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HAL Authorization

1 **Populational and metabolic shifts induced by acetate, butyrate and**
2 **lactate in dark fermentation**

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8 **Abstract**

9 Dark fermentation is subject to inhibition by end products. In this study, the effects of
10 acetate, butyrate and lactate on fermentation routes of glucose were investigated for
11 concentrations ranging from 25 to 400 mM. Whatever the acid considered, an
12 inhibition threshold of hydrogen production was observed at acid concentration as
13 low as 50 mM. 300 mM of acetate, 200 mM of butyrate and 400 mM of lactate were
14 critical concentrations resulting exclusively in lactate production. At these high
15 concentrations, bacterial communities shifted from *Clostridiaceae* to *Lactobacillaceae*
16 family after acetate or lactate addition, and to *Bacillaceae* after butyrate addition. At
17 lower acid concentrations, the nature and the concentration of the added acid shaped
18 metabolic and populational changes. Specifically, *Clostridium butyricum* was able to
19 grow up to 250 mM, 150 mM and 300 mM of acetate, butyrate and lactate
20 respectively, but was suspected to shift its metabolism towards lactate production.

21 **Keywords:** acid inhibition, biohydrogen, microbial diversity, undissociated acid

22

23 **1. Introduction**

24 Low carbon footprint hydrogen (H₂) could play a major role in the future to reduce
25 carbon dioxide emissions and prevent global warming. Among the existing green H₂
26 production technologies, biological production by dark fermentation (DF) presents
27 one of the lowest global warming potentials [1]. When performed with mixed cultures,
28 DF presents also the advantages of being light-independent, having high H₂
29 production rates and using various low-cost raw materials and waste as feedstocks
30 [2]. This promising biological process relies on the degradation of carbohydrate-rich
31 organic matter by H₂-producing bacteria in anaerobic conditions. Efficient H₂
32 production in DF is mostly associated to acetate and butyrate metabolic pathways
33 with the stoichiometry from Equation (1) [3],



35 together with the dominance of species belonging to the *Clostridiaceae* family.
36 Although the microbial diversity in mixed cultures can provide robustness and
37 function redundancies, it could also disfavour the H₂ production by introducing
38 microorganisms engaged in substrate competition (*Lactobacillus sp.*), in the release
39 of inhibitory co-products (bacteriocins, lactate) or in H₂ consumption (bacteria with
40 homoacetogenic activities or hydrogenotrophic methanogens) [4].

41 The effects of end-products accumulation on H₂ production have been widely
42 studied in mixed cultures [5]. Overall, acetate, butyrate, propionate and ethanol were
43 found to have inhibitory effects on H₂ yields, hydrogen production rates (HPR) and
44 substrate degradation [5]. Inhibitory threshold for H₂ yield (the lowest concentration
45 that does not result in a significant reduction in H₂ yield) by butyrate varies from < 10
46 mM in batch at pH 7 using heat-treated digested sludge and glucose as substrate [6]
47 to 38 mM in continuous reactor at pH 5.5 from glucose using baked agricultural soil

48 as inoculum [7]. Similarly, inhibition threshold for H₂ yield by acetate was reported to
49 vary from < 10 mM [6] to 98 mM [7].

50 Furthermore, the activity of lactic acid producing bacteria (LAB) was associated
51 with DF instability [8]. Regarding the specific effect of lactate, Baghchehsaraee et al.
52 [9] showed that up to 55 mM of lactate did not inhibit H₂ production. Consistently,
53 Noblecourt et al. [10] did not observe a negative impact of 300 mM endogenously
54 produced lactate during the DF of pre-fermented food wastes. However, the latter
55 observed that 44 mM of exogenously added L-lactate induced a 35 % decrease in H₂
56 production, highlighting that enantiomeric form of lactate plays a key role in its
57 inhibitory effect. In addition, Kim et al. [11] observed that 178 mM of lactate
58 (unknown enantiomeric form) induced a 40 % increase in lag phase. Therefore, the
59 different results and conclusions found in literature concerning inhibition by acetate,
60 butyrate and lactate are very dependent on the experimental conditions as well as
61 the reactor feeding modes applied, hindering efficient prevention of end-products
62 inhibition.

63 The cellular mechanisms of such end-products inhibition on the microbial activity
64 was deeply studied with pure cultures. Past experiments showed that organic acids
65 can affect the bacterial activity and thus inhibit H₂ production by various means. In
66 their undissociated form, the organic acids can freely cross the cytoplasmic
67 membranes and easily dissociate inside the cell, thus acidifying the cytoplasm and
68 dissipating the proton motive force [12]. Active proton extrusion by ATPases
69 counteracts this effect, but at the expense of the energy available for growth. In their
70 dissociated form, the organic acids can increase the ionic strength, triggering cell
71 lysis, and thus reducing the microbial activity [13,14]. Furthermore, depending on the

72 microorganism studied, the impact of the undissociated form compared to the
73 dissociated form may vary, as well as the extent of the inhibition [15,16].

74 Therefore, in mixed cultures, it can be hypothesised that the variable resistance of
75 the microorganisms to end-products would cause microbial communities shifts, but
76 the knowledge in the literature on this subject is rather poor. Recently, Chen et al.
77 [17] studied the impact of butyrate from 6 to 284 mM on microbial communities with
78 glucose as substrate and at pH 5.5, 6, 6.5 and 7. They showed that H₂ production
79 reduction was associated with a decrease in the proportion of H₂-producing
80 microorganisms, such as *Clostridium sp. (Clostridiaceae)*, and an increase in the
81 proportion of other bacteria, including *Pseudomonas sp. (Pseudomonadaceae)*,
82 *Klebsiella sp. (Enterobacteriaceae)*, *Acinetobacter sp. (Moraxellaceae)*, and *Bacillus*
83 *sp. (Bacillaceae)*. However, the analysis of the microbial communities conducted by
84 the authors was limited, replicates of the trials are not presented and only the effect
85 of butyrate was studied. In contrast, under comparable conditions, i.e, at pH 6 with
86 glucose as substrate, Noblecourt et al. [18] observed that an endogenously produced
87 mixture of 191 mM butyrate and 80 mM acetate decreased H₂ production rate but
88 had no impact on the composition of the highly enriched microbial communities from
89 sewage sludge. These results show that H₂ production inhibition by end-products
90 could result from populational changes as well as from metabolic changes. However,
91 the conditions that lead to either of these phenomena are unknown.

92 A deeper understanding of the mechanisms of end-product inhibition in mixed
93 culture could then contribute to a more effective prevention of these inhibitions.

94 The objective of this work was to provide further insights on populational and
95 metabolic mechanisms behind the impact of end-products on H₂ fermentation routes

96 in a mixed culture. More specifically, the novelty of this investigation is attributed to
97 the qualitative and quantitative comparison of bacterial communities through a beta
98 diversity analysis to distinguish between metabolic inhibition (i.e within the same
99 community, changes in metabolic activity are responsible for reduced H₂ production)
100 from populational inhibition (i.e changes in the bacterial community are responsible
101 for reduced H₂ production). For that, the impact of the addition of acetate (from 0 to
102 300 mM), butyrate (from 0 to 250 mM) and lactate (from 0 to 400 mM) on DF of
103 glucose was investigated.

104 **2. Materials and Methods**

105 **2.1. Seed sludge**

106 Activated sludge was used as inoculum. The sludge was collected from an aerobic
107 tank at the wastewater treatment plant (WWTP) of Narbonne (France). pH of the
108 freshly collected sludge was 6.9. The sludge was first centrifuged 10 minutes at
109 16,800 g (20 °C), frozen, freeze-dried, mixed, aliquoted and stored at - 80 °C before
110 use. Freeze-drying storage was selected as it has recently been shown to be an
111 effective strategy for preserving the H₂ production potential of WWTP sludge [19].
112 After storage, the sludge was composed of 0.96 ± 0.02 gTS.g⁻¹ and 0.75 ± 0.04
113 gVS.g⁻¹. Prior to the experimental procedures, the sludge was rehydrated in 200 mL
114 of osmosed water and pre-treated by heating at 90°C for 15 min to select spore
115 forming organisms, as heat-treatment was reported as a great strategy to achieve
116 this purpose [20].

117 **2.2. Experimental setup**

118 Experiments were performed in 600 mL bottles with a 200 mL working volume. The
119 medium was composed of 9.34 ± 0.26 g.L⁻¹ (i.e., 10.0 ± 0.3 gDCO.L⁻¹) glucose, 100

120 mM 2-(N-morpholino)ethanesulfonic acid (MES buffer) ($pK_a = 6.15$), $0.8 \text{ g.L}^{-1} \text{ NH}_4\text{Cl}$,
121 $0.5 \text{ g.L}^{-1} \text{ K}_2\text{HPO}_4$ and 1 mL of a micronutrient solution (in mg.L^{-1} : $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$: 1500;
122 H_3BO_3 : 60; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 117; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$: 25; ZnCl_2 : 70; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$: 25;
123 $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$: 15; $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$: 25; HCl : 1750). The glucose-to-inoculum ratio
124 (g/g_{VS}) was set at 10, by adjusting the amount of inoculum. The effects of acetate,
125 butyrate and lactate addition were separately evaluated, in three distinct experiments.
126 Sodium acetate ($\geq 99\%$, Sigma Aldrich) was added at 0, 50, 100, 150, 200, 250 and
127 300 mM; sodium butyrate (98 %, Sigma Aldrich) was added at 0, 25, 50, 100, 150,
128 200 and 250 mM and L (+) sodium lactate ($> 99\%$, PanReac AppliChem) was added
129 at 0, 50, 100, 150, 250, 300 and 400 mM. The concentration ranges evaluated for
130 each acid were chosen based on their inhibitory effects reported in the literature
131 [6,21,22]. Temperature was maintained at $37 \pm 1 \text{ }^\circ\text{C}$ by means of a water bath with
132 no stirring. As the H_2 yield in the present study were similar to the yield obtained
133 under similar conditions with stirring ($1.56 \pm 0.19 \text{ mol}_{\text{H}_2}/\text{mol}_{\text{glucose added}}$ in the present
134 study and $1.4 \pm 0.1 \text{ mol}_{\text{H}_2}/\text{mol}_{\text{glucose}}$ with glucose as substrate, pH 6, 250 mL working
135 volume, 300 RPM [23]), it was considered that stirring was not essential in the
136 present study.

