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1 **Acetate accumulation enhances mixed culture fermentation of biomass to lactic acid**

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ABSTRACT

Lactic acid is a high-in-demand chemical, which can be produced through fermentation of lignocellulosic feedstock. However, fermentation of complex substrate produces a mixture of products at efficiencies too low to justify a production process. We hypothesized that the background acetic acid concentration plays a critical role in lactic acid yield, therefore its retention via selective extraction of lactic acid or its addition would improve overall lactic acid production and eliminate net production of acetic acid. To test this hypothesis, we added 10 g/L of acetate to fermentation broth to investigate its effect on products composition and concentration, and bacterial community evolution using several substrate-inoculum combinations. With rumen fluid inoculum, lactate concentrations increased by 80 ± 12 % (corn starch, $p < 0.05$) and 16.7 ± 0.4 % (extruded grass, $p < 0.05$) while with pure culture inoculum (*L. delbrueckii* and genetically modified (GM) *E. coli*), 4 to 23 % increase was observed. Using rumen fluid inoculum, the bacterial community was enriched within 8 days to >69 % lactic acid bacteria (LAB), predominantly *Lactobacillaceae*. Higher acetate concentration promoted a more diverse LAB population, especially on non-inoculated bottles. In subsequent tests, acetate was added in a semi-continuous percolation system with grass as substrate. These tests confirmed our findings producing lactate at concentrations 26 ± 5 % ($p < 0.05$) higher than the control reactor over 20 days operation. Overall, our work shows that recirculating acetate has the potential to boost lactic acid production from waste biomass to levels more attractive for application.

Keywords: lactic acid – acetate – lignocellulosic biomass – mixed culture.

37 **Introduction**

38 Lactic acid is a compound with versatile applications in many industries including food
39 production, chemistry, textile, pharmaceutics and cosmetics. The global lactic acid market is
40 forecasted to reach 367.3 kton by 2017, primarily due to the drive and demand from industry
41 and new applications (GIA 2012). During the past decades, interest has grown in polylactic
42 acid as a renewable and biodegradable plastic. However, due to the high cost of its precursor,
43 lactic acid, use of this polymer has been limited. Lactic acid is currently produced at industrial
44 scale through pure culture fermentation using filamentous fungi (e.g. *Rhizopus* spp.), bacteria
45 (e.g. *Bacillus coagulans*) or yeast, and using costly feedstock such as glucose (Taskila and
46 Ojamo 2013a). Lactic acid accumulation acidifies the fermentation broth, therefore much
47 research has been carried out to genetically engineer microorganisms that are more tolerant of
48 acids and low pH (below pH 4), but also utilize both pentose and hexose sugars, which are
49 commonly found in lignocellulosic biomass (Taskila and Ojamo 2013b). Depending on the
50 product application, mixed culture fermentation of lignocellulosic biomass can be an
51 attractive alternative since it would eliminate the need for sterilization and utilizes a cheaper
52 substrate.
53
54 Lactic acid bacteria (LAB) usually ferment glucose into lactic acid. During homolactic
55 fermentation two molecules of lactate is produced per molecule of glucose via the formation
56 of two molecules of pyruvate. During heterolactic fermentation, which typically occurs under
57 substrate limitation, one molecule of lactate is produced via pyruvate, while one molecule
58 each of ethanol and carbon dioxide are produced via Acetyl-CoA. At either low pH or high
59 substrate concentration bacteria will undergo the shorter pathway of homolactic fermentation,
60 to decrease demand for reducing power (Thomas et al. 1979). Many LAB are also able to
61 degrade lactic acid to acetate under anoxic conditions in the presence of alternative electron
62 acceptors or even under strict anaerobic conditions without supporting cell growth (Oude
63 Elferink et al. 2001). When oxygen is present, pyruvate may be converted directly to acetate
64 to benefit the cell (Quatravaux et al. 2006). Temudo et al. (2007) investigated open mixed
65 culture fermentation of glucose under different pH to direct specific product formation since
66 normally a mixture of formate, acetate, butyrate and ethanol is produced. Acetate is typically
67 the main side-product because production of acetate is energetically more favourable than
68 lactate production (Hunt et al. 2010). Typically acetate concentrations vary widely in mixed
69 culture fermentation (Elsden 1945; Wang et al. 2012). To completely eliminate acetate
70 production, research has been done to genetically modifying microorganisms such as *E. coli*,

71 however small amounts are still detected even when the gene for acetate production is
72 knocked out (De Mey et al. 2007). Bobillo and Marshall (1992) found that addition of salt (6
73 % NaCl) could inhibit acetate production in *Lactobacillus plantarum* at pH 4.5, without
74 inhibiting lactate production. It has also been demonstrated that acetate can inhibit bacterial
75 growth when present at high concentrations (>5 g/L) and low pH (<7) (Luli and Strohl 1990;
76 Roe et al. 2002; Russell 1992), depending on the bacteria strain and operating conditions. If
77 high but not toxic concentrations of acetate are present in open culture, it is possible that
78 acetate accumulation will direct the biochemical processes towards other reactions. This may
79 be a possible strategy to steer the bacterial community towards net lactate production. To
80 enable this, a process which is able to extract and separate lactic acid and acetic acid is
81 necessary.
82
83 Regarding the separation of lactic acid from acetic acid, numerous separation technologies
84 have been investigated including electrodialysis, ion exchange, extractive distillation.
85 Separation of lactic acid from acetic acid has also been demonstrated using a four zone
86 simulated moving bed process (Lee et al. 2004). Recently a membrane electrolysis approach
87 was developed that provides both specific extraction of unbranched fatty acids and pH control
88 of the fermentation broth without chemical dosing (Andersen et al. 2014). This approach
89 might be used to boost lactate production by separating acetate and lactate downstream of the
90 fermentation, and returning the acetate.
91
92 The objective of the current study was thus to investigate the effect of high acetate
93 concentration on the fermentation products (both batch and semi-continuous mode) and
94 bacterial community (batch mode) with the aim of driving the fermentation to lactic acid. A
95 target of 10 g/L acetate was chosen as a realistic value to reach considering typical production
96 values in mixed cultures (2.5 g/L acetate in the study of Wang et al. (2012)), and an extraction
97 system where lactate is removed and acetate is recycled back to the reactor. To compare the
98 performance between different substrates and inocula, fermentation of both a simple (corn
99 starch) and complex (extruded grass) substrate were tested using three different inocula: a
100 pure culture of *L. delbrueckii*, a genetically modified (GM) *E. coli* and a mixed microbial
101 community from rumen fluid.

