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1 **Sub-dominant bacteria as keystone species in microbial communities producing bio-**
2 **hydrogen**

3

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20

21 **Abstract**

22

23 Nowadays mixed cultures are considered as a serious alternative to pure cultures in biotechnological
24 processes. Mixed cultures can use various organic substrates and be operated under unsterile and
25 continuous conditions. Although most of studies dealing with fermentative hydrogen production have
26 focused on dominant species, sub-dominant bacteria can also have a significant effect despite their low
27 abundance. The determination of their exact ecological role is essential for better understanding
28 microbial metabolic networks in mixed cultures.

29 In this work, the contribution of sub-dominant bacteria to fermentative H₂ production was investigated
30 using chemostats continuously fed with a glucose-based medium. Interestingly, *Clostridium*
31 *pasteurianum* was dominant in six assays on seven at steady state, and only bacterial populations in
32 low abundance differed. Acting as keystone species, these bacteria impacted substantially the
33 microbial metabolic network of the overall ecosystem despite their low abundance. While *Bacillus*
34 spp. and *Lactobacillus* spp. lowered the H₂ yields by diverting a part of the H₂ potential to lactate
35 production, the presence of *E. coli* increased the H₂ yield by redirecting the metabolic network to
36 acetate and butyrate hydrogen-producing pathways.

37

38 **Keywords**

39 Biohydrogen / Chemostat / Dark fermentation / Microbial interactions / Minority bacteria /

40 Mixed cultures

41

42

43 **1 Introduction**

44

45 Hydrogen (H₂) is considered as one of the most interesting energy carriers in a global context of
46 exploring new sources of renewable energy and reducing greenhouse gases. H₂ has a high energetic
47 yield (122 kJ.g⁻¹), can be stored and its combustion produces only water. Nowadays, H₂ is mainly
48 produced by chemical processes such as natural gas reforming or water electrolysis. These processes
49 produce large amounts of H₂ but are high energy-consuming and release high amounts of CO₂ into the
50 environment [1].

51 Hydrogen can also be produced in biological processes and becomes more environmental friendly.
52 Especially, in anaerobic digestion bioprocesses, H₂ is a key metabolic intermediate which is produced
53 by acetogenic bacteria during fermentation of organic compounds, and is then immediately consumed
54 by microorganisms coupling the oxidation of H₂ to the reduction of more oxidized compounds [2].
55 Such H₂ consumption can be avoided by using appropriate operating conditions [3,4]. In addition,
56 fermentative processes operated with mixed bacterial cultures can use a large range of renewable,
57 complex, non-sterile and inexpensive substrates to produce H₂ [5–7]. Compared to pure cultures, these
58 bioprocesses are more robust and susceptible to adapt themselves more easily to changes of
59 environmental conditions [4,7]. Finally, such complex microbial communities are able to provide
60 useful combinations of metabolic pathways for the degradation and the transformation of complex
61 substrates [8].

62 In counterpart, maximal conversion yields of carbohydrates are limited to 2.5 mol H₂.mol⁻¹ hexose
63 under experimental conditions [3], whereas the maximal theoretical yield is 4 mol H₂.mol⁻¹ hexose
64 through the acetate pathway. Instability problems of these processes are also often encountered.
65 Instability is mainly due to either a shift of microbial metabolic pathways in response to environmental
66 conditions, or the emergence competitive non hydrogen-producing bacteria for carbohydrates or direct
67 H₂-consuming bacteria [9].

68 To limit this instability and thus optimize bioprocesses, many operational parameters have already
69 been tested, such as pH, temperature and hydraulic retention time [10,11]. It is recommended to

70 maintain pH lower than 6.5, and more generally between 5.5 and 6.0 [8,12,13]. Hydrogen production
71 occurs at mesophilic (around 37°C), thermophilic (around 55°C) or even hyperthermophilic (60 –
72 80°C) conditions, although most of the studies found in the literature have been carried out under
73 mesophilic conditions [12,14]. When reactors are operated in a continuous mode, especially with
74 glucose as single substrate, a short hydraulic retention time ranging from 2 to 12 hours is
75 recommended to avoid the presence of methanogenic *Archaea* which consumes efficiently H₂ and
76 fermentation end-products to produce methane and carbon dioxide [3,10,15]. Another way to limit the
77 presence of H₂-consuming microorganisms is to apply several pre-treatment methods of the inoculum
78 such as heat-shock treatment, acidic and alkaline treatments and microaerobic treatment. These
79 treatments, especially heat-shock, favour specifically the growth of spore-forming H₂-producing
80 bacteria while H₂-consuming microorganisms are strongly inhibited, such as methanogens or
81 homoacetogenic bacteria.

