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1 **Rates of production and utilisation of lactate by microbial communities from the**

2 **human colon**

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12 **Running title:** Lactate metabolism by colonic microbiota

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21 ## Abstract

22 Lactate metabolism was studied in mixed bacterial communities using single stage
23 continuous flow fermentors inoculated with faecal slurries from four different volunteers
24 and run for 6 days at pH 5.5 and 6.0, using carbohydrates, mainly starch, as substrates. A
25 continuous infusion of [U-^{13}C]starch and L-[3-^{13}C]lactate was performed on day 5 and a
26 bolus injection of L-[3-^{13}C]lactate plus DL-lactate on day 6. Short chain fatty acids and
27 lactate concentrations plus enrichments and numbers of lactate producing and utilizing
28 bacteria on day 5 were measured. Faecal samples were also collected weekly over a 3-
29 month period to inoculate 24h-batch culture incubations at pH 5.9 and 6.5 with
30 carbohydrates alone or with 35 mmol L^{-1} lactate. In the fermentors, potential lactate
31 disposal rates were more than double formation rates, and lactate concentrations usually
32 remained below detection. Lactate formation was greater ($P < 0.05$) at the lower pH with a
33 similar tendency for utilization. Up to 20% of butyrate production was derived from
34 lactate. In batch cultures lactate was also efficiently used at both pH values, especially at
35 6.5, although volunteer and temporal variability existed. Under healthy gut environmental
36 conditions, bacterial lactate disposal seems to markedly exceed production.

37

38 ## Introduction

39 The metabolic activities of gut bacteria have a considerable influence in human health
40 and disease (Guarner & Malagelada, 2003). Dietary carbohydrate substrates, including
41 starch (Jacobasch *et al*., 1999), that escape digestion by host enzymes may be fermented by
42 microbes to short chain fatty acids (SCFA) in the colon. Acetate is the predominant
43 product of such fermentation but may also be converted to butyrate by several bacterial
44 species in the colon by the action of butyryl CoA: acetate CoA transferase (Pryde *et al*.,
45 2002; Duncan *et al*., 2004a; Louis *et al*., 2004). Butyrate, which is not further metabolised
46 by microbes in the colon (Belenguer *et al*., 2008), is the preferred energy source for the
47 colonocytes (Pryde *et al*., 2002; Gill & Rowland., 2002) and may help ameliorate
48 inflammation and prevent colorectal cancer (McIntyre *et al*., 1993; Tazoe *et al*., 2008;
49 Hamer *et al*., 2008; Louis & Flint, 2009).

50 Propionate is the other major fermentation product detected in the colon whilst lactate
51 is an intermediate product usually found in low concentrations in faecal samples from
52 healthy subjects (< 5 mmol L^{-1}) due to further microbial utilization and conversion to
53 butyrate, propionate or acetate (Belenguer *et al*., 2007). Lactate is a product of several
54 bacterial groups, including bifidobacteria (Florent *et al*., 1985) and certain anaerobes
55 (Mcfarlane & Gibson, 1991; Duncan *et al*., 2002). At low concentrations lactate is
56 considered beneficial in the colon as the low pKa makes it inhibitory to pathogens. Lactate,
57 however, may accumulate to high concentrations (up to 90 mmol L^{-1}) in the colonic lumen
58 of ulcerative colitis sufferers (Vernia *et al*., 1988) with detrimental effects, including
59 neurotoxic responses (Ewarschuk *et al*., 2005).

60 Among the factors that affect the gut microbial ecosystem, pH impacts markedly on the
61 composition and metabolism of the colonic microbiota (Walker *et al*., 2005; Duncan *et al*.,
62 2009). This is also the case for lactate metabolism and previous studies have shown that
63 lactate production and utilization are maintained in balance by mixed human faecal

64 bacteria (Bourriaud *et al*., 2005; Morrison *et al*., 2006), within the normal physiological pH
65 range (Belenguer *et al*., 2007). At pH 5.2, however, lactate utilization was curtailed and
66 this metabolite accumulated (Belenguer *et al*., 2007). This may explain high lactate
67 concentrations in severe colitis (Vernia *et al*., 1988) where the colonic pH can approach
68 that of the stomach (Fallingborg *et al*., 1993). The contribution of various bacterial species
69 to lactate utilization remains ill-defined, however, but several are known to convert lactate
70 to propionate or butyrate (Duncan *et al*., 2004b; Morrison *et al*., 2006; Falony *et al*., 2006).
71 These include *Eubacterium hallii*, *Anaerostipes caccae* and an un-named species (Duncan
72 *et al*., 2004b) that are butyrate-producing bacteria and belong to the dominant core group
73 of species in the human intestinal microbiota (Tap *et al*., 2009; Walker *et al*., 2010).
74
75 Maintenance of low amounts of lactate within the colon represents a balance between
76 utilization and production and imbalances in either can cause lactate accumulation. The
77 current study uses two approaches, long-term (6 days) continuous fermentors and short
78 term (24h) batch cultures to estimate rates of lactate production and utilization and
79 determine if these link to certain bacterial groups. The pH of the culture media was shown
80 to modify rates of lactate metabolism and stable isotope approaches were used to allow
81 quantification of flow from starches to lactate and to end-product metabolites.
82

83 ## Material and methods

84 ### Collection of faecal samples

85 These were provided by four adult volunteers (two male and two female), aged 32-62
86 years and all consuming a Western style diet. The volunteers (referred to as donors A, B, C
87 and D) did not take any antibiotics or drugs known to influence faecal microbiota for the
88 last 6 months prior to the start of the studies.
89

90 **Continuous flow fermentor incubations**

91 Single-stage continuous fermentor systems were operated as described previously
92 (Duncan *et al*., 2003) using a medium based on that of Macfarlane *et al*. (1989) as
93 modified by Walker *et al*. (2005). The carbon sources present in the mixed substrate
94 medium were potato starch (0.5% weight in volume, w/v) in addition to xylan, pectin,
95 amylopectin and arabinogalactan each at 0.06% (w/v). The total peptide concentrations
96 (comprising equal amounts of casein hydrolysate and peptone water) were 0.2%. The
97 fermentor growth medium was maintained under a stream of CO_2 with a flow rate of fresh
98 medium equating to one pool per day, giving a dilution rate of 0.042 h^{-1}. Prime doses of
99 SCFA were added to give initial concentrations of approximately 35 mmol L^{-1} acetate, 9
100 mmol L^{-1} propionate, 5 mmol L^{-1} butyrate and 1 mmol L^{-1} each of valerate, iso-valerate,
101 and iso-butyrate, but were not included in the supplied medium. The pH was maintained at
102 either 5.5 ± 0.1 (vessel 1) or 6.0 ± 0.1 (vessel 2). The temperature was maintained at 37ºC
103 using a thermal jacket. Faecal suspensions (20%) were prepared by suspending fresh faecal
104 samples in 50 mmol L^{-1} phosphate buffer (pH 6.5) containing 0.05% cysteine under O_2-
105 free CO_2 to give a faecal inoculum of 2% (w/v) in the vessel. Substrate (mixture of
106 carbohydrates) was infused continuously, with potato soluble starch being supplied at
107 approximately 16 and 22 mg h^{-1} (which would be equivalent to approximately 92 and 124
108 µmol glucose h^{-1}) in vessels 1 and 2, respectively.

