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

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Abstract

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

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

1. Introduction

Low carbon footprint hydrogen (H_2) could play a major role in the future to reduce carbon dioxide emissions and prevent global warming. Among the existing green H_2 production technologies, biological production by dark fermentation (DF) presents one of the lowest global warming potentials [1]. When performed with mixed cultures, DF presents also the advantages of being light-independent, having high H_2 production rates and using various low-cost raw materials and waste as feedstocks [2]. This promising biological process relies on the degradation of carbohydrate-rich organic matter by H_2-producing bacteria in anaerobic conditions. Efficient H_2 production in DF is mostly associated to acetate and butyrate metabolic pathways with the stoichiometry from Equation (1) [3],

$$4C_6H_{12}O_6 + 2H_2O \rightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 10H_2 \quad \text{(Equation 1)}$$

together with the dominance of species belonging to the *Clostridiaceae* family. Although the microbial diversity in mixed cultures can provide robustness and function redundancies, it could also disfavour the H_2 production by introducing microorganisms engaged in substrate competition (*Lactobacillus sp.*), in the release of inhibitory co-products (bacteriocins, lactate) or in H_2 consumption (bacteria with homoacetogenic activities or hydrogenotrophic methanogens) [4].

The effects of end-products accumulation on H_2 production have been widely studied in mixed cultures [5]. Overall, acetate, butyrate, propionate and ethanol were found to have inhibitory effects on H_2 yields, hydrogen production rates (HPR) and substrate degradation [5]. Inhibitory threshold for H_2 yield (the lowest concentration that does not result in a significant reduction in H_2 yield) by butyrate varies from < 10 mM in batch at pH 7 using heat-treated digested sludge and glucose as substrate [6] to 38 mM in continuous reactor at pH 5.5 from glucose using baked agricultural soil

as inoculum [7]. Similarly, inhibition threshold for H_2 yield by acetate was reported to vary from < 10 mM [6] to 98 mM [7].

Furthermore, the activity of lactic acid producing bacteria (LAB) was associated with DF instability [8]. Regarding the specific effect of lactate, Baghchehsaraee et al. [9] showed that up to 55 mM of lactate did not inhibit H_2 production. Consistently, Noblecourt et al. [10] did not observe a negative impact of 300 mM endogenously produced lactate during the DF of pre-fermented food wastes. However, the latter observed that 44 mM of exogenously added L-lactate induced a 35 % decrease in H_2 production, highlighting that enantiomeric form of lactate plays a key role in its inhibitory effect. In addition, Kim et al. [11] observed that 178 mM of lactate (unknown enantiomeric form) induced a 40 % increase in lag phase. Therefore, the different results and conclusions found in literature concerning inhibition by acetate, butyrate and lactate are very dependent on the experimental conditions as well as the reactor feeding modes applied, hindering efficient prevention of end-products inhibition.

The cellular mechanisms of such end-products inhibition on the microbial activity was deeply studied with pure cultures. Past experiments showed that organic acids can affect the bacterial activity and thus inhibit H_2 production by various means. In their undissociated form, the organic acids can freely cross the cytoplasmic membranes and easily dissociate inside the cell, thus acidifying the cytoplasm and dissipating the proton motive force [12]. Active proton extrusion by ATPases counteracts this effect, but at the expense of the energy available for growth. In their dissociated form, the organic acids can increase the ionic strength, triggering cell 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 H_2 production
79 reduction was associated with a decrease in the proportion of H_2-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 H_2 production rate but
88 had no impact on the composition of the highly enriched microbial communities from
89 sewage sludge. These results show that H_2 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 H_2 fermentation routes

in a mixed culture. More specifically, the novelty of this investigation is attributed to the qualitative and quantitative comparison of bacterial communities through a beta diversity analysis to distinguish between metabolic inhibition (i.e within the same community, changes in metabolic activity are responsible for reduced H_2 production) from populational inhibition (i.e changes in the bacterial community are responsible for reduced H_2 production). For that, the impact of the addition of acetate (from 0 to 300 mM), butyrate (from 0 to 250 mM) and lactate (from 0 to 400 mM) on DF of glucose was investigated.

