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1 **Glucose electro-fermentation with mixed cultures: a key role of**  
2 **the *Clostridiaceae* family**

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11

12 **Abstract**

13 Electro-fermentation is a new type of bioprocess combining the concepts of fermentation  
14 and electro-microbiology to improve the conversion of organic substrates into valuable  
15 fermentation products. During electro-fermentation metabolic profiles could be redirected  
16 by the presence of polarized electrodes through changes in the microbial communities in  
17 the dark fermentation. This paper aims to investigate the influence of the bacterial  
18 community composition on glucose electro-fermentation in batch electro-systems. Our  
19 results showed that the initial microbial community significantly impacted the final  
20 microbial community and related metabolic patterns. During electro-fermentation, the H<sub>2</sub>  
21 yield was increased using anaerobic sludge but decreased using activated sludge as inocula.  
22 While using other inocula from similar origins, no differences between electro-fermentation  
23 and traditional fermentation were evidenced. The relative abundance of *Clostridiaceae*  
24 family members in the inoculum appeared to be a determining factor affecting the global  
25 performances. These findings provide new insights on electro-fermentation mechanisms  
26 occurring in mixed cultures.

27

28 **Keywords:** Biohydrogen; Bio-Electrochemical System (BES); Dark fermentation;  
29 Microbial community; Electro-assisted fermentation.

## 30 1. INTRODUCTION

31 Bio-hydrogen (bioH<sub>2</sub>) production by dark fermentation involves the activity of anaerobic  
32 microorganisms carrying fermentative pathways. Because a wide variety of substrates can  
33 be used, biosystems are preferably operated with mixed cultures for their cheaper operation  
34 and easier control [1]. Moreover, these bioprocesses are flexible and can easily adapt to  
35 environmental changes, *e.g.* substrate variability or abrupt changes in pH and temperature  
36 [2–4]. The microbial species able to produce H<sub>2</sub> are widely present in the environment,  
37 such as in anaerobic and aerobic sludge, composts, soils, sediments, leachates, organic  
38 waste, among others [1]. However, in complex H<sub>2</sub>-producing microbial communities,  
39 microorganisms with different functions coexist, and can be defined as H<sub>2</sub> producers, H<sub>2</sub>  
40 consumers, and competitive bacteria that are not capable of producing H<sub>2</sub> but can ferment  
41 the same substrate [4]. At cellular level, during fermentation, the cells seeks to recycle their  
42 electrons in excess and regenerate ferredoxin and NADH through the release of H<sub>2</sub> and  
43 other soluble metabolites. Metabolic pathways greatly depend on the operating conditions  
44 of the system that influence the cellular metabolism of the fermentative bacteria. In  
45 addition, at populational level, the selection of a specific microbial community is affected  
46 by the operating conditions [5]. Thus, high H<sub>2</sub> yields are associated with the butyrate and  
47 acetate pathways, while lower yields are associated with lactate and alcohols-producing  
48 pathways [6,7]. In general, when *Clostridium* are abundant or dominant in H<sub>2</sub>-producing  
49 communities, higher yields in H<sub>2</sub> and butyrate are observed [2,4,6].

50 Electro-fermentation (EF) is a new type of bioprocess combining the concepts of  
51 conventional fermentation and electromicrobiology. It aims to control the conversion of  
52 organic substrates into valuable fermentative end-products in presence of polarized

53 electrodes. This technology allows to redirect the metabolic pathways through the  
54 supply/removal of small amount of electrons to/from the fermentation medium. Compared  
55 to microbial electrosynthesis, the amount of energy provided is low and consequently the  
56 amount of electrons exchanged at the polarized electrode as well. Thus, during EF the main  
57 source of electrons remains an organic source [8–11]. Microorganisms could interact with  
58 the polarized electrode through so-called direct (DIET) or indirect (MIET) interspecies  
59 electron transfer mechanisms. [12,13]. DIET has been extensively studied with *Geobacter*  
60 *sufurreducens* and *Shewanella oneidensis* as models of electroactive bacteria. DIET  
61 consists of an electron transfer mechanism carried by electrically conductive pili or proteins  
62 on the outer membranes of the cells, such as cytochromes [14,15]. Meanwhile, MIET  
63 involves the use of electron shuttles/mediators produced by cells (*e.g.* phenazines, flavins,  
64 H<sub>2</sub> and formate) [13,16].

65 EF has been successfully applied in both pure and mixed cultures to increase the 1,3-  
66 propanediol production from glycerol [9,11,17] and for butanol or H<sub>2</sub> production from  
67 glucose [9,10]. However, the mechanisms involved in EF are not yet fully understood and  
68 several hypotheses based on pure culture behaviour have been formulated. The first  
69 hypothesis is related to a direct conversion of the substrate and electrons to a product of  
70 interest. Here, the polarized electrodes could act as an unlimited source or sink of electrons,  
71 depending on the working potential [8]. The second one considers a modification of the  
72 oxidation-reduction potential through a partial dissipation of the electrons in excess issued  
73 from fermentation or a small supply of extra electrons to the fermentation medium. In both  
74 cases, a slight change in the NADH/NAD<sup>+</sup> balance can occur, contributing to metabolic  
75 modifications in central cellular functions, including genetic expression and enzymatic

76 synthesis [5,8,16]. The third possibility can result from a syntrophic interaction between  
77 fermentative and electroactive bacteria. Here, the fermentative partner provides a co-  
78 substrate to the electroactive bacteria that, in return, makes the fermentation  
79 thermodynamically favourable by removing inhibitory end-products [8,11]. Finally, some  
80 authors also proposed that polarized electrodes can generate changes in cell structures and  
81 membrane zeta potentials that can generate metabolic shifts, as observed in *Clostridium*  
82 *pasteurianum* [9]. All these hypothetical mechanisms could also have an impact on the  
83 microbial community selection when working with mixed cultures. For example, the  
84 polarized electrode could favour the selection of electroactive bacteria, which could  
85 establish specific interactions with partner species, motivating their growth. As a result, the  
86 fermentation products could be modified through changes in the microbial communities  
87 [8,18].

88 Previous investigations on EF with mixed cultures reported changes in the metabolic  
89 patterns in association with changes in microbial communities [10,11,19,20]. It was  
90 concluded that EF could affect the microbial community by giving a competitive advantage  
91 to some bacteria. Thus, the initial composition of the microbial community as well as the  
92 interactions between microbial species and the polarized electrode are probably key aspects  
93 of EF. This paper aims to investigate the influence of the initial bacterial community  
94 composition on glucose EF. Mixed cultures from different origins, issued from H<sub>2</sub>-  
95 producing systems or anaerobic reactors treating different substrates, were tested in batch  
96 EF reactors.

97

98

99 **2. MATERIAL AND METHODS**

100

101 **2.1 Inocula**

102 Five different types of inoculum were used to compare the EF process in batch reactors:

103 **AnSlu:** Heat-treated anaerobic sludge sampled from a lab-scale anaerobic digester treating

104 sewage sludge (37.7 gVS.l<sup>-1</sup>); **ActSlu:** Activated sludge sampled from sewage treatment

105 plant in Narbonne – France (10.0 gVS.l<sup>-1</sup>); **AciSlu:** Acidogenic sludge sampled from lab-

106 scale H<sub>2</sub>-producing reactor fed with glucose (1.5 gVS.l<sup>-1</sup>); **AnSlu2:** Heat-treated anaerobic

107 sludge sampled from a lab-scale anaerobic digester treating food waste (7.1 gVS.l<sup>-1</sup>);

108 **AnSlu3:** Anaerobic sludge sampled from a lab-scale anaerobic digester treating volatile

109 fatty acids (9.5 gVS.l<sup>-1</sup>). **AnSlu** and **AnSlu2** were heat-treated at 90°C for 30 minutes using

110 water bath before inoculation.

