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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 H₂
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 (bioH₂) 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 H₂ 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 H₂-producing microbial communities,
39 microorganisms with different functions coexist, and can be defined as H₂ producers, H₂
40 consumers, and competitive bacteria that are not capable of producing H₂ 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 H₂ 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 H₂ 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 H₂-producing
49 communities, higher yields in H₂ 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 H₂ 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 H₂ 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⁺ 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 H₂-
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⁻¹); **ActSlu:** Activated sludge sampled from sewage treatment

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

106 scale H₂-producing reactor fed with glucose (1.5 gVS.l⁻¹); **AnSlu2:** Heat-treated anaerobic

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

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

109 fatty acids (9.5 gVS.l⁻¹). **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⁻¹ glucose and other nutrients as follows (g.l⁻¹): 2.0 NH₄Cl, 0.5 K₂HPO₄, 0.0086

115 FeCl₂·4H₂O, 19.5 MES buffer (100 mM) and 1.0 mL.l⁻¹ oligoelements solution. The latter

116 was composed as follows (g.l⁻¹): 60.0 CaCl₂·2H₂O, 55.0 MgCl₂·6H₂O, 7.0

117 FeSO₄(NH₄)₂SO₄·6H₂O, 1.3 CoSO₄·7H₂O, 1.2 MnCl₂·4H₂O, 1.0 ZnCl₂·2H₂O, 1.0

118 Mo₇O₂₄(NH₄)₆·4H₂O, 0.4 CuSO₄·5H₂O, 0.1 BO₃H₃, 0.05 NiCl₂·6H₂O, 0.01 Na₂SeO₃·5H₂O

119 and 46.0 ml.l⁻¹ 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 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 °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 (g.l^{-1}) and X is the initial biomass in the reactor
135 (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 μm
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 H_2SO_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 CO_2 , H_2 , and 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 FastDNATM 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 (η_{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 ± 0.2 gCOD.l⁻¹). Total chemical
200 oxygen demand (COD) mass balance calculated from soluble products and accumulated H₂,
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, H₂ production was favoured 2.6 times in EF (1.80 ± 0.31
208 molH₂.mol⁻¹_{glucose}) with respect to F (0.70 ± 0.12 molH₂.mol⁻¹_{glucose}). In contrast, when
209 ActSlu was used, H₂ production was disfavoured about 50% in EF (0.57 ± 0.17 molH₂.mol⁻¹
210 _{glucose}) compared to F (1.14 ± 0.09 molH₂.mol⁻¹_{glucose}). Interestingly, no difference was

211 observed in H₂ production between EF and F when AciSlu, AnSlu2 and AnSlu3 were used
212 as inoculum. The molar H₂ yields in these EF batches were 0.87 molH₂.mol⁻¹_{glucose} (F= 0.90
213 molH₂.mol⁻¹_{glucose}), 0.48 molH₂.mol⁻¹_{glucose} (F= 0.50 molH₂.mol⁻¹_{glucose}) and 0.73 molH₂.mol⁻¹
214 _{glucose} (F= 0.72 molH₂.mol⁻¹_{glucose}), respectively. Our results achieved H₂ yields between
215 24% and 90% of the theoretical maximum yield, considering butyrate as the only soluble
216 metabolite [23]. These differences in H₂ 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 H₂ 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%_{COD}), acetate
223 (14.3±1.2%_{COD}) and ethanol (22.7±2.4%_{COD}) increased during EF, with regard to F where
224 lactate (56.9±2.1%_{COD}) was the main accumulated metabolite. When ActSlu was used, the
225 main EF metabolites were butyrate (26.8±9.2%_{COD}) and lactate (25.7±33.7%_{COD}), while in
226 F, butyrate reached up to 57.5±0.1%_{COD}. 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%_{COD}) and ethanol (20.2±2.2%_{COD}). In AnSlu3, the main metabolites were
234 propionate (35.5±1.8%_{COD}) and ethanol (30.5±0.2%_{COD}).