109 For the lactate metabolism studies a continuous infusion of [U-^{13}C]starch (equivalent to
110 7.35-8.83 and 10.95-17.00 µmol glucose h^{-1} in vessels 1 and 2, respectively) and L-[3-
111 ^{13}C]lactate (5.29-6.58 and 6.58-7.88 µmol h^{-1} in vessels 1 and 2, respectively) was
112 performed for 10 h on day 5, with a prime injection of [1-^{13}C]acetate (184.2-384.8 and
113 219.4-298.6 µmol in vessels 1 and 2, respectively), [1,2-^{13}C]acetate (176.9-402.3 and
114 195.7-296.4 µmol in vessels 1 and 2, respectively) and [1-^{13}C]propionate (9.4-22.2 and
115 7.6-21.7 µmol in vessels 1 and 2, respectively) given approximately 4 h after the start of

116 the infusion of labelled starch and lactate. The following day (day 6) both vessels received
117 a bolus injection of L-[3-^{13}C]lactate (approximately 23 and 28 μmol in vessels 1 and 2,
118 respectively) plus DL-lactate (approximately 230 and 275 μmol in vessels 1 and 2,
119 respectively).
120 Daily samples were taken from each vessel to monitor SCFA and lactate concentrations.
121 On the infusion day (day 5) samples were taken every 30 min for the first 3 h of the
122 infusion and hourly thereafter until 10 h to measure SCFA and lactate concentrations and
123 metabolite ^{13}C enrichments. On the injection day (day 6) samples were taken at 30 min
124 intervals from just before until 4 h after the bolus injection, with lactate concentrations and
125 metabolite ^{13}C enrichments measured.
126
127 **Batch culture incubations**
128 Fresh faecal samples from the same four volunteers (A, B, C and D) were collected
129 weekly at 12 occasions over a three month period. Slurries of this material were used for
130 batch culture incubations with an anaerobic medium similar to that used for the continuous
131 flow fermentor incubations, based on Macfarlane *et al*. (1989) as modified by Walker *et al*.
132 (2005). The carbohydrate sources present in the mixed substrate medium were potato
133 starch (0.14% w/v) in addition to xylan, pectin, amylopectin, and arabinogalactan each at
134 0.015% (w/v). The total peptide concentrations (comprising equal amounts of casein
135 hydrolysate and peptone water) were 0.2%. Samples were inoculated at two different pH
136 values (mean ± standard deviation 5.9 ± 0.2 and 6.5 ± 0.2) and with either a carbohydrate
137 mixture alone or with DL-lactate (approximately 35 mmol L^{-1} initial concentration) also
138 present. SCFA were also added to the medium to give initial concentrations of
139 approximately 33 mmol L^{-1} acetate, 9 mmol L^{-1} propionate, 5 mmol L^{-1} butyrate and 1
140 mmol L^{-1} each of valerate, iso-valerate, and iso-butyrate. The fermentor medium was
141 dispensed into Hungate tubes under a stream of CO_2 (Miyazaki *et al*., 1997) and heat

142 sterilised at 121 °C (15 min). After cooling, heat-labile vitamins were added and the

143 medium was inoculated with the faecal slurry under CO_2 and incubated at 37 ºC. Faecal

144 slurries (20%) were prepared within 2 h of collection in anaerobic phosphate buffer saline

145 to give a final concentration of approximately 0.2%. Tubes were inoculated in duplicate

146 and samples were taken at 24 h to measure SCFA and lactate concentrations. Samples of

147 uninoculated medium were also taken to measure initial concentrations and initial pH

148 values.

149

150 **Quantification of bacteria in faecal and continuous fermentor samples by**

151 **fluorescent in situ hybridization analysis**

152 Samples were taken from faeces (0.5 g) and the fermentor incubations on day 5 (1 ml)

153 for fluorescent in situ hybridization (FISH) analysis. Faecal samples were diluted with

154 phosphate buffer (1:10), and all samples were fixed by mixing 1:3 in 4% (w/v)

155 paraformaldehyde at 4°C for 16 h and stored at -20°C. FISH analysis was performed as

156 described by Harmsen *et al*. (2002). Diluted cell suspensions were applied to gelatin-

157 coated slides and the slides were hybridized overnight with the appropriate probes. 50 µl of

158 Vectashield (Vector Laboratories, Burlingame, CA) was applied to each slide to prevent

159 fading. Cells were counted automatically using image analysis software CellF (Olympus

160 Soft Imaging Solutions GmbH, Germany) with an Olympus microscope, except when the

161 number of cells was less than 10 per field of view, in which case the cells were counted

162 manually. For each sample 30 microscopic fields were counted and the data averaged. All

163 samples were assessed with the following probes: total bacteria (Eub338, Amann *et al*.,

164 1990), *Bifidobacterium* spp. (Bif164, Langendijk *et al*., 1995), as lactate-producing

165 bacteria, and the *Eubacterium hallii* (Ehal1469, Harmsen *et al*., 2002) and *Anaerostipes*

166 *caccae* (Acac194, Hold *et al*., 2002) groups, as potential lactate utilisers.

