

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

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4 Myrsini Sakarika^{1,2,†}, Alberte Regueira^{1,2,3,†}, Korneel Rabaey^{1,2}, Ramon Ganigué^{1,2,*}

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

7 ² Center for Advanced Process Technology for Urban Resource recovery (CAPTURE), Frieda Saeystraat,
8 9052 Ghent, Belgium

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

11 [†]Equal contribution

12 * Correspondence to: Ramon Ganigué, Ghent University; Faculty of Bioscience Engineering; Center for
13 Microbial Ecology and Technology (CMET); Coupure Links 653; B-9000 Gent, Belgium; phone: +32 (0)9 264
14 59 76; fax: +32 (0)9 264 62 48; e-mail: ramon.ganigue@ugent.be.

15

16 **Abstract**

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

- 37 **Keywords:** Thermophilic fermentation; Chain elongation; Grass; Green biorefinery; Carboxylate platform;
- 38 Caproic acid

39 1. Introduction

40 The use of side-streams and waste as feedstocks for the production of commodity chemicals *via* microbial
41 fermentation could be a more sustainable alternative to conventional (petro)chemical processes
42 (Ioannidou et al., 2020). Among the many potential end-products, organic acids such as short chain
43 carboxylic acids (SCCA) and medium chain carboxylic acids (MCCA) have attracted considerable interest.
44 MCCA contain 6 – 12 carbon atoms, and have applications as animal feed supplements and antimicrobials,
45 flavor-enhancing food additives, organic herbicides, or can be used as feedstocks for the production of
46 fragrances, bioplastics and lubricants (ChainCraft, 2021). Conventional MCCA production is rather
47 unsustainable since it is based on petrochemicals and vegetable oils (Anneken et al., 2006). Microbial chain
48 elongation – a process by which microorganisms elongate carboxylic acids by adding 2 carbon atoms per
49 step using organics as carbon and electron donors (de Groof et al., 2019) – could be a more sustainable
50 alternative MCCA production route that could be based on waste and industrial side-streams, thereby
51 being independent from agriculture and fossil resources and contributing to a more circular economy.
52 MCCA are more hydrophobic than SCCA, with the solubility drastically decreasing as a result of the increase
53 in carbon chain length (Xu et al., 2018). Each elongation step results in a considerable increase in the price
54 thereby increasing the value of the final product (*e.g.* 0.33 – 0.67€/kg for acetic acid; 1.9 – 2.1€/kg for
55 caproic acid), which makes them a more attractive end-product than SCCA (Moscoviz et al., 2018). Due to
56 the large number of applications of MCCA mixtures and/or individual MCCA such as caproic acid
57 (ChainCraft, 2021), their market size is expected to rise by 60% (2.76 billion USD) by 2028 compared to
58 2020 (1.73 billion USD) (Fior Markets, 2021).

59 Chain elongation can use, among others, lactic acid (Carvajal-Arroyo et al., 2019), ethanol (Candry et al.,
60 2020b), methanol (Chen et al., 2016), carbon monoxide (Liu et al., 2022), hydrogen (Baleeiro et al., 2021)
61 and sugars/carbohydrates (Wang et al., 2022) as electron donors. Substrates and feedstocks commonly
62 used for chain elongation include synthetic media containing the desired electron donor(s) (Candry et al.,

63 2020b), organic side-streams (Carvajal-Arroyo et al., 2019), food waste (Reddy et al., 2020; Zhang et al.,
64 2022) and green biomass (Khor et al., 2017). In particular, the use of green biomass such as grass holds
65 great potential as feedstock for biotechnological applications. Grass is one of the most abundant plant
66 families, it is inexpensive (Osterburg et al., 2010) and its liquid fraction, grass juice, has a suitable
67 composition for fermentation (*i.e.* high content in fermentable organics and nutrients) (Dien et al., 2006;
68 Sakarika et al., 2022). Hence, grass can be used as feedstock for the production of higher value products
69 such as platform chemicals (Khor et al., 2017) or microbial protein (Sakarika et al., 2022) allowing for their
70 decoupling from the use of fossil resources (Corona et al., 2018). Furthermore, the high sugar content in
71 grass juice (>25 g/L) (Sakarika et al., 2022) can enable high product titers. Therefore, grass juice can serve
72 as the sole substrate for chain elongation without the need for external addition of substrate components.

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

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

93 In this work, we systematically investigated the thermophilic production of caproic acid from grass juice in
94 an open-culture chain elongating system, with the aim of providing a first substantiated study on the effect
95 of the main operational parameters. First, we studied the effect of (i) pH (5.5 – 6.5) and (ii) temperature
96 (45 – 55°C) under continuous mode, as these parameters are known to heavily influence the outcome of
97 mixed-culture bioprocesses. Subsequently we assessed the effect of (iii) substrate composition, (iv)
98 substrate availability and (v) potential caproic acid toxicity on the product profile and end concentration.
99 Additionally, in parallel to the process performance we assessed how the microbial community changed
100 as a result of the aforementioned factors to gain further insights on community shifts and the links to the
101 observed chain elongation performance.

102 **2. Materials and methods**

103 **2.1 Grass juice fractionation and characteristics**

104 Freshly cut grass was fractionated to grass juice and cake using a biomass extruder equipped with an
105 internal twin screw. Right after fractionation the grass juice was stored at –20°C, and only defrosted ca.
106 24h prior to its use. The grass juice composition was analyzed and contained among others, *ca.* 87 g/L
107 chemical oxygen demand (COD), 28 g/L total suspended solids (TSS), 25 g/L carbohydrates and 4.1 g/L
108 protein (**Table 1**). The latter is expected to be dominated by glucose (46-62%) and xylose (18-36%) (Dien
109 et al., 2006).