111

112 **2.2 Fermentation medium**

113 The fermentation medium was adapted from Rafrafi *et al.*, (2013) and was composed of 5.0

114 g.l<sup>-1</sup> glucose and other nutrients as follows (g.l<sup>-1</sup>): 2.0 NH<sub>4</sub>Cl, 0.5 K<sub>2</sub>HPO<sub>4</sub>, 0.0086

115 FeCl<sub>2</sub>·4H<sub>2</sub>O, 19.5 MES buffer (100 mM) and 1.0 mL.l<sup>-1</sup> oligoelements solution. The latter

116 was composed as follows (g.l<sup>-1</sup>): 60.0 CaCl<sub>2</sub>·2H<sub>2</sub>O, 55.0 MgCl<sub>2</sub>·6H<sub>2</sub>O, 7.0

117 FeSO<sub>4</sub>(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>·6H<sub>2</sub>O, 1.3 CoSO<sub>4</sub>·7H<sub>2</sub>O, 1.2 MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.0 ZnCl<sub>2</sub>·2H<sub>2</sub>O, 1.0

118 Mo<sub>7</sub>O<sub>24</sub>(NH<sub>4</sub>)<sub>6</sub>·4H<sub>2</sub>O, 0.4 CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.1 BO<sub>3</sub>H<sub>3</sub>, 0.05 NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.01 Na<sub>2</sub>SeO<sub>3</sub>·5H<sub>2</sub>O

119 and 46.0 ml.l<sup>-1</sup> HCl 37%.

120

### 121 **2.3 Electro-fermentation systems and start-up**

122 Batch EF tests were performed in dual-chamber reactors with 0.9 liters of working volume.  
123 A cation exchange membrane (FKE-50, FuMA-Tech GmbH, Germany) was placed  
124 between the chambers. Working and counter electrodes corresponded to 90% platinum –  
125 10% iridium grids with a size of 3.5 cm x 3.5 cm (Heraeus Deutschland GmbH & Co. KG,  
126 Hanau – Germany). A saturated calomel reference electrode (SCE) connected to a VSP  
127 Potentiostat/Galvanostat interfaced to a VMP3B-80 Current Booster unit (BioLogic  
128 Science Instruments, France) was used as reference electrode to maintain constant the  
129 applied potential at the working electrode in  $-0.4\text{ V vs SCE}$ . This value was set according  
130 to the study published by Toledo–Alarcon *et al.*, (2019).

131 Batch EF experiments were carried out at  $37\text{ }^{\circ}\text{C}$  using a bath water and 250 rpm, for a  
132 maximum of 24 hours. Initial pH was adjusted at 6.0 with 2 M NaOH [10,22,23]. In the  
133 working electrode chamber the fermentation medium and inoculum were added at  $S/X =$   
134 10, where S is glucose concentration ( $\text{g.l}^{-1}$ ) and X is the initial biomass in the reactor  
135 ( $\text{gVS.l}^{-1}$ ). For the counter electrode chamber, only a glucose free fermentation medium was  
136 added. Batch control experiments, as conventional fermentation (F), were also performed  
137 under similar operating conditions, using a single-chamber reactor and in absence of  
138 polarized electrodes. Only experiments using AnSlu and ActSlu as inoculum were  
139 performed in duplicate.

140

### 141 **2.4 Analytical methods**

142 Liquid samples were taken from both compartments of the reactor, *i.e.* working and counter  
143 electrode chamber. The samples were prepared by centrifugation at 12,000 g for 15 min,

144 the pellet was reserved for DNA extraction while the supernatant was filtered with 0.2  $\mu\text{m}$   
145 syringe filters. The latter was analyzed in an High Performance Liquid Chromatography  
146 (HPLC) coupled to a refractive index detector (Waters R410) to determine the  
147 concentrations of glucose, alcohols and organic acids. HPLC analyses were conducted on  
148 an Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad) column at a temperature of 35°C, using  
149  $\text{H}_2\text{SO}_4$  (4 mM) as mobile phase at a flow rate of 0.3  $\text{mL}\cdot\text{min}^{-1}$ . Biogas production was  
150 continuously monitored during operation using a liquid displacement system. Percentages  
151 of  $\text{CO}_2$ ,  $\text{H}_2$ , and  $\text{CH}_4$  in the biogas were quantified by gas chromatography (Clarus 580 GC,  
152 Perkin Elmer) equipped with a thermal conductivity detector (TCD).

### 153 **2.5 Microbial community analysis**

154 DNA was extracted with FastDNA<sup>TM</sup> SPIN Kit following the instructions provided by the  
155 manufacturer (MP Biomedical; Santa Ana, California – USA). Extractions were confirmed  
156 and DNA concentration was obtained using Infinite 200 PRO NanoQuant (Tecan Group  
157 Ltd., Männedorf, Switzerland). The V3 – V4 region of the *16S rRNA* gene was amplified  
158 according to Carmona-Martínez *et al.*, (2015). The community composition was evaluated  
159 using MiSeq v3 (Illumina) with 2x300 bp paired-end reads at the GenoToul platform  
160 (<http://www.genotoul.fr>). Sequences were retrieved after demultiplexing, cleaning and  
161 affiliation of the raw sequences using Mothur v1.39.5. For alignment, the SILVA 132  
162 database was used. Sequences were submitted to GenBank under the accession No.  
163 MT000996-MT001185.

164



165 **2.6 Data analysis**

166 **Pearson correlation.** A Pearson correlation matrix was carried out from metabolites  
167 distribution and the composition of the final microbial community at family level. All data  
168 from F and EF were used for this analysis.

169 **Principal component analysis (PCA).** Two principal component analysis were carried out  
170 using variance-covariance matrices from: (i) metabolites distribution and final microbial  
171 community composition; (ii) Initial microbial community in the inocula and final microbial  
172 community composition. Data from F and EF were used for this analysis.

173 **Mantel test and partial Mantel test.** Mantel tests were performed to evaluate the  
174 correlation existing between the inoculum microbial communities, the metabolic patterns  
175 and the final microbial communities. Then, a Partial Mantel test was performed to  
176 determine whether the inoculum microbial community affected the correlation between the  
177 metabolites produced and the final microbial community [25,26].

178 **Non-metric multidimensional scaling (NMDS), Analysis of Similarities (ANOSIM)**  
179 **and Similarity Percentage (SIMPER).** A NMDS test was performed to represent the  
180 gradient of inoculum microbial community from different sources. ANOSIM test was  
181 performed to determine whether the differences between the inoculum microbial  
182 communities were statistically significant using 9999 permutations and a Bray Curtis  
183 similarity index. Then, a SIMPER test was performed to determine which families of the  
184 inoculum microbial community contribute the most percentage to the differences between  
185 inoculums compared [27].

186 **Electro-fermentation efficiency ( $\eta_{EF}$ ).** This coefficient corresponds to the ratio between  
187 the number of electrons passing through the electrical circuit and the number of extra-

188 electrons recovered in the fermentation products. For EF systems, this value should be as  
189 low as possible, showing that electricity production or consumption was not predominating  
190 the metabolites production. A value close to 1 indicates direct bioelectrosynthesis or  
191 electrolysis [8].

192 All statistical analyses of the data were carried out using the PAST (**PA**laeontological  
193 **ST**atistics) software v3.22 (<https://folk.uio.no/ohammer/past/>).

