Thermophilic caproic acid production from grass juice by sugar-based chain elongation

Myrsini Sakarika[1,2,†], Alberte Regueira[1,2,3,†], Korneel Rabaey[1,2], Ramon Ganigué[1,2,*]

[1] Center for Microbial Ecology and Technology (CMET), Faculty of Bioscience Engineering Ghent University, Coupure Links 653, 9000 Ghent, Belgium

[2] Center for Advanced Process Technology for Urban Resource recovery (CAPTURE), Frieda Saeysstraat, 9052 Ghent, Belgium

[3] Cross-disciplinary Research in Environmental Technologies (CRETUS), Department of Chemical Engineering, Universidade de Santiago de Compostela

[†]Equal contribution

* Correspondence to: Ramon Ganigué, Ghent University; Faculty of Bioscience Engineering; Center for Microbial Ecology and Technology (CMET); Coupure Links 653; B-9000 Gent, Belgium; phone: +32 (0)9 264 59 76; fax: +32 (0)9 264 62 48; e-mail: ramon.ganigue@ugent.be.

Abstract

Medium chain carboxylic acids (MCCA) such as caproic acid have a plethora of applications, ranging from food additives to bioplastics. MCCA can be produced *via* microbial chain elongation using waste and side-streams as substrates, a process that can be more sustainable than conventional production routes. Most chain elongation studies have focused on mesophilic conditions, with only two recent studies hinting at the possibility of thermophilic chain elongation, but a systematic study of its mechanisms is lacking. Here, we investigated thermophilic chain elongation from grass juice, to understand the effect of key operational parameters (pH, temperature, substrate) on the process performance and to establish the key microbial genera and their role in the system. The genus *Caproiciproducens* was identified as responsible for thermophilic chain elongation, and caproic acid production was most favorable at pH 6.0 and 50°C amongst the conditions tested, reaching an average concentration of 3.4 g/L. Batch experiments showed that the substrate for caproic acid production were glucose and xylose, while lactic acid led to the production of only butyric acid. Fed-batch experiments showed that substrate availability and the presence of caproic acid in the system play a major role in shaping the profile of thermophilic chain elongation. The increase of the total sugar concentration by glucose addition (without changing the organic load) during continuous operation led to a microbial community dominated (75%) by *Caproiciproducens* and increased by 76% the final average caproic acid concentration to 6.0 g/L (13 g_{COD}/L) which represented 32% (g/g) of the total carboxylic acids. The highest concentration achieved was 7.2 g/L (day 197) which is the highest concentration reported under thermophilic conditions thus far. The results of this work pave the way to the potential development of thermophilic systems for upgrading various underexplored abundant and cheap sugar-rich side-streams to caproic acid.

Keywords: Thermophilic fermentation; Chain elongation; Grass; Green biorefinery; Carboxylate platform; Caproic acid

1. Introduction

The use of side-streams and waste as feedstocks for the production of commodity chemicals *via* microbial fermentation could be a more sustainable alternative to conventional (petro)chemical processes (Ioannidou et al., 2020). Among the many potential end-products, organic acids such as short chain carboxylic acids (SCCA) and medium chain carboxylic acids (MCCA) have attracted considerable interest. MCCA contain 6 – 12 carbon atoms, and have applications as animal feed supplements and antimicrobials, flavor-enhancing food additives, organic herbicides, or can be used as feedstocks for the production of fragrances, bioplastics and lubricants (ChainCraft, 2021). Conventional MCCA production is rather unsustainable since it is based on petrochemicals and vegetable oils (Anneken et al., 2006). Microbial chain elongation – a process by which microorganisms elongate carboxylic acids by adding 2 carbon atoms per step using organics as carbon and electron donors (de Groof et al., 2019) – could be a more sustainable alternative MCCA production route that could be based on waste and industrial side-streams, thereby being independent from agriculture and fossil resources and contributing to a more circular economy. MCCA are more hydrophobic than SCCA, with the solubility drastically decreasing as a result of the increase in carbon chain length (Xu et al., 2018). Each elongation step results in a considerable increase in the price thereby increasing the value of the final product (*e.g.* 0.33 – 0.67€/kg for acetic acid; 1.9 – 2.1€/kg for caproic acid), which makes them a more attractive end-product than SCCA (Moscoviz et al., 2018). Due to the large number of applications of MCCA mixtures and/or individual MCCA such as caproic acid (ChainCraft, 2021), their market size is expected to rise by 60% (2.76 billion USD) by 2028 compared to 2020 (1.73 billion USD) (Fior Markets, 2021).

Chain elongation can use, among others, lactic acid (Carvajal-Arroyo et al., 2019), ethanol (Candry et al., 2020b), methanol (Chen et al., 2016), carbon monoxide (Liu et al., 2022), hydrogen (Baleeiro et al., 2021) and sugars/carbohydrates (Wang et al., 2022) as electron donors. Substrates and feedstocks commonly used for chain elongation include synthetic media containing the desired electron donor(s) (Candry et al.,

2020b), organic side-streams (Carvajal-Arroyo et al., 2019), food waste (Reddy et al., 2020; Zhang et al., 2022) and green biomass (Khor et al., 2017). In particular, the use of green biomass such as grass holds great potential as feedstock for biotechnological applications. Grass is one of the most abundant plant families, it is inexpensive (Osterburg et al., 2010) and its liquid fraction, grass juice, has a suitable composition for fermentation (*i.e.* high content in fermentable organics and nutrients) (Dien et al., 2006; Sakarika et al., 2022). Hence, grass can be used as feedstock for the production of higher value products such as platform chemicals (Khor et al., 2017) or microbial protein (Sakarika et al., 2022) allowing for their decoupling from the use of fossil resources (Corona et al., 2018). Furthermore, the high sugar content in grass juice (>25 g/L) (Sakarika et al., 2022) can enable high product titers. Therefore, grass juice can serve as the sole substrate for chain elongation without the need for external addition of substrate components.

While there are numerous reports of mesophilic (20 – 45°C) chain elongation for caproic acid production (de Groof et al., 2019), thermophilic (45 – 70°C) chain elongation is understudied, with only two reports showing caproic acid production (>0.50 g/L), without investigating the effect of relevant parameters (*e.g.* pH, temperature, substrate). Specifically, 0.74 g/L caproic acid were produced during grass juice fermentation targeting lactic acid at 45°C (Sakarika et al., 2022) and 4.7 g/L caproic acid on food waste fermentation at 55°C targeting caproic acid (Zhang et al., 2022). Thermophilic processes have the potential to present a number of benefits compared to the mesophilic ones. Higher temperatures could allow for a higher substrate hydrolysis rate (Kim et al., 2003), which is the rate-limiting factor in consolidated bioprocesses. The high activity and stability of thermophilic enzymes, coupled with the better enzyme penetration at elevated temperatures can result in higher substrate conversion efficiency (Paës and O'Donohue, 2006). Thermophilic bioprocesses often exhibit higher production rates (Ryue et al., 2020) and end-product titers (Hao and Wang, 2015), which is of high importance when using real streams with high solid content as substrates (*e.g.* grass juice). Additionally, thermophilic processes result in more efficient pathogen removal (Ryue et al., 2020), which could broaden and/or facilitate the uses of the

produced MCCA in industrial applications. At the same time, thermophilic bioprocesses often harbor less diverse microbial communities (Niu et al., 2015), which may limit the function and robustness of the system, but potentially allow to minimize competing pathways in mixed culture fermentations. Therefore, thermophilic temperatures could be a tool to increase the selectivity of caproic acid production and, hence, potentially increase the economic viability of chain elongation. Nevertheless, to enable the development of such a competitive thermophilic process, the basic mechanisms need to be understood.

In this work, we systematically investigated the thermophilic production of caproic acid from grass juice in an open-culture chain elongating system, with the aim of providing a first substantiated study on the effect of the main operational parameters. First, we studied the effect of (i) pH (5.5 – 6.5) and (ii) temperature (45 – 55°C) under continuous mode, as these parameters are known to heavily influence the outcome of mixed-culture bioprocesses. Subsequently we assessed the effect of (iii) substrate composition, (iv) substrate availability and (v) potential caproic acid toxicity on the product profile and end concentration. Additionally, in parallel to the process performance we assessed how the microbial community changed as a result of the aforementioned factors to gain further insights on community shifts and the links to the observed chain elongation performance.