167

168 **Determination of concentrations and ^{13}C enrichments in short chain fatty acids**
169 **and lactate**

170 Daily samples from the single-stage continuous fermentors were derivatised in duplicate
171 for estimation of concentrations of SCFA and lactate by capillary gas chromatography
172 (Richardson *et al*., 1989). Similar analyses were performed for blank and 24h samples
173 from the batch culture incubations to measure lactate concentrations. Samples from the
174 fermentors on the infusion day (day 5) were analysed for lactate and SCFA concentrations
175 and enrichments but only lactate concentrations and enrichments were determined in the
176 samples collected during the injection day (day 6). For samples from both day 5 and 6
177 concentrations were quantified by isotope dilution, while enrichments were measured by
178 gas chromatography-mass spectrometry analysis of the *tert*-butyldimethylsilyl derivatives,
179 as described previously (Duncan *et al*., 2004a; Belenguer *et al*., 2006). Analyses were
180 under electron impact ionisation conditions; for acetate, the ions M+, M+1 and M+2 at
181 mass/charge (m/z) 117, 118 and 119 were monitored; for butyrate, M+, M+1, M+2 and
182 M+4 (i.e. m/z 145, 146, 147 and 149) were determined, the latter to quantify butyrate
183 formation from two [1,2-^{13}C] acetate molecules; for propionate, M+, M+1, M+2 and M+3
184 (i.e. m/z 131, 132, 133 and 134) were measured; for lactate, M+, M+1, M+2 and M+3 ion
185 fragments were analysed (m/z 261, 262, 263 and 264). For the concentration
186 determinations appropriate corrections were applied for the enrichments of the samples.
187

188 **Metabolic activities of pure cultures of bacteria**

189 Based on data reported previously (Belenguer *et al*., 2006) rates of conversion of 0.2%
190 (w/v) starch substrate to lactate in batch cultures were calculated at pH 5.7 and 6.7 between
191 4 and 8 h of incubation. Similarly the activity of *E. hallii* to utilise lactate was determined
192 in the presence of 45 mmol L^{-1} lactate at pH 5.7 and 6.7 between 8 and 24 h of incubation.

193 For both species, the number of bacteria in the respective incubations was determined by
194 optical density (1 $OD_{600}=10^9$ cells; Lech *et al.*, 1987).
195
196 **Kinetic modelling**
197 The model structure and fates of the various isotopes are shown in Fig. 1. Let q, Q, and
198 E denote the labelled amount (µmol), the total (labelled plus unlabelled) amount (µmol)
199 and enrichment (0.01 molar % excess, MPE) of either acetate, butyrate, propionate, lactate,
200 or starch, denoted by subscripts 'a', 'b', 'p', 'l' and 's', respectively. Let i denote the
201 interval between any two times t_0 and t_1, with $t_1 > t_0$, and let F(i) denote the flow of a
202 metabolite (labelled plus unlabelled) during i. Eff(i) denotes the loss to the effluent during
203 interval i, and E(i) denotes the average enrichment during i. Subscript 'in' refers to inflow
204 (production) and subscript 'out' refers to use in further metabolic processes (e.g. acetate
205 used to produce butyrate). For example, $F_{a.in}$ refers to acetate production, while $F_{a.out}$ stands
206 for acetate outflow, etc. Flows to pool y from pool x are denoted by F_{yx}. Q, q, E and Eff
207 were measured, whilst the F_{yx}, F_{in} and F_{out} were unknown.
208 Data are expressed in terms of two carbon (C_2) units, to allow for 'molar equivalent'
209 transfers. To achieve this, the concentration of butyrate is multiplied by 2 and the
210 enrichment divided by 2. The enrichments of propionate and lactate, in terms of C_2 units,
211 are given as 0.01 (MPE(M+2) + MPE(M+3)). The concentrations of acetate, propionate
212 and lactate and their M+1 enrichments are as measured directly.
213
214 *Infusion day (day 5):*
215 Calculations are based on time points during the continuous infusion of labelled starch
216 and lactate between 4 and 10 h, after the prime doses of labelled acetate and propionate.
217 Lactate formation ($F_{l.in}$) and utilisation ($F_{l.out}$) were obtained from the changes in labelled

218 (M+1) and total (labelled plus unlabelled) lactate as observed during the continuous

219 infusion of [3-^{13}C]lactate:

220 $$q_{l.m+1}(t_1) = q_{l.m+1}(t_0) + \text{Infusion}(i) - E_{l.m+1}(i)\, F_{l.out}(i) - E_{l.m+1}(i)\, \text{Eff}_l(i) \quad (1)$$

221 $$Q_l(t_1) = Q_l(t_0) + \text{Infusion}(i) + F_{l.in}(i) - F_{l.out}(i) - \text{Eff}_l(i) \quad (2)$$

222

223 It was assumed that butyrate was formed ($F_{b.in}$) via two pathways, either through the

224 extracellular acetate pool (F_{ba}) or directly from lactate (F_{bl}). First, from the changes in the

225 total butyrate concentration $F_{b.in}$ was obtained:

226 $$Q_b(t_1) = Q_b(t_0) + F_{b.in}(i) - \text{Eff}_b(i) \quad (3)$$

227 Then, changes in the M+1 enriched butyrate were modelled as

228 $$q_{b.m+1}(t_1) = q_{b.m+1}(t_0) + E_{l.m+1}(i)\, F_{bl}(i) + E_{a.m+1}(i)\, F_{ba}(i) - E_{b.m+1}(i)\, \text{Eff}_b(i) \quad (4)$$

229 Writing $F_{ba} = F_{b.in} - F_{bl}$ and substituting in equation (4) then provides F_{bl}, and F_{ba}

230 follows.

231

232 The total production of propionate ($F_{p.in}$) was obtained from

233 $$Q_p(t_1) = Q_p(t_0) + F_{p.in}(i) - \text{Eff}_p(i) \quad (5)$$

234 where it was assumed that propionate has no further metabolic fates, i.e. $F_{p.out} = 0$.

235 Changes in labelled propionate derived from 3-^{13}C-lactate were modelled as:

236 $$q_{p.m+1}(t_1) = q_{p.m+1}(t_0) + E_{l.m+1}(i)\, F_{pl}(i) - E_{p.m+1}(i)\, \text{Eff}_p(i) \quad (6)$$

237 which then provided an estimate for F_{pl}.

238

239 The incorporation of lactate into acetate (F_{al}) is obtained from assuming that lactate may

240 be utilised only to produce acetate, butyrate and propionate: $F_{l.out} = F_{al} + F_{bl} + F_{pl}$.

241 Furthermore, the M+1 acetate movements yield an estimate for $F_{a.out}$, based on:

242 $$q_{a.m+1}(t_1) = q_{a.m+1}(t_0) + E_{l.m+1}(i)\, F_{al}(i) - E_{a.m+1}(i)\, F_{a.out}(i) - E_{a.m+1}(i)\, \text{Eff}_a(i) \quad (7)$$

243 $F_{a.in}$ follows from

244 $Q_a(t_1) = Q_a(t_0) + F_{a.in}(i) – F_{a.out}(i) – Eff_a(i)$ (8)

245

246 Incorporation of starch into acetate (F_{as}), lactate (F_{ls}) and propionate (F_{ps}) was obtained

247 from the changes in labelled metabolites that were produced from the infused [U-^{13}C]

248 starch. For F_{ls}:

249 $q_{l.m+2}(t_1) = q_{l\ m+2}(t_0) + E_s F_{ls}(i) – E_{l.m+2}(i) F_{l.out}(i) – E_{l.m+2}(i) Eff_l(i)$ (9)

