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Highlights

- Mixed culture low reproducibility was related to severe population shifts
- Bioaugmentation with pure strains reduced the ecosystem variability
- Despite remaining in minor abundance, *E. coli* and *C. necator* led to reproducible H_2 production
- Best performances correlated with the predominance of *C. acetobutylicum* and *C. butyricum*

Abstract

Process stability is a key operational issue when operating dark fermentation with mixed microbial cultures for hydrogen production. This study aimed at mitigating the instability of hydrogen production by separately adding exogenous pure strains suspected to have key roles in fermentative cultures. Among them, *Clostridium acetobutylicum, Clostridium pasteurianum* and *Lactobacillus bulgaris* which became predominant within the mixed culture strongly reduced the spectrum of produced metabolites and H_2 production variability. Interestingly, *Escherichia coli* and *Cupriavidus necator*, which remained in minor abundance, maintained a high and stable H_2 production while lowering the metabolic variability. 16S rRNA revealed that this could correlate to a simplification of the microbial diversity and the non-emergence of spore-forming competitors such as *Sporolactobacillus* sp. These results illustrate the potential beneficial role of minor OTUs as keystone species on H_2-producing complex ecosystem and support the possibility of using them to engineer the ecosystem and maintain high and stable performances.

Keywords:

- Biohydrogen
- Bioaugmentation
- Dark fermentation
- Ecological engineering

1 Introduction

Due to the current context of global warming as well as the growing scarcity of non-renewable fossil fuels, more research efforts focusing on sustainable energy production are required. Among the possibilities for alternative energy development, hydrogen (H_2) has attracted considerable attention over the past decade due to its high energetic yield (267 $kJ.mol^{-1}$). Nowadays, H_2 is mainly generated through energetically expensive thermochemical processes, such as natural gas reforming and water electrolysis. Therefore, development of clean, sustainable, and cost-competitive H_2 production processes is of major concern (Hosseini and Wahid, 2016).

Encouraging alternatives to produce environmentally friendly H_2 are driven by microbial processes that present the lowest environmental impacts (Dincer and Acar, 2015). Among the biological options, dark fermentation appears to be the least restrictive technology compared to biophotolysis and photofermentation since it can be operated as an intensive process and without any external supply of light (Ghimire et al., 2015). Additionally, strict anaerobic or facultative anaerobic bacteria involved in dark fermentation provide higher productivities than photo-fermentative bacteria (Ghimire et al., 2015; Manish and Banerjee, 2008). Converting organic biomass in dark fermenters into H_2 is basically a truncated anaerobic digestion process. Indeed, H_2 is a key metabolic intermediate produced mainly during acidogenesis and is then immediately consumed to reduce by-products into mainly methane or acetate. A combination of suitable operational parameters such as moderately low pH and short solids retention time favor H_2 production and suppress the principal consumers: the methanogens. Moreover, the ability of dark fermentation to generate H_2 from a variety of renewable non-sterile and inexpensive feedstock's such as organic waste makes it suitable for

62 the demand of a sustainable development strategy (Hosseini and Wahid, 2016; Łukajtis et al.,
63 2018; Wong et al., 2014).

64 Various pure cultures of fermentative bacteria having the ability to produce H_2 from
65 simple substrates have been identified (Cabrol et al., 2017; Lee et al., 2011). As the most
66 dominant H_2-producing species found in microbial ecosystems, *Clostridium* sp. and
67 *Enterobacter* sp. were the most widely hydrogen-producing bacteria (HPB) used as inoculum
68 (Cabrol et al., 2017; Mishra et al., 2019). Even though pure bacterial strains often exhibit
69 better yields than mixed natural communities, bioprocess operation requires sterile conditions
70 and well controlled conditions that make their development at industrial scale too costly.
71 Moreover, using pure cultures for producing H_2 restricts feedstock's possibilities and does not
72 take advantage of the wide metabolic possibilities inherent to the use of complex ecosystems.
73 Thereby, mixed cultures-based bioprocesses appear as a more suitable solution for H_2
74 production from complex organic wastes. In theory, their robustness allows them to degrade a
75 wide range of substrates, due to their higher resistance and resilience to environmental
76 changes (Cabrol et al., 2017; Chandrasekhar et al., 2015).

77 However, complex microbial ecosystems present numerous interactions between the
78 different communities involved in fermentation. Illustratively, and contrary to the competitive
79 exclusion principle stating that microbial diversity could not be maintained in a chemostat at
80 steady-state, it is regularly observed that low abundant bacterial species are always present.
81 Their roles remain unclear but many recent studies tend to consider them as keystone species
82 (Cabrol et al., 2017; Rafrafi et al., 2013a). Despite their low abundance, they are supposed to
83 significantly participate to ecosystem functioning through various interactions such as trophic
84 competition. Ubiquitous microorganisms such as lactic acid bacteria (LAB) reduce H_2
85 production yields due to substrate competition (replacement of H_2 fermentation by lactic acid

86 fermentation) and to the excretion of bacteriocins (Gomes et al., 2016). Homoacetogenic
87 bacteria which grow on H_2 and CO_2 can also alter the performance of H_2 bioreactors. Besides,
88 a larger range of interactions can occur between microorganisms. Direct exchanges of
89 molecules or electrons via cytoplasmic, pilis or nanotubes connections, with or without
90 conductive materials have also been recently investigated in anaerobic environments (also
91 called DIET for Direct Interspecies Electron Transfer (Baek et al., 2018)). Cellular
92 communication between microorganisms called "quorum sensing" is also attracting more and
93 more attention in fermentation (Nguyen et al., 2019). Whatever the type of interaction,
94 fermentation patterns could be impacted thus redirecting the fermentation pathways towards
95 H_2 production (Benomar et al., 2015). Hence, whether physical or chemical, intra or
96 extracellular, all these interactions are evidences of a complex bacterial communication
97 network in which all species, and even the least abundant, would be able to drive the
98 functioning of the whole ecosystem.