2. Materials and methods

2.1 Grass juice fractionation and characteristics

Freshly cut grass was fractionated to grass juice and cake using a biomass extruder equipped with an internal twin screw. Right after fractionation the grass juice was stored at −20°C, and only defrosted ca. 24h prior to its use. The grass juice composition was analyzed and contained among others, *ca*. 87 g/L chemical oxygen demand (COD), 28 g/L total suspended solids (TSS), 25 g /L carbohydrates and 4.1 g/L protein (**Table 1**). The latter is expected to be dominated by glucose (46-62%) and xylose (18-36%) (Dien et al., 2006).

2.2 Experimental procedures

2.2.1 Continuous fermentation

Continuous grass juice fermentation experiments were conducted in a 0.5L jacketed glass continuous stirred-tank reactor (CSTR) with a 0.5 L working volume. The reactor was operated under non-axenic conditions and continuous stirring at 400 rpm. Inoculation was performed at 10% v/v using the effluent of a non-axenic thermophilic (45°C) CSTR converting grass juice to lactic acid, when the abundance of *Caproiciproducens* was ca. 30% and the production of up to 0.74 g/L caproic acid was detected (Sakarika et al., 2022). The reactor was operated in batch mode for the initial 8 days after which it was switched to continuous mode at a hydraulic retention time (HRT) of 4 days, corresponding to an organic loading rate (OLR) of *ca.* 22 g_{COD}/L/d. Different temperatures in the thermophilic range (45 – 55°C) and pH values (5.5 – 6.5) were tested to establish the performance of the system (**Table 2**). At the last phase of continuous operation, grass juice was supplemented with glucose by mixing 3 parts of grass juice with one part of a glucose solution with the same chemical oxygen demand (COD of *ca*. 87 g_{COD}/L). The organic load (COD) of the resulting substrate remained the same, but the concentration of sugars/carbohydrates increased by 50%.

To avoid a shock during the temperature transitions, a gradual adaption was done (0.50 °C every *ca*. 12h). The reactor pH was controlled by dosing NaOH or HCl (2M). Samples (10 mL) were taken three times per week. The pH was immediately measured in raw samples which were subsequently centrifuged at 20,817 g for 5 min. The supernatant was immediately filtered (0.2 µm PVDF filters, Chromafil®) and the liquid samples and pellet were stored at -20°C until further analyses of carboxylic acids and microbial community composition, respectively.

2.2.2 Batch and fed-batch tests

A series of batch and fed-batch experiments were performed in parallel with the continuous experiments, to establish (i) the substrate used for caproic acid production, (ii) the effect of substrate availability and

the potential for achieving high titers of caproic acid and (iii) the potential inhibiting effect of caproic acid. To ensure high microbial activity, these experiments were performed using the most recent reactor effluent as inoculum, which was incubated under the same conditions of the running reactor. All experiments were performed in triplicate using 120 mL serum vials with a working volume of 50 mL under 120 rpm orbital shaking. The pH was set at 6.0, buffered using 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) and was daily corrected using NaOH or HCl (5M). To ensure anaerobic conditions all the serum vials were flushed with a mixture of N_2 and CO_2 (90:10% v/v) for 5 min and sealed with rubber stopper. For all batch and fed-batch experiments, serum vials containing the inoculum supplemented with MES buffer and distilled water (instead of substrate) that were incubated under the same conditions served as controls. In addition, in all cases, the substrate (or distilled water) was added at the same volume, to achieve the same dilution effect. The duration of the experiments was 7 – 18 days and the pressure of all serum vials was daily set to atmospheric levels. Samples (2 mL) for carboxylic acid and microbial community composition analysis were taken every 1 – 3 days and were treated as described in section 2.1.1. The specific experiments and associated goals are described below:

- To establish the substrate for chain elongation, batch tests were performed at 45°C using glucose, xylose, cellulose or lactic acid supplied at 10 g_{COD}/L. Glucose, xylose and cellulose were provided as substrates since they are the main sugars/carbohydrates usually present in grass (Dien et al., 2006). Lactic acid was also tested as it can be a fermentation product of grass juice (Sakarika et al., 2022) and is a well-established substrate in chain elongation (Candry et al., 2020a).
- To assess whether the substrate composition and availability affect the product profile identify the limit of caproic acid concentration of the community using a real and a synthetic substrate, fed-batch experiments were performed at 50°C using glucose or grass juice. The initial substrate concentration was 10 g_{COD}/L and the serum vials were spiked with 10 g_{COD}/L every 2 days to a total COD addition of 60 g_{COD}/L.

- Finally, to establish the potential product inhibition provoked by caproic acid, fed-batch experiments were conducted at 50°C using glucose, without and with the initial presence of caproic acid. The initial substrate concentration was 10 g_{COD}/L and each serum vial was spiked once with 10 g_{COD}/L to a total COD load of 20 g_{COD}/L. When caproic acid was added, its initial concentration was around 3.7 g/L (titer at which caproic acid production ceased in the previous fed-batch experiment), while when no additional caproic acid was provided, its initial concentration was 1.5 g/L.

2.3 Analytical techniques

The pH of raw samples was measured using a pH sensor (SP10T, Consort, BE). Carboxylic acids (acetic, butyric, isobutyric, propionic, valeric, isovaleric, caproic, and isocaproic acids) were quantified using a gas chromatograph (GC-2014, Shimadzu®, NL) equipped with a flame ionization detector (FID) and a DB-FFAP 123-3232 column (30m x 0.32 mm x 0.25 µm; Agilent, BE). Sulfuric acid, sodium chloride and 2-methyl hexanoic acid (internal standard) were added in diluted and filtered liquid samples (0.2 µm PVDF filters, Chromafil®) and carboxylic acids were extracted with diethyl ether. Prepared sample (1 µL) was injected at 200°C with a split ratio of 60 and a purge flow of 3 mL/min. The oven temperature increased from 110°C to 165°C at a rate of 6°C/min where it was kept for 2 min. The FID temperature was set at 220°C and the carrier gas (nitrogen) was supplied at a flow rate of 2.49 mL/min. Lactic and formic acids were determined in filtered samples (0.2 µm PVDF filters, Chromafil®) using an ion chromatograph (930 Compact IC Flex; Metrohm, CH), equipped with a Metrosep organic acids 250/7.8 column, a Metrosep organic acids guard column/4.6 and a 850 IC conductivity detector (Metrohm, CH). These organic acids were eluted at a flow rate of 0.5 mL/min using H_2SO_4 1 mM (95-98%, Carl Roth).

2.4 Molecular techniques

DNA from the batch, fed-batch and continuous experiments was extracted by means of bead beating with a PowerLyzer (Qiagen, Venlo, the Netherlands) and phenol/chloroform extraction (De Paepe et al., 2017).

10 µL of the DNA extract was sent out to LGC genomics GmbH (Berlin, Germany) for library preparation and sequencing on an Illumina MiSeq platform with v3 chemistry. The primers used were 341F (5'-CCT ACG GGN GGC WGC AG −3') and 785Rmod (5'-GAC TAC HVG GGT ATC TAA KCC-3'). Read assembly and cleanup was based on the MiSeq SOP described by Schloss et al. (2009). In brief, mothur (v.1.40.3) was used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED alignment, v. 123), remove chimeras, assign taxonomy using a naïve Bayesian classifier and SILVA NR v132 and cluster contigs into OTUs at 97% sequence similarity. All sequences that were classified as Eukaryota, Archaea, Chloroplasts and Mitochondria were removed. In addition, sequences that could not be classified at all (even at (super) Kingdom level) were removed. After the above-mentioned filtering, for each OTU representative sequences were picked as the most abundant sequence within that OTU.

