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

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Abstract

Electro-fermentation is a new type of bioprocess combining the concepts of fermentation and electro-microbiology to improve the conversion of organic substrates into valuable fermentation products. During electro-fermentation metabolic profiles could be redirected by the presence of polarized electrodes through changes in the microbial communities in the dark fermentation. This paper aims to investigate the influence of the bacterial community composition on glucose electro-fermentation in batch electro-systems. Our results showed that the initial microbial community significantly impacted the final microbial community and related metabolic patterns. During electro-fermentation, the H_2 yield was increased using anaerobic sludge but decreased using activated sludge as inocula. While using other inocula from similar origins, no differences between electro-fermentation and traditional fermentation were evidenced. The relative abundance of *Clostridiaceae* family members in the inoculum appeared to be a determining factor affecting the global performances. These findings provide new insights on electro-fermentation mecanisms occurring in mixed cultures.

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

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1. INTRODUCTION

Bio-hydrogen (bioH_2) production by dark fermentation involves the activity of anaerobic microorganisms carrying fermentative pathways. Because a wide variety of substrates can be used, biosystems are preferably operated with mixed cultures for their cheaper operation and easier control [1]. Moreover, these bioprocesses are flexible and can easily adapt to environmental changes, *e.g.* substrate variability or abrupt changes in pH and temperature [2–4]. The microbial species able to produce H_2 are widely present in the environment, such as in anaerobic and aerobic sludge, composts, soils, sediments, leachates, organic waste, among others [1]. However, in complex H_2-producing microbial communities, microorganisms with different functions coexist, and can be defined as H_2 producers, H_2 consumers, and competitive bacteria that are not capable of producing H_2 but can ferment the same substrate [4]. At cellular level, during fermentation, the cells seeks to recycle their electrons in excess and regenerate ferredoxin and NADH through the release of H_2 and other soluble metabolites. Metabolic pathways greatly depend on the operating conditions of the system that influence the cellular metabolism of the fermentative bacteria. In addition, at populational level, the selection of a specific microbial community is affected by the operating conditions [5]. Thus, high H_2 yields are associated with the butyrate and acetate pathways, while lower yields are associated with lactate and alcohols-producing pathways [6,7]. In general, when *Clostridium* are abundant or dominant in H_2-producing communities, higher yields in H_2 and butyrate are observed [2,4,6].

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

electrodes. This technology allows to redirect the metabolic pathways through the supply/removal of small amount of electrons to/from the fermentation medium. Compared to microbial electrosynthesis, the amount of energy provided is low and consequently the amount of electrons exchanged at the polarized electrode as well. Thus, during EF the main source of electrons remains an organic source [8–11]. Microorganisms could interact with the polarized electrode through so-called direct (DIET) or indirect (MIET) interspecies electron transfer mechanisms. [12,13]. DIET has been extensively studied with *Geobacter sufurreducens* and *Shewanella oneidensis* as models of electroactive bacteria. DIET consists of an electron transfer mechanism carried by electrically conductive pili or proteins on the outer membranes of the cells, such as cytochromes [14,15]. Meanwhile, MIET involves the use of electron shuttles/mediators produced by cells (*e.g.* phenazines, flavins, H_2 and formate) [13,16].

EF has been successfully applied in both pure and mixed cultures to increase the 1,3-propanediol production from glycerol [9,11,17] and for butanol or H_2 production from glucose [9,10]. However, the mechanisms involved in EF are not yet fully understood and several hypotheses based on pure culture behaviour have been formulated. The first hypothese is related to a direct conversion of the substrate and electrons to a product of interest. Here, the polarized electrodes could act as an unlimited source or sink of electrons, depending on the working potential [8]. The second one considers a modification of the oxidation-reduction potential through a partial dissipation of the electrons in excess issued from fermentation or a small supply of extra electrons to the fermentation medium. In both cases, a slight change in the $NADH/NAD^+$ balance can occur, contribuiting to metabolic modifications in central cellular functions, including genetic expression and enzymatic

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

Previous investigations on EF with mixed cultures reported changes in the metabolic patterns in association with changes in microbial communities [10,11,19,20]. It was concluded that EF could affect the microbial community by giving a competitive advantage to some bacteria. Thus, the initial composition of the microbial community as well as the interactions between microbial species and the polarized electrode are probably key aspects of EF. This paper aims to investigate the influence of the initial bacterial community composition on glucose EF. Mixed cultures from different origins, issued from H_2-producing systems or anaerobic reactors treating different substrates, were tested in batch EF reactors.