102 **Materials and Methods**

103 **Substrate**

104 A readily fermentable substrate such as corn starch (total solid (TS) 86.77 ± 0.05 %, volatile
105 solid (VS, % of fresh mass) 86.35 ± 0.01 %, sold under the commercial name Basak) and a
106 more lignocellulosic and complex substrate such as extruded grass (TS 64.14 ± 0.04 % and
107 VS 60.35 ± 0.01 %) were used. Landscape grass was harvested on October 2012, kindly
108 provided by Inagro vzw (West Flanders, Belgium). To make the grass more accessible for
109 fermentation, it was extruded with a pilot scale twin-screw extruder (model MSZK,
110 Laborextruder 4 kW, Lehmann, Germany), provided by Bioliquid (Raalte, the Netherlands),
111 as performed by Khor et al. (2015). No further hydrolysis was performed. Substrates were
112 stored at 4 °C until used.

113

114 **Microorganisms and cultivation**

115 *Lactobacillus delbrueckii* LMG 6412 strain was obtained from the Belgian Co-ordinated
116 Collections of Micro-organisms (BCCM) and grown in 1 L 'de Man, Rogosa and Sharpe'
117 (MRS) medium at 37 ºC. GM *Escherichia coli* strain (3KO: *E. coli* K12 MG1655 δ (ackA-
118 pta) δ (poxB), where the carbon flow to acetate is directly reduced), was provided by Marjan
119 de Mey (InBio, Ghent University) and grown in 1 L Luria Bertani (LB) medium (De Mey et
120 al. 2007) at 37 ºC. Both strains were chosen as they have the ability to utilize both pure sugars
121 and more complex substrates (Dumbrepatil et al. 2007; Colunga and Antonio, 2014). Prior to
122 inoculation, the cells were washed with M9 medium and concentrated to 50 mL through
123 centrifugation (*1500 x g* for 5 min) (De Weirdt 2013). Optical density (OD_{610}) was 30.1 for *L.*
124 *delbrueckii* and 10.2 for GM *E. coli*. M9 medium was composed of 8.5 g/L Na_2HPO_4, 3.0 g/L
125 KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 0.24 g/L $MgSO_4$, and 0.011 g/L $CaCl_2$. Rumen fluid
126 (0.06 % bacterial protein) was provided by Institute for agricultural and fisheries research
127 (ILVO), Ghent University. It was sieved and stored in a thermoflask before use on the same
128 day.

129

130 **Fermentation tests**

131 *Batch mode*

132 Fermentations were carried out in 120 mL serum bottles for 8 days. In total, 20 different
133 conditions and corresponding negative controls without substrate, each of them in triplicate,
134 were performed, as shown in Table S1. For pure culture tests, each serum bottle contained 49
135 mL of M9 medium, 1 mL of concentrated bacteria (for *L. delbrueckii* and GM *E. coli)*, 5 g of

136 substrate (corn starch or extruded grass) and 0.03 g of sodium bicarbonate to mitigate the
137 stripping of CO_2 into headspace. For mixed culture tests, each serum bottle was filled with 40
138 mL of M9 medium and 10 mL of rumen fluid. For tests with acetate addition, 683.5 mg
139 sodium acetate (10 g/L acetate) was added. During the addition of substrate and inoculum,
140 bottles were sparged with nitrogen to ensure anaerobic condition. The bottles were then
141 flushed with nitrogen, except for a subset that were flushed with hydrogen. Gas samples were
142 taken from the headspace of each bottle immediately after flushing to confirm that oxygen
143 was removed. The pH was initially adjusted to either 5.5 for tests with rumen fluid and *L.*
144 *delbrueckii*, or 7 for tests with GM *E. coli*. The pH was not controlled throughout the
145 experiment, but was measured at the end. As controls, auto-fermentation of extruded grass
146 without inoculum addition was evaluated at pH 5.5 and pH 7, with and without acetate
147 addition. All bottles were placed on a shaker (130 rpm) at 30 °C for 8 days. Gas production
148 was monitored by means of pressure measurements, and liquid and gas samples were taken
149 and analysed on day 0, 1, 2, 4 and 8 for each replicate. Samples for bacterial community
150 analysis were taken on day 8.
151
152 *Semi-continuous mode*
153 Two vertical up-flow tubular acrylic glass reactors of 400 mL were packed with 30 g of
154 extruded grass each and inoculated with 60 mL of rumen fluid. They were run under
155 anaerobic condition for 50 days in a 20 °C room. M9 medium was circulated through the
156 reactors at a flow rate of 0.1 mL/min. Half of the reactor outlet was recycled back to the
157 reactor while the other half was purged as effluent (2 days of hydraulic retention time). For
158 the test reactor, concentrated sodium acetate (100 g/L sodium acetate) was added in the inlet
159 at a rate of 5 µL/min to replenish the loss of acetate in the effluent, achieving 10 g/L acetate in
160 the reactor, while for the control reactor there was no acetate addition. Half of the substrate
161 was replaced with fresh substrate every two days, the substrate was not mixed to ensure four
162 days solid retention time. The reactors were sparged continuously with nitrogen during
163 substrate replacement to ensure anaerobic environment. Sampling for chemical analysis was
164 performed before the substrate was replaced (every two days) and average concentrations and
165 standard deviations were calculated for all collected values.
166
167 **Bacterial community analysis**
168 *DNA extraction*

169 DNA extraction was performed using the FastPrep method described by Vilchez-Vargas et al.
170 (2013). Samples were taken of inoculum, substrate and end of fermentation broth for analysis.
171 Samples of 0.5 mL were centrifuged at 11 000 g for 5 min in a 2 mL Lysing Matrix E tube
172 (Qbiogene, Alexis Biochemicals, Carlsbad, CA). Cell pellets were re-suspended in 1 mL of
173 lysis buffer containing Tris/HCl (100 mM pH 8.0), 100 mM EDTA, 100 mM NaCl, 1 % (w/v)
174 polyvinylpyrrolidone and 2 % (w/v) sodium dodecyl sulphate. Cells were lysed in a Fast Prep-
175 96 homogenizer (40 s, 1600 rpm). Samples were then centrifuged at 18 000 g for 1 min at
176 room temperature and washed once with one volume phenol/chloroform (1:1) and the second
177 time with one volume chloroform. After centrifugation, nucleic acids (aqueous phase) were
178 precipitated with one volume of ice-cold isopropanol and 1:10 volume of 3 M sodium acetate.
179 After centrifugation and washing with 80 % ethanol, the pellet was re-suspended in 20 µL of
180 milliQ water. The quality and quantity of the DNA samples were analysed on 1 % agarose
181 gels.
182
183 *DNA sequencing and bioinformatics processing*
184 The V3–4 region of the bacterial 16S rRNA gene was sequenced by Illumina sequencing
185 Miseq and v3 Reagent kit (http://www.illumina.com/products/miseq-reagent-kit-v3.ilmn, by
186 LGC Genomics GmbH, Berlin, Germany) using 2 x 300 bp paired-end reads and primers
187 341F (5'-NNNNNNNNTCCTACGGGNGGCWGCAG) and 785R (5'-
188 NNNNNNNNTGACTACHVGGGTATCTAAKCC) described in Stewardson et al. (2015).
189 Each polymerase chain reaction (PCR) included approximately 5 ng of DNA extract and 15
190 pmol of each forward and reverse primer, in 20 µL volume of MyTaq buffer containing 1.5
191 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Prokopenko et
192 al.). For each sample, the forward and reverse primers had the same 8-nt barcode sequence.
193 PCRs included a pre-denaturation step of 2 min at 96 °C pre-denaturation step; followed by
194 30 cycles of the following: 96 °C for 15 s, 50 °C for 30 s, and 72 °C for 60 s. DNA
195 concentration of the amplicons of interest were determined by gel electrophoresis. About 20
196 ng of amplicon DNA from each sample were pooled for a total of 48 samples each carrying
197 different barcodes. PCRs showing low yields were further amplified for 5 cycles. The
198 amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove
199 primer dimer and other small mispriming products, followed by an additional purification on
200 MinElute columns (Qiagen). About 100 ng of each purified amplicon pool DNA was used to
201 construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN).