2. Materials and Methods

2.1. Seed sludge

Activated sludge was used as inoculum. The sludge was collected from an aerobic tank at the wastewater treatment plant (WWTP) of Narbonne (France). pH of the freshly collected sludge was 6.9. The sludge was first centrifuged 10 minutes at 16,800 g (20 °C), frozen, freeze-dried, mixed, aliquoted and stored at - 80 °C before use. Freeze-drying storage was selected as it has recently been shown to be an effective strategy for preserving the H_2 production potential of WWTP sludge [19]. After storage, the sludge was composed of 0.96 ± 0.02 gTS.g $^{-1}$ and 0.75 ± 0.04 gVS.g $^{-1}$. Prior to the experimental procedures, the sludge was rehydrated in 200 mL of osmosed water and pre-treated by heating at 90°C for 15 min to select spore forming organisms, as heat-treatment was reported as a great strategy to achieve this purpose [20].

2.2. Experimental setup

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

mM 2-(N-morpholino)ethanesulfonic acid (MES buffer) (pKa = 6.15), 0.8 $g.L^{-1}$ NH_4Cl, 0.5 $g.L^{-1}$ K_2HPO_4 and 1 mL of a micronutrient solution (in $mg.L^{-1}$: $FeCl_2,4H_2O$: 1500; H_3BO_3 : 60; $MnSO_4,H_2O$: 117; $CoCl_2,6H_2O$: 25; $ZnCl_2$: 70; $NiCl_2,6H_2O$: 25; $CuCl_2,2H_2O$: 15; $NaMoO_4,2H_2O$: 25; HCl: 1750). The glucose-to-inoculum ratio (g/g_{VS}) was set at 10, by adjusting the amount of inoculum. The effects of acetate, butyrate and lactate addition were separately evaluated, in three distinct experiments. Sodium acetate (≥ 99 %, Sigma Aldrich) was added at 0, 50, 100, 150, 200, 250 and 300 mM; sodium butyrate (98 %, Sigma Aldrich) was added at 0, 25, 50, 100, 150, 200 and 250 mM and L (+) sodium lactate (> 99 %, PanReac AppliChem) was added at 0, 50, 100, 150, 250, 300 and 400 mM. The concentration ranges evaluated for each acid were chosen based on their inhibitory effects reported in the literature [6,21,22]. Temperature was maintained at 37 ± 1 °C by means of a water bath with no stirring. As the H_2 yield in the present study were similar to the yield obtained under similar conditions with stirring (1.56 ± 0.19 $mol_{H2}/mol_{glucose\ added}$ in the present study and 1.4 ± 0.1 $mol_{H2}/mol_{glucose}$ with glucose as substrate, pH 6, 250 mL working volume, 300 RPM [23]), it was considered that stirring was not essential in the present study.

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

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 H_2,
148 O_2, N_2, CH_4 analysis and a PoraPlot U (PPU) 8 m column running at 70 °C, 20 PSI
149 with helium as carrier gas for CO_2 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 N_2 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 H_2SO_4 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

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

2.4. Microbial community analyses and sequencing

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

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

2.5. Modelling and statistical data analysis

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

194 production starts (λ, days). These three parameters were deducted from the fit as
195 follows: HPR was the maximal slope, Hc was the highest cumulated volume of H_2 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 $$HPR = \frac{HPR_{ctrl}}{1 + (C/K_c)^n}$$ (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 Kc 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 booststrap 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 H_2 production: maximal hydrogen production Hc ($mLH_2 \cdot g_{glucose\ added}^{-1}$), maximal hydrogen production rate HPR ($mL \cdot g_{glucose\ added}^{-1} \cdot day^{-1}$) 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		
	Hc ($mL \cdot g_{glucose}$	HPR ($mL \cdot g_{glucose\ added-}$	λ (days)	Hc ($mL \cdot g_{glucose}$	HPR ($mL \cdot g_{glucose\ added-}$	λ (days)	Hc ($mL \cdot g_{glucose}$	HPR ($mL \cdot g_{glucose\ added-}$	λ (days)

3. Results and Discussion

3.1. Adverse effect of acid addition on biohydrogen production

Fermentation was monitored over a period of 4 days, after which a plateau in H_2 production was reached in all conditions (Supplementary material 2). No methane production was observed in all experiments. H_2 production was modelled with a spline fit used to estimate the volume of cumulated H_2 produced per gram of glucose added (Hc, $mL_{H2} \cdot g_{glucose\ added}^{-1}$), the maximal H_2 production rate (HPR, $mL_{H2} \cdot g_{glucose\ added}^{-1} \cdot day^{-1}$) and the lag phase before H_2 production starts (λ, days) as reported in Table 1. Without acid addition, Hc reached 193 ± 23 $mL_{H2} \cdot g_{glucose\ added}^{-1}$ (1.56 ± 0.19 $mol_{H2} \cdot mol_{glucose\ added}^{-1}$), HPR 248 ± 47 $mLH_2 \cdot g_{glucose\ added}^{-1} \cdot day^{-1}$ (2.0 ± 0.38 $mol_{H2} \cdot mol_{glucose\ added}^{-1} \cdot day^{-1}$) and λ 0.56 ± 0.13 day. Less than 20 % variability was observed between the replicates for Hc, HPR and λ in each condition, exception made