137 Each condition was carried out in quadruplicate. pH was initially adjusted at 6.0 ± 0.2
138 with 8M NaOH or 8M HCl. Finally, bottles were sealed with a rubber stopper, locked
139 with an aluminium screw, and purged with N_2 to ensure anaerobic conditions. 2 mL
140 samples were manually taken from each bottle at the beginning, after 2 days and
141 after 4 days of fermentation. Samples were centrifuged at 13,000 g for 15 min.
142 Supernatant and pellet were separated and stored at -20°C prior to metabolite,
143 glucose and microbial analysis.

144 **2.3. Analytical procedures**

145 Composition of the headspace was analysed every two hours with an automatic
146 sampler coupled to a micro-gas chromatograph (SRA I-GC R3000) equipped with a
147 Molsieve 5A 10 m column running at 80 °C, 30 PSI with argon as carrier gas for H₂,
148 O₂, N₂, CH₄ analysis and a PoraPlot U (PPU) 8 m column running at 70 °C, 20 PSI
149 with helium as carrier gas for CO₂ analysis. Component detection was realised with a
150 micro-thermal conductivity detector (TCD) set at 90 °C. The volume of gas was
151 estimated every two hours by automatic measurement of the total pressure. The
152 pressure was automatically controlled in each bottle to not exceed 1.2 bar in order to
153 limit inhibition by gaseous products [24]. Data were automatically stored in dedicated
154 internal database.

155 Volatile fatty acids were measured by gas chromatography (GC) while glucose
156 and other metabolites (i.e., lactate, formate, ethanol, butanol) were analysed by high-
157 performance liquid chromatography (HPLC). Samples were filtrated through 0.2 µm
158 prior to analysis. The Perkin Elmer Clarus 580 GC was equipped with an Alltech-
159 FFAP EC™1000 column coupled to a flame ionization detector (FID) set at 280 °C.
160 N₂ was used as carrier gas with a flow of 6 mL.min⁻¹. The HPLC was equipped with a
161 protective precolumn (Microguard cation H refill catbridges, Biorad) and an HPX-87H
162 column (300 × 7.8 mm, Biorad) running at 45 °C, with a 4 mM H₂SO₄ solution as
163 eluent at a flow of 0.3 mL.min⁻¹. Component detection was carried out with a
164 refractive index detector (Waters 2414) running at 45 °C.

165 At the end of the experiment, the fermentation medium of each bottle was
166 collected to assess the amount of biomass produced. Medium was centrifuged at
167 11,900 g for 20 minutes at 20 °C. Supernatant was removed and the pellet was
168 resuspended in deionized water to eliminate liquid metabolic products and preserve
169 biomass. Total dry mass and volatile solids analysis were performed according to the

170 APHA standard methods [25]. Biomass formula was considered to be $C_5H_7NO_2$ and
171 $1.42g_{COD}/g_{biomass}$. [26].

172 **2.4. Microbial community analyses and sequencing**

173 Microbial communities were analysed at start and at the end of the fermentation. At
174 start, three samples were randomly selected for microbial community analyses, for
175 each of the three experiments (one per type of acid addition). At the end of the
176 fermentation, if significant H_2 production was observed, at least two samples were
177 analysed, for a total of 51 samples. More samples were analysed in case of
178 significant variability in H_2 production per replicate. DNA extraction was made with a
179 FastDNA™ SPIN kit (MP biomedical) following the manufacturer's instructions.
180 Bacterial members identification was carried out by amplification of the V3–V4 region
181 of the 16S rRNA gene as reported by Braga Nan et al. [27]. Sequences were
182 grouped in operational taxonomic units (OTUs) having 98 % similarity. Sequences
183 were submitted to GenBank, under the accession number PRJNA785481.

184 Beta diversity analysis was conducted after rarefying the samples at the smallest
185 number of reads (21340). A multiple sequence alignment was performed using the
186 Clustal Omega online tool from the European Bioinformatics Institute (EMBL-EBI)
187 (<https://www.ebi.ac.uk/>).

188 **2.5. Modelling and statistical data analysis**

189 The most common Gompertz model did not fit the experimental data (Supplementary
190 material 1). Therefore, H_2 production was modelled using a spline fit with the grofit()
191 function of the grofit package in R [28]. The function returned the maximal H_2
192 production rate (HPR, $mLH_2 \cdot g_{glucose\ added}^{-1} \cdot day^{-1}$), the maximal volume of cumulated
193 H_2 per gram of glucose added (H_c , $mLH_2/g_{glucose\ added}$) and the lag time before H_2

194 production starts (λ , days). These three parameters were deduced from the fit as
195 follows: HPR was the maximal slope, Hc was the highest cumulated volume of H₂ per
196 gram of glucose added and λ corresponded to the x-intercept of the line of slope
197 HPR that passes through the inflection point (visual representation can be found in
198 Supplementary material 1).

199 Modelling of the inhibitory impact of acetate, lactate, butyrate on HPR was
200 performed with the following non-competitive inhibition equation (Equation 2) [22] :

$$201 \quad HPR = \frac{HPR_{ctrl}}{1 + (C/K_c)^n} \quad \text{(Equation 2)}$$

202

203 where HPR_{ctrl} is HPR without acid addition, C is the concentration of the added acid
204 (mM), n is the degree of inhibition and K_c is the inhibition constant (mM).

205 Statistical analysis of the data was performed with R software. Differences in
206 bacterial community composition were assessed using average linkage cluster
207 analysis (UPGMA) and the weighted UniFrac distance metric [29]. An edited version
208 of *pvclust* was used [30] to assess the uncertainty in hierarchical cluster analysis
209 through a multiscale bootstrap resampling (n= 10,000). The bootstrap probability
210 value (BP) and approximately unbiased probability values (AU) were also calculated.
211 Nine clusters showing a AU > 95 % were considered as relevant.

212 Visual representation of bacterial relative abundance was done with phyloseq
213 package [31]. Analysis of variance (ANOVA) and Kruskal wallis tests were performed
214 using `aov()` and `kruskal.test()` function in R software, respectively. ANOVA was
215 performed in case of validation of the assumptions associated with the test, otherwise
216 a Kruskal wallis test was performed.

Table 1 – Kinetic constants describing H₂ production: maximal hydrogen production H_c (mLH₂·g_{glucose added}⁻¹), maximal hydrogen production rate HPR (mL·g_{glucose added}⁻¹·day⁻¹) and lag phase λ (days) at different concentrations of acetate, butyrate and lactate. The data are shown for the different groups of quadruplicate (mean values and standard deviation). NA: not applicable.

Concentration of added acid (mM)	LACTATE			ACETATE			BUTYRATE		
	H _c (mL·g _{glucose}	HPR (mL·g _{glucose added}	λ (days)	H _c (mL·g _{glucose}	HPR (mL·g _{glucose added}	λ (days)	H _c (mL·g _{glucose}	HPR (mL·g _{glucose added}	λ (days)

217 **3. Results and Discussion**

218 **3.1. Adverse effect of acid addition on biohydrogen production**

219 Fermentation was monitored over a period of 4 days, after which a plateau in H₂
 220 production was reached in all conditions (Supplementary material 2). No methane
 221 production was observed in all experiments. H₂ production was modelled with a
 222 spline fit used to estimate the volume of cumulated H₂ produced per gram of glucose
 223 added (H_c, mL_{H2}·g_{glucose added}⁻¹), the maximal H₂ production rate (HPR, mL_{H2}·g_{glucose}
 224 added⁻¹·day⁻¹) and the lag phase before H₂ production starts (λ, days) as reported in
 225 Table 1. Without acid addition, H_c reached 193 ± 23 mL_{H2}·g_{glucose added}⁻¹ (1.56 ± 0.19
 226 mol_{H2}·mol_{glucose added}⁻¹), HPR 248 ± 47 mL_{H2}·g_{glucose added}⁻¹·day⁻¹ (2.0 ± 0.38
 227 mol_{H2}·mol_{glucose added}⁻¹·day⁻¹) and λ 0.56 ± 0.13 day. Less than 20 % variability was
 228 observed between the replicates for H_c, HPR and λ in each condition, exception
 229 made

230 for 300 mM of lactate, 200 mM and 250 mM of acetate (Supplementary material 3).

	added ⁻¹)	1.day ¹)	added ⁻¹)	1.day ¹)	added ⁻¹)	1.day ¹)	added ⁻¹)	1.day ¹)	
0	201 ± 7	279 ± 18	0.62 ± 0.03	197 ± 5	240 ± 50	0.42 ± 0.04	162 ± 30	278 ± 36	0.50 ± 0.1
25	/	/	/	/	/	/	153 ± 27	242 ± 29	0.70 ± 0.14
50	203 ± 30	412 ± 19	0.69 ± 0.02	197 ± 17	327 ± 50	0.74 ± 0.1	164 ± 32	223 ± 11	0.70 ± 0.12
100	141 ± 18	336 ± 18	0.73 ± 0.05	161 ± 12	297 ± 27	0.88 ± 0.08	109 ± 30	136 ± 43	1.28 ± 0.39
150	134 ± 22	243 ± 46	0.88 ± 0.23	126 ± 13	219 ± 18	1.09 ± 0.17	75 ± 6	92 ± 4	1.78 ± 0.04
200	/	/	/	81 ± 49	137 ± 89	1.42 ± 0.11	0 ± 0	0 ± 0	NA
250	123 ± 23	172 ± 16	1.37 ± 0.17	37 ± 71	57 ± 95	2.24 ± 1.37	0 ± 0	0 ± 0	NA
300	63 ± 55	92 ± 70	2.31 ± 0.56	0 ± 0	0 ± 0	NA	/	/	/
400	0 ± 0	0 ± 0	NA	/	/	/	/	/	/

231 Cumulated hydrogen production - Hc

232 A significant inhibition threshold for Hc was observed at 50 mM when acetate
233 (ANOVA, $p = 0.0005$), butyrate (ANOVA, $p = 0.047$) and lactate (Kruskal Wallis, $p =$
234 0.0400) were individually added. From this threshold, Hc declined progressively until
235 it reached 0 at different concentrations depending on the acid added: 400 mM for
236 lactate addition, 300 mM for acetate addition and 200 mM for butyrate addition. This
237 result shows that acetate, butyrate and lactate can inhibit the total volume of H₂
238 produced and that this inhibitory effect is dose dependent.