99 Since structure and interactions within microbial communities are crucial for the
100 efficiency of bioprocesses, many studies aimed at better managing complex ecosystems by
101 inoculating exogenous functional microbial consortia or specific strains to mixed culture. This
102 strategy, called bioaugmentation, has been used for several purposes such as withstanding
103 bioprocess fluctuations, accelerating substrate degradation, restoring reactor performance or
104 relieving toxicant inhibition (Bartrolí et al., 2011; Bouchez et al., 2000; Fotidis et al., 2014;
105 Herrero and Stuckey, 2015; Jianlong et al., 2002; Kotay and Das, 2009; Yang and Wang,
106 2018).

107 Bioaugmentation has notably been performed by several authors to enhance the
108 hydrogen production as reviewed by Yang and Wang (2018). Interestingly, Goud *et al.,*
109 (2014) demonstrated that bioaugmentation with three various acidogenic bacteria (*Bacillus*

110 *subtilis*, *Pseudomonas stutzeri* and *Lysinibacillus fusiformis*) can enhance hydrogen
111 productivity from food waste despite overloaded substrate condition (Goud et al., 2014).
112 Moreover, Kumar *et al.,* (2015) showed that bioaugmentation with facultative anaerobes such
113 as *Escherichia coli* XL1-Blue and *Enterobacter cloacae* DSM 16657 can improve hydrogen
114 yield of a sewage sludge inoculum by creating strict anaerobic conditions for *Clostridium*
115 species (Kumar et al., 2015). The potentialities of bioaugmentation with hydrogen producing
116 facultative anaerobes was also pointed by Marone *et al.,* (2012). They reported that the
117 addition of *Rahnella* sp. 10, *Raoultella* sp. 47 and *Butiauxiella* sp. 4 increased hydrogen
118 production rate and yield compared to the indigenous ecosystem during the fermentation of
119 vegetable food waste (Marone et al., 2012). They also suggested synergistic interactions
120 induced by the triple co-culture of these strains. Benomar et al., (2013) demonstrated that
121 physical interactions, including an exchange of molecules such as proteins, between in a co-
122 culture of two anaerobic partners considered as competitors (*Clostridium acetobutylicum* and
123 *Desulfovibrio vulgaris Hildenborough*) allowed a substantial increase in hydrogen production
124 without requiring genetic engineering. Similarly, Laocharoen et al., (2015) also indicated that
125 the addition of a lactic acid-producing strain (*Lactobacillus delbrueckii* ssp. *bulgaricus*
126 TISTR 895) could avoid lactic acid accumulation in the fermentation system and enhance
127 hydrogen production by *Rhodobacter sphaeroides* KKU-PS5 (Laocharoen et al., 2015). The
128 importance of these non-hydrogen producing bacteria, often sub-dominant in bioreactors has
129 also been highlighted by Rafrafi et al., (2013) (Rafrafi et al., 2013b).

130 In this framework, the aim of this study was to investigate the impact of the addition of
131 seven exogenous strains, reported as keystone species, on H_2 production performances and on
132 the microbial ecosystem balance of a complex community. Based on the literature and on
133 previous work, seven (*Clostridium acetobutylicum, Clostridium pasteurianum, Escherichia*

134 *coli, Lactobacillus bulgaris, Enterococcus casseliflavus, Desulfovibrio vulgaris* and
135 *Cupriavidus necator*) were selected for their potential interaction with H_2 producers.
136 Microbial dynamics were monitored with 16S rRNA gene sequencing. Inoculum originating
137 from an industrial methanogenic reactor was tested.

2 Materials and methods

2.1 Experimental set-up

Experiments were carried out in 600 mL batch flasks with a working volume of 200 mL. Growth medium composition is detailed in Rafrafi et al. (2013). Main components were 2g/L of yeast extract, 10 g/L of glucose, 7.4 g/L MES Buffer, oligo-element solution 40 mL/L, H_2O *q.s.p* 1 L. pH was initially adjusted to 6.0 using NaOH (5N). Bioreactors were sealed with a screw cap and a rubber septum and headspaces were flushed with N_2 (purity > 99.99%, Linde gas SA) during 10 min in order to ensure strict anaerobic conditions. They were then autoclaved (121°C, 20 min) and stored at 4°C prior to inoculation.

An anaerobic inoculum was sampled from the outlet of a lab-scale methanogenic reactor fed with wine distillery wastewater (Ornaisons, France). To avoid hydrogen consumption by methanogenic populations, a heat-shock treatment (90°C, 10 min) was performed on this anaerobic sludge. It was then incubated at 35°C in a 1000 mL flask. A set of 24 anaerobic batch flasks were initially seeded with 10 mL of this anaerobic inoculum with a syringe through the rubber septum. One pure strain was added to each bioreactor. A set of seven pure strains were used for bioaugmentation assays on the basis of previous works, ie. *Clostridium acetobutylicum, Clostridium pasteurianum, Escherichia coli, Lactobacillus bulgaris, Enterococcus casseliflavus, Desulfovibrio vulgaris* and *Cupriavidus necator* (Benomar et al., 2015; Rafrafi et al., 2013). A total of 10 mL of each pure strain culture, corresponding to 10^7 CFU and 0.1% of the total biomass of the mixed culture, was sampled during their respective exponential growth phase and added to the bioreactor using a syringe through the rubber septum. Control flasks were supplemented with sterile water. Three replicates were carried out for each condition.

Time zero (T_0) samples were taken and all reactors were incubated at 37°C. Agitation was manually performed before and after each sampling campaign. Liquid samples (2 mL) were periodically taken through the septum and centrifuged at 10,000 g for 10 min. The pellets and supernatant obtained were stored separately at -20°C for analysis of biomass and chemical indicators respectively. The incubations were run up to 120 hours at 35 °C until all daily biogas productions decreased below 1 mL/h.

2.2 Analytical methods

Biogas production and composition (H_2, CO_2, O_2, CH_4 and N_2 content) were analyzed using a gas chromatograph R3000 (SRA Instruments) as detailed in Chatellard *et al*., (2016). The volume of produced biogas was normalized according to the ambient temperature and pressure.

To determine H_2 production parameters, the cumulative H_2 production (H (t)) data was fitted to a modified Gompertz equation (Eq. (1)):

$$H\,(t) = P\ \times exp\left\{-exp\left[\frac{R_{max}\times e}{P}\ \times (\lambda - t) + 1\right]\right\} \qquad \text{(Eq.1)}$$

where P is the maximum cumulative H_2 production (mLH_2/L), R_{max} is the maximum H_2 production rate ($mLH_2/L/day$), λ is the lag-phase time (day), t is the incubation time (day) and e is exp (1). The cumulative H_2 production was expressed in mL per L of culture taking into account the variations in volume due to gas and liquid sampling. The values of P, R_{max} and λ were estimated using a non-linear regression algorithm developed with Matlab software (version 6.5, MathWorks). H_2 production yield was calculated by dividing the maximum H_2 production (P) by the molar amount of glucose consumed.