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})	
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	/	/	/	/	/	/

Cumulated hydrogen production - Hc

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

Consistently, inhibitory threshold lower than 83 mM of acetate was observed at pH 5.5 with sucrose (25 gCOD·L^{-1}) [22]. Thresholds below 10 mM were found at pH 6 [32] and 7 [6] but in these study, pH was unregulated; and potentially insufficiently buffered (48 mM $NaHCO_3$ with 10 g·L^{-1} glucose), respectively.

Concerning butyrate, the inhibitory threshold observed here is consistent with the 71 mM threshold found by Zheng and Yu [33] in similar conditions, i.e, at pH 6 with 10 g.L^{-1} glucose. Again, lower thresholds of 17mM and < 10mM found by Chen et al. [17] at pH 6 and Wang et al. [6] at pH 7 were obtained with potentially insufficient buffering (48 mM $NaHCO_3$ with 10 g·L^{-1} glucose again) and no buffering of the fermentation medium.

With regards to lactate, Noblecourt et al. [10] found a lower inhibitory threshold of < 44 mM of L-lactate at pH 6. In contrast, Kim et al. [11] found that lactate addition from 11 to 178 mM at pH 4.5 improved or did not affect the overall H_2 production. Finally, the 24h pre-treatment of the inoculum at pH 2 carried by Kim et al. [11] selected acid-resistant strains, which would explain the higher tolerance to lactic acid.

Interestingly, comparing the different studies, inhibitory thresholds for H_2 production do not increase with an increase in pH. Also, in this study, inhibitory threshold for H_2 production were 50 mM for acetate, butyrate and lactate, although these acids present variable pka (4.76, 4.82 and 3.86, resp.). This may be surprising as some authors have stated that the undissociated form of the acid was the main cause of inhibition of H_2 production [7,17]. However, in Chen et al. [17] and Van Ginkel and Logan [7] studies, the coefficient of determination associated with the linear regression between H_2 yield and undissociated butyric acid concentration were quite low, achieving 0.54 and 0.04 (calculated from the data shown in the article). Furthermore, the inhibition thresholds found in the two studies were also different, reaching 6 mM [7] and less than 2 mM of undissociated butyric acid [17]. Considered as a whole, this suggests that considering undissociated acid concentrations alone is insufficient to explain inhibitions in mixed cultures. The extent of inhibition is probably also related to the different operational parameters applied and the microbial communities used as inoculum.

Hydrogen production rate - HPR

Inhibitory threshold values of HPR were estimated at 200 mM for acetate (ANOVA, $p = 0.007$), 50 mM for butyrate (ANOVA, $p = 0.00223$), and 250 mM for lactate (ANOVA, $p = 0.00011$). Thus, acetate, butyrate and lactate exhibit an inhibitory activity on H_2 production rate, which could be related either to metabolic

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

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

In addition, 50 mM of acetate induced a significant 36 % improvement of HPR (ANOVA, $p = 0.0367$). Similarly, Colin and Moulin [35] observed that acetate addition up to 210 mM induced an increase in *Clostridium butyricum* growth rate. This effect and associated increase in the production of H_2 in presence of acetate was also observed by Heyndrickx et al. [36] with *C. butyricum* and *Clostridium pasteurianum*. These authors suggested that acetate was used as an indirect proton and electron acceptor, stimulating substrate fermentation by providing intermediate acetyl-CoA.

Interestingly, 50 mM and 100 mM of lactate induced in this study significant improvements of the HPR of 48 % and 20 % compared to HPR without lactate (ANOVA, $p = 0.00005$ and 0.004 respectively). This result could be attributed to the simultaneous consumption of glucose and lactate to produce H_2. Consistently, Kim et al. [11] also reported HPR increase after lactate addition from 11 to 178 mM.

