Investigating the Stability of a Chain Elongation Synthetic Microbial Community Under pH Disturbances in Bioreactor Systems

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Abstract

This thesis examines the stability of a 5-membered chain-elongation SynCom between LAB and CEB, when exposed to pH disturbances under different cultivation methods. Microbial communities have helped us shift from fossil fuels to an environmentally friendly economy. However, monoculture systems and mixed open microbial communities both have their drawbacks. Synthetic communities try to be the best of both worlds; however, there are still uncertainties regarding the stability of these SynComs. Although it is suspected that the addition of multiple strains with similar functionalities results in functional redundancy within the SynCom, and increases the overall stability of the SynCom.

Three different experiments were performed with each different cultivation methods or pH regimes. Firstly, a community assembly was performed without pH alteration to serve as a validation of the designed chain-elongation SynCom and baseline for further experiments. Next, a batch and a fed-batch bioreactor experiment were performed with pH disturbances to explore the stability of the SynCom under different cultivation methods via MCCA production and relative abundance

Analysis of the batch and fed-batch experiments suggests a strong correlation between the relative abundance of *M. elsdenii* and *M. hexanoica* and the pH disturbance. Introduction of the pH disturbance resulted in a reduction of the relative abundance of *M. elsdenii*, while an increase in the relative abundance of *M. hexanoica* was observed. During this shift, MCCA production did not decrease significantly, suggesting *M. hexanoica* was partly taking over the role of the dominant MCCA producer, suggesting functional redundancy between the CEB. This indicates that functional redundancy might help the stability of a chain-elongation SynCom. This first exploration of the understanding of SynCom stability under stress will provide a foundation for further understanding of SynCom stability, improving on cultivation strategies to enhance yield and efficiency in SynCom bioprocesses.

Dedication & Acknowledgements

I would like to say a special thank you to my supervisor Pieter Candry, and everybody in the Systems & Synthetic Biology group. Your support, guidance and overall insight into this field have helped me throughout the time of research and writing of this thesis. Thank you for making this such an enjoyable and educational experience for me.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's regulations and Code of Practice for Research Degree Programmes, and that it has not been submitted for any other academic awards. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in this dissertation are those of the author.

Signed:

Date: 22-08-2025

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Chapter 1: Introduction

1.1 Synthetic communities

Shifting the chemical industry away from its dependence on fossil feedstocks has been a key objective in transitioning to a more sustainable economy. Adopting a sustainable model could not only reduce the heavy reliance on fossil fuels but also broaden the range of available production methods. A promising approach involves converting bio-waste into bio-based chemicals using microorganisms. This removes the heavy reliance on fossil fuels, but also saves costs and energy in treating the many agricultural biomass waste streams. This provides a viable pathway towards a more sustainable industry. While this solution is already being partially integrated into the chemical supply chain, certain limitations and challenges remain to be addressed (Chen et al., 2017; Guo et al., 2019).

Bio-based chemical production currently primarily occurs through monoculture production systems or open mixed culture systems (naturally occurring microbial communities). Each system has its advantages and disadvantages. Monocultures are generally easier to control and can be genetically engineered to enhance the specific production of cell metabolites and products. However, they lack robustness and resilience against (a)biotic stressors. In contrast, open mixed cultures tend to be more resilient and robust and are capable of metabolising more complex substrates. However, open mixed cultures are more difficult to steer towards specific products due to being inherently complex (Diender et al., 2021).

In recent years, researchers have explored synthetic microbial communities (SynComs) to address the shortcomings of both monocultures and open mixed cultures. SynComs involve the design of a synthetic co-culture aimed at achieving novel functions that cannot be performed by monocultures. In SynComs, microbial species are selected to form a community where cross-feeding and division of labour occur, enhancing overall metabolism and resulting in products of interest. Thus, SynComs can overcome the metabolic limitations of single-cell cultures and genetically engineered microbial cell factories. Furthermore, SynComs allow a greater level of control compared to monocultures, eliminate strains that adversely affect mixed cultures and are also simpler than mixed cultures (Diender et al., 2021; Johnston et al., 2020; Shahab et al., 2020).

Synthetic communities can be designed in two distinct ways: top-down or bottom-up. In a top-down approach, the function of the synthetic community (SynCom) to be designed must first be determined to characterise its structure and dynamics. Comparing this to ‘natural’ microbial communities used in biotechnological fields provides a better understanding of the community's structure and properties, as well as an opportunity to study long-term stability. Conversely, a bottom-up approach uses knowledge about specific interactions and pathways between bacteria to design an overall community structure and dynamics. This approach allows for a better understanding of the growth conditions necessary for specific microbe interactions (Großkopf & Soyer, 2014; Lyu et al., 2024). In this project, a combination of top-down and bottom-up

approaches was used to design a SynCom for further research on the stability of the bacterial community.

1.2 Chain elongation

For this project, a combination of top-down and bottom-up approaches was used to design a SynCom for the production of medium-chain carboxylic acids (MCCAs). MCCAs are saturated fatty acids with a carbon chain of 6 to 12 that have a wide range of applications in the food, medical, agricultural, and chemical sectors. Currently, MCCAs—specifically, the six-carbon MCCA caproic acid—are produced from food crops such as coconut and palm oils. However, coconut and palm oil contain only low concentrations of caproic acid and directly compete with human food supply. Consequently, replacing current MCCA production with SynCom-based processes using bio-waste would contribute to the implementation of a more sustainable economic model (Guo et al., 2019).

Microbes produce MCCAs through chain elongation. In this pathway, chain-elongation bacteria (CEB) anaerobically convert short-chain fatty acids (acetate) and electron donors, such as ethanol and lactate, into MCCAs. The most common pathway for chain elongation is the cyclical reverse β-oxidation (RBO) pathway, which adds an acetyl-CoA molecule (C2) to the carbon backbone of the short fatty acid in each cycle. This process can be repeated several times to produce caproic acid (C6) or a longer saturated fatty acid. Nonetheless, additional biochemical pathways for chain elongation are under investigation (Angenent et al., 2016; Bevilacqua et al., 2021; Candry & Ganigué, 2021). This understanding of chain elongation and MCCA production was utilised as a foundation for a top-down approach to a SynCom design.

To design a SynCom, another bacterium is required that can provide one of the substrates essential for chain elongation. Lactic acid bacteria (LAB) have been linked to CEB to form a SynCom. LAB produce lactic acid, which can be taken up by some CEB in its conjugate base form, lactate (Apostolova & Pearce, 2022). In this manner, the metabolism of LAB can be associated with the metabolism of CEB to yield MCCAs from simple sugars. This was also proven by other research that found that caproate production in mixed settings, like anaerobic wastewater digestion, found both CEB and LAB bacteria present. Especially since LAB are tolerant to high concentrations of caproate, which allows cohabitation with CEB. Additionally, it was found that while LAB show a faster growth rate than CEB, eventually CEB take over the system, reaching biomass concentrations twice as high as LAB (Kang et al., 2021; Ulčar et al., 2024). Employing information from both pathways to devise specific interactions between the two bacterial groups contributes to a bottom-up approach to our final SynCom design.

1.2.1 Chain elongation SynCom

For this study, 2 CEB and 3 LAB previously used in the SSB lab have been proposed to form a chain elongation SynCom. The 2 CEB have slightly different metabolic networks related to chain elongation.

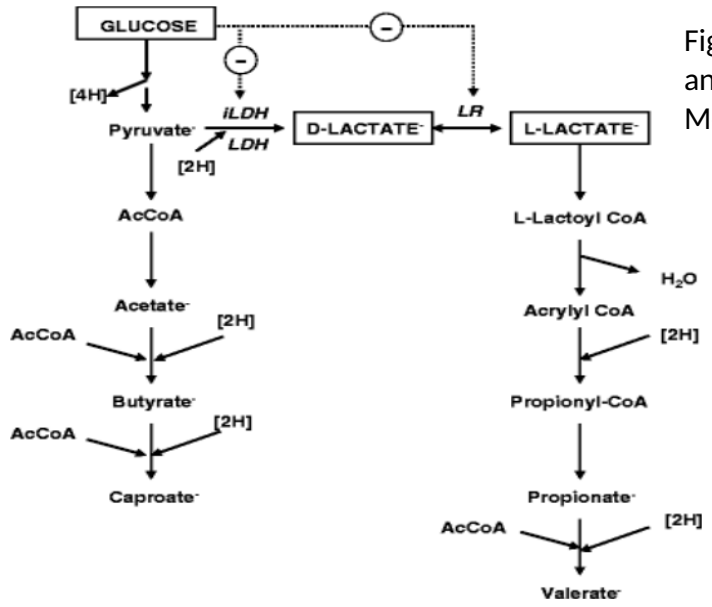


Figure 1: Metabolic pathway of glucose and lactate in M. elsdenii from Weimer & Moen (2012)

Megasphaera elsdenii is a CEB that utilises the RBO pathway to produce caproate, the conjugate base of caproic acid (Figure 1). *M. elsdenii* uses both glucose and lactate as substrates but prefers lactate consumption over glucose consumption. Notably, *M. elsdenii* performs chain elongation when growing on glucose, but performs propionate and valerate fermentation when growing on lactic acid (Weimer & Moen, 2012; Da Silva Cabral & Weimer, 2024). Choi et al. (2013) determined that pH 7.0 is optimal for M. elsdenii in caproate production. Da Silva Cabral and Weimer (2024) determined that *M. elsdenii* was able to grow between a pH of 4.65 and 7.

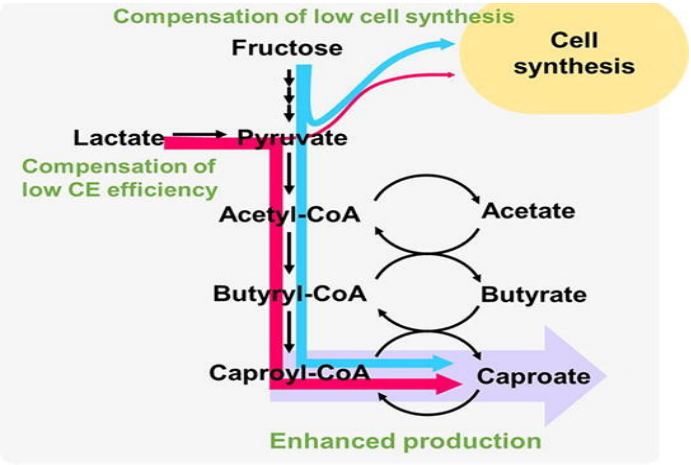


Figure 2: Metabolic flux pathway of M. hexanoica adapted from Kang et al. (2021)

Megasphaera hexanoica is the other CEB proposed and also uses the reverse beta-oxidation pathway to produce caproic acid (Figure 2). The difference between *M. hexanoica* and *M. elsdenii* is that *M. hexanoica* uses a different carbohydrate to produce caproic acid. *M. hexanoica* uses both fructose and lactate as substrates for caproic acid production. However, lactate concentration is more important for the production rate of caproate. Excess lactate resulted in the highest production rate of caproic acid (Kang et al., 2021). No pH optimum has yet been

tested for *M. hexanoica*, but Jeon et al. (2017) have shown *M. hexanoica* to be able to grow between pH 5.5-7.5.