Fermentation end products (lactate, ethanol) were quantified using high performance liquid chromatography (HPLC) coupled to a refractometer (Waters R410). The chromatograph was equipped with an HPX 87 column (Biorad) and the eluent corresponded to a H_2SO_4 solution (0.222 ml/L) under isocratic elution at 0.4 mL/min. The operating conditions were: temperature of column, 35°C; temperature of refractometer, 40°C.

2.3 Molecular analyses of microbial communities

2.3.1 DNA extraction and PCR amplification

Total DNA was extracted from the pellet using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. The purity and amount of DNA in the extracts were measured by spectrophotometry (Infinite NanoQuant M200, Tecan). DNA were stored at -20°C prior sequencing. The procedure for 16S rRNA gene sequencing and raw data processing was described elsewhere by Carmona-Martinez (2015). The V3-V4 region of the 16S rRNA gene was amplified with the forward primer CTTTCCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and the reverse primer GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT plus the respective linkers over 30 amplification cycles at an annealing temperature of 65.0°C. In a second PCR reactor of 12 cycles, an index sequence was added using the primers AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC and CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT. The PCR products were then sent for sequencing on a Illumina MySeq cartridge in GeT PlaGe sequencing center of the genotoul life science network in Toulouse, France (get.genotoul.fr) according to the procedure described in (Carmona-Martínez et al., 2015).

2.3.2 Data processing and statistical analysis

A slightly modified version of the Standard Operation Procedure was used to process the raw data for alignment and assignation of the sequences in MOTHUR version 1.33.0. SILVA release 102. This version is more detailed by Carmona-Martinez et al. (2015). R CRAN software (version 3.3.3) was used to examine 16S abundance file. Considering the dispersion in the total number of reads identified in each sample, bacterial OTUs abundances were normalized. Only OTUs that exceeded 0.5% in at least one sample have been taken into account for the histogram representation.

212 # 3 Results and discussion

213 ## 3.1 H2 production dynamics

214 To accurately assess the H_2 production performances, cumulative H_2 production curves
215 were fitted to a modified Gompertz equation for each batch experiment. Gompertz model
216 showed a good fit with the experimental H_2 production data, with determination coefficients
217 r^2 over 0.99 for all batch tests. H_2 production curves obtained with the mixed culture were
218 decomposed to two distinct fitting curves, each corresponding to a different H_2 production
219 phase. Gompertz parameters determined for all incubations are presented in Table. 1.

220 ### 3.1.1 Mixed culture (controls)

221 Cumulated H_2 production dynamics obtained from the different replicates of the mixed
222 culture are detailed in Fig. 1A. Kinetics evidenced a low reproducibility between the three
223 replicates. H_2 production started in all bioreactors after a lag time of 6 hours and consisted of
224 2 phases. The duration of this first production phase was variable between the three replicates.
225 It lasted respectively from 15 hours in replicates 2 and 3 ending with a plateau phase, up to 45
226 hours in replicate 1. The plateau phase duration ranged from 25 hours to 50 hours in replicates
227 3 and 2, respectively. Cumulated H_2 production volumes at the end of this first phase varied
228 between 180 mL and 280 mL. For all the replicates, a second H_2 production phase followed
229 the plateau whose duration was also variable from one replicate to another. Total H_2
230 production was also not consistent, ranging from 70 mL to 280 mL during this phase.
231 Consequently, ultimate H_2 production yield (H_{max}) varied from 271 mL up to 562 mL. The
232 variability observed in the analysis of H_2 production kinetics could be explained by an
233 instability of the bacterial communities during both growth phases that directly affected the

234 H_2 production of the consortium as already described by a recent study (Chatellard et al.,
235 2016).

236 3.1.2 Pure strains behaviours

237 H_2 production was highly reproducible among each triplicate of hydrogen producing
238 strains (*Clostridium acetobutylicum*, *Clostridium pasteurianum* and *Escherichia coli*). Data
239 are respectively presented in presented in Supplementary Fig. S1-S3. H_2 production dynamics
240 revealed that *Clostridium pasteurianum* reached higher maximum production yields
241 (514.4±32.5 mL, 2.02±0.04 molH_2/mol glucose) than *Clostridium acetobutylicum*
242 (463.1±26.6 mL, 1.94±0.14 molH_2/mol glucose) and *Escherichia coli* (54.0±7.7 mL,
243 0.90±0.12 molH_2/mol glucose). These yields are similar to those recovered in many studies
244 (Cabrol et al., 2017).

245 Both *Clostridia* reached comparable H_2 yield as those obtained with replicates 2 and 3
246 of the mixed culture, ie. 471 mL and 562 mL of H_2, respectively. Pure bacterial strains such as
247 *Clostridiales* are known to give better yields when producing H_2 than mixed natural
248 communities suggesting that, despite a low reproducibility, two out of the three replicates of
249 the mixed culture used for this study were able to reach good performances.

250 3.1.3 Confrontation between mixed culture and pure strains

251 The mixed culture confrontation with pure strains induced the establishment of new H_2
252 production kinetics. Three tendencies were observed:

253 Interestingly, adding strains such as *Clostridium acetobutylicum* (Fig. 1B), *Clostridium*
254 *pasteurianum* (Fig. 1C) and *Lactobacillus bulgaris* (Fig. 1D) generated the disappearance of
255 the first plateau phase. In presence of exogenous *Clostridies*, the entire substrate was
256 consumed after 60 hours of incubation which implied that the addition of these two pure

strains increased substrate consumption rate. However, lower H_2 production yields were obtained. In presence of *Clostridium acetobutylicum*, a yield of only 271.1±42.4 mL was reached, which was still comparable to the H_{max} obtained with one of the mixed cultures replicate. *Clostridium pasteurianum* led to higher H_{max} values ranging between 330 mL and 370 mL of H_2. Addition of *Lactobacillus bulgaris* within the mixed culture generated a strong reduction in H_2 production. Only 15 hours after seeding this strain, H_2 productivity reached zero. As a result, the global H_2 yield reached only 169.5±18.1 mL. This suggested that *Lactobacillus bulgaris* addition caused trophic competition between this lactic acid bacteria (LAB) and the native ecosystem, likely favored by a release of bacteriocins in the medium as already reported by several authors (Cabrol et al., 2017; Gomes et al., 2016; Noike et al., 2002; Palomo-Briones et al., 2018). Considering that H_2 generation completely stopped after only 15 hours after this strain addition, this observation well supports the fact that *Lactobacillus bulgaris* outcompeted efficiently hydrogen-producing bacteria.