250 Here it is assumed that both M+2 and M+3 lactate were formed from [U-^{13}C]starch (i.e.

251 $E_{l.m+2} = 0.01\ MPE_{lactate}(M+2) + 0.01\ MPE_{lactate}(M+3)$). Making similar assumptions for

252 propionate, F_{ps} follows from:

253 $q_{p.m+2}(t_1) = q_{p.m+2}(t_0) + E_{l.m+2}(i) F_{pl}(i) + E_s(i) F_{ps}(i) – E_{p.m+2}(i) Eff_p(i)$ (10)

254 Incorporation of starch into acetate follows from:

255 $q_{a.m+2}(t_1) = q_{a.m+2}(t_0) + E_{l.m+2}(i) F_{al}(i) + E_s F_{as}(i) – E_{a.m+2}(i) F_{a.out}(i) – E_{a.m+2}(i) Eff_a(i)$

256 (11)

257

258 *Bolus injection of labelled lactate (day 6):*

259 Except in the vessel at the lower pH (5.5) inoculated with a faecal suspension from

260 volunteer A, no lactate was detected so that lactate enrichments $E_{l.m+1}$ and $E_{l.m+2}$ could not

261 be determined. Estimates of lactate formation were based, instead, on samples collected

262 following the bolus injection of [3-^{13}C]lactate on day 6. Lactate utilisation ($F_{l.out}$) on day 6

263 was obtained from the changes in labelled (M+1) lactate (based on equation (1), with

264 'Infusion' set equal to zero). This was then used to obtain $F_{l.in}$, based on changes in total

265 (labelled plus unlabelled) lactate (equation (2), with 'Infusion' set to zero). The remaining

266 calculations are based on the day 5 measurements, as follows. It was assumed that $F_{l.in}$ was

267 the same on days 5 and 6 and this was substituted in equation (2) to derive $F_{l.out}$ on day 5.

268 Subsequently, an estimate of the lactate M+1 enrichment on day 5, denoted by $E^*_{l.m+1}$, was

269 obtained from $E^*_{l.m+1}$ = Infusion rate / ($F_{l.in}$ + Infusion rate), assuming that the infusate was

270 fully labelled. In subsequent calculations, $E^*_{l.m+1}$ replaced $E_{l.m+1}$, so that F_{bl}, F_{ba}, F_{pl}, F_{al},

271 $F_{a.out}$ and $F_{a.in}$ could be calculated as before, based on equations (4), (6)-(8). To estimate

272 the fates of starch, it was assumed that 40% of the lactate came from starch, so that the

273 lactate M+2 enrichment was assumed to be 0.4 of the starch enrichment. This estimate of

274 the lactate M+2 enrichments, denoted as $E^*_{l.m+2}$, then replaced $E_{l.m+2}$ in equations (9) – (11)

275 to provide estimates for F_{ls}, F_{ps} and F_{as}. Note that $F_{b.in}$ and $F_{p.in}$ (equations (3) and (5)

276 respectively) are unaffected by any of the assumptions.

277 The assumption of $F_{l.in}$ on day 5 being equal to that on day 6 was supported by data from

278 donor A at pH 5.5 (0.29 and 0.28 μmol mL^{-1} h^{-1} respectively. Data from the same volunteer

279 showed that approximately 40% of the lactate came from starch and this value was applied

280 to the other volunteers. Based on the model adopted, this assumption should only influence

281 the calculation of F_{ls}, F_{ps} and F_{as}. In practice, sensitivity analysis with the proportion of

282 lactate from starch varied from 1 to 99% only impacted seriously on F_{as}, F_{ps} and F_{px}, with

283 the coefficient of variation lower than 36%, except for F_{px} that showed flows lower than

284 0.15 μmol mL^{-1} h^{-1}.

285

286 **Statistical analysis**

287 Where SCFA data were replicated the average values were used. The daily SCFA data

288 from the continuous fermentors were analysed as repeated measures, with volunteer and

289 time point nested within volunteer as random effects, while time point, pH and their

290 interaction were taken as fixed effects. The weekly lactate data from the batch culture

291 incubations were analysed using the same random structure, but with fixed effects now

292 consisting of time point, pH, substrate and their interactions. To account for dependency on

293 previous time points, a suitable covariance structure (compound symmetry) was fitted on

294 the basis of Schwarz's Bayesian information model fit criterion**.** Quantities (such as carbon

295 flows, numbers of bacteria) obtained from the day 5 (or day 6) data in the continuous

296 fermentors were analysed as one-way analysis of variance with volunteer as random effect
297 and pH as fixed effect. Pure culture data on lactate formation and utilization were also
298 analysed as one-way analysis of variance with pH as fixed effect. $P < 0.05$ was regarded as
299 statistically significant. All data were analysed using the MIXED procedure of the SAS
300 software package, version 9.1 (SAS Inst. Inc., Cary, NC). In addition, the linear
301 relationships between variables of interest were analysed using the REG procedure of the
302 SAS software.
303

304 ## Results

305 **Concentrations of SCFA and lactate over time in continous flow fermenters**

306 Daily concentrations of SCFA (acetate, propionate, butyrate) and lactate in the single
307 stage fermentors are presented in Fig. 2. SCFA and lactate concentrations required 3 to 4
308 days to stabilise in all vessels. Lactate was usually detectable only during the first two days
309 and reduced to negligible amounts by day 3. For other SCFA (data not shown) succinate
310 was occasionally detected, albeit at low concentrations, whereas formate had variable
311 initial values (up to 4 mmol L^{-1}) on day 1 but these decreased to zero by day 3. Volunteer
312 A at pH 5.5 showed a different pattern to the other volunteers, with butyrate nearly
313 undetectable (< 0.4 mmol L^{-1}) by 3 days, while lactate was detectable throughout and
314 formate was present at approximately 11 mmol L^{-1} from day 3 onwards.
315

316 **Rates of lactate formation and utilisation**

317 Originally it was expected that lactate concentrations would be above the limits of
318 detection but, in practice, this only occurred at day 5 for volunteer A at pH 5.5. This
319 volunteer provided the only direct comparison of metabolism on days 5 and 6, with
320 endogenous lactate formation similar on both days (0.29 and 0.28 µmol mL^{-1} h^{-1}
321 respectively). For the other samples, therefore, the various rates of lactate metabolism were

322 calculated based on formation determined on day 6 (Table 1) plus metabolite masses and
323 enrichments from day 5. These parameters of endogenous lactate metabolism are presented
324 in Table 2.
325 Lactate formation was consistently greater at the lower pH ($P < 0.05$; Tables 1 and 2)
326 and a similar trend ($P = 0.053$) was also seen for endogenous lactate utilization (Table 2).
327 As expected, rates of production and utilization were closely matched to maintain constant
328 lactate concentrations, even below the limit of detection.
329

