Glucose electro-fermentation as main driver for efficient H2-producing bacteria selection in mixed cultures

Javiera Belen Toledo-Alarcon, Roman Moscoviz, Eric Trably, Nicolas Bernet

Glucose electro-fermentation as main driver for efficient H_2-producing bacteria selection in mixed cultures

Javiera Toledo-Alarcón[a], Roman Moscoviz[a], Eric Trably[a,*], Nicolas Bernet[a]

[a] LBE, Univ Montpellier, INRA, 102 Avenue des étangs, 11100 Narbonne, France

*corresponding author: *eric.trably@inra.fr*

Abstract

Electro-fermentation has been recently proposed as a new operational mode of bioprocess control using polarized electrodes. This paper aims to evaluate how polarized electrodes are affecting microbial metabolic fermentative pathways, with a special focus on how the bacterial populations are affected during hydrogen production by dark fermentation. Four different potentials were applied on the working electrode in batch electro-fermentation tests operated with mixed culture and using glucose as a substrate. Two different metabolic behaviours for H_2 production were observed in electro-fermentation. The first one led to a higher H_2 production compared to conventional fermentation with a strong selection of *Clostridium sp.* The second behaviour led to lower H_2 production along with ethanol, and strongly correlated with the selection of *Escherichia* and *Enterobacter* genera. However, the effect of the applied potential on population selection was mostly non-linear and no simple relationship was found between these two parameters. Overall, electro-fermentation process has shown its potential as a new type of control for mixed-culture bioprocesses with significant effects of polarized electrodes on glucose fermentation.

Keywords: Biohydrogen; Bio-Electrochemical System (BES); Microbial consortium; Dark fermentation; Oxidation Reduction Potential (ORP).

1. INTRODUCTION

Nature is governed by different microbial communities fulfilling key roles in nutrient recycling and organic matter decomposition. These communities are considered as fundamental in the perfect balance of terrestrial, aquatic and aerial ecosystems. Many of these communities have been extensively studied, revealing how different microbial populations can coexist through complex interactions and efficient cooperative relationships. These microbial interactions make mixed cultures more attractive than pure cultures for biotechnological purposes. In particular, mixed culture-based bioprocesses are generally more robust, overcoming sudden environmental changes and carrying out more complex activities. Additionally, from an economic point of view, maintaining costly sterile conditions in a fermentation process is not fully necessary [1]. In this context, mixed cultures have been widely used for H_2 production by dark fermentation. However, many different microorganisms are able to grow in fermentation media and no selection pressure can be directly applied for selecting bacteria carrying efficient H_2-producing pathways, such as *Clostridium* sp. Inoculum pre-treatment is thus generally required to prevent methanogenic activity during dark fermentation. Heat shock is the most applied pre-treatment, aiming to eliminate the non-spore-forming microorganisms (*e.g.* methanogenic archaea), and favor species from *Clostridium* genus, well known as the most efficient H_2 producers [2], [3]. Another strategy when H_2 is produced in continuous mode consists in selecting species according to their growth rate by fixing a short hydraulic retention time (HRT). Usually the HRT is set at values less than 24 h and, consequently, methanogenic

45 archaea are washed out from the reactor since they need more time to grow. pH is another
46 major operational parameter for microbial selection and, according to the literature, low pH
47 along with short HRT is enough to obtain an efficiently H_2-producing microbial
48 community, so-called biokinetic control [4], [5]. Recently, Silva-Illanes et al. (2017)
49 reported that an optimal microbial community for H_2 production can be obtained using pH
50 5.5 and 12h of HRT using glycerol as substrate. Additionally, these authors showed that pH
51 variations generate important changes in the microbial community, particularly in dominant
52 species, while different HRT values affected mainly the subdominant species [6]. To date,
53 lot of efforts have been made on optimizing the different operating parameters, including:
54 carbon sources, macro-micro nutrients, temperature, pH, HRT, organic loading rates, H_2
55 partial pressure [7]–[9]. However, only few controllers (pH, OLR, HRT) are available to
56 maintain stable the dark fermentation process, *i.e.* metabolic patterns and H_2 performances
57 [10], [11].

58 Electro-fermentation (EF) has recently been proposed as a new type of bioprocess control
59 in presence of polarized electrodes. Depending on the applied potential, EF can occur at the
60 anode or at the cathode, acting either as electron sink (*i.e.* passing the electrons excess from
61 fermentation medium towards circuit electric) or additional energy source (*i.e.* passing
62 electrons towards fermentation medium from the circuit), respectively [11], [12]. EF is a
63 kind of bioelectrochemical system (BES), where a high current density is not necessary to
64 have a strong effect on cellular metabolism, and only a small amount of electrons has a
65 significant impact on the metabolic patterns [10]–[13]. Indeed, EF relies on the

66 modification of cellular metabolism with a low amount of electrons. Thus, the main source
67 of electrons to generate the expected product comes from the organic substrate, as found in
68 conventional fermentation process [12]. That differentiates EF from microbial
69 electrosynthesis (MET) where the main source of electrons is the electrode. As an
70 illustration, EF could be considered as a control tool of H_2-producing dark fermentation
71 process, while H_2 is chemically produced at the cathode in microbial electrolysis cells [14].

72 The action mechanisms in EF are still not completely known. Moscoviz et al. (2016)
73 proposed three hypothetical mechanisms: the first one considers a direct conversion of the
74 substrate to the product where the electrodes act as an unlimited source or sink of electrons,
75 depending on the working potential. The second one considers a modification in the
76 oxidation-reduction potential through (i) a partial dissipation of the electrons in excess (*i.e.*
77 towards polarized electrode) produced by fermentation or (ii) a small addition of extra
78 electrons (*i.e.* from polarized electrode) to the fermentation medium. In both cases, a
79 change in $NADH/NAD^+$ ratio is promoted, that contributes to metabolic regulation of many
80 important cellular functions including genetic expression and enzymatic synthesis [11],
81 [15], [16]. The third mechanism considers the syntrophic interaction between fermentative
82 (*e.g. Clostridia* species) and electroactive bacteria (*e.g. Geobacter* species) according to
83 interspecies electron transfer mechanisms [17]. To date, mixed cultures EF has only been
84 tested on glycerol with the aim of increasing 1,3-propanediol production [18]–[21].
85 Moscoviz et al. (2017) showed that changes in the metabolic pathways highly correlated
86 with a microbial community selection due to the presence of polarized electrodes. Our

87 research aims to use polarized electrodes in glucose fermentation to evaluate their influence
88 in the fermentation medium on metabolic pathways and H_2 production, with a special focus
89 on how the bacterial populations are affected.

90

91 ## 2. MATERIALS AND METHODS

92 ### 2.1 Inoculum and fermentation medium

93 The inoculum corresponded to an anaerobic sludge sampled from a lab-scale anaerobic
94 digester treating sewage sludge (37.7 $g_{VS}.L^{-1}$). The sludge was heat-treated at 90°C for 30
95 minutes using water bath before inoculation at a ratio of $S_{substrate}/X_{biomass}$ ($g_{glucose}/g_{VS}$) = 10.

96 The fermentation medium was composed of 4.9 ± 0.2 $g.L^{-1}$ glucose and other nutrients as
97 follows: 2.0 $g.L^{-1}$ NH_4Cl, 0.5 $g.L^{-1}$ K_2HPO_4, 8.6 $mg.L^{-1}$ $FeCl_2{\cdot}4H_2O$, 1.0 $mL.L^{-1}$
98 oligoelements solution (46.0 $ml.L^{-1}$ HCl 37%, 60.0 $g.L^{-1}$ $CaCl_2{\cdot}2H_2O$, 55.0 $g.L^{-1}$
99 $MgCl_2{\cdot}6H_2O$, 7.0 $g.L^{-1}$ $FeSO_4(NH_4)_2SO_4{\cdot}6H_2O$, 1.3 $g.L^{-1}$ $CoSO_4{\cdot}7H_2O$, 1.2 $g.L^{-1}$
100 $MnCl_2{\cdot}4H_2O$, 1.0 $g.L^{-1}$ $ZnCl_2{\cdot}2H_2O$, 1.0 $g.L^{-1}$ $Mo_7O_{24}(NH_4)_6{\cdot}4H_2O$, 0.4 $g.L^{-1}$
101 $CuSO_4{\cdot}5H_2O$, 0.1 $g.L^{-1}$ BO_3H_3, 0.05 $g.L^{-1}$ $NiCl_2{\cdot}6H_2O$ and 0.01 $g.L^{-1}$ $Na_2SeO_3{\cdot}5H_2O$) and
102 9.8 $g.L^{-1}$ MES buffer (50 mM). This medium was adapted from Rafrafi *et.al*., 2013 [22].