Lag phase - λ

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

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

3.2. Hydrogen production rate inhibition modelling

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

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

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

The higher inhibitory impact of butyrate on HPR with regards to acetate found in this study is consistent with the study of Van Ginkel and Logan and Zhang et al. [7,37]. In contrast, Kc of 104 mM of butyrate was 2-fold lower than the Kc of ~ 225 mM estimated from Wang et al. [6]. The latter is close to the Kc of 220 mM estimated from Zheng and Yu [33], although initial pH varied, set at 6 and 7, respectively. Chen et al. [17] observed a highly lower Kc of 17 mM of butyrate at pH 6, but, again, pH may have been potentially insufficiently buffered (48 mM NaHCO3 with 10 $g \cdot L^{-1}$ glucose). Somehow, the microbial community composition may affect resistance to organic acids, as suggested by Chen et al. [17] who investigated the impact of butyric acid in two studies; indeed, under the same operating conditions, a distinct inhibition of the H_2 production is reported [6,17].

Regarding lactate, 44 mM of L-Lactate addition by Noblecourt et al. [10] resulted in a reduction of 41 % of HPR, decreasing from 2.36 $L.h^{-1}$ to 1.39 $L.h^{-1}$. Such effect of lactate is much more detrimental than the effect reported in the present 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	Kc (mM)	Cmax (mM)	n	Initial pH - Regulation	Reference
Acetate	Freeze-dried activated sludge	93 $mL \cdot h^{-1}$	209	300	7.56	6 – Buffered with 100mM MES	This study
	Sludge from UASB treating vinasse	8.7 ± 0.3 $mL \cdot h^{-1}$	86	167	/	6 - None	[32]
	Sludge from USAB treating citrate-producing wastewater	221.7 $mL \cdot gVSS^{-1} \cdot h^{-1}$	157	>833	1.52	5.5 - Regulated	[22]
	Clostridium bifermentans,	58 $mL \cdot L \cdot h^{-1}$	/	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 $mL \cdot L \cdot h^{-1}$	/	>200	/	7, Buffered with 20 mM MOPS	[38]
	Anaerobic sludge	12.9 $mL \cdot h^{-1}$	~80	471	2.9	7 - 48mM $NaHCO_3$	[6]
Butyrate	Freeze-dried activated sludge	100 $mL \cdot h^{-1}$	104	200	3.07	6– Buffered with 100mM MES	This study
	Anaerobic sludge from WWTP	61.5 $ml \cdot gVSS^{-1} \cdot h^{-1}$	220	291	0.34	6 – Buffered with 59 mM H_2PO4^- and HPO_4^{2-}	[33]
	Sludge from anaerobic digester	112 $mLH_2 \cdot L^{-1} \cdot h^{-1}$	~225	306	0.6	7 - 48mM $NaHCO_3$	[6]
	Sludge from anaerobic digester	42 $mLH_2 \cdot L^{-1} \cdot h^{-1}$	~284	>284	/	7 - 48mM $NaHCO_3$	[17]
	Clostridium bifermentans	60.1$mL \cdot L \cdot h^{-1}$		334	2.35	7	[37]
	Sludge from anaerobic digester	27$mLH_2 \cdot L^{-1} \cdot h^{-1}$	~17	~34	/	6 - 48mM $NaHCO_3$	[17]
Lactate	Freeze-dried activated sludge	102 $mL \cdot h^{-1}$	273	400	7.58	6 – Buffered with 100mM MES	This study
	Derived mix of thermophilic sludge and sludge from WWTP, enriched with *Clostridium cellulolyticum*	2360$mL \cdot h^{-1}$	~50	>50	/	6 - Regulated	[10]