194

### 195 **3. RESULTS AND DISCUSSION**

#### 196 **3.1 Reactor performances during glucose electro-fermentation**

197 Five different inocula were used to study the influence of the inoculum source on glucose  
198 EF. All EF experiments were compared to conventional fermentation (F). At the end of  
199 operation, glucose was totally consumed in all reactors ( $5.3 \pm 0.2$  gCOD.l<sup>-1</sup>). Total chemical  
200 oxygen demand (COD) mass balance calculated from soluble products and accumulated H<sub>2</sub>,  
201 ranged between 72.5% and 87.7%, except for AnSlu2 (only  $42.1 \pm 1.2\%$ ), where some of the  
202 metabolites were probably unknown. Here, some COD could have been destined to the  
203 production of extracellular polysaccharides, such as exopolysaccharides (EPS) [28].  
204 However, despite the analyses performed, not all fermentation products were determined.  
205 Approximately 10-15% of the initial COD was attributed to the production of microbial  
206 biomass. Besides, no methane production was detected in any of the operated reactors.

207 When AnSlu was used, H<sub>2</sub> production was favoured 2.6 times in EF ( $1.80 \pm 0.31$   
208 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>) with respect to F ( $0.70 \pm 0.12$  molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>). In contrast, when  
209 ActSlu was used, H<sub>2</sub> production was disfavoured about 50% in EF ( $0.57 \pm 0.17$  molH<sub>2</sub>.mol<sup>-1</sup>  
210 <sub>glucose</sub>) compared to F ( $1.14 \pm 0.09$  molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>). Interestingly, no difference was

211 observed in H<sub>2</sub> production between EF and F when AciSlu, AnSlu2 and AnSlu3 were used  
212 as inoculum. The molar H<sub>2</sub> yields in these EF batches were 0.87 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub> (F= 0.90  
213 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>), 0.48 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub> (F= 0.50 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>) and 0.73 molH<sub>2</sub>.mol<sup>-1</sup>  
214 <sub>glucose</sub> (F= 0.72 molH<sub>2</sub>.mol<sup>-1</sup><sub>glucose</sub>), respectively. Our results achieved H<sub>2</sub> yields between  
215 24% and 90% of the theoretical maximum yield, considering butyrate as the only soluble  
216 metabolite [23]. These differences in H<sub>2</sub> yields show the importance of the microbial  
217 community selected at the end of the operation from inocula with different origins. This  
218 also seems to have an impact during EF process, because not all microbial communities  
219 responded in the same way. Interestingly, our results show that H<sub>2</sub> production could be  
220 increased, decreased or unaffected by the presence of polarized electrodes.

221 Fig. 1 shows the metabolite distribution expressed in relative COD content, for each of the  
222 inoculum. With AnSlu, the production of butyrate (19.0±10.4%<sub>COD</sub>), acetate  
223 (14.3±1.2%<sub>COD</sub>) and ethanol (22.7±2.4%<sub>COD</sub>) increased during EF, with regard to F where  
224 lactate (56.9±2.1%<sub>COD</sub>) was the main accumulated metabolite. When ActSlu was used, the  
225 main EF metabolites were butyrate (26.8±9.2%<sub>COD</sub>) and lactate (25.7±33.7%<sub>COD</sub>), while in  
226 F, butyrate reached up to 57.5±0.1%<sub>COD</sub>. In this case, a strong difference was observed in  
227 lactate and propionate concentrations between the EF duplicates. Some microbial species  
228 are able to consume lactate and produce propionate as end product, and could explain the  
229 high variability of these two metabolites in the duplicates due to the emergence of lactate  
230 consumers in the microbial communities [29].

231 When AciSlu, AnSlu2 and AnSlu3 were used, no difference in the metabolic patterns  
232 between EF and F was observed. In AciSlu, the main metabolites were butyrate

233 (24.5±1.6%<sub>COD</sub>) and ethanol (20.2±2.2%<sub>COD</sub>). In AnSlu3, the main metabolites were  
234 propionate (35.5±1.8%<sub>COD</sub>) and ethanol (30.5±0.2%<sub>COD</sub>).

235 Overall, the maximum H<sub>2</sub> yields were associated with an increase of butyrate (AnSlu-EF  
236 and ActSlu-F) and a decrease of lactate. This is consistent with data from literature which  
237 often reports higher H<sub>2</sub> yields when butyrate and acetate are the most important metabolites  
238 [6,30,31]. In addition, lactate production in H<sub>2</sub> production reactors with mixed cultures is  
239 mainly related to lactic acid bacteria. These compete for the substrate and reoxidize NADH  
240 through the non-H<sub>2</sub> producing pathway. As a result, the microbial community achieves  
241 lower H<sub>2</sub> yields [4,10]. Despite this, during this research no significant linear correlation  
242 was found between H<sub>2</sub> production and butyrate or lactate production (see Fig. 4). In this  
243 context, the ActSlu-EF, AciSlu-F and AciSlu-EF reactors showed low H<sub>2</sub> yields even with  
244 high butyrate production. As can be seen in Fig. 2, the inocula used in this research are  
245 quite different, as well as the species selected after EF. Clearly, these differences in the  
246 microbial communities, including members of dominant and sub-dominant families, affect  
247 the metabolic pathways used to release excess electrons and thereby determine final H<sub>2</sub>  
248 yields. In short, the metabolic products of any fermentation are highly influenced by the  
249 microbial species involved, as they interact with each other and with the polarized electrode  
250 [4,32].

251 The total electrical current that passed through the polarized electrodes during the operation  
252 of the EF reactors was – 0.17±0.11 C, – 4.94±1.11 C, – 12.92 C, – 8.03 C and – 10.90 C for  
253 the inoculum AnSlu, ActSlu, AciSlu, AnSlu2 and AnSlu3, respectively. The EF efficiency  
254 coefficient ( $\eta_{EF}$ ) was then calculated according to Moscoviz *et al.* (2016). When  
255 considering all the fermentation products, the  $\eta_{EF}$  was between 0.0007% and 0.033%.

256 Interestingly, the amount of electrons transferred through the polarized electrode was very  
257 low in all cases, as already shown in the literature and cannot explained the metabolic  
258 changes occurring in EF [8,10,11]. For instance, Moscoviz *et al* (2017) reported that during  
259 EF, ethanol production was increased and lactate decreased, compared to traditional  
260 fermentation. Here the amount of electrons supplied by the cathode only represented 0.2%  
261 of the total feed (calculated based on the electrons fed in the carbon source plus electric  
262 current). In addition, as discussed above, the EF could be motivated by changes in the  
263 redox potential of the system. However, these data are difficult to obtain because the sensor  
264 also detects the electrical gradient caused by electrodes polarization.

265

### 266 **3.2 Link between final bacterial communities and metabolic patterns**

267 Microbial communities were analysed for each condition studied at the end of batch  
268 operation. Figure 2 shows the microbial community distribution, which is represented  
269 between 90.5 and 98.6% by the most abundant families, that is, with a relative abundance  
270  $\geq 10\%$ . The low abundance families were grouped as "Others". In all reactors, most of the  
271 microbial diversity at the end of operation was represented by the families  
272 *Enterobacteriaceae*, *Streptococcaceae* and *Clostridiaceae*. In AnSlu, the *Streptococcaceae*  
273 family dominated in F ( $57.9 \pm 5.1\%$ ) while in EF, members of the *Enterobacteriaceae*  
274 ( $57.7 \pm 12.1\%$ ) and *Clostridiaceae* ( $36.5 \pm 5.5\%$ ) families were dominant. In ActSlu, the  
275 *Streptococcaceae* family was dominant in both F ( $42.1 \pm 3.2\%$ ) and EF ( $56.2 \pm 23.7\%$ )  
276 although different metabolic patterns were observed. Despite the differences observed in  
277 the EF duplicates, lower relative abundances of *Enterobacteriaceae* ( $10.6 \pm 4.7\%$ ) and  
278 *Clostridiaceae* ( $23.7 \pm 19.9\%$ ) than in F were observed. In AciSlu, AnSlu2 and AnSlu3 the

279 dominant family in F and EF was *Enterobacteriaceae*, representing  $86.0\pm 5.6\%$ ,  $95.3\pm 0.3\%$   
280 and  $97.9\pm 0.9\%$ , respectively.