110 2.2 Experimental procedures

111 2.2.1 Continuous fermentation

112 Continuous grass juice fermentation experiments were conducted in a 0.5L jacketed glass continuous
113 stirred-tank reactor (CSTR) with a 0.5 L working volume. The reactor was operated under non-axenic
114 conditions and continuous stirring at 400 rpm. Inoculation was performed at 10% v/v using the effluent of
115 a non-axenic thermophilic (45°C) CSTR converting grass juice to lactic acid, when the abundance of
116 *Caproiciproducens* was ca. 30% and the production of up to 0.74 g/L caproic acid was detected (Sakarika
117 et al., 2022). The reactor was operated in batch mode for the initial 8 days after which it was switched to
118 continuous mode at a hydraulic retention time (HRT) of 4 days, corresponding to an organic loading rate
119 (OLR) of ca. 22 g_{COD}/L/d. Different temperatures in the thermophilic range (45 – 55°C) and pH values (5.5
120 – 6.5) were tested to establish the performance of the system (**Table 2**). At the last phase of continuous
121 operation, grass juice was supplemented with glucose by mixing 3 parts of grass juice with one part of a
122 glucose solution with the same chemical oxygen demand (COD of ca. 87 g_{COD}/L). The organic load (COD) of
123 the resulting substrate remained the same, but the concentration of sugars/carbohydrates increased by
124 50%.

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

131 2.2.2 Batch and fed-batch tests

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

134 the potential for achieving high titers of caproic acid and (iii) the potential inhibiting effect of caproic acid.
135 To ensure high microbial activity, these experiments were performed using the most recent reactor
136 effluent as inoculum, which was incubated under the same conditions of the running reactor. All
137 experiments were performed in triplicate using 120 mL serum vials with a working volume of 50 mL under
138 120 rpm orbital shaking. The pH was set at 6.0, buffered using 50 mM 2-(N-morpholino)ethanesulfonic
139 acid (MES) and was daily corrected using NaOH or HCl (5M). To ensure anaerobic conditions all the serum
140 vials were flushed with a mixture of N₂ and CO₂ (90:10% v/v) for 5 min and sealed with rubber stopper. For
141 all batch and fed-batch experiments, serum vials containing the inoculum supplemented with MES buffer
142 and distilled water (instead of substrate) that were incubated under the same conditions served as
143 controls. In addition, in all cases, the substrate (or distilled water) was added at the same volume, to
144 achieve the same dilution effect. The duration of the experiments was 7 – 18 days and the pressure of all
145 serum vials was daily set to atmospheric levels. Samples (2 mL) for carboxylic acid and microbial
146 community composition analysis were taken every 1 – 3 days and were treated as described in section
147 2.1.1. The specific experiments and associated goals are described below:

- 148 • To establish the substrate for chain elongation, batch tests were performed at 45°C using glucose,
149 xylose, cellulose or lactic acid supplied at 10 g_{COD}/L. Glucose, xylose and cellulose were provided
150 as substrates since they are the main sugars/carbohydrates usually present in grass (Dien et al.,
151 2006). Lactic acid was also tested as it can be a fermentation product of grass juice (Sakarika et al.,
152 2022) and is a well-established substrate in chain elongation (Candry et al., 2020a).
- 153 • To assess whether the substrate composition and availability affect the product profile identify
154 the limit of caproic acid concentration of the community using a real and a synthetic substrate,
155 fed-batch experiments were performed at 50°C using glucose or grass juice. The initial substrate
156 concentration was 10 g_{COD}/L and the serum vials were spiked with 10 g_{COD}/L every 2 days to a total
157 COD addition of 60 g_{COD}/L.

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

165 **2.3 Analytical techniques**

166 The pH of raw samples was measured using a pH sensor (SP10T, Consort, BE). Carboxylic acids (acetic,
167 butyric, isobutyric, propionic, valeric, isovaleric, caproic, and isocaproic acids) were quantified using a gas
168 chromatograph (GC-2014, Shimadzu®, NL) equipped with a flame ionization detector (FID) and a DB-FFAP
169 123-3232 column (30m x 0.32 mm x 0.25 µm; Agilent, BE). Sulfuric acid, sodium chloride and 2-methyl
170 hexanoic acid (internal standard) were added in diluted and filtered liquid samples (0.2 µm PVDF filters,
171 Chromafil®) and carboxylic acids were extracted with diethyl ether. Prepared sample (1 µL) was injected
172 at 200°C with a split ratio of 60 and a purge flow of 3 mL/min. The oven temperature increased from 110°C
173 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
174 carrier gas (nitrogen) was supplied at a flow rate of 2.49 mL/min. Lactic and formic acids were determined
175 in filtered samples (0.2 µm PVDF filters, Chromafil®) using an ion chromatograph (930 Compact IC Flex;
176 Metrohm, CH), equipped with a Metrosep organic acids 250/7.8 column, a Metrosep organic acids guard
177 column/4.6 and a 850 IC conductivity detector (Metrohm, CH). These organic acids were eluted at a flow
178 rate of 0.5 mL/min using H₂SO₄ 1 mM (95-98%, Carl Roth).

179 **2.4 Molecular techniques**

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

182 10 µL of the DNA extract was sent out to LGC genomics GmbH (Berlin, Germany) for library preparation
183 and sequencing on an Illumina MiSeq platform with v3 chemistry. The primers used were 341F (5'-CCT
184 ACG GGN GGC WGC AG -3') and 785Rmod (5'-GAC TAC HVG GGT ATC TAA KCC-3'). Read assembly and
185 cleanup was based on the MiSeq SOP described by Schloss et al. (2009). In brief, mothur (v.1.40.3) was
186 used to assemble reads into contigs, perform alignment-based quality filtering (alignment to the mothur-
187 reconstructed SILVA SEED alignment, v. 123), remove chimeras, assign taxonomy using a naïve Bayesian
188 classifier and SILVA NR v132 and cluster contigs into OTUs at 97% sequence similarity. All sequences that
189 were classified as Eukaryota, Archaea, Chloroplasts and Mitochondria were removed. In addition,
190 sequences that could not be classified at all (even at (super) Kingdom level) were removed. After the
191 above-mentioned filtering, for each OTU representative sequences were picked as the most abundant
192 sequence within that OTU.