103 ### 2.2 Electro-fermentation systems and start-up

104 Batch EF experiences were performed using a double-chamber reactor with 0.5 L of
105 working volume and 0.5 L of headspace in each cell. A cation exchange membrane (FKE-
106 50, FuMA-Tech GmbH, Germany) was placed between the chambers and 90% platinum –

10% iridium grids with a size of 3.5 cm x 3.5 cm (Heraeus Deutschland GmbH & Co. KG, Hanau – Germany) were used as working and counter electrodes. This type of electrodes made of biologically inert material were used to limit microbial electrosynthesis. 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 to maintain a constant the applied potential at the working electrode. Values of applied potentials at working electrode were –0.9, –0.4, +0.4 and +0.9 V *vs* SCE. The Fig. 1 shown the schematic diagram of experimental start-up for the EF tests.

All batch experiments were carried out for 20 hours, at 37 °C using a bath water and 250 rpm. Initial pH was adjusted at 6.0 with 2 M NaOH. Fermentation medium and inoculum were added in the working electrode chamber, while in the counter electrode chamber only fermentation medium free of glucose was added. To remove oxygen traces, the reactor medium and headspace were bubbled with N_2 gas (purity ≥ 99.9%) for 30 minutes before experiments, using a commercial air stone.

Since the presence of unpolarised electrodes did not show any impact on the fermentation process (data not shown), control experiments, as conventional fermentation tests, were performed in a single-chamber reactor in absence of polarized electrodes and under similar operating conditions, *i.e.* 0.5L of working volume and 0.5 L of headspace, 37 °C, pH 6.0, 250 rpm and the fermentation medium with glucose.

The experiments were carried out in duplicates (–0.9V and –0.4V), triplicates (+0.4V and +0.9V) and quintuplicates (controls).

2.3 Analytical methods

Concentrations of glucose, alcohols and organic acids were measured by HPLC with a refractive index detector (Waters R410). Samples were first centrifuged at 12,000g for 15 min and then supernatants were filtered with 0.2 µm syringe filters. HPLC analysis were performed on an Aminex HPX-87H, 300 x 7.8 mm (Bio-Rad) column at a temperature of 35°C and a flow rate of 0.4 $mL.min^{-1}$. H_2SO_4 (4 mM) was used as mobile phase. Biogas production was measured during all the operation time using a liquid displaced system. Biogas composition (CO_2, H_2, O_2, N_2 and CH_4) was quantified by gas chromatography (GC Clarus 580, Perkin Elmer) equipped with a thermal conductivity detector (TCD) [23].

2.4 Microbial community analysis

DNA was extracted with the QIAamp fast DNA stool mini kit in accordance with manufacturer's instructions (Qiagen, Hilden, Germany). Extractions were confirmed using Infinite 200 PRO NanoQuant (Tecan Group Ltd., Männedorf, Switzerland). The V3-4 region of the *16S rRNA* gene was amplified according to Carmona-Martinez et al. 2015 [24]. The community composition was evaluated using the MiSeq v3 chemistry (Illumina) with 2x300 bp paired-end reads at the GenoToul platform (http://www.genotoul.fr). Sequences were retrieved after demultiplexing, cleaning, clustering (97 %) and affiliating sequences using Mothur [25]. Sequences have been submitted to GenBank with accession No. KX632761-KX632946.

2.5 Data analysis

Pearson correlation matrix. Two Pearson correlation matrix were built from: (i) the metabolite profiles including H_2 yields, after 20 hours of fermentation and the microbial community compositions at the family level, using all reactors (control and EF) and (ii) abiotic parameters (applied potential and ηEF) and microbial community composition, using only the EF reactors. Correlation and significance calculations were assessed with PASW Statistics 18 (www.spss.com.hk).

Principal component analysis (PCA). A PCA was performed using microbial community composition at the family level. COD mass balance and H_2 yields were used to find correlations with the principal components of the PCA. All replicated data issued from the EF reactors were used. Meanwhile only two replicates of the control reactors (n=5) were used since the microbial community structure in all controls did not show significant differences among them. The PCA was made using PASW Statistics 18 (www.spss.com.hk).

3. RESULTS AND DISCUSSION

3.1 Increase in H_2 production and changes in metabolite distribution during glucose electro-fermentation

To evaluate the effect of polarized electrodes, four different values of applied potential at working electrode were set up during glucose EF. After 20 hours of batch operation, the

glucose was totally consumed in all conditions (5.2±0.2 $g_{COD}.l^{-1}$). Total COD mass balance, measured as soluble products and H_2 gas, was between 73.2 and 80.6%. Approximately 10-15% of missing COD was likely corresponding to biomass growth. Whatever the condition, no methane was detected in headspace.

First, a low H_2 yield of 0.74±0.09 $mol_{H2}.mol^{-1}_{glucose}$ was observed in the control (conventional fermentation). H_2 production was significantly enhanced by a factor between 1.8 and 2.5 in presence of the polarized electrodes. The highest H_2-yield was 1.81±0.32 $mol_{H2}.mol^{-1}_{glucose}$ and was reached at the applied potentials of –0.4V and +0.9V. Lower H_2 yields were observed at –0.9V and +0.4V, *i.e.* 1.49±0.06 and 1.34±0.12 $mol_{H2}.mol^{-1}_{glucose}$, respectively. The H_2 yields were statistically different only between the control and the EF experiments (ANOVA: $F=20.68$, $P<0.0001$), but not between EF tests. This result suggests that placing a polarized electrode, whatever the applied potential, in the fermentation medium is enough to observe a clear effect on H_2 production. The maximum H_2 yield obtained in this study is comparable to reported for glucose dark fermentation in batch operation using mixed cultures as inoculum (2.5 $mol_{H2}.mol^{-1}_{glucose}$) [26]. However, depending on inoculum source and the pre-treatment employed, the H_2 yields could be between 0.1 and 3.0 $mol_{H2}.mol^{-1}_{glucose}$ [9], [27]–[30].

Fig. 2 shows the metabolites distribution according to the COD mass balance. In the control, lactate was the main metabolite reaching to 66.9±4.9%$_{COD}$. Ethanol and acetate were also observed representing 14.0±2.5%$_{COD}$ and 9.5±1.0%$_{COD}$, respectively. In less

187 quantity butyrate and propionate were produced, representing 1.6±3.1%$_{COD}$ and
188 0.4±0.9%$_{COD}$, respectively.

189 In EFs tests, lactate production significantly decreased representing only between 1.1 –
190 32.1%$_{COD}$. Regardless of the applied potential, ethanol and acetate productions increased
191 representing between 24.7 – 38.6%$_{COD}$ and 14.0 – 18.5%$_{COD}$, respectively. Particularly, in –
192 0.4V and +0.9V experiments, butyrate production increased by 14.6 and 16.8 fold, when
193 compared to the control. While it represented about 24.6 and 29.3%$_{COD}$ at –0.4V and
194 +0.9V, respectively. As minority compounds, succinate production (not detected in the
195 control) also increased to a lesser extent, representing 1.0 – 6.9%$_{COD}$. Propionate was
196 produced at only very low concentrations (<1.6%$_{COD}$).