82 So far, most of literature studies have focused on dominant hydrogen-producing species such as
83 *Clostridium* spp. Nevertheless, the presence of low abundant bacterial species are always observed in
84 reactors operated under continuous mode, but their exact role is still unknown [3]. These experimental
85 observations are not consistent with the competitive exclusion principle stating that microbial diversity
86 should not be maintained in a chemostat at steady-state, unless the microbial populations are subject to
87 product inhibition, cross-feeding or other direct microbial interactions [16–18]. Thus, low abundant
88 bacteria should participate significantly to ecosystem functioning despite their low abundance, and
89 therefore some of them may be considered as keystone species as defined by Smeets [19]. The influence
90 of these keystone species occurs through interactions between microorganisms. These interactions are
91 often trophic: a microorganism is dependent on another microorganism for the degradation of specific
92 substrates or products [17,20], or different microorganisms outcompete for the same substrate [21]. In
93 amensalism cases, a microorganism has an adverse effect on other microorganisms, for example by
94 producing antibiotics or toxic compounds [22]. Other physical or chemical, intra and extracellular
95 mechanisms can also occur, including cellular communication between microorganisms, so called
96 "quorum sensing" or through direct exchange of molecules or electrons *via* cytoplasmic connections,
97 pilis or nanotubes [23,24].

98 The aim of this study was to determine the ecological role of low abundant bacteria in mixed cultures
99 producing H₂ by dark fermentation. Seven inocula originated from anaerobic sludge, caecotrophs,
100 fermented cassava, or a mixture of them were tested. Heat shock treatment was also used to reduce the
101 microbial diversity in initial inoculum. Hydrogen-producing microbial communities were
102 characterized after 40 hydraulic retention times at steady state in continuous chemostats operated
103 under same conditions.

104

105 2 Material and methods

106 107 2.1 Inoculum sources

108
109 Three different sources of anaerobic inoculum were selected: (i) an anaerobic sludge (AS) was
110 sampled from the outlet of a lab-scale methanogenic reactor fed with wine distillery wastewater (ii)
111 cassava (Cas) was previously fermented in water and then homogenised with a sterile mixer, and (iii)
112 cæcotrophs (Cæ) (*i.e.*, soft fæces of rabbits) were collected from the digestive system of rabbits and
113 diluted in autoclaved physiological water solution (9 g NaCl.L⁻¹). For each inoculum, a heat treatment
114 shock (90°C, 10 min) was additionally tested (named AS^{ht}, Cas^{ht} and Cæ^{ht}). Finally, a mixture of the
115 three inoculum sources (Mix) was prepared in the same ratio of volatile solids (VS).

116 117 2.2 Feeding solution

118
119 A glucose solution of 10 g.L⁻¹ was used as sole carbon source in a feeding medium composed by the
120 following nutrients (in mg.L⁻¹): K₂HPO₄, 500; NH₄Cl, 2 000; yeast extract, 200; HCl 37%, 55; MgCl₂,
121 55; FeSO₄(NH₄)₂SO₄, 7; ZnCl₂, 1; MnCl₂, 1.2; CuSO₄, 0.4; CoSO₄, 1.3; BO₃H₃, 0.1; Mo₇O₂₄(NH₄)₆, 1;
122 NiCl₂, 0.05; Na₂SeO₃, 0.01; CaCl₂, 60.

123 The glucose and nutrient solutions were autoclaved separately at 121°C for 20 minutes. The feeding
124 solution was prepared daily under sterile conditions in an Esco Labculture Class II Type A2 Biological
125 Safety Cabinet, sparged with nitrogen gas and stored at 4°C prior to use.

126 127 2.3 Experimental set-up

128
129 Seven experiments were carried out using the different sources of inoculum (AS, Cas, Cæ, AS^{ht}, Cas^{ht},
130 Cæ^{ht} and Mix). Continuous stirred tank reactors, with a working volume of 1.5 L, were used. Before
131 each experiment, reactors and sampling tubes were autoclaved at 121°C for 20 minutes. Each
132 bioreactor was filled with the feeding solution, and was then inoculated with an inoculum to reach a
133 final volatile solid content of 0.6 g VS.L⁻¹. pH was then adjusted at 5.5 using HCl, and the reactor was

134 flushed for 15 minutes with N₂ to achieve anaerobic conditions. Each assay started with a batch period
135 of 24 h. Then, the reactors were operated in a continuous mode (24 h after inoculation). The hydraulic
136 retention time (HRT) was fixed at 6 h for all experiments. The feed was stored at 4°C and was added
137 continuously in the reactor using a peristaltic pump. A level sensor and a peristaltic pump were used to
138 keep constant the liquid volume inside the reactor. Control of pH at 5.5, using NaOH 2 M, started with
139 the continuous feeding of the reactor. The reactor was heated at 37°C using a silicon heating blanket
140 wrapped around the vessel. The agitation speed was fixed at 300 rpm. Reactor was equipped with
141 temperature and pH probes. The gas flow rate was monitored using an electronic gas volumeter. All
142 continuous experiments lasted from 10 to 12 days, equivalent to more than 40-48 hydraulic retention
143 times.