Overall, all pure strain bioaugmentations led to a decrease in H_2 yields. However, these seeding generated an increase of the reproducibility of the H_2 production dynamics when compared to the mixed culture control. It can be assumed that adding these pure strains led to new interactions in the global ecosystem. With regard to the hydrogen yields, it was presumed that trophic interactions could have taken place in the mixed culture leading to an increased competition for the substrate.

In addition, bioaugmentation of *Escherichia coli* (Fig. 1E) and *Cupriavidus necator* (Fig. 1F) did not prevent H_2 production kinetics from being decomposed into two phases. However, it greatly reduced the variability of H_2 production during the second phase. While the average production of this phase remained stable within these three replicates, the standard deviation dropped from 159.3 mL for the mixed culture alone down to 79.9 mL in presence of *C.*

281 *necator* and even to 49.5 mL in presence of *E. coli*. The addition of these two strains may
282 have led to new interactions taking place during the second phase of production and
283 suggesting that they managed to remain active in the global ecosystem.

284 Finally, *E. casseliflavus* (Fig. 1G) and *D. vulgaris* (Fig. 1H) did not have any beneficial
285 influence on the variability within the mixed culture during the second phase of H_2
286 production. It could either indicate that both strains did not grow throughout the incubation or
287 that their growth did not modify the interactions between the key hydrogen producers and
288 consumers.

289 3.2 Metabolites accumulation

290 Fig. 2 presents the distribution of the soluble metabolites produced concomitantly with
291 H_2.

292 3.2.1 Mixed culture (controls)

293 Metabolite production kinetics by the mixed culture is presented in Supplementary Fig.
294 S4. It was divided into two bar charts corresponding to two H_2 production phases. High
295 variability observed within the triplicate of bioreactors required to distinguish the results for
296 each replicate. Contrary to what could be expected, a great reproducibility was revealed in the
297 metabolic pathways used by the mixed culture during the first H_2 production phase. All
298 replicates produced four types of metabolite (acetate, butyrate, formate and lactate) in
299 relatively unchanged proportions. Acetate and butyrate production were the major
300 fermentation products (3.22±0.05 mmol and 3.58±0.11 mmol, respectively) suggesting that
301 *Clostridium spp* could be predominant within the mixed culture during the first phase.
302 Replicate 1 revealed a high lactate accumulation of 8.66 mmol during the second phase that
303 consequently led to lower H_2 production. In replicate 2, caproate and ethanol that were not

304 detected in the first phase, accumulated at a level of 2.32 mmol and 1.20 mmol respectively.
305 This result suggests that additional metabolic pathways were used for substrate conversion
306 during the second phase. Interestingly, replicate 3 did not reveal any metabolic shift during
307 the second phase. An increase in acetate (4.6 mmol) and butyrate (8.5 mmol) production was
308 observed confirming the shape of the metabolite distribution present in the first phase. The
309 difference observed in the three replicates indicated that the ecosystem was governed by a
310 high variability in metabolic patterns with subsequent changes in metabolite distribution.
311 Compared to the other replicates, the metabolites distribution of replicate 3 was consistent
312 between both phases and led to the highest H_2 production. Instability found in replicates 1 and
313 2 could be the consequence of a trophic competition between the communities leading to
314 different shifts in bacterial distribution of the ecosystem.

315 3.2.2 Pure strains

316 Final metabolite production of all pure strains is presented in supplementary Fig. S5.
317 Both *Clostridies* used similar metabolic pathways and produced four types of metabolites
318 (acetate, butyrate, propionate and formate), in relatively unchanged proportions. *C.*
319 *acetobutylicum* produced 3.37±0.50 mmol of acetate, 5.03±0.21 mmol of butyrate, 1.20±0.17
320 mmol of propionate and 2.17±0.06 mmol of formate while *C. pasteurianum* produced
321 3.90±0.20 mmol of acetate, 6.17±0.11 mmol of butyrate, 0.30±0.01 mmol of propionate and
322 1.76±0.06 mmol of formate. It could be noted that *C. acetobutylicum* produced more
323 propionate than *C. pasteurianum.* Propionic acid pathway is a hydrogen consuming pathway
324 that leads to limited hydrogen production. Here, it seems that in these conditions *C.*
325 *acetobutylicum* metabolic pathways were less efficient for hydrogen production than *C.*
326 *pasteurianum* which is underlined by a lower butyrate level and consequently to lower H_2
327 yield.

328 *E. coli* synthesized 1.64±0.15 mmol of acetate, 1.65±0.05 mmol of propionate and
329 0.73±0.06 mmol of lactate while no butyrate was detected during these incubations. Hosseini
330 and Wahid (2016) demonstrated that *Escherichia coli* was able to use acetate and formate
331 pathways to synthesize H_2. However, formate is hardly detectable. The presence of lactate
332 suggested that *E. coli* partly shunted theoretical hydrogen potential towards solventogenesis.
333 This could be induced by acetate inhibition which could orientate *E. coli* metabolic pathways
334 towards non-acetate producing pathways.

335 *L. bulgaris* and *E. casseliflavus* produced respectively between 8.7±0.1 mmol and
336 6.5±0.5 mmol of lactate from glucose while *D. vulgaris* and *C. necator* did not produce any
337 metabolite confirming their incapacity to produce H_2.