2. MATERIAL AND METHODS

2.1 Inocula

Five different types of inoculum were used to compare the EF process in batch reactors: **AnSlu:** Heat-treated anaerobic sludge sampled from a lab-scale anaerobic digester treating sewage sludge (37.7 $gVS.l^{-1}$); **ActSlu:** Activated sludge sampled from sewage treatment plant in Narbonne – France (10.0 $gVS.l^{-1}$); **AciSlu:** Acidogenic sludge sampled from lab-scale H_2-producing reactor fed with glucose (1.5 $gVS.l^{-1}$); **AnSlu2:** Heat-treated anaerobic sludge sampled from a lab-scale anaerobic digester treating food waste (7.1 $gVS.l^{-1}$); **AnSlu3:** Anaerobic sludge sampled from a lab-scale anaerobic digester treating volatile fatty acids (9.5 $gVS.l^{-1}$). **AnSlu** and **AnSlu2** were heat-treated at 90°C for 30 minutes using water bath before inoculation.

2.2 Fermentation medium

The fermentation medium was adapted from Rafrafi *et al*., (2013) and was composed of 5.0 $g.l^{-1}$ glucose and other nutrients as follows ($g.l^{-1}$): 2.0 NH_4Cl, 0.5 K_2HPO_4, 0.0086 $FeCl_2 \cdot 4H_2O$, 19.5 MES buffer (100 mM) and 1.0 $mL.l^{-1}$ oligoelements solution. The latter was composed as follows ($g.l^{-1}$): 60.0 $CaCl_2 \cdot 2H_2O$, 55.0 $MgCl_2 \cdot 6H_2O$, 7.0 $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 1.3 $CoSO_4 \cdot 7H_2O$, 1.2 $MnCl_2 \cdot 4H_2O$, 1.0 $ZnCl_2 \cdot 2H_2O$, 1.0 $Mo_7O_{24}(NH_4)_6 \cdot 4H_2O$, 0.4 $CuSO_4 \cdot 5H_2O$, 0.1 BO_3H_3, 0.05 $NiCl_2 \cdot 6H_2O$, 0.01 $Na_2SeO_3 \cdot 5H_2O$ and 46.0 $ml.l^{-1}$ HCl 37%.

2.3 Electro-fermentation systems and start-up

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

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

2.4 Analytical methods

Liquid samples were taken from both compartments of the reactor, *i.e.* working and counter 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 $mL.min^{-1}$. 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 FastDNA™ 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

2.6 Data analysis

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

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

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

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

Electro-fermentation efficiency (η_{EF}). This coefficient corresponds to the ratio between the number of electrons passing through the electrical circuit and the number of extra-

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

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

3. RESULTS AND DISCUSSION

3.1 Reactor performances during glucose electro-fermentation

Five different inocula were used to study the influence of the inoculum source on glucose EF. All EF experiments were compared to conventional fermentation (F). At the end of operation, glucose was totally consumed in all reactors (5.3 ± 0.2 $gCOD.l^{-1}$). Total chemical oxygen demand (COD) mass balance calculated from soluble products and accumulated H_2, ranged between 72.5% and 87.7%, except for AnSlu2 (only 42.1±1.2%), where some of the metabolites were probably unknown. Here, some COD could have been destined to the production of extracellular polysaccharides, such as exopolysaccharides (EPS) [28]. However, despite the analyses performed, not all fermentation products were determined. Approximately 10-15% of the initial COD was attributed to the production of microbial biomass. Besides, no methane production was detected in any of the operated reactors.