202 Illumina libraries were pooled and size selected by preparative gel electrophoresis.
203 Sequencing was done on an Illumina MiSeq using v3 Chemistry (Illumina).
204
205 Bioinformatics was conducted with 16S rRNA targeted metagenomics analysis (QIIME;
206 (Caporaso et al. 2010)). Data were pre-processed by first de-multiplexing of all samples using
207 Illumina's CASAVA data analysis software version 1.8.4. The reads were then sorted by
208 amplicon inline barcodes, no barcode mismatches were allowed. The barcode sequence was
209 clipped from the sequence after sorting and reads with missing barcodes, one-sided barcodes
210 or conflicting barcode pairs were discarded. Sequencing adapters in all reads were removed
211 and reads with final lengths below 100 nt were discarded (Clipping of Illumina TruSeq^{TM}
212 adapters in all reads). Combination of forward and reverse reads was done using BBMerge
213 34.30 (http://bbmap.sourceforge.net/). The sequence fragments were turned into forward-
214 reverse primer orientation after removing the primer sequences. FastQC report was creating
215 for every FASTQ files. 16S pre-processing and OTU picking from amplicons were performed
216 using Mothur v1.33 software package (Schloss et al. 2009). Sequences containing ambiguous
217 bases (Ns), with homo-polymer stretches of more than 8 bases or with an average Phred
218 quality score below 33 were removed. Reads were aligned against the 16S Silva reference
219 alignment release 102. Truncated and unspecific PCR products were removed. 15 000
220 sequences per sample were subsampled. Pre-clustering allowed up to 3 differing bases in a
221 cluster. Chimera were removed using uchime algorithm (Edgar et al. 2011). Taxonomical
222 classification of sequences and removal of non-bacterial sequences were done using the Silva
223 database. OTU were picked by clustering at the 97 % identity level using the cluster split
224 method.
225
226 The raw sequence dataset was deposited on the European Nucleotide Archive (ENA) of
227 European Bioinformatics Institute, with accession number LT006862-LT009376
228 (http://www.ebi.ac.uk/ena/data/view/LT006862-LT009376). Information regarding the OTUs
229 are included in the Supplementary Material – OTU table (xls format).
230
231 *Statistical analysis*
232 Representation of Principal Coordinate Analysis (PCoA) of Bray-Curtis dissimilarity indexes
233 was performed. The Vegan package in R (R version 2.13.2, http://www.r-project.org/) was
234 used to calculate Bray-Curtis dissimilarity matrices (vegdist function) and the data was
235 represented by PCoA function using the ape package.

236

237 **Analytical methods**

238 Determination of fermentation products including lactate, acetate, propionate and butyrate,
239 was performed with Dionex ion chromatography equipped with IonPac ICE-AS1 column
240 (Dionex) using 4 mM H_2SO_4 as eluent at a flow rate of 0.8 mL/min and an ED50 conductivity
241 detector. The gas phase composition was analysed with a compact gas chromatography
242 (Global Analyser Solutions, Breda, The Netherlands), equipped with a Molsieve 5A pre-
243 column and Porabond column (for CH_4, H_2 and N_2) and a Rt-Q-bond pre-column and column
244 (for CO_2). Nitrogen was used as carrier gas for H_2 analysis while helium was used for CH_4
245 and N_2. Concentrations of gases were determined by means of a thermal conductivity
246 detector. Bacterial protein of rumen fluid was analysed according to Makkar et al. (1982).
247 Optical density (OD_{610}) of pure culture inoculum was measured with a UV-VIS
248 spectrophotometer (ISIS 9000, Dr Lange, Germany) at 610 nm.

249 **Results**

250 **Impact of acetate addition on lactic acid concentration**

251 *Batch mode*

252 Fig. 1 depicts the results of fermentation tests with different inocula and substrates. When
253 acetate was not added (w/o acetate), it was still detected as an intermediate at the end of the
254 tests. However, in the test with acetate addition (w/ acetate), a smaller acetate production over
255 the initially supplied was obtained (the difference between initial and end point was smaller
256 than in w/o acetate tests) in all combinations. Using corn starch, there was no statistical
257 difference in terms of lactic acid production between tests with and without acetate addition
258 when pure cultures were used as inoculum. However, with rumen fluid as inoculum, the final
259 lactic acid concentration was 80 ± 12 % ($p < 0.05$) higher when acetate was added *vs.* control
260 bottles. In experiments with extruded grass, acetate addition increased the lactic acid
261 concentration after 8 days of fermentation by 16.7 ± 0.4 % ($p < 0.05$) with rumen fluid
262 inoculum, and by 4 to 23 % with either pure culture or genetically modified inoculum. There
263 was no statistical difference in lactate production for non-inoculated extruded grass tests at pH
264 5.5 ($p > 0.05$) with or without acetate addition (Table 1), due to . Although initially the pH
265 was adjusted to 5.5, the final pH of all bottles supplemented with acetate reached an average
266 of 4.4, while those without acetate addition reached 3.9, which roughly corresponds to the
267 pKa of the respective major species present in solution (pKa acetic acid 4.75: pKa lactic acid
268 3.8).
269
270 The highest final lactate concentration (14.7 ± 0.3 g/L) was achieved with rumen fluid
271 inoculum and extruded grass as substrate, coupled with 2.7 ± 0.1 g/L acetate production over
272 that supplied (Fig. 1; Table S2). Elevated acetate concentrations did not alter the spectrums of
273 other fermentation products such as butyrate and propionate (Figure S1). The initial rates of
274 lactate production did not differ much with acetate addition (Figure S2). No methane was
275 detected in all tests with nitrogen in the headspace.
276
277 *Semi-continuous mode*