281 For a better visualization and identification of the relationship between fermentation  
282 products and microbial communities after EF, a PCA analysis was performed using  
283 variance-covariance matrices. All data from F and EF reactors were used in the analysis,  
284 even though the biplot representation shows only the most important variables of each  
285 component. More than 85% of the variance was explained by the two first axes, as shown  
286 in Fig. 3A. The PCA shows that the tests performed with AciSlu, AnSlu2 and AnSlu3 are  
287 located within the same area, on the left side. Interestingly, these reactors were all related to  
288 the emergence of members of the *Enterobacteriaceae* family and ethanol production, and  
289 none of these inocula showed significant differences between F and EF.

290 At the right side of the PCA (Fig. 3A), the effects of the EF process are shown. The AnSlu-  
291 EF samples are grouped on the top right, and along the horizontal axis are the ActSlu-F  
292 samples. Both are related to high H<sub>2</sub> yields, high relative abundance of the *Clostridiaceae*  
293 family and high butyrate accumulation. On the right bottom, are represented the AnSlu-F and  
294 ActSlu-EF samples, performing lactate fermentation carried by members of the  
295 *Streptococcaceae* family. This was confirmed by a significant and positive Pearson  
296 correlation, as discussed below.

297 In addition, a Pearson test was performed to evaluate the correlations existing between the  
298 metabolic patterns and the final microbial community structures (Fig. 4). The H<sub>2</sub> yield  
299 positively correlates with the abundance of *Clostridiaceae* and *Prevotellaceae* families.  
300 Consistently, high H<sub>2</sub> yields are commonly associated with members of the *Clostridiaceae*  
301 family [4,6]. Although members of the *Prevotellaceae* have been frequently reported as

302 subdominant in H<sub>2</sub> production reactors, their function are not entirely clear. *Prevotellaceae*  
303 have been reported to contribute to the breakdown of complex substrates, but also to  
304 outcompete for glucose consumption [4]. In addition, butyrate production positively  
305 correlates with the relative abundances in *Clostridiaceae* and *Prevotellaceae*, and  
306 negatively correlates with *Enterobacteriaceae*. This is consistent with literature, since  
307 butyrate production is a typical metabolic product of members of the *Clostridiaceae* family  
308 but not for organisms from the *Enterobacteriaceae* family [33]. In addition, the lactate  
309 production correlates positively with the *Streptococcaceae* family and negatively with  
310 ethanol production. Members of the *Streptococcaceae* family are known as lactate  
311 producers and are commonly reported in H<sub>2</sub> production reactors [4,34,35]. More  
312 particularly, lactate production could be negatively correlated with ethanol production  
313 because they are produced by bacteria that commonly outcompete for the same substrate  
314 [4].

315 Finally, ethanol production correlates positively with *Enterobacteriaceae* and negatively  
316 with butyrate and the *Streptococcaceae* family. Although ethanol production could be  
317 linked to solventogenic fermentation by some species from the *Clostridium* genus, this  
318 result suggests that another pathway was preferentially carried by some members of the  
319 *Enterobacteriaceae* family that also co-produced ethanol during H<sub>2</sub> production [36–38].

320 In Fig. 4, information about ecological interactions occurring between species are also  
321 provided. As *Enterobacteriaceae* negatively correlates with *Clostridiaceae* and  
322 *Streptococcaceae*, a possible competitive interaction is here likely evidenced. Moreover,  
323 *Clostridiaceae* positively correlates with *Prevotellaceae*, suggesting a cooperative  
324 interaction between these two families.

325

### 326 **3.3 Bacterial characterization of inoculum sources**

327 To understand the behaviour of each inoculum during F and EF, the initial bacterial  
328 communities were analysed, as is presented in Fig. 2. Here, the families with a relative  
329 abundance  $\geq 10\%$  are represented and the rest were classified as "Others". For more details  
330 see Table S.1 in supplementary material. Dominant family in AnSlu was *Clostridiaceae*  
331 representing  $17.6 \pm 1.6\%$ . As this inoculum was heat pre-treated, spore-forming bacteria  
332 were preferentially selected [39–41]. In ActSlu the dominant families were *Saprospiraceae*  
333 and *Rhodocyclaceae*, representing  $14.6 \pm 1.4\%$  and  $11.3 \pm 0.6\%$ , respectively. Both families  
334 have been reported in wastewater treatment systems, as performing important functions  
335 such as the degradation of complex organic matter and in denitrification processes,  
336 respectively [42–44]. In AciSlu, *Sporolactobacillaceae* were dominant, representing  
337  $83.8 \pm 2.4\%$ . Generally, these lactate-producing bacteria are not dominant in H<sub>2</sub>-producing  
338 reactors but here the inoculum was sampled from a reactor outlet storage tank, and,  
339 probably, uncontrolled pH conditions favoured their development. In AnSlu2, the dominant  
340 groups were Clostridia\_unclassified, *Bacteroidaceae* and *Ruminococcaceae*, representing  
341  $15.8 \pm 0.3\%$ ,  $13.9 \pm 0.1\%$  and  $13.3 \pm 0.2\%$ , respectively. Although the members of  
342 *Bacteroidaceae* family are not spore-forming, they survived the heat pre-treatment  
343 performed in AnSlu2, as already reported in literature [45]. Members of the  
344 *Ruminococcaceae* family has been reported with an important hydrolytic activity when  
345 complex substrates are used during H<sub>2</sub> production [30,41]. Finally, organisms classified as  
346 Bacteroidetes\_unclassified were dominant in AnSlu3, representing  $19.5 \pm 0.4\%$ . Particularly,



347 members of this group has been reported as dominant in a UASB reactor treating poultry  
348 slaughterhouse wastewater [46].

349 As already evidenced, the five inocula were different in the composition of their microbial  
350 communities. Graphically, through a non-metric multidimensional scaling (NMDS)  
351 performed with Bray Curtis similarity index matrix, it was observed that all inocula were  
352 significantly distant from each other (See supplementary materials). An ANOSIM analysis  
353 confirmed that these distances were statistically significant (Mean rank within  
354 inoculum=5.5, mean rank between inoculum=38.5, R=1 and  $p_{value}=0.0001$ ).

355

#### 356 **3.4 Impact of inoculum source on electro-fermentation performances**

357 A PCA was performed based on the family distribution of the microbial communities at the  
358 beginning and after the reactors' operation. This allowed a better visualization of the  
359 changes in the microbial community from the inoculum and after the EF. About 75% of the  
360 variance was explained by the two first axes, as shown in Fig. 3B. This PCA shows that all  
361 inocula are grouped in the lower left quadrant. After F and EF the microbial communities  
362 changed and were distributed through the PCA depending on their H<sub>2</sub> yields. In the upper  
363 left of the PCA are represented the reactors inoculated with ActSlu in F and EF, where the  
364 families *Clostridiaceae* and *Streptococcaceae* were selected. At the top centre are the  
365 reactors inoculated with AnSlu in F and EF with the highest H<sub>2</sub> production, and where the  
366 families *Enterobacteriaceae* and *Clostridiaceae* were dominant. Finally, in the lower right  
367 quadrant are located the reactors that did not show any significant differences between F  
368 and EF in the fermentation products distribution. This is observed in reactors inoculated  
369 with AciSlu, AnSlu2 and AnSlu3, where the final microbial communities are dominated by

370 members of the *Enterobacteriaceae* family. Within this group there are species with  
371 different metabolic pathways, so different species could have been selected from each  
372 inoculum at the end of EF [4]. Moreover, members of families with low relative abundance  
373 could also contribute significantly to reactor performance, as has been reported in the  
374 literature [4,21]. Both aspects could explain the different metabolic products observed in  
375 these reactors.