Two out of the 3 LAB proposed for the chain elongation SynCom originate from the *Lactobacilliacea* family. *Lacticaseibacillus casei* and *Lactiplantibacillus pentosus* are facultative anaerobic *Lactobacillaceae*, exhibiting homolytic and heterolytic fermentative processes, respectively. While homolytic fermentation yields two lactate molecules from one glucose molecule, heterolytic fermentation results in one lactate molecule, one ethanol molecule, and one molecule of CO2 (Soundharrajan et al., 2021; Zanoni et al., 1987). Furthermore, prior research conducted in the SSB lab has demonstrated that these LABs can utilise various substrates. *L. casei* is capable of fermenting glucose and fructose, whereas *L. pentosus* can utilise glucose, fructose, and small quantities of xylose as fermentation substrates (Peeters, 2024). The optimum pH values for *L. casei* and *L. pentosus* are pH 6.7 and pH 6.0, respectively (Kondybayev et al., 2022; Tabacof et al., 2023b).

Lastly, *Bifidobacterium animalis subsp. Lactis* will be proposed for inclusion in a chain elongation SynCom. Although *B. animalis* is not classified within the *Lactobacilliacea* family, it possesses similar metabolic functionalities. *B. animalis* is a strictly anaerobic strain that ferments sugars, including glucose, into acetic acid and lactic acid (González-Rodríguez et al., 2013). Previous studies by Bujna et al. (2017) and van Kampen (2024) have shown that SynComs combining *B. animalis* with *Lactobacilliacea* strains facilitate the growth of both. Roy (2019) has indicated that the optimum pH range for *B. animalis* lies between pH 6.5 and pH 7.0.

1.3 Microbial community stability

The stability of a microbial community is defined here as its ability to endure disturbances while preserving its ecological function. This concept can be divided into the community's insensitivity to disturbances (resistance) and its capacity to recover following a disturbance (resilience). These disturbances are regarded as stressors and can significantly affect the growth, composition, and productivity of a microbial community (Philippot et al., 2021; Shade et al., 2012).

While the information on the stability of chain elongation SynComs is sparse, more information is available on the stability of open cultures containing CEB. In mixed open cultures, a lot of members share overlapping metabolic niches. This introduces competition for substrates into the community. In batch bioreactors, this favours the domination of the fastest-growing bacteria due to resource limitations. Continuous models provide more accurate simulations but are dynamically more complex (Liu et al., 2020; Liu et al., 2023).

Another aspect impacting the stability of a microbial community is the type of disturbance affecting the microbial community. Disturbances occur in various forms, depending on the environmental context, which, in this study, is a bioreactor environment. Disturbances can be classified as biotic stressors (such as contamination and competition) or abiotic stressors (such as shear stress, temperature, and pH) (Shade et al., 2012). Control and knowledge over these parameters, especially the abiotic parameters, allow for better optimisation, stability and productivity (Trummer et al., 2006).

The primary types of disturbances are pulse, ramp, and press, each having different temporal characteristics. A pulse represents a sharp, short-term disturbance; a press signifies a sharp but constant disturbance; whereas a ramp is characterised by a gradual increase or decrease over time (Figure 3; Lake, 2000). In contrast to nature, where parameters can change both quickly and slowly, in a bioreactor, the most likely disturbances are due to sharp increases or decreases in parameters. In a bioreactor, parameters are more easily controlled and monitored than in nature, which can help prevent a slow drift in a certain parameter. Therefore, sharp parameter fluctuations and thus pulse and press disturbances are more common in bioreactors.

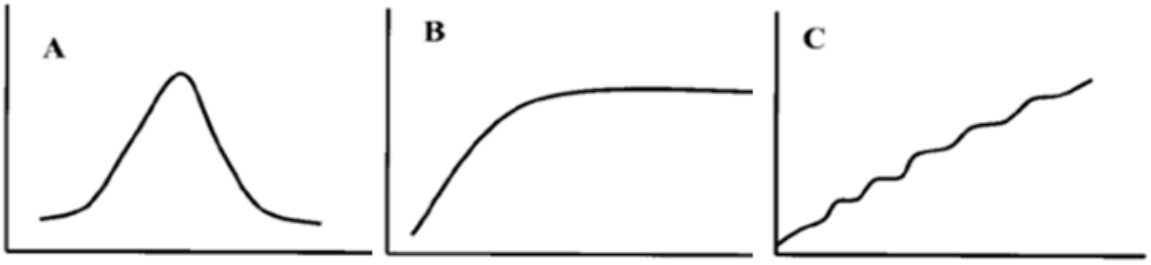


Figure 3: Three types of stability disturbance (A: Pulse, B: Press, C: Ramp) distinguished by temporal trends in the strength (y-axis) and duration (x-axis) of the disturbing force, adapted from Lake (2000)

1.4 Research objective

Given the significance of the stability of SynComs, this study investigates the long-term stability of a chain elongation SynCom when introduced to perturbations in the environment, specifically sharp pH disturbances.

A recent study by Liu et al. (2023) provides insight into the resilience of a natural mixed chain elongation culture when pH alterations are introduced to the system. In this study, long-term observations were conducted on community composition and product formation while multiple stepwise pH disturbances (Δ0.5) were introduced. Liu et al. (2023) noted shifts in community composition following pH disturbances, while product formation recovered to its previous state after initial fluctuations. These results could be explained by changes in dominance in LAB and CEB strains at varying pH levels due to functional redundancy. Different LAB and CEB will be more prevalent if the pH value favours them over their competitors. This aligns with the principle that, although competition between microbes negatively influences the efficiency of the overall microbial community, it also enhances the stability of the community by mitigating destabilising effects (Coyte et al., 2015).

Based on the findings of Liu et al. (2023), it is hypothesised that SynComs will exhibit less stability and resilience to pH disturbances as fewer species are competing with one another, lowering functional redundancy compared to open mixed cultures. This functional redundancy could improve resistance and resilience towards stressors. However, the limited competition may be sufficient for the chain elongation SynCom to recover from the pH disturbance when compared to disturbances in monoculture systems, which often result in complete cell death. chai

While dissolved oxygen and temperature perturbations are bioreactor parameters that can also influence SynCom stability, this study specifically selected sharp pH disturbances as the most interesting focus. Temperature is crucial for the initiation of chain elongation in a mixed community. Nonetheless, temperature has primarily been linked to species competition and preference, and is considered more important for community productivity and composition rather than community stability (Krieger et al., 2021; Ren et al., 2024). Furthermore, the anaerobic nature of the chain elongation process will decrease the importance of dissolved oxygen towards the stability of the SynCom to a certain level. Gemeinhardt et al. (2025) have shown that oxygen supplementation in the form of micro-aeration plays a critical role in caprylate (C8) chain elongation. Similarly, previous research within the SSB lab has shown that some of the bacterial strains used can tolerate small amounts of dissolved oxygen and therefore still impact the system's overall stability (Peeters, 2024). However, due to the uncertainties regarding oxygen controllability by the SSB bioreactors and DO probes, this parameter was not chosen to be the main objective.

To answer the research question, this project has been divided into three main experiments. The first objective is to establish a small-scale community assembly experiment to determine whether the designed SynCom can grow, to assess the composition of the SynCom bacteria, and to evaluate their production of MCCAs. This work will act as a baseline for comparison to later experiments. Additionally, the results of the small-scale community assembly of the SynCom design will be reviewed and, if necessary, adjusted for experiments two and three.

In experiment two, the (adapted) SynCom will be cultivated in a batch bioreactor where pH disturbances of Δ1 will be introduced after the initial growth has settled. This change will also be reversed, allowing the environment to revert to the baseline pH value. An analysis of the production and composition of the SynCom before, during, and after pH disturbances throughout the batch bioreactor growth will provide insights into the behaviour and stability of the chain elongation SynCom when pH disturbances occur. In Experiment 3, the same experimental setup will be repeated, using a fed-batch mode bioreactor to reduce domination by the fastest-growing strain in a pure batch reactor. Together, this work will provide a first exploration of the stability of a 5-member biotechnological SynCom.

Chapter 2: Methods

2.1 Strains and cultivation media

The strains used in this project: *Lacticaseibacillus casei* (DSM 20011), *Lactiplantibacillus pentosus* (DSM 20314), *Bifidobacterium animalis subsp. lactis* (DSM 10140), *Megasphaera hexanoica* (DSM 106893) and *Megasphaera elsdenii* (DSM 20460) originate from DSMZ cell lines. The SSB lab group performed initial cultivation of the original DSMZ strains and stored viable -80°C glycerol stock cultures, which were used for further cultivation.

Before SynCom assembly, individual strains were cultivated at 37°C for at least 24 hours in their respective media, starting from -80°C glycerol stock cultures. The respective media used for each strain can be found in Table 1. This initial cultivation was performed in duplicate to have a higher rate of success.

L. casei and *L. pentosus* were grown on aerobic MRS media, *M. hexanoica* and *M. elsdenii* were grown on anaerobic Peptone-Yeast media (PYG) with glucose (5 g/L) as a carbon source, and *B. animalis* was grown on anaerobic bifidobacterium media with glucose (10 g/L) as a carbon source. While initial cultivation of the LAB in MRS media was done in aerobic conditions, the other bacteria were grown anaerobically. Therefore, cysteine (0.5 g/L) was added to the media of both CEB and *B. animalis* as a reducing agent to maintain the medium's anaerobic condition. Growth experiments of the SynCom were only performed in anaerobic Peptone-Yeast media with glucose (5 g/L) as a carbon source and cysteine (0.5 g/L) as a reducing agent to maintain the anaerobic nature of the SynCom medium. Complete media compositions can be found in Appendix A.

2.2 Experiment 1: SynCom assembly & baselining

First, the SynCom was assembled to test the coexistence of the SynCom members and provide a baseline for further experiments. 0.1 mL of each 5 bacterial strains obtained from the initial cultivation was combined in a 15 mL belch tube and grown in triplicate for 48 hours in 10 mL anaerobic PYG medium. Samples were taken every 24 hours by removing 3 mL of the media. From this sample, 1.5 mL was spun down, where the supernatant was used for HPLC analysis and the pellet was used for genomic DNA extraction. The other 1.5 mL was used for pH analysis and OD600 measurements using an OD600 DiluPhotometer™ (Implen) spectrophotometer. These samples were frozen at -20°C until used. This experiment was performed to validate the cohabitation of the strains, as well as to provide benchmarks of HPLC analysis and relative abundance for later experiments.