193 **2.5 Data analysis and availability**

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

201 3. Results

202 3.1 Continuous thermophilic caproic acid production using grass juice at different pH, temperature 203 and sugar availability

204 3.1.1 Effect of pH

205 The effect of pH was tested at 45°C in a continuous reactor (HRT 4 days) fed with grass juice (**Figure 1(a)**).
206 At a pH of 5.5, lactic acid accumulated (18 ± 2.1 g/L) as the main fermentation product ($63 \pm 4.0\%$ of total
207 carboxylic acids in mass). The maximum caproic acid achieved under these conditions was 1.9 ± 0.2 g/L,
208 representing only $6.6 \pm 0.7\%$ of the total carboxylic acids in mass (**Figure 1(b)**). Transient accumulation of
209 propionic acid was also noted (up to *ca.* 3.5 g/L). The initial microbial community at the start of the
210 continuous operation was clearly dominated (80%) by lactic acid bacteria belonging to the genus
211 *Pediococcus* (**Figure 1(c)**). By the time caproic acid production was first observed in continuous mode (at
212 45°C, pH 5.5), the community composition changed substantially and was dominated (23%) by
213 *Clostridium_sensu_stricto_7* followed by *Lactobacillus* (13%), while the abundance of *Caproiciproducens*
214 increased to 10%. At the end of the cultivation at 45°C and pH 5.5 the community was dominated (61%)
215 by *Lactobacillus*, and the abundance of *Caproiciproducens* was 26%, which correlates with the dominance
216 of lactic acid in the product spectrum and the consolidation of caproic acid titer at 1.9 g/L.

217 When the pH was increased to 6.0 the product profile shifted drastically: lactic acid was barely detected
218 and the main product was butyric acid ($61 \pm 3.1\%$ of total carboxylic acids) at a concentration of 11 ± 2.0
219 g/L. The caproic acid concentration (1.7 ± 0.15 g/L) was not substantially affected with this change,
220 however, its selectivity increased to $9.6 \pm 1.8\%$ of the total carboxylic acids due to the overall lower
221 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).
222 The pH increase to 6.0 additionally caused a shift in the community, with *Caproiciproducens* being now
223 the dominant genus (40%) and *Lactobacillus* almost disappearing from the system (0.44%), which is in line
224 with the disappearance of lactic acid. Finally, the abundance of *Caldicoprobacter* sharply increased to 17%.

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

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

232 **3.1.2 Effect of temperature**

233 After fixing pH to 6.0, the temperature of the reactor was gradually elevated ($1.0^\circ\text{C}/\text{day}$) to 50°C (**Figure**
234 **1(a)**). The caproic acid concentration increased to 3.4 ± 0.16 g/L representing $21 \pm 0.61\%$ of total carboxylic
235 acids, a 2.4 times higher selectivity than 45°C ($8.7 \pm 0.96\%$) (**Figure 1(b)**). At the same time, butyric acid
236 decreased to 7.8 ± 0.29 g/L ($48 \pm 1.0\%$ of total carboxylic acids). The abundance of *Caproiciproducens*
237 slightly increased (42%), the abundance of *Caldicoprobacter* drastically decreased (1.8%) whereas the
238 abundances of Family_XI_unclassified (9.6%) and *Herbinix* (9.5%) increased compared to 45°C and pH 6.0
239 (**Figure 1(c)**).

240 When the temperature was further increased to 55°C , the total concentration of carboxylic acids
241 decreased by $8.2 \pm 0.72\%$ compared to 50°C , while lactic acid started accumulating. In particular, at 55°C
242 caproic acid concentration decreased down to 0.89 ± 0.33 g/L and its selectivity ($6.5 \pm 3.4\%$) was 3.3 times
243 lower than at 50°C . This decrease was accompanied by a decrease in the abundance of *Caproiciproducens*
244 (29%), indicating the possibility that this temperature was close to the maximum threshold of this genus.
245 The abundance of Family_XI_unclassified further increased to 29%, while *Herbinix* remained at same levels
246 (10%).

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

250 **3.1.3 Effect of sugar availability**

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

260 **3.2 Batch thermophilic caproic acid production using different substrates**

261 A series of batch tests (**Figure 2**) were performed to pinpoint the real substrate used for caproic acid
262 production under the selected conditions, using the main sugars/carbohydrates present in grass juice and
263 lactic acid which can be the main primary fermentation product (section 3.1). The use of glucose and xylose
264 resulted in similar total carboxylic acid production (3.7 ± 0.30 and 4.1 ± 0.29 g/L, respectively) and similar
265 product profiles, dominated by caproic and acetic acids at the end of the experiment. The net caproic acid
266 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
267 production) was $41 \pm 3.9\%$ and $38 \pm 2.9\%$ in the case of glucose and xylose, respectively, which is
268 substantially higher than the values obtained under continuous operation using non-amended grass juice
269 (**Figure 1(b)**). The main difference between the product profiles was the concentration of acetic and
270 butyric acids, with 1.5 times more acetic acid and roughly half the butyric acid concentration when xylose

271 was used compared to glucose. The community composition was affected by the selected substrate
272 (**Figure 2(e)-(g)**). When glucose was used, during the 4th day of the experiment, the dominant species was
273 *Caproiciproducens* ($44 \pm 3.9\%$) followed by *Caldicoprobacter* ($12 \pm 0.33\%$) and *Sporanaerobacter* ($32 \pm$
274 4.1%). When the substrate was xylose the abundance of *Caproiciproducens* sharply increased to $63 \pm 3.4\%$,
275 the second most dominant species was *Sporanaerobacter* ($15 \pm 2.3\%$), and *Caldicoprobacter* remained at
276 the same levels ($12 \pm 0.88\%$). The community in the case where xylose is used as a substrate was narrower,
277 as reflected by the lowest value of the alpha diversity (4.3 ± 0.44 compared to 7.4 ± 0.21 for glucose and
278 7.2 ± 0.65 for lactic acid; **Figure S3**).