3.3. Glucose removal

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

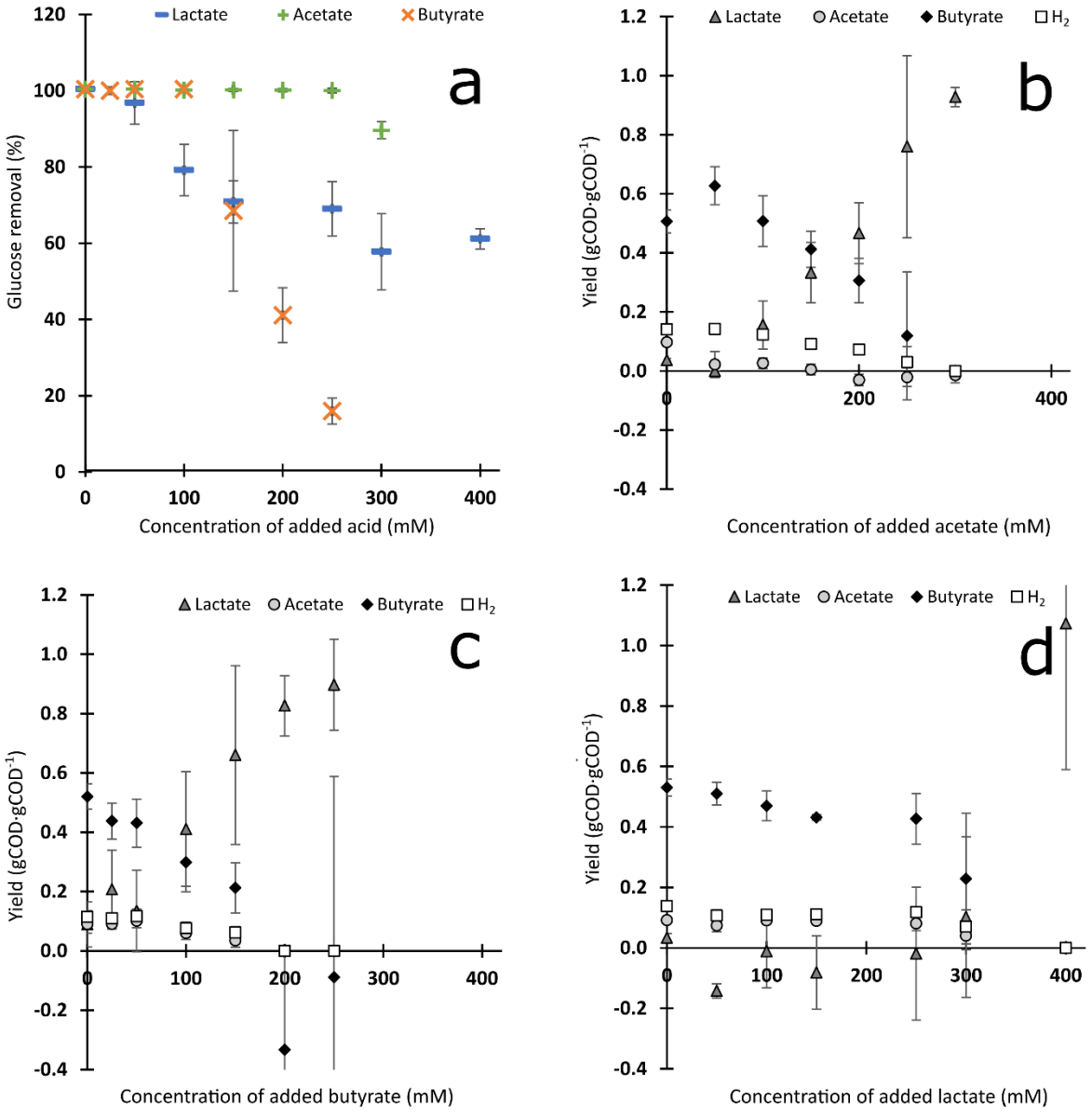


Fig. 1 - Effects of acetate, butyrate and lactate on metabolic activity. (a) mean glucose removal (%) ± standard deviation. Mean yields of products ± standard deviation as a function of acetate (b), butyrate (c) and lactate (d) concentrations. Yields were calculated in COD of products (H2, acetate, butyrate 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).

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

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

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

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

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

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

Therefore, glucose removal was hence affected differently depending on the nature of the acid and its concentration. The reduction in H_2 production in the presence of butyrate and lactate can already be explained, at least in part, by a

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

3.4. Metabolic pathways

For each flask, a COD mass balance was performed at the end of the H_2 production phase, and varied between 78 and 112 %, with an average of 97 ± 6 % (Supplementary material 4). These results clearly show that all major metabolites were considered in the analysis.

Considering the concentrations of acetate, butyrate and lactate at the end of the experiment, it was observed that a partial consumption of the added acids occurred during the fermentation. It was not surprising to observe lactate [8] and acetate [32,35] consumption, but the decrease in butyrate concentration was more surprising, although Chen et al. [17] also observed it. Metabolic pathways were investigated taking into account the consumption of glucose and of the exogenously added acid and calculating yields in $gCOD \cdot gCOD^{-1}$ (Fig. 1 b - d). Without acid addition (i.e., glucose is the only source of substrate), H_2 yield reached 0.13 ± 0.02 $gCOD \cdot gCOD^{-1}$ (1.6 ± 0.2 $mol \cdot mol_{glucose}^{-1}$) and acetate and butyrate were the major products, with 0.09 ± 0.02 $gCOD \cdot gCOD^{-1}$ (14 ± 3 mM, 0.28 ± 0.05 $mol \cdot mol_{glucose}^{-1}$) and 0.52 ± 0.04 $gCOD \cdot gCOD^{-1}$ (30 ± 3 mM, 0.59 ± 0.06 $mol \cdot mol_{glucose}^{-1}$), respectively. Other metabolites were notably produced but in smaller quantities: lactate, formate, propionate, succinate and butanol.