197 In general, a high lactate yield is consistent with low H_2 performances like in our control
198 tests. Because lactate is directly produced from pyruvate (not by the acetyl-coA pathway)
199 by consuming NADH, allowing the cell to quickly get rid of the excess in electrons through
200 NAD^+ regeneration without H_2 production [30]. Increasing H_2 yields were observed in EF
201 tests and correlated with ethanol and acetate accumulation. Acetate is a key molecule for H_2
202 production due to the high energy gain of this pathway through ATP production [9], [31].
203 Although ethanol is a more reduced molecule than glucose and its production allows to
204 release the excess of electrons by direct NAD^+ regeneration, ethanol is also associated with
205 H_2 production [12]. Finally, this study show the maximum H_2 was linked with the butyrate
206 production. This metabolite has been often associated with additional ATP production and
207 butyrate production was suggested as the most thermodynamically favourable reaction

208 during dark fermentative H_2 production [32]. As a consequence, butyrate production is
209 often related to high H_2 producing reactors [9].

210 **3.2 Microbial community analysis**

211 DNA samples were taken from inoculum and after 20 hours of batch operation to analyse
212 the effect of the polarized electrodes on the microbial communities. A total of 589
213 operational taxonomic units (OTUs) were found after MiSeq sequencing in all samples.
214 The dominant family found in the inoculum was *Clostridiaceae* representing 17.6±1.6% of
215 the microbial community (Fig. 3). Families with an abundance relative lower than 5.0%
216 represented 46.9±3.1% of the microbial community in the inoculum, evidencing a high
217 diversity (Simpson index of 0.955±0.003) at start of the experiments.

218 After batch operation, the Simpson diversity index decreased about 26.1 – 39.4% and only
219 three families (*Streptococcaceae, Enterobacteriaceae* and *Clostridiaceae)* dominated the
220 microbial community, representing about 93.8 – 97.8% of the total abundance (Fig. 3).
221 Only 8 OTUs showed a relative abundance higher than 1.0% in at least one sample (Table
222 1). In the control, *Streptococcaceae* (57.9±5.1%) and *Enterobacteriaceae* (34.0±5.2%)
223 families were the most representative families, and OTU2 and OTU1 were dominant with
224 57.7±5.2 and 28.9±4.9% of the total bacterial community, respectively. These two OTUs
225 were related to *Escherichia fergusonii* (99% 16S rRNA sequence similarity with OTU1)
226 and *Streptococcus equinus* (99% 16S rRNA sequence similarity with OTU2).

227 The *Clostridiaceae* family was mainly represented by OTU3 and was enriched at –0.4V
228 (35.7±4.2%) and +0.9V (38.1±11.2%). OTU3 had 99% of 16S rRNA sequence similarity

229 with *Clostridium butyricum.* The *Enterobacteriaceae* family was the most abundant in all
230 EF reactors, reaching 77.8±2.9, 57.7±12.0, 56.9±17.5 and 42.2±12.8% of total microbial
231 community at –0.9V, –0.4V, +0.4V and +0.9V, respectively. Two main OTUs represented
232 this family, *i.e.* OTU1 and OTU4. OTU1 was already described at the beginning of this
233 section and OTU4 had 99% of 16S rRNA sequence similarity with *Enterobacter cloacae*.

234 In all the samples, H_2-producing bacteria were selected, and their relative abundances
235 increased, and more specially members of the *Enterobacteriaceae* and *Clostridiaceae*
236 families. Despite the inoculum was heat-treated before reactor inoculation, none-spore
237 forming bacteria of the *Enterobacteriaceae* family such as OTU1 and OTU4 were selected
238 and dominated at the end of operation. Probably these species can survive due to limitations
239 in heat transfer depending on the system used for pre-treatment. Consistently, it has been
240 reported in literature that even methanogens can survive after some operation days after
241 heat shock pre-treatment, and the type of inoculum source plays a key role in its
242 effectiveness [26], [33].

243 **3.3 The three mains metabolic pathways for H_2 production in EF resulted from**
244 **microbial community selection**

245 To represent the relationships between microbial communities and reactor performances, a
246 principal component analysis (PCA) was performed. Fig. 4 shows the PCA based on
247 bacterial population in EF and control reactors evidencing the categorical differences
248 between using or not polarized electrodes. Three main H_2 production pathways correlated
249 well with the selection of specific microbial communities. In the first pathway (Fig. 4 on

250 the right side) observed in control reactors, *Streptococcaceae* family abundance was
251 correlated with high lactate production (R^2 = 0.92, P<0.01) and with a low H_2 production
252 (R^2 = –0.73, P<0.01). Details of the correlation matrix are presented as supplementary
253 materials (Fig S.1). *Streptococcus equinus* belongs to a known lactic acid bacteria group
254 [34], that was previously found in low H_2-producing reactors [30], [35]–[37]. Overall, lactic
255 acid bacteria act as a suppressor of H_2 production through substrate competition (*i.e.*
256 pyruvate) and produce lactate at the expense of H_2, resulting in lower yields or the release
257 of bactericides inhibiting the growth of H_2 producing bacteria [2], [34].

258 In the second pathway (Fig. 4 on the left upper side), as observed at –0.4V and +0.9V,
259 *Clostridiaceae* abundance correlated well with high H_2 yields (R^2 = 0.79, P<0.01) and high
260 butyrate production (R^2 = 0.94, P<0.01). Additionally, a negative correlation was evidenced
261 with lactate production (R^2 = –0.63, P<0.05). By selecting *Clostridium* species, up to 45%
262 of the theoretical H_2 – yield (4 $mol_{H2}.mol_{glucose}^{-1}$) was reached (See section 3.1) [9], [32],
263 along with an important increase in the butyrate production. In this case, OTU3 (related to
264 *Clostridium butyricum*) was greatly favoured when compared to the control. This species
265 was the most abundant in only one condition (+0.9V) but, at the family level, the
266 *Enterobacteriaceae* dominated in all other EF experiences. *Clostridium butyricum* is a well-
267 known efficient H_2 producer producing butyrate as main metabolite [9], [38], [39] which is
268 consistent with the present findings. More generally, species belonging to the *Clostridia*
269 genus are considered as efficient H_2 producers [30] and they have been found in most of the
270 mixed culture-based H_2 producing fermentative systems. [9].

A third pathway (Fig. 4 on the left bottom) was observed at –0.9V and +0.4V and was related to the *Enterobacteriaceae* abundance which positively correlated with succinate (R^2 = 0.86, P<0.01), ethanol (R^2 = 0.93, P<0.01) and acetate (R^2 = 0.71, P<0.05). The *Enterobacteriaceae* selection in EF tests led to a lower H_2 production than when the second pathway was promoted, but H_2 yields were still higher than in the control. In these reactors the OTU3 related to *Clostridium butyricum* was not favoured and the *Enterobacteriaceae* family was largely dominating. OTUs 1 and 4, related to *Escherichia fergusonii* and *Enterobacter cloacae*, respectively, were the main species and both belong to genera that have been widely used in pure cultures for H_2 production [40]–[46]. However, species from the *Enterobacteriaceae* family were already found during periods of poor H_2 production [47]. Consistently, our results show that this family is positively correlated with succinate and ethanol accumulation [30], [48].

3.4 Electro-fermentation patterns: low current is enough to trigger high changes in fermentation patterns

During EF, the total electric charges transferred from (*i.e.* negative sign) / to (*i.e.* positive sign) the electrodes were –615.4 ±378.6 C, –0.17 ±0.11 C, +2.85 ±1.75 C and +1.89 ±0.83 C at an applied potential of –0.9V, –0.4V, +0.4V and +0.9V, respectively. Experimental curves of the charge over time are detailed in supplementary material (Fig. S.2). Although the measured current was significant, the quantity of electrons at –0.9V represented only 1.9% of the total electrons, *i.e.* the electrons issued from glucose or from and the electric current. Current was close to zero in all other EF conditions (Table S.2). To differentiate EF

292 from other bioelectrochemical systems, an efficiency coefficient of EF (η_{EF}), analogous to
293 the coulombic efficiency in conventional BES but focusing on the targeted product, could
294 be estimated, as previously proposed by Moscoviz et al. (2016). The η_{EF} values range
295 between 0 and 1 in EF systems and values higher than 1 indicate the possible occurrence of
296 direct bio-electrosynthesis. When considering H_2 as targeted product, the η_{EF} value at –
297 0.9V was 0.16, meaning that the electric current could not directly contribute to more than
298 16.0% of the total accumulated H_2. Thus, hydrogen production mainly resulted from
299 glucose catabolism. In other EF conditions, the η_{EF} was <0.001, meaning that the electric
300 current did not significantly contribute to the whole metabolic reaction (<0.1% of H_2
301 production). Consequently, only a small amount of energy was sufficient to generate a great
302 impact, as evidenced by the low values of η_{EF} [12]. This is consistent with EF articles
303 already published, where current was not the main source of energy for microbial
304 metabolism [12], [49]. Overall, such low amount of energy could explain the relative
305 independence of the H_2 yields to the applied potential since electrons are not directly
306 converted to H_2 and polarized electrodes rather contribute to change the local environment
307 around the electrode leading to microbial selection and subsequently different H_2 yields.