338 3.2.3 Confrontation of mixed culture with pure strains

339 Results presented in Figure 2 showed that *C. acetobutylicum, C. pasteurianum, E. coli;*
340 and *C. necator* additions strongly directed ecosystem metabolic pathways towards acetate and
341 butyrate, although small amounts of other metabolites (lactate, formate and ethanol) were still
342 produced. Interestingly, metabolites synthesized by these four triplicates as well as by the
343 triplicate consisting of the mixed culture confronted with *L. bulgaris* were more reproducible
344 within each other than for the mixed culture alone. *Lactobacillus bulgaris*, promoted the
345 synthesis of lactate, repressed acetate and butyrate pathways thus inducing a decrease in H_2
346 production. This strain addition could moreover limit the growth of bacterial population
347 degrading lactate to butyrate, which apparently occurred in replicates 2 and 3 of the mixed
348 culture. *Clostridium pasteurianum* promoted acetate production. However, the presence of
349 ethanol and lactate in some replicates may indicate that some metabolic instability persisted in
350 the ecosystem. Similarly, *E. coli* and *C. necator* induced an increase in butyrate production
351 leading to higher and more stable H_2 production levels. Contrastingly, *E. casseliflavus* and *D.*

352 *vulgaris,* which do not produce H_2 led to highly diverse metabolites production suggesting
353 that they do not influence H_2 production pathways.

354 The repeatability of the results obtained when adding these five strains contrasted with
355 the variability of metabolites in the mixed culture replicates. This finding tended to imply that
356 these strains were metabolically active in the ecosystem and that their inclusion would have a
357 stabilizing effect on the overall consortium metabolism. Except when adding *Lactobacillus*,
358 which seemed to activate lactate pathway while preventing the acetate and butyrate pathways,
359 no trend appeared to emerge about the influence of *Clostridium* sp. on the metabolite patterns
360 of the ecosystem. Indeed, the excessive variability of microbial communities observed during
361 the mixed culture growth induced significant fluctuations in the production of metabolites
362 which prevent us to conclude about a possible impact of adding *Clostridium* sp. on the choice
363 of the ecosystem metabolic pathways.

364 3.3 Microbial population dynamics

365 Microbial community dynamics were investigated with 16S rRNA gene high-
366 throughput sequencing. The objective was to identify key microorganisms involved in
367 structural variations of the microbiota. A total of 15 samples were selected. One sample was
368 used to characterize the initial composition of the mixed culture (inoculum). A second sample
369 was collected after 15 hours of incubation of the mixed culture, during the first exponential H_2
370 production phase. Due to the high variability observed within H_2 production kinetics of the
371 mixed culture, two samples per replicate were subsequently collected at the end of each
372 plateau phase. For the incubation confronting the seven exogenous strains tested, one sample
373 per triplicate, collected at the end of the H_2 production was sequenced.

Several thousands of sequences were identified for each sample. They were assembled into 493 bacterial OTUs. OTUs belonging to the same bacterial order were represented with the same color palette. Bacterial community composition dynamics were represented in histograms at the OTU level in Fig. 3.

3.3.1 Mixed culture instability

Inoculum bacterial composition was highly diverse with a total of 486 different OTUs. For sake of clarity, OTUs for which relative abundance did not exceed 1% were aggregated and considered as minor OTUs.

Mixed culture was mainly composed of OTUs assigned to phyla *Firmicutes* (18%), *Bacteroidetes* (16%) and *Proteobacteria* (12%). Two H_2 producing OTUs were notably predominant: *Clostridium acetobutylicum* (8%) and *Clostridium butyricum* (3%).

After 15 hours of incubation, *Clostridium butyricum* became highly predominant reaching a relative abundance of 81%. At the end of the first plateau phase, this OTU was equally represented within each replicate, with a relative abundance varying between 96% and 97%. This great microbial stability confirmed the good reproducibility observed within H_2 production kinetics.

Interestingly, at the end of the second plateau phase, the microbial composition of the three replicates greatly varied. In all cases, *Clostridium butyricum* proportion dropped while different hydrogen producers took advantage on substrate consumption.

In the first replicate (R1), *Clostridium acetobutylicum* became predominant, representing 51% of the total OTUs, while *Clostridium algifaecis* and *Clostridium sporogenes* reached comparable proportions of 12-13%. *Clostridium butyricum* relative abundance dropped down to 19%. Moreover, two OTUs belonging to *Sporolactobacillus* genus became

detectable in the ecosystem with a total proportion of 4%. Their appearance can thus be directly correlated to the presence of lactate within the final metabolic pattern and to the inhibition of the second H_2 production phase. Although, these lactic acid bacteria were not detectable in the inoculum, their ability to form spore could explain their resistance to the initial heat treatment.

In the second replicate, *Clostridium butyricum* proportion decreased down to 51% while two other hydrogen producers, *Clostridium pasteurianum and Clostridium acetobutylicum* emerged, reaching relative abundance of 29% and 19%, respectively. Meanwhile, a minor OTU assigned to *Clostridium autoethanogenum* also emerged. This genus has been reported to perform medium-chain fatty acids synthesis via chain elongation, utilizing acetate and ethanol as main substrates. Its presence within the ecosystem could explain the production of caproate and ethanol in the final metabolites of this bioreactor.

Contrastingly, the third bioreactor was almost exclusively dominated by two OTUs assigned to *Clostridium acetobutylicum* (50%) and to *Clostridium butyricum* (45%). A small amount of lactate was produced and could be linked to the presence of *Sporolactobacillus vinae* (1%). However, contrary to the first replicate, this lactic acid bacterium did not seem to reach sufficient proportion to impact the H_2 production.

The 16S rRNA sequences confirmed trends observed for H_2 production and metabolites accumulation. It also evidenced the instability in microbial community composition and related metabolic patterns occurring during the second H_2 production phase. This highlights the microbial rearrangements occurring between H_2 producers and the emergence of competitors such as lactic acid bacteria. Considering their ability to form spores of *Sporolactobacillus and Clostridiaceae*, this instability could be explained by the fact that after

420 a certain lag phase, these bacteria managed to emerge and to create new competitions within
421 the ecosystem.