235 Overall, the maximum H₂ 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 H₂ yields when butyrate and acetate are the most important metabolites
238 [6,30,31]. In addition, lactate production in H₂ 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-H₂ producing pathway. As a result, the microbial community achieves
241 lower H₂ yields [4,10]. Despite this, during this research no significant linear correlation
242 was found between H₂ production and butyrate or lactate production (see Fig. 4). In this
243 context, the ActSlu-EF, AciSlu-F and AciSlu-EF reactors showed low H₂ 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 H₂
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 (η_{EF}) was then calculated according to Moscoviz *et al.* (2016). When
255 considering all the fermentation products, the η_{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 H₂ 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 H₂ yield
299 positively correlates with the abundance of *Clostridiaceae* and *Prevotellaceae* families.
300 Consistently, high H₂ 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 H₂ 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 H₂ 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 H₂ 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 H₂-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 H₂ 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 $p_{\text{value}}=0.0001$).

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_2 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_2 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 H₂ production and according to the effects
392 observed during EF, three groups were distinguished representing a positive effect (E_p;

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 H₂ 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 H₂ and butyrate
431 productions associated with the selection of H₂-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 H₂-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 ≤ 0.05 (*) and p-values ≤ 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 ^a	Final microbial community ^a
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 ^a 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 (%) ^b		E_P & E_0 (%) ^b		E_N & E_P (%) ^b	
	Dissimilarity contrib. ^{c*}	Total ^d	Dissimilarity contrib. ^{c*}	Total ^d	Dissimilarity contrib. ^{c*}	Total ^d
<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> ^a	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> ^a	1.2	71.3	0.0	76.9	2.0	73.8

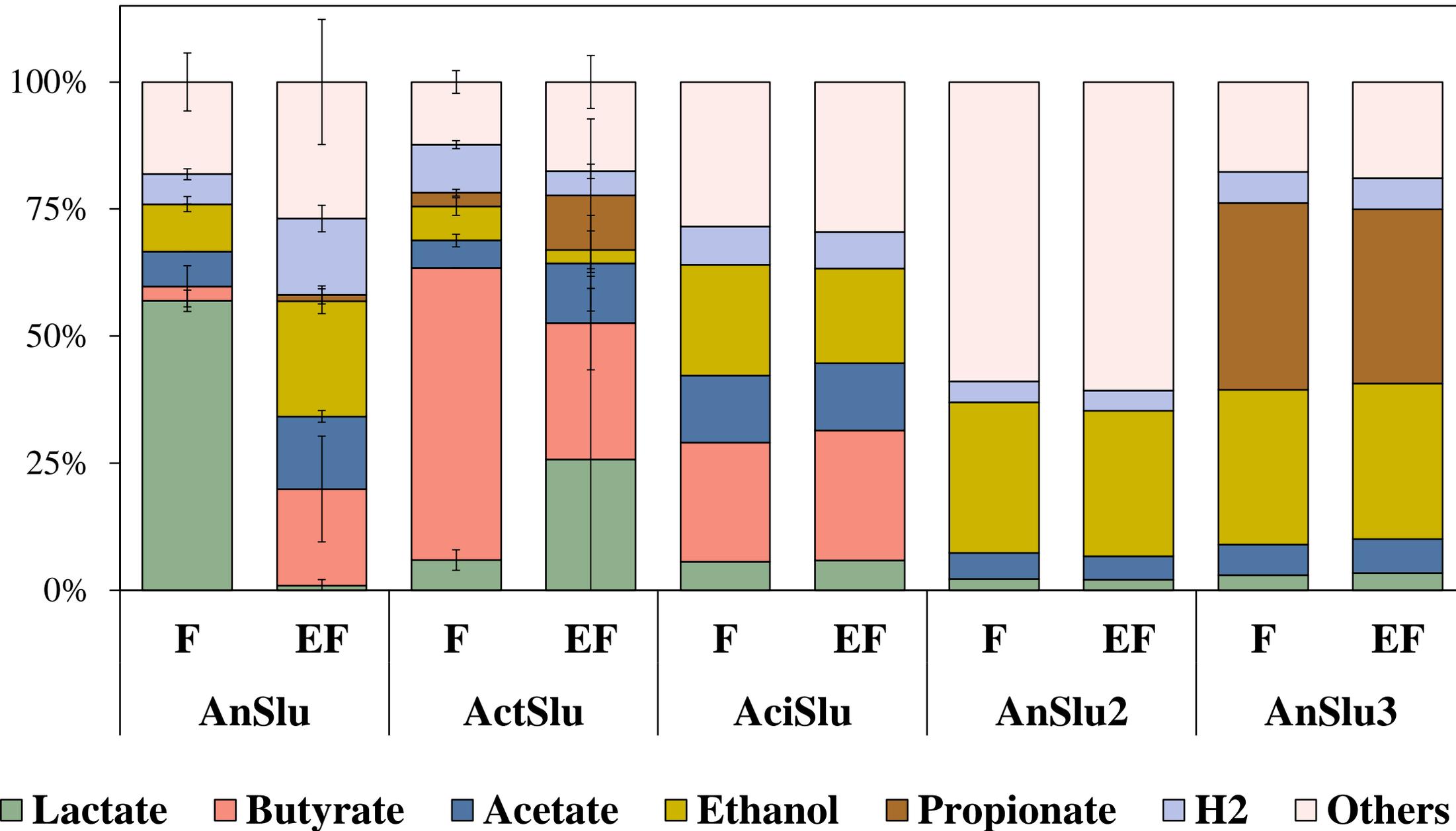
654 ^{*}Only families that contribute $\geq 2.0\%$, in at least one sample, to the dissimilarity are
 655 included in the table.