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

284 **3.3 Fed-batch thermophilic caproic acid production using glucose and grass juice**

285 A series of fed-batch experiments (**Figure 3**) was performed to assess whether the type of substrate and
286 its availability affect the product profile, and to identify the caproic acid concentration limit using glucose
287 or grass juice. Glucose was chosen as substrate over xylose since it resulted in a higher caproic acid
288 productivity during the batch tests (0.73 for glucose vs 0.68 g/L/d for xylose, not considering the lag phase,
289 (section 3.2)). When glucose was the substrate of fed-batch experiments, 2.4 ± 0.11 g/L of caproic acid
290 were produced (**Figure 3(a)**). The lactic acid concentration peaked on day 11 (6.9 ± 0.97 g/L), after which
291 it was gradually fully consumed and the most dominant product at the end of the experiment was butyric
292 acid (6.1 ± 0.43 g/L). The community was dominated ($48 \pm 3.7\%$, day 5) by *Caproiciproducens* and the
293 second most dominant genus ($20 \pm 2.7\%$) was Incertae_Sedis from the *Ethanoligenenaceae* family (**Figure**
294 **3(c)**).

295 In contrast, when grass juice was used, neither substantial net caproic acid production was noted ($0.21 \pm$
296 0.074 g/L) nor a lactic acid peak, and the final concentration of the most dominant product, butyric acid,
297 was 8.0 ± 0.12 g/L. The abundance of *Caproiciproducens* was substantially lower ($14 \pm 1.6\%$, day 5)
298 compared to glucose **Figure 3(d)**). The most dominant genus in this case was Family_XI_unclassified ($24 \pm$
299 2.9%), and substantial increase in the abundance of *Clostridium_sensu_stricto_7* ($12 \pm 6.3\%$) was noted.
300 In this case, the community was richer compared to the case where glucose was the substrate (**Figure S3**).
301 Given that a plateau in the caproic acid concentration was quickly reached during the fed-batch tests, an
302 additional fed-batch experiment where glucose was used as a substrate was performed, to assess whether
303 the caproic acid concentration plays a role in the product profile (**Figure 4**). The results show that, at the
304 initial absence of caproic acid, 1.0 ± 0.19 g/L caproic acid were produced, while the main net products
305 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
306 present (3.5 g/L) no additional caproic acid was produced, and the supplied glucose was channeled to
307 lactic acid (up to 8.7 ± 1.0 g/L) followed by its gradual consumption and concurrent increase in butyric acid
308 concentration (6.4 ± 0.52 g/L).

309 **4. Discussion**

310 **4.1 Effect of pH and temperature on the thermophilic production of caproic acid**

311 Given the relevance of pH as a process parameter in shaping the product profile and microbial community
312 composition in chain elongation processes at mesophilic conditions (Candry et al., 2020a; Lim et al., 2008),
313 we explored its effect at thermophilic conditions. The results show that the process behaved in a different
314 way than at mesophilic conditions as the caproic acid concentration remained relatively constant ($1.7 \pm$
315 $0.15 - 1.9 \pm 0.16$ g/L) across the tested pH range (5.5 – 6.5) and the highest selectivity ($9.6 \pm 1.8\%$) was
316 noted at pH 6.0. The main differences in the product profile are that pH < 6 led to lactic acid accumulation
317 (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 $0.89 \pm$
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

366 not linked with caproic acid production. This was suggested in the batch tests performed with individual
367 monomers (section 3.2) and validated by the last phase of the continuous operation, where increased
368 glucose levels yielded additional caproic acid (section 3.1.3). In the latter case, where the
369 sugar/carbohydrate content of grass juice was increased by 50% while maintaining the same organic (COD)
370 load (50°C, pH 6.0), an average concentration of 6.0 ± 1.0 g/L caproic acid was achieved in the reactor
371 representing 40% of the carboxylic acid COD. The peak concentration was 7.2 g/L (day 197) which, together
372 with the average titer achieved here, is the highest concentration reported so far under thermophilic
373 conditions. Specifically, Zhang et al. (2022) achieved 4.7 g/L ($10 \text{ g}_{\text{COD}}/\text{L}$) under semi-continuous
374 fermentation of food waste at 55°C, while other reports mention concentrations below 1.0 g/L – *i.e.* 0.14
375 – 0.74 g/L at 45 – 55°C (**Table S4**). The negligible carboxylic acid production during batch fermentation of
376 cellulose (**Figure 2(c)**) hinted that hydrolysis did not take place in our system, which appears to have the
377 genetic potential to utilize cellulose, since members of the *Herbinix* genus are thermophilic cellulose
378 degraders (Koeck et al., 2015). The lack of carboxylic acid production from cellulose in the batch tests could
379 be ascribed to the lower temperature of the experiment (45°C) compared to the optimal temperature
380 (55°C) of members of the genus (Koeck et al., 2015), or potentially due to the much slower hydrolysis
381 kinetics (Dionisi et al., 2015; Kataeva et al., 2013).