2.3 Experiment 2: Batch mode pH disturbance

The (adapted) chain-elongation SynCom was cultivated in batchwise bioreactors (triplicate) for 168 hours. In the first 24 hours, the chain-elongation SynCom was grown at a pH of 6.5. Afterwards, the pH was sharply lowered to pH 5.5 to introduce a disturbance to the community. Finally, after 48 hours, the pH was changed back to the original value of 6.5. Samples were taken at 0 h, 24 h, 48 h and 168 h. Sampling was done by removing 10 mL of media using a 10 mL syringe

and the bioreactor sampling port. Afterwards, the same methods used in Experiment 1 were used for further analysis and storage, except for pH analysis, which was no longer necessary. This experiment was performed to get a basic understanding of the stability and resilience of the designed chain-elongation SynCom under pH disturbances.

However, before Experiment 2 could be performed, bioreactors had to be set up, which was beyond the experimental scope of this project. The bioreactor setup played a significant role and took up significant time. This extra time allocated to setting up the bioreactor will not result in extra data, and no conclusion can be drawn from it. However, due to its significant part in this thesis and its importance for further studies within the SSB group, a short description will be given here.

Due to the long-dormant time of the bioreactors, the equipment had to be checked thoroughly. First of all, tubing of the bioreactors was checked and, if needed, replaced. Additionally, some tubing had to be rerouted as the bioreactors were previously set up for aerobic conditions, while anaerobic conditions were needed for this project. Next, the other equipment and probes were tested. pH and Do probes had to be recalibrated, and the stirrer, buffer pumps and thermal jacket were tested. A test run with demi water was performed to confirm the functionality of the bioreactors. Two sets of DO probes were available in the SSB lab: a membrane-based DO probe and an optical sensor-based DO probe. In Experiment 2, the DO probes with optical sensors were tested, but they resulted in bad readings of the DO. In Experiment 3, the DO probes with membrane-based technology were used and resulting in clear readings of the DO. Therefore, for further experiments within the SSB lab, it is advised to use the DO sensors with membrane-based technology.

2.4 Experiment 3: Fed-batch pH disturbance

The chain-elongation SynCom was cultivated in fed-batch bioreactors (triplicate) for 70 hours. In the first 47 hours, the chain-elongation SynCom was grown at a pH of 6.5. Afterwards, the pH was sharply lowered to pH 5.5 to introduce a disturbance to the community until the end of the bioreactor run. Samples were taken at 0 h, 20 h, 21 h, 48 h, 49 h and 70 h.

To achieve a fed-batch system, old media was discarded and fresh media was added after 20 and 48 hours. This was done by removing 100 mL of old media via the sampling port of the bioreactor using a 60 mL syringe. Afterwards, 100 mL of fresh media was added the same way to the bioreactor via the septum. Sampling was done the same as Experiment 2 at 21 h, 48 h and 70 h. For 20 h and 48 h, the sampling was done the same, but directly from the removed media for feeding instead of the bioreactor port.

This experiment was performed to get a further understanding of the stability of the designed chain-elongation SynCom. Additionally, a fed-batch run was performed to account for the domination of the fastest growing bacteria, as well as limitation by carbon source depletion present in batch mode cultivation.

2.5 Chemical and molecular analyses

2.5.1 HPLC

HPLC was performed by adapting the SSB protocol “HPLC-ICS5000+-Organic acids". A previous student optimised the pre-existing HPLC program for detecting organic acids to detect longer-chain compounds. The run time was extended to 47 minutes to allow for the detection of caproate, which would otherwise remain undetected. An Aminex HPX-87H (BioRad) column was used within a Dionex ICS-5000 system (ThermoFischer) with a 0.6 ml/min flow rate using 0.016 NH_2SO_4 as the eluent. Samples were measured with an RI sensor at a temperature of 35°c. The chromatogram was automatically integrated using Chromeleon 7.3.2 (ThermoFischer), although manual baseline correction for integration was performed when necessary. The area under the curve of each compound was used to measure the concentrations present. The area was then compared to the standard curves of each component, with calibration of concentrations ranging from 0.1 g/L to 5 g/L. Standard curves can be found in Appendix B.

2.5.2 DNA extraction

The GenElute™ Bacterial Genomic DNA Kit was utilised to extract genomic DNA from 1.5 mL pelleted cells following the Sigma-Aldrich protocol (GenElute™ Bacterial Genomic DNA Kit Protocol (NA2100, NA2110, NA2120), n.d.). The final DNA concentration and purity in the eluate obtained from the kit were measured using a Nanodrop spectrophotometer for further experiments.

2.5.3 Nanopore sequencing

Whole genome sequencing was performed using kits and software obtained from Oxford Nanopore Technologies. First, DNA samples were processed using the Native Barcoding Kit 24 V14 (SQK-NBD114.24) to allow for multiplexed sequencing. This was done following the Oxford Nanopore protocol (Ligation Sequencing gDNA - Native Barcoding Kit 24 V14 (SQK-NBD114.24), 2025). The same protocol was followed to perform sequencing, using a Flongle™ flow cell (FLO-FLG114), a Flongle adapter and MinION Mk1B device. MinKNOW (Version 24.11.10) was used to control sequencing and perform basecalling of the sequencing reads, obtaining fastq sequencing data for each specific barcode. Nanopore whole genome sequencing using MinION MK1B software uses a cut-off value Phred Q-Score of >= 9 (i.e., 0.126% chance of false basecall) for a read to count as a passed read. Both passed reads and failed reads were separately exported as fastq files, where only the passed reads were used for further analysis. Furthermore, sequencing data which has not been basecalled was also exported as pod5 data. This data cannot be directly analysed, but was stored anyway if further analysis was proved to be necessary.

The Fastq data generated by MinKNOW Flongle™ sequencing could then be analysed using a metagenomics workflow (wf-metagenomics v2.11.0 & v2.13.0) in EPI2ME (V5.2.5), specific for Oxford Nanopore Technologies sequencing data. This workflow utilises Kraken2 (v2.0.8-beta) to obtain taxonomic classification and relative abundance (Appendix C) from the sequencing data, which is then analysed further by plotting the data in bar charts.

2.5.4 Statistical analysis

Average and standard deviation were calculated from triplicate samples using Excel. Additionally, an unpaired two-tailed Student's t-test was performed between batch, fed-batch, and baseline experiments for 0, 24, and 48 hours to compare the OD600 values. However, due to different exact starting cultures, different pH regimes and slight differences in sampling times, observed differences may reflect multiple factors beyond the cultivation method alone.

Chapter 3: Results

The stability and MCCA-production capacity of a designed synthetic chain-elongation community were evaluated over multiple experiments and analyses. First, baseline experiments without pH disturbance were performed to characterise the SynCom and provide data for further comparison. Next, the SynCom stability and resilience were tested by comparing data from a controlled batch and fed-batch experiment with pH disturbances to the baseline experiment.

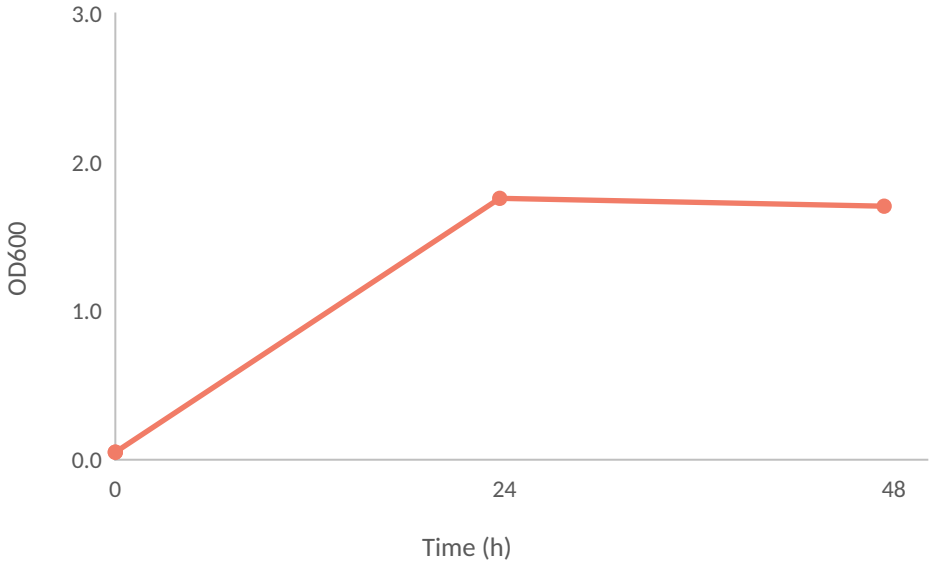
3.1 Experiment 1: Syncom assembly & baselining

In this section, the results of growing the designed chain-elongation SynCom are presented, which will help to assess the performance of the designed SynCom and serve as a comparison for the following experiments.

3.1.1 Growth curve & pH curve

To evaluate the biological activity of the designed chain-elongation SynCom, a growth curve and pH profile were established for the duration of Experiment 1.

A)



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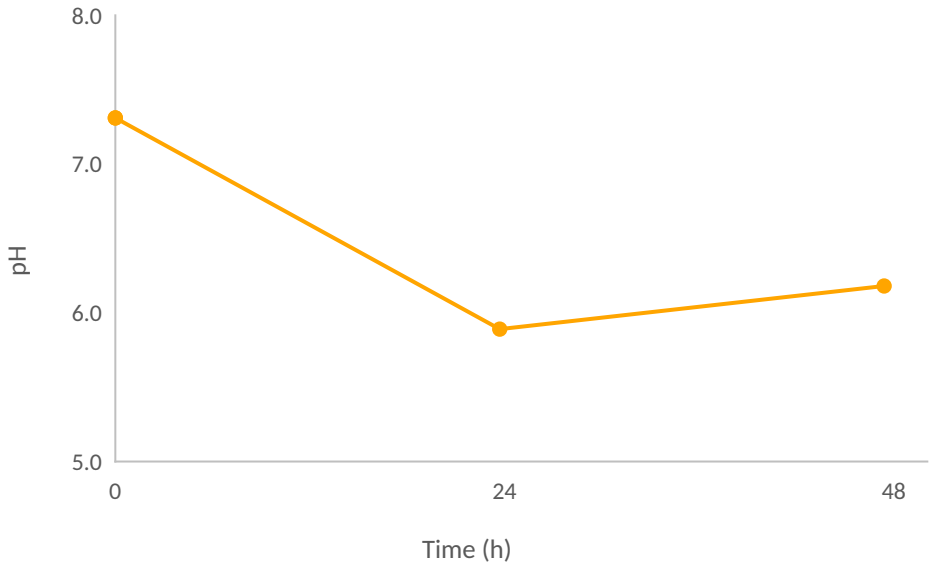


Figure 4: A) Growth curve and B) pH curve of the designed chain-elongation SynCom cultured in uncontrolled batch mode in PYG medium during Experiment 1

During Experiment 1, clear growth of the designed chain-elongation SynCom was detected. Within 24 hours, the SynCom reached the highest concentration of biomass measured during this experiment (Figure 4A). The pH value during Experiment 1 can be divided into two distinct regions. First, a decrease in pH indicates acidification of the PYG medium. Next, the medium slowly increases in pH again, indicating a return to alkalinity (Figure 4B).