308 **3.5 Hypothetical action mechanisms during glucose electro-fermentation**

309 To better understand the mechanisms that drive the EF process, Arunasri et al. (2016)
310 reported that the microbial community could be affected by exposure to different applied
311 potentials during the H_2 production in a single microbial electrolysis cell (MEC). These
312 authors showed that members of the *Firmicutes* phylum were favored by increasing the

313 applied potentials [50]. In contrast, in the present study, no significant linear relationship
314 between the applied potential and population selection was observed. The only OTU which
315 had an abundance that linearly and negatively correlated with the applied potential was
316 OTU 1 (*Escherichia fergusonii*) which was selected at low applied potential (R^2 = –0.71,
317 $P<0.05$, Table S.1). However, substantial changes in the microbial community were
318 triggered by small amounts of current that could not sustain an electrochemical H_2
319 production that would explain the difference between controls and EF conditions.

320 One hypothetical action in EF is related to the changes that could occur on the net charge of
321 the cell surface. Choi et al. (2014) reported that the zeta potential of *C. pasteurianum* DSM
322 525 cells grown with electricity was near to zero, whereas cells were electronegative in
323 open circuit [49]. The selection of OTU1 could have resulted from a change in the net
324 charge of the cell surface in response to the potential applied on the working electrode. This
325 would have likely caused physiological changes on growth rate and cell division and would
326 consequently disadvantage it in substrate competition while giving the opportunity to other
327 species to emerge [49], [51]. In addition, changes in cell surface net charge towards values
328 close to zero in microorganisms such as *Clostridium* could have made them more resistant
329 to bactericide produced by OTU 2 (related to *Streptococcus equinus*). As an illustration,
330 nisin is a known toxin released by lactic acid bacteria causing a depolarization of energized
331 bacterial membranes, especially affecting *Clostridia* species. This toxin stimulates the
332 formation of voltage-dependent multi-state pores when membrane potential is negative (–
333 80 to –100 mV). Through these pores, vital gradients equilibrate with the extracellular

medium and metabolites and salts can be lost causing cell deaths [52]–[55]. By modifying the cell surface net charge, the EF process could help sensitive bacterial species to counter this effect.

A second hypothetical mechanism is related to small changes in the extracellular redox potential caused by the polarized electrodes. That would affect the regulation of key membrane-bound enzymes involved in H_2 production (hydrogenases), due to their high sensitivity to redox potential variations [48], [50], [56]. However, such mechanisms are still difficult to clearly identify in mixed cultures. In all cases, interactions among species within microbial community are crucial to structure this community and the subsequent metabolism.

4. CONCLUSION

This article evidences a clear effect of polarized electrodes on both metabolic pathways and microbial community structure in dark fermentation. A strong correlation was observed between the microbial community selected and the metabolites produced including H_2. In all EF conditions, an increase in the H_2-yields was shown, independently on the applied potential. The effects on microbial community were mostly non-linear except for specific OTUs related to *Escherichia fergusonii* and *Streptococcus equinus*. Such interaction mechanisms between polarized electrodes and microbial community remain however still unclear but open new field of investigation in mixed cultures fermentation.

ACKNOWLEDGMENTS

We thank the Chilean National Commission for Scientific and Technology Research (CONICYT) for the award of a scholarship to J.T-A. RM was supported by the French National Research Agency (BIORARE Project: ANR-10-BTBR-02).

REFERENCES

[1] C.-H. Hung, Y.-T. Chang, and Y.-J. Chang, "Roles of microorganisms other than Clostridium and Enterobacter in anaerobic fermentative biohydrogen production systems--a review.," *Bioresour. Technol.*, vol. 102, no. 18, pp. 8437–44, Sep. 2011.

[2] M. A. Z. Bundhoo and R. Mohee, "Inhibition of dark fermentative bio-hydrogen production : A review," *Int. J. Hydrogen Energy*, vol. 41, pp. 6713–6733, 2016.

[3] J. Wang and W. Wan, "Factors influencing fermentative hydrogen production: A review," *Int. J. Hydrogen Energy*, vol. 34, no. 2, pp. 799–811, Jan. 2009.

[4] B. Si, J. Li, B. Li, Z. Zhu, R. Shen, Y. Zhang, and Z. Liu, "The role of hydraulic retention time on controlling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR)," *Int. J. Hydrogen Energy*, vol. 40, no. 35, pp. 11414–11421, 2015.

[5] J. E. Ramírez-Morales, E. Tapia-Venegas, J. Toledo-Alarcón, and G. Ruiz-Filippi, "Simultaneous production and separation of biohydrogen in mixed culture systems by continuous dark fermentation," *Water Sci. Technol.*, vol. 71, no. 9, pp. 1271–1285, 2015.

[6] F. Silva-Illanes, E. Tapia-venegas, M. C. Schiappacasse, E. Trably, and G. Ruiz-filippi, "Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol," *Energy*, 2017.

[7] G. Kumar, P. Bakonyi, T. Kobayashi, K. Q. Xu, P. Sivagurunathan, S. H. Kim, G.

377 Buitrón, N. Nemestóthy, and K. Bélafi-Bakó, “Enhancement of biofuel production
378 via microbial augmentation : The case of dark fermentative hydrogen,” *Renew.*
379 *Sustain. Energy Rev.*, vol. 57, pp. 879–891, 2016.

380 [8] K. Chandrasekhar, Y. Lee, and D.-W. Lee, “Biohydrogen Production : Strategies to
381 Improve Process Efficiency through Microbial Routes,” *Int. J. Mol. Sci.*, vol. 16, no.
382 4, pp. 8266–8293, 2015.

383 [9] A. Ghimire, L. Frunzo, F. Pirozzi, E. Trably, R. Escudie, P. N. L. Lens, and G.
384 Esposito, “A review on dark fermentative biohydrogen production from organic
385 biomass : Process parameters and use of by-products,” *Appl. Energy*, vol. 144, pp.
386 73–95, 2015.

387 [10] R. D. Hoelzle, B. Virdis, and D. J. Batstone, “Regulation mechanisms in mixed and
388 pure culture microbial fermentation.,” *Biotechnol. Bioeng.*, vol. 111, no. 11, pp.
389 2139–54, Nov. 2014.

390 [11] A. Schievano, T. P. Sciarria, K. Vanbroekhoven, H. De Wever, S. Puig, S. J.
391 Andersen, K. Rabaey, and D. Pant, “Electro-Fermentation - Merging
392 Electrochemistry with Fermentation in Industrial Applications,” *Trends Biotechnol.*,
393 vol. xx, pp. 1–13, 2016.

394 [12] R. Moscoviz, J. Toledo-Alarcón, E. Trably, and N. Bernet, “Electro-Fermentation:
395 How To Drive Fermentation Using Electrochemical Systems,” *Trends Biotechnol.*,
396 vol. 34, no. 11, pp. 856–865, 2016.

397 [13] K. Rabaey and R. A. Rozendal, “Microbial electrosynthesis - revisiting the electrical
398 route for microbial production.,” *Nat. Rev. Microbiol.*, vol. 8, no. 10, pp. 706–16,
399 Oct. 2010.

400 [14] P. Parkhey and P. Gupta, “Improvisations in structural features of microbial
401 electrolytic cell and process parameters of electrohydrogenesis for efficient
402 biohydrogen production: a review,” *Renew. Sustain. Energy Rev.*, vol. 69, no.
403 September 2016, pp. 1085–1099, 2017.