376 Some statistical analyses were performed to determine how the microbial community  
377 composition in the inoculum affected the metabolic patterns and the final microbial  
378 communities. First, a Mantel test was carried out to evaluate the correlations between the  
379 initial inoculum microbial community, the final metabolites produced and the final  
380 microbial community. For that, three matrices with Euclidean distance data were  
381 calculated, including F and EF data. As observed in Table 1, only one positive linear  
382 correlation of  $r_M = 0.283$  (Pearson correlation) with a significance of  $p_{value} = 0.004$ , was  
383 found between the metabolic patterns matrix and the final microbial community matrix. To  
384 determine whether the initial microbial community distance matrix affected the correlation  
385 between the other two matrices *i.e.* metabolic patterns and final microbial community, a  
386 Partial Mantel test was then performed. Interestingly, this test showed a significant impact  
387 of the inoculum source on the correlation existing between the final microbial community  
388 and the metabolites produced ( $Z_M = 6.33$ ,  $r_M = 0.301$  and  $p_{value} = 0.003$ ).

389 As the initial microbial community of the inoculum influenced the EF behaviour, further  
390 investigation was performed to identify the bacterial families contributing to differences  
391 and similarities between the inocula. Based on H<sub>2</sub> production and according to the effects  
392 observed during EF, three groups were distinguished representing a positive effect (E<sub>p</sub>;

393 AnSlu), a negative effect ( $E_N$ ; ActSlu) and a neutral effect ( $E_0$ ; AciSlu, AnSlu2 and  
394 AnSlu3). A similarity of percentages (SIMPER) test was performed to determine which  
395 families mainly contributed to the differences between inocula (Table 2).

396 Table 2 summarizes the results obtained in the SIMPER test, showing only families with a  
397 contribution to dissimilarity  $\geq 2.0\%$ . Comparing  $E_N$  and  $E_0$ , a dissimilarity of 92.7% was  
398 observed, mainly due to *Sporolactobacillaceae* (22.3%) and *Saprospiraceae* (6.3%)  
399 families. *Sporolactobacillaceae* was abundantly present in AciSlu, inoculum belonging to  
400 the  $E_0$  group, but was absent in  $E_N$ . Well-known families producing  $H_2$  such as  
401 *Enterobacteriaceae* and *Clostridiaceae* were found in very low concentrations in both  
402 inoculum groups and contributed to less than 2.0% in the dissimilarity of these groups  
403 [2,4].

404 By comparing  $E_P$  and  $E_0$ , a dissimilarity of 84.3% was observed, mainly caused by the  
405 *Sporolactobacillaceae* (23.4%) and *Clostridiaceae* (7.3%) families. *Clostridiaceae* family  
406 was present in  $E_P$  with a greater abundance than  $E_0$ , while *Sporolactobacillaceae* were only  
407 present in  $E_0$ . Besides, *Enterobacteriaceae* family was contributing with 2.1% in  
408 dissimilarity between these two groups. By comparing  $E_P$  y  $E_N$ , a dissimilarity of 85.6%  
409 was observed, determined mainly by the *Clostridiaceae* (12.2%) and *Saprospiraceae*  
410 (10.7%) families. The *Enterobacteriaceae* family only contributed with 3.0% in  
411 dissimilarity between these two inoculum groups. In particular, *Clostridiaceae* family was  
412 more abundant in  $E_P$  inoculum.

413 Statistical analyses showed that the relative abundance in *Clostridiaceae* family members  
414 was crucial on the effect of EF. In particular, and because the *Clostridiaceae* family was  
415 underrepresented in the AciSlu, AnSlu2 and AnSlu3 inocula, no effect of the polarized

416 electrodes was observed. However, members of this family were significantly present in  
417 AnSlu and ActSlu, but with a higher relative abundance in AnSlu. These inocula showed a  
418 positive response to the presence of polarized electrodes increasing the H<sub>2</sub> production.  
419 Interestingly, as previously reported in literature, some *Clostridium* species could transfer  
420 electrons from or to a polarized electrode, either directly or using electron mediators  
421 [47,48]. Choi *et al.*, (2014) reported that a pure culture of *C. pasteurianum* received  
422 electrons directly from a polarized cathode with changes in the metabolic profiles,  
423 increasing the production of 1,3-propanediol and butanol from glycerol and glucose  
424 respectively. Choi *et al.*, (2012) reported an increase in butyrate production from sucrose  
425 using a pure culture of *C. tyrobutyricum* in a fermentation medium containing the electron  
426 mediator methyl viologen. Kumar *et al.*, (2017) also worked with an enriched microbial  
427 consortium for the production of bioelectricity in dual-chamber microbial fuel cells.  
428 *Clostridium* was the dominant genus in the reactor bulk and was responsible for  
429 fermentation as well as the transfer of electrons from the fermentation medium to the  
430 electrode mediated by ferredoxin. Finally, and more recently, an increase in H<sub>2</sub> and butyrate  
431 productions associated with the selection of H<sub>2</sub>-producing bacteria, including *Clostridia*  
432 species, during glucose EF using mixed cultures was reported, supporting the observations  
433 of the present study [10].

434

### 435 **3.5 Conclusion**

436 Our results show that inocula with a diverse bacterial composition from different sources  
437 had a significant impact on the electro-fermentation of glucose by selecting different  
438 microbial communities and metabolic patterns at the end of the operation. The relative

439 abundance of H<sub>2</sub>-producing bacteria from the *Clostridiaceae* family present in the inoculum  
440 appears to be a key parameter affecting the final behaviour in electro-fermentation. These  
441 findings provide new insights on electro-fermentation mechanisms occurring in mixed  
442 cultures, attributing a key role to *Clostridium* sp. Electro-fermentation is a new process and  
443 a tool with great potential to control bio-processes. However, the mechanisms involved,  
444 especially when working with mixed cultures, are not fully determined. In this context,  
445 more research is needed using pure cultures and testing different operational parameters.  
446 This would clarify the mechanisms of electro-fermentation with pure cultures and expand  
447 knowledge of electro-fermentation with mixed cultures.

448

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455

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620

621 Figure legends

622 **Fig. 1: Metabolite distribution based on COD mass balance in final samples of glucose**  
623 **electro-fermentation using different inocula source.** F: conventional fermentation as  
624 control. EF: electro-fermentation test. Error bars represent the standard deviation of the data  
625 replications where applicable.

626 1.5-column image.

627 **Fig. 2: Familial distribution of the microbial community both initially and after batch**  
628 **operation using different inocula.** Initial: Refers to the initial inocula. F: conventional  
629 fermentation as control. EF: electro-fermentation test. Error bars represent the standard  
630 deviation of the data replications where applicable.

631 1.5-column image.

632 **Fig. 3: Principal component analysis (PCA) performed with variance-covariance**  
633 **matrix from: A) microbial population distribution after batch operation and**  
634 **metabolic patterns; B) microbial population distribution both initial and after batch**  
635 **operation.**

636 2-column image.

637 **Fig. 4: Pearson correlation matrix from metabolic patterns and microbial population**  
638 **distribution after glucose electro-fermentation.** All data were used including F and EF in  
639 duplicates when correspond. In bold were marked the significant correlations with p-values  
640  $\leq 0.05$  (\*) and p-values  $\leq 0.01$  (\*\*). Positive (■ for 1.0), negative (■ for -1.0) and null (□ for  
641 zero) correlations were marked with gradient colour depending on value.

642 2-column image.