239 Consistently, inhibitory threshold lower than 83 mM of acetate was observed
240 at pH 5.5 with sucrose (25 gCOD·L⁻¹) [22]. Thresholds below 10 mM were found at
241 pH 6 [32] and 7 [6] but in these study, pH was unregulated; and potentially
242 insufficiently buffered (48 mM NaHCO₃ with 10 g·L⁻¹ glucose), respectively.

243 Concerning butyrate, the inhibitory threshold observed here is consistent with
244 the 71 mM threshold found by Zheng and Yu [33] in similar conditions, i.e, at pH 6
245 with 10 g·L⁻¹ glucose. Again, lower thresholds of 17mM and < 10mM found by Chen
246 et al. [17] at pH 6 and Wang et al. [6] at pH 7 were obtained with potentially
247 insufficient buffering (48 mM NaHCO₃ with 10 g·L⁻¹ glucose again) and no buffering
248 of the fermentation medium.

249 With regards to lactate, Noblecourt et al. [10] found a lower inhibitory threshold
250 of < 44 mM of L-lactate at pH 6. In contrast, Kim et al. [11] found that lactate addition
251 from 11 to 178 mM at pH 4.5 improved or did not affect the overall H₂ production.
252 Finally, the 24h pre-treatment of the inoculum at pH 2 carried by Kim et al. [11]
253 selected acid-resistant strains, which would explain the higher tolerance to lactic acid.

254 Interestingly, comparing the different studies, inhibitory thresholds for H₂
255 production do not increase with an increase in pH. Also, in this study, inhibitory
256 threshold for H₂ production were 50 mM for acetate, butyrate and lactate, although
257 these acids present variable pka (4.76, 4.82 and 3.86, resp.). This may be surprising
258 as some authors have stated that the undissociated form of the acid was the main
259 cause of inhibition of H₂ production [7,17]. However, in Chen et al. [17] and Van
260 Ginkel and Logan [7] studies, the coefficient of determination associated with the
261 linear regression between H₂ yield and undissociated butyric acid concentration were
262 quite low, achieving 0.54 and 0.04 (calculated from the data shown in the article).
263 Furthermore, the inhibition thresholds found in the two studies were also different,
264 reaching 6 mM [7] and less than 2 mM of undissociated butyric acid [17]. Considered
265 as a whole, this suggests that considering undissociated acid concentrations alone is
266 insufficient to explain inhibitions in mixed cultures. The extent of inhibition is probably
267 also related to the different operational parameters applied and the microbial
268 communities used as inoculum.

269 **Hydrogen production rate - HPR**

270 Inhibitory threshold values of HPR were estimated at 200 mM for acetate
271 (ANOVA, p =0.007), 50 mM for butyrate (ANOVA, p = 0.00223), and 250 mM for
272 lactate (ANOVA, p = 0.00011). Thus, acetate, butyrate and lactate exhibit an
273 inhibitory activity on H₂ production rate, which could be related either to metabolic

274 disruption or to changes in microbial communities. In addition, this result highlights
275 that butyrate showed the strongest inhibitory effect on HPR, and lactate the lowest.

276

277 In comparison, the threshold values for HPR inhibition for acetate and butyrate were
278 lower, < 83 mM in the experiment of Wang et al. [22] and < 48 mM in the experiment
279 of Zheng and Yu , respectively [33]. The higher HPR inhibitory threshold reported in
280 the present study could be interestingly attributed to the unstirred fermentation.
281 Indeed, flocculation was observed and extracellular polymeric substances
282 surrounding the cells in biofilms and flocs are known to protect them from toxic
283 substance [34]. Thus, flocculation may have been associated with a higher resistance
284 of bacteria to organic acids.

285 In addition, 50 mM of acetate induced a significant 36 % improvement of HPR
286 (ANOVA, $p = 0.0367$). Similarly, Colin and Moulin [35] observed that acetate addition
287 up to 210 mM induced an increase in *Clostridium butyricum* growth rate. This effect
288 and associated increase in the production of H₂ in presence of acetate was also
289 observed by Heyndrickx et al. [36] with *C. butyricum* and *Clostridium pasteurianum*.
290 These authors suggested that acetate was used as an indirect proton and electron
291 acceptor, stimulating substrate fermentation by providing intermediate acetyl-CoA.

292 Interestingly, 50 mM and 100 mM of lactate induced in this study significant
293 improvements of the HPR of 48 % and 20 % compared to HPR without lactate
294 (ANOVA, $p = 0.00005$ and 0.004 respectively). This result could be attributed to the
295 simultaneous consumption of glucose and lactate to produce H₂. Consistently, Kim et
296 al. [11] also reported HPR increase after lactate addition from 11 to 178 mM.

297 **Lag phase - λ**

298 Inhibitory threshold of lag phase was < 50 mM for the three acids (ANOVA, $p =$
299 $0.0003, 0.039, 0.0097$ for acetate, butyrate and lactate addition, respectively).
300 Butyrate caused the highest lag phases, with a 3.5-fold increase from 0 to 250 mM,
301 compared to a 2.6- and a 1.4-fold increase with the addition of 250 mM of acetate
302 and lactate, respectively.

303 Consistently, Siqueira and Reginatto [32] found an inhibitory threshold value for
304 lag phase of 42 mM of acetate and Wang et al. [22] reported a value lower than 83
305 mM. Concerning butyrate and lactate, inhibitory threshold of lag phase was estimated
306 at 95 mM and 89 mM, respectively [11,33]. In conclusion, the present results are
307 consistent with an inhibitory threshold of the lag phase below 100 mM in batch for
308 acetate, butyrate and lactate.

309

310 **3.2. Hydrogen production rate inhibition modelling**

311 Inhibition thresholds indicate the concentrations at which acids have an effect on
312 bacterial physiology. However, they do not provide information on the extent of the
313 inhibitory effect as a function of acid concentration. To access this information, HPR
314 modelling was performed according to (Equation 2). Results of the inhibition
315 modelling from this study and from literature data in mixed cultures are presented in
316 Table 2. Inhibition of HPR by 50 % (half maximal inhibitory concentration, K_c) was
317 achieved for 209 mM of acetate, 104 mM of butyrate and 273 mM of lactate. Again,
318 butyrate shows the highest inhibitory impact, and acetate the lowest. Total inhibition
319 of HPR and H_2 production was achieved at values named C_{max} shown in Table 2
320 (i.e, at 300 mM of acetate, 200 mM of butyrate and 400 mM of lactate).

321 To the authors knowledge, K_c of 209 mM of acetate found in the present study
322 is the highest value found in the literature for mixed cultures. Siqueira and Reginatto ,

323 Wang et al. and Wang et al. [6,22,32] found K_c of 86, ~ 80 mM and 157 mM of
 324 acetate at pH 6, 7 and 5.5, respectively. Unfavourable pH conditions [6,32] and the
 325 use of sucrose as a substrate [22], a sugar which requires hydrolytic activities for its
 326 degradation and therefore a more diverse microbial community than for glucose
 327 degradation, could explain the differences observed.

328 The higher inhibitory impact of butyrate on HPR with regards to acetate found in this
 329 study is consistent with the study of Van Ginkel and Logan and Zhang et al. [7,37].

330 In contrast, K_c of 104 mM of butyrate was 2-fold lower than the K_c of ~ 225 mM
 331 estimated from Wang et al. [6]. The latter is close to the K_c of 220 mM estimated from
 332 Zheng and Yu [33], although initial pH varied, set at 6 and 7, respectively. Chen et al.
 333 [17] observed a highly lower K_c of 17 mM of butyrate at pH 6, but, again, pH may
 334 have been potentially insufficiently buffered (48 mM NaHCO_3 with $10 \text{ g}\cdot\text{L}^{-1}$ glucose).
 335 Somehow, the microbial community composition may affect resistance to organic
 336 acids, as suggested by Chen et al. [17] who investigated the impact of butyric acid in
 337 two studies; indeed, under the same operating conditions, a distinct inhibition of the
 338 H_2 production is reported [6,17].

339 Regarding lactate, 44 mM of L-Lactate addition by Noblecourt et al. [10]
 340 resulted in a reduction of 41 % of HPR, decreasing from $2.36 \text{ L}\cdot\text{h}^{-1}$ to $1.39 \text{ L}\cdot\text{h}^{-1}$. Such
 341 effect of lactate is much more detrimental than the effect reported in the present
 342 study, as 250 mM of lactate was needed to lower the HPR by 39%.