[15] C. G. Liu, C. Xue, Y. H. Lin, and F. W. Bai, “Redox potential control and applications in microaerobic and anaerobic fermentations.,” *Biotechnol. Adv.*, vol. 31, no. 2, pp. 257–65, 2013.

[16] J. C. Thrash and J. D. Coates, “Review : Direct and Indirect Electrical Stimulation of Microbial Metabolism,” *Environ. Sci. Technol.*, vol. 42, no. 11, pp. 3921–3931, 2008.

[17] A. Kouzuma, S. Kato, and K. Watanabe, “Microbial interspecies interactions : recent findings in syntrophic consortia,” *Front. Microbiol.*, vol. 6, no. May, pp. 1–8, 2015.

[18] M. Zhou, J. Chen, S. Freguia, K. Rabaey, and J. Keller, “Carbon and electron fluxes during the electricity driven 1,3-propanediol biosynthesis from glycerol.,” *Environ. Sci. Technol.*, vol. 47, no. 19, pp. 11199–205, Oct. 2013.

[19] M. Zhou, S. Freguia, P. G. Dennis, J. Keller, and K. Rabaey, “Development of bioelectrocatalytic activity stimulates mixed-culture reduction of glycerol in a bioelectrochemical system,” *Microb. Biotechnol.*, vol. 8, no. 3, pp. 483–489, 2015.

[20] P. G. Dennis, F. Harnisch, Y. K. Yeoh, G. W. Tyson, and K. Rabaey, “Dynamics of cathode-associated microbial communities and metabolite profiles in a glycerol-fed bioelectrochemical system.,” *Appl. Environ. Microbiol.*, vol. 79, no. 13, pp. 4008–14, Jul. 2013.

[21] R. Moscoviz, E. Trably, and N. Bernet, “Electro-fermentation triggering population selection in mixed-culture glycerol fermentation,” *Microb. Biotechnol.*, vol. 0, pp. 000–000, 2017.

[22] Y. Rafrafi, E. Trably, J. Hamelin, E. Latrille, I. Meynial-Salles, S. Benomar, M.-T. Giudici-Orticoni, and J.-P. Steyer, “Sub-dominant bacteria as keystone species in microbial communities producing bio-hydrogen,” *Int. J. Hydrogen Energy*, vol. 38, no. 12, pp. 4975–4985, Apr. 2013.

[23] R. Moscoviz, E. Trably, and N. Bernet, “Consistent 1,3-propanediol production from

glycerol in mixed culture fermentation over a wide range of pH," *Biotechnol. Biofuels*, vol. 9, no. 1, p. 32, 2016.

[24] A. A. Carmona-Martínez, E. Trably, K. Milferstedt, R. Lacroix, L. Etcheverry, and N. Bernet, "Long-term continuous production of H2 in a microbial electrolysis cell (MEC) treating saline wastewater," *Water Res.*, vol. 81, pp. 149–156, 2015.

[25] P. D. Schloss, S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber, "Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities," *Appl. Environ. Microbiol.*, vol. 75, no. 23, pp. 7537–7541, Dec. 2009.

[26] Y. M. Wong, T. Y. Wu, and J. C. Juan, "A review of sustainable hydrogen production using seed sludge via dark fermentation," *Renew. Sustain. Energy Rev.*, vol. 34, pp. 471–482, Jun. 2014.

[27] H. Liu, G. Wang, D. Zhu, and G. Pan, "Enrichment of the hydrogen-producing microbial community from marine intertidal sludge by different pretreatment methods," *Int. J. Hydrogen Energy*, vol. 34, no. 24, pp. 9696–9701, 2009.

[28] Y.-Y. Wang, P. Ai, C.-X. Hu, and Y.-L. Zhang, "Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose," *Int. J. Hydrogen Energy*, vol. 36, no. 1, pp. 390–396, Jan. 2011.

[29] J. L. Wang and W. Wan, "Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge," *Int. J. Hydrogen Energy*, vol. 33, no. 12, pp. 2934–2941, 2008.

[30] L. Cabrol, A. Marone, E. Tapia-Venegas, J. P. Steyer, G. Ruiz-Filippi, and E. Trably, "Microbial ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the ecosystem function," *FEMS Microbiol. Rev.*, vol. 41,

457 no. 2, pp. 158–181, 2017.

458 [31] S. K. Khanal, W. H. Chen, L. Li, and S. Sung, “Biological hydrogen production:
459 Effects of pH and intermediate products,” *Int. J. Hydrogen Energy*, vol. 29, no. 11,
460 pp. 1123–1131, 2004.

461 [32] H. Lee, M. B. Salerno, and B. E. Rittmann, “Thermodynamic Evaluation on H2
462 Production in Glucose Fermentation,” *Environ. Sci. Technol.*, vol. 42, no. 7, pp.
463 2401–2407, 2008.

464 [33] G. Kumar, G. Zhen, T. Kobayashi, P. Sivagurunathan, S. H. Kim, and K. Q. Xu,
465 “Impact of pH control and heat pre-treatment of seed inoculum in dark H2
466 fermentation: A feasibility report using mixed microalgae biomass as feedstock,” *Int.*
467 *J. Hydrogen Energy*, vol. 41, no. 7, pp. 4382–4392, 2016.

468 [34] J. Ferrer-Valenzuela, L. A. Pinuer, A. García-Cancino, and R. Bórquez-Yáñez,
469 “Metabolic Fluxes in Lactic Acid Bacteria — A Review,” *Food Biotechnol.*, vol. 29,
470 no. 2, pp. 185–217, 2016.

471 [35] C. Hung, C. Cheng, D. Guan, and S. Wang, “Interactions between Clostridium sp .
472 and other facultative anaerobes in a self-formed granular sludge hydrogen-producing
473 bioreactor,” *Int. J. Hydrogen Energy*, vol. 36, no. 14, pp. 8704–8711, 2010.

474 [36] Z. Song, Y. Dai, Q. Fan, X. Li, Y. Fan, and H. Hou, “Effects of pretreatment method
475 of natural bacteria source on microbial community and bio-hydrogen production by
476 dark fermentation,” *Int. J. Hydrogen Energy*, vol. 37, no. 7, pp. 5631–5636, 2012.

477 [37] C. Chu, S. Wu, Y. Wu, B. Sen, and C. Hung, “Phase holdups and microbial
478 community in high-rate fermentative hydrogen bioreactors,” *Int. J. Hydrogen*
479 *Energy*, vol. 36, no. 1, pp. 364–373, 2011.

480 [38] P. Sivagurunathan, B. Sen, and C. Lin, “Batch fermentative hydrogen production by
481 enriched mixed culture : Combination strategy and their microbial composition,” *J.*
482 *Biosci. Bioeng.*, vol. 117, no. 2, pp. 222–228, 2014.

[39] H. H. P. Fang, T. Zhang, and H. Liu, “Microbial diversity of a mesophilic hydrogen-producing sludge,” *Appl. mi*, vol. 58, pp. 112–118, 2002.

[40] O. Elsharnouby, H. Hafez, G. Nakhla, and M. H. El Naggar, “A critical literature review on biohydrogen production by pure cultures,” *Int. J. Hydrogen Energy*, vol. 38, no. 12, pp. 4945–4966, 2013.

[41] J. J. Seppala, J. A. Puhakka, O. Yli-Harja, M. T. Karp, and V. Santala, “Fermentative hydrogen production by Clostridium butyricum and Escherichia coli in pure and co-cultures,” *Int. J. Hydrogen Energy*, vol. 36, pp. 10701–10708, 2011.

[42] D. Ghosh and P. C. Hallenbeck, “Fermentative hydrogen yields from different sugars by batch cultures of metabolically engineered Escherichia coli DJT135,” *Int. J. Hydrogen Energy*, vol. 34, no. 19, pp. 7979–7982, Oct. 2009.

[43] L. M. Rosales-Colunga, E. Razo-Flores, and A. De León Rodríguez, “Fermentation of lactose and its constituent sugars by Escherichia coli WDHL: Impact on hydrogen production,” *Bioresour. Technol.*, vol. 111, pp. 180–184, May 2012.