643 **Table 1:** Mantel tests performed with Euclidean distance matrix from inoculum microbial  
 644 composition, metabolite production and final microbial composition.

645

	Inoculum microbial community	Metabolic patterns <sup>a</sup>	Final microbial community <sup>a</sup>
Inoculum microbial community		$Z_M=56.3$ $r_M= -0.179$ $p_{value}=0.300$	$Z_M=46.9$ $r_M= 0.070$ $p_{value}=0.458$
Metabolic patterns			$Z_M=89.0$ $r_M=0.283$ $p_{value}=0.004$
Final microbial community			

646 <sup>a</sup> $Z_M$  is the Mantel statistic;  $r_M$  value is simply the Pearson's correlation coefficient and  
 647 ranges from  $- 1.0$  to  $+ 1.0$ . Significance of the test was calculated from 9999 permutation.  
 648 In this study a  $p_{value} < 0.01$  was considered statically significant to refuse the null  
 649 hypothesis.

650

651

652 **Table 2:** Similarity of percentage analysis (SIMPER) performed to compare the family  
 653 microbial composition of all inoculum data.

Family	$E_N$ & $E_0$ (%) <sup>b</sup>		$E_P$ & $E_0$ (%) <sup>b</sup>		$E_N$ & $E_P$ (%) <sup>b</sup>	
	Dissimilarity contrib. <sup>c*</sup>	Total <sup>d</sup>	Dissimilarity contrib. <sup>c*</sup>	Total <sup>d</sup>	Dissimilarity contrib. <sup>c*</sup>	Total <sup>d</sup>
<i>Sporolactobacillaceae</i>	22.3	22.3	23.4	23.4	0.0	0.0
<i>Clostridiaceae</i>	1.8	24.1	7.3	30.7	12.2	12.2
<i>Saprospiraceae</i>	6.3	30.4	0.0	30.7	10.7	22.9
<i>Rhodocyclaceae</i>	4.9	35.3	0.1	30.8	8.1	31.0
Bacteroidetes_unclassified	5.6	40.9	5.7	36.5	0.8	31.8
Family_XI	1.5	42.4	3.2	39.7	5.6	37.4
<i>Clostridia_unclassified</i>	4.6	47.0	4.8	44.5	0.0	37.4
Unknown	1.9	48.9	3.0	47.4	4.3	41.7
<i>Ruminococcaceae</i>	4.2	53.1	4.1	51.5	0.5	42.2
<i>Draconibacteriaceae</i>	1.1	54.2	2.5	54.0	4.1	46.2
<i>Planococcaceae</i>	0.1	54.3	2.4	56.4	4.1	50.3
<i>Bacteroidaceae</i>	3.8	58.1	3.9	60.4	0.0	50.3
<i>Christensenellaceae</i>	0.1	58.2	1.8	62.2	3.0	53.3
<i>Enterobacteriaceae</i>	0.6	58.7	2.1	64.3	3.0	56.3
<i>Peptostreptococcaceae</i>	1.0	59.7	2.0	66.3	2.9	59.2
uncultured	1.8	61.5	0.4	66.7	2.8	62.1
<i>Acidimicrobiales_I.S.</i> <sup>a</sup>	0.3	61.8	1.8	68.5	2.6	64.6
<i>Rikenellaceae</i>	2.3	64.1	2.6	71.1	1.2	65.9
<i>Rhodospirillaceae</i>	1.5	65.5	0.0	71.1	2.5	68.4
<i>Porphyromonadaceae</i>	2.4	68.0	2.3	73.4	1.2	69.6
<i>Intrasporangiaceae</i>	0.2	68.1	1.5	74.8	2.2	71.8
<i>Desulfuromonadaceae</i>	1.9	70.1	2.0	76.9	0.0	71.8
<i>Xanthomonadales_I.S.</i> <sup>a</sup>	1.2	71.3	0.0	76.9	2.0	73.8

654 <sup>a</sup>Only families that contribute  $\geq 2.0\%$ , in at least one sample, to the dissimilarity are  
 655 included in the table.

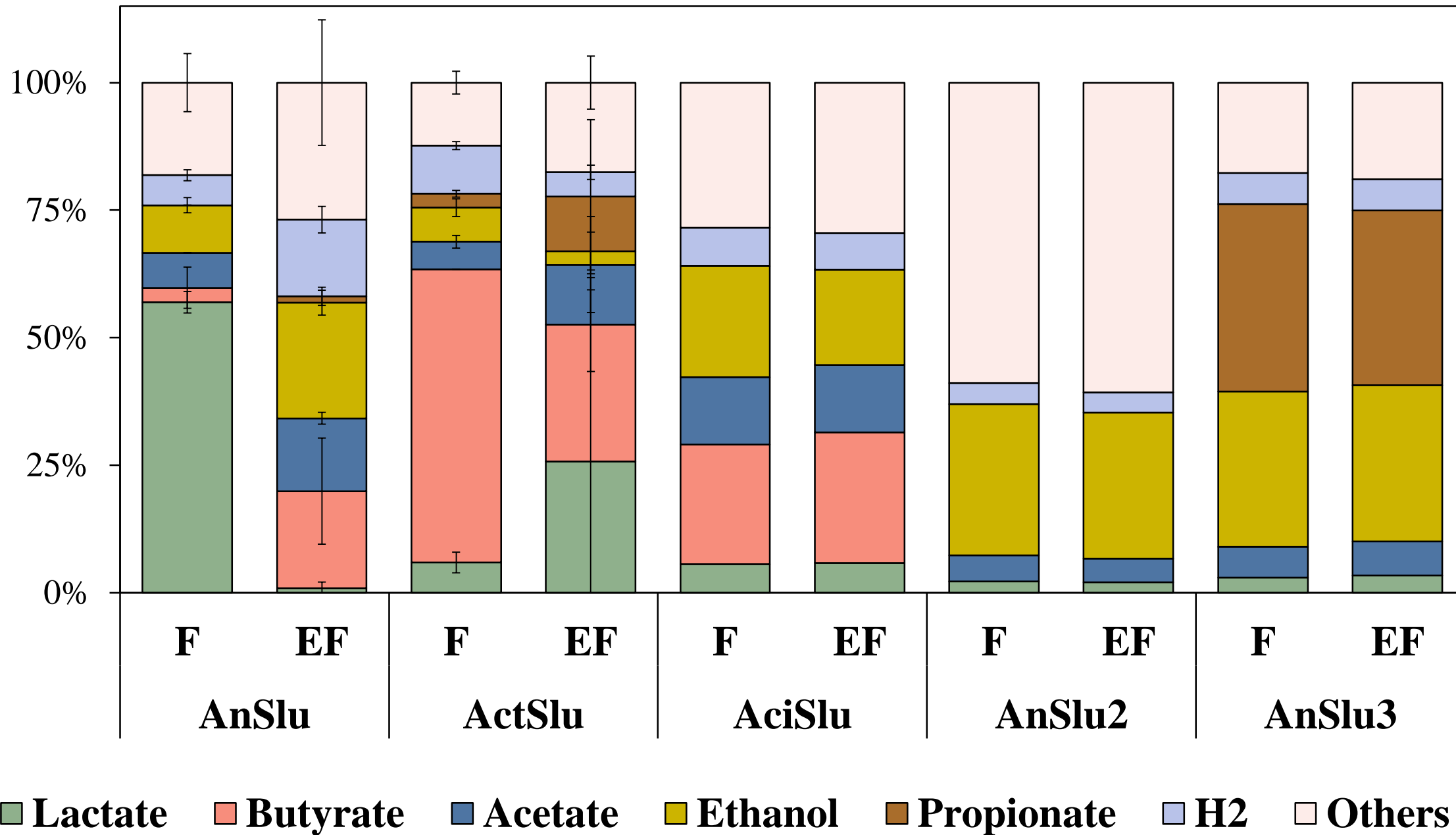
656 <sup>a</sup>I.S: abbreviation of Incertae Sedis.