144

145 *2.4 Chemical analysis*

146

147 Biogas composition (H₂, CO₂, O₂, CH₄ and N₂ content) and volatile fatty acids (VFAs) were analyzed
148 according to Aceves-Lara et al. [10]. Residual glucose as well as fermentation end-products (lactate,
149 ethanol, acetone...) were quantified using high performance liquid chromatography (HPLC) coupled
150 to a refractometer (Waters R410). The chromatograph was equipped with a HPX 87 column (Biorad)
151 and the eluent corresponded to a H₂SO₄ solution (0.222 µL.L⁻¹) under isocratic elution at 0.4 mL.min⁻¹.
152 The operating conditions were: temperature of column, 35°C; temperature of refractometer, 40°C.

153 The biomass concentration of reactor outlet was assessed by the determination of volatile suspended
154 solids (VSS) by filtration at 0.2 µm and drying at 500°C overnight, according to Standard Methods
155 [25].

156

157 *2.5 DNA extraction and PCR amplification*

158

159 Microbial cells were collected after centrifugation at 12,100 x g for 15 min of two milliliters of
160 culture. Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification kit
161 (Promega). The amount and purity of DNA in the extracts were measured by spectrophotometry
162 (Infinite NanoQuant M200, Tecan).

163 The 16S rRNA genes were amplified according to Quéméneur et al. [13] for further CE-SSCP
164 analysis. Peaks were assigned by cloning and sequencing as previously described by Godon et al. [26].
165

166 2.6 *CE-SSCP fingerprinting*

167
168 CE-SSCP fingerprinting method was previously described by Quéméneur et al. [13]. CE-SSCP
169 profiles were aligned with an internal standard to consider any electrophoretic variability. CE-SSCP
170 profiles were then normalized using the StatFingerprints library [27] from R version 2.9.2 [28] and
171 according to standard procedure [29]. On average, each peak of the CE-SSCP profile corresponded to
172 the gene fragment of a unique 16S rDNA sequence, and thus represented a specific species within the
173 microbial community. The area under a peak represented the relative abundance of the corresponding
174 microbial species in the community.

175

176 2.7 *Assignment of the CE-SSCP peaks*

177

178 PCR products were purified using a PCR Purification Kit (Qiagen). The clone libraries were built
179 using the TA Cloning kit (Invitrogen). After PCR amplification using plasmid-targeted primers T7 and
180 P13, the PCR-CE-SSCP profiles of each PCR product, corresponding to an unique sequence and
181 therefore an unique peak, were compared with the mixed profiles of the H₂-producing cultures for
182 peak assignment. After assignment, the corresponding cloned inserts were sent for sequencing
183 (MilleGen company, Toulouse, France). The nucleotide sequences, were aligned with reference
184 sequences retrieved from the Genbank database using the CLUSTALW program [30], and were
185 further refined manually using the BioEdit program [31]. The 16S rRNA gene sequences were
186 deposited in the European Nucleotide Archive database and available in Genbank database under the
187 accession numbers HE613257 to HE613267.

188

189 2.8 *COD mass balance and statistical analysis*

190

191 A COD mass balance of the glucose fermentation was calculated for all reactors at steady state to
192 validate that all metabolic end-products were quantified. During H₂ fermentation, glucose was
193 converted to VFAs, alcohols, hydrogen, carbon dioxide and biomass. In order to convert bacterial
194 growth in COD or mmol flow, the chemical formula for biomass was assumed as C₅H₇NO₂ [32].
195 Significant differences between H₂ performances and metabolic network of the different chemostats
196 were assessed by Student's t-tests and ANOVA using R software.

197

198

199 **3 Results**

200 201 *3.1 Bacterial communities at steady state*

202
203 At the end of the batch period (24 h after inoculation), a significant reduction of the microbial
204 diversity was observed compared to the initial inoculum with the emergence of one dominant species
205 and several minor species. Simplification of all ecosystems was observed during the continuous
206 culture to reach a steady state after about 30 generations (hydraulic retention time, HRT). Throughout
207 the steady state period representing at least 10 HRT, the microbial community remained stable. As an
208 illustration of microbial community dynamics over experimental time, the behavior of the microbial
209 community structure in the reactor inoculated with cassava (Cas) is presented in Figure 1. The
210 behavior of all ecosystems inoculated with the seven different microbial inocula is presented as
211 Supplementary Data (Figure S1).

212
213 [FIGURE 1]

214
215 The stable microbial community structures of the seven reactors at steady state are presented in Figure
216 2. All the CE-SSCP profiles were composed of one major *Clostridium* spp. peak (*peak 1* or *peak 2*)
217 representing between 67 and 89 % of the community abundance, and several other minor peaks (from
218 *peak 3* to *peak 11*) never exceeding 19% of the total abundance. Interestingly, except in the culture
219 inoculated with the heat-treated anaerobic sludge (AS^{ht}), a single dominant bacterial species was found
220 whatever the initial inoculum. Thus, only the nature, the number and the relative abundance of the low
221 abundant bacterial populations differed from one ecosystem to another, at steady state.