[44] N. Khanna, S. M. Kotay, J. J. Gilbert, and D. Das, “Improvement of biohydrogen production by Enterobacter cloacae IIT-BT 08 under regulated pH,” *J. Biotechnol.*, vol. 152, no. 1–2, pp. 9–15, Mar. 2011.

[45] N. Kumar and D. Das, “Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08,” *Process Biochem.*, vol. 35, no. 6, pp. 589–593, Jan. 2000.

[46] K. Nath, A. Kumar, and D. Das, “Effect of some environmental parameters on fermentative hydrogen production by *Enterobacter cloacae* DM11,” *Can. J. Microbiol.*, vol. 52, no. 6, pp. 525–532, Jun. 2006.

[47] Y. Cho and T. Lee, “Variations of hydrogen production and microbial community with heavy metals during fermentative hydrogen production,” *J. Ind. Eng. Chem.*, vol. 17, no. 2, pp. 340–345, 2011.

[48] J. Mathews and G. Wang, “Metabolic pathway engineering for enhanced

509 biohydrogen production," *Int. J. Hydrogen Energy*, vol. 34, no. 17, pp. 7404–7416,
510 Sep. 2009.

511 [49] O. Choi, T. Kim, H. M. Woo, and Y. Um, "Electricity-driven metabolic shift through
512 direct electron uptake by electroactive heterotroph Clostridium pasteurianum," *Sci.*
513 *Rep.*, vol. 4, no. 6961, 2014.

514 [50] K. Arunasri, J. Annie Modestra, D. K. Yeruva, K. Vamshi Krishna, and S. Venkata
515 Mohan, "Polarized potential and electrode materials implication on electro-
516 fermentative di-hydrogen production: Microbial assemblages and hydrogenase gene
517 copy variation," *Bioresour. Technol.*, vol. 200, pp. 691–698, 2016.

518 [51] W. W. Wilson, M. M. Wade, S. C. Holman, and F. R. Champlin, "Status of methods
519 for assessing bacterial cell surface charge properties based on zeta potentil
520 measurements," *J. Microbiol. Methods*, vol. 43, pp. 153–164, 2001.

521 [52] T. Noike, H. Takabatake, O. Mizuno, and M. Ohba, "Inhibition of hydrogen
522 fermentation of organic wastes by lactic acid bacteria," *Int. J. Hydrogen Energy*, vol.
523 27, no. 11–12, pp. 1367–1371, 2002.

524 [53] A. Sikora, M. Błaszczyk, M. Jurkowski, and U. Zielenkiewicz, "Lactic Acid Bacteria
525 in Hydrogen-Producing Consortia: On Purpose or by Coincidence?," in *Lactic Acid*
526 *Bacteria - R & D for Food, Health and Livestock Purposes*, M. Kongo, Ed. Rijeka:
527 InTech, 2013.

528 [54] M. A. Riley and J. E. Wertz, "Bacteriocins: Evolution, Ecology, and Application,"
529 *Annu. Rev. Microbiol.*, vol. 56, no. 1, pp. 117–137, 2002.

530 [55] F. Schuller, R. Benz, and H. Sahl, "The peptide antibiotic subtilin acts by formation
531 of voltage-dependent multi-state pore in bacterial and artificial membanes," *Eur. J.*
532 *Biochem.*, vol. 186, pp. 181–186, 1989.

533 [56] D. Xing, N. Ren, and B. E. Rittmann, "Genetic diversity of hydrogen-producing
534 bacteria in an acidophilic ethanol-H2-coproducing system, analyzed using the [Fe]-

hydrogenase gene," *Appl. Environ. Microbiol.*, vol. 74, no. 4, pp. 1232–1239, 2008.

Figure legends

Fig. 1: Schematic diagram of experimental start-up for electro-fermentation tests

Fig. 2: Metabolite distribution based on COD mass balance in final samples of glucose electro-fermentation. Values were calculated based on total glucose consumed. Values represent the average from quintuplicates (Control), triplicates (+0.4V and +0.9V) or duplicates (–0.9V and –0.4V). Error bars represent the standard deviation of the data.

Fig. 3: Family distribution of the microbial communities found in final samples of glucose electro-fermentations and fermentation controls. Values represent the average of triplicates (+0.4V and +0.9V) or duplicates (Control, –0.9V and –0.4V). Error bars represent the standard deviation of the data.

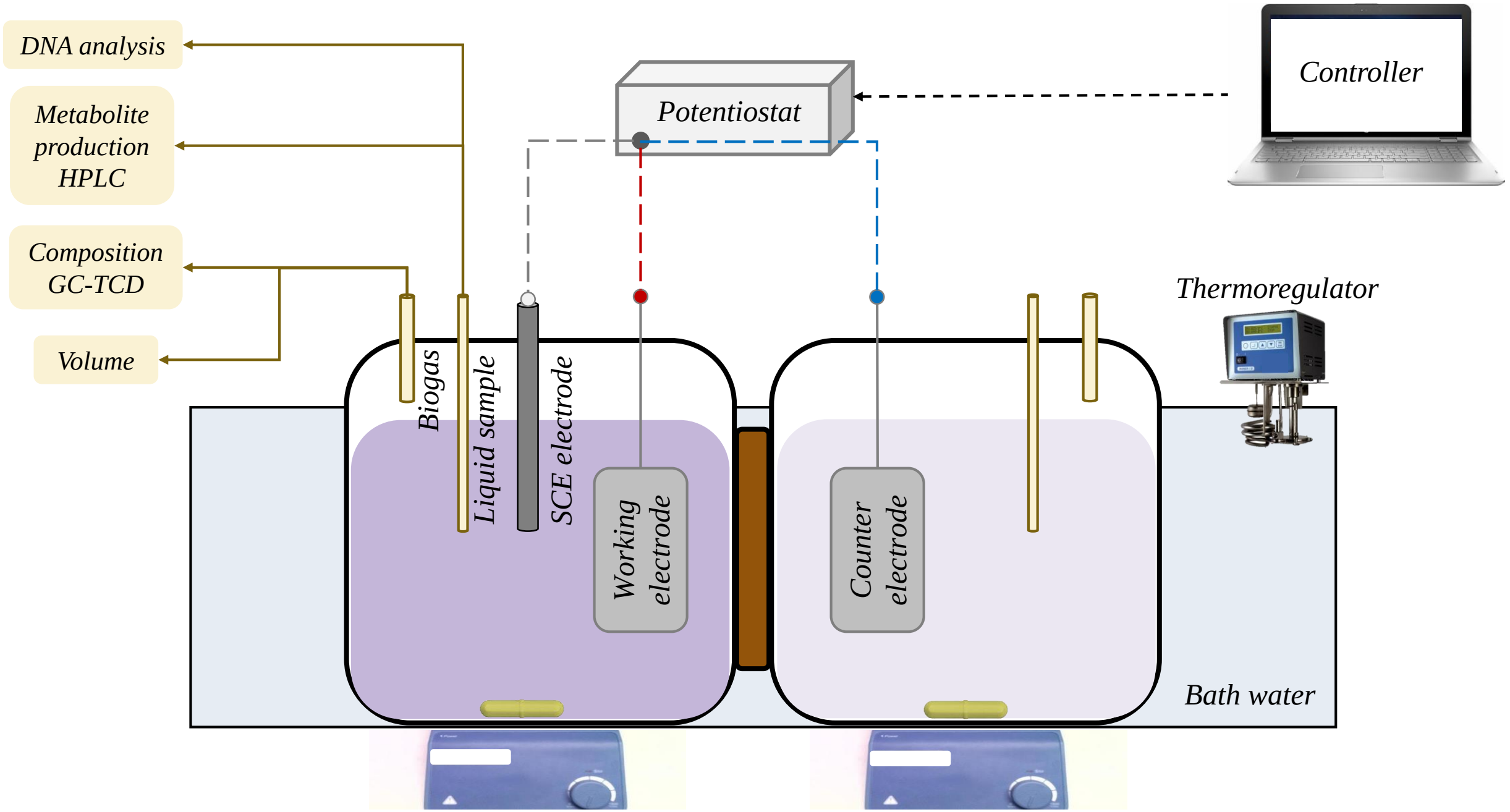
Fig. 4: Principal component analysis (PCA) based on microbial population distribution. Black plain lines and dotted lines represent correlations between PCA axes and taxonomic families and metabolic yields, respectively. Stars, squares, triangles, circles and diamonds represent control (n=2), –0.9V, +0.4V, –0.4V and +0.9V reactors, respectively.