3.1.2 Substrate consumption and product formation by the SynCom

To evaluate the biochemical activity of the designed chain-elongation SynCom, HPLC was performed to monitor substrate consumption and MCCA production for the duration of Experiment 1.

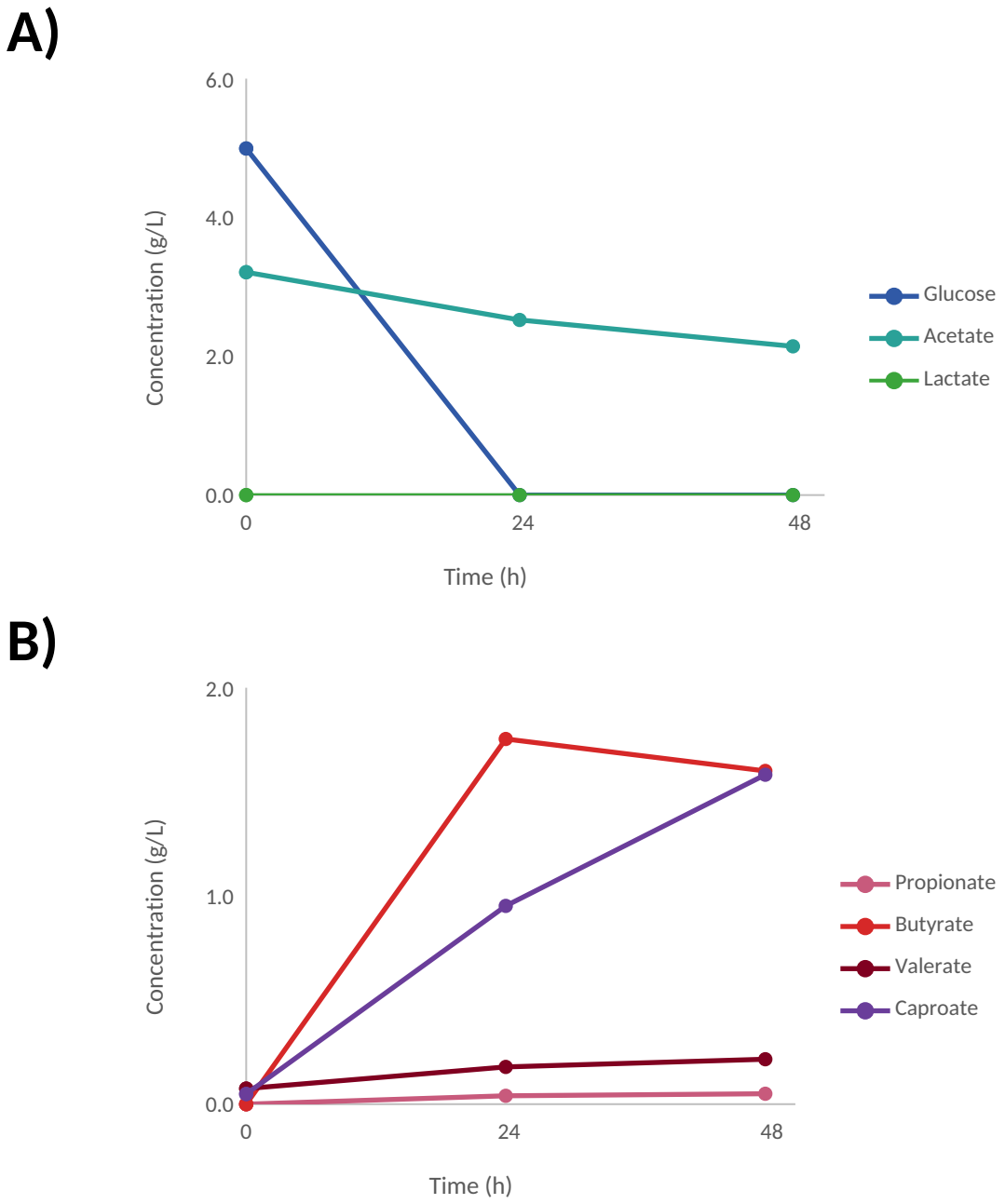


Figure 5: A) Consumption of substrates and B) production of MCCAs by the designed chain-elongation SynCom cultured in uncontrolled batch mode in PYG medium during Experiment 1

Glucose, the main carbon source for the bacteria in the designed SynCom, is rapidly depleted within 24 hours. The more specific substrates for chain elongation, acetate and lactate, show different patterns. Acetate, which is added at 0 h, is slowly being consumed by the chain-elongation SynCom, but is not depleted after 48 hours. Lactate, which is not added at 0 h, is not detected throughout the full duration of the chain-elongation SynCom assembly experiment (Figure 5A).

The production of different MCCAs is plotted during the uncontrolled SynCom batch run. This graph shows that mainly butyrate and caproate, the main MCCA for a chain-elongation SynCom, are produced in higher concentrations. Propionate and valerate, on the other hand, do not reach similar concentrations (Figure 5B).

3.1.3 Syncom relative abundance

To determine the relative abundance of the designed chain-elongation SynCom throughout Experiment 1, metagenomics was performed on the sequencing data.

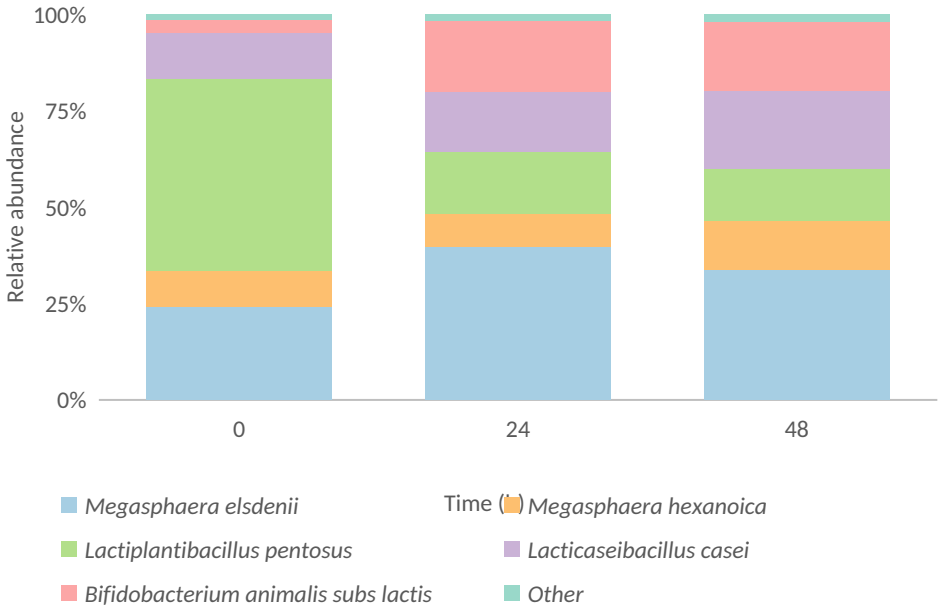


Figure 6: Relative abundance of the designed chain-elongation SynCom cultured in uncontrolled batch mode in PYG during Experiment 1

The relative abundance changed markedly in the first 24 hours. Initially, the community was dominated by *L. pentosus* (50%), followed by *M. elsdenii* (24%), *L. casei* (12%), *M. hexanoica* (9%) and *B. animalis* (3%). By 24 h, a new composition was observed, which was now dominated by *M. elsdenii* (40±3%). *L. pentosus* decreased to a relative abundance of 16±1%, while *L. casei* and *B. animalis* increased to a relative abundance of 16±1% and 18±0% respectively. *M. hexanoica* had a similar relative abundance at 8±1%. After 48 hours, the strains kept roughly equal relative abundance and standard deviations. Furthermore, additional strains of other taxa (~1-2%) were observed during the experiment (Figure 6).

Analysing the results from Experiment 1, it was decided not to change the bacteria strains used in the SynCom for Experiments 2 and 3, as all strains were sufficiently present.

3.2 Experiment 2: Batch mode pH disturbance

In this section, data gathered from Experiment 2 are analysed and compared to the results from Experiment 1. This will give a first look into the resistance and resilience of a 5-member chain-elongation SynCom in a batch-mode bioreactor operation where pH disturbances are introduced.

3.2.1 Growth curve

Similar to experiment 1, to evaluate the biological activity of the chain-elongation SynCom, a growth curve was established for a controlled batch system over 168 hours. pH values no longer indicate the biological activity of the chain-elongation SynCom, since they were manually set at a certain level for different time intervals.

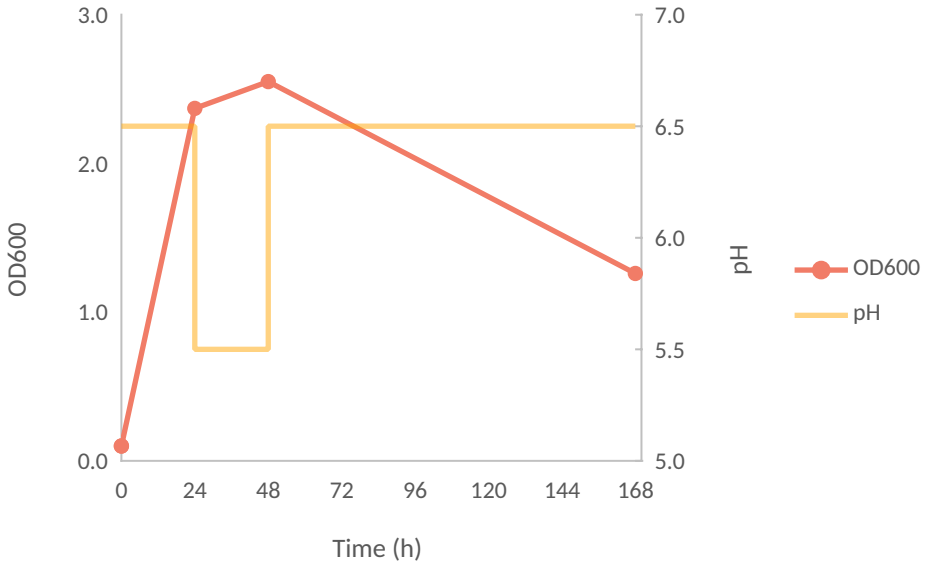


Figure 7: Growth curve of the chain-elongation SynCom, cultured in controlled batch mode in PYG medium during Experiment 2, with the pH regimes during growth on the secondary axis

The growth curve of Experiment 2 exhibits similar trends to those of the uncontrolled batch mode experiment. First, a growth phase is observed, followed by a more stationary phase. However, more growth was observed compared to Experiment 1 during the stationary phase. This is interesting, as the pH disturbance took place during this stationary phase. Due to the longer runtime of this experiment, a drop in biomass concentration is visible after the 48-hour mark when the pH is reversed to the original baseline value (Figure 7).