222 The 16S rDNA sequence affiliations of the clones that match with the main different peaks are shown
223 in Table 1. Sequence affiliations were always above 99% of similarity with sequences of known
224 species. In the seven ecosystems, the most abundant peaks were assigned to known H₂-producing
225 *Clostridium* spp., either *C. butyricum* (*peak 1*) in AS^{ht} culture, or *C. pasteurianum* (*peak 2*) in all other
226 cultures. Among lower abundant bacterial populations, *peak 3* was detected in all chemostats, except
227 in the culture inoculated with anaerobic sludge (AS). *Peak 3* was related to *Clostridium beijerinckii*.

228 Other minority bacteria corresponded mainly to lactate-producing bacteria: *peak 4* and *peak 5*,
229 respectively affiliated to *Sporolactobacillus laevolacticus* and *Bacillus coagulans*, were detected in the
230 reactor inoculated with AS^{ht}. *Bacillus racemilacticus* (*peak 6*) was detected in the reactors inoculated
231 with AS and AS^{ht}. In the cultures generated from cassava (Cas), heat treated cassava (Casth) and heat-
232 treated cæcotroph (Cæth), the sub-dominant populations were all related to *Lactobacillus* spp.:
233 *L. paracasei*, *L. casei*, *L. nagelii* and *L. ghanensis* respectively for *peak 7*, *peak 8*, *peak 9* and *peak 10*.
234 Finally, the only minority species (*peak 11*) neither belonging to *Clostridium* genus nor lactate-
235 producing genera was affiliated to *Escherichia coli*, and was obtained in the reactor inoculated with
236 untreated cæcotrophs (Cæ).

237

238 [TABLE 1]

239 [FIGURE 2]

240

241 3.2 Hydrogen production performances

242

243 Hydrogen productivities and yields were assessed at steady state for each culture, and are presented in
244 Table 2. In this study, an HRT of 6 hours was applied and steady state was considered when H₂
245 production was stable for more than 2 days (*i.e.*, 8 HRT): the average values of H₂ productivity and
246 yield were thus calculated over this period. At steady state, hydrogen productivity ranged from 5.47 to
247 9.07 mmol H₂.L⁻¹.h⁻¹ in AS^{ht} and AS cultures, respectively. Five different levels of H₂ production were
248 observed (p<0.05, t-test). Hydrogen productivities were similar in Cæ and Cas cultures with 6.56 and
249 6.42 mmol H₂.L⁻¹.h⁻¹ (p>0.05, t-test), respectively. The Mix and Cas^{ht} cultures presented almost the
250 same H₂ production rates of 8.06 and 8.04 mmol H₂.L⁻¹.h⁻¹, respectively. Finally, the Cæ^{ht} culture
251 presented an intermediate productivity of 7.45 mmol H₂.L⁻¹.h⁻¹.

252 The H₂ yield was calculated by considering both hydrogen productivity and glucose consumption at
253 steady state. A minimum H₂ yield of 1.21 mol H₂.mol⁻¹ glucose_consumed was observed in AS^{ht}
254 culture. A similar value was found in Cas culture with 1.41 mol H₂.mol⁻¹ glucose_consumed. The Cæ^{ht}
255 and Cas^{ht} cultures presented an intermediate H₂ yield of 1.72 and 1.79 mol H₂.mol⁻¹

256 glucose_consumed, respectively. Higher results were obtained for AS and Mix cultures with about
257 1.9 mol H₂.mol⁻¹ glucose_consumed. Finally, the highest hydrogen yield was obtained in Cæ culture
258 with 2.32 mol H₂.mol⁻¹ glucose_consumed. This H₂ yield was significantly different from the yields of
259 all other cultures (p<0.05, t-test). In Table 3, the H₂ production and yields obtained from different
260 carbon sources using different inocula are reported and are mainly consistent with our results (Table
261 3).

262

263 [TABLE 2]

264 [TABLE 3]

265

266 3.3 *Metabolic pathways*

267

268 Figure 3 presents the COD mass balance of the different cultures at steady state. By considering the
269 composition of reactor outlet in biomass, gas and liquid, the COD mass balance was identical to the
270 inlet with a reasonable variability error of 10%. It confirmed that no major metabolite was missing
271 during the analytical procedure.

272

273 [FIGURE 3]

274

275 Figure 4 presents the distribution of metabolic end-products for each reactor. All the cultures produced
276 acetate, butyrate and lactate but in different proportions. Ethanol production was also detected in Cæ^{ht},
277 Cas and Cas^{ht} cultures with respectively 0.45, 0.86 and 0.86 mmol.L⁻¹.h⁻¹. The lactate production
278 ranged from 0.75 to 3.44 mmol.L⁻¹.h⁻¹ in Cæ and AS^{ht} cultures, respectively. The lowest acetate
279 production was observed in AS^{ht} culture with 1.06 mmol.L⁻¹.h⁻¹ and the lowest butyrate production
280 was observed in Cæ culture with 1.59 mmol.L⁻¹.h⁻¹. Meanwhile, the highest production of acetate and
281 butyrate was obtained in AS culture with 1.90 and 2.18 mmol.L⁻¹.h⁻¹, respectively. In addition, the
282 Acetate/Butyrate ratio varied in all cultures (Table 2). It was lower than one for the AS^{ht}, AS and Cas^{ht}
283 cultures (0.65, 0.84 and 0.86 respectively) and close to one for the Cas, Cæ^{ht} and Mix cultures.