When AnSlu was used, H_2 production was favoured 2.6 times in EF (1.80 ±0.31 $molH_2.mol^{-1}_{glucose}$) with respect to F (0.70±0.12 $molH_2.mol^{-1}_{glucose}$). In contrast, when ActSlu was used, H_2 production was disfavoured about 50% in EF (0.57±0.17 $molH_2.mol^{-1}_{glucose}$) compared to F (1.14±0.09 $molH_2.mol^{-1}_{glucose}$). Interestingly, no difference was

observed in H_2 production between EF and F when AciSlu, AnSlu2 and AnSlu3 were used as inoculum. The molar H_2 yields in these EF batches were 0.87 $molH_2.mol^{-1}_{glucose}$ (F= 0.90 $molH_2.mol^{-1}_{glucose}$), 0.48 $molH_2.mol^{-1}_{glucose}$ (F= 0.50 $molH_2.mol^{-1}_{glucose}$) and 0.73 $molH_2.mol^{-1}_{glucose}$ (F= 0.72 $molH_2.mol^{-1}_{glucose}$), respectively. Our results achieved H_2 yields between 24% and 90% of the theoretical maximum yield, considering butyrate as the only soluble metabolite [23]. These differences in H_2 yields show the importance of the microbial community selected at the end of the operation from inocula with different origins. This also seems to have an impact during EF process, because not all microbial communities responded in the same way. Interestingly, our results show that H_2 production could be increased, decreased or unaffected by the presence of polarized electrodes.

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

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

(24.5±1.6%$_{COD}$) and ethanol (20.2±2.2%$_{COD}$). In AnSlu3, the main metabolites were propionate (35.5±1.8%$_{COD}$) and ethanol (30.5±0.2%$_{COD}$).

Overall, the maximum H_2 yields were associated with an increase of butyrate (AnSlu-EF and ActSlu-F) and a decrease of lactate. This is consistent with data from literature which often reports higher H_2 yields when butyrate and acetate are the most important metabolites [6,30,31]. In addition, lactate production in H_2 production reactors with mixed cultures is mainly related to lactic acid bacteria. These compete for the substrate and reoxidize NADH through the non-H_2 producing pathway. As a result, the microbial community achieves lower H_2 yields [4,10]. Despite this, during this research no significant linear correlation was found between H_2 production and butyrate or lactate production (see Fig. 4). In this context, the ActSlu-EF, AciSlu-F and AciSlu-EF reactors showed low H_2 yields even with high butyrate production. As can be seen in Fig. 2, the inocula used in this research are quite different, as well as the species selected after EF. Clearly, these differences in the microbial communities, including members of dominant and sub-dominant families, affect the metabolic pathways used to release excess electrons and thereby determine final H_2 yields. In short, the metabolic products of any fermentation are highly influenced by the microbial species involved, as they interact with each other and with the polarized electrode [4,32].

The total electrical current that passed through the polarized electrodes during the operation 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 the inoculum AnSlu, ActSlu, AciSlu, AnSlu2 and AnSlu3, respectively. The EF efficiency coefficient (η_{EF}) was then calculated according to Moscoviz *et al*. (2016). When considering all the fermentation products, the η_{EF} was between 0.0007% and 0.033%.

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

3.2 Link between final bacterial communities and metabolic patterns

Microbial communities were analysed for each condition studied at the end of batch operation. Figure 2 shows the microbial community distribution, which is represented between 90.5 and 98.6% by the most abundant families, that is, with a relative abundance ≥10%. The low abundance families were grouped as "Others". In all reactors, most of the microbial diversity at the end of operation was represented by the families *Enterobacteriaceae*, *Streptococcaceae* and *Clostridiaceae*. In AnSlu, the *Streptococcaceae* family dominated in F (57.9±5.1%) while in EF, members of the *Enterobacteriaceae* (57.7±12.1%) and *Clostridiaceae* (36.5±5.5%) families were dominant. In ActSlu, the *Streptococcaceae* family was dominant in both F (42.1±3.2%) and EF (56.2±23.7%) although different metabolic patterns were observed. Despite the differences observed in the EF duplicates, lower relative abundances of *Enterobacteriaceae* (10.6±4.7%) and *Clostridiaceae* (23.7±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±5.6%, 95.3±0.3%
280 and 97.9±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_2 yields, high relative abundance of the *Clostridiaceae*
293 family and high butyrate accuulation. 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_2 yield
299 positively correlates with the abundance of *Clostridiaceae* and *Prevotellaceae* families.
300 Consistently, high H_2 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_2 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_2 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_2 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 ≥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±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±1.4% and 11.3±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±2.4%. Generally, these lactate-producing bacteria are not dominant in H_2-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±0.3%, 13.9±0.1% and 13.3±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_2 production [30,41]. Finally, organisms classified as
346 Bacteroidetes_unclassified were dominant in AnSlu3, representing 19.5±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_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 selectioned. 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 H_2 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 ≥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