422 3.3.2 Confrontation with exogenous pure strains

423 Three strains (*C. acetobutylicum, C. pasteurianum*, and *L. bulgaris*) completely
424 annihilated the second H_2 production phase, thus reducing the global performance variability
425 of the microbiota. The 16S rRNA sequences revealed that after its addition in the bioreactor,
426 *C. acetobutylicum* became ultra predominant in the final ecosystem, reaching a final
427 proportion of 99%. Furthermore, the addition of *C. pasteurianum* induced equilibrium
428 between this strain (59%) and the major H_2 producer during the first production phase of the
429 mixed culture, *C. butyricum* (39%). This result suggested that *C. acetobutylicum* outcompeted
430 for the substrate against *C. butyricum* in higher extends than *C. pasteurianum*. However, in
431 terms of performance, higher production yields were observed in presence of *C. pasteurianum*
432 which tended to indicate that this co-culture consisted of two *Clostridies* was more efficient in
433 producing as the single strain. *L. bulgaris* also became predominant within the ecosystem,
434 which completely simplified the final composition of the microbiota. It was exclusively
435 dominated by this lactic acid bacterium (45%) and *C. butyricum* (54%). Nevertheless, besides
436 the metabolic stability of this triplicated reactors, *L. bulgaris* growth partly rerouted glucose
437 fermentation towards lactate production, which greatly reduced the H_2 production yields.
438 Thus, the annihilation of the second H_2 production phase seemed to be explained by the
439 extreme simplification of the ecosystem induced by the addition of good competitors for the
440 substrate consumption.

441 Moreover, adding the two strains (*E. coli* and *C. necator*) significantly stabilized the
442 performances during the second H_2 production phase. Sequencing results evidenced that *E.*
443 *coli* slightly emerged (2%). Nevertheless, its presence seemed to induce beneficial effects

444 leading to a simplified microbiota quasi exclusively composed of *C. acetobutylicum* (67%)
445 and *C. butyricum* (27%). Similarly, while *C. necator* was not even detected in the final
446 ecosystem, its addition in the mixed culture led to a great simplification of its composition
447 tending towards a co-culture consisting of *C. acetobutylicum* (40%) and *C. butyricum* (54%).
448 These results suggested that the initial addition of these strains drove the microbiota towards a
449 more stable composition similar to the one observed in the third replicate of the mixed culture
450 alone. This type of ecosystem only consisted of two *Clostridium* sp. strains showed the best
451 performance indicating that both strains had positive effects in terms of stability and final H_2
452 production volumes.

453 In presence of *E. casseliflavus* or *D. vulgaris*, performance instability was not mitigated.
454 Samples were selected in bioreactors for which the second H_2 production phase did not occur
455 to evidence potential OTUs that could be responsible for the inhibition of this phase.
456 Sequencing results indicated that although *E. casseliflavus* reached a final proportion of 3%,
457 its presence was also associated to the emergence of *Sporolactobacillus* which became
458 predominant (28%). This lactic acid bacterium was also recovered in the first replicate of the
459 mixed culture for which no second H_2 production phase was observed. As a result, H_2
460 producers such as *C. acetobutylicum* and *C. butyricum* were less represented in the ecosystem
461 (40% and 22 %, respectively). Interestingly, *C. algifaecis* was significantly present with a
462 relative abundance of 8%. *D. vulgaris* was not detected in the final ecosystem. But similarly
463 to the bioreactors within which no second H_2 production phase occurred, *Sporolactobacillus*
464 and *C. algifaecis* gained importance (34% and 8%, respectively) at the expense of *C.*
465 *acetobutylicum* (28%) and *C. butyricum* (30%). Thus, it can be hypothesized that either these
466 strains were not metabolically active or that they did not influence the interactions within the
467 mixed culture.

Nevertheless, it confirms that the mixed culture instability could lead to situations where strong spore forming competitors such as lactic acid bacteria could emerge and greatly affect H_2 production. Interestingly, this instability always appeared after a first H_2 production phase during which rearrangements occurred within the ecosystem, probably because of a shift in substrate composition from glucose to metabolites such as VFA. It can be hypothesized that acetate production could create inhibitory conditions that were less favorable to *C. butyricum*. During this shift, new interactions were implemented and spore-forming bacteria could grow. Results prove that this instability could be avoided in batch reactors by two strategies: either by bioaugmenting with specific H_2 producers which were able to resist to acetate production and pH drop, or by using other strains (eg., *E. coli* or *C. necator*) which seemed to be able to prevent the growth of competitors such as lactic acid bacteria.

4 Conclusions

The distinct addition of *E. coli* and *C. necator* was proved to reduce metabolic and H_2 production instability of a mixed culture. Bioaugmentations with *C. acetobutylicum*, *C. pasteurianum* and *L. bulgaris* also mitigated this variability but induced stronger trophic competition that greatly limited final H_2 production. Divergence in performances were associated to populations shifts in the mixed culture but also to the appearance of spore forming, lactate producing genera such as *Sporolactobacillus.* This outcome highlighted potentialities to use pure strains to enhance process stability and limit the stochastic emergence of substrate competitors within mixed cultures.

E-supplementary data of this work can be found in online version of the paper.

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614

Figure caption

Figure 1: Cumulated H_2 production (mL) over time (number of hours) of the mixed culture (A), of the mixed culture bioaugmented with *Clostridium acetobutylicum* (B), with *Clostridium pasteurianum* (C), with *Lactobacillus bulgaris* (D), with *Escherichia coli* (E), with *Cupriavidus necator* (F) with *Enterococcus casseliflavus* (G) and with *Desulfovibrio vulgaris (H).*

Replicates 1 are represented in blue while replicates 2 are represented in red and replicates 3 in green.

Figure 2: Ultimate metabolites concentration (mmol) of the different reactors inoculated with the mixed alone or bioaugmented with the different pure strains after the total conversion of the substrate.

Figure 3: Relative abundances of the bacterial genera generated by 16S rRNA gene sequencing of the different reactors inoculated with the mixed alone or bioaugmented with the different pure strains.

Figure S1: Cumulated H_2 production (mL) over time (number of hours) of a pure culture of *Clostridium acetobutylicum.*

Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in green.