Comparing amounts of biomass produced between Experiments 1 and 2, there was significantly more biomass produced after 24 hours in Experiment 2 than in Experiment 1 (OD600 Exp 1, 1.76±0.04; Exp 2, 2.37±0.12; $p = 0.003$). This was also true for 48 hours (OD600 Exp 1, 1.71±0.04; Exp 2, 2.55±0.26; $p = 0.010$).

3.2.2 Substrate consumption and product formation by the SynCom

Similar to Experiment 1, the biochemical activity of the chain-elongation SynCom during Experiment 2 was measured using HPLC to monitor substrate consumption and MCCA production.

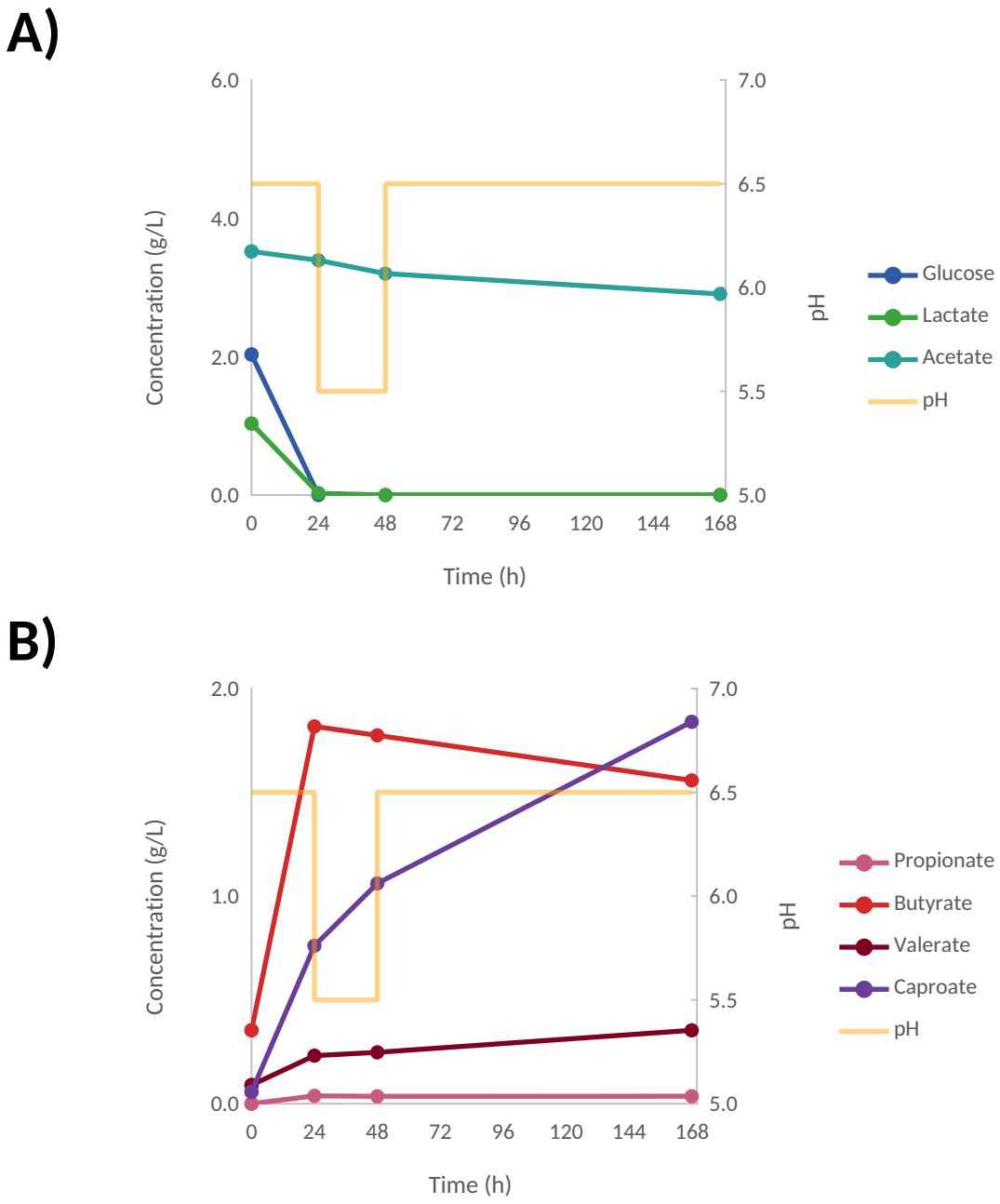


Figure 8: A) Consumption of substrates and B) production of MCCAs by the designed chain-elongation SynCom, cultured in controlled batch mode in PYG medium during Experiment 2, with the pH regimes during growth shown on the secondary axis

The consumption of substrates and production of MCCA follow similar trends in Experiment 2 compared to Experiment 1. Glucose is rapidly depleted within 24 hours, along with lactate, which is now detected at 0 hours. Acetate is again slowly consumed, and although the runtime of Experiment 2 was longer, it was still not fully depleted by the end of the run. During the pH disturbance, acetate consumption was slightly increased, while in Experiment 1, at similar time points, acetate consumption decreased somewhat (Figure 8A).

Also, production of MCCAs followed similar trends in Experiment 2 compared to Experiment 1. No clear differences were observed during the pH disturbance. Furthermore, it is remarkable that caproate is still being produced after glucose is fully depleted (Figure 8B).

3.2.3 Syncom relative abundance

To answer how the relative abundance of the chain-elongation SynCom changes when introduced to pH disturbances, metagenomics was performed on the sequencing data throughout Experiment 2.

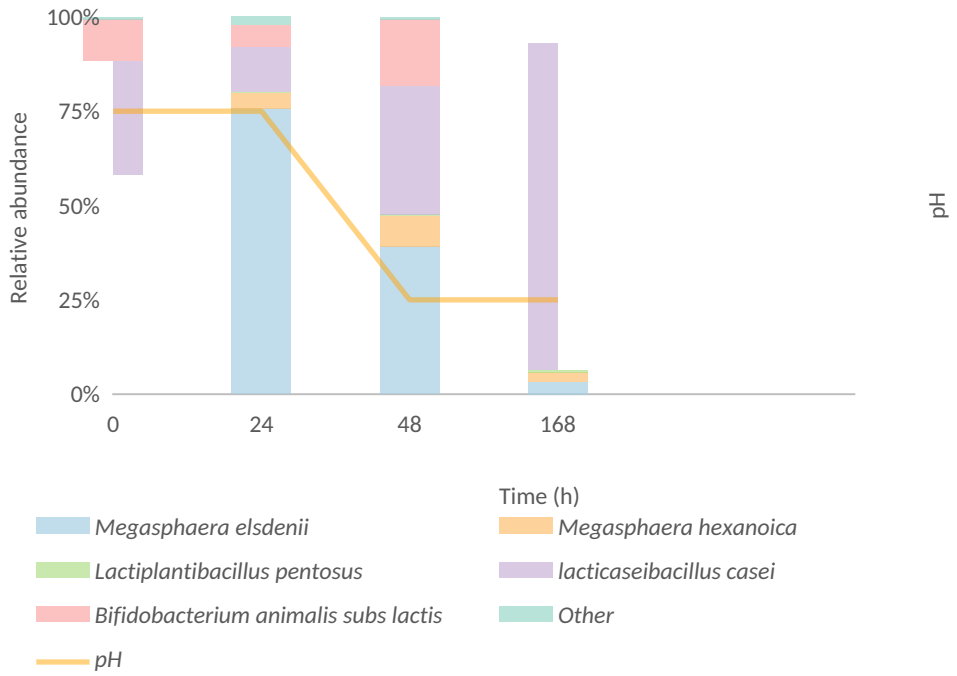


Figure 9: Relative abundance of the chain-elongation SynCom cultured in controlled batch mode in PYG during Experiment 2, with the pH regimes during growth shown on the secondary axis

Initially, the community was dominated by *M. elsdenii* (50%) instead of *L. pentosus*, which was the dominant strain in Experiment 1. Other strains present included *L. casei* (30%), *B. animalis* (11%), *M. hexanoica* (4%), and *L. pentosus* (4%). After 24 hours, *M. elsdenii's* relative abundance increased to 76±5%. *L. casei* and *B. animalis* decreased to 12±1% and 6±2%, respectively. *M. hexanoica* remained at a similar level ±1% of relative abundance as initially, while *L. pentosus* was barely detectable (~0-1±0%) (Figure 9).

After the pH disturbance, the relative abundance shifted again. Although M. elsdenii remained the most abundant at 39±5%, it was no longer as dominant as before. This created an opportunity for L. casei, B. animalis, and M. hexanoica to grow, reaching relative abundances of 34±2%, 18±3%, and 8±1%, respectively. *L. pentosus* was still barely detectable (~0-1%±0%). After 168 hours, the SynCom was almost completely dominated by *L. casei* (87±3%). The other 4 members of the SynCom were still detected: *B. animalis* (5±2%), *M. elsdenii* (3±2%), *M. hexanoica* (3±0%) and *L. pentosus* (1±0%). Similar to Experiment 1, throughout the whole duration of Experiment 2, other taxa were detected at levels around 1-2% (Figure 9).

3.3 Experiment 3: Fed-Batch mode pH disturbance

In this section, data gathered from Experiment 3 are analysed and compared to the results from Experiment 1 & 2. This will give a further look into the resistance and resilience of a 5-member chain-elongation SynCom and a comparison between a batch-mode and fed-batch mode bioreactor operation, where pH disturbances are introduced.

3.3.1 Growth curve

Similar to Experiment 1 & Experiment 2, to evaluate the biological activity of the chain-elongation SynCom, a growth curve was established for a controlled fed-batch system over 72 hours. pH values no longer indicate the biological activity of the chain-elongation SynCom, since they were manually set at a certain level for different time intervals. Additionally, two hypothetical measurements of OD600 were calculated and plotted after dilution with fresh media at 21 and 49 hours, since no real measurements were taken at this timepoint.