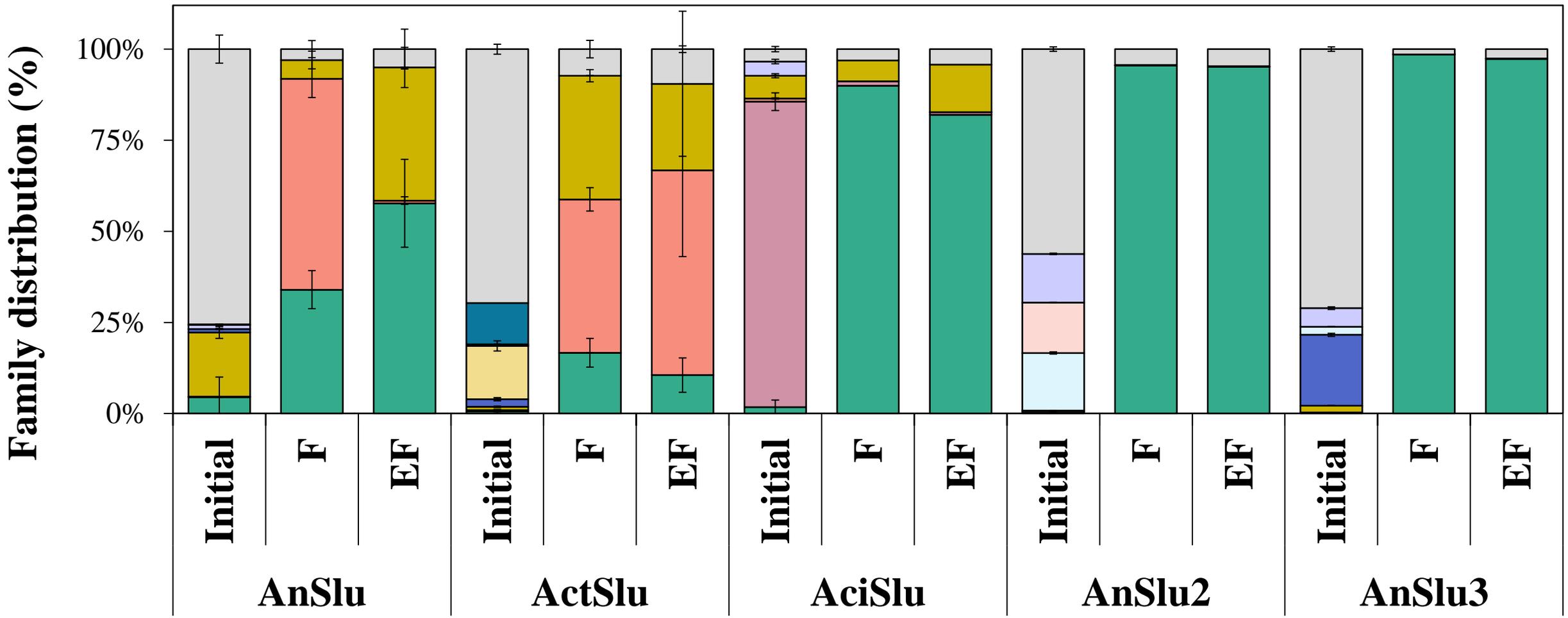
656 ^aI.S: abbreviation of Incertae Sedis.