Table 2 - Inhibition parameters of maximum hydrogen production rate HPR by acids and associated initial pH of fermentation in this study and in the literature

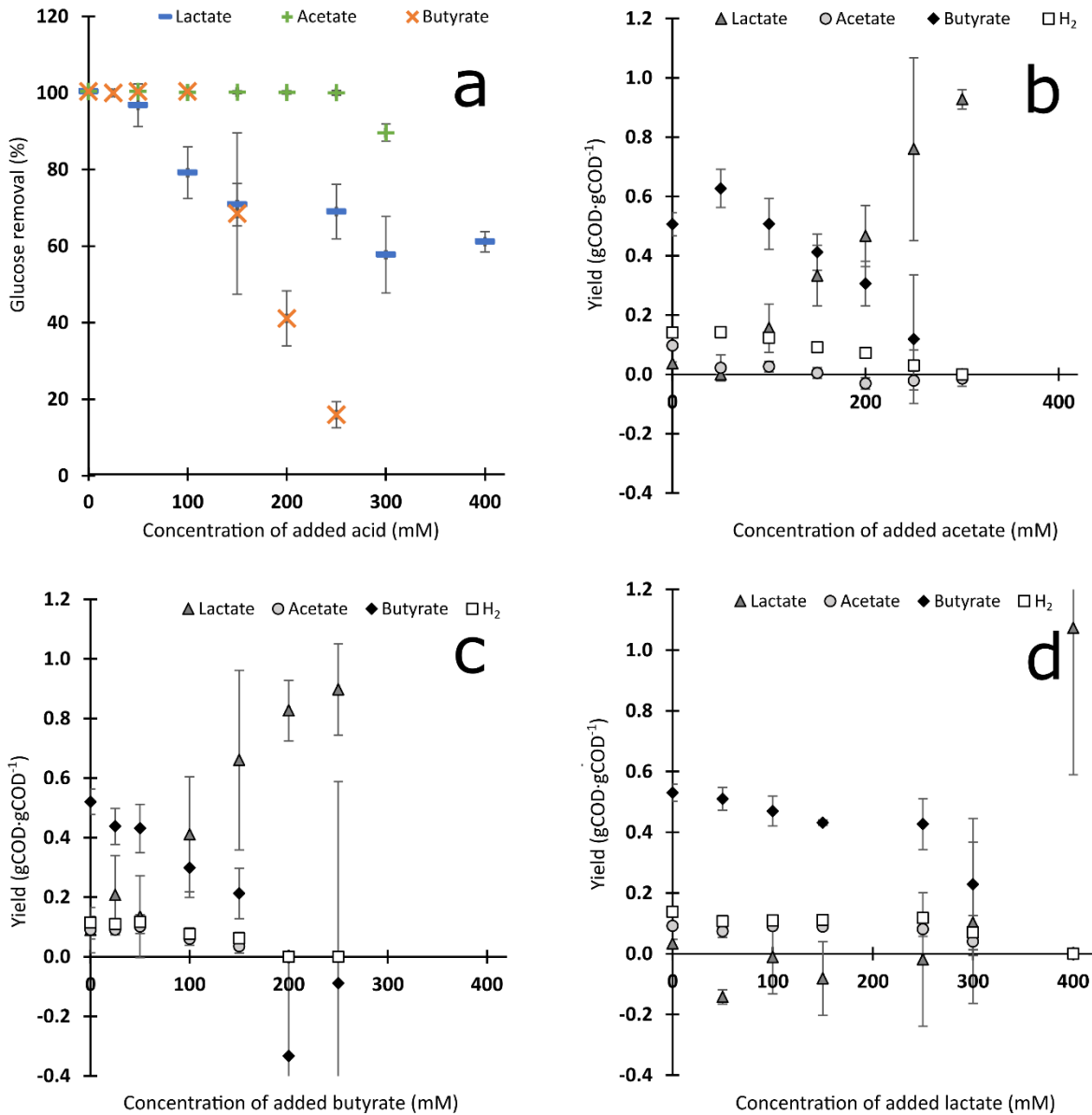
Inhibitor	Inoculum	HPR max	K_c (mM)	C_{max} (mM)	n	Initial pH - Regulation	Reference
Acetate	Freeze-dried activated sludge	$93 \text{ mL}\cdot\text{h}^{-1}$	209	300	7.56	6 – Buffered with 100mM MES	This study
	Sludge from UASB treating vinasse	$8.7 \pm 0.3 \text{ mL}\cdot\text{h}^{-1}$	86	167	/	6 - None	[32]
	Sludge from USAB treating citrate-producing wastewater	$221.7 \text{ mL}\cdot\text{gVSS}^{-1}\cdot\text{h}^{-1}$	157	>833	1.52	5.5 - Regulated	[22]
	<i>Clostridium bifermentans</i> ,	$58 \text{ mL}\cdot\text{L}\cdot\text{h}^{-1}$	/	502	0.52	7 - None	[37]

	cultivated with 30g/L NaCl, isolated from anaerobic digester of WWTP						
	Anaerobic sludge adapted for cultivation at 70°C	~ 70 mL·L ⁻¹ ·h ⁻¹	/	>200	/	7, Buffered with 20 mM MOPS	[38]
	Anaerobic sludge	12.9 mL·h ⁻¹	~80	471	2.9	7 - 48mM NaHCO ₃	[6]
Butyrate	Freeze-dried activated sludge	100 mL·h ⁻¹	104	200	3.07	6– Buffered with 100mM MES	This study
	Anaerobic sludge from WWTP	61.5 mL·gVSS ⁻¹ ·h ⁻¹	220	291	0.34	6 – Buffered with 59 mM H ₂ PO ₄ ⁻ and HPO ₄ ²⁻	[33]
	Sludge from anaerobic digester	112 mLH ₂ ·L ⁻¹ ·h ⁻¹	~225	306	0.6	7 - 48mM NaHCO ₃	[6]
	Sludge from anaerobic digester	42 mLH ₂ ·L ⁻¹ ·h ⁻¹	~284	>284	/	7 - 48mM NaHCO ₃	[17]
	<i>Clostridium bifermentans</i>	60.1 mL·L·h ⁻¹		334	2.35	7	[37]
	Sludge from anaerobic digester	27mLH ₂ ·L ⁻¹ ·h ⁻¹	~17	~34	/	6 - 48mM NaHCO ₃	[17]
Lactate	Freeze-dried activated sludge	102 mL·h ⁻¹	273	400	7.58	6 – Buffered with 100mM MES	This study
	Derived mix of thermophilic sludge and sludge from WWTP, enriched with <i>Clostridium cellulolyticum</i>	2360mL·h ⁻¹	~50	>50	/	6 - Regulated	[10]

343

344 **3.3. Glucose removal**

345 Glucose removal, an indicator of microbial activity, was calculated as a ratio of
 346 glucose consumed to initial glucose, in percentage, and presented in Fig. 1 a.
 347 Without acid addition, glucose consumption was complete, as it reached 100 ± 0
 348 %.



349 Fig. 1 - Effects of acetate, butyrate and lactate on metabolic activity. (a) mean glucose removal (%) \pm standard deviation. Mean yields of products \pm standard deviation as a function of acetate (b), butyrate (c) and lactate (d) concentrations. Yields were calculated in COD of products (H₂, acetate, butyrate and lactate) per COD of consumed glucose and consumed exogenous added acid (viz. glucose + acetate consumed, glucose + butyrate consumed and glucose + lactate consumed for (b), (c) and (d) respectively).

350 Acetate concentrations from 50 to 250 mM did not influence glucose removal,
 351 which was only slightly affected at 300 mM (89 \pm 2 %). This fact suggested that the
 352 global microbial activity was only slightly affected by acetate. In contrast, Wang et al.
 353 [22] reported a threshold value for the reduction of sucrose removal estimated at < 83

354 mM acetate. Wang et al. [6] found an inhibitory threshold of glucose removal at a
355 concentration of acetate lower than 10 mM.

356 Regarding butyrate, a concentration of 100 mM was estimated as the threshold value
357 for inhibition of glucose removal (ANOVA, $p = 0.02$). Above this concentration, a
358 linear dose-dependent inhibition of glucose removal was observed, reaching 16 ± 3
359 % at 250 mM. Consistently, Zhang et al. [37] showed that in presence of 250 mM
360 butyrate, glucose removal was only 18 % while it was still 76 % in the presence of
361 250 mM acetate.

362 In contrast, Chen et al. [17] showed that at pH 6, the inhibitory threshold for
363 glucose removal with butyrate was as low as 6 mM, but glucose removal was already
364 low in control conditions, reaching ~ 45 %. Wang et al. [6] also found a low inhibitory
365 threshold for glucose removal at a concentration of butyrate < 10 mM, pH 7, but the
366 extent of the inhibition was far lower than in the present study, since more than 70 %
367 of the substrate was still consumed in presence of 300 mM of butyrate. In
368 contradiction, Zheng and Yu [33] assessed this threshold around 190 mM.

369 Finally, the threshold for glucose removal inhibition by lactate was 50 mM (ANOVA, p
370 = 0.000747), with 79 ± 6 % at 100 mM and 61 ± 3 % at 400 mM. Similarly, the
371 addition of 222 mM lactate also reduced glucose degradation by *Clostridium*
372 *saccharoperbutylacetonicum* from 85 % to 47.5 % [39].

373 In comparison, Kim et al. [11] did not find any effect of lactate on glucose removal
374 up to 178 mM.

375 Therefore, glucose removal was hence affected differently depending on the
376 nature of the acid and its concentration. The reduction in H₂ production in the
377 presence of butyrate and lactate can already be explained, at least in part, by a

378 reduction in glucose consumption, but the inhibitory effect of 100-200 mM acetate
379 must be explained by other mechanisms, such as alteration of metabolic pathways or
380 microbial communities.

381 **3.4. Metabolic pathways**

382 For each flask, a COD mass balance was performed at the end of the H₂ production
383 phase, and varied between 78 and 112 %, with an average of 97 ± 6 %
384 (Supplementary material 4). These results clearly show that all major metabolites
385 were considered in the analysis.

386 Considering the concentrations of acetate, butyrate and lactate at the end of the
387 experiment, it was observed that a partial consumption of the added acids occurred
388 during the fermentation. It was not surprising to observe lactate [8] and acetate
389 [32,35] consumption, but the decrease in butyrate concentration was more surprising,
390 although Chen et al. [17] also observed it. Metabolic pathways were investigated
391 taking into account the consumption of glucose and of the exogenously added acid
392 and calculating yields in $\text{gCOD}\cdot\text{gCOD}^{-1}$ (Fig. 1 b - d). Without acid addition (i.e.,
393 glucose is the only source of substrate), H₂ yield reached $0.13 \pm 0.02 \text{ gCOD}\cdot\text{gCOD}^{-1}$
394 ($1.6 \pm 0.2 \text{ mol}\cdot\text{mol}_{\text{glucose}}^{-1}$) and acetate and butyrate were the major products, with
395 $0.09 \pm 0.02 \text{ gCOD}\cdot\text{gCOD}^{-1}$ ($14 \pm 3 \text{ mM}$, $0.28 \pm 0.05 \text{ mol}\cdot\text{mol}_{\text{glucose}}^{-1}$) and 0.52 ± 0.04
396 $\text{gCOD}\cdot\text{gCOD}^{-1}$ ($30 \pm 3 \text{ mM}$, $0.59 \pm 0.06 \text{ mol}\cdot\text{mol}_{\text{glucose}}^{-1}$), respectively. Other
397 metabolites were notably produced but in smaller quantities: lactate, formate,
398 propionate, succinate and butanol.