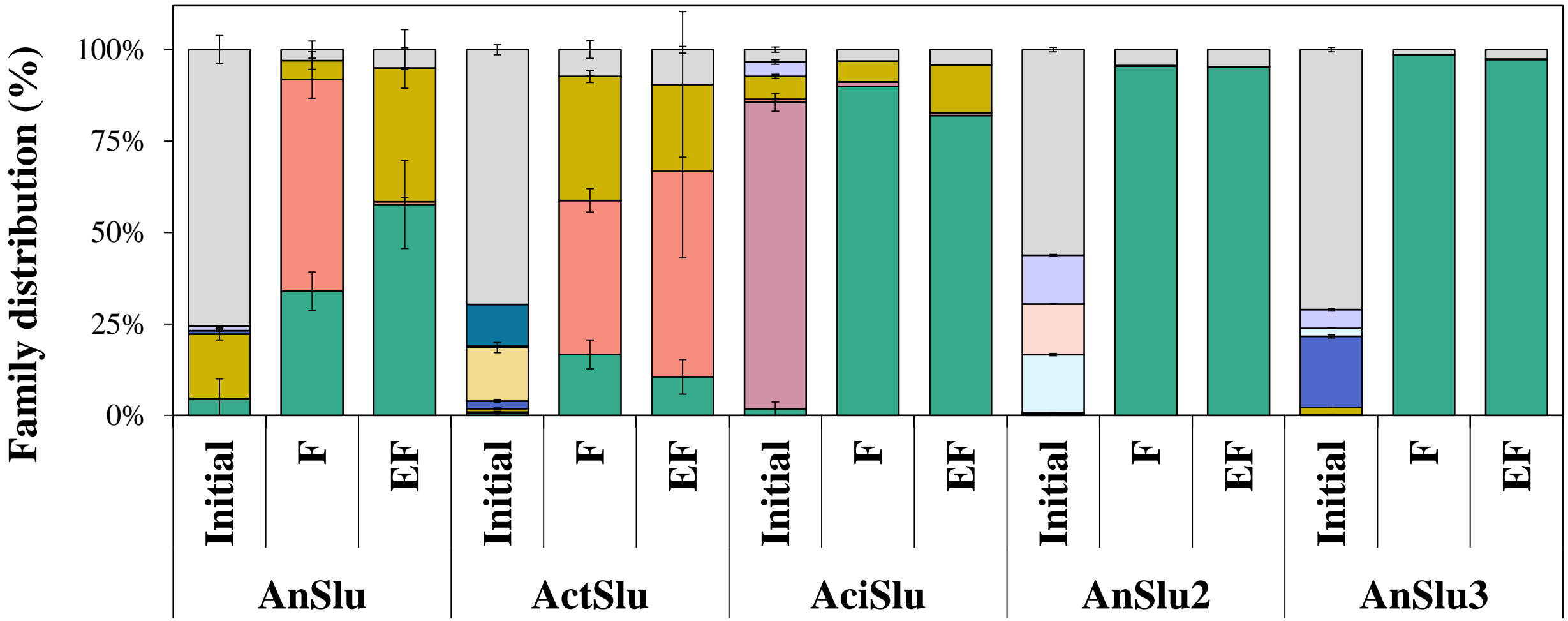
657 <sup>b</sup> $E_N$ : negative effect on H<sub>2</sub> production;  $E_P$ : positive effect on H<sub>2</sub> production;  $E_0$ : neutral  
 658 effect on H<sub>2</sub> production.

659 <sup>c</sup>Dissimilarity contrib.: correspond to percentage that each family is contributing to  
660 dissimilarity between the groups compared.  
661 <sup>d</sup>Total: correspond to accumulative contribution of each family to dissimilarity percentage.