2.5 Data analysis and availability

Data analysis was performed using R (v4.0.2). Regarding the amplicon sequencing data, the 10 most abundant genera were displayed using the *phyloseq* package (v 1.32.0). Alpha diversity was calculated using the observed richness and the inverse Simpson diversity index based on the OTU abundance matrix. The calculation of the taxonomic beta diversity was based on Bray-Curtis dissimilarity index, using the *phyloseq* package. A non-metric multidimensional scaling (NMDS) analysis was then performed to illustrate the community structure similarities. Raw 16S rRNA gene sequences were deposited on the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA853508.

3. Results

3.1 Continuous thermophilic caproic acid production using grass juice at different pH, temperature and sugar availability

3.1.1 Effect of pH

The effect of pH was tested at 45°C in a continuous reactor (HRT 4 days) fed with grass juice (**Figure 1(a)**). At a pH of 5.5, lactic acid accumulated (18 ± 2.1 g/L) as the main fermentation product (63 ± 4.0% of total carboxylic acids in mass). The maximum caproic acid achieved under these conditions was 1.9 ± 0.2 g/L, representing only 6.6 ± 0.7% of the total carboxylic acids in mass (**Figure 1(b)**). Transient accumulation of propionic acid was also noted (up to *ca*. 3.5 g/L). The initial microbial community at the start of the continuous operation was clearly dominated (80%) by lactic acid bacteria belonging to the genus *Pediococcus* (**Figure 1(c)**). By the time caproic acid production was first observed in continuous mode (at 45°C, pH 5.5), the community composition changed substantially and was dominated (23%) by *Clostridium*_sensu_stricto_7 followed by *Lactobacillus* (13%), while the abundance of *Caproiciproducens* increased to 10%. At the end of the cultivation at 45°C and pH 5.5 the community was dominated (61%) by *Lactobacillus*, and the abundance of *Caproiciproducens* was 26%, which correlates with the dominance of lactic acid in the product spectrum and the consolidation of caproic acid titer at 1.9 g/L.

When the pH was increased to 6.0 the product profile shifted drastically: lactic acid was barely detected and the main product was butyric acid (61 ± 3.1% of total carboxylic acids) at a concentration of 11 ± 2.0 g/L. The caproic acid concentration (1.7 ± 0.15 g/L) was not substantially affected with this change, however, its selectivity increased to 9.6 ± 1.8% of the total carboxylic acids due to the overall lower concentration of carboxylic acids in the system (18 ± 2.6 g/L at pH 6.0 compared to 29 ± 1.8 g/L at pH 5.5). The pH increase to 6.0 additionally caused a shift in the community, with *Caproiciproducens* being now the dominant genus (40%) and *Lactobacillus* almost disappearing from the system (0.44%), which is in line with the disappearance of lactic acid. Finally, the abundance of *Caldicoprobacter* sharply increased to 17%.

When increasing the pH further to 6.5, the concentrations of butyric, caproic and lactic acids remained relatively unaffected, but the concentration of acetic acid doubled compared to pH 6.0. At this point, the dominance of *Caproiciproducens* decreased to 24% and the second most dominant genus was *Tepidanaerobacter* (14%).

Considering the higher selectivity (9.6 ± 1.8%) compared to the other values tested here, pH 6.0 was deemed as the optimal pH value for thermophilic caproic acid production. This choice was further validated by the higher relative abundance of *Caproiciproducens* (40%) compared to pH 5.5 and 6.5.

3.1.2 Effect of temperature

After fixing pH to 6.0, the temperature of the reactor was gradually elevated (1.0°C/day) to 50°C (**Figure 1(a)**). The caproic acid concentration increased to 3.4 ± 0.16 g/L representing 21 ± 0.61% of total carboxylic acids, a 2.4 times higher selectivity than 45°C (8.7 ± 0.96%) (**Figure 1(b)**). At the same time, butyric acid decreased to 7.8 ± 0.29 g/L (48 ± 1.0% of total carboxylic acids). The abundance of *Caproiciproducens* slightly increased (42%), the abundance of *Caldicoprobacter* drastically decreased (1.8%) whereas the abundances of Family_XI_unclassified (9.6%) and *Herbinix* (9.5%) increased compared to 45°C and pH 6.0 (**Figure 1(c)**).

When the temperature was further increased to 55°C, the total concentration of carboxylic acids decreased by 8.2 ± 0.72% compared to 50°C, while lactic acid started accumulating. In particular, at 55°C caproic acid concentration decreased down to 0.89 ± 0.33 g/L and its selectivity (6.5 ± 3.4%) was 3.3 times lower than at 50°C. This decrease was accompanied by a decrease in the abundance of *Caproiciproducens* (29%), indicating the possibility that this temperature was close to the maximum threshold of this genus. The abundance of Family_XI_unclassified further increased to 29%, while *Herbinix* remained at same levels (10%).

In summary, considering the increased concentration (3.4 ± 0.16 g/L) and selectivity of caproic acid production (21 ± 0.61%), it can be concluded that 50°C was the optimal temperature tested for thermophilic caproic acid production.

3.1.3 Effect of sugar availability

Based on the hypothesis that caproic acid production is linked with glucose, an additional experiment was performed at 50°C and pH = 6.0, where grass juice was supplemented with glucose without increasing the COD concentration (**Figure 1(a)**). The caproic acid concentration increased up to 7.2 g/L (day 197) with an average value of 6.0 ± 1.0 g/L (74% increase compared to non-amended grass juice) representing 32% of the total carboxylic acids. The butyric acid concentration remained at the same levels (7.3 g/L). The selectivity in COD yielded equal shares for butyric and caproic acids (40 ± 6.1 and 39 ± 3.8% in COD), corresponding to 30% of the incoming COD (15 ± 1.4 % for butyric acid; 15 ± 2.4 % for caproic acid) (**Table S1**). At this point, the abundance of *Caproiciproducens* sharply increased to 75% (**Figure 1(c)**) and the alpha diversity – a measure of the within-sample diversity or richness presented the lowest value (**Figure S3**).

3.2 Batch thermophilic caproic acid production using different substrates

A series of batch tests (**Figure 2**) were performed to pinpoint the real substrate used for caproic acid production under the selected conditions, using the main sugars/carbohydrates present in grass juice and lactic acid which can be the main primary fermentation product (section 3.1). The use of glucose and xylose resulted in similar total carboxylic acid production (3.7 ± 0.30 and 4.1 ± 0.29 g/L, respectively) and similar product profiles, dominated by caproic and acetic acids at the end of the experiment. The net caproic acid production during the batch was 1.5 ± 0.071 g/L and 1.6 ± 0.042 g/L and the selectivity (based on this net production) was 41 ± 3.9% and 38 ± 2.9% in the case of glucose and xylose, respectively, which is substantially higher than the values obtained under continuous operation using non-amended grass juice (**Figure 1(b)**). The main difference between the product profiles was the concentration of acetic and butyric acids, with 1.5 times more acetic acid and roughly half the butyric acid concentration when xylose

was used compared to glucose. The community composition was affected by the selected substrate (**Figure 2(e)-(g)**). When glucose was used, during the 4th day of the experiment, the dominant species was *Caproiciproducens* (44 ± 3.9%) followed by *Caldicoprobacter* (12 ± 0.33%) and *Sporanaerobacter* (32 ± 4.1%). When the substrate was xylose the abundance of *Caproiciproducens* sharply increased to 63 ± 3.4%, the second most dominant species was *Sporanaerobacter* (15 ± 2.3%), and *Caldicoprobacter* remained at the same levels (12 ± 0.88%). The community in the case where xylose is used as a substrate was narrower, as reflected by the lowest value of the alpha diversity (4.3 ± 0.44 compared to 7.4 ± 0.21 for glucose and 7.2 ± 0.65 for lactic acid; **Figure S3**).