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

390 concentration did not seem to be affected by the changes in lactic acid concentration in any of these cases.
391 These facts suggest that lactic acid could be an intermediate in butyric acid production and not in caproic
392 acid production. Similar results were noted by Zhang et al. (2022), where the product profile was
393 dominated by butyric acid instead of caproic acid when lactic acid was the only substrate in a thermophilic
394 chain elongation process. These facts strongly suggest the possibility that the thermophilic chain
395 elongators yielding caproic acid in our system cannot utilize lactic acid as substrate and that the
396 microorganisms using lactic acid for chain elongation yield mainly butyric acid as end-product. These
397 findings do not correlate with the observations described in mesophilic chain elongation processes where
398 lactic acid can be used for extensive caproic acid production (Carvajal-Arroyo et al., 2019; Wang et al.,
399 2022), presenting notable production (0.81 g/L caproic acid) even at initial concentrations as low as 7.2
400 g/L lactic acid (Xie et al., 2021). Finally, lactic acid consumption was also linked to propionic acid
401 production, as shown by the production 2.0 ± 0.11 g_{PA}/L during the lactic acid-fed batches (**Figure 2(d)**),
402 and its transient accumulation (up to *ca.* 3.5 g/L) during continuous operation at pH 5.5 (**Figure 1**).

403 Our results also show that substrate availability (here sugar monomers) plays a major role in shaping the
404 product profile of the process. For instance, during fed-batch tests (**Figure 3(a)-(b)**), the 233% higher
405 availability of glucose (10 g_{COD}/L/feeding) when it was provided as a pure substance led to 2.4 g/L caproic
406 acid production compared to marginal production (0.21 g/L) when using grass juice containing glucose (*ca.*
407 3 g_{COD}/L/feeding). In this case, it is likely that caproic acid-producers could not get a significant share of the
408 sugar monomers due to competition with other microbial groups such as lactic acid-producers (and
409 subsequently butyric acid-producers from lactic acid), which consumed most of the substrate. More
410 specifically, we hypothesize that the answer to this question lies within the ecological r/K framework
411 (Favere et al., 2021), with lactic acid bacteria being r-strategists – thereby having high growth rates at high
412 substrate availability, and chain elongators being K-strategists – characterized by high substrate affinity.
413 This can be further explained by the fact that lactic acid bacteria likely have shorter metabolic pathways

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

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

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

441 **4.3 Microbial community structure during caproic acid production under thermophilic conditions**

442 Similar to the main performance indicators, the microbial community composition was largely affected by
443 substrate (**Figure 1(c); Figure 2(e)-(g); Figure 3(c)-(d)**), temperature and pH (**Figure 1(c)**). In general, the
444 use of sugars (*i.e.* glucose, xylose) promoted the presence of *Caproiciproducens* which was represented by
445 130 different OTU, and we believe was the main caproic acid-producer in our system. During the batch
446 tests, the use of xylose resulted in the highest abundance of *Caproiciproducens* ($63 \pm 3.4\%$ compared to
447 $44 \pm 3.9\%$ for glucose) (**Figure 2(e)-(g)**). In this case the community was less diverse and potentially more
448 specialized in caproic acid production (**Figure S3**). The same was observed in the last part of the continuous
449 operation, where additional sugars were provided (*via* glucose addition), and the abundance of
450 *Caproiciproducens* increased to 75% (**Figure 1(c)**). The fact that the presence of *Caproiciproducens* is
451 promoted by the presence of sugars was also demonstrated under mesophilic conditions (34 – 35°C) where
452 *Caproiciproducens* dominated the culture (80%) in a medium with xylose (10 g/L) (Tang et al., 2022), and
453 in thin-stillage fermentation with and without glucose amendment (up to 86% abundance in granular
454 biomass) (Mariën et al., 2022). The presence of *Caproiciproducens* has also been noted in other
455 thermophilic reactors producing caproic acid from sugar-rich substrates such as grass juice at 45°C
456 (Sakarika et al., 2022) and food waste at 55°C (Zhang et al., 2022).

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

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

472 The increase of *Clostridium_sensu_stricto_7* during fed-batch experiments using grass juice where butyric
473 acid was the main organic acid produces suggests that this genus was the main butyric acid producer in
474 this system (**Figure 3(d)**). Similar to our work, Zhang et al. (2022) identified *Clostridium_sensu_stricto_7*
475 as the main genus responsible for lactic acid-driven butyric acid production under thermophilic conditions
476 (55°C). Nevertheless, in our work, it is likely that also some of the butyric acid stemmed from the activity
477 of *Caproiciproducens*. It is also described that mesophilic chain elongators like *Caproicibacterium*
478 *lactatifermentans* (Wang et al., 2022) can often use both sugars and lactic acid as substrates. A similar
479 observation was made by Ingle et al. (2021), where during fermentation of hydrolyzed dairy manure under
480 mesophilic conditions (35°C, pH 5.0) lactic acid was first produced and subsequently consumed together
481 with the remaining sugars, and the abundance of *Caproiciproducens* (and *Clostridium_sensu_stricto_12*)
482 was positively correlated with butyric acid production.

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

486 the most dominant genera were *Caproiciproducens* (40%) and *Caldicoprobacter* (17%) – a genus that is
487 reported to produce lactic and acetic acids and ethanol from sugars under thermophilic conditions
488 (Yokohama et al., 2010). Further increase to pH 6.5 yielded a culture dominated by *Caproiciproducens*
489 (24%) and *Tepidanaerobacter* (14%), with members of this genus able to consume lactic acid at pH 6.0 –
490 7.0 and 45 – 50°C and produce mainly acetic acid, propionic acid and valeric acid (Sekiguchi et al., 2006).
491 The increase of temperature from 45 to 50°C did not substantially affect the abundance of
492 *Caproiciproducens* (42%), nevertheless, the abundance of *Caldicoprobacter* decreased from 17 to 1.8%.
493 The uncharacterized genus Family_XI_unclassified and *Herbinix* – which can produce acetic acid, small
494 amounts of propionic acid and ethanol from cellulose (Koeck et al., 2015) – increased to 9.6 and 9.5%
495 respectively. *Sporanaerobacter*, which is reported to produce acetic acid, isobutyric and isovaleric acids
496 and H₂ from sugars was mostly present at experiments conducted at 45°C (**Figure 1(a); Figure 2(e)-(g)**),
497 which is expected since this genus is mesophilic, but can withstand mildly thermophilic conditions (up to
498 50°C) (Hernandez-Eugenio et al., 2002). Further increase to 55°C decreased the abundance of
499 *Caproiciproducens* to 29%, indicating the that this temperature is beyond its physiological optimum which
500 appears to be close to 50°C. At this point the abundance of Family_XI_unclassified increased to 29% and
501 the genus *Herbinix*, composed of thermophilic cellulose degraders with optimal temperature 55°C (Koeck
502 et al., 2015), remained at the same levels (10%).