electrodes was observed. However, members of this family were significantly present in AnSlu and ActSlu, but with a higher relative abundance in AnSlu. These inocula showed a positive response to the presence of polarized electrodes increasing the H_2 production. Interestingly, as previsouly reported in literature, some *Clostridium* species could transfer electrons from or to a polarized electrode, either directly or using electron mediators [47,48]. Choi *et al*., (2014) reported that a pure culture of *C. pasteurianum* received electrons directly from a polarized cathode with changes in the metabolic profiles, increasing the production of 1,3-propanediol and butanol from glycerol and glucose respectively. Choi *et al*., (2012) reported an increase in butyrate production from sucrose using a pure culture of *C. tyrobutyricum* in a fermentation medium containing the electron mediator methyl viologen. Kumar *et al*., (2017) also worked with an enriched microbial consortium for the production of bioelectricity in dual-chamber microbial fuel cells. *Clostridium* was the dominant genus in the reactor bulk and was responsible for fermentation as well as the transfer of electrons from the fermentation medium to the electrode mediated by ferredoxin. Finally, and more recently, an increase in H_2 and butyrate productions associated with the selection of H_2-producing bacteria, including *Clostridia* species, during glucose EF using mixed cultures was reported, supporting the observations of the present study [10].

3.5 Conclusion

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

439 abundance of H_2-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 mecanisms occuring 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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Figure legends

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

1.5-column image.

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

1.5-column image.

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

2-column image.

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

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.