The production of carboxylic acids when cellulose was used was negligible. Finally, when lactic acid was the substrate, its fermentation was highly directed to butyric acid (net selectivity of 62 ± 4.7%) and final titer of 4.0 ± 0.23 g/L) as well as to propionic acid (net selectivity of 30 ± 2.1%, final titer of 2.0 ± 0.11 g/L), while no caproic acid production was noted. In this case, the dominance of *Caproiciproducens* drastically decreased to 13 ± 2.3%, and the most dominant species was *Caldicoprobacter* (24 ± 3.0%) (**Figure 2(g)**).

3.3 Fed-batch thermophilic caproic acid production using glucose and grass juice

A series of fed-batch experiments (**Figure 3**) was performed to assess whether the type of substrate and its availability affect the product profile, and to identify the caproic acid concentration limit using glucose or grass juice. Glucose was chosen as substrate over xylose since it resulted in a higher caproic acid productivity during the batch tests (0.73 for glucose vs 0.68 g/L/d for xylose, not considering the lag phase, (section 3.2)). When glucose was the substrate of fed-batch experiments, 2.4 ± 0.11 g/L of caproic acid were produced (**Figure 3(a)**). The lactic acid concentration peaked on day 11 (6.9 ± 0.97 g/L), after which it was gradually fully consumed and the most dominant product at the end of the experiment was butyric acid (6.1 ± 0.43 g/L). The community was dominated (48 ± 3.7%, day 5) by *Caproiciproducens* and the second most dominant genus (20 ± 2.7%) was Incertae_Sedis from the *Ethanoligenenaceae* family (**Figure 3(c)**).

In contrast, when grass juice was used, neither substantial net caproic acid production was noted (0.21 ± 0.074 g/L) nor a lactic acid peak, and the final concentration of the most dominant product, butyric acid, was 8.0 ± 0.12 g/L. The abundance of *Caproiciproducens* was substantially lower (14 ± 1.6%, day 5) compared to glucose **Figure 3(d)**). The most dominant genus in this case was Family_XI_unclassified (24 ± 2.9%), and substantial increase in the abundance of *Clostridium*_sensu_stricto_7 (12 ± 6.3%) was noted. In this case, the community was richer compared to the case where glucose was the substrate (**Figure S3**).

Given that a plateau in the caproic acid concentration was quickly reached during the fed-batch tests, an additional fed-batch experiment where glucose was used as a substrate was performed, to assess whether the caproic acid concentration plays a role in the product profile (**Figure 4**). The results show that, at the initial absence of caproic acid, 1.0 ± 0.19 g/L caproic acid were produced, while the main net products were butyric acid (4.7 ± 0.49 g/L) and acetic acid (1.3 ± 0.17 g/L). In contrast, when caproic acid was initially present (3.5 g/L) no additional caproic acid was produced, and the supplied glucose was channeled to lactic acid (up to 8.7 ± 1.0 g/L) followed by its gradual consumption and concurrent increase in butyric acid concentration (6.4 ± 0.52 g/L).

4. Discussion

4.1 Effect of pH and temperature on the thermophilic production of caproic acid

Given the relevance of pH as a process parameter in shaping the product profile and microbial community composition in chain elongation processes at mesophilic conditions (Candry et al., 2020a; Lim et al., 2008), we explored its effect at thermophilic conditions. The results show that the process behaved in a different way than at mesophilic conditions as the caproic acid concentration remained relatively constant (1.7 ± 0.15 – 1.9 ± 0.16 g/L) across the tested pH range (5.5 – 6.5) and the highest selectivity (9.6 ± 1.8%) was noted at pH 6.0. The main differences in the product profile are that pH < 6 led to lactic acid accumulation (18 ± 2.1 g/L), pH ≥ 6.0 promoted butyric acid production (11 ± 1.2 g/L), and pH 6.5 led to higher acetic

318 acid concentrations (**Figure 1**). During fermentation of food waste at mesophilic conditions, pH 5.0 led to
319 the production of up to 2.0 g/L caproic acid, pH 5.5 halved its concentration (0.97 g/L) and at pH 6.0 caproic
320 acid production ceased (Lim et al., 2008). Candry et al. (2020a) found that during lactic acid-driven
321 mesophilic chain elongation, pH > 6.0 led to lactic acid being mainly converted to propionic and acetic
322 acids, while pH < 6.0 favored chain elongation products (*e.g.* caproic and butyric acids). Here, an increase
323 in the pH did not favor the lactic acid oxidation pathway to yield propionic acid. Specifically, propionic acid
324 titers remained low (<1.0 g/L) and constant throughout the operation at pH 6.0 – 6.5 and it was only
325 transiently accumulated in the system at pH 5.5 (**Figure 1**).

326 Another important difference between the results obtained here and the common observations in
327 mesophilic systems is the fact that lactic acid consumption was very sensitive to low pH values and appears
328 to be halted at pH values below 6.0. Toxicity of organic acids is higher at lower pH, since pH values in the
329 vicinity (*i.e.* one unit above) of the pk_a of the acid (**Table S2**) or lower increase the abundance of the
330 undissociated acid form, which freely diffuses through the membrane causing inhibition (Warnecke and
331 Gill, 2005). This has a higher importance under elevated temperatures, since thermophiles are more
332 sensitive to changes in environmental conditions compared to mesophiles (Yenigün and Demirel, 2013).
333 Lowering the pH, thereby increasing the free acid form of carboxylic acids, under thermophilic conditions
334 might then impact the process significantly more compared to mesophilic conditions.

335 Similar to pH, temperature changes substantially affect the product profile and community composition
336 of chain elongation processes (de Groof et al., 2019). Therefore, the effect of temperature at the
337 thermophilic range was investigated at pH 6.0. The increase of temperature from 45°C to 50°C positively
338 impacted caproic acid production as indicated by the 2-fold increase in its concentration (from 1.7 ± 0.15
339 g/L to 3.4 ± 0.2 g/L) (**Figure 1**). The positive effect of increasing the fermentation temperature in mesophilic
340 food waste fermentation was demonstrated by Lim et al. (2008). No caproic acid was produced at 25°C,
341 while the elevation at 35°C yielded up to 0.97 g/L caproic acid and further increase to 45°C increased 5-

342 fold the caproic acid concentration (up to 5.0 g/L) (Lim et al., 2008). Here, with further temperature
343 increase to 55°C the system showed signs of inhibition as indicated by the 8.2% drop in the concentration
344 of total carboxylic acids, the lactic acid accumulation and the caproic acid concentration drop to 0.89 ±
345 0.33 g/L. This indicates that at 55°C sugars were mainly converted to lactic acid which was not further
346 converted to butyric acid, potentially due to lower activity or lower presence of lactic acid-driven chain
347 elongators. This can be ascribed to a deviation from the physiological optimum temperature of caproic
348 acid-producers present in the system, which appears to be 50°C. Therefore, from the values tested, the
349 most favorable temperature for caproic acid production was 50°C. Similar observations on the impact of
350 temperature on caproic acid concentrations have been previously reported in experiments for hydrogen,
351 methane and/or SCCA production. For instance, during fermentation of water hyacinths, the caproic acid
352 concentration decreased 10-fold when the fermentation temperature increased from 40°C (0.50 g/L) to
353 55°C (0.050 g/L) (Forrest et al., 2010). Similarly, during syngas fermentation, the production of caproic acid
354 (0.88 g/L) was noted at 35°C while no caproic acid was produced at 55°C (Shen et al., 2018). Nevertheless,
355 it should be noted that the temperature tested in these studies did not fall within the same temperature
356 range (mesophilic or thermophilic), while no prior adaptation was carried out. This may not have allowed
357 for the best performance (or even tolerance) by the autochthonous microbial communities. Another
358 possible explanation for the stark influence of temperature on the process performance is its effect on
359 membrane properties. At elevated temperatures, the membrane fluidity and permeability can drastically
360 increase as a result of the inability to maintain a liquid crystalline lipid phase (Siliakus et al., 2017). This is
361 counteracted by altering the composition of lipids in the membrane. This could negatively affect the
362 proton permeability, increasing thus the inhibiting effect of free carboxylic acid molecules.