284 Interestingly the Ac/But ratio of the Cæ culture was the only one greater than one, indicating a shift in
285 microbial metabolic pathway in this culture.

286 Biomass concentration also differed significantly from one culture to another. The highest biomass
287 concentration was observed in AS culture with $1 \text{ mmol.L}^{-1}.\text{h}^{-1}$, which is twice the concentration
288 observed in AS^{ht} culture.

289

290 [FIGURE 4]

291

292

293 **4 Discussion**

294 295 *4.1 Correlation between hydrogen production performances and metabolic network*

296
297 In this study, a large range of H₂ yields and productivities were observed, but all were consistent with
298 those previously reported in literature with mixed cultures [7-9; 12; 33]. Significant differences on H₂
299 production performances were observed and were directly related to the community structure of the
300 microbial ecosystems present at steady state which also exhibited distinct metabolisms. Interestingly,
301 community structures of the microbial ecosystems were not only influenced by the initial inoculum,
302 but were also substantially different after batch incubation, with different proportions of sub-dominant
303 bacteria. Therefore, initial conditions, i.e. inoculum diversity and microbial community structure, after
304 the batch incubation had a high influence on the final microbial community found after 40 HRT.

305 In terms of reactor performances, the Cæ culture showed the highest H₂ yield with 2.32 mol H₂.mol⁻¹
306 glucose_consumed, but the total amount of metabolites in Cæ culture was much lower than those
307 observed with other inocula. Indeed, bacterial populations of Cæ culture degraded only 25% of inlet
308 glucose suggesting a higher specificity and efficiency of the microbial pathways to produce hydrogen.
309 The main end-products observed in Cæ culture were acetate (37%) and butyrate (34%), which are the
310 two main metabolites linked to dark fermentative H₂ production pathways. According to
311 stoichiometric reactions of dark fermentation, the formation of one mole of acetate or one mole of
312 butyrate lead both to the release of two moles of H₂ (eq. 1 and 2). In contrast, there is no direct
313 relationship between H₂ production and lactate or ethanol production (eq. 3 and 4). In Cæ culture,
314 lactate represented only 13% of the end-product flow, and no ethanol production was observed. These
315 results indicated that the degradation in the Cæ culture was more specific than with the other initial
316 inoculum.

317 The other reactors produced also H₂ using the acetate and butyrate metabolic pathways, but showed
318 lower H₂ yields. Two hypotheses could be formulated to explain this observation: Either a direct
319 consumption of H₂ through homoacetogenesis (eq. 5) or a shift of microbial metabolisms towards non-
320 H₂-production pathways, such as ethanol or lactate. In all reactors, the ratio of H₂ production and the

321 sum of acetate and butyrate remained almost constant around 2 mol H₂.mol⁻¹ VFAs_{produced} which
322 corresponded to the theoretical value. This suggested that there was no direct H₂ consumption due to
323 homoacetogenesis, since acetate did not accumulate. The lower H₂ yield observed for the other
324 reactors is mainly explained by less specific hydrogen-producing pathways and a higher production of
325 other end-products, such as lactate and ethanol.

326



328



330



332



334



336

337 4.2 Role of dominant bacteria in hydrogen production

338

339 Interestingly, metabolites distribution as well as H₂ production was significantly different in all
340 continuous cultures although the operating parameters were constant and similar. The only parameter
341 that varied from one experiment to another was the bacterial community at steady state and, therefore,
342 microbial community structure is the sole parameter that can explain such differences in metabolic
343 network and H₂ performances. Consistently, when two cultures showed the same species distribution
344 at steady state (Cas^{ht} and Mix), metabolic network and H₂ performances were similar.

345 In this study, whatever the inoculum source, the final microbial ecosystems were simplified with only
346 one majority bacterial species and few low abundant minority species. At steady state, the main H₂-
347 producing bacteria found in all the ecosystems selected belonged to *Clostridium* genus, which is in
348 accordance with the literature where clostridia are the main dominant bacteria producing hydrogen

349 [12,38-39]. Furthermore, the type of *Clostridium* species had an effect on the overall H₂ production.
350 Indeed, the lowest H₂ production was observed in AS^{ht} culture which was the only experiment with
351 *Clostridium butyricum* as dominant species. This is explained by the preferential use of the butyrate
352 pathway (acetate/butyrate ratio of 0.64) by *C. butyricum* as compared to *C. pasteurianum*. According
353 to eq.1 and 2, the butyrate pathway leads to half of the amount of H₂ produced through acetate
354 pathway. Such preferential usage of butyrate pathway of *C. butyricum* was already reported in
355 previous studies with similar acetate/butyrate ratios ranging from 0.45 to 0.67 [40–42].
356 In contrast, *Clostridium pasteurianum* led to a more equilibrated byproducts distribution
357 (acetate/butyrate ratio between 0.87 and 1.08). Therefore, *C. pasteurianum* favoured H₂ production by
358 using the acetate pathway compared to *C. butyricum*. This explains partly why H₂ yields were higher
359 in the six other cultures when *C. pasteurianum* was the dominant species.