399 Metabolic pathways were greatly influenced by acid addition. A same trend was
400 observed for increasing addition of acetate and butyrate, with a progressive decline
401 of acetate and butyrate pathways in favour of lactate pathway, associated with a

402 decrease in H₂ yield. Ultimately, for the three acids, increasing concentration resulted
403 in a total shift from acetate/butyrate to lactate fermentation, i.e, at 300 mM of acetate,
404 200 mM of butyrate and 400 mM of lactate, which coincides with the previously
405 reported concentration associated with a total inhibition of H₂ production in this study.

406 Acetate addition induced a specific decrease of acetate production pathway.
407 Indeed, butyrate-to-acetate (B/A, mol/mol) ratio was 2.1 ± 0.1 without acid addition,
408 and increased up to 21 at 100 mM. Above 100 mM acetate, endogenous acetate
409 reached a maximum of 4.6 mM and was often consumed, while it was 15 ± 2 mM
410 without acid addition. This specific inhibition of acetate pathway and its consumption
411 was already observed elsewhere [6,32,35]. Such total inhibition of the acetate
412 pathway (Fig. 1 – b) was not associated with a total inhibition of the butyrate
413 pathway.

414 Butyrate addition caused a reduction of the butyrate pathway, but less selective
415 as B/A decreased from 2.6 ± 1.1 to 1.8 ± 0.5 above 100 mM butyrate. Above 150 mM
416 butyrate, butyrate production was restricted to a maximum of 11 mM, while it was 31
417 ± 3 mM without acid addition. Interestingly, when butyrate pathway was totally
418 inhibited, acetate pathway was also totally suppressed. This observation is consistent
419 with the idea supported by Van Ginkel and Logan [7] suggesting that the reduction of
420 butyrate pathway is more deleterious for the cell due to the reduction of NAD⁺
421 regenerating pathways. Indeed, butyrate production allows the regeneration of NAD⁺
422 through Crotonyl coA reduction, Acetoacetyl coA reduction and H₂ production while
423 acetate production allow this regeneration only through H₂ producing pathways. To
424 ensure adequate regeneration of NAD⁺ when butyrate pathway is thermodynamically
425 restricted due to an excess of butyrate, electrons are likely redirected towards the

426 production of solvent, such as lactate or propionate [7]. Some of the lactate observed
427 in presence of acids could be produced through this mechanism.

428 Conversely, Lactate from 0 to 250 mM had a low impact on metabolic spectrum
429 as it reduced only slightly the butyrate yield from $0.53 \pm 0.03 \text{ gCOD}\cdot\text{gCOD}^{-1}$ to $0.43 \pm$
430 $0.08 \text{ gCOD}\cdot\text{gCOD}^{-1}$ and the H_2 yield from $0.14 \pm 0 \text{ gCOD}\cdot\text{gCOD}^{-1}$ to 0.12 ± 0.0
431 $\text{gCOD}\cdot\text{gCOD}^{-1}$. However, above 250 mM of lactate, H_2 , butyrate and acetate yield
432 decreased as lactate yield increased, as for acetate and butyrate addition.

433 In addition, endogenous lactate production and lactate yields at 200 mM of
434 acetate and 100 mM of butyrate, i.e at concentrations close to the K_c of 209 mM of
435 acetate and 104 mM of butyrate, were $50 \pm 12 \text{ mM}$, $0.47 \pm 0.1 \text{ gCOD}\cdot\text{gCOD}^{-1}$ and 42
436 $\pm 20 \text{ mM}$, $0.41 \pm 0.19 \text{ gCOD}\cdot\text{gCOD}^{-1}$, respectively. In other words, 50 % inhibition of
437 HPR in presence of acetate and butyrate was associated with a similar lactate
438 production induction.

439 In conclusion, metabolic pathways were significantly affected by the addition of
440 organic acids, and the inhibition was strongly related to the nature and concentration
441 of the added acid. These results show that inhibition by organic acids in mixed culture
442 cannot be explained solely by undissociated acid concentrations. Analyses of
443 bacterial communities was then performed to distinguish cellular metabolic pathways
444 alteration and microbial communities alteration caused by organic acids, which is
445 currently poorly understood.

446 **3.5. Bacterial communities**

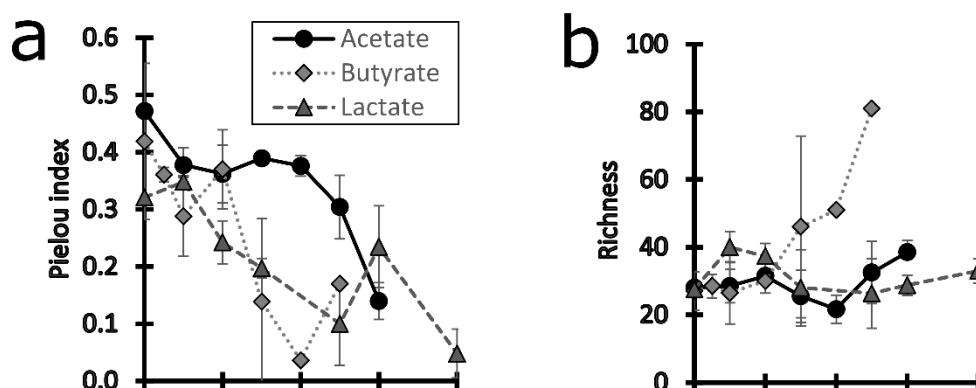
447 2714 OTUs were identified in all the experiments and grouped in 33 phyla, 70
448 classes, 153 orders, 310 families and 657 genera. Only three classes showed a
449 relative abundance higher than 1 %: Clostridia, Bacillii and Gammaproteobacteria.

450 Among them, 12 genera showed a relative abundance higher than 1 % and only 9
451 genera showed a relative abundance higher than 10 %. After rarefication, 477 OTUs
452 were kept, grouped in 12 phyla, 28 classes, 63 orders, 127 family and 198 genera.

453

454

Fig. 2 - Bacterial community analysis. Effects of acetate, butyrate and lactate on (a) pielou and (b) richness indexes. (c) Mean relative abundance of bacteria at family level depending on the concentration of acetate, butyrate and lactate.



456 Acid concentration had a significant negative effect on sample evenness (Kruskal
457 Wallis, $p = 0.002$ for pielou index) and an insignificant effect on sample richness (32
458 ± 11 species considering all samples, Kruskal Wallis, $p = 0.27$) (Fig. 2 a, b). This
459 result means that acid addition did not reduce the number of species in the microbial
460 community, but favoured an uneven growth of the species present. In comparison,
461 Chen et al. [17] found that inhibition of H_2 production by butyrate was associated with
462 an increase in microbial diversity but a decrease in richness. These differences can
463 be explained by the higher richness in Chen et al. [17] experiments, which they
464 estimated with the Chao1 index to be 132 ± 28 in the control conditions, whereas we
465 estimate Chao1 to be 32 ± 5 in the control experiments of this study. The lower
466 richness in the present study might be due to the combination of inoculum storage
467 process (freeze-drying and storage at $-80\text{ }^\circ\text{C}$) and of the pretreatment (15 min at 90
468 $^\circ\text{C}$) carried, while Chen et al. (2021a) only performed a 15 min pretreatment at 100
469 $^\circ\text{C}$.

470 Microbial diversity at family level is presented in Fig. 2 c, depending on the nature
471 and the concentration of added acid. With no acid addition, *Clostridiaceae* family
472 represented $78 \pm 11\%$ of the microbial community. This is consistent with high
473 abundances of the Firmicutes phylum and *Clostridiaceae* family in high-yielding H_2
474 producing reactor as reported by Etchebehere et al. [40]. In addition to the strong
475 dominance of *Clostridiaceae* family, bacteria from the *Bacillaceae*, *Lactobacillaceae*
476 and *Aeromonadaceae* families are also emerging in a variable extent, from 4 to 13 %,
477 0 to 17 % and 0 to 19 %, respectively. Such variability in minor OTUs is not unusual
478 in mixed culture [41] and is not associated in this study with significant differences in
479 metabolic products.

480 At a concentration at which a total inhibition of H₂ production was observed, i.e.,
481 300 mM, 200 mM and 400 mM for acetate, butyrate and lactate addition respectively,
482 the microbial community analysis revealed a drastic shift in families relative
483 abundances. 300 mM of acetate resulted in 87 ± 3 % *Lactobacillaceae*. 200 mM of
484 butyrate led to 98 % *Bacillaceae* while 250 mM of butyrate led to 71 % *Bacillaceae*
485 and 27 % *Enterococcaceae*. 400mM of lactate induced the selection of 98.9 ± 0.1 %
486 *Lactobacillaceae* in 3 flasks and the growth of 89 % *Enterococcaceae* in one flask.