363 **4.2 Substrate composition and availability play a key role in thermophilic caproic acid production**

364 The results of the present study indicate that, at thermophilic conditions, sugar monomers (*i.e.* glucose
365 and xylose) have the potential to yield caproic acid at high selectivity and that lactic acid consumption is

not linked with caproic acid production. This was suggested in the batch tests performed with individual monomers (section 3.2) and validated by the last phase of the continuous operation, where increased glucose levels yielded additional caproic acid (section 3.1.3). In the latter case, where the sugar/carbohydrate content of grass juice was increased by 50% while maintaining the same organic (COD) load (50°C, pH 6.0), an average concentration of 6.0 ± 1.0 g/L caproic acid was achieved in the reactor representing 40% of the carboxylic acid COD. The peak concentration was 7.2 g/L (day 197) which, together with the average titer achieved here, is the highest concentration reported so far under thermophilic conditions. Specifically, Zhang et al. (2022) achieved 4.7 g/L (10 g_{COD}/L) under semi-continuous fermentation of food waste at 55°C, while other reports mention concentrations below 1.0 g/L – *i.e.* 0.14 – 0.74 g/L at 45 – 55°C (**Table S4**). The negligible carboxylic acid production during batch fermentation of cellulose (**Figure 2(c)**) hinted that hydrolysis did not take place in our system, which appears to have the genetic potential to utilize cellulose, since members of the *Herbinix* genus are thermophilic cellulose degraders (Koeck et al., 2015). The lack of carboxylic acid production from cellulose in the batch tests could be ascribed to the lower temperature of the experiment (45°C) compared to the optimal temperature (55°C) of members of the genus (Koeck et al., 2015), or potentially due to the much slower hydrolysis kinetics (Dionisi et al., 2015; Kataeva et al., 2013).

Another finding of our work is that lactic acid consumption directly correlates with mainly butyric acid production. The use of lactic acid led to butyric acid production (4.0 ± 0.23 g/L) in batch experiments (**Figure 2(d)**). In fed-batch experiments using glucose (**Figure 3(a)**) butyric acid was only a relevant product after the accumulated lactic acid was consumed, confirming again the tight link between lactic acid consumption and butyric acid production. During continuous operation, this fact was further confirmed as the concentration of these two acids showed an inverse relationship (**Figure 1(a)**). When lactic acid was barely detected in the system the main product was butyric acid, and an increase in lactic acid concentration coincided with a decrease in butyric acid concentration (days 130 – 134). The caproic acid

concentration did not seem to be affected by the changes in lactic acid concentration in any of these cases. These facts suggest that lactic acid could be an intermediate in butyric acid production and not in caproic acid production. Similar results were noted by Zhang et al. (2022), where the product profile was dominated by butyric acid instead of caproic acid when lactic acid was the only substrate in a thermophilic chain elongation process. These facts strongly suggest the possibility that the thermophilic chain elongators yielding caproic acid in our system cannot utilize lactic acid as substrate and that the microorganisms using lactic acid for chain elongation yield mainly butyric acid as end-product. These findings do not correlate with the observations described in mesophilic chain elongation processes where lactic acid can be used for extensive caproic acid production (Carvajal-Arroyo et al., 2019; Wang et al., 2022), presenting notable production (0.81 g/L caproic acid) even at initial concentrations as low as 7.2 g/L lactic acid (Xie et al., 2021). Finally, lactic acid consumption was also linked to propionic acid production, as shown by the production 2.0 ± 0.11 g_{PA}/L during the lactic acid-fed batches (**Figure 2(d)**), and its transient accumulation (up to *ca*. 3.5 g/L) during continuous operation at pH 5.5 (**Figure 1**).

Our results also show that substrate availability (here sugar monomers) plays a major role in shaping the product profile of the process. For instance, during fed-batch tests (**Figure 3(a)**-**(b)**), the 233% higher availability of glucose (10 g_{COD}/L/feeding) when it was provided as a pure substance led to 2.4 g/L caproic acid production compared to marginal production (0.21 g/L) when using grass juice containing glucose (*ca*. 3 g_{COD}/L/feeding). In this case, it is likely that caproic acid-producers could not get a significant share of the sugar monomers due to competition with other microbial groups such as lactic acid-producers (and subsequently butyric acid-producers from lactic acid), which consumed most of the substrate. More specifically, we hypothesize that the answer to this question lies within the ecological r/K framework (Favere et al., 2021), with lactic acid bacteria being r-strategists – thereby having high growth rates at high substrate availability, and chain elongators being K-strategists – characterized by high substrate affinity. This can be further explained by the fact that lactic acid bacteria likely have shorter metabolic pathways

than chain elongators, which may in turn lead to faster growth rates, whereas chain elongators potentially having longer pathways could be characterized by high ATP yields (Kreft et al., 2020). In this context, chain elongators are likely outcompeted by lactic acid producers in (fed-)batch tests where the initial substrate concentration is high, but thrive under continuous operation where the oligotrophic microorganisms (characterized by low Ks values) can prevail. Nevertheless, this hypothesis could be valid if both microbial groups are present in the community and remains to be proven. Furthermore, during the last phase of continuous operation where the abundance of sugars/carbohydrates was 50% increased by glucose addition, the caproic acid concentration increased by 74%. The positive impact of increased OLR was also observed under mesophilic (37°C) fermentation of food waste, where OLR of 5 $g_{substrate}$/L/d did not result in caproic acid production while caproic acid production was noted at OLR 9 $g_{substrate}$/L/d (up to 0.97 g/L) and 13 $g_{substrate}$/L/d (up to 3.7 g/L), with the latter increase leading to 52% higher selectivity (35°C, pH 5.5) (Lim et al., 2008).

Finally, we have shown that, similar to mesophilic conditions (Andersen et al., 2017), the presence of caproic acid affects the product profile under thermophilic conditions. In fed-batch experiments using glucose (**Figure 3(a)**) the highest concentration achieved was 3.7 g/L (net production of 2.4 g/L). After this concentration was reached, lactic acid accumulated which was further converted to butyric acid, meaning that glucose was channeled to other end-products. This shows that caproic acid increased until a concentration in which it started to be inhibiting for caproic acid producers. At that point, lactic acid bacteria had the chance of consuming a higher share of the substrate and their product (*i.e.* lactic acid) was eventually converted to butyric acid. This was also verified by a fed-batch experiment using glucose where 3.7 g/L caproic acid was initially added, and no additional caproic acid was produced (**Figure 4**). It can therefore be concluded that concentrations of *ca*. 3.7 g/L caproic acid are approaching the inhibition limit under semi-continuous thermophilic conditions, whereas under continuous mode, higher concentrations can be achieved, however, with fluctuations (both at pH 6.0 and 50°C). Similar results were

noted under mesophilic conditions (30°C), where inhibition was noted at caproic acid concentration of 4.0 g/L (initial pH 5.5, 30°C) (Pan et al., 2020). Similar to our work, Pan et al. (2020) report fluctuations during continuous operation between 1.6 and 5.4 g/L as a result of caproic acid inhibition.

4.3 Microbial community structure during caproic acid production under thermophilic conditions

Similar to the main performance indicators, the microbial community composition was largely affected by substrate (**Figure 1(c)**; **Figure 2(e)-(g)**; **Figure 3(c)-(d)**), temperature and pH (**Figure 1(c)**). In general, the use of sugars (*i.e.* glucose, xylose) promoted the presence of *Caproiciproducens* which was represented by 130 different OTU, and we believe was the main caproic acid-producer in our system. During the batch tests, the use of xylose resulted in the highest abundance of *Caproiciproducens* (63 ± 3.4% compared to 44 ± 3.9% for glucose) (**Figure 2(e)-(g)**). In this case the community was less diverse and potentially more specialized in caproic acid production (**Figure S3**). The same was observed in the last part of the continuous operation, where additional sugars were provided (*via* glucose addition), and the abundance of *Caproiciproducens* increased to 75% (**Figure 1(c)**). The fact that the presence of *Caproiciproducens* is promoted by the presence of sugars was also demonstrated under mesophilic conditions (34 – 35°C) where *Caproiciproducens* dominated the culture (80%) in a medium with xylose (10 g/L) (Tang et al., 2022), and in thin-stillage fermentation with and without glucose amendment (up to 86% abundance in granular biomass) (Mariën et al., 2022). The presence of *Caproiciproducens* has also been noted in other thermophilic reactors producing caproic acid from sugar-rich substrates such as grass juice at 45°C (Sakarika et al., 2022) and food waste at 55°C (Zhang et al., 2022).