657 ^b E_N : negative effect on H₂ production; E_P : positive effect on H₂ production; E_0 : neutral
 658 effect on H₂ production.

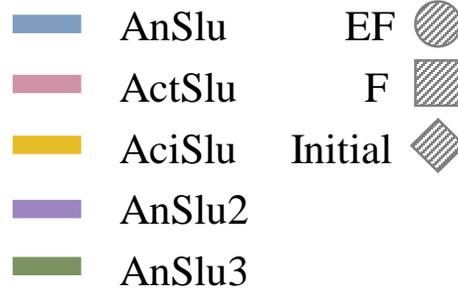
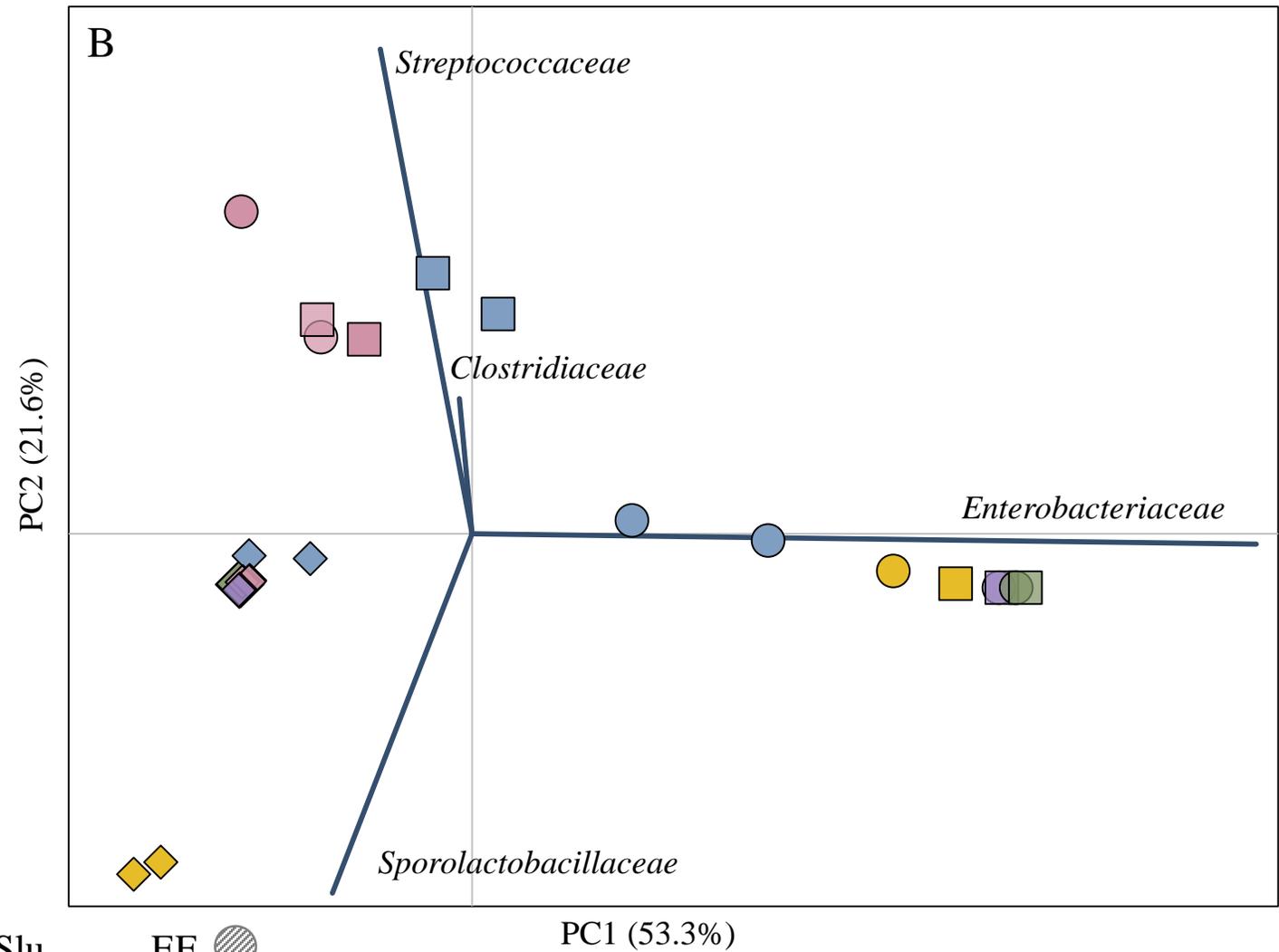
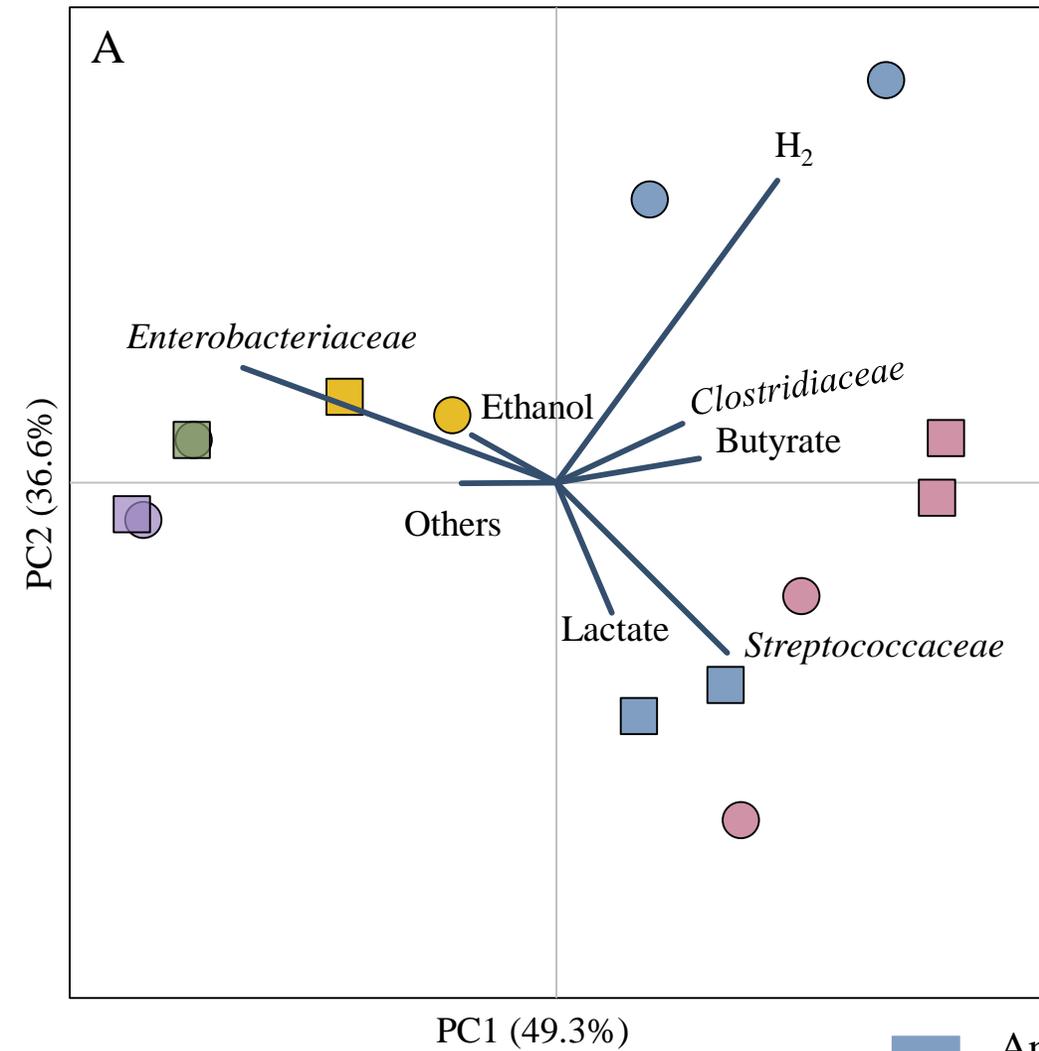
659 ^cDissimilarity contrib.: correspond to percentage that each family is contributing to
660 dissimilarity between the groups compared.
661 ^dTotal: correspond to accumulative contribution of each family to dissimilarity percentage.

