0	1	1
butyrate	0	71	71	0	283	283	0	84	84	0	105	105	0	116	116	0	212	212	0	83	83	0	249	249	0	80	80
i-valerate	0	14	14	0	11	11	0	1	1	0	8	8	0	3	3	0	4	4	0	10	10	0	6	6	0	3	3
valerate	0	56	56	0	82	82	0	32	32	0	1	1	0	0	0	0	1	1	0	27	27	0	23	23	0	1	1
i-caproate	0	1	1	0	2	2	0	1	1	0	2	2	0	0	0	0	0	0	0	3	3	0	2	2	0	0	0
caproate	0	14	14	0	137	137	0	490	490	0	1	1	0	1	1	0	1	1	0	6	6	0	55	55	0	2	2
heptanoate	0	1	1	0	2	2	0	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
caprylate	4	0	-4	0	2	2	0	0	0	0	1	1	0	0	0	4	1	-4	0	0	0	0	1	1	0	2	2
H_2	0	0	0	0	0	0	0	16	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total mmol/L e^-	506	511	5	734	861	126	1017	1013	-4	470	305	-166	686	545	-141	938	849	-89	425	413	-12	661	678	17	913	915	2
Electron recovery [%]	101			117			99.6			64.9			79.4			90.5			97.2			102.6			100.2		

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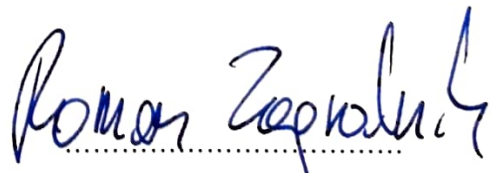

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W pracy/*In the article*:

Duber, A., Zagrodnik, R., Gutowska, N., Łężyk, M., Oleskowicz-Popiel, P., 2022. Lactate and ethanol chain elongation in presence of lactose: Insight into Product Selectivity and Microbiome Composition. ACS Sustain. Chem. Eng. 10, 3407−3416. https://doi.org/10.1021/acssuschemeng.1c05869

Anna Duber: zainicjowała i kierowała planowaniem badań, prowadziła badania, wykonywała analizy chromatograficzne i mikrobiologiczne, interpretowała wyniki i napisała manuskrypt. Roman Zagrodnik: planował badania, znacząco przyczynił się do interpretacji wyników i ulepszenia manuskryptu, Natalia Gutowska: wykonała analizy chromatograficzne, Mateusz Łężyk: wykonał analizę bioinformatyczną, Piotr Oleśkowicz-Popiel sprawował opiekę merytoryczną, wspomagał interpretację wyników oraz poprawił ostateczną wersję artykułu.

***Anna Duber**: initiated and led the planning of the research, conceptualised and conducted the research, performed the chromatographic and microbiological analyses, interpreted the results and wrote the manuscript. Roman Zagrodnik: conceptualised the research, significantly contributed to the interpretation of the results and the improvement of the manuscript. Natalia Gutowska: performed chromatographic analyses. Mateusz Łężyk: performed the bioinformatic analysis. Piotr Oleśkowicz-Popiel supervised the study, supported the interpretation of the results and corrected the final version of the article.*

The percentage contribution is as followed:

Współautor/*Co-author*	
Anna Duber	75%
Roman Zagrodnik	10%
Natalia Gutowska	5%
Mateusz Łężyk	5%
Piotr Oleśkowicz- Popiel	5%

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(podpis współautora, *signature)*

List of scientific achievements

Articles

- Juzwa, W., **Duber, A.**, Myszka, K., Białas, W., Czaczyk, K., 2016. Identification of microbes from the surfaces of food-processing lines based on the flow cytometric evaluation of cellular metabolic activity combined with cell sorting. Biofouling 32, 841–851. https://doi.org/10.1080/08927014.2016.1201657

- Jankowska, E., **Duber, A.**, Chwialkowska, J., Stodolny, M., Oleskowicz-Popiel, P., 2018. Conversion of organic waste into volatile fatty acids – The influence of process operating parameters, Chem. Eng. J. 345, 395–403. https://doi.org/10.1016/j.cej.2018.03.180

- **Duber, A.**, Jaroszynski, L., Zagrodnik, R., Chwialkowska, J., Juzwa, W., Ciesielski, S., Oleskowicz-Popiel, P., 2018. Exploiting the real wastewater potential for resource recovery – n-caproate production from acid whey. Green Chem. 20, 3790–3803. https://doi.org/10.1039/C8GC01759J

- Chwialkowska, J., **Duber, A.**, Zagrodnik, R., Walkiewicz, F., Łężyk, M., Oleskowicz-Popiel, P., 2019. Caproic acid production from acid whey via open culture fermentation – Evaluation of the role of electron donors and downstream processing. Bioresour. Technol. 279, 74–83. https://doi.org/10.1016/j.biortech.2019.01.086