330 **Carbon flows between starch, lactate and SCFA in continous fermentors**

331 The continuous infusion of [U-^{13}C]starch and [3-^{13}C]lactate, together with the bolus
332 injection of [1,2-^{13}C]acetate, [1-^{13}C]acetate and [1-^{13}C]propionate allowed estimation of
333 flows (expressed as C_2 units) between lactate and the main SCFA (acetate, propionate,
334 butyrate; Table 2). Labelled starch also allowed quantification of the flow to lactate.
335 Carbon flow through the acetate pool ($F_{a.in}$) was considerable (1.7-2.1 μmol mL^{-1} h^{-1}), with
336 most (> 53%) derived from sources other than starch (contribution 16 to 42%) or lactate
337 (contribution < 14%). Flows from starch to lactate (F_{ls}) and from lactate to acetate (F_{al})
338 were greater at the lower pH ($P < 0.05$). Propionate formation (approximately 0.5 μmol
339 mL^{-1} h^{-1}) was independent of pH ($P > 0.10$) and with the majority derived from starch (>
340 51% F_{ps}:$F_{p.in}$) . Butyrate formation ($F_{b.in}$) was similar to propionate formation and was at
341 least two-fold greater for volunteers C and D than A or B at the lower pH. Most butyrate
342 derived from lactate (estimated as $F_{al} \times F_{ba}/F_{a.out}$) was via the external acetate pool (>
343 78%), and involved the action of acetyl-CoA transferase. The exception was volunteer A at
344 the lower pH where no acetate utilisation or butyrate formation was observed and most
345 propionate derived from sources (63%) other than starch (21%) or lactate (16%).
346 The proportions of lactate carbon converted to acetate, propionate and butyrate were
347 also estimated (Table 3). The proportion of lactate converted to propionate was always

348 greater at pH 6 ($P < 0.05$). In contrast, the proportion of lactate metabolised to acetate and
349 butyrate was independent of pH. The fate of lactate also appeared volunteer-dependent. For
350 one subject (C) butyrate was the main end product whereas for two other volunteers (A and
351 D) a substantial amount of the lactate (37-68%) was converted to propionate. The
352 proportion of butyrate formed from lactate, either directly or via the external acetate pool,
353 varied between 0-20%.
354

355 **FISH quantification of bacteria that produce or utilize lactate**

356 *Bifidobacterium* spp. accounted for 3.8 to 6.1% of the total bacteria present in the fecal
357 inocula, whereas the populations of the *E. hallii* group were low and more variable (0.04 to
358 0.61%) and *A. caccae* was below the limit of detection (< 0.01%). By d 5 of inoculation,
359 total bacterial numbers had increased at least 4-fold (Table 4). By this time, for three
360 volunteers the bifidobacteria accounted for only 0.3 to 4.2% of total bacteria whereas for
361 volunteer A the *Bifidobacterium* spp. contribution was 47% at the lower pH (an increase
362 of 8.5×10^7 g^{-1}). Overall, the $\log_{10}$ numbers of *Bifidobacterium* spp. only tended to show a
363 weak relationship with the rate of lactate formation (adjusted $r^2 = 0.41$, $P = 0.05$). The
364 populations of the *E. hallii* group increased over time by 160-fold but these still accounted
365 for less than 0.7% of the total bacteria and were not affected by pH.
366

367 **Effect of pH on lactate metabolism in batch cultures**.

368 A similar mixture of dietary polysaccharides was used for the batch cultures, in the
369 presence of either 0 or 35 mmol L^{-1} DL-lactate. The two pH studied were similar, but not
370 identical, to the fermentor study (5.9 and 6.5). Over the 24h of batch culture, the pH
371 remained relatively stable (difference between initial and final pH < 0.4).
372 In the absence of added lactate, net lactate formation or utilisation was in balance for
373 most cultures at both pH. When lactate was added to the initial medium, net disposal was

374 complete in most incubations at pH 6.5 and was always greater ($P < 0.001$) than at pH 5.9
375 (Table 5). In the absence of lactate, acetate was the main end product, whereas butyrate
376 accumulated ($P < 0.001$) when lactate was present. The presence of lactate also decreased
377 net production of acetate ($P < 0.001$) but increased net formation of propionate ($P <$
378 0.001). Furthermore, net production of all three of these SCFA was enhanced at the higher
379 pH ($P < 0.005$), although for propionate and butyrate this effect was more pronounced with
380 the mixture plus lactate than the mixture alone (interaction of substrate x pH, $P < 0.001$).
381 Net lactate utilization was also greater at the higher pH, but again this occurred mainly in
382 the presence of lactate (interaction of substrate x pH, $P < 0.001$). At the lower pH (5.9) and
383 with the mixture plus lactate cultures, a linear relationship was observed between net
384 lactate utilisation and butyrate formation ($P < 0.001$; adjusted $r^2 = 0.79$; Fig. 3).
385
386 Responses varied between volunteers and weeks. For example, net lactate utilisation
387 and butyrate production were lower for volunteers C and D than A and B, at the lower pH,
388 and net formation of propionate and butyrate differed between weeks.
389
390 **Activity of pure cultures**

391 Estimates of the equi-cell abilities of *B. adolescentis* L2-32 to convert starch to lactate
392 and *E. hallii* L2-7 to metabolise lactate (to butyrate) are given in Table 6. Both types of
393 bacteria were more active ($P < 0.01$) at the lower pH. Nonetheless, at both pH the ability of
394 *E. hallii* L2-7 to dispose of lactate exceeded formation by *B. adolescentis* L2-32 by at least
395 5-fold.
396

397 Discussion

398 Kinetics of lactate formation and utilization

399 Although lactate is a known fermentation product of carbohydrate metabolism within
400 the colon, the concentrations are usually low or undetectable in faecal samples from
401 healthy donors (Mcfarlane & Cummings, 1991; Vernia *et al*., 1988; Duncan *et al*., 2007).
402 Thus rapid metabolism must also occur. When lactate accumulates, however, as in patients
403 with severe ulcerative colitis (Vernia *et al*., 1988; Hove *et al*., 1994) then, in the absence of
404 altered rates of absorption (Umesaki *et al*., 1979), this must be due to changes in either rate
405 of formation and(or) disposal. Of the many factors that influence microbial lactate
406 utilisation and production the most important probably include substrate supply
407 (Cummings *et al*., 1989; Duncan *et al*., 2007), pH (Belenguer *et al*., 2007; Duncan *et al*.,
408 2009) and abundance of appropriate bacteria (Roberfroid, 2005). In the present study
409 substrate supply was fixed and the effect of pH was tested, both on direct metabolism and
410 via changes in bacterial populations. Use of stable isotopes allowed direct quantification of
411 lactate production and utilisation as well as the conversion of lactate to propionate or
412 butyrate, the latter either via butyrate kinase or via the butyryl CoA: acetate CoA
413 transferase route. Furthermore, co-operative actions between bacteria have been identified
414 (Wolin *et al*., 1991; Flint *et al*., 2007), and including those that produce and utilize lactate
415 (Duncan *et al*., 2004b; Belenguer *et al*., 2006). Therefore, changes in the activity and
416 population abundances of these bacteria need to be considered alongside the dynamic
417 quantification of inflows and outflows of specific metabolites. This work suggested that up
418 to 20% of butyrate production in the mixed community could be derived from lactate
419 rather than produced directly from carbohydrates.