Figure S2: Cumulated H_2 production (mL) over time (number of hours) of a pure culture of *Clostridium pasteurianum.*

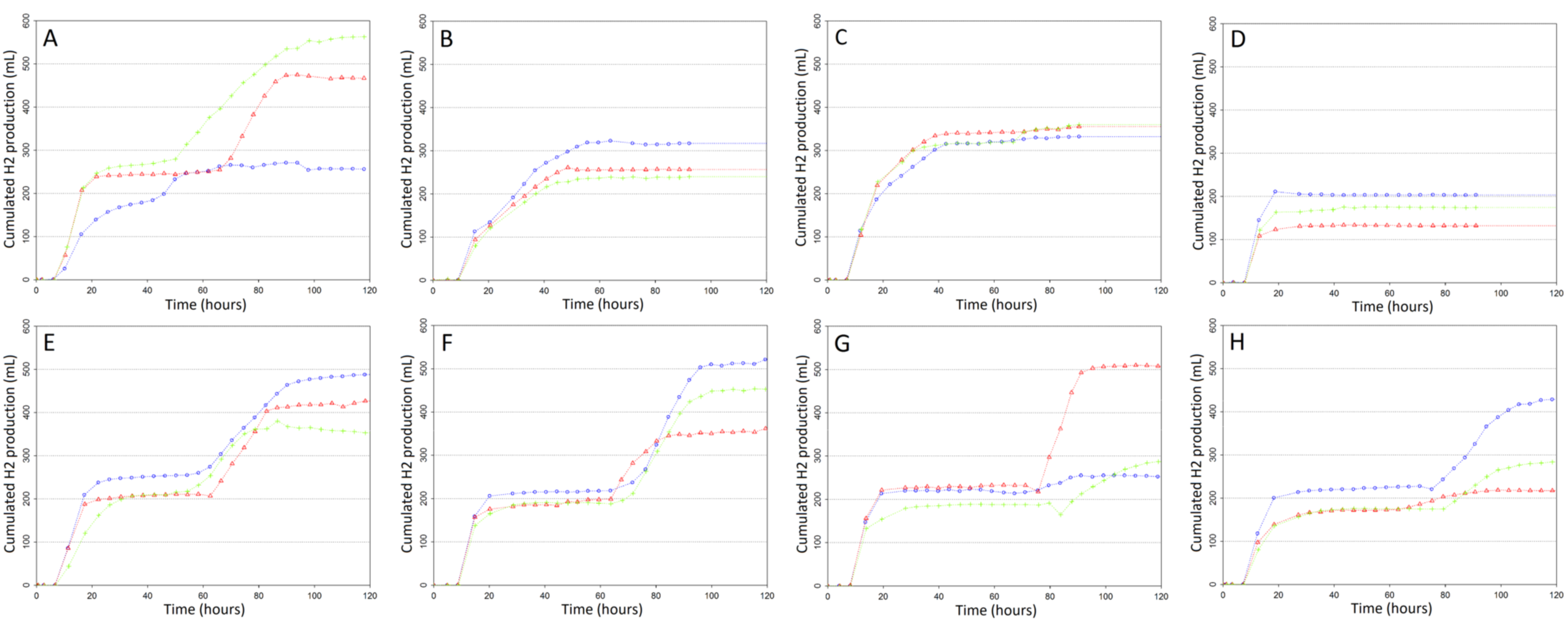
Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in green.

Figure S3: Cumulated H_2 production (mL) over time (number of hours) of a pure culture of *Escherichia coli.*

Replicate 1 is represented in blue while replicate 2 is represented in red and replicate 3 in green.

Figure S4: Metabolites concentration (mmol) of the different reactors inoculated with the mixed culture after the first and the second plateau phase of H_2 production.

Figure S5: Ultimate metabolites concentration (mmol) of the different reactors inoculated with the different pure strains after the total conversion of the substrate.


A
B
C
D
E
F
G
H
Cumulated H2 production (mL)
Time (hours)
0
100
200
300
400
500
600
0
20
40
60
80
100
120

Metabolite concentration (mmol)