503 **5. Conclusions**

504 We have shown that caproic acid production under thermophilic conditions using grass juice was favored
505 at pH 6.0 and 50°C, amongst the conditions tested. In our system, the substrate for caproic acid production
506 under thermophilic conditions was sugars (glucose, xylose), while lactic acid resulted in high butyric acid
507 yields. The use of xylose favored the presence of *Caproiciproducens*, as indicated by its *ca.* 20% higher
508 relative abundance compared to the use of glucose and the 50% higher abundance compared to
509 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 ($32 \pm 3.1\%$) was substantially increased compared to the use of non-amended grass juice ($21 \pm$
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.

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693

694 **Figure captions**

695 **Figure 1:** (a) Concentration of the different carboxylic acids during continuous fermentation of grass juice.

696 Carboxylic acids with concentrations ≤ 1 g/L are lumped here (*i.e.* minor products, including formic,

697 isobutyric, isovaleric and valeric acids) and their individual concentration is presented in **Figure S2**. The

698 shaded area indicates the transition periods between different operational conditions. Black arrows

699 illustrate the samples where the microbial community composition analysis was performed. (b) Selectivity

700 of butyric, caproic and lactic acids in total carboxylic acid mass throughout the experimental period.

701 Average values \pm standard deviation are presented. (c) Microbial community composition at the selected

702 timepoints of the continuous operation. Relative abundance of top 10 genera was calculated based on

703 their combined relative abundance across all samples.

704

705 **Figure 2:** Net production and/or consumption of the different carboxylic acids during batch fermentation

706 of (a) glucose, (b) xylose, (c) cellulose and (d) lactic acid. Carboxylic acids with concentrations lower than

707 1.0 g/L (*i.e.* minor products) are summed. The concentration of carboxylic acids in the control experiments

708 (addition of distilled water instead of substrate) were subtracted to illustrate the net production and/or

709 consumption. Average values \pm standard deviation are presented. Black arrows illustrate the samples

710 where microbial community composition analysis was performed. Microbial community composition at

711 the selected timepoints of the batch tests using (e) glucose, (f) xylose and (g) lactic acid. Relative

712 abundance of top 10 genera was calculated based on their combined relative abundance across all

713 samples.

714

715 **Figure 3:** Net production and/or consumption of the different carboxylic acids during fed-batch

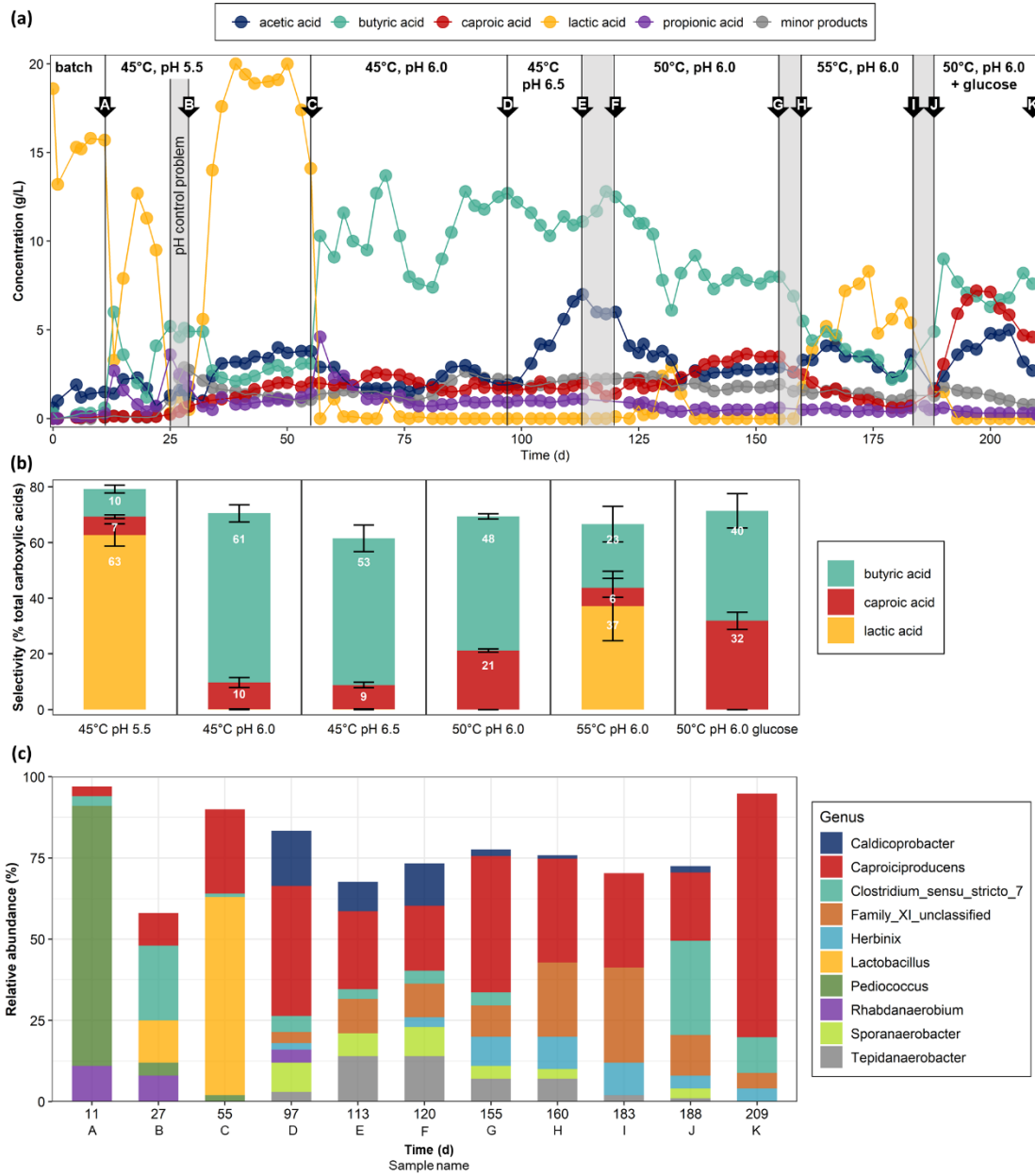
716 fermentation of (a) glucose and (b) grass juice. Carboxylic acids with concentrations *ca.* 0.10 g/L (*i.e.* minor