- **Duber, A.**, Zagrodnik, R., Chwialkowska, J., Juzwa, W., Oleskowicz-Popiel, P., 2020. Evaluation of the feed composition for an effective medium chain carboxylic acid production in an open culture fermentation. Sci. Total Environ. 728, 138814. https://doi.org/10.1016/j.scitotenv.2020.138814

- Zagrodnik, R., **Duber, A.**, Łężyk, M., Oleskowicz-Popiel, P., 2020. Enrichment Versus Bioaugmentation—Microbiological Production of Caproate from Mixed Carbon Sources by Mixed Bacterial Culture and Clostridium kluyveri. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.9b07651

- Brodowski, F., **Duber, A.**, Zagrodnik, R., Oleskowicz-Popiel, P., 2020. Co-production of hydrogen and caproate for an effective bioprocessing of waste. Bioresour. Technol. 318, 123895. https://doi.org/10.1016/j.biortech.2020.123895

- Zagrodnik, R., **Duber, A.**, Seifert, K., 2021. Hydrogen production during direct cellulose fermentation by mixed bacterial culture: The relationship between the key process parameters using response surface methodology. J. Clean. Prod. 314, 127971. https://doi.org/10.1016/j.jclepro.2021.127971

- **Duber, A.**, Zagrodnik, R., Gutowska, N., Łężyk, M., Oleskowicz-Popiel, P., 2022. Lactate and ethanol chain elongation in presence of lactose: Insight into Product Selectivity and Microbiome Composition. ACS Sustain. Chem. Eng. 10, 3407−3416. https://doi.org/10.1021/acssuschemeng.1c05869

Scientific activity

- **Researcher** in WasteValue project funded by the Polish National Centre for Research and Development under the POLNOR programme (Norway grants and EEA) Anaerobic biorefinery for resource recovery from waste feedstock, contract no. NOR/POLNOR/WasteValue/0002/2019-00 (PI: Prof. dr hab. inż. Piotr Oleśkowicz-Popiel), 12/2020 – 12/2023

- **Researcher** in the 'CaproBiome' project funded by the Polish National Centre for Research and Development under the LIDER V programme for young scientists, contract no.: LIDER/013/261/L-5/13/NCBR/2014, PI: Prof. dr hab. inż. Piotr Oleśkowicz-Popiel, 01/2015 – 06/2018

- **Principal Investigator** in the project 'Impact of bioaugmentation on medium-chain fatty acids and microbiome formation in mixed culture fermentation processes' financed by the Polish National Science Centre under the Preludium 13 programme for young scientists, contract no.: 2017/25/N/ST8/01795, PI: Anna Duber), 02/2018 – 02/2022

- **Researcher** in SBMK 2020/2021 project 'Badanie wstępne potencjału sekwencjonowania nanoporowego jako przystępnej metody analizy składu wybranych kultur mieszanych', project no.: 504101/0713/SBAD/0934, PI: dr inż. Mateusz Łężyk

- **Principal Investigator** in DSMK 2018 'Biologiczna produkcja kwasów karboksylowych ze strumieni odpadowych', project no.: 01/13/DSMK/0884, PI: mgr inż., Anna Duber

- **Researcher** in DSMK 2017 'Wykorzystanie nowoczesnych metod identyfikacji mikroorganizmów do badań biologicznie aktywnych filtrów węglowych', project no.: 01/13/DSMK/0864, PI: dr Beata Mądrecka

- **Researcher** in DSMK 2016 'Metody usuwania mikrozanieczyszczeń i utylizacja osadów w systemach oczyszczania ścieków', project no.: 01/13/DSMK/0836, PI: dr inż. Wojciech Góra

Traineeships

- Traineeship under the Erasmus+ Programme (09/2016 - 12/2016) within the **ERC Advanced Grant 'Novel anaerobes for the biobased economy'**. The research topic was 'study of anaerobic microorganisms for the valorisation of waste glycerol in the research group BRIDGE – Bioresources, Bioremediation and Biorefinery, Centre of Biological Engineering, University of Minho, Braga, Portugal

- Traineeship on molecular biology methods used in environmental biotechnology (04/2016) Department of Environmental Biotechnology, University of Warmia and Mazury, Olsztyn, Poland

- Traineeship on PCR-DGGE method (04/2015) Environmental Biotechnology Department, Silesian University of Technology, Gliwice, Poland

Oral conference presentations

- **Duber A.**, Zagrodnik R., Oleskowicz-Popiel P.: Acid whey wastewater valorisation to caproate using reactor microbiome. IWA Wastewater, Water and Resource Recovery (WWRR) Conference, 10-13.04.2022, Poznan University of Technology, Poznan, Poland. Presenting author.

- **Duber A.**, Chwialkowska J., Jaroszynski L., Zagrodnik R., Juzwa W., Ciesielski S., Oleskowicz-Popiel P.: Biologiczna produkcja kwasu kapronowego z serwatki w procesie fermentacji przy użyciu kultur mieszanych, II Ogólnopolskie Sympozjum Chemii Bioorganicznej, Organicznej i Biomateriałów BioOrg 2017, 02.12.2017, Poznan University of Technology, Poland. Presenting author.