The results under thermophilic conditions contradict findings under mesophilic conditions using sugar-rich substrates, where caproic acid production is often ascribed to the consumption of both sugars and lactic acid, therefore the communities are often rich in lactic acid-producers. This was noted during thin-stillage fermentation (34°C) where the community was dominated by *Caproiciproducens* and the lactic acid-producing *Olsenella* (Mariën et al., 2022). Similarly, during switchgrass stillage fermentation using a

pretreated stream rich in xylose at 35°C, *Pseudoramibacter* and *Roseburia* were identified as the responsible microbial genera for the production of caproic acid, in a community dominated by *Lactobacillus* (Scarborough et al., 2018). Under mesophilic conditions members of the genus *Megasphaera* (Veillonellaceae family) have been reported to utilize sugars for caproic acid production (Jeon et al., 2017). This family was not detected in our system, which was to be expected given that their reported growth temperatures fall within the mesophilic range (30 – 40°C) (Jeon et al., 2017). Additionally, in thermophilic fermentations (55°C) of pretreated corn fiber the genus *Thermosinus,* which belongs to Veillonellaceae, was correlated with the production of caproic acid from lactic acid (main product was butyric acid), indicating that thermophilic chain elongators of this family do exist (Agler et al., 2012), but were not present in our system.

The increase of *Clostridium*_sensu_stricto_7 during fed-batch experiments using grass juice where butyric acid was the main organic acid produces suggests that this genus was the main butyric acid producer in this system (**Figure 3(d)**). Similar to our work, Zhang et al. (2022) identified *Clostridium*_sensu_stricto_7 as the main genus responsible for lactic acid-driven butyric acid production under thermophilic conditions (55°C). Nevertheless, in our work, it is likely that also some of the butyric acid stemmed from the activity of *Caproiciproducens*. It is also described that mesophilic chain elongators like *Caproicibacterium lactatifermentans* (Wang et al., 2022) can often use both sugars and lactic acid as substrates. A similar observation was made by Ingle et al. (2021), where during fermentation of hydrolyzed dairy manure under mesophilic conditions (35°C, pH 5.0) lactic acid was first produced and subsequently consumed together with the remaining sugars, and the abundance of *Caproiciproducens* (and *Clostridium*_sensu_stricto_12) was positively correlated with butyric acid production.

Apart from the substrate, pH and temperature also shaped the microbial community composition, as indicated by the large community turnover when varying these parameters (**Figure S4**). Specifically, experiments conducted at pH 5.5 (45°C) promoted the presence of *Lactobacillus* (61%), whereas at pH 6.0

the most dominant genera were *Caproiciproducens* (40%) and *Caldicoprobacter* (17%) – a genus that is reported to produce lactic and acetic acids and ethanol from sugars under thermophilic conditions (Yokohama et al., 2010). Further increase to pH 6.5 yielded a culture dominated by *Caproiciproducens* (24%) and *Tepidanaerobacter* (14%), with members of this genus able to consume lactic acid at pH 6.0 – 7.0 and 45 – 50°C and produce mainly acetic acid, propionic acid and valeric acid (Sekiguchi et al., 2006). The increase of temperature from 45 to 50°C did not substantially affect the abundance of *Caproiciproducens* (42%), nevertheless, the abundance of *Caldicoprobacter* decreased from 17 to 1.8%. The uncharacterized genus Family_XI_unclassified and *Herbinix* – which can produce acetic acid, small amounts of propionic acid and ethanol from cellulose (Koeck et al., 2015) – increased to 9.6 and 9.5% respectively. *Sporanaerobacter*, which is reported to produce acetic acid, isobutyric and isovaleric acids and H_2 from sugars was mostly present at experiments conducted at 45°C (**Figure 1(a)**; **Figure 2(e)-(g)**), which is expected since this genus is mesophilic, but can withstand mildly thermophilic conditions (up to 50°C) (Hernandez-Eugenio et al., 2002). Further increase to 55°C decreased the abundance of *Caproiciproducens* to 29%, indicating the that this temperature is beyond its physiological optimum which appears to be close to 50°C. At this point the abundance of Family_XI_unclassified increased to 29% and the genus *Herbinix*, composed of thermophilic cellulose degraders with optimal temperature 55°C (Koeck et al., 2015), remained at the same levels (10%).

5. Conclusions

We have shown that caproic acid production under thermophilic conditions using grass juice was favored at pH 6.0 and 50°C, amongst the conditions tested. In our system, the substrate for caproic acid production under thermophilic conditions was sugars (glucose, xylose), while lactic acid resulted in high butyric acid yields. The use of xylose favored the presence of *Caproiciproducens*, as indicated by its *ca.* 20% higher relative abundance compared to the use of glucose and the 50% higher abundance compared to batch tests using lactic acid. When grass juice was supplemented with glucose (50°C, pH 6.0), we achieved

510 the highest caproic acid concentration reported thus far (7.2 g/L at day 197) under thermophilic
511 conditions. At this point, *Caproiciproducens* dominated the microbial community (75%) and caproic acid
512 selectivity (32 ± 3.1%) was substantially increased compared to the use of non-amended grass juice (21 ±
513 0.61%), indicating the role of substrate availability in the product profile. To position thermophilic chain
514 elongation as a mature and robust technology and with potential to outcompete the performance
515 achieved under mesophilic conditions it is imperative to fill the existing knowledge gaps. These include
516 elucidating the role of different substrates, defining the microbial species involved in thermophilic chain
517 elongation and their metabolic characteristics as well as the role of possible competing, commensal or
518 mutualistic communities in this environment.

519 **Acknowledgements**

520 The authors gratefully acknowledge Tim Lacoere for his assistance on the molecular analyses. A.R.
521 acknowledges the support of the Xunta de Galicia through a postdoctoral fellowship (ED481B-2021-012).
522 A.R. belongs to a Galician Competitive Research Group (GRC ED431C 2021/37), co-funded by ERDF (UE).
523 R.G. gratefully acknowledges support from the Special Research Fund of Ghent University
524 (BOF19/STA/044).

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Figure captions

Figure 1: **(a)** Concentration of the different carboxylic acids during continuous fermentation of grass juice. Carboxylic acids with concentrations ≤1 g/L are lumped here (*i.e.* minor products, including formic, isobutyric, isovaleric and valeric acids) and their individual concentration is presented in **Figure S2**. The shaded area indicates the transition periods between different operational conditions. Black arrows illustrate the samples where the microbial community composition analysis was performed. **(b)** Selectivity of butyric, caproic and lactic acids in total carboxylic acid mass throughout the experimental period. Average values ± standard deviation are presented. **(c)** Microbial community composition at the selected timepoints of the continuous operation. Relative abundance of top 10 genera was calculated based on their combined relative abundance across all samples.

Figure 2: Net production and/or consumption of the different carboxylic acids during batch fermentation of **(a)** glucose, **(b)** xylose, **(c)** cellulose and **(d)** lactic acid. Carboxylic acids with concentrations lower than 1.0 g/L (*i.e.* minor products) are summed. The concentration of carboxylic acids in the control experiments (addition of distilled water instead of substrate) were subtracted to illustrate the net production and/or consumption. Average values ± standard deviation are presented. Black arrows illustrate the samples where microbial community composition analysis was performed. Microbial community composition at the selected timepoints of the batch tests using **(e)** glucose, **(f)** xylose and **(g)** lactic acid. Relative abundance of top 10 genera was calculated based on their combined relative abundance across all samples.