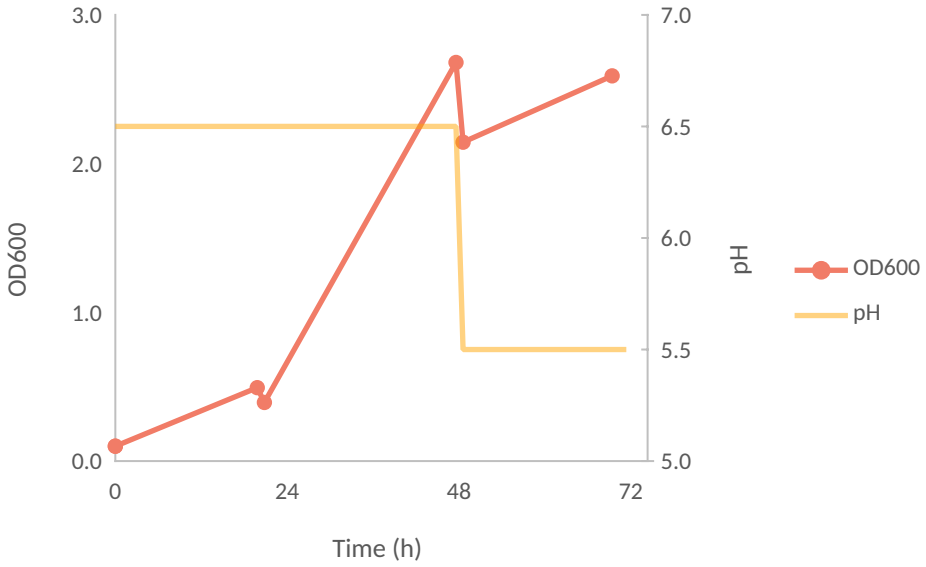


Figure 10: Growth curve of the chain-elongation SynCom, cultured in controlled fed-batch mode in PYG medium during Experiment 3, with the pH regimes during growth shown on the secondary axis. OD600 at 21 hours and 49 hours are theoretically calculated

The growth curve of Experiment 3 follows a different curve compared to Experiments 1 and 2. During Experiment 3, growth is not as quick and only reaches its highest biomass concentration after 48 hours. After dilution at 48 hours, growth is occurring but more slowly than previously. Due to this longer growth phase and a later pH disturbance, the stationary phase takes place during the pH disturbance. In Experiment 2, this resulted in a stationary phase with more growth, which is no longer the case in Experiment 3. Furthermore, after 72 hours variation between biomass concentration was observed between the three samples, resulting in a larger standard deviation than otherwise measured (Figure 10).

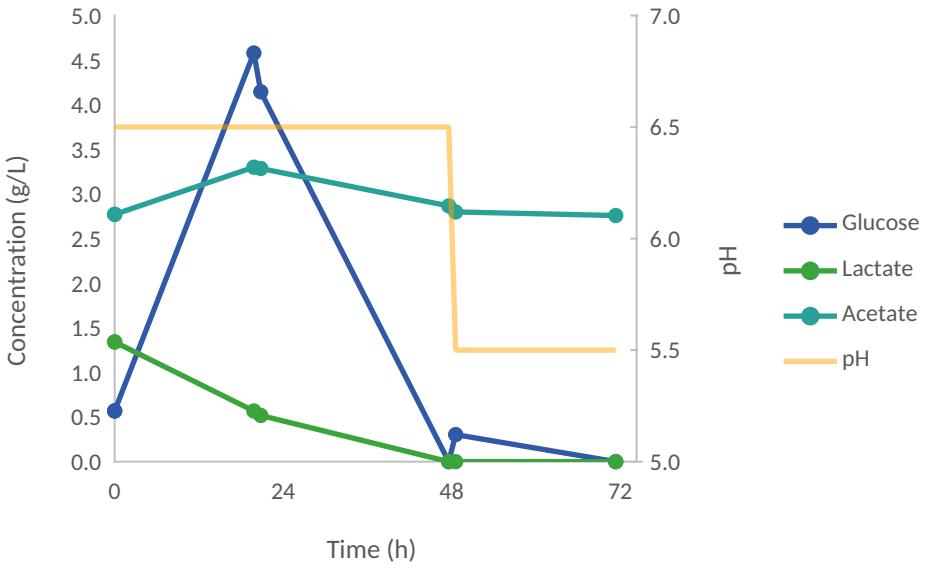
Comparing biomass concentration between Experiments 1 and 3, there was significantly less biomass produced after 24 hours in Experiment 3 than in Experiment 1 (OD600 Exp 1, 1.76±0.04; Exp 3, 0.49±0.03; $p < 0.001$). However, after 48 hours, a higher biomass concentration was observed (OD600 Exp 1, 1.71±0.04; Exp 3, 2.68±0.05; $p < 0.001$).

Comparing biomass concentration between Experiment 2 and 3, there was significantly less biomass produced after 24 hours in Experiment 3 than Experiment 2 (OD600 Exp 2, 2.37±0.12; Exp 3, 0.49±0.03; $p < 0.001$). However, after 48 hours, an insignificantly higher biomass concentration was observed (OD600 Exp 2, 2.55±0.26; Exp 3, 2.68±0.05; $p = 0.525$).

3.3.2 Substrate consumption and product formation by the SynCom

The biochemical activity of the chain-elongation SynCom during Experiment 3 was once again measured using HPLC to monitor substrate consumption and MCCA production. However, in this experiment, substrates were regularly added, which might impact the results compared to Experiment 2.

A)



B)

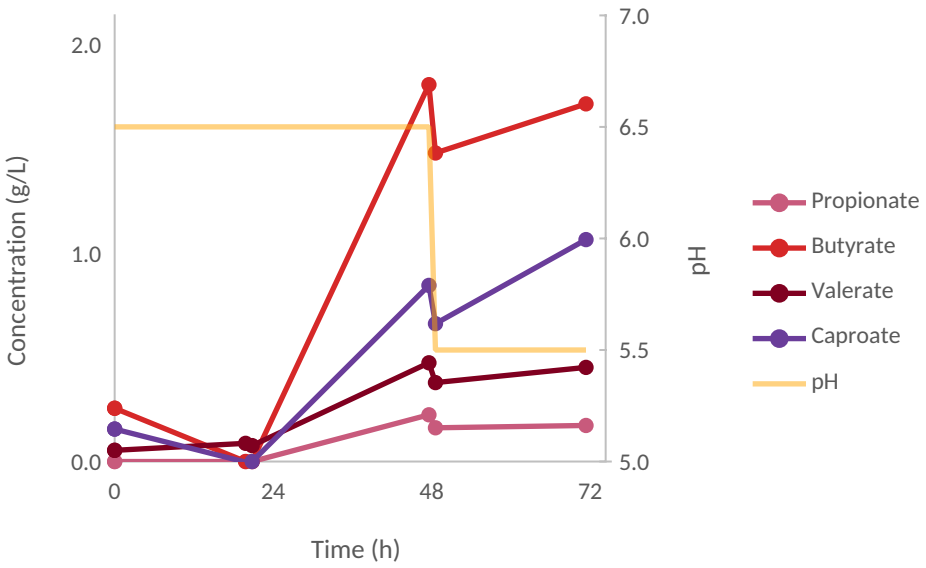


Figure 11: A) Consumption of substrates and B) production of MCCAs by the designed chain-elongation SynCom, cultured in controlled fed-batch mode in PYG medium during Experiment 3, with the pH regimes during growth shown on the secondary axis

Compared to the batch mode experiment, the consumption of substrates in a fed-batch mode follows a different trend. Within the first 20 hours, where previously rapid consumption of glucose was observed, an increase in glucose concentration is observed. A similar, but smaller, trend is observed in the acetate concentration. Lactate, however, is slowly consumed during the first 20 hours. After 20 hours, new media is added, which contains more substrates. From this point onwards, a similar trend is observed with rapid consumption of glucose and moderate consumption of acetate and lactate (Figure 11A).

Production of MCCAs follows a different curve compared to Experiment 1 and Experiment 2; however, it follows it later. In Experiment 1 and Experiment 2, MCCA production was seen immediately after inoculation; in Experiment 3, the concentration of MCCAs started to increase after 20 hours. Another decrease in MCCA concentration is shown after 48 hours when new media is added (Figure 11B).

3.3.3 Syncom relative abundance

To answer how the relative abundance of the chain-elongation SynCom changes when introduced to pH disturbances, metagenomics was performed on the sequencing data throughout Experiment 2.

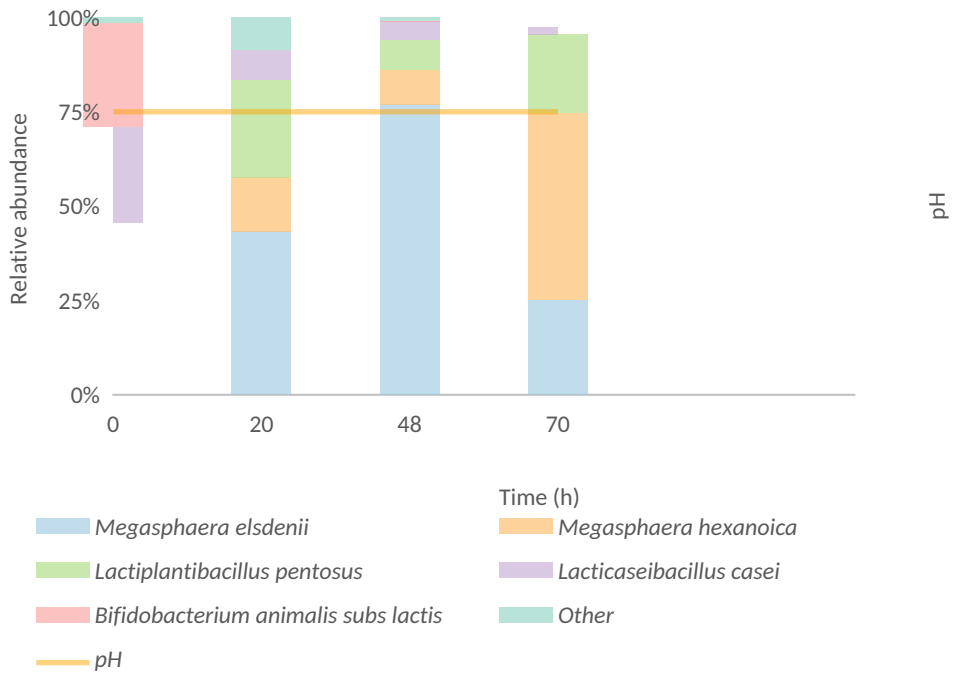


Figure 12: Relative abundance of the chain-elongation SynCom cultured in controlled fed-batch mode in PYG during Experiment 3, with the pH regimes during growth shown on the secondary axis

Initially, the community was roughly evenly distributed among four strains: *L. pentosus* at 31%, *B. animalis* at 27%, *L. casei* at 25%, and M. elsdenii at 13%. *M. hexanoica*, however, was only present at a relative abundance of 2%. After 20 hours, *M. elsdenii* began to dominate the SynCom, increasing to a relative abundance of 43±6%. *L. pentosus* and *L. casei* decreased to 26±5% and 8+2%, respectively, while *M. hexanoica* grew to 14±4%. *B. animalis* was no longer detected at this stage and remained absent for the rest of the experiment. Other taxa, normally present around 1-2%, increased to an average of 9±9% due to one bioreactor reaching a level of 21% other taxa. After 48 hours, the SynCom was even more dominated by *M. elsdenii*, now at 77±6% relative abundance. *M. hexanoica* stood at 9±2%, L. pentosus at 8±4%, and *L. casei* at 5±1%. Other taxa reverted to levels between 1-2% (Figure 12).