420 For the fermentor study, the infusion of carbohydrate was equivalent to approximately
421 1.7 µmol glucose mL^{-1} h^{-1} with a theoretical maximal lactate formation > 3 µmol mL^{-1} h^{-1}.
422 In practice, observed rates were much lower (0.06-0.34 µmol mL^{-1} h^{-1}) indicating that only
423 a small fraction of the carbohydrate (and peptide) substrates were converted to lactate. In
424 contrast, the capacity to dispose of lactate appears greater, as shown from the rates of

425 disposal observed following a bolus injection of lactate (0.36-0.86 μmol mL^{-1} h^{-1}, data not
426 shown). A high estimated minimal rate of disposal (1.47 μmol mL^{-1} h^{-1}) was observed for
427 the batch culture incubations with 35 mmol L^{-1} lactate at both pH 5.9 and 6.5. In both the
428 fermentor and batch approaches, these values represent a capacity for a rapid response and,
429 therefore, the inherent disposal capacity of the microorganisms involved exceeds the
430 ability to produce lactate under the substrate conditions employed with these healthy
431 volunteers. Nonetheless changes in either process can alter lactate concentrations.
432 Increased lactate formation has also been observed previously in batch cultures at mild to
433 moderate acidic pH (studied between 5.2 to 6.4; Belenguer *et al*., 2007). These earlier data
434 (Belenguer *et al*., 2007) also showed that lactate utilisation was strongly inhibited at pH
435 5.2 and this would help explain lactate accumulation in colitis patients, where a similar low
436 pH occurs (Nugent *et al*., 2001). At higher pH (5.9), however, the mixed faecal microbiota
437 were able to rapidly utilise lactate (Belenguer *et al*., 2007) and thus prevent excessive
438 accumulation. The current data show that even at a more acidic pH (5.5), but still within
439 the range reported for the proximal large intestine in healthy people (Bown *et al*., 1974;
440 Macfarlane *et al*., 1992), the capacity for lactate utilization still exceeded lactate formation.
441 Nonetheless, changes in type and supply of fermentable substrate and environmental
442 conditions influence both bacterial populations and products of their metabolism. For
443 example, for stool samples collected weekly over 3 months from the free-living volunteers
444 in this study only in 29/41 cases was lactate detected (at > 1 mmol per kg faecal water). All
445 volunteers had at least five stools with detectable lactate, and the maximum number of
446 stools with lactate for any one volunteer was eight (out of 12 collections). Thus, all the
447 volunteers possessed lactate producers.
448
449 **Potential lactate producers and utilizers**

450 Considering lactate producers, *Bifidobacterium* spp. (Florent *et al*., 1985) are major
451 starch-utilisers within the human colon (Macfarlane & Englyst, 1986; Leitch *et al*., 2007).
452 Furthermore, in pure culture, lactate production by bifidobacteria is stimulated at slightly
453 acidic pH (Table 6; Belenguer *et al*., 2006). Therefore, it was expected, based on earlier
454 observations (Levrat *et al*., 1991; Silvi *et al*., 1999; Belenguer *et al*., 2006), that lactate-
455 producing bacteria, such as bifidobacteria, and lactate formation would both be increased
456 in the fermentors at the lower pH with starch as a substrate. Nonetheless, the increase in
457 bifidobacteria at the lower pH was less, relatively, than the change in lactate production
458 and raises the question of the importance of the bifidobacteria to lactate metabolism.
459 Although 41% of the variance in lactate formation within the fermentors could be
460 explained by the numbers (log_{10}) of *Bifidobacterium* spp. present, the actual numbers of
461 those bacteria, both in absolute terms and as a percentage of total bacteria, varied between
462 individuals, as observed previously (Flint *et al*., 2007). Indeed, when these bacterial
463 numbers were combined with the rates of lactate production from a starch substrate for
464 specific *Bifidobacterium* species (Table 6) then this would account for between 2.8-70% of
465 lactate formation within the fermentors. The largest contribution occurred with volunteer A
466 at pH 5.5, who had the greatest abundance of *Bifidobacterium* spp. (47% at pH 5.5)
467 whereas for this volunteer at the higher pH and the other three volunteers at both pH only a
468 maximum of 21% of lactate formation could be accounted by bifidobacteria. These
469 observations show that other microorganisms make a very important contribution to
470 lactate-production. Apart from other lactic acid bacteria such as *Lactobacillus* spp.,
471 additional bacterial groups known to synthesise lactate are *Collinsella* spp., *E.*
472 *rectale/Roseburia* spp., *Faecalibacterium prausnitzii*, and *Bacteroides* spp. (Macfarlane &
473 Gibson, 1991; Barcenilla *et al*., 2000; Duncan *et al*., 2002). The latter four groups include
474 the most abundant bacterial species found within the human intestinal microbiota (Tap *et*

475 *al*., 2009, Walker *et al*., 2010) and typically account for >50% of total faecal bacteria (e.g.

476 Duncan *et al*., 2007).

477 *E. hallii, A. caccae* and the new species *A. coli* (Walker *et al*., 2010) have been

478 identified as lactate utilizers (Duncan *et al*., 2004b) that form butyrate as the end product in

479 the presence of fermentable polysaccharides (Belenguer *et al*., 2007). On an equi-cell

480 basis, the ability of *E. hallii* to metabolise lactate exceeds considerably the capacity for

481 lactate production by bifidobacteria (Table 6), but there was no relationship between *E.*

482 *hallii* abundance and total lactate utilisation ($P > 0.10$). Furthermore, the near-maximal rate

483 of lactate disposal by *E. hallii* (Table 6) when combined with the numbers present in the

484 fermentors, would only account for 1.2-18.0% of lactate total disposal, with < 4.8% in

485 most cases. The situation is somewhat different when only lactate converted to butyrate is

486 considered, however, and where 0-47% could be attributed to the action of *E. hallii.* Thus,

487 other bacteria must play important roles in the utilization of lactate, including conversion

488 to butyrate. Interestingly, recent evidence indicates that *A. coli*, that may only utilise D-

489 lactate, is of similar abundance to *E. hallii* in the human colon (Walker *et al*., 2010). Other

490 candidates not detected by the FISH probes used here include *Coprococcus catus* (Louis &

491 Flint, 2009) and bacteria related to *Megasphaera elsdenii* and *Eubacterium limosum* (Sato

492 *et al*., 2008). Involvement of these other bacteria would explain why lactate disposal in the

493 fermentors (Table 1) was not pH-sensitive and why butyrate was not always the dominant

494 end-product.