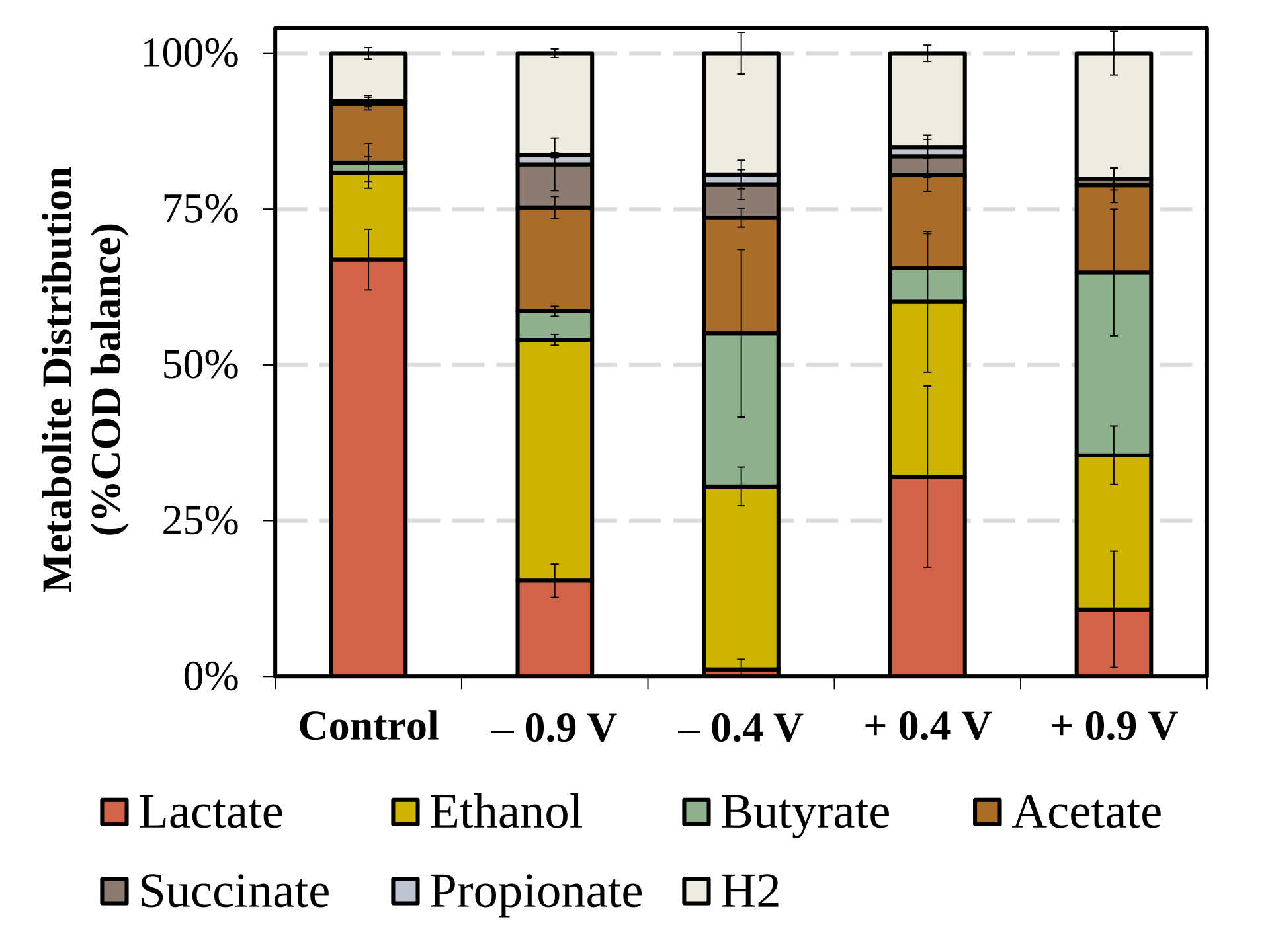
555 Table 1: Relative abundance (%) of microbial community after 20 hours of batch operation

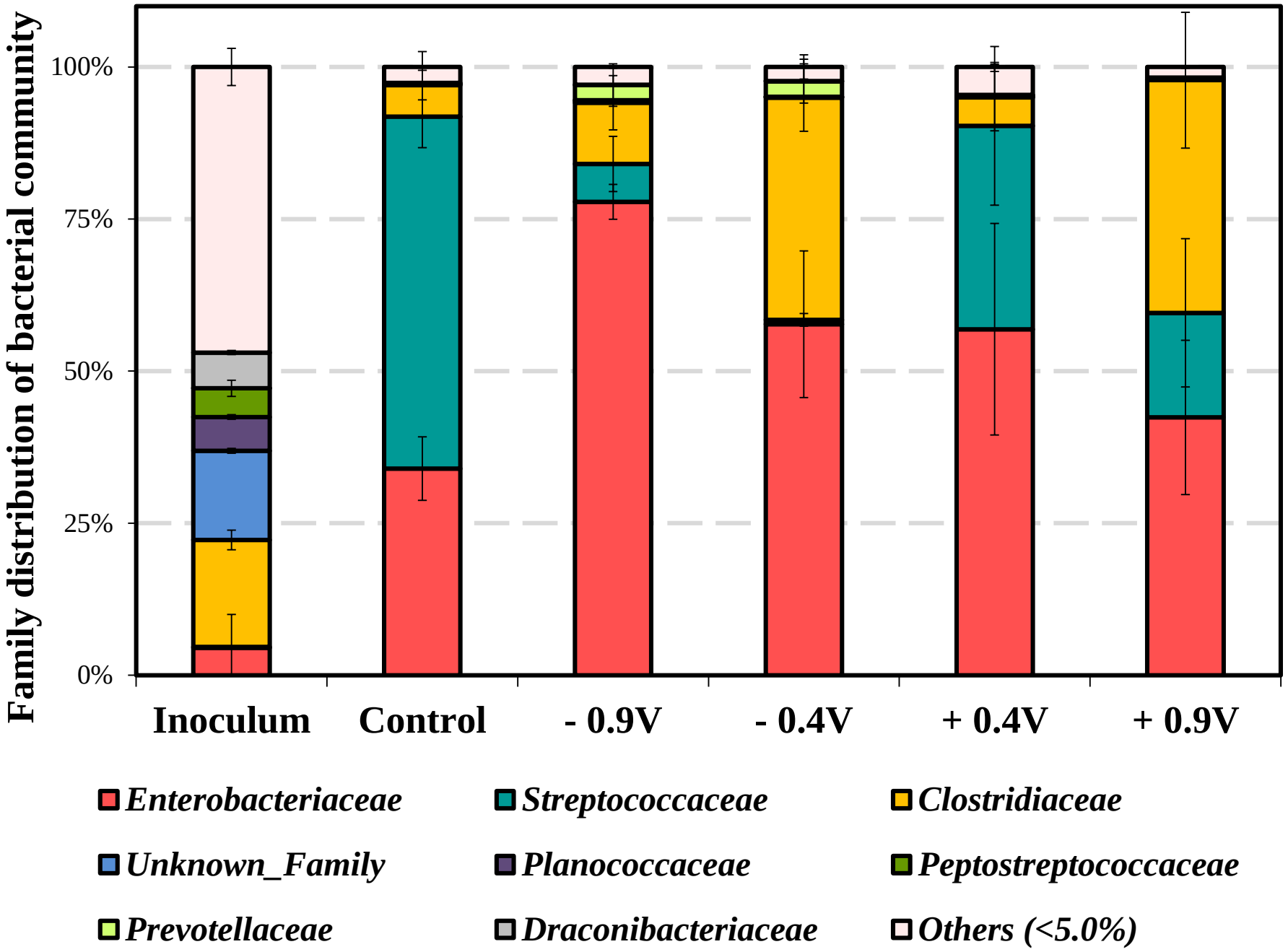
556 based on MiSeq sequencing of 16S rRNA gene