278 A semi-continuous lactic acid fermentation of extruded grass gave results similar to those
279 observed in batch mode, where supplemental acetate resulted in a higher final lactate
280 concentration (Fig. 2). Reactors were started up allowed to acclimate during the first 30 days
281 after which operation continued until day 50. On average over the latter fermentation period
282 (20 days), the lactic acid concentration was 26 ± 5 % ($p < 0.05$) higher in the reactor with

283 acetate addition, compared to that of the control reactor. The spectrums of fermentation
284 products were similar to those observed in the batch fermentation tests, where they did not
285 differ with or without the addition of acetate. Also, no methane was detected during the
286 fermentation. Since the purpose of this test was to investigate the effect of elevated acetate
287 concentration, process optimization was not performed, hence the low conversion and
288 production rate. The average conversion achieved was 0.14 ± 0.01 g lactate/g VS extruded
289 grass fed for the control reactor and 0.17 ± 0.01 g lactate/g VS extruded grass fed for the test
290 reactor ($p < 0.05$). The average production rate was 2.0 ± 0.1 g/L.d for control reactor and 2.6
291 ± 0.2 g/L.d ($p < 0.05$) for test reactor. The operational conditions for the semi-continuous
292 reactors were defined from the batch test outcomes. A solids retention time (grass) of 4 days
293 was chosen since lactate concentration had reached a plateau after 4 days of fermentation in
294 batch tests (Figure S2). Propionate and butyrate profiles are presented in Figure S3.
295

296 **Effect of operational parameters on lactic acid production**

297 Additional batch tests were carried out to evaluate the effect of operational conditions on
298 lactate production, including headspace composition, pH, and inoculum choice. First,
299 hydrogen presence to create reductive conditions was tested. Corn starch was used as
300 substrate and rumen fluid as inoculum, with and without acetate addition, with either nitrogen
301 or hydrogen in the headspace (Table 2). Hydrogen addition did not enhance lactate production
302 but, even with a low working pH of 5.5, generated methane with and without acetate addition
303 (3.18 ± 0.12 mmol and 3.26 ± 0.36 mmol, respectively).
304

305 To evaluate pH and inocula, tests with extruded grass were performed with different inocula
306 and their respective optimal pH (5.5 or 7). Controls without inoculum addition at pH 5.5 and 7
307 were also included to evaluate the performance of the autochthonous bacteria in the grass
308 (Table 1). No differences in lactate (only about 4 g L^{-1}) were detected in the bottles without
309 inoculum at different pH. However, when acetate was supplied, lactate only increased about 1
310 g L^{-1} at pH 5.5 (not statistically significant) while it improved by nearly 5 g L^{-1} at pH 7, which
311 accounted for an increase of 111.1 ± 42.4 %. Thus, lactate titres were improved at higher pH,
312 either with or without inocula. In most cases, inoculated bottles reached higher lactic acid
313 concentration compared to the non-inoculated ones. When acetate was supplied, lactate
314 production was again improved. Fermentation of extruded grass by inoculated with either
315 rumen microbial community or *L. delbrueckii* maintained higher lactic acid concentrations
316 than with GM *E. coli*. When looking at production rates (Figure S2), a 1-day lag time was

317 observed before lactate concentration increased significantly in tests without inoculum, while
318 lactate increased from day 0 in the inoculated bottles. Apart from the lag phase, the rate of
319 production for both inoculated (3.9 ± 0.1 g/L.d, between day 0 and day 1) and non-inoculated
320 bottles (4.1 ± 0.3 g/L.d, between day 1 and day 2) were similar.
321
322 **Impact of acetate addition on bacterial community structure**
323 *Community shift with different substrates*
324 The effect of elevated acetate on bacterial community of bottles inoculated with rumen fluid
325 (both extruded grass and corn starch) or without any inoculation (extruded grass only) was
326 investigated and compared. A mixed microbial community such as ones found in rumen fluid
327 can be modified from its initial structure by application of different operational conditions.
328 Firstly, we measured the impact of acetate addition on fermentation of corn starch and
329 extruded grass as substrates (Fig. 3). *Lactobacillus* spp. became the most dominant species,
330 with and without acetate addition for both substrates tested. *Lactobacillus* spp. is a lactic acid
331 bacterium (LAB) able to convert sugars such as glucose and xylose, present in lignocellulosic
332 biomass, to lactic acid. When no acetate was added, there was no significant differences in the
333 microbial community between the tests with corn starch and extruded grass (Fig. 3a versus
334 Fig. 3c). When acetate was supplemented, the bacterial diversity increased, resulting in a
335 decrease in the relative abundance of *Lactobacillus* spp. (e.g. 69 ± 3 %, Fig. 3b *versus* 84 ± 4
336 % in control bottles, Fig. 3a, $p < 0.05$). Also with acetate addition, a higher relative abundance
337 of *Prevotella* spp. was found in tests with corn starch (20 %) compared to extruded grass (2
338 %). Furthermore, when the two substrates both with acetate addition were compared, extruded
339 grass gave a more diverse lactic acid producers compared to corn starch at the end of
340 fermentation batch test (e.g. Fig. 3b and Fig. 3d), which likely correlates to higher feed
341 complexity.
342
343 In rumen fluid inoculum itself, *Prevotellaceae* family was the most dominant (e.g. 85 %
344 relative abundance, Figure S4a). *Acidaminococcaceae* (6 %) and *Ruminococcaceae* (2 %)
345 were also present in lower abundance (Figure S4a). While on the extruded grass itself, a very
346 diverse inherent bacterial community was found, with *Curvibacter* spp. (23 %), *Massilia* spp.
347 (14 %) and *Bacillus* spp. (9 %) being the three most abundant species (Figure S4d). After 8
348 days of batch fermentation, most test and control bottles consisted mainly of LAB, especially
349 *Lactobacillus* spp. (69 % to 99 %), while its presence in both the rumen inoculum (0.03 %)
350 and extruded grass (Figure S4d, below detection limit) were extremely low.

351

352 *Effect of headspace composition on the microbiome*

353 The bacterial community distribution in fermentations carried out on corn starch as substrate
354 and inoculated with rumen fluid was analysed under different headspace gas composition and
355 acetate presence (Fig. 4). As we observed with different substrates, gas composition did not
356 have any effect on the community structure and *Lactobacillus* spp. was by far the dominant
357 species without acetate addition. Also in this case, regardless of the gas composition in the
358 headspace, the addition of acetate increased the microbial diversity with the presence of 3-4%
359 *Prevotella* spp. (Fig. 4b and 4d). Primers used in these tests were specific to bacterial DNA,
360 hence methanogens were not detected in the sequencing.