Following the pH disturbance, the relative abundance changed. *M. hexanoica* became the most dominant strain at 49±11%, followed by *M. elsdenii* at 25±11%, *L. pentosus* at 21±6%, and *L. casei* at 2±0%. When comparing the standard deviation from Experiment 3 to that from Experiments 1 and 2, it is evident that there was greater variability between the bioreactors in Experiment 3 (Figure 12).

Chapter 4: Discussion & Conclusion

This study aimed to evaluate the stability and MCCA-production capacity of a designed synthetic chain-elongation community throughout cultivation experiments with pH disturbances. Optimising cultivation strategies and understanding SynCom stability is essential for improving yield and process efficiency in bioprocessing applications. The results of this project provide insight into the stability and performance of a chain-elongation SynCom under pH disturbances and different cultivation strategies, highlighting factors for further studies.

4.1 Experiment 1: SynCom assembly & baselining

SynCom assembly and baselining confirmed the growth of all members of the designed chain-elongation SynCom and were biologically active. Acidification of the media and consumption of glucose during the first 24 hours indicate LAB bacteria are converting the glucose into acids like lactic acid. Lactate is, however, not detected during the whole run, which might indicate faster consumption of lactate by the CEB than is produced by the LAB. This is supported which is supported by the consumption of acetate, as lactate and acetate are the main substrates for CEB to produce MCCAs. Growth curves and pH curves obtained showed similar results compared to similar SynCom experiments done in the SSB group (Peeters, 2024; Van Kampen, 2024).

Additionally, increasing MCCA concentrations and a slight increase in pH further suggest the SynCom to be functionally working as intended. While lactate and MCCAs are both acids, conversion should increase the pH. Lactate has a pKa of 3.85, while MCCAs have a pKa around 4.8 (Handy, 2006; Kanicky & Shah, 2002). This means lactate the stronger acid is being converted into weaker acids, which increases the pH of the system slightly.

One of the observations was that some MCCAs are detected at a higher concentration than others. This can be explained by the fact that the RBO pathway adds C2 to the carbon backbone. Therefore, if acetate (C2) is the starting substrate and lactate exchange is taking place between LAB & CEB, butyrate (C4) and caproate (C6) will be the products formed. However, *M. elsdenii* has another pathway for MCCA production, which it prefers over the RBO pathway to produce caproate. *M. elsdenii* can also use lactate to produce C2-C5 molecules, including propionate (C3) and valerate (C5) (Weimer & Moen, 2012; Da Silva Cabral & Weimer, 2024). But the data from HPLC suggest both pathways happen at the same time, with dominance of the RBO pathway to produce caproate. These competitive pathways and metabolic switches may explain the low propionate and valerate concentrations present in the SynCom system.

Outside of the 5 SynCom strains, other taxa were observed from the sequencing data in the relative abundance. However, most strains that were found were closely related to one of the 5 SynCom strains. Therefore, it does not seem like contamination was an issue, but sequencing errors resulted in these other taxa emerging from the sequencing data.

4.2 Experiment 2: Batch mode pH disturbance

Comparing data from Experiment 2 to data from Experiment 1 will give an insight into how constant pH and a pH disturbance will affect the SynCom. As calculated in the results section, at

both 24 and 48 hours, significantly more biomass growth was observed in Experiment 2 at constant pH levels. Looking at the community composition between 0 and 20 hours, both Experiments experienced the most dominant growth by *M. elsdenii*. After 24 hours, both systems were at similar pH levels. Experiment 1 naturally reached a pH of 5.89±0.05, while Experiment 2 was manually set at a pH of 5.5. In both systems, the lowered pH resulted in *M. elsdenii* lowering in relative abundance. In Experiment 2, this effect was more noticeable as the pH was at 5.5 until 48 hours, while in Experiment 1, this effect was less noticeable as the pH rose slightly again to 6.18±0.02. Da Silva Cabral and Weimer (2024) showed that *M. elsdenii* could grow at these pH ranges, so this might indicate that the other strains present were more resistant to the sudden pH disturbance. *M.* hexanoica grew slightly in relative abundance after the lower pH was present during Experiment 1 and significantly after the pH disturbance in Experiment 2. Because both *M. elsdenii* and *M. hexanoica* are functionally the same in the SynCom, the rise of relative abundance of *M. hexanoica* after pH disturbance/lowering and the decrease of relative abundance of *M. elsdenii* after pH disturbance/lowering might indicate functional redundancy. As soon as the pH drops, which could favour *M. hexanoica*, it partly takes over the chain-elongation task from *M. elsdenii*. After the pH disturbance, when the pH was again set at 6.5, the relative abundance of the CEB was reduced even further. However, this measurement was done after quite some time, so this might not be as reliable.

Comparing caproate production, it might be that *M. hexanoica* is less efficient in chain elongation. In Experiment 2, where the functional redundancy is observed more strongly, the total amount of caproate being produced after 48 hours is lower than that of Experiment 1. However, after the pH disturbance and 168 hours, the caproate concentration measured was similar to the caproate concentration after 48 hours. Glucose and acetate consumption seem relatively similar between Experiments 1 and 2.

Functional redundancy is not observed for the LAB strains, as after pH disturbance/lowering, there is no clear difference in the ratio between the strains. They do, however, increase in total relative abundance compared to the CEB. So this might indicate that a lower pH is preferred by the LAB or that they are more resistant to pH disturbances. The residue of Lactate, detected at 0 hours in the serum bottle, is rapidly consumed after inoculation. This further strengthens the argument that lactate is consumed faster by CEB than it is produced by the LAB.

One difference between Experiment 2 and Experiment 1 is that sample measurements at 0 hours are not from the bioreactor, but from a preliminary 200 ml serum flask containing the 5-member Syncom in PYG medium without substrates. This was done to avoid concentrations too low for detection for sequencing at 0 hours. However, this affects the substrate concentrations at 0 hours. The measured concentrations at 0 hours in the bioreactor should be: glucose at 5 g/L and lactate at 0 g/L. However, the concentrations measured from the serum flask are: glucose at 2 g/L and lactate at 1 g/L. This is expected as glucose in the serum flask was mostly consumed, so a lower concentration was observed, while lactate was produced, so a higher concentration was observed. This would also be the case in Experiment 3.

4.3 Experiment 3: Fed-Batch

Comparing data from Experiment 3 to data from Experiments 1 & 2 will give an insight into how constant pH and a pH disturbance, together with a feeding regime, will affect the SynCom. After 20 hours, significantly less biomass was observed in Experiment 3 compared to Experiments 1 & 2. However, the biomass concentration recovered to a similar level compared to Experiment 2, and was significantly higher than Experiment 1. Looking at the composition in the first 24 hours, similarly, *M. elsdenii* was the dominating strain in the SynCom. This again shows that a pH of 6.5 was more optimal for the growth of *M. elsdenii* than the other strains. This is even further strengthened by the fact that in this Experiment, the pH was kept longer at 6.5, and that *M. elsdenii* had an even bigger relative abundance after 48 hours. After 48 hours, the pH disturbance was introduced, and the pH was sharply lowered to 5.5. This resulted again in a decrease in the relative abundance of *M. elsdenii* and an increase in the relative abundance of *M. hexanoica*. This again points towards *M. hexanoica* taking over a bigger role in chain elongation at lower pH due to functional redundancy, as also hypothesised in Experiment 2.

Caproate and other MCCA production also experienced a delay in increase. During the first 20 hours, no additional MCCA production was observed, and there was even a slight decrease in concentration. This can be explained by the fact that the initial measurement was taken from the 200 mL serum flask, which probably contained MCCAs due to preculturing. After inoculation from this serum flask, the system was heavily diluted, resulting in a decrease in MCCA concentration. After 20 hours, caproate and MCCA production followed a similar curve compared to the concentrations found in Experiment 2. Comparing Experiments 2 and 3 to Experiment 1, it does not seem like the pH disturbance and feeding regime had an impact on the caproate and MCCA production, even though it seemed to have an impact on relative abundance. This further points towards functional redundancy between the two CEB strains happening during pH disturbances.

In Experiment 3, there is also no clear functional redundancy observed between the LAB strains, as the ratio between the relative abundance of the strains is similar before and after the pH disturbance. The total amount of LAB strains again increased after the pH disturbance, similar to Experiment 2, indicating they prefer a lower pH or are more resistant to pH disturbance. Different ratios of relative abundance were found between all three Experiments, with the total SynCom not behaving differently. In all three experiments, a different LAB strain was (co)dominant and/or a strain was hardly present at all. Therefore, functional redundancy between the LAB strains could be the case. However, which strain(s) will be more dominant seems to be more dependent on the initial relative abundance instead of the pH disturbance, which is the case for the CEB strains. Initial lactate is, similar to Experiment 2, consumed until a concentration of 0 is reached during the Experiment.

Overall, Experiment 3 produces similar results to Experiment 2, but with a delay. This suggests that it took longer for the SynCom to establish itself and activate its metabolic network in Experiment 3. In all three experiments, different pre-cultures, which were not tested, were prepared. This variation in pre-cultures could have influenced the time it takes for the SynCom to stabilise and the specific relative abundance of LAB and CEB within the system.

4.4 Summarised conclusion

This study proves that the designed chain-elongation SynCom is stable, functionally active, and capable of producing caproate and other MCCAs under pH disturbances and different cultivation strategies. In all experiments, *M. elsdenii* was the dominant CEB strain under more neutral pH ranges (pH~6.5). However, the role of *M. elsdenii* was partly taken over by *M. hexanoica* when the pH suddenly dropped, which might indicate *M. elsdenii* to be less resistant to pH disturbances. This functional redundancy between the two CEB strains allowed MCCA production to continue despite shifts in community composition. Lower yields were, however, observed when *M. hexanoica* was more abundant, suggesting it is less efficient than *M. elsdenii*. In contrast, LAB strains did not show clear functional redundancy linked to pH shifts, even though their relative abundance compared to CEB did increase, suggesting greater resistance to pH disturbances. However, the difference in relative abundance between LAB strains observed might suggest functional redundancy linked to the initial community composition.

Fed-batch cultivation delayed biomass growth and MCCA production compared to batch cultivation, but eventually showed similar trends. Both experiments had different pre-cultures, highlighting the impact of pre-culture variability on SynCom establishment rather than on overall metabolic performance. Despite differences in strain dominance, community dynamics and pH regimes across experiments, glucose and acetate consumption, lactate consumption, and MCCA production patterns remained consistent, underscoring the stability of the SynCom's metabolic network.

Overall, the findings confirm that pH disturbances influence community composition but not the overall metabolic output due to functional redundancy among CEB strains. These insights strengthen the understanding of SynCom stability under stress and provide a foundation for optimising cultivation strategies to enhance yield and efficiency in SynCom bioprocesses.