495 In summary, lactate was efficiently used at two physiological pH, 5.5 and 6.0, in

496 continuous fermentor systems and, in most cases, exceeded rates of lactate production by

497 species such as *Bifidobacterium*. This ability to dispose of lactate in excess of the amounts

498 normally produced should be viewed as a beneficial trait for the human colon where

499 moderate to high accumulation of lactate are usually associated with detrimental responses

500 (Ewaschuck *et al*., 2005). While some of the key players have been identified, the relative

501 importance of different bacterial species in lactate formation and disposal within the
502 microbial community has still to be established.
503
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655

656

657 **Table 1.** Lactate formation ($F_{l.in}$) rates in single stage continuous fermentors inoculated

658 with faecal suspensions from four different volunteers estimated after a bolus injection of

659 labelled [3-^{13}C]-lactate plus DL-lactate at two different pH values (5.5 and 6.0) on day 6 of

660 study.

	Bolus size[a] (µmol mL^{-1})	$F_{l.in}$ (µmol mL^{-1} h^{-1})
Volunteer A		
pH 5.5	2.67	0.28
pH 6.0	2.32	0.07
Volunteer B		
pH 5.5	2.36	0.19
pH 6.0	2.32	0.05
Volunteer C		
pH 5.5	2.25	0.09
pH 6.0	2.56	0.06
Volunteer D		
pH 5.5	2.24	0.34
pH 6.0	2.26	0.12
SED		0.044
P for pH		0.043

661 [a] Includes both DL-lactate plus L[^{13}C]lactate.

662 Data were analysed by analysis of variance, with volunteer as random effect and pH as

663 fixed effect.

664 SED, standard error of the difference.

665

666 **Table 2.** Estimated carbon flows (µmol C_2 mL^{-1} h^{-1}) between starch, lactate, acetate,
667 propionate and butyrate estimated from the continuous infusion of labelled [$^{13}C_6$]-starch
668 and [3-^{13}C]-lactate and the bolus injection of [1-^{13}C]-acetate, [1,2-^{13}C]-acetate and [1-^{13}C]-
669 propionate in single stage continuous fermentors inoculated with faecal suspensions from
670 four different volunteers at two different pH values (5.5 and 6.0).

	pH 5.5	pH 6.0	SED	*P* for pH
Lactate production ($F_{l.in}$)	0.23	0.08	0.044	0.044
From starch (F_{ls})	0.09	0.03	0.017	0.043
From other sources (F_{lx})	0.14	0.05	0.027	0.044
Lactate utilization ($F_{l.out}$)	0.25	0.13	0.038	0.053
Acetate production ($F_{a.in}$)	2.13	1.72	0.417	0.401
From starch (F_{as})	0.60	0.38	0.207	0.358
From lactate (F_{al})	0.18	0.06	0.027	0.023
From other sources (F_{ax})	1.34	1.28	0.261	0.843
Acetate utilization ($F_{a.out}$)	1.09	1.08	0.475	0.987
Propionate production ($F_{p.in}$)	0.48	0.50	0.040	0.657
From starch (F_{ps})	0.24	0.33	0.067	0.285
From lactate (F_{pl})	0.07	0.06	0.012	0.669
From other sources (F_{px})	0.17	0.11	0.061	0.438
Butyrate production ($F_{b.in}$)	0.58	0.35	0.200	0.346
From acetate (F_{ba})	0.58	0.35	0.202	0.347
From lactate (via acetate)	0.071	0.024	0.0220	0.120
From lactate (direct; F_{bl})	<0.002	<0.003	0.0025	0.677

671 Data were analysed by analysis of variance, with volunteer as random effect and pH as
672 fixed effect.
673 SED, standard error of the difference.
674

675 **Table 3.** Proportion of the different fates of lactate carbon (acetate, propionate, butyrate)

676 and proportion of butyrate derived from lactate in single stage continuous fermentors

677 inoculated with faecal suspensions from four different volunteers at two different pH

678 values (5.5 and 6.0). The proportions of lactate going to butyrate and of butyrate derived

679 from lactate includes both direct and indirect (via extracellular acetate) routes.

Volunteer	A		B		C		D		Means			
pH	5.5	6.0	5.5	6.0	5.5	6.0	5.5	6.0	5.5	6.0	SED	*P* for pH
Fates of lactate (%)												
To propionate	37.5	50.5	18.2	37.3	4.8	29.0	36.6	68.5	24.2	46.3	4.00	0.012
To butyrate (direct and via acetate)	0.0	13.1	35.2	11.0	66.9	38.6	28.8	19.6	32.7	20.6	9.36	0.285
To acetate[a]	62.5	36.4	46.6	51.7	28.3	32.4	34.6	11.8	43.0	33.1	8.41	0.324
Sources of butyrate (%)												
From lactate (direct and via acetate)	0.0	6.1	20.0	3.8	8.9	12.9	13.4	7.0	10.6	7.5	4.62	0.549

680 [a] Accounts for the lactate-C remaining in acetate, not further metabolized into other

681 products.

682 Data were analysed by analysis of variance, with volunteer as random effect and pH as

683 fixed effect.

684 SED, standard error of the difference.

685

686 **Table 4.** Total counts (log_{10}) per ml from inoculation of total bacteria (using the universal

687 probe Eub338) and the *Bifidobacterium* spp. and *Eubacterium hallii* groups (using the

688 probes Bif164 and Ehal1469) initially and after 5 days of incubating faecal slurries from

689 four different volunteers in continuous flow fermenters.