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

724

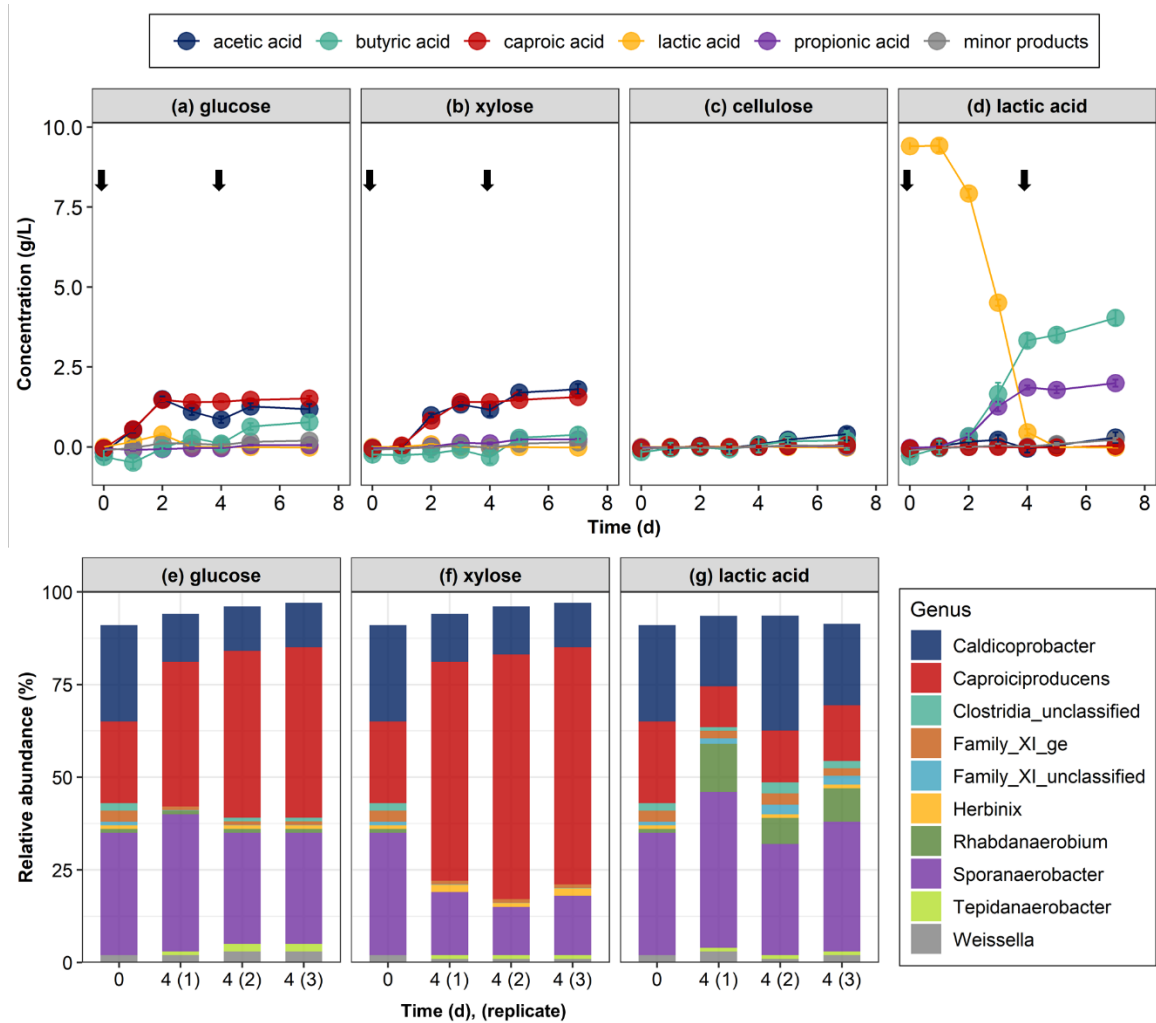
725 **Figure 4:** Net production and/or consumption of the different carboxylic acids during fed-batch
726 fermentation of **(a)** glucose and **(b)** glucose with the addition of caproic acid. Carboxylic acids with
727 concentrations *ca.* 0.10 g/L (*i.e.* minor products) are summed. Asterisks indicate substrate addition (10
728 $\text{g}_{\text{COD}}/\text{L}$). The concentration of carboxylic acids in the control experiments (addition of distilled water instead
729 of substrate) were subtracted to illustrate the net production and/or consumption. Average values \pm
730 standard deviation are presented.