4.5 Further recommendations

Based on the findings of this project, several recommendations can be made to further research the stability of SynComs. First of all, errors made during this project can be prevented. The difference in pre-culture seems to have an impact on the further development of the SynCom community composition and growth speed. If this can be standardised, it will allow for less pre-culture bias. Additionally, sampling at 0 hours from only the serum flask is not representative of the concentration of substrates and MCCAs in the bioreactor. If an additional sample is taken directly from the bioreactor at 0 hours to determine these concentrations, potential baseline bias can be prevented.

Since this project was only a first look into the stability of a 5-member chain-elongation SynCom, this could be further explored with similar experiments. In this project, no clear data was gathered on the resilience of the chain elongation SynCom, which was initially also planned. In Experiment 2, the time between the measurement after reestablishing the pH at the baseline was long, and with no extra substrates added to the bioreactor, no clear conclusion can be drawn from this data. Additionally, in Experiment 3, the baseline was never reestablished at the baseline

pH. Therefore, an experiment with measurements closer to the reestablishment of the baseline pH could provide interesting data on the resilience of a chain-elongation SynCom.

In this project, only 1 kind of pH disturbance was explored. Different kinds and different durations of pH disturbances, like ramp, stepwise or multiple and longer disturbances, could provide interesting results, further exploring the stability of chain-elongation SynComs. Similarly, different cultivation methods could be explored. A continuous-mode bioreactor resolves the problem of substrate limitation, especially glucose, observed in all three experiments. This might improve overall growth in the system and increase the effects of functional redundancy observed when pH disturbances are introduced.

Because limited research was done on the effect and impact of pH disturbances, the amount of disturbance could also be explored. Growth curves of the different LAB and CEB strains under different pH levels might give a better look at which pH level the disturbances could be set. Additionally, other parameters can also be tested, like temperature and DO levels.

Finally, this project revealed interesting results concerning the competitive pathways M. elsdenii can utilise. This chain-elongation system can also offer a fundamental understanding of these metabolic pathways and how you can use a metabolic switch for further optimisation in bioprocess engineering systems.

Appendix

A. Media recipes

Media recipes described are based on 1 L of medium filled with demi water.

1.A Salt solution for PYG and Bifidobacterium medium

$CaCl_2$ x 2 H_2O	0.25 g
$MgSO_4$ x 7 H_2O	0.50 g
K_2HPO_4	1.00 g
KH_2PO_4	1.00 g
$NaHCO_3$	10.00 g
NaCl	2.00 g

2.A PYG medium

Tryptone	5 g
Peptone	5 g
Yeast extract	10 g
Beef extract	5 g
K_2HPO_4	2 g
Na-Acetate	5 g
Salt solution	40 mL
Resazurin	1mg (1 mL of 1 g/L solution)

Added after autoclaving:

Glucose	5 g
Cysteine	0.5 g

3.A Bifidobacterium medium

Tryptone	10.00 g
Yeast extract	5.00 g
Beef extract	5.00 g
Bacto Soytone	5.00 g
K_2HPO_4	2.00 g
$MgSO_4$ x 7 H_2O	0.20 g
$MnSO_4$ x H_2O	0.05 g
NaCl	5.00 g
Salt solution	40.00 ml
Resazurin	1 mg (1 ml of 1 g/L solution)

Added after autoclaving:

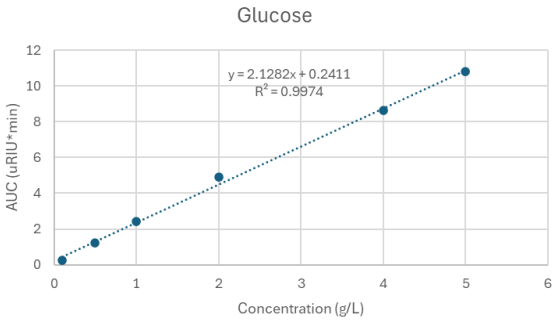
Cysteine	0.50 g
Glucose	10.00 g

4.A MRS medium

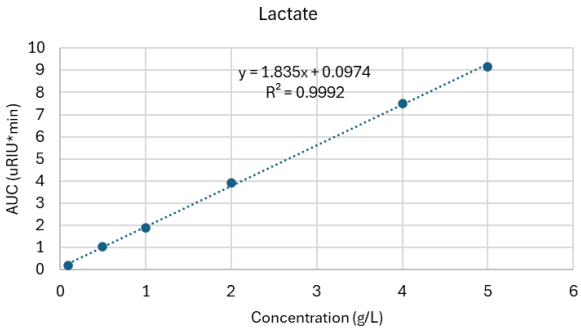
Tryptone	10.00 g
Beef extract	10.00 g
Yeast extract	5.00 g
Glucose	20.00 g
Tween 80	1.00 g
K_2HPO4	2.00 g
Na-acetate	5.00 g
$(NH_4)_3$ citrate	2.00 g
$MgSO_4 \times 7\ H_2O$	0.20 g
$MnSO_4 \times H_2O$	0.05 g

B. Standard curves

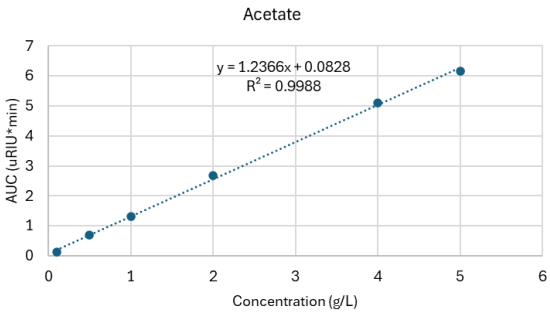
A)



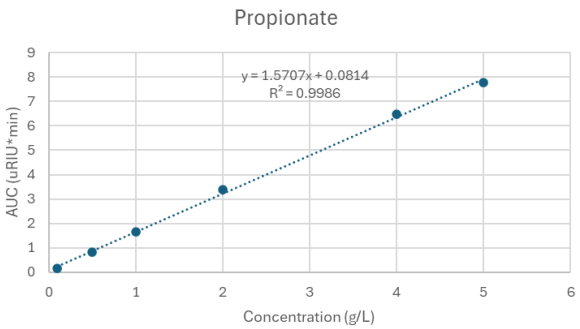
B)



C)



D)



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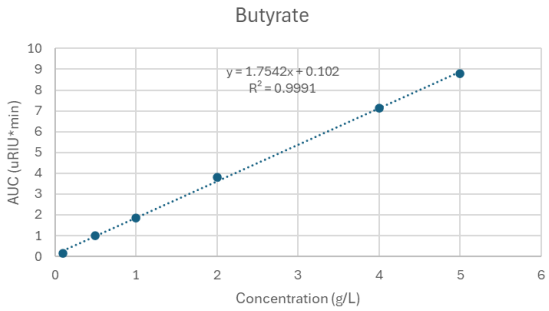

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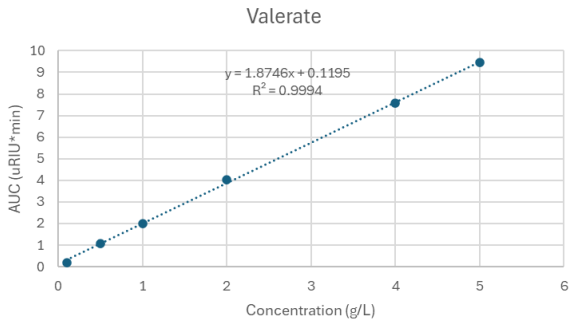

G)

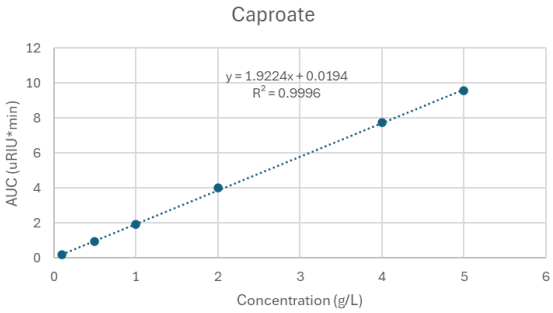

Figure S 1: Standard curves for quantification of metabolites by HPLC. Calibration curves for (A) Glucose, (B) Lactate, (C) Acetate, (D) Propionate, (E) Valerate, and (F) Caproate were generated by samples with known concentrations to determine the concentrations of each compound in experimental samples

C. EPI2ME report

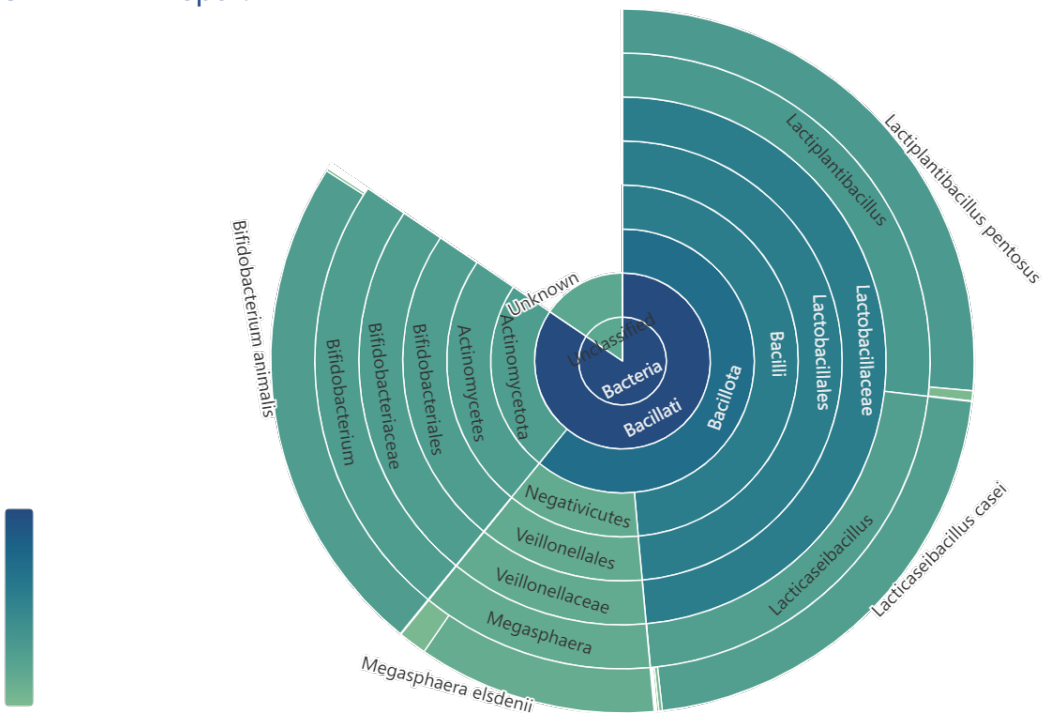

Figure S 2: Example of Sunburst plot generated from sequencing data analysis by EPI2ME for a single barcoded sample, which was further used for relative abundance analysis

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