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

decrease in H_2 yield. Ultimately, for the three acids, increasing concentration resulted in a total shift from acetate/butyrate to lactate fermentation, i.e, at 300 mM of acetate, 200 mM of butyrate and 400 mM of lactate, which coincides with the previously reported concentration associated with a total inhibition of H_2 production in this study.

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

Butyrate addition caused a reduction of the butyrate pathway, but less selective as B/A decreased from 2.6 ± 1.1 to 1.8 ± 0.5 above 100 mM butyrate. Above 150 mM butyrate, butyrate production was restricted to a maximum of 11 mM, while it was 31 ± 3 mM without acid addition. Interestingly, when butyrate pathway was totally inhibited, acetate pathway was also totally suppressed. This observation is consistent with the idea supported by Van Ginkel and Logan [7] suggesting that the reduction of butyrate pathway is more deleterious for the cell due to the reduction of NAD+ regenerating pathways. Indeed, butyrate production allows the regeneration of NAD+ through Crotonyl coA reduction, Acetoacetyl coA reduction and H_2 production while acetate production allow this regeneration only through H_2 producing pathways. To ensure adequate regeneration of NAD+ when butyrate pathway is thermodynamically 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 ± 0.03 gCOD·gCOD^{-1} to 0.43 ±
430 0.08 gCOD·gCOD^{-1} and the H_2 yield from 0.14 ± 0 gCOD·gCOD^{-1} to 0.12 ± 0.0
431 gCOD·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 ± 12 mM, 0.47 ± 0.1 gCOD·gCOD^{-1} and 42
436 ± 20 mM, 0.41 ± 0.19 gCOD·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.

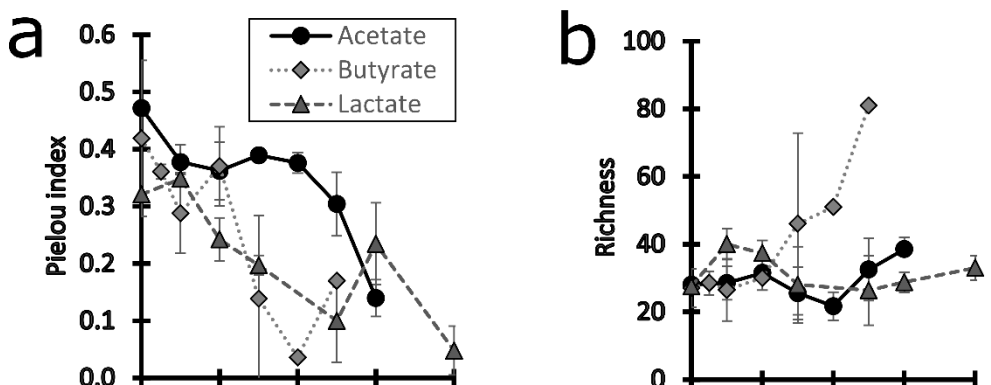
439 In conclusion, metabolic pathways were significantly affected by the addition of
440 organic acids, and the inhibition was strongly related to the nature and concentration
441 of the added acid. These results show that inhibition by organic acids in mixed culture
442 cannot be explained solely by 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.

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

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.



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

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

At a concentration at which a total inhibition of H_2 production was observed, i.e., 300 mM, 200 mM and 400 mM for acetate, butyrate and lactate addition respectively, the microbial community analysis revealed a drastic shift in families relative abundances. 300 mM of acetate resulted in 87 ± 3 % *Lactobacillaceae*. 200 mM of butyrate led to 98 % *Bacillaceae* while 250 mM of butyrate led to 71 % *Bacillaceae* and 27 % *Enterococcaceae*. 400mM of lactate induced the selection of 98.9 ± 0.1 % *Lactobacillaceae* in 3 flasks and the growth of 89 % *Enterococcaceae* in one flask.