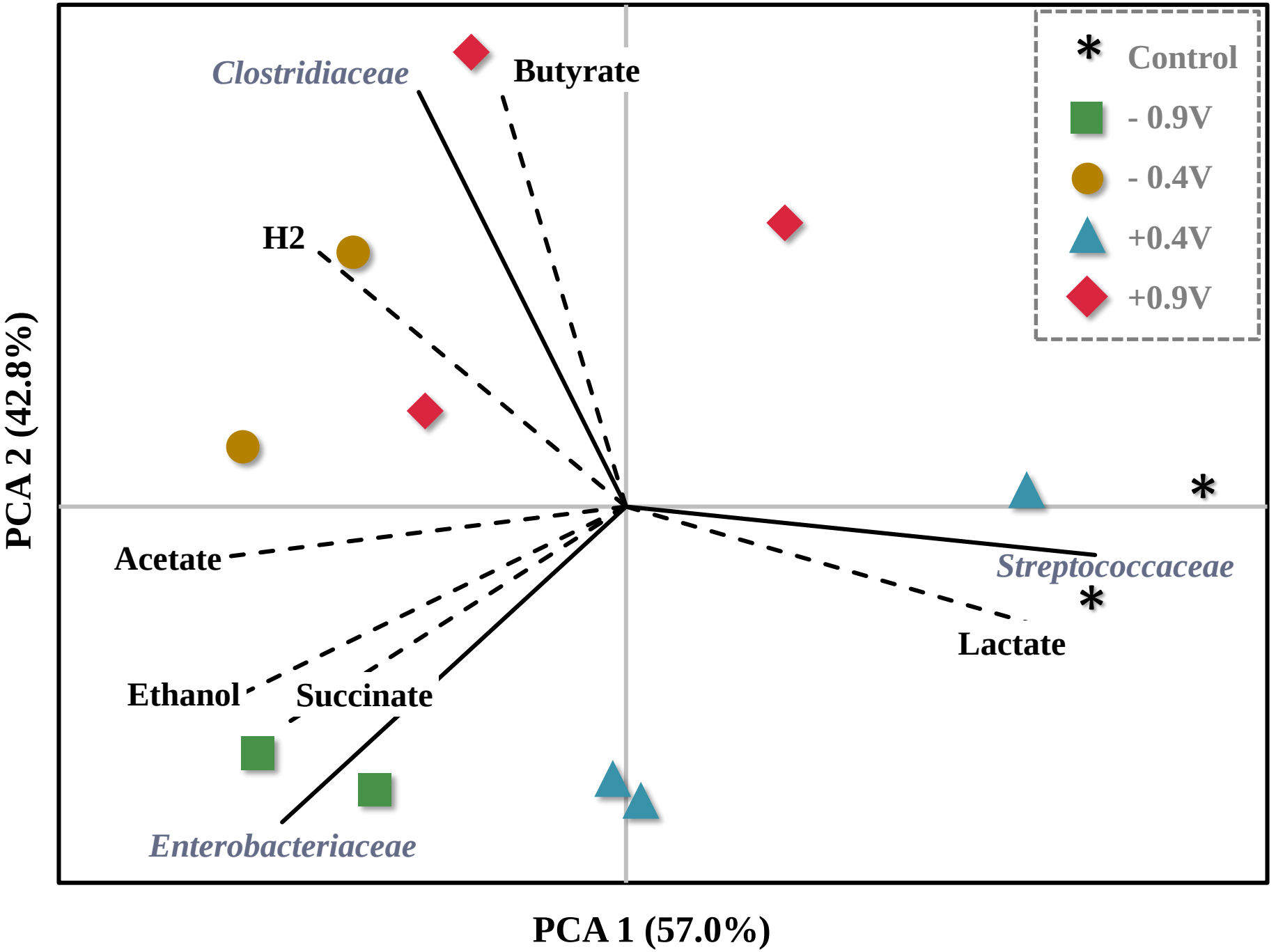
OTU	Putative identification (16S rRNA sequence similarity)	**Control**		**–0.9V**		**–0.4V**		**0.4V**			**0.9V**		
		n=1	*n=2*	*n=1*	*n=2*	*n=1*	*n=2*	*n=1*	*n=2*	*n=3*	*n=1*	*n=2*	*n=3*
Enterobacteriaceae													
1	*Escherichia fergusonii* (99)	25.4	32.3	70.8	32.4	64.7	45.7	40.6	18.6	29.2	38.5	9.6	15.0
4	*Enterobacter cloacae* (99)	4.9	5.3	9.1	43.4	1.5	3.5	26.6	48.1	7.5	18.4	27.5	17.9
Total[a]		*30.8*	*38.6*	*80.7*	*76.5*	*66.5*	*49.9*	*68.0*	*67.3*	*36.9*	*57.2*	*38.5*	*33.6*
Streptococcaceae													
2	*Streptococcus equinus* (99)	61.4	54.0	3.0	9.4	0.0	1.4	24.6	27.3	48.5	10.7	9.6	31.1
Clostridiaceae													
3	*Clostridium butyricum* (99)	6.7	2.3	10.8	6.4	29.8	37.8	1.8	0.3	10.6	29.9	49.3	33.3
7	*Clostridium intestinale* (97)	0.0	0.0	1.6	0.1	2.2	1.7	0.1	0.0	0.0	0.1	1.5	0.1
Enterococcaceae													
8	*Enterococcus casseliflavus* (100)	0.0	2.0	0.0	0.0	0.0	0.8	0.6	0.0	0.1	0.0	0.0	0.0
Total[a]		*68.5*	*60.3*	*16.9*	*16.6*	*32.8*	*43.5*	*30.1*	*27.8*	*59.6*	*41.0*	*60.7*	*65.2*
Corynebacteriaceae													
5	*Corynebacterium vitaeruminis* (99)	0.1	0.7	1.8	1.5	0.4	0.8	1.2	4.1	3.1	1.0	0.1	0.7
Total[a]		*0.2*	*0.8*	*2.0*	*1.7*	*0.5*	*0.9*	*1.4*	*4.2*	*3.2*	*1.2*	*0.2*	*0.8*
Prevotellaceae													
6	*Prevotella paludivivens* (99)	0.0	0.0	0.0	5.0	0.0	5.1	0.2	0.4	0.1	0.2	0.5	0.2
Total[a]		*0.3*	*0.2*	*0.2*	*5.1*	*0.1*	*5.7*	*0.4*	*0.6*	*0.3*	*0.5*	*0.5*	*0.3*
Others		0.2	0.2	0.3	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0

557 [a] including OTUs with <1.0% relative abundance.


DNA analysis
Metabolite production HPLC
Composition GC-TCD
Volume
Potentiostat
Controller
Thermoregulator
Biogas
Liquid sample
SCE electrode
Working electrode
Counter electrode
Bath water


Metabolite Distribution
(%COD balance)
100%
75%
50%
25%
0%
Control
– 0.9 V
– 0.4 V
+ 0.4 V
+ 0.9 V
Lactate
Ethanol
Butyrate
Acetate
Succinate
Propionate
H2


Family distribution of bacterial community
100%
75%
50%
25%
0%
Inoculum
Control
- 0.9V
- 0.4V
+ 0.4V
+ 0.9V
Enterobacteriaceae
Streptococcaceae
Clostridiaceae
Unknown_Family
Planococcaceae
Peptostreptococcaceae
Prevotellaceae
Draconibacteriaceae
Others (<5.0%)


Control
- 0.9V
- 0.4V
+0.4V
+0.9V
Clostridiaceae
Butyrate
H2
Acetate
Streptococcaceae
Lactate
Ethanol
Succinate
Enterobacteriaceae
PCA 2 (42.8%)
PCA 1 (57.0%)