360

361 4.3 Sub-dominant bacteria as keystone species within the metabolic network

362

363 The presence of the same single dominant species in these ecosystems constituted a major driving
364 force for H₂ production. Nevertheless, substantial differences were observed in metabolic pathways,
365 although operating conditions remained the same. When considering the six ecosystems with
366 *C. pasteurianum* as dominant species (AS, Cæ, Cæ^{ht}, Cas, Cas^{ht} and Mix), the significant differences
367 in H₂ production and in end-products distribution were attributed to the persistence of different sub-
368 dominant species after 40 HRT. Interestingly, each of these species never exceeded 19% of the total
369 abundance. According to the competitive exclusion principle [43], which states that a simple
370 unstructured environment containing a single resource can only support the growth of one competitor,
371 these sub-dominant bacteria should have been washed out unless they interacted with their
372 environment. Despite their low abundance, the sub-dominant bacteria had the highest influence on the
373 overall ecosystem metabolism. In that sense, these sub-dominant bacteria may be viewed as true
374 keystone species [19,44].

375

376 *4.3.1 Role of competitive Clostridium spp.*

377
378 *Clostridium beijerinckii* was one of the sub-dominant bacteria in the Cas, Cas^{ht}, Cæ, Cæ^{ht} and Mix
379 cultures. A competitive (trophic) interaction with the dominant *Clostridium* was expected. No
380 significant effect of *C. beijerinckii* was shown on H₂ production. However, this species is known to
381 produce H₂ through both acetate and butyrate pathways. In some cases it may become a substrate
382 competitor and produces propionate, butanol, lactate or ethanol [45]. In this study, the exact role of the
383 different species of *Clostridium* genus cannot be distinguished. Whatever the experiment, the
384 abundance of bacteria belonging to the *Clostridium* genus ranged from 79-97%. Therefore, the
385 differences in H₂ production resulted from the microbial activity of only 3 to 21% of the bacteria that
386 belong to other species.

387

388 *4.3.2 Role of homolactic bacteria through either competition or amensalism.*

389
390 Five cultures (AS, Cas^{ht}, Cæ^{ht}, Cas and Mix) contained lactic acid bacteria, although heat treatment
391 should have drastically reduced the amount of lactic acid bacteria in the initial inoculum [46]. The
392 presence of non-spore-forming lactic acid bacteria at steady state was likely due to low survival by
393 heat resistance of these microorganisms in initial heat-treated inocula. In the five cultures, the lactate
394 concentration was higher with negative effects on H₂ production. Karadag and Puhakka [47] also
395 found high lactate production co-occurred with low H₂ production in presence of *Bacillus coagulans*.
396 Indeed, homolactic bacteria are known to produce lactate with no associated H₂ production, according
397 to eq. 3. [36]. These bacteria do not use H₂ directly but consume glucose which is then no longer
398 available for H₂ production. These lactic bacteria outcompete with *Clostridium* spp. for glucose.
399 Ethanol production was observed in cultures Cas^{ht}, Cas and Cæ^{ht}. For *Clostridium* species, such
400 ethanol production can be caused by a physiological stress [9]. Noike et al. [48] showed that
401 *Lactobacillus* spp. can decrease H₂ production by secreting bacteriocins in the culture media. The
402 presence of bacteriocins is stressful for clostridia which leads to a switch in the metabolic pathway
403 from H₂ production to solvent production (ethanol and lactate mainly) [9]. Bacteriocins can also affect

404 the presence of other bacteria in the ecosystem. In fact, the only culture (Cæ) where *E. coli* was found
405 was the only one containing no lactic bacteria. As amensalistic interaction, similar observations were
406 reported in the human gastrointestinal tract where lactic acid bacteria have an antimicrobial activity
407 against coliforms like *E. coli* [49].

408 In addition, some homolactic bacteria might have a positive effect on H₂ production. Indeed, *Bacillus*
409 *racemilacticus* was found as sub-dominant bacteria in AS culture where the highest biomass
410 concentration was observed, and consequently the highest H₂ productivity. Such influence on biomass
411 accumulation within the AS culture could be explained by the presence of such *Bacillus* sp. since
412 lactic acid bacteria are known to produce exopolysaccharides (EPS) [50]. Fang et al. [34] showed that
413 several *Bacillus* sp. produce EPS which can favour floc formation and thus increase biomass
414 concentration. In our study, biomass accumulation in flocs would have led to higher H₂ production. In
415 nature, EPS generated by lactic acid bacteria play a wide role in flocs and biofilm development for
416 colonization of environmental niches, such as in fermentative human gastrointestinal tract. In addition,
417 EPS protects the bacteria against hostile environmental conditions [51-52].