487 Below C_{max}, trends at family level are not fully clear, as *Clostridiaceae* accounted in
488 average for more than 50 % of the bacterial community, in all conditions excepted at
489 200 mM of acetate. Therefore, the impact of acid addition was investigated at genus
490 level (Table 3). Addition of acetate, butyrate or lactate below C_{max} inhibited
491 *Clostridium_sensu_stricto_11* growth (OTU 4, *Clostridium neuense* / *acetobutylicum* /
492 *hydrogeniproducens*, 99.5 % identity), as its abundance decreased from 29 ± 8 % in
493 control conditions to 2 ± 3 %. Also, *Bacillus* (OTU 10, *Bacillus thuringiensis* 99.3%
494 identity) was inhibited by the addition of acetate, butyrate or lactate as its abundance
495 decreased from 8 ± 4 % to 0 % below C_{max}. In contrast, *Rumellibacillus* (OTU 12,
496 *Rummeliibacillus pycnus* 99.8 % identity) abundance increased as acetate
497 concentration increased, from 0 % to 8 % below C_{max}. *Bacillus sp.* and *Lactobacillus*
498 *sp.* favoured in presence of high concentration of acids were identified as
499 *Weizmannia coagulans* (100 % identity) and *Lactobacillus plantarum* (97.9 %
500 identity), respectively. Interestingly, acetate, butyrate and lactate addition did not
501 inhibit the emergence of *Clostridium_sensu_stricto_1* (OTU 1, *C. butyricum*, 98.5 %
502 identity) as its abundance reached 45 ± 8 % in control conditions and varied between
503 0.1 % and 83.4 % at 250 mM of acetate, 150 mM of butyrate and 300 mM of lactate.
504 Acid addition could even favour the growth of *C. butyricum* as its abundance reached

505 57 % for 150 mM of acetate, 66 % for 50 mM of butyrate and 91 % for 250 mM of
 506 lactate.

507 From these results, it was hypothesised that the diminution in abundance of
 508 *Clostridium* sp. (OTU 4) and the emergence of *L. plantarum* and *W. coagulans* were
 509 responsible for the diminution of the H₂ yield at concentrations below C_{max}. Globally,
 510 the emergence of *C. butyricum* instead of *Clostridium* sp. (OTU 4) below C_{max}, did
 511 not permit to maintain H₂ yield although the abundance in *Clostridiaceae* was stable.

512 Interestingly, H₂ yield reached 0.11 gCOD·gCOD⁻¹ when the community was
 513 composed of 53 % *C. butyricum* and 26 % *Clostridium* sp. (OTU 4) (at 50 mM of
 514 lactate) and when the community was composed of 84 % *C. butyricum* and 1 %
 515 *Clostridium* sp. (OTU 4) (at 150 mM of lactate). This result show that the same H₂
 516 yield can be reached with and without *Clostridium* sp. (OTU 4), due to the growth of
 517 *C. butyricum*. In addition, 25 mM of butyrate resulted in the selection of 21 % *W.*
 518 *coagulans* and 53 % *C. butyricum* with a lactate yield of 0.07 gCOD·gCOD⁻¹
 519 (producing 7 mM of lactate), while at 100 mM of butyrate 13% *W. coagulans*, 5 % *L.*
 520 *plantarum* and 55 % *C. butyricum* with a lactate yield of 0.44 gCOD·gCOD⁻¹

Table 3 - Relative abundances of bacteria at genus or species level (%) of most represented OTUs depending on the concentration of acetate, butyrate and lactate. For each condition, all replicates for which a microbial community analysis was conducted are presented and separated by a vertical line.

Concentration of added acid (mM)	<i>Clostridium butyricum</i> (%)	<i>Clostridium</i> sp. (OTU 4) (%)	<i>Clostridium</i> sp. (%)	<i>Weizmannia coagulans</i> (%)	<i>Lactobacillus plantarum</i> (%)	<i>Enterococcus hirae</i> (%)	<i>Bacillus thuringiensis</i> (%)	<i>Rummeliibacillus pycnus</i> (%)	Others (%)	
Acetate	0	35 43	29 22	65 66	1 1	0 0	0 0	3 7	0 0	32 26
	50	39 59	7 9	49 73	0 11	0 3	49 12	0 0	0 0	2 1
	100	49 63	7 4	58 67	29 17	9 14	3 0	0 0	1 1	1 1
	150	52 61	12 1	65 63	19 12	14 12	0 12	0 0	1 1	1 0
	200	51 11 41	1 2 0	52 29 42	10 8 8	35 60 48	0 0 1	0 0 0	3 2 2	0 0 0
	250	68 0	8 1	76 1	8 20	10 67	1 0	0 0	5 11	0 1
	300	0 0	0 0	1 0	5 7	91 84	0 0	0 0	3 8	1 1
Butyrate	0	37 53	17 35	74 88	4 1	17 0	0 0	4 9	0 0	0 2
	25	53 55	23 2	77 72	21 24	0 0	1 0	0 1	0 0	1 2
	50	61 71	14 21	76 92	23 6	0 0	0 0	0 0	0 0	1 2
	100	70 40 56	16 3 19	86 53 75	1 35 13	6 8 5	1 3 0	0 0 0	0 0 0	5 1 6
	150	0 65	0 3	0 69	98 31	0 0	0 0	0 0	0 0	2 0
	200	0	0	0	98	0	0	0	0	1
250	0	0	0	71	0	27	0	0	2	
Lactate	0	54 48	32 40	86 88	0 0	0 0	0 0	13 11	0 0	1 1
	50	38 52 55	29 28 25	97 95 89	0 0 0	0 0 0	0 2 10	0 1 1	0 0 0	2 2 1
	100	77 77 61	3 0 6	84 78 67	0 0 0	14 18 29	0 0 0	1 3 2	0 0 0	1 1 2
	150	84 84	3 0	89 86	0 0	9 8	0 4	1 1	0 0	0 1
	250	86 86 99	0 0 0	87 86 99	1 1 0	12 13 0	0 0 0	0 0 0	0 0 0	1 1 0
	300	0 18 83 78	0 0 0 0	68 19 83 79	31 27 1 7	0 54 15 4	0 0 0 9	0 0 0 0	0 0 0 0	0 1 1 0
	400	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	99 99 9 99	0 0 89 0	0 0 0 0	0 0 0 0	1 1 1 1

521 (producing 44mM of lactate) was observed. As it is hardly possible that 5 % *L.*
522 *plantarum* would be responsible for the 6-fold improvement in lactate concentration
523 and yield, this result suggests that some lactate was produced by *C. butyricum*.
524 Considered all together, these results suggest that *C. butyricum* shifted its
525 metabolism from acetate/butyrate pathway to lactate pathway in presence of acids.
526 The comparison of spearman correlation coefficient between H₂ yield and *Clostridium*
527 *sp.* (OTU 4) abundance ($\rho = 0.59$, p-value = 6.53×10^{-5}) on one hand and between
528 H₂ yield and *C. butyricum* abundance ($\rho = 0.16$, p-value = 0.32) in another hand
529 reinforced this hypothesis. This metabolic shift could be explained by the perturbation
530 of the NADH/NAD⁺ ratio after acid addition [42]. This hypothesis is consistent with
531 the findings of Payot et al. [43], who showed that *Clostridium cellulolyticum* shifted its
532 metabolism from acetate-butyrate pathways to lactate in case of accumulation of
533 intracellular NADH.

534 To decipher the impact of acid addition at populational or metabolic levels,
535 bacterial communities of each sample were compared qualitatively (i.e. considering
536 which species grew) and quantitatively (i.e. at what relative abundance). This beta
537 diversity analysis was performed by calculating the distance between microbial
538 communities at genus level in pairs (UniFrac distance metric) and then by applying a
539 hierarchical clustering method to the distance matrix (Unweighted pair group method
540 with arithmetic mean, UPGMA). The result is presented as a dendrogram in Fig. 3.
541 Nine clusters were distinguished and bootstrapping (n=10,000) revealed that these
542 clusters were strongly supported by the data (approximately unbiased probability
543 value > 95 %). In this Figure, the distance between two microbial communities is
544 represented by the height of the dendrogram.

545 All conditions for which no H₂ production was observed are grouped in the
546 cluster 8. This cluster 8 is subdivided in two clusters, with samples associated to
547 butyrate addition on one side, and samples associated to acetate or lactate addition
548 on the other side. This observation is consistent with the observations of populational
549 shifts above C_{max}. In addition, samples with no acid addition or in presence of 50
550 mM butyrate and 50 mM lactate were grouped in cluster 2. The operating conditions
551 of the communities grouped in this cluster are those associated with the highest H₂
552 production performances, with $207 \pm 13 \text{ mLH}_2/\text{g}_{\text{glucose added}}$. Thus, for the extreme
553 conditions of high H₂ production and total inhibition of H₂ production, the
554 performances were clearly associated to populational shifts with clear and selection
555 of distinct microbial communities.