Figure 3: Net production and/or consumption of the different carboxylic acids during fed-batch fermentation of **(a)** glucose and **(b)** grass juice. Carboxylic acids with concentrations *ca.* 0.10 g/L (*i.e.* minor

products) are summed. Asterisks indicate substrate addition (10 g_{COD}/L). Black arrows illustrate the samples where microbial community composition analysis was performed. The concentration of carboxylic acids in the control experiments (addition of distilled water instead of substrate) were subtracted to illustrate the net production and/or consumption. Average values ± standard deviation are presented. Microbial community composition at the selected timepoints of the fed-batch tests using **(c)** glucose and **(d)** grass juice. Relative abundance of top 10 genera was calculated based on their combined relative abundance across all samples.

Figure 4: Net production and/or consumption of the different carboxylic acids during fed-batch fermentation of **(a)** glucose and **(b)** glucose with the addition of caproic acid. Carboxylic acids with concentrations *ca.* 0.10 g/L (*i.e.* minor products) are summed. Asterisks indicate substrate addition (10 g_{COD}/L). The concentration of carboxylic acids in the control experiments (addition of distilled water instead of substrate) were subtracted to illustrate the net production and/or consumption. Average values ± standard deviation are presented.

Figures

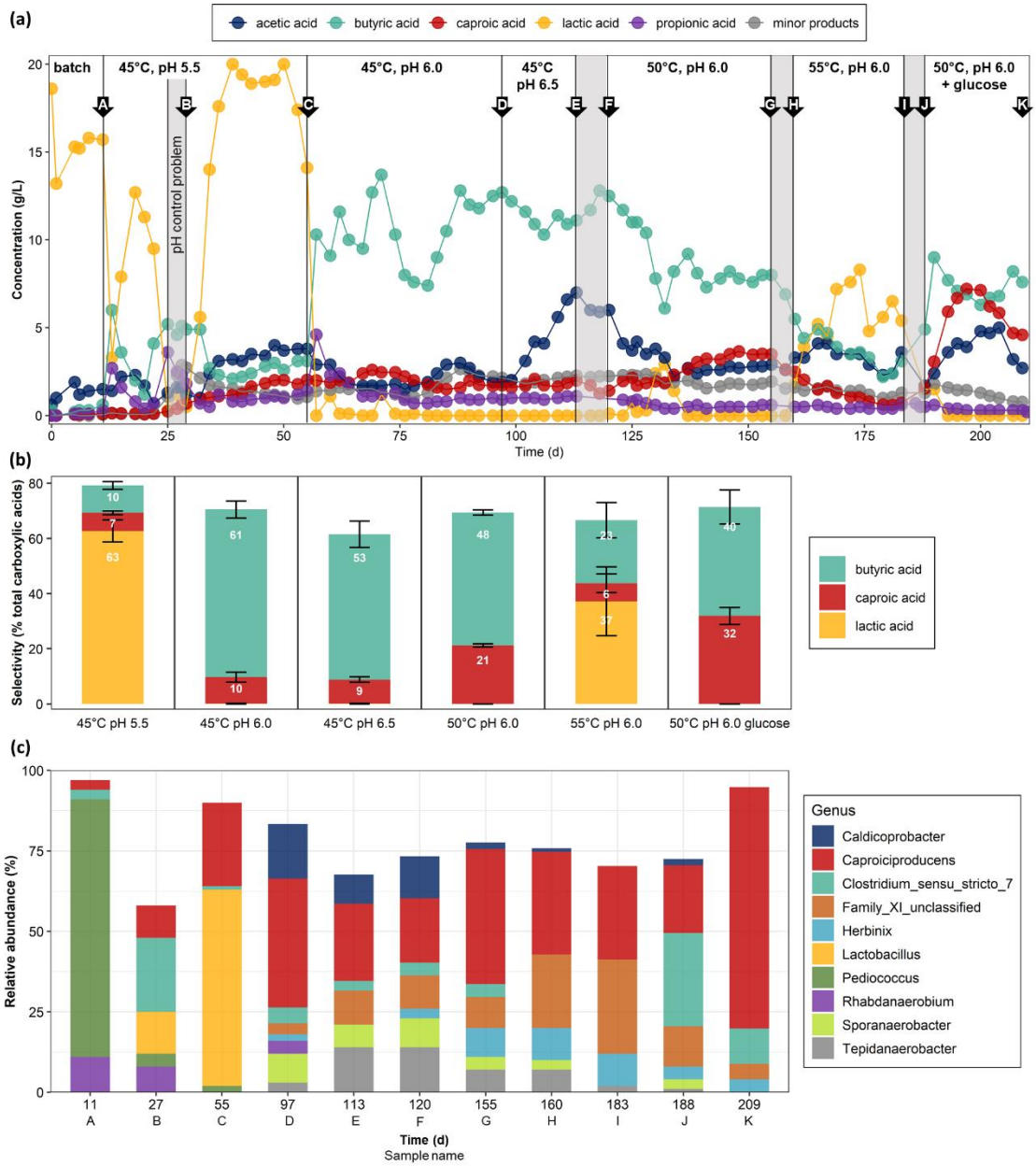
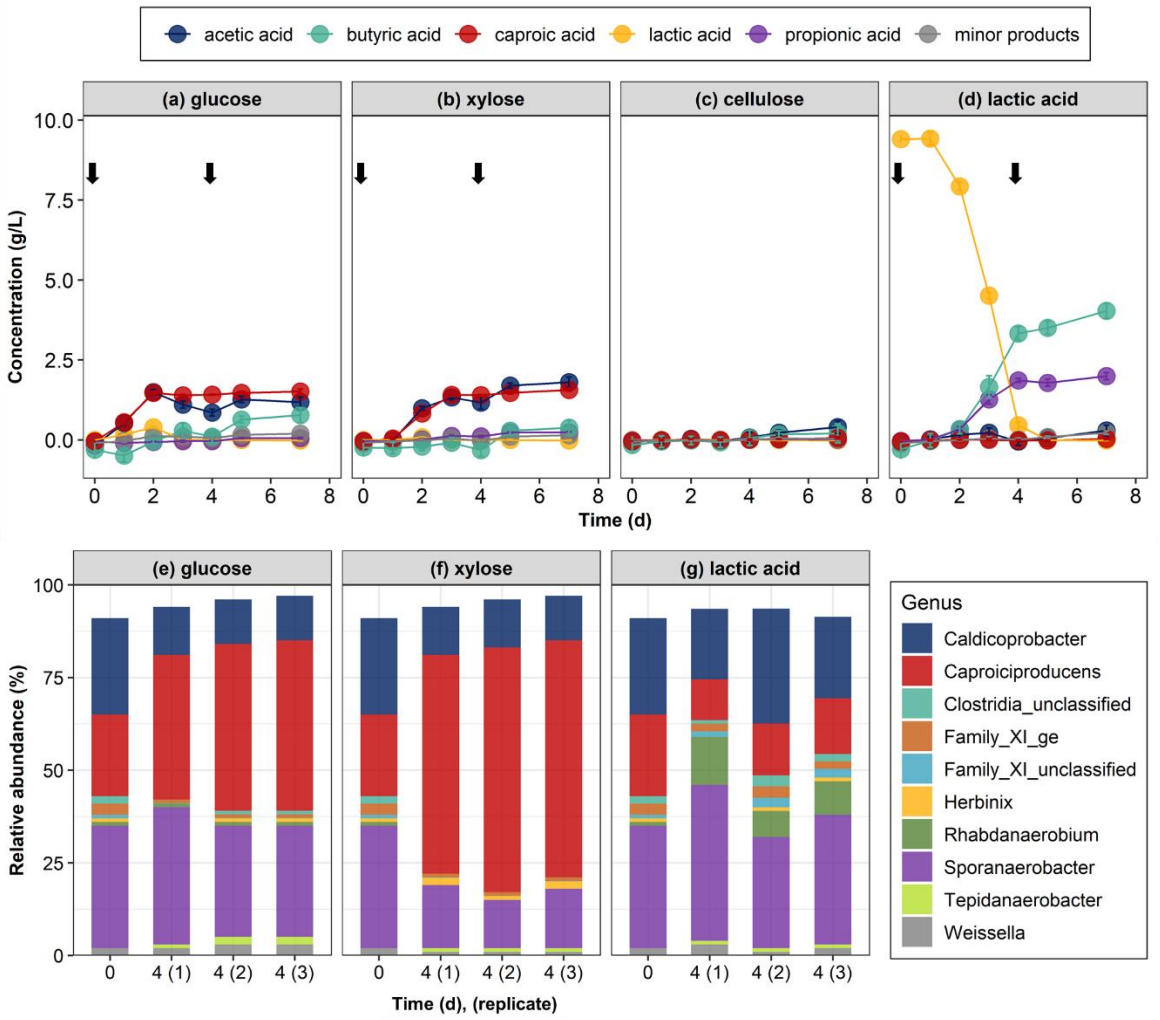