Below Cmax, trends at family level are not fully clear, as *Clostridiaceae* accounted in average for more than 50 % of the bacterial community, in all conditions excepted at 200 mM of acetate. Therefore, the impact of acid addition was investigated at genus level (Table 3). Addition of acetate, butyrate or lactate below Cmax inhibited *Clostridium_sensu_stricto_11* growth (OTU 4, *Clostridium neuense / acetobutylicum / hydrogeniproducens,* 99.5 % identity), as its abundance decreased from 29 ± 8 % in control conditions to 2 ± 3 %. Also, *Bacillus* (OTU 10, *Bacillus thuringiensis* 99.3% identity) was inhibited by the addition of acetate, butyrate or lactate as its abundance decreased from 8 ± 4 % to 0 % below Cmax. In contrast, *Rumellibacillus* (OTU 12, *Rummeliibacillus pycnus* 99.8 % identity) abundance increased as acetate concentration increased, from 0 % to 8 % below Cmax. *Bacillus sp.* and *Lactobacillus sp.* favoured in presence of high concentration of acids were identified as *Weizmannia coagulans* (100 % identity) and *Lactobacillus plantarum* (97.9 % identity), respectively. Interestingly, acetate, butyrate and lactate addition did not inhibit the emergence of *Clostridium_sensu_stricto_1* (OTU 1, *C. butyricum*, 98.5 % identity) as its abundance reached 45 ± 8 % in control conditions and varied between 0.1 % and 83.4 % at 250 mM of acetate, 150 mM of butyrate and 300 mM of lactate. 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 H_2 yield at concentrations below Cmax. Globally,
510 the emergence of *C. butyricum* instead of *Clostridium sp.* (OTU 4) below Cmax, did
511 not permit to maintain H_2 yield although the abundance in *Clostridiaceae* was stable.

512 Interestingly, H_2 yield reached 0.11 $gCOD \cdot gCOD^{-1}$ 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 H_2
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 $gCOD \cdot gCOD^{-1}$
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 $gCOD \cdot gCOD^{-1}$

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)		*Clostridium butyricum (%)*	*Clostridium sp. (OTU 4) (%)*	Clostridium sp. (%)	*Weizmannia coagulans (%)*	*Lactobacillus plantarum (%)*	*Enterococcus hirae (%)*	*Bacillus thuringiensis (%)*	*Rummeliibacillus pycnus (%)*	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

(producing 44mM of lactate) was observed. As it is hardly possible that 5 % *L. plantarum* would be responsible for the 6-fold improvement in lactate concentration and yield, this result suggests that some lactate was produced by *C. butyricum*. Considered all together, these results suggest that *C. butyricum* shifted its metabolism from acetate/butyrate pathway to lactate pathway in presence of acids. The comparison of spearman correlation coefficient between H_2 yield and *Clostridium sp.* (OTU 4) abundance ($\rho = 0.59$, p-value = 6.53×10^{-5}) on one hand and between H_2 yield and *C. butyricum* abundance ($\rho = 0.16$, p-value = 0.32) in another hand reinforced this hypothesis. This metabolic shift could be explained by the perturbation of the NADH/NAD+ ratio after acid addition [42]. This hypothesis is consistent with the findings of Payot et al. [43], who showed that *Clostridium cellulolyticum* shifted its metabolism from acetate-butyrate pathways to lactate in case of accumulation of intracellular NADH.

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

All conditions for which no H_2 production was observed are grouped in the cluster 8. This cluster 8 is subdivided in two clusters, with samples associated to butyrate addition on one side, and samples associated to acetate or lactate addition on the other side. This observation is consistent with the observations of populational shifts above Cmax. In addition, samples with no acid addition or in presence of 50 mM butyrate and 50 mM lactate were grouped in cluster 2. The operating conditions of the communities grouped in this cluster are those associated with the highest H_2 production performances, with 207 ± 13 $mLH_2/g_{glucose\ added}$. Thus, for the extreme conditions of high H_2 production and total inhibition of H_2 production, the performances were clearly associated to populational shifts with clear and selection of distinct microbial communities.