418

419 4.3.3 *Escherichia coli*, a keystone species driving *Clostridium* spp. metabolism.

420
421 The highest H₂ yield, i.e. 2.3 mol H₂.mol⁻¹ glucose_consumed, was observed in the Cæ culture. In this
422 culture, only two low abundant species were found: *Clostridium beijerinckii* and *Escherichia coli*.

423 The presence of *E. coli* in this continuous reactor was likely due to interaction with *Clostridium* sp.,
424 but the exact role of *E.coli* in terms of H₂ production remains to be elucidated.

425 Microorganisms belonging to the genera *Escherichia* can carry out a mixed acid fermentation and
426 produce lactate, acetate, succinate, ethanol, formate, CO₂ and H₂ [53]. Thus *E. coli* potentially
427 produces H₂ using the formate pathway [53-54]. Nevertheless, this is not coherent with the results
428 obtained for the H₂ productivity since the quantity of acetate and butyrate produced in the Cæ culture
429 was highly correlated to H₂ production. Indeed, the ratio between H₂ production and acetate/butyrate
430 concentration was equal to 1.9, slightly lower than theoretical ratio. This suggests that H₂ was
431 exclusively produced by acetate and butyrate pathways, but not by formate pathway.

432 Some studies use the association between *E. coli* and *Clostridium* to maintain an anaerobic
433 environment by oxygen depletion and thus increase the H₂ yield [55]. But for all our experiments the
434 redox potential was highly negative (-330 mV), suggesting that this commensalistic interaction did not
435 occur in Cæ culture.

436 Another hypothesis, even though very speculative, can be formulated in order to explain such high H₂
437 yield in Cæ culture: Recently, Dubey and Ben-Yehuda [24] showed that some bacteria, of which *E.*
438 *coli* belongs, could form nanowires with Gram+ bacteria and interact through direct electrons transfer
439 or metabolites exchanges. That type of interaction could also be considered between *Clostridium* spp.
440 and *E. coli*. This interaction could disturb the metabolism of *Clostridium* spp. and redirect it to the
441 acetate pathway under unknown cellular mechanisms. It might explain why the Cæ culture was the
442 only one to reach an acetate/butyrate ratio higher than one. This type of interaction would also explain
443 why *E. coli* was not washed out after 40 hydraulic retention times, according to the competitive
444 exclusion principle, by attachment to *Clostridium* sp. cells, after division. However, further
445 investigations are needed to clarify the exact interaction existing between *E.coli* and *Clostridium* sp.

446

447

448 **5 Conclusions**

449

450 In this study, H₂ production by dark fermentation in mixed cultures was clearly influenced by the
451 structure of the bacterial community. Even though dominant bacteria played a role in H₂ production,
452 the metabolic network of the ecosystem seemed to be driven by low-abundant sub-dominant bacteria.
453 These bacteria in low abundance influenced H₂ production from a benefic or a negative way.
454 Particularly, *E. coli* seemed to have a positive effect on H₂ production, by redirecting the global
455 metabolism of the ecosystem towards the acetate pathway and by increasing the H₂ yield. In contrast,
456 the homolactic bacteria had a negative impact by using a part of the carbohydrates for lactate
457 production which is not linked to H₂ production. These bacteria are also known for their ability to
458 produce bacteriocins which could stress clostridia or prevent the implantation of other bacteria, such
459 as *E.coli*. In this case, the influence of lactic acid bacteria on the overall ecosystem is high even at low
460 abundance. In conclusion, it was shown that bacteria in low abundance can be considered as keystone
461 species controlling the metabolism of the global microbial ecosystem in fermentative mixed cultures.

462

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468 inoculum.

469

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- 619

620 **Table and Figure captions**

621 **Table 1:** Phylogenetic affiliation of bacterial 16S rDNA cloned from samples of the different cultures
622 at steady state. The number of each clone indicates the peak of the CE-SSCP profile, to which the
623 clone was assigned. The relative abundance of each species in the ecosystem was calculated from the
624 area under each peak. The different cultures at steady state were: heat-treated anaerobic sludge (AS^{ht}),
625 anaerobic sludge (AS), heat-treated cassava (Casth), cassava (Cas), heat-treated cæcotroph (Cæth),
626 cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

627
628 **Table 2:** H₂ production performances at steady state for the seven selected ecosystems: heat-treated
629 anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat treated cassava (Casth), cassava (Cas), heat-
630 treated cæcotroph (Cæth), cæcotroph (Cæ) and mixture of the three non heat-treated inocula (Mix).
631 Statistical differences are indicated by letters (p<0.05, t-tests).

632
633 **Table 3:** Hydrogen productivity and yields observed in the literature for mixed cultures in continuous
634 reactor.