361

362 *Bacterial community under different pH and acetate presence*

363 Chemical analysis already showed a lower lactic acid production in non-inoculated bottles
364 compared to the inoculated during fermentation test with extruded grass. The bacterial
365 community under fermentation at different pH and acetate concentrations was evaluated (Fig.
366 5). During fermentation at pH 5.5 with extruded grass and no inoculum, a more diverse
367 bacterial community was obtained compared to samples inoculated with rumen fluid (e.g. Fig.
368 5a vs Fig. 3c). Initially on extruded grass, the *Comamonadaceae* family (26 %, Figure S4d)
369 dominated the diverse mix bacterial community but over time, the fermentation resulted in a
370 bacterial community mainly composed of LAB (52 ± 25 %, Fig. 5a). When acetate was
371 elevated, LAB abundance increased further to 82 ± 29 % (Fig. 5b).

372

373 In batch experiments where extruded grass was fermented at pH 7 without inoculum, lower
374 abundance of LAB was obtained, with *Lactobacillus* spp. Making up 17 ± 15 % (Fig. 5c) and
375 *Leuconostoc* spp. Making up 4 ± 4 % (Fig. 5c). When acetate was supplemented, multiple
376 strains of LAB dominated the bacterial community (89 ± 76 %, Fig. 5d). Apart from
377 *Lactobacillus* spp., the other LAB detected were *Leuconostoc* spp., *Lactococcus* spp. and
378 *Pediococcus* spp. By comparing the non-inoculated extruded grass fermentation, *Leuconostoc*
379 spp. (LAB) represented a much higher proportion (34 ± 25 %, Fig. 5a) at pH 5.5 compare to
380 at pH 7 (4 ± 4 %, Fig. 5c). Presence of *Leuconostoc* spp. was further stimulated by decrease
381 of pH with the control bottles (Fig. 5b vs Fig. 5d), while the phenomenon was different for
382 *Lactobacillus* spp., which was not perturbed by the pH (15 ± 3 % at pH 7, Fig. 5a *versus* 17 ±
383 15 % at pH 5.5, Fig. 5b). Data regarding alpha and beta diversity are presented in the
384 supplementary material (Figure S5 and S6).

385 **Discussion**

386 **Impact of acetate addition on lactic acid concentration**

387 In most cases, elevated acetate concentrations decreased the net production of acetic acid.
388 When different metabolic pathways are possible (i.e. lactate or acetate production), high
389 product concentration will thermodynamically favour alternative pathways according to the
390 Van't Hoff equation ($\Delta G = \Delta G° + RT\ln Q$). With regards to physico-chemical properties, acetate
391 addition will increase the conductivity of the system and high salt concentration can inhibit
392 the growth of microorganisms (McCarty and McKinney 1961). Furthermore, acetate addition
393 will bring the pH of the system towards its acidic buffer capacity (pKa 4.75), thus possibly
394 favouring the route of the lactic acid production by LAB. However, a high proportion of the
395 total acetate will be in equilibrium with the acid form of acetic acid, which may inhibit some
396 microorganisms. By considering these effects, addition of acetate should have a positive
397 impact on lactic acid fermentation if toxic concentrations are not reached. Takahashi et al.
398 (1999) found that increasing acetate concentration (from 2 g/L to 12 g/L) reduced the GM *E.*
399 *coli* biomass concentration, however the fermentation product (ethanol) concentration was not
400 affected. In this study, addition of 10 g/L sodium acetate did not negatively affect the lactic
401 acid production rate. On the contrary, initial rates seemed to be the same (Figure S2) and the
402 addition of acetate increased the final lactic acid concentration. In the fermentation pathway,
403 lactate production takes place via a relatively short and simple pathway to reduce pyruvate,
404 generating one NAD^+ for each pyruvate reduced. This pathway can quickly relieve the cell of
405 reducing power by using lactate as electron sink, e.g. when substrate is over-abundant or
406 during pH perturbation, although at the cost of lower energy gain (2.50-2.66 ATP per
407 molecule glucose reduced) compared to acetate production. While producing acetate can yield
408 more energy (4 ATP per molecule glucose reduced), lactate production can become the
409 preferred pathway of microorganisms under these circumstances. Addition of acetate may
410 perturb the reducing state of the environment, and hence stretch the lactate production further,
411 as in this study.

412

413 Lactic acid can be degraded to acetate by many LAB, requiring electron acceptors and
414 producing hydrogen (Quatravaux et al. 2006; Thauer et al. 1977). Thus, hydrogen presence
415 should, by thermodynamically increasing the energy requirements of acetate production
416 (Van't Hoff equilibrium), assist in the shift from acetate to lactate production. However, no
417 improvement was observed in the tests performed. While acetoclastic methanogenesis was
418 inhibited during the fermentation tests under low pH (5.5), presence of carboxylates, or short

419 experimental time (8 days), low concentrations of methane were detected when hydrogen was
420 supplied. Costa (2013) studied the electron flow and energy conservation in hydrogenotrophic
421 methanogens and showed that they are capable of using other substrates such as formate for
422 growth, independently of hydrogen. Hence, this may result in substrate competition for lactate
423 production and thus not notably increase its production.
424
425 Short hydraulic retention time (2 days) in combination with low pH (5.5) also allowed the
426 suppression of methanogenesis. The tested reactor mimicked a continuous recirculation of
427 acetate while lactate was harvested in the effluent. This is the first proof of concept that a
428 selective extraction of lactate from acetate will enhance the titres and make the fermentation
429 from (ligno)cellulosic biomass more feasible towards lactate production.
430
431 **Effect of operational parameters: pH, inoculum and headspace gas composition**
432 Fermentations inoculated with rumen culture or *L. delbrueckii* inoculum were set at pH 5.5
433 while those inoculated with GM *E. coli* were set at pH 7, based on the optimal working pH for
434 the inoculum. This difference in pH could have an effect on the kinetics of the fermentation
435 and caused the difference in lactic acid concentration (Fig. 1). In the test without inoculum,
436 the end lactate concentration was similar without acetate addition at both pH tested. Lactic
437 production was higher at pH 7 compared to pH 5.5 when acetate was added (Table 1), likely
438 due to enhanced bacterial growth.
439
440 The pH only had a major impact when inoculum was not supplied. When the pH was low,
441 more carboxylic acids (e.g. acetic acid and lactic acid) are in their protonated form. This
442 allows the compounds to diffuse more easily into the bacteria cells, lowering the inner pH of
443 bacterial cell. To combat this, bacteria have to invest more energy in cell maintenance and
444 transporting the compounds out of the cell to maintain the inner cell pH. This caused energy
445 loss which could otherwise be used to grow. Inoculation ensured that the fermentation was not
446 inhibited due to low number of bacteria cells initially. Inoculation shortened the lag phase and
447 allowed immediate conversion of readily digestible substrates into lactic acid (Figure S2).
448 Comparing the three inoculums tested, rumen fluid inoculum had the best performance in
449 terms of lactate concentration for both corn starch and extruded grass as substrate. The mixed
450 culture inoculum proved to be more effective for lactic acid fermentation on this complex
451 substrate, probably due to a higher capacity to deal with the complex substrate.
452