**Metabolite Distribution
(% COD balance)**





- Enterobacteriaceae*
- Clostridiaceae*
- Saprospiraceae*
- Rhodocyclaceae*
- Sporolactobacillaceae*
- Bacteroidetes_unclassified*
- Bacteroidaceae*
- Others (<10.0%)*
- Streptococcaceae*
- Clostridia_unclassified*
- Ruminococcaceae*



	H₂Y	Succinate	Lactate	Ethanol	Acetate	Butyrate	Other+Biomass	Diversity	<i>Enterobacteriaceae</i>	<i>Streptococcaceae</i>	<i>Clostridiaceae</i>	<i>Prevotellaceae</i>
H₂Y		0.41	-0.35	-0.05	0.49	0.40	-0.35	0.44	-0.14	-0.26	0.74**	0.67**
Succinate	0.41		-0.49	0.73**	0.29	-0.39	0.29	-0.49	0.66*	-0.77**	-0.05	-0.14
Lactate	-0.35	-0.49		-0.57*	-0.33	-0.24	-0.22	0.23	-0.48	0.79**	-0.31	-0.30
Ethanol	-0.05	0.73**	-0.57*		-0.03	-0.60*	0.53	-0.80**	0.95**	-0.91**	-0.44	-0.25
Acetate	0.49	0.29	-0.33	-0.03		0.20	-0.23	0.32	-0.05	-0.27	0.54*	0.42
Butyrate	0.40	-0.39	-0.24	-0.60*	0.20		-0.48	0.73**	-0.62*	0.30	0.76**	0.56*
Other+Biomass	-0.35	0.29	-0.22	0.53	-0.23	-0.48		-0.54*	0.56*	-0.44	-0.45	-0.38
Diversity	0.44	-0.49	0.23	-0.80**	0.32	0.73**	-0.54*		-0.86**	0.59*	0.80**	0.57*
<i>Enterobacteriaceae</i>	-0.14	0.66*	-0.48	0.95**	-0.05	-0.62*	0.56*	-0.86**		-0.87**	-0.60*	-0.43
<i>Streptococcaceae</i>	-0.26	-0.77**	0.79**	-0.91**	-0.27	0.30	-0.44	0.59*	-0.87**		0.14	0.03
<i>Clostridiaceae</i>	0.74**	-0.05	-0.31	-0.44	0.54*	0.76**	-0.45	0.80**	-0.60*	0.14		0.77**
<i>Prevotellaceae</i>	0.67**	-0.14	-0.30	-0.25	0.42	0.56*	-0.38	0.57*	-0.43	0.03	0.77**	