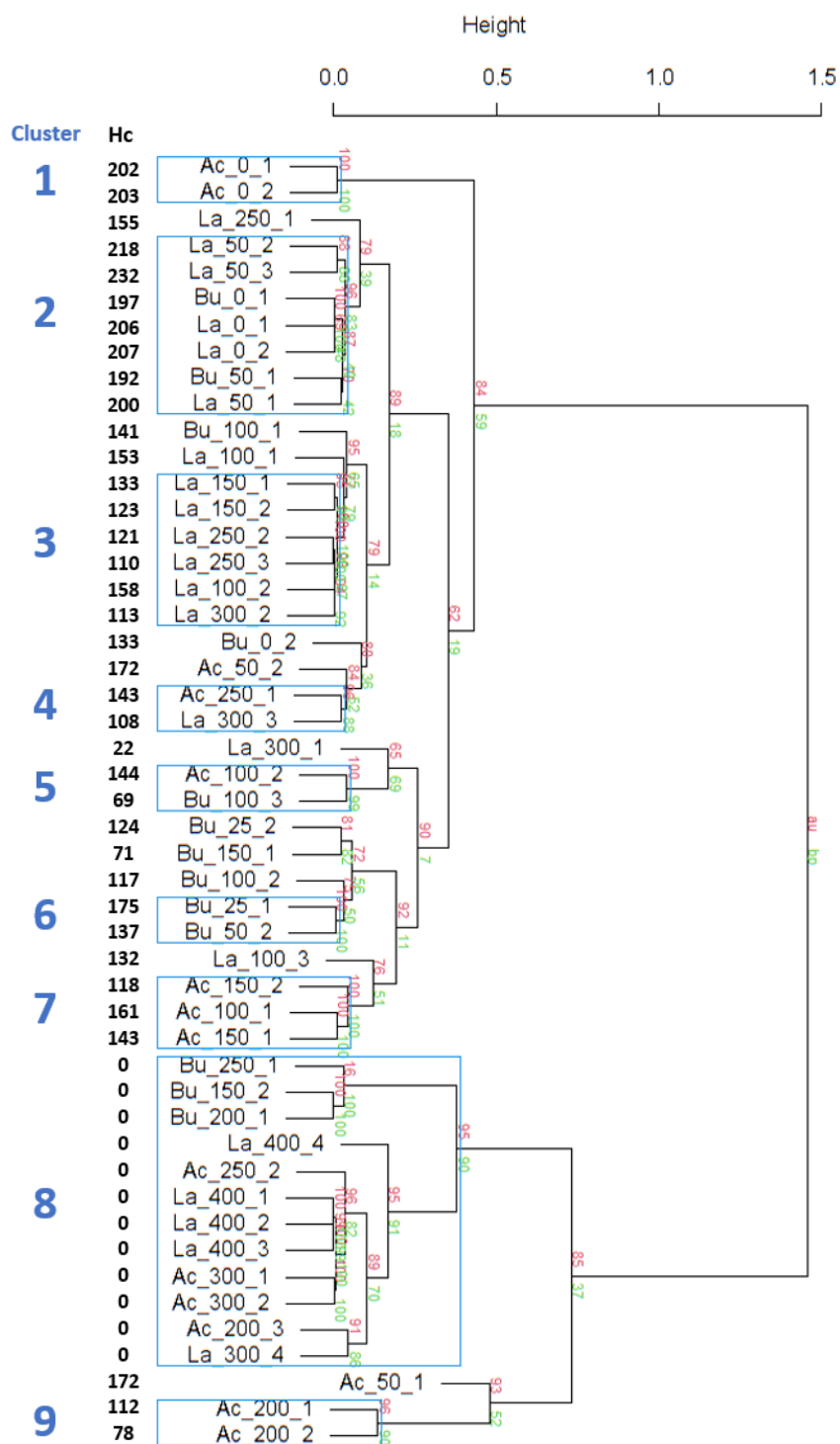


Fig. 3 - Bootstrapped dendrogram of Beta diversity. Bootstrap probability value (BP) and approximately unbiased probability values (AU) appear in green and red respectively above each node. Clusters associated with an AU > 95 % circled in blue and numbered from 1 to 9. The maximal volume of cumulated H₂ per gram of glucose added (Hc, mLH₂/g_{glucose added}) measured in each condition are presented. For each sample, the added acid is abbreviated as “La”, “Bu” and “Ac” for lactate, butyrate and acetate respectively. The initial concentration of the added acid is indicated between two underscores. The end number, from 1 to 4, is used to distinguish the replicates.

557 Interestingly, almost all samples with an exogenous addition of lactate at 100
558 to 300 mM and with H₂ yield between 110 mLH₂/g_{glucose added} and 158 mLH₂/g_{glucose}
559 _{added} are grouped in cluster 3, with a low height between each sample (< 0.1).
560 Therefore, it was concluded that the inhibition of H₂ production by adding lactate from
561 100 mM to 300 mM was not related to population changes but to a lower substrate
562 degradation only. In addition, clusters 4, 5, 7 and 9 do not group samples sharing a
563 similar H₂ production performance. For example, cluster 7 groups three samples in
564 presence of 100 and 150 mM of acetate, although H₂ production varies from 118
565 mLH₂/g_{glucose added} to 161 mLH₂/g_{glucose added} within these conditions. Thus, the
566 differences in H₂ yield within these conditions are not due to changes in populations,
567 but more probably to changes in metabolic pathways. These results support again
568 the conclusion that below C_{max}, a combination of metabolic shifts and to a lesser
569 extent populational changes explained the observed reduction in H₂ yield.

570 Inhibition by organic acids is one of the main causes of low H₂ production
571 during DF in mixed cultures [5] and a deeper understanding of the mechanisms
572 behind these inhibitions will contribute to select appropriate solutions to relieve this
573 inhibition. Indeed, inhibition associated with metabolic shifts within a bacterial
574 community could be addressed with acid tolerance response induction [44] or with
575 organic acids extractions.

576 However, acid extraction, although often chosen, can be a major source of
577 process failure due to the formation of cake layer or fouling and increase the energy
578 and financial input [45]. Alternatively, inhibition associated with a populational shift
579 could be mitigated at a lower cost and more easily through operational parameters
580 modification (pH, temperature, organic loading rate) to disfavour the undesirable
581 bacteria or with bioaugmentation with H₂-producing strains. Thus, the present study

582 highlights that above 250 mM of acetate, 150 mM of butyrate and 300 mM of lactate,
583 the strategies implemented to alleviate inhibition must address the finding that
584 populational change is the main factor responsible for a decrease in H₂ production.

585 **4. Conclusions**

586 In this study, the initial addition of acetate, butyrate and lactate in DF was
587 investigated. A significant reduction on H₂ production was shown. Butyrate and
588 lactate affected glucose removal, whereas 100 % glucose degradation efficiency was
589 observed at high acetate concentration of 250 mM. For all acids, significant
590 diminution of bacterial diversity was observed when increasing the concentration. 300
591 mM of acetate, 200 mM of butyrate and 400 mM of lactate resulted in total
592 community shift through the inhibition of *Clostridiaceae* growth in favour of
593 *Lactobacillaceae* and *Bacillaceae*. Below these concentrations, metabolic changes
594 were mostly responsible of the decrease in H₂ production performances. More
595 specifically, *C. butyricum* was suspected to shift its metabolism towards lactate
596 production. Overall, this work shows that both the nature and the concentration of the
597 inhibitors should be considered, as well as the microbial communities involved in the
598 process. These new insights on the mechanisms of inhibition by end-products in
599 mixed culture can be useful to efficiently select strategies for overcoming inhibition.

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605 **6. CRediT authorship contribution statement**

606 M. Noguera: Investigation, formal analysis, visualization, writing - original draft.
607 R. Escudie: Conceptualization, supervision, validation, writing - review & editing
608 N. Bernet : Validation, writing - review & editing.
609 E. Trably: Conceptualization, funding acquisition, supervision, validation, writing -
610 review & editing.

611 7. Declaration of Competing Interest

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

615 8. References

- 616 [1] Dincer I, Acar C. Review and evaluation of hydrogen production methods for
617 better sustainability. *Int J Hydrogen Energy* 2014;40:11094–111.
618 <https://doi.org/10.1016/j.ijhydene.2014.12.035>.
- 619 [2] Łukajtis R, Hołowacz I, Kucharska K, Glinka M, Rybarczyk P, Przyjazny A, et
620 al. Hydrogen production from biomass using dark fermentation. *Renew Sustain*
621 *Energy Rev* 2018;91:665–94. <https://doi.org/10.1016/j.rser.2018.04.043>.
- 622 [3] Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark
623 fermentative hydrogen production by mesophilic microflora: Principles and
624 progress. *Int J Hydrogen Energy* 2007;32:172–84.
625 <https://doi.org/10.1016/j.ijhydene.2006.08.014>.
- 626 [4] Castelló E, Nunes Ferraz-Junior AD, Andreani C, Anzola-Rojas M del P,
627 Borzacconi L, Buitrón G, et al. Stability problems in the hydrogen production by
628 dark fermentation: Possible causes and solutions. *Renew Sustain Energy Rev*
629 2020;119:109602. <https://doi.org/10.1016/j.rser.2019.109602>.
- 630 [5] Chen Y, Yin Y, Wang J. Recent advance in inhibition of dark fermentative
631 hydrogen production. *Int J Hydrogen Energy* 2021;46:5053–73.
632 <https://doi.org/10.1016/j.ijhydene.2020.11.096>.
- 633 [6] Wang B, Wan W, Wang J. Inhibitory effect of ethanol, acetic acid, propionic
634 acid and butyric acid on fermentative hydrogen production. *Int J Hydrogen*
635 *Energy* 2008;33:7013–9. <https://doi.org/10.1016/j.ijhydene.2008.09.027>.
- 636 [7] Van Ginkel S, Logan BE. Inhibition of biohydrogen production by undissociated
637 acetic and butyric acids. *Environ Sci Technol* 2005;39:9351–6.
638 <https://doi.org/10.1021/es0510515>.