453 **Impact of acetate addition on bacterial community structure**
454 Free acid inhibition of biochemical reactions is well documented (Colin et al. 2001). At high
455 concentrations and low pH, carboxylate equilibrium shifts to the protonated form, which can
456 penetrate the cell membrane and reduce the activity of most bacteria. Many studies have been
457 performed on single strains of bacteria, such as Clostridium (Tang et al. 1989), and models
458 have been developed to study the inhibitory effect of acetate (Zeng et al. 1994). However, the
459 effect can be different for mixed microbial communities. In a study of a mixed community
460 consisting of methanogens and acetogens, Fotidis et al. (2013) increased the acetate
461 concentration stepwise up to 9 g/L and found that the growth of some cultures was inhibited,
462 while that of others was either similar or significantly higher. In this study, a similar
463 behaviour may occur as some bacteria only perform homolactic fermentation while many
464 others can undergo heterolactic fermentation, and even lactate degradation, that will be
465 affected by acetate concentration. Elevated acetate levels during fermentation of corn starch
466 promoted the presence of a more diverse bacterial community. A more specialized bacterial
467 community, mainly composed of LAB, was found in the bottles without acetate addition
468 (Table S3). However, the lactate concentration was higher in the bottles with acetate addition.
469 This indicates that with acetate addition, more capacity for lactic acid production arose.
470
471 For fermentation of extruded grass without rumen fluid inoculum, an elevated amount of
472 acetate promoted the presence of multiple species of LAB at pH 7, but not at pH 5.5 which
473 may be due to the aforementioned higher relative proportion of its free acid form. Bobillo and
474 Marshall (1992) studied the effect of acidic pH and salt on acid end products and found
475 that *Lactobacillus plantarum* is capable of altering its metabolic pathways of acid production
476 as the environmental pH changes. Here we observed rather a shift towards other bacteria
477 maintaining the lactic acid fermentation. During fermentation of corn starch, *Lactobacillus*
478 *spp.* dominated the bacterial community and elevated acetate concentration gave rise to a
479 more diverse bacterial community. Also, when rumen inoculum was used with extruded grass,
480 the diversity of the bacterial community increased with elevated acetate concentration.
481 Although the experiments performed here were likely too short to draw many conclusions on
482 diversity in the longer term, the fact that similar chemical results were obtained for the semi-
483 continuous operation could indicate that this likely remains the case in longer term. In the
484 rumen fluid inoculum, *Prevotellaceae* family was the most dominant, followed by
485 *Acidaminococcaceae* and *Ruminococcaceae*. These bacteria are commonly found among the
486 species belonging to the rumen fluid core microbiome (Huws et al. 2015). Some *Prevotella*

487 species have been reported to breakdown carbohydrates such as in Kabel et al. (2011), and
488 they may have an important role in ruminal biohydrogenation (Huws et al. 2011).
489
490 Overall, it can be concluded that the addition of acetate to mixed culture fermentations geared
491 at lactic acid production improves the rate and yield of the process while selecting for a
492 community dominated by LAB. Although addition of acetate on mono-cultures fermentation
493 increased lactate production, the difference was not significant compared to bottles without
494 acetate addition in some cases. The findings might be related to the capacity of mixed-cultures
495 to undergo different reactions and the thermodynamic impediment caused by acetate addition
496 to heterolactic fermentation or lactic acid degradation.

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513

514 **Conflict of interest**

515 The authors declare that they have no competing interests.

516

517 **Compliance with ethical standards**

518 This article does not contain any studies with human participants or animals performed by any
519 of the authors.

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656 **Fig. 1** Lactate and acetate concentrations after 8 days of fermentation batch test. (Error bars
657 represent standard deviation between experimental replicates; ‘grey’ horizontal lines represent
658 the starting lactate concentration; ‘black’ horizontal lines represent the starting acetate
659 concentration)
660
661 **Fig. 2** Lactate and acetate profile of fermentation semi-continuous test.
662
663 **Fig. 3** Bacterial community of batch fermentation at pH 5.5, under N_2 headspace, inoculated
664 with rumen fluid. (a) corn starch; (b) corn starch with acetate addition; (c) extruded grass; (d)
665 extruded grass with acetate addition
666
667 **Fig. 4** Bacterial community of batch fermentation of corn starch at pH 5.5, inoculated with
668 rumen fluid. (a) N_2 headspace; (b) N_2 headspace with acetate addition; (c) H_2 headspace; (d)
669 H_2 headspace with acetate addition
670
671 **Fig. 5** Bacterial community of batch fermentation extruded grass under N_2 headspace,
672 without inoculation. (a) pH 5.5; (b) pH 5.5 with acetate addition; (c) pH 7; (d) pH 7 with
673 acetate addition
674

675 Table 1 Lactate concentration of extruded grass fermentation with or without inoculum
676 at different pH

	Lactate concentration (g/L)			
	pH 5.5		pH 7.0	
	w/o acetate	w/ acetate	w/o acetate	w/ acetate
No inoculum	4.6 ± 0.3	5.6 ± 0.2	4.3 ± 1.6	9.1 ± 0.6
Rumen culture	12.6 ± 0.1	14.7 ± 0.3	N/T	N/T
E.coli	N/T	N/T	7.4 ± 0.2	9.0 ± <0.1