25
20
15
10
5
0

R1 R2 R3

Mixed culture

Mixed culture + *Clostridium acetobutylicum*

Mixed culture + *Clostridium pasteurianum*

Mixed culture + *Lactobacillus bulgaris*

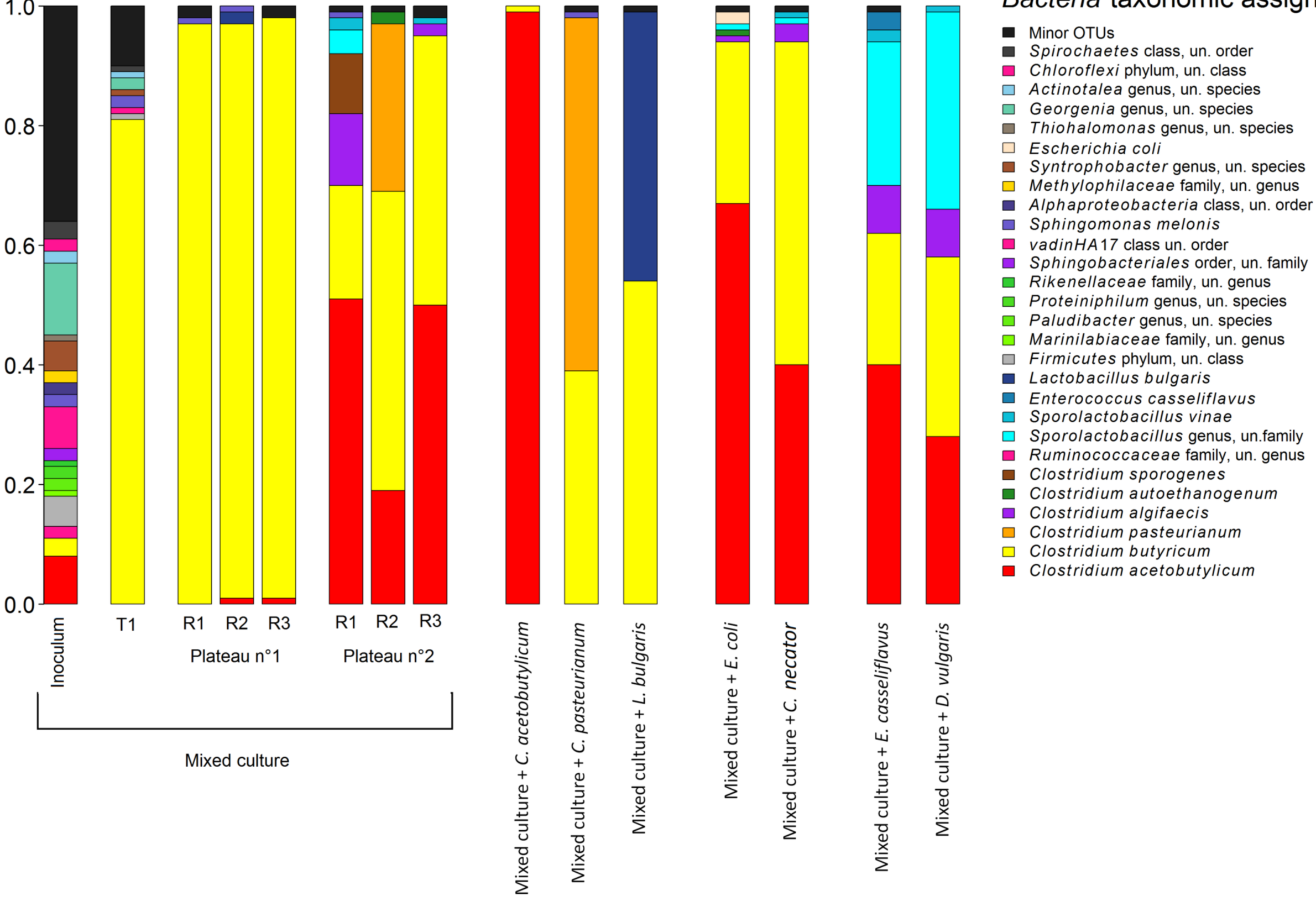
Mixed culture + *Escherichia coli*

Mixed culture + *Cupriavidus necator*

Mixed culture + *Enterococcus casseliflavus*

Mixed culture + *Desulfovibrio vulgaris*

Acetate *Butyrate* *Lactate* *Formate* *Caproate* *Ethanol*


1.0
0.8
0.6
0.4
0.2
0.0
Inoculum
T1
R1
R2
R3
Plateau n°1
R1
R2
R3
Plateau n°2
Mixed culture
Mixed culture + C. acetobutylicum
Mixed culture + C. pasteurianum
Mixed culture + L. bulgaris
Mixed culture + E. coli
Mixed culture + C. necator
Mixed culture + E. casseliflavus
Mixed culture + D. vulgaris
Bacteria taxonomic assignation
Minor OTUs
Spirochaetes class, un. order
Chloroflexi phylum, un. class
Actinotalea genus, un. species
Georgenia genus, un. species
Thiohalomonas genus, un. species
Escherichia coli
Syntrophobacter genus, un. species
Methylophilaceae family, un. genus
Alphaproteobacteria class, un. order
Sphingomonas melonis
vadinHA17 class un. order
Sphingobacteriales order, un. family
Rikenellaceae family, un. genus
Proteiniphilum genus, un. species
Paludibacter genus, un. species
Marinilabiaceae family, un. genus
Firmicutes phylum, un. class
Lactobacillus bulgaris
Enterococcus casseliflavus
Sporolactobacillus vinae
Sporolactobacillus genus, un.family
Ruminococcaceae family, un. genus
Clostridium sporogenes
Clostridium autoethanogenum
Clostridium algifaecis
Clostridium pasteurianum
Clostridium butyricum
Clostridium acetobutylicum

		V_{max} (mL H_2.L^{-1}.h^{-}	H_{max} (mL H_2)	λ (hour)	Carbon conversion yield (mol H_2.mol glucose^{-1})
Mixed culture	*Production phase n°1*	*126.8 ± 52.6*	*224.2 ± 49.2*	*8.4 ± 0.3*	*1.95 ± 0.10*
	Production phase n°2	*49.5 ± 23.8*	*176.3 ± 151.5*	-	*1.50 ± 1.28*
	Total	-	431.9 ± 159.3	-	*1.68 ± 0.62*
Clostridium acetobutylicum		303.0 ± 40.4	463.1 ± 26.6	19.4 ± 0.3	*1.65 ± 0.10*
Clostridium pasteurianum		77.5 ± 7.0	514.4 ± 32.5	14.2 ± 0.5	*1.79 ± 0.12*
Escherichia coli		18.3 ± 0.7	54.0 ± 7.7	19.2 ± 0.6	*0.18 ± 0.03*
Mixed culture + *Clostridium acetobutylicum*		*50.1 ± 3.5*	*274.8 ± 42.4*	*9.7 ± 0.2*	*1.17 ± 0.21*
Mixed culture + *Clostridium pasteurianum*		*93.9 ± 23.1*	*349.1 ± 14.8*	*8.3 ± 0.1*	*1.45 ± 0.06*
Mixed culture + *Lactobacillus bulgaris*		*142.7 ± 8.3*	*169.5 ± 36.1*	*8.7 ± 0.4*	*1.21 ± 0.23*
Mixed culture + *Escherichia coli*	*Production phase n°1*	*112.0 ± 40.1*	*224.5 ± 23.9*	*7.9 ± 0.1*	*1.61 ± 0.27*
	Production phase n°2		*207.2 ± 33.6*	-	*2.04 ± 0.09*
	Total	-	*436.0 ± 49.5*	-	*1.77 ± 0.28*
Mixed culture + *Cupriavidus necator*	*Production phase n°1*	*164.0 ± 26.4*	*197.7 ± 15.4*	*9.7 ± 0.1*	*1.47 ± 0.16*
	Production phase n°2		*247.3 ± 68.3*	-	*2.08 ± 0.55*
	Total	-	*445.6 ± 79.9*	-	*1.75 ± 0.31*
Mixed culture + *Enterococcus casseliflavus*	*Production phase n°1*	*106.3 ± 36.5*	*189.6 ± 26.7*	*8.3 ± 0.2*	*1.46 ± 0.20*
	Production phase n°2		*120.9 ± 81.7*	-	*1.00 ± 0.65*
	Total	-	*310.5 ± 107.4*	-	*1.24 ± 0.42*
Mixed culture + *Desulfovibrio vulgaris*	*Production phase n°1*	*169.5 ± 36.4*	*212.2 ± 21.3*	*9.4 ± 0.1*	*1.47 ± 0.18*
	Production phase n°2		*138.4 ± 126.5*	-	*1.21 ± 1.05*
	Total	-	*350.6 ± 138.5*	-	*1.35 ± 0.51*