Figure 1



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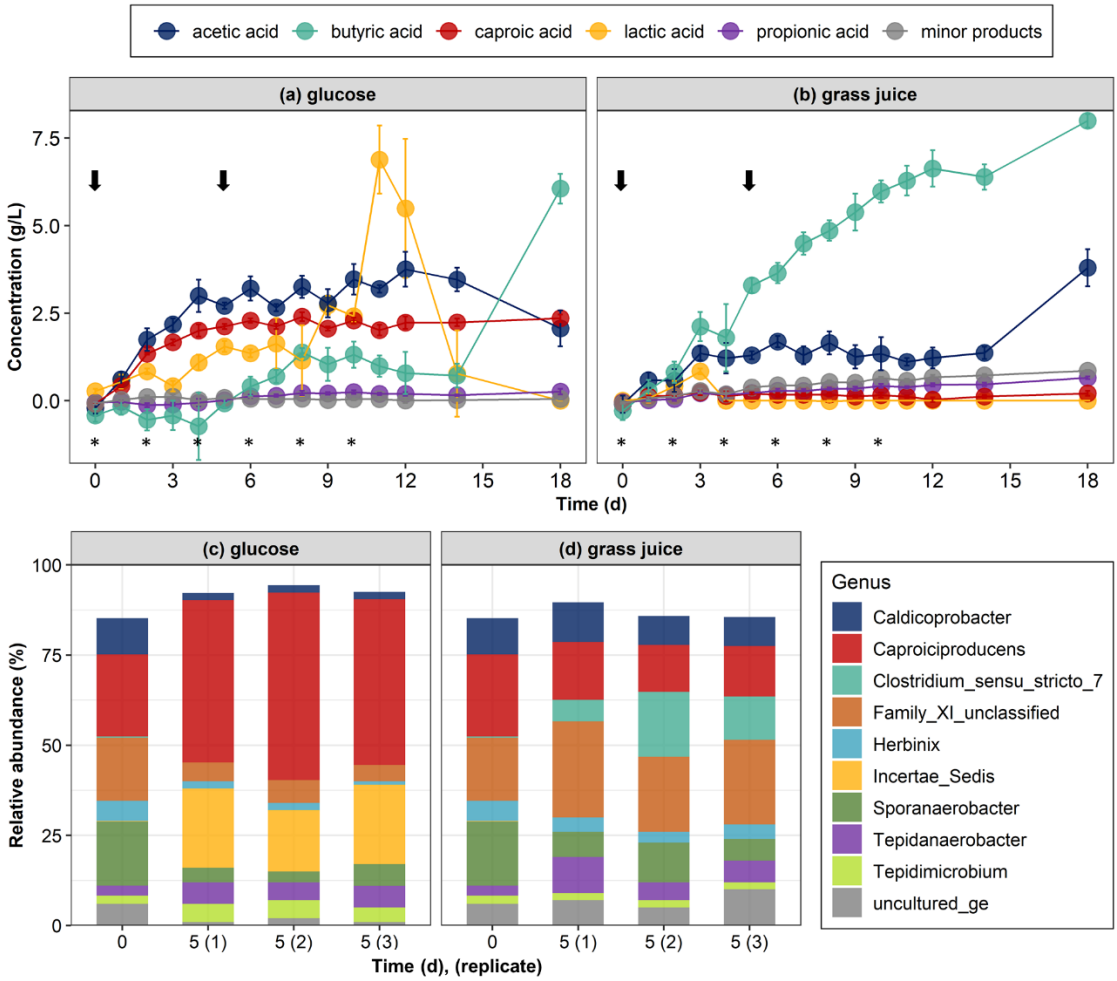
736 **Figure 2**



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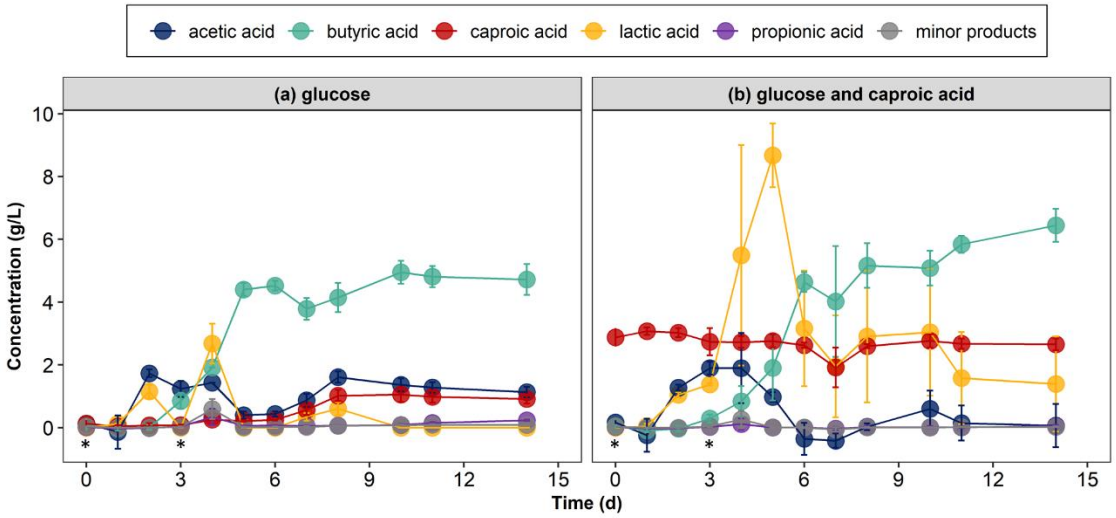
738 **Figure 3**



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740 **Figure 4**

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742 **Tables**

743 Table 1. Characteristics of green grass juice used in this work. Parenthesis indicates the number of
744 determinations.

Parameter	Unit	Value
pH	-	6.0 ± 0.4 (n=12)
Electrical conductivity	mS/cm	12 ± 2 (n=2)
Total suspended solids (TSS)	g/L	28 ± 13 (n=11)
Volatile suspended solids (VSS)	g/L	24 ± 11 (n=11)
Chemical oxygen demand (COD)	g/L	87 ± 24 (n=6)
Total carbohydrates	$g_{glucose}$/L	25 ± 6 (n=7)
Soluble carbohydrates	$g_{glucose}$/L	26 ± 3 (n=2)
Total protein (TN × 6.25)	g/L	4.1 ± 1.5 (n=6)
Total organic acids	g/L	0.77 ± 0.48 (n=13)
Acetic acid	mg/L	189 ± 112 (n=13)
Butyric acid	mg/L	0.0 ± 0.0 (n=13)
Caproic acid	mg/L	0.0 ± 0.0 (n=13)
Formic acid	mg/L	47 ± 47 (n=13)
Propionic acid	mg/L	6.0 ± 10 (n=13)
Lactic acid	mg/L	523 ± 408 (n=13)
Total nitrogen (TN)	g_N/L	0.65 ± 0.24 (n=6)
Total phosphorus (TP)	g_P/L	0.58 ± 0.20 (n=2)

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747 **Table 2**: Operational temperature and pH in each condition tested in continuous mode

Phase	Substrate	Chemical oxygen demand in feed (g_{COD}/L)	Temperature (°C)	pH
A	grass juice	~87	45	5.5
B				6.0
C				6.5
D			50	6.0
E			55	
F	grass juice and glucose		50	

748