677 N/T : not tested

678

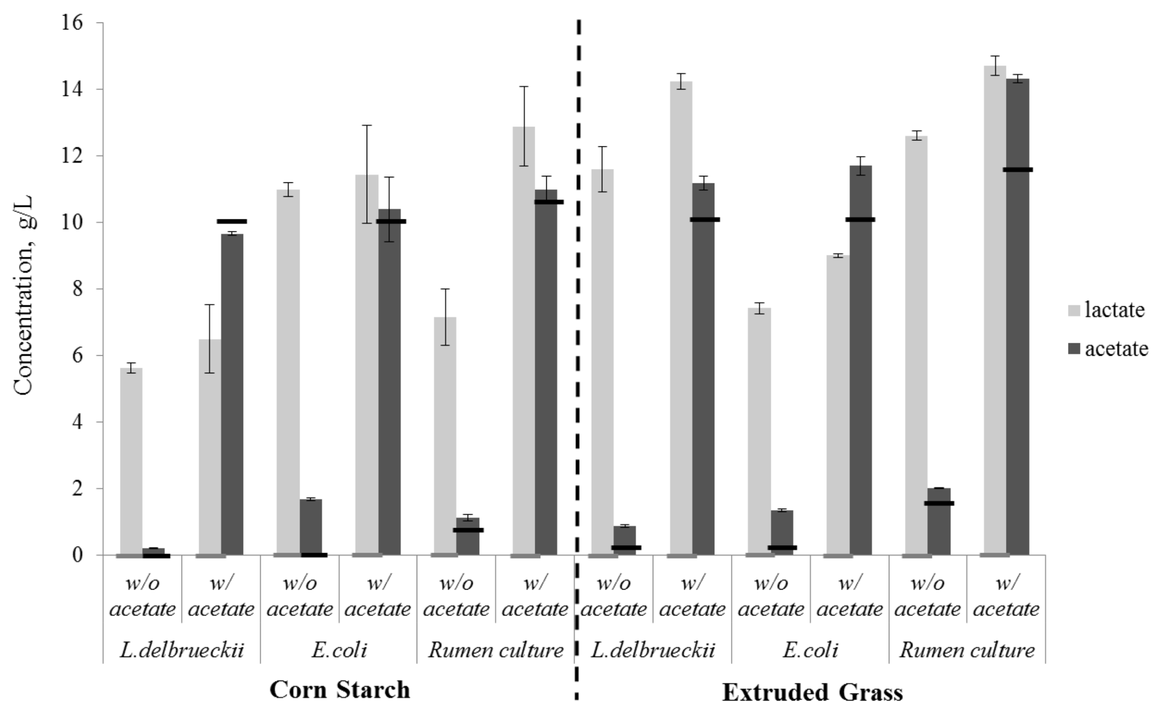
679 Table 2 Lactate concentration of corn starch fermentation inoculated with rumen fluid
680 under different headspace

Headspace gas	Lactate concentration (g/L)	
	without acetate	with acetate
N_2	6.7 ± 0.1	14.0 ± 0.4
H_2	6.7 ± 0.6	13.5 ± 0.1