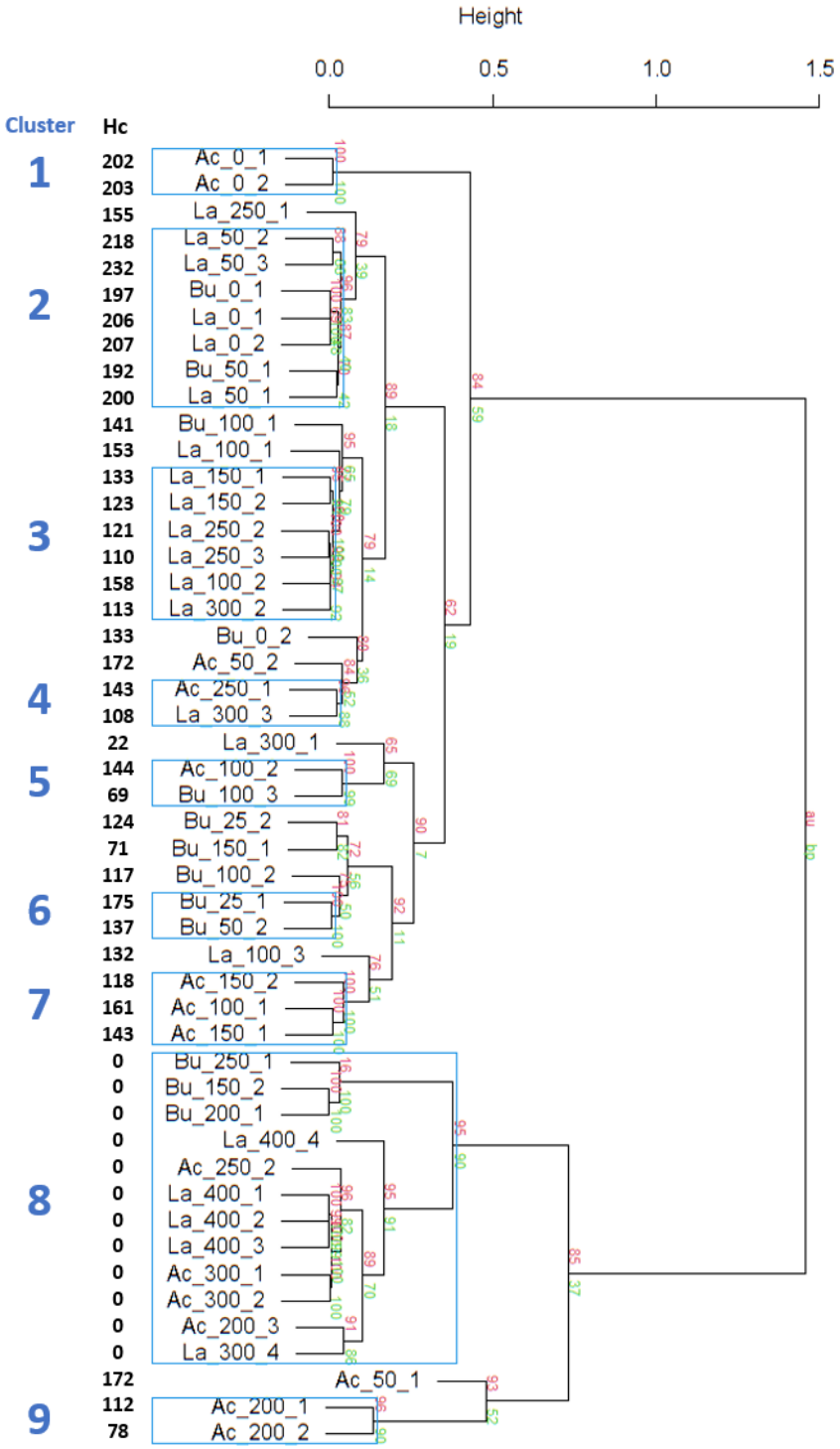


Fig. 3 - Bootstrapped dendrogram of Beta diversity. Booststrap probability value (BP) and approximately unbiased probability values (AU) appear in green and red respectively above each node. Clusters associated with an AU > 95 % circled in blue and numbered from 1 to 9. The maximal volume of cumulated H_2 per gram of glucose added (Hc, $mLH_2/g_{glucose\ added}$) 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.

556

557 Interestingly, almost all samples with an exogenous addition of lactate at 100
558 to 300 mM and with H_2 yield between 110 $mLH_2/g_{glucose\ added}$ and 158 $mLH_2/g_{glucose}$
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 H_2 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 H_2 production performance. For example, cluster 7 groups three samples in
564 presence of 100 and 150 mM of acetate, although H_2 production varies from 118
565 $mLH_2/g_{glucose\ added}$ to 161 $mLH_2/g_{glucose\ added}$ within these conditions. Thus, the
566 differences in H_2 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 Cmax, a combination of metabolic shifts and to a lesser
569 extent populational changes explained the observed reduction in H_2 yield.

570 Inhibition by organic acids is one of the main causes of low H_2 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 H_2-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 H_2 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 H_2 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 H_2 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.

600 ## 5. Acknowledgements

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605 ## 6. CRediT authorship contribution statement

M. Noguer: Investigation, formal analysis, visualization, writing - original draft.

R. Escudie: Conceptualization, supervision, validation, writing - review & editing

N.Bernet : Validation, writing - review & editing.

E. Trably: Conceptualization, funding acquisition, supervision, validation, writing - review & editing.

7. Declaration of Competing Interest

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

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770