635
636 **Figure 1:** Dynamics of the microbial community structure: example of the culture inoculated with
637 cassava (Cas). The first CE-SSCP profile corresponds to the structure of the inoculum, followed by the
638 profile of the community structure after a batch period of 24 hours. The last two profiles correspond to
639 the microbial community structure at steady state after 32 and 40 hydraulic retention times (HRT),
640 respectively. The CE-SSCP profiles were aligned on the basis of the common ROX internal standard,
641 and areas normalized. The X- and Y-axes represent the relative peak migration distance and the
642 relative peak intensity, respectively. The dynamics of all cultures are given in Supporting Information
643 (Figure S1).

644
645 **Figure 2:** CE-SSCP profiles of the seven hydrogen-producing ecosystems at steady state: heat-treated
646 anaerobic sludge (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Casth), cassava (Cas), heat-
647 treated cæcotroph (Cæth), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

648 The CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and areas
649 normalized. The X- and Y-axes represent the relative peak migration distance and the relative peak
650 intensity, respectively. A representative profile of the ecosystem at steady state is presented for each
651 culture. Phylogenetic affiliation of the clone sequences corresponding to the numbered peaks is given
652 in Table 1.

653
654 **Figure 3:** COD mass balance of the different cultures at steady state: heat-treated anaerobic sludge
655 (AS^{ht}), anaerobic sludge (AS), heat-treated cassava (Casth), cassava (Cas), heat-treated cæcotroph
656 (Cæth), cæcotroph (Cæ), and mixture of the three non heat-treated inocula (Mix).

657
658 **Figure 4:** Metabolites and hydrogen produced by the different cultures at steady state. The Y-Axes
659 have been scaled to represent the difference between the H₂ productivity and the associated
660 metabolites (theoretically if H₂ is produced by acetate and butyrate pathways: H₂ Productivity = 2 (Ac
661 + Bu)). The different cultures at steady state were: heat-treated anaerobic sludge (AS^{ht}), anaerobic
662 sludge (AS), heat-treated cassava (Casth), cassava (Cas), heat-treated cæcotroph (Cæth), cæcotroph
663 (Cæ), and mixture of the three non heat-treated inocula (Mix).

Clone number	Closely related to	Sequence similarity (%)	Relative abundance of species in each ecosystem (%)						
			AS ^{ht}	AS	Cas ^{ht}	Cas	Cæ ^{ht}	Cæ	Mix
1	<i>Clostridium butyricum</i>	100	74.0	-	-	-	-	-	-
2	<i>Clostridium pasteurianum</i>	100	-	89.1	67.7	66.8	74.0	78.8	73.2
3	<i>Clostridium beijerinckii</i>	99	15.7	-	18.3	12.6	15.8	18.8	16.8
4	<i>Sporolactobacillus laevolacticus</i>	99	3.5	-	-	-	-	-	-
5	<i>Bacillus coagulans</i>	99	1.8	-	-	-	-	-	-
6	<i>Bacillus racemilacticus</i>	100	5.0	10.9	-	-	-	-	-
7	<i>Lactobacillus paracasei</i>	99	-	-	14.0	13.6	-	-	10.0
8	<i>Lactobacillus casei</i>	100	-	-	-	7.0	-	-	-
9	<i>Lactobacillus nagelii</i>	99	-	-	-	-	7.3	-	-
10	<i>Lactobacillus ghanensis</i>	99	-	-	-	-	2.9	-	-
11	<i>Escherichia coli</i>	100	-	-	-	-	-	2.4	-

	AS ^{ht}	AS	Cas ^{ht}	Cas	Cæ ^{ht}	Cæ	Mix
H ₂ productivity (mmol.L ⁻¹ .h ⁻¹)	5.47 ^a	9.07 ^e	8.04 ^d	6.42 ^b	7.45 ^c	6.56 ^b	8.06 ^d
Y (mole H ₂ .mole ⁻¹ Glc _{consumed})	1.21 ^a	1.92 ^c	1.79 ^{b,c}	1.41 ^a	1.72 ^b	2.32 ^d	1.87 ^c
acetate / butyrate ratio	0.64 ^a	0.87 ^b	0.88 ^b	0.99 ^d	0.99 ^{c,d}	1.08 ^e	0.94 ^c

Letters indicated the statistical confidence for grouping (p < 0.05, t-tests)

Inoculum	Substrate	Productivity (mmol H ₂ .L ⁻¹ .h ⁻¹)	H ₂ Yield (mol H ₂ .mol hexose ⁻¹)	Reference
Different types heat treated or not	Glucose	5.5 – 9.1	1.2 – 2.3	This study
Sewage sludge	Glucose	0.3 – 15	0.6 – 1.7	[33]
Sludge of hydrogen reactor	Glucose	4.3	2.1	[34]
Agricultural soil heat treated	Glucose	3.6 – 96.7	1.7 – 2.8	[35]
Sludge from an anaerobic digester heat treated	Sucrose	14.2	1.2	[36]
Sludge from an anaerobic digester	Sucrose	-	0.1 – 1.7	[37]