681

682

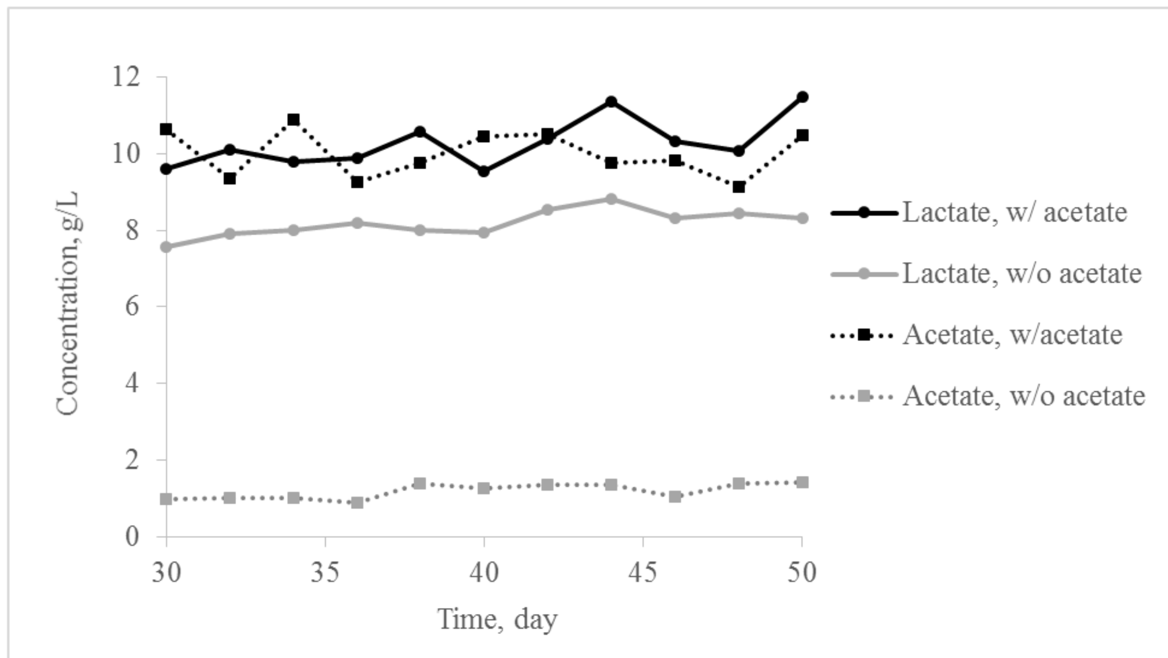
683 FIGURE 1



684

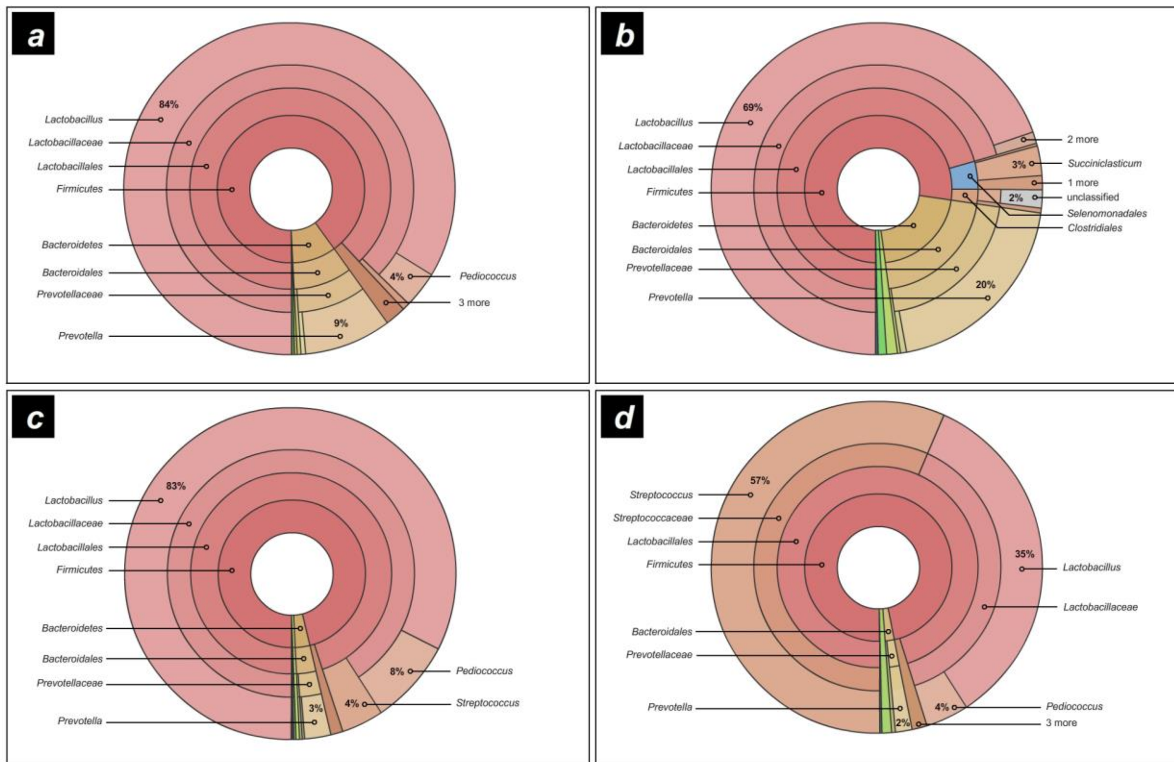
685

686 FIGURE 2



687

688 FIGURE 3



689

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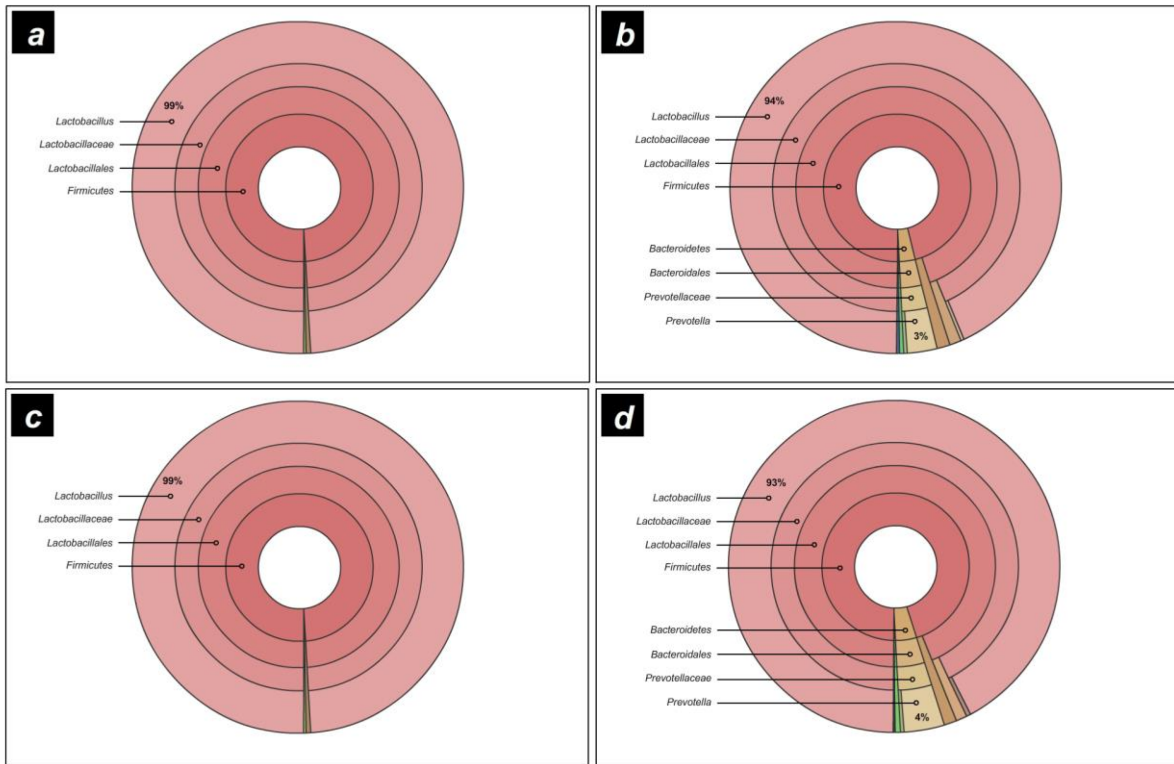
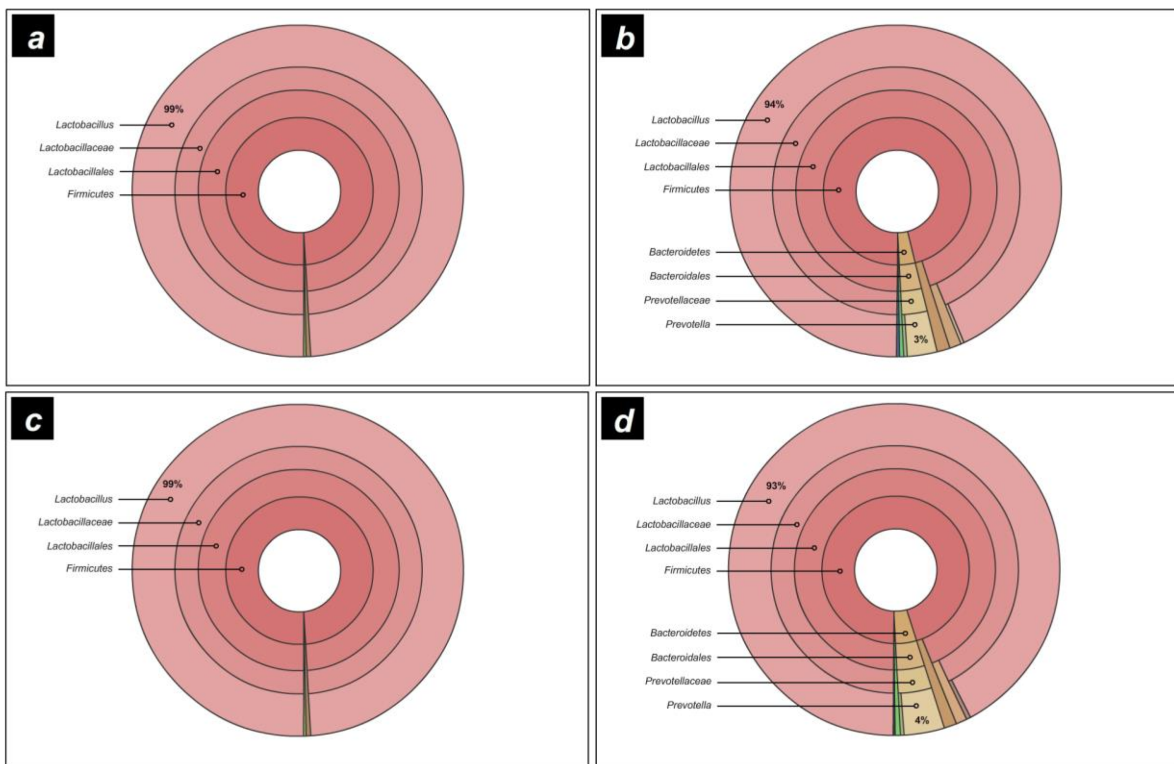
691 FIGURE 4



692

693 FIGURE 5



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