- 639 [8] García-Depraect O, Castro-Muñoz R, Muñoz R, Rene ER, León-Becerril E,
640 Valdez-Vazquez I, et al. A review on the factors influencing biohydrogen
641 production from lactate: The key to unlocking enhanced dark fermentative
642 processes. *Bioresour Technol* 2021;324:124595.
643 <https://doi.org/10.1016/j.biortech.2020.124595>.
- 644 [9] Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A. Effect of extrinsic
645 lactic acid on fermentative hydrogen production. *Int J Hydrogen Energy*
646 2009;34:2573–9. <https://doi.org/10.1016/j.ijhydene.2009.01.010>.
- 647 [10] Noblecourt A, Christophe G, Larroche C, Fontanille P. Hydrogen production by
648 dark fermentation from pre-fermented depackaging food wastes. *Bioresour*
649 *Technol* 2018;247:864–70. <https://doi.org/10.1016/j.biortech.2017.09.199>.
- 650 [11] Kim TH, Lee Y, Chang KH, Hwang SJ. Effects of initial lactic acid
651 concentration, HRTs, and OLRs on bio-hydrogen production from lactate-type
652 fermentation. *Bioresour Technol* 2012;103:136–41.
653 <https://doi.org/10.1016/j.biortech.2011.09.093>.
- 654 [12] Baronofsky JJ, Schreurs WJA, Kashket ER. Uncoupling by acetic acid limits
655 growth and acetogenesis by *Clostridium thermoaceticum*. *Appl Environ*
656 *Microbiol* 1984;48:1134–9. <https://doi.org/10.1128/aem.48.6.1134-1139.1984>.
- 657 [13] Russell JB. Another explanation for the toxicity of fermentation acids at low pH:
658 anion accumulation versus uncoupling. *J Appl Bacteriol* 1992;73:363–70.
659 <https://doi.org/10.1111/j.1365-2672.1992.tb04990.x>.
- 660 [14] van Niel EWJ, Claassen PAMM, Stams AJMM. Substrate and product inhibition
661 of hydrogen production by the extreme thermophile, *Caldicellulosiruptor*
662 *saccharolyticus*. *Biotechnol Bioeng* 2003;81:255–62.
663 <https://doi.org/10.1002/bit.10463>.
- 664 [15] Eklund T. The antimicrobial effect of dissociated and undissociated sorbic acid
665 at different pH levels. *J Appl Bacteriol* 1983;54:383–9.
666 <https://doi.org/10.1111/j.1365-2672.1983.tb02632.x>.
- 667 [16] Kovanda L. In vitro antimicrobial activities of organic acids and their derivatives
668 on several species of gram-negative and Gram-positive bacteria. *Molecules*
669 2019;24:3770. <https://doi.org/10.3390/molecules24203770>.
- 670 [17] Chen Y, Yin Y, Wang J. Influence of butyrate on fermentative hydrogen
671 production and microbial community analysis. *Int J Hydrogen Energy*
672 2021;46:26825–33. <https://doi.org/10.1016/j.ijhydene.2021.05.185>.
- 673 [18] Noblecourt A, Christophe G, Larroche C, Santa-Catalina G, Trably E, Fontanille
674 P. High hydrogen production rate in a submerged membrane anaerobic
675 bioreactor. *Int J Hydrogen Energy* 2017;42:24656–66.
676 <https://doi.org/10.1016/j.ijhydene.2017.08.037>.
- 677 [19] Dauptain K, Schneider A, Noguier M, Fontanille P, Escudie R, Carrere H, et al.
678 Impact of microbial inoculum storage on dark fermentative H₂ production.
679 *Bioresour Technol* 2021;319:124234.
680 <https://doi.org/10.1016/j.biortech.2020.124234>.
- 681 [20] Bundhoo MAZ, Mohee R, Hassan MA. Effects of pre-treatment technologies on

- 682 dark fermentative biohydrogen production: A review. *J Environ Manage*
683 2015;157:20–48. <https://doi.org/10.1016/j.jenvman.2015.04.006>.
- 684 [21] Wang H, Fang M, Fang Z, Bu H. Effects of sludge pretreatments and organic
685 acids on hydrogen production by anaerobic fermentation. *Bioresour Technol*
686 2010;101:8731–5. <https://doi.org/10.1016/j.biortech.2010.06.131>.
- 687 [22] Wang Y, Zhao QB, Mu Y, Yu HQ, Harada H, Li YY. Biohydrogen production
688 with mixed anaerobic cultures in the presence of high-concentration acetate. *Int*
689 *J Hydrogen Energy* 2008;33:1164–71.
690 <https://doi.org/10.1016/j.ijhydene.2007.12.018>.
- 691 [23] Park W, Hyun SH, Oh S-E, Logan BE, Kim IS. Removal of Headspace CO₂
692 Increases Biological Hydrogen Production. *Environ Sci Technol* 2005;39:4416–
693 20. <https://doi.org/10.1021/es048569d>.
- 694 [24] Chang S, Li J, Liu F, Yu Z. Effect of different gas releasing methods on
695 anaerobic fermentative hydrogen production in batch cultures. *Front Environ*
696 *Sci Eng China* 2012;6:901–6. <https://doi.org/10.1007/s11783-012-0403-1>.
- 697 [25] APHA. Standard Methods for the Examination of Water and Wastewater. vol.
698 20th ed. 2017.
- 699 [26] Lakatos G. Biological wastewater treatment. 2018.
700 <https://doi.org/10.1201/b18368-4>.
- 701 [27] Braga Nan L, Trably E, Santa-Catalina G, Bernet N, Delgenès JP, Escudié R.
702 Biomethanation processes: New insights on the effect of a high H₂ partial
703 pressure on microbial communities. *Biotechnol Biofuels* 2020;13:1–17.
704 <https://doi.org/10.1186/s13068-020-01776-y>.
- 705 [28] Kahm M, Hasenbrink G, Lichtenberg-Fraté H, Ludwig J, Kschischo M. Grofit:
706 Fitting biological growth curves with R. *J Stat Softw* 2010;33:1–21.
707 <https://doi.org/10.18637/jss.v033.i07>.
- 708 [29] Lozupone C, Knight R. UniFrac: A new phylogenetic method for comparing
709 microbial communities. *Appl Environ Microbiol* 2005;71:8228–35.
710 <https://doi.org/10.1128/AEM.71.12.8228-8235.2005>.
- 711 [30] Cope EK, Goldberg AN, Pletcher SD, Lynch S V. Compositionally and
712 functionally distinct sinus microbiota in chronic rhinosinusitis patients have
713 immunological and clinically divergent consequences. *Microbiome* 2017;5:1–
714 16. <https://doi.org/10.1186/s40168-017-0266-6>.
- 715 [31] McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive
716 Analysis and Graphics of Microbiome Census Data. *PLoS One* 2013;8:e61217.
717 <https://doi.org/10.1371/journal.pone.0061217>.
- 718 [32] Siqueira MR, Reginatto V. Inhibition of fermentative H₂ production by hydrolysis
719 byproducts of lignocellulosic substrates. *Renew Energy* 2015;80:109–16.
720 <https://doi.org/10.1016/j.renene.2015.01.070>.
- 721 [33] Zheng XJ, Yu HQ. Inhibitory effects of butyrate on biological hydrogen
722 production with mixed anaerobic cultures. *J Environ Manage* 2005;74:65–70.
723 <https://doi.org/10.1016/j.jenvman.2004.08.015>.

- 724 [34] Suresh A, Grygolowicz-Pawlak E, Pathak S, Poh LS, Abdul Majid M bin,
725 Dominiak D, et al. Understanding and optimization of the flocculation process in
726 biological wastewater treatment processes: A review. *Chemosphere*
727 2018;210:401–16. <https://doi.org/10.1016/j.chemosphere.2018.07.021>.
- 728 [35] Colin T, Bories A, Lavigne C, Moulin G. Effects of acetate and butyrate during
729 glycerol fermentation by *Clostridium butyricum*. *Curr Microbiol* 2001;43:238–43.
730 <https://doi.org/10.1007/s002840010294>.
- 731 [36] Heyndrickx M, De Vos P, Vancanneyt M, De Ley J. The fermentation of
732 glycerol by *Clostridium butyricum* LMG 1212t2 and 1213t1 and *C.*
733 *pasteurianum* LMG 3285. *Appl Microbiol Biotechnol* 1991;34:637–42.
734 <https://doi.org/10.1007/BF00167914>.
- 735 [37] Zhang S, Kim T-H, Lee Y, Hwang S-J. Effects of VFAs concentration on bio-
736 hydrogen production with *Clostridium Bifermentans* 3AT-ma. *Energy Procedia*
737 2012;14:518–23. <https://doi.org/10.1016/j.egypro.2011.12.968>.
- 738 [38] Liu D, Min B, Angelidaki I. Biohydrogen production from household solid waste
739 (HSW) at extreme-thermophilic temperature (70 °C) - Influence of pH and
740 acetate concentration. *Int J Hydrogen Energy* 2008;33:6985–92.
741 <https://doi.org/10.1016/j.ijhydene.2008.08.059>.
- 742 [39] Oshiro M, Hanada K, Tashiro Y, Sonomoto K. Efficient conversion of lactic acid
743 to butanol with pH-stat continuous lactic acid and glucose feeding method by
744 *Clostridium saccharoperbutylacetonicum*. *Appl Microbiol Biotechnol*
745 2010;87:1177–85. <https://doi.org/10.1007/s00253-010-2673-5>.
- 746 [40] Etchebere C, Castelló E, Wenzel J, Del M, Anzola-Rojas P, Borzacconi L, et
747 al. Microbial communities from 20 different hydrogen-producing reactors
748 studied by 454 pyrosequencing. *Appl Microbiol Biotechnol* 2016;100:3371–84.
749 <https://doi.org/10.1007/s00253-016-7325-y>.
- 750 [41] Poirier S, Steyer JP, Bernet N, Trably E. Mitigating the variability of hydrogen
751 production in mixed culture through bioaugmentation with exogenous pure
752 strains. *Int J Hydrogen Energy* 2020;45:2617–26.
753 <https://doi.org/10.1016/j.ijhydene.2019.11.116>.
- 754 [42] Srikanth S, Venkata Mohan S. Regulating feedback inhibition caused by the
755 accumulated acid intermediates during acidogenic hydrogen production
756 through feed replacement. *Int J Hydrogen Energy* 2014;39:10028–40.
757 <https://doi.org/10.1016/j.ijhydene.2014.04.152>.
- 758 [43] Payot S, Guedon E, Gelhaye E, Petitdemange H. Induction of lactate
759 production associated with a decrease in NADH cell content enables growth
760 resumption of *Clostridium cellulolyticum* in batch cultures on cellobiose. *Res*
761 *Microbiol* 1999;150:465–73. [https://doi.org/10.1016/S0923-2508\(99\)00110-2](https://doi.org/10.1016/S0923-2508(99)00110-2).
- 762 [44] Huang Z, Yu X, Miao H, Ren H, Zhao M, Ruan W. Enzymatic dynamics of
763 microbial acid tolerance response (ATR) during the enhanced biohydrogen
764 production process via anaerobic digestion. *Int J Hydrogen Energy*
765 2012;37:10655–62. <https://doi.org/10.1016/j.ijhydene.2012.04.116>.
- 766 [45] Tharani D, Ananthasubramanian M. Process intensification in separation and
767 recovery of biogenic volatile fatty acid obtained through acidogenic

768 fermentation of organics-rich substrates. *Chemical Eng Process Process Intensif*
769 2021;169:108592. <https://doi.org/10.1016/j.cep.2021.108592>.
770