	Total numbers (log_{10})		
	Eub	Bif	Ehal
Volunteer A			
Initial count[a]	7.50	6.13	4.46
pH 5.5	8.27	7.94	5.36
pH 6.0	8.34	6.25	5.82
Volunteer B			
Initial count	7.06	5.69	4.84
pH 5.5	7.91	6.36	5.70
pH 6.0	8.31	6.27	5.84
Volunteer C			
Initial count	7.53	6.11	4.22
pH 5.5	8.28	6.91	5.48
pH 6.0	8.60	6.17	6.43
Volunteer D			
Initial count	7.56	6.35	4.13
pH 5.5	8.43	6.97	5.76
pH 6.0	8.35	6.49	5.74
Means			
Initial count	7.41	6.07	4.41
pH 5.5	8.22	7.04	5.57
pH 6.0	8.40	6.30	5.96
SED	0.111	0.336	0.184
P-value	0.208	0.112	0.128

690 [a] Estimated from faecal counts and taking into account the slurry preparation.

691 SED, standard error of the difference.

692

693 **Table 5.** Net formation or utilization of lactate, acetate, propionate and butyrate (in C_2

694 units) in 24 h-incubated batch cultures inoculated with faecal slurries prepared from 4

695 different healthy volunteers (A, B, C and D) with a mixture of carbohydrates plus 35 mmol

696 L^{-1} lactate and at two pH values (5.9 and 6.7).

697

		Lactate	Acetate	Propionate	Butyrate
Mix	5.9	0.54	9.66	1.35	7.47
	6.5	-0.09	12.6	3.74	6.16
Mix+lactate	5.9	-21.6	2.85	2.80	30.0
	6.5	-33.6	4.91	8.03	38.8
	SED	0.911	0.598	0.319	1.370
	pH	<0.001	<0.001	<0.001	0.007
P-value	substrate	<0.001	<0.001	<0.001	<0.001
	Week	0.73	0.14	0.003	0.019
	Substrate x pH	<0.001	0.49	<0.001	<0.001

698 SED, standard error of the difference.

699

700 **Table 6.** Pure culture data for metabolic rates of lactate formation from starch by

701 *Bifidobacterium adolescentis* (L2-32) and utilization of lactate by *Eubacterium hallii* (L2-

702 7).

703

pH	Formation μmol 10^9cells^{-1} h^{-1}	Utilization μmol 10^9cells^{-1} h^{-1}
5.7	2.36	12.07
6.7	1.23	7.69
SED	0.219	0.844
P for pH	0.007	0.007

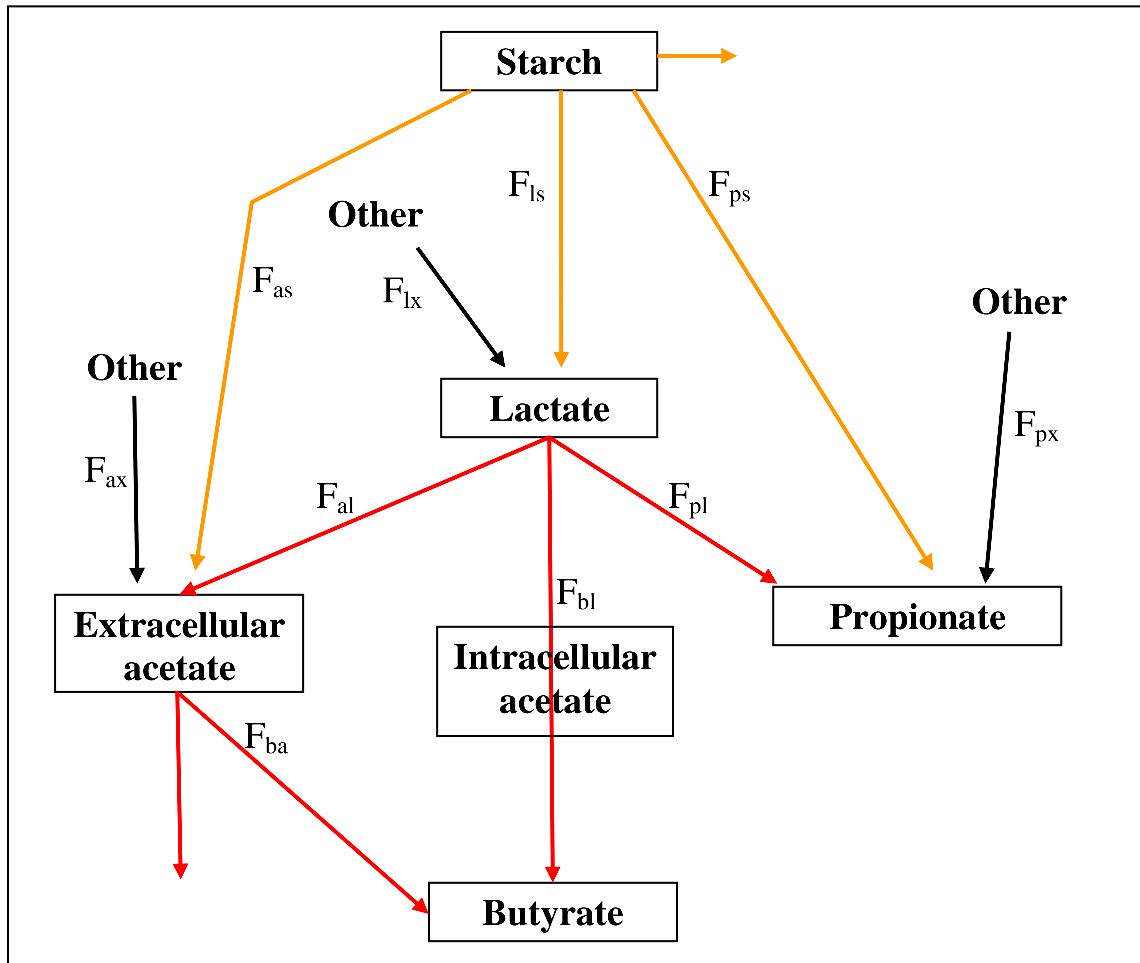
704 SED, standard error of the difference

705

706 **Fig. 1.** Tracer and tracee flows. Assumed to be in C_2 units. Black: tracee flow; Orange:
707 M+2 and tracee flows; Red: M+2, M+1 and tracee flows. All pools also have loss of
708 material via the effluent, but this has been omitted from the schematic below.
709
710 **Fig. 2.** Time course of the concentrations of acetate (diamond), propionate (triangle),
711 butyrate (circle) and lactate (square) in single stage-fermentor systems at two different pH
712 values (5.5 and 6.0) using four different volunteers (A, B, C, and D).
713
714 **Fig. 3.** Relationship between 24h lactate utilisation (mmol L^{-1}) and butyrate formation
715 (mmol L^{-1}) in batch cultures inoculated with faecal samples from 4 volunteers (different
716 symbols for each volunteer), with a mixture of carbohydrates and DL-lactate (35.6 mmol
717 L^{-1}) as substrates at pH 5.9. ($P < 0.001$ and adjusted $r^2 = 0.79$)
718
719

720 **Fig. 1.**

721



722