- **Duber A.**, Chwialkowska J., Zagrodnik R., Jaroszynski L., Ciesielski S., Oleskowicz-Popiel P.: The microbiome structure in mixed culture fermentation for caproic acid production from acid whey. Symposium "Novel Anaerobes 2017", 10th November 2017, University of Minho, Braga, Portugal. Presenting author.

- **Duber A.**, Chwialkowska J.,Jaroszynski L., Juzwa W., Ciesielski S., Oleskowicz-Popiel P.:Biological production of caproate from whey in an up-flow anaerobic sludge blanket reactor**.** 5th IWA Young Water Professionals BeNeLux Conference, 05-07 July 2017, Ghent, Belgium. Presenting author.

- **Duber A.**, Chwialkowska J., Jaroszynski L., Oleskowicz-Popiel P.: Resource recovery from organic-rich waste streams – does it have to be biogas? VII International Conference of Biotechnology Students, 20-22.11.2015, Poznan University of Life Sciences, Poznan. Presenting author.

Poster presentations

- **Duber A.**, Zagrodnik R., Gutowska N., Oleskowicz-Popiel P.: Impact Of The Carbon Substrates Ratio On The Carboxylic Acid Production In Open Culture Fermentation. 25 May - 04 June 2021, IWA Digital World Water Congress, Copenhagen, Denmark, on-line

- **Duber A.**, Zagrodnik R., Gutowska N., Oleskowicz-Popiel P.: Evaluation of a bioaugmentation technique for biochemicals production. 26-28 April 2021, 43rd Symposium on Biomaterials, Fuels and Chemicals, USA, on-line

- Lezyk M., **Duber A**., Brodowski F., Gutowska N., Chwialkowska J., Zagrodnik R., Oleskowicz-Popiel P.: Open culture fermentation for biofuels and biochemicals production – caproic acid production. 21-24 July 2019, SIMB Annual Meeting, Marriott Wardman Park Hotel, Washington, DC, UAS

- **Duber A.**, Zagrodnik R., Chwialkowska J., Oleskowicz-Popiel P.: The role of ethanol in carboxylic fatty acids production from acid whey. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability Biorestech, 16-19 September 2018, Sitges, Spain

- Jankowska E., Chwialkowska J., **Duber A**., Oleskowicz-Popiel P.: Conversion of organic wastes and wastewaters to carboxylic acids. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability Biorestech, 16-19 September 2018, Sitges, Spain

- Łężyk M., Gutowska N., **Duber A**., Jankowska E., Kabasakal T., Oleskowicz Popiel P.: Designing reactor microbiomes for chemical production from organic waste. 2nd International Conference on Bioresource Technology for Bioenergy, Bioproducts & Environmental Sustainability Biorestech, 16-19 September 2018, Sitges, Spain

- **Duber A.**, Chwiałkowska J., Jaroszyński Ł., Juzwa W., Ciesielski S., Oleśkowicz-Popiel P.: Caproic acid production from whey in an up-flow anaerobic sludge blanket reactor. 39th Symposium on Biotechnology for Fuels and Chemicals, 1-4 May 2017, San Francisco, USA

- **Duber A.**, Chwiałkowska J., Jaroszyński Ł., Juzwa W., Oleśkowicz-Popiel P.: Chain elongation by undefined mixed microbial cultures. IWA Specialist Conference Microbial Ecology and Water Engineering: A joint conference of the MEWE and Biofilm specialist groups, 4-7 Sept 2016, Copenhagen, Denmark

- **Duber A**., Chwiałkowska J., Jaroszyński Ł., Oleśkowicz-Popiel P.: Organic acids recovery from sludge and agri-industrial residues. 3rd IWA Specialised International Conference Ecotechnologies for Wastewater Treatment, 27-30 June 2016, Cambridge, UK

- **Duber A.**, Chwiałkowska J., Jaroszyński L., Oleskowicz-Popiel P.: From ethanol to caproic acid – evaluation of process re-integration. Eco-BIO2016 – Challenges in Building a Sustainable Biobased Economy, 6-9 March 2016, Rotterdam, the Netherlands

- **Duber A.**, Chwiałkowska J., Jaroszyński L., Oleskowicz-Popiel P.: Caproic acid production with use of microbiome. 14th World Congress of Anaerobic Digestion, 15-18 Nov. 2015 Vina del Mar, Chile

- **Duber A.**, Chwiałkowska J., Jaroszyński Ł., Oleśkowicz-Popiel P Produkcja kwasu kapronowego za pomocą mikrobiomu. Hydromikro conference, 14-16 Sept. 2015, Silesian University of Technology, Gliwice, Poland

- Jankowska E., Chwiałkowska J., **Janicka A.**, Stodolny M., Oleśkowicz-Popiel P. Impact of external factors on the catabolic pathways in mixed-culture fermentation. Environmental Technology for Impact conference. 29-30 April 2015, Wageningen, Netherlands