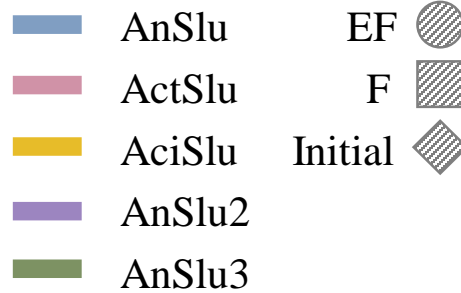
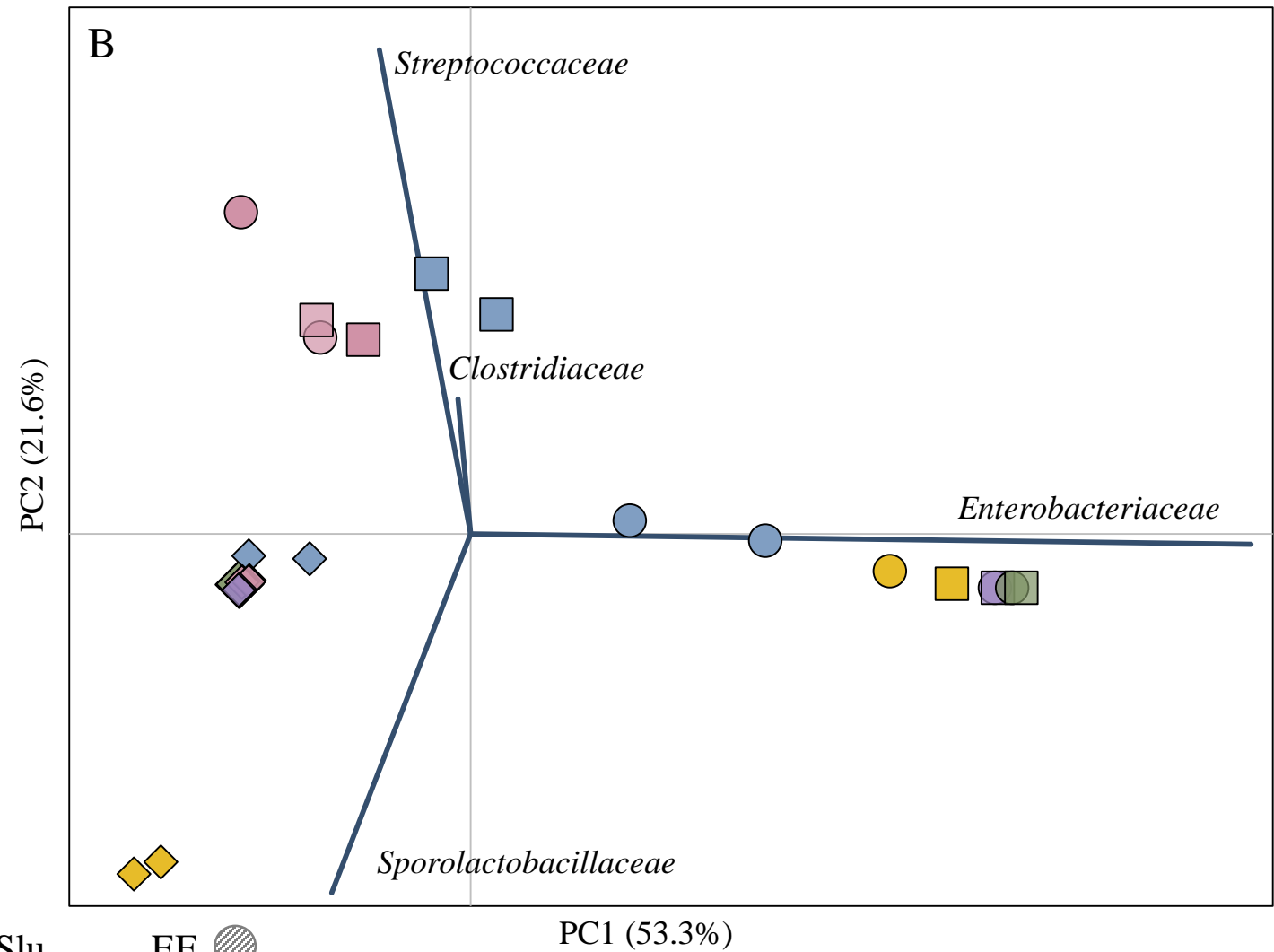
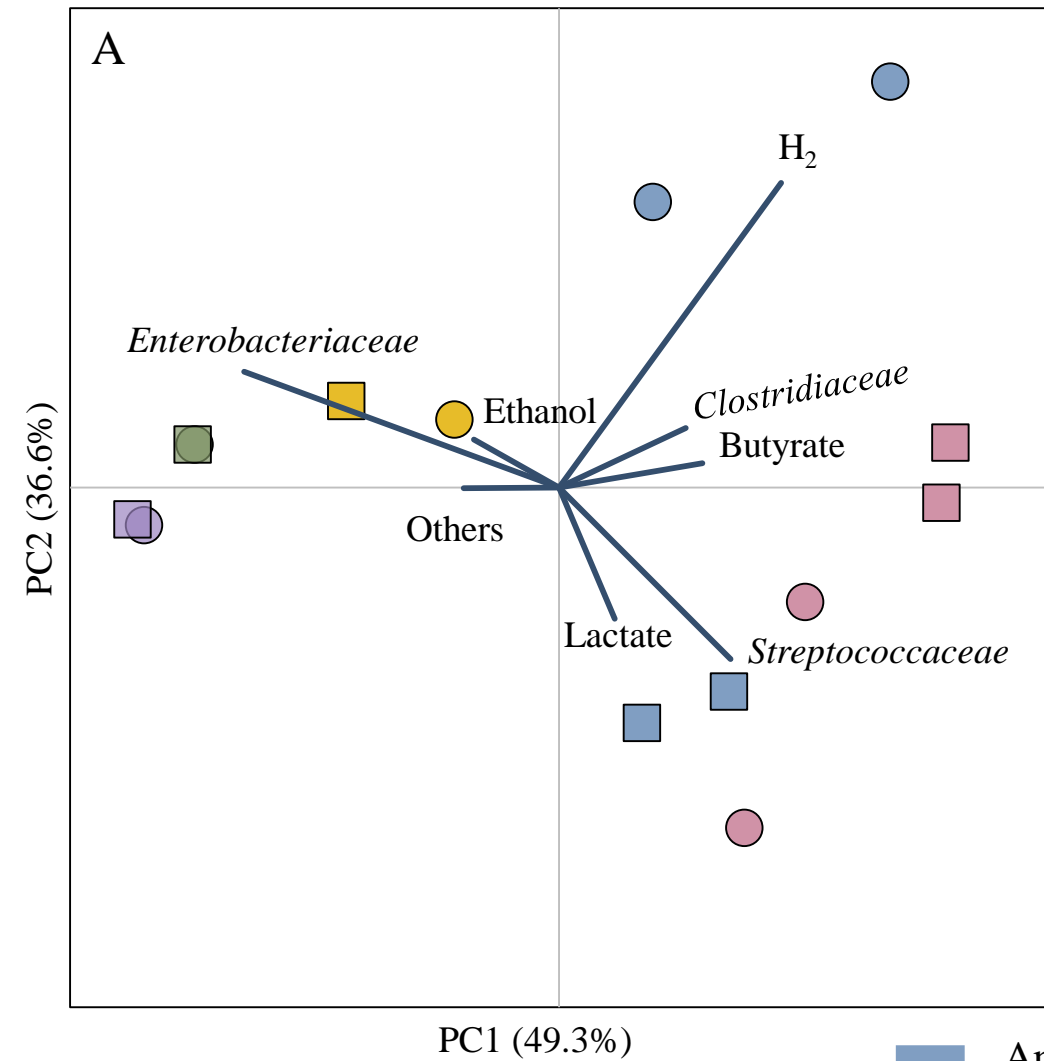


**Metabolite Distribution  
(% COD balance)**





- Enterobacteriaceae*
- Clostridiaceae*
- Saprospiraceae*
- Rhodocyclaceae*
- Sporolactobacillaceae*
- Bacteroidetes\_unclassified*
- Bacteroidaceae*
- Others (<10.0%)*
- Streptococcaceae*
- Clostridia\_unclassified*
- Ruminococcaceae*



	<b>H<sub>2</sub>Y</b>	<b>Succinate</b>	<b>Lactate</b>	<b>Ethanol</b>	<b>Acetate</b>	<b>Butyrate</b>	<b>Other+Biomass</b>	<b>Diversity</b>	<i>Enterobacteriaceae</i>	<i>Streptococcaceae</i>	<i>Clostridiaceae</i>	<i>Prevotellaceae</i>
<b>H<sub>2</sub>Y</b>		0.41	-0.35	-0.05	0.49	0.40	-0.35	0.44	-0.14	-0.26	<b>0.74**</b>	<b>0.67**</b>
<b>Succinate</b>	0.41		-0.49	<b>0.73**</b>	0.29	-0.39	0.29	-0.49	<b>0.66*</b>	<b>-0.77**</b>	-0.05	-0.14
<b>Lactate</b>	-0.35	-0.49		<b>-0.57*</b>	-0.33	-0.24	-0.22	0.23	-0.48	<b>0.79**</b>	-0.31	-0.30
<b>Ethanol</b>	-0.05	<b>0.73**</b>	<b>-0.57*</b>		-0.03	<b>-0.60*</b>	0.53	<b>-0.80**</b>	<b>0.95**</b>	<b>-0.91**</b>	-0.44	-0.25
<b>Acetate</b>	0.49	0.29	-0.33	-0.03		0.20	-0.23	0.32	-0.05	-0.27	<b>0.54*</b>	0.42
<b>Butyrate</b>	0.40	-0.39	-0.24	<b>-0.60*</b>	0.20		-0.48	<b>0.73**</b>	<b>-0.62*</b>	0.30	<b>0.76**</b>	<b>0.56*</b>
<b>Other+Biomass</b>	-0.35	0.29	-0.22	0.53	-0.23	-0.48		<b>-0.54*</b>	<b>0.56*</b>	-0.44	-0.45	-0.38
<b>Diversity</b>	0.44	-0.49	0.23	<b>-0.80**</b>	0.32	<b>0.73**</b>	<b>-0.54*</b>		<b>-0.86**</b>	<b>0.59*</b>	<b>0.80**</b>	<b>0.57*</b>
<i>Enterobacteriaceae</i>	-0.14	<b>0.66*</b>	-0.48	<b>0.95**</b>	-0.05	<b>-0.62*</b>	<b>0.56*</b>	<b>-0.86**</b>		<b>-0.87**</b>	<b>-0.60*</b>	-0.43
<i>Streptococcaceae</i>	-0.26	<b>-0.77**</b>	<b>0.79**</b>	<b>-0.91**</b>	-0.27	0.30	-0.44	<b>0.59*</b>	<b>-0.87**</b>		0.14	0.03
<i>Clostridiaceae</i>	<b>0.74**</b>	-0.05	-0.31	-0.44	<b>0.54*</b>	<b>0.76**</b>	-0.45	<b>0.80**</b>	<b>-0.60*</b>	0.14		<b>0.77**</b>
<i>Prevotellaceae</i>	<b>0.67**</b>	-0.14	-0.30	-0.25	0.42	<b>0.56*</b>	-0.38	<b>0.57*</b>	-0.43	0.03	<b>0.77**</b>	