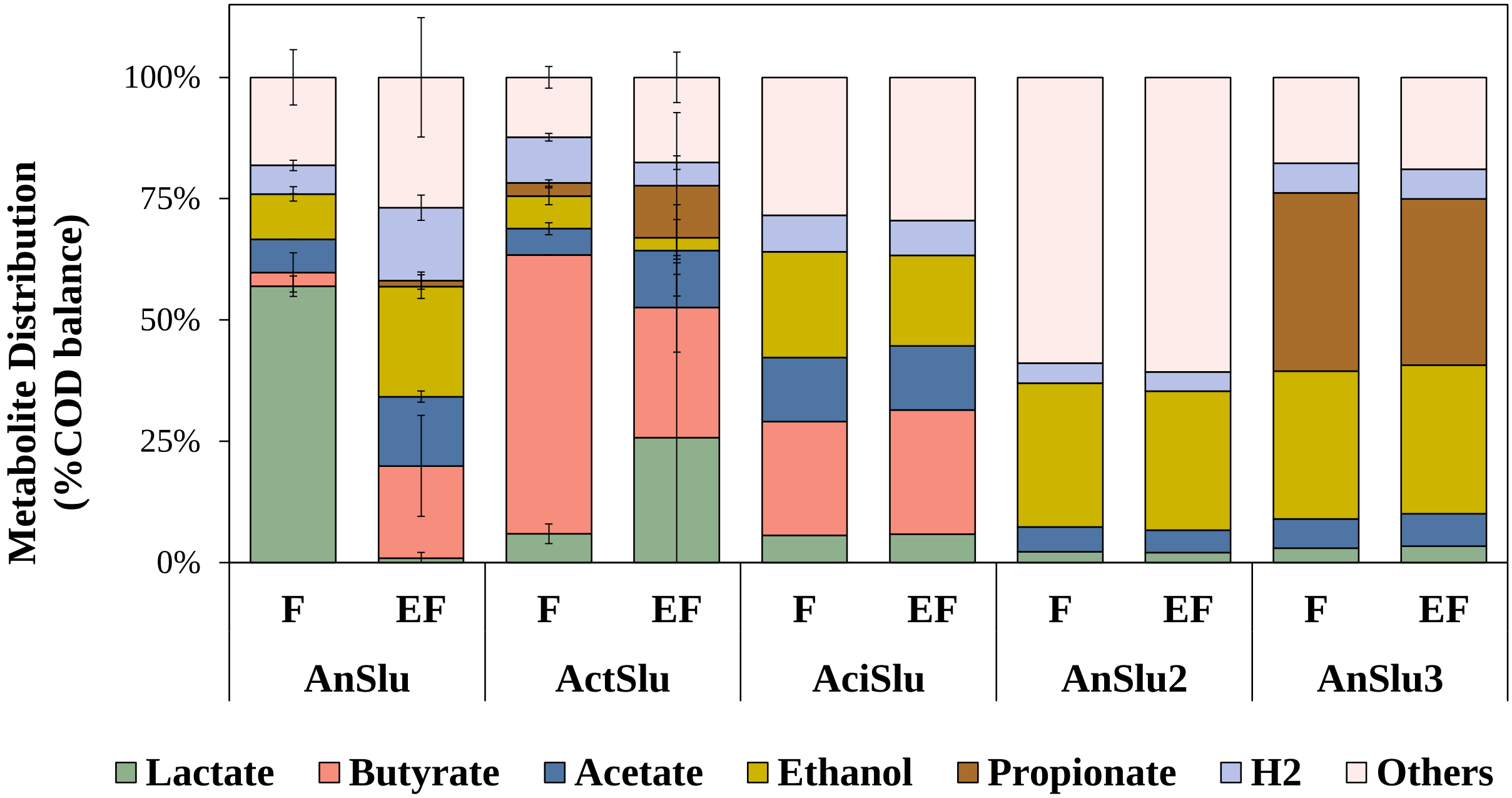
Family	E_N & E_0 (%)[b]		E_P & E_0 (%)[b]		E_N & E_P (%)[b]	
	Dissimilarity contrib.[c*]	Total[d]	Dissimilarity contrib.[c*]	Total[d]	Dissimilarity contrib.[c*]	Total[d]
Sporolactobacillaceae	22.3	22.3	23.4	23.4	0.0	0.0
Clostridiaceae	1.8	24.1	7.3	30.7	12.2	12.2
Saprospiraceae	6.3	30.4	0.0	30.7	10.7	22.9
Rhodocyclaceae	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
Clostridia_unclassified	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
Ruminococcaceae	4.2	53.1	4.1	51.5	0.5	42.2
Draconibacteriaceae	1.1	54.2	2.5	54.0	4.1	46.2
Planococcaceae	0.1	54.3	2.4	56.4	4.1	50.3
Bacteroidaceae	3.8	58.1	3.9	60.4	0.0	50.3
Christensenellaceae	0.1	58.2	1.8	62.2	3.0	53.3
Enterobacteriaceae	0.6	58.7	2.1	64.3	3.0	56.3
Peptostreptococcaceae	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
Acidimicrobiales_I.S.[a]	0.3	61.8	1.8	68.5	2.6	64.6
Rikenellaceae	2.3	64.1	2.6	71.1	1.2	65.9
Rhodospirillaceae	1.5	65.5	0.0	71.1	2.5	68.4
Porphyromonadaceae	2.4	68.0	2.3	73.4	1.2	69.6
Intrasporangiaceae	0.2	68.1	1.5	74.8	2.2	71.8
Desulfuromonadaceae	1.9	70.1	2.0	76.9	0.0	71.8
Xanthomonadales_I.S.[a]	1.2	71.3	0.0	76.9	2.0	73.8