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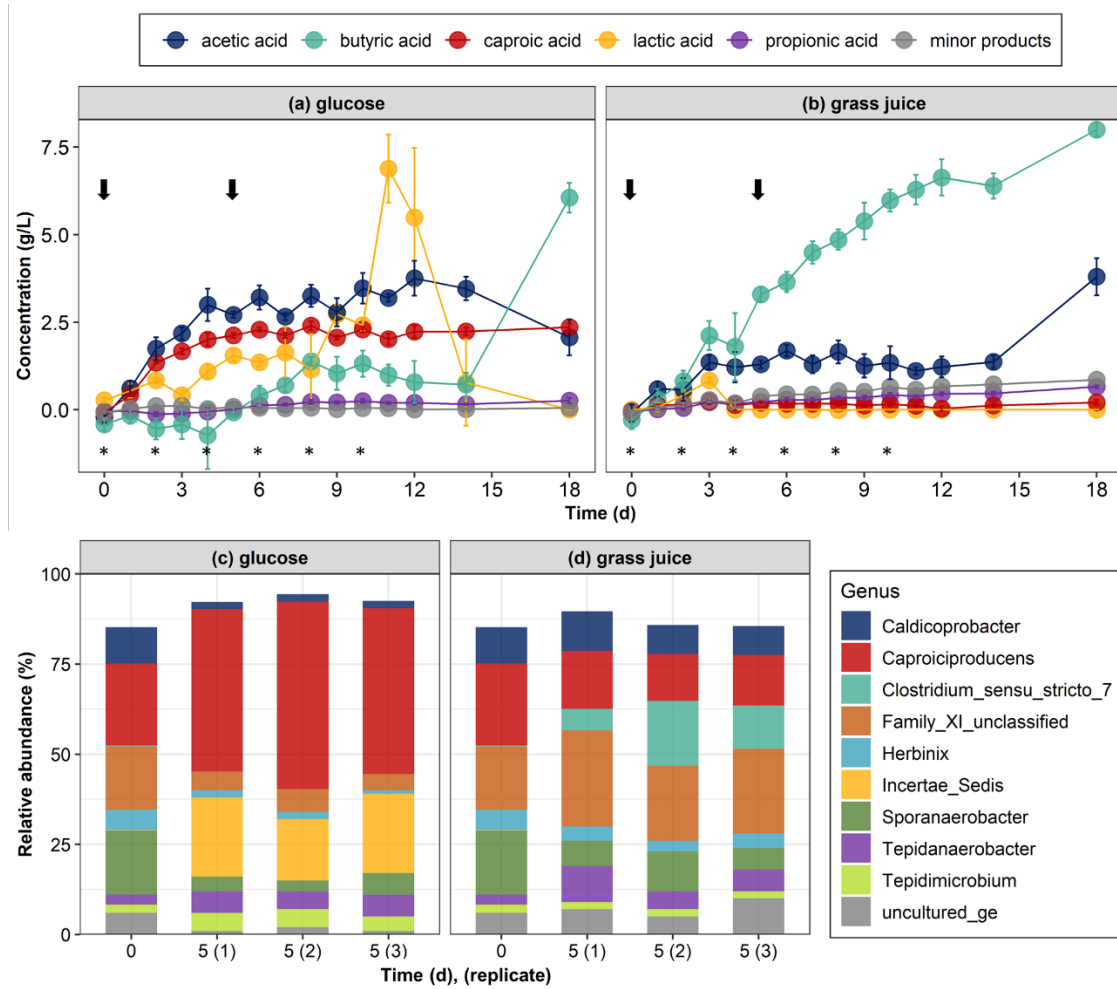
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734 **Figure 1**



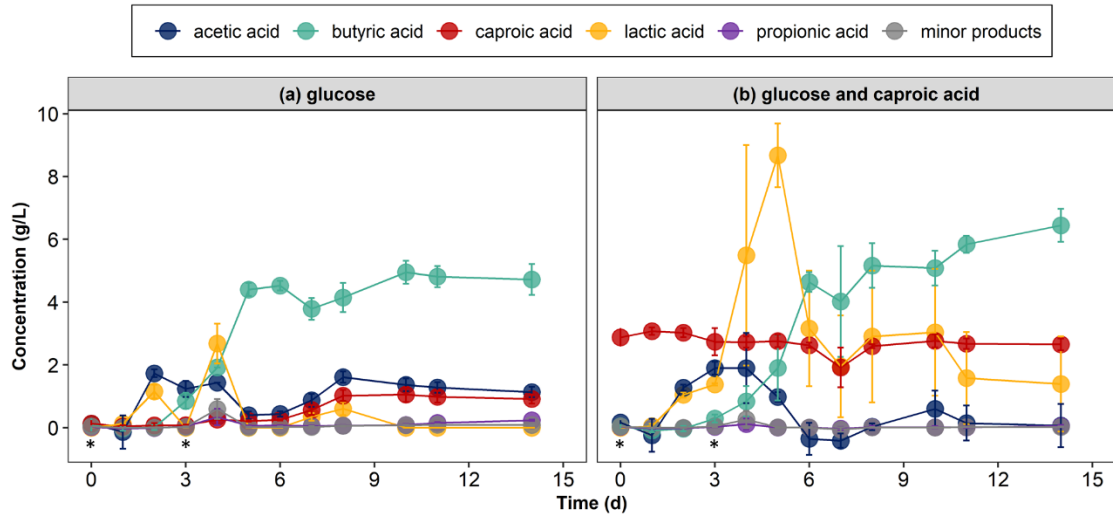
735

736 **Figure 2**



737

738 **Figure 3**



739

740 **Figure 4**

741

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)

745

746

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				5.5
B			45	6.0
C	grass juice	~87		6.5
D			50	
E			55	6.0
F	grass juice and glucose		50	

748