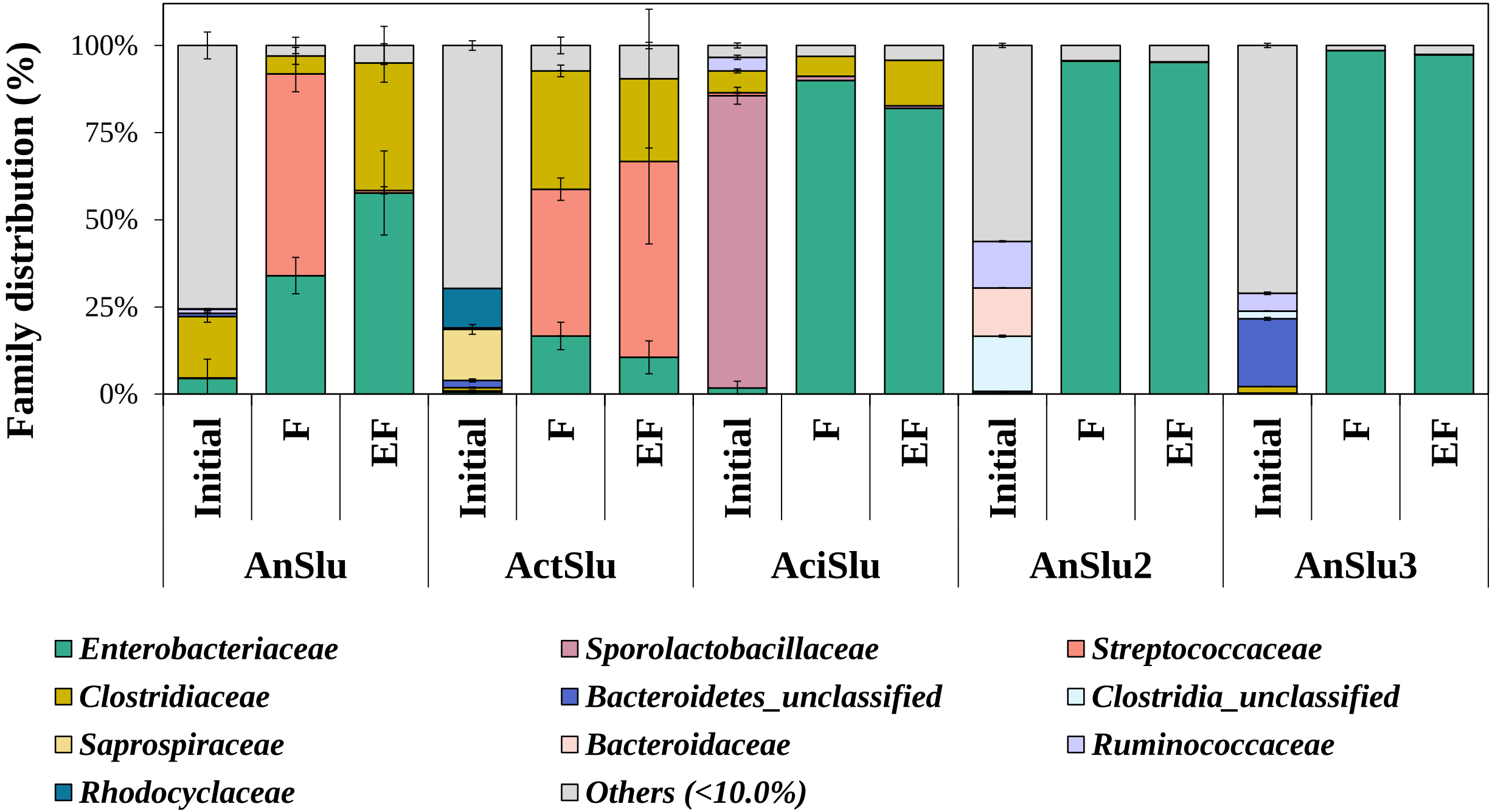
654 [*]Only families that contribute ≥ 2.0%, in at least one sample, to the dissimilarity are
655 included in the table.

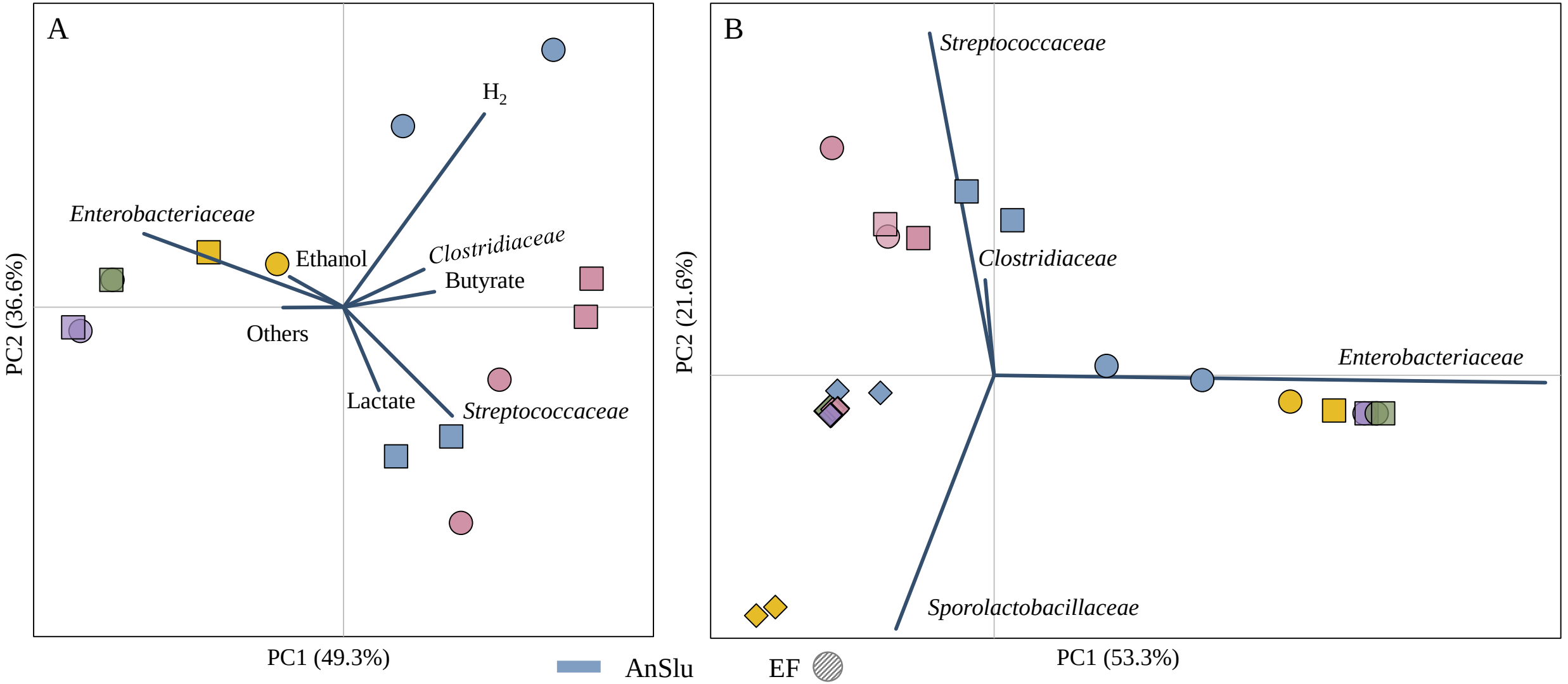
656 [a]I.S: abreviation of Incertae Sedis.

657 [b]E_N: negative effect on H_2 production; E_P: positive effect on H_2 production; E_0: neutral
658 effect on H_2 production.

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


Metabolite Distribution
(%COD balance)
100%
75%
50%
25%
0%
F
EF
AnSlu
ActSlu
AciSlu
AnSlu2
AnSlu3
Lactate
Butyrate
Acetate
Ethanol
Propionate
H2
Others


Family distribution (%)
100%
75%
50%
25%
0%
Initial
F
EF
AnSlu
ActSlu
AciSlu
AnSlu2
AnSlu3
Enterobacteriaceae
Clostridiaceae
Saprospiraceae
Rhodocyclaceae
Sporolactobacillaceae
Bacteroidetes_unclassified
Bacteroidaceae
Others (<10.0%)
Streptococcaceae
Clostridia_unclassified
Ruminococcaceae


A
H2
Enterobacteriaceae
Ethanol
Clostridiaceae
Butyrate
Others
Lactate
Streptococcaceae
PC2 (36.6%)
PC1 (49.3%)
B
Streptococcaceae
Clostridiaceae
Enterobacteriaceae
Sporolactobacillaceae
PC2 (21.6%)
PC1 (53.3%)
AnSlu
ActSlu
AciSlu
AnSlu2
AnSlu3
EF
F
Initial

	H_2Y	Succinate	Lactate	Ethanol	Acetate	Butyrate	Other+Biomass	Diversity	*Enterobacteriaceae*	*Streptococcaceae*	*Clostridiaceae*	*Prevotellaceae*
H_2Y		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***
Enterobacteriaceae	-0.14	**0.66***	-0.48	**0.95****	-0.05	**-0.62***	**0.56***	**-0.86****		**-0.87****	**-0.60***	-0.43
Streptococcaceae	-0.26	**-0.77****	**0.79****	**-0.91****	-0.27	0.30	-0.44	**0.59***	**-0.87****		0.14	0.03
Clostridiaceae	**0.74****	-0.05	-0.31	-0.44	**0.54***	**0.76****	-0.45	**0.80****	**-0.60***	0.14		**0.77****
Prevotellaceae	**0.67****	-0.14	-0.30	-0.25	0.42	**0.56***	-0.38	**0.57***	-0.43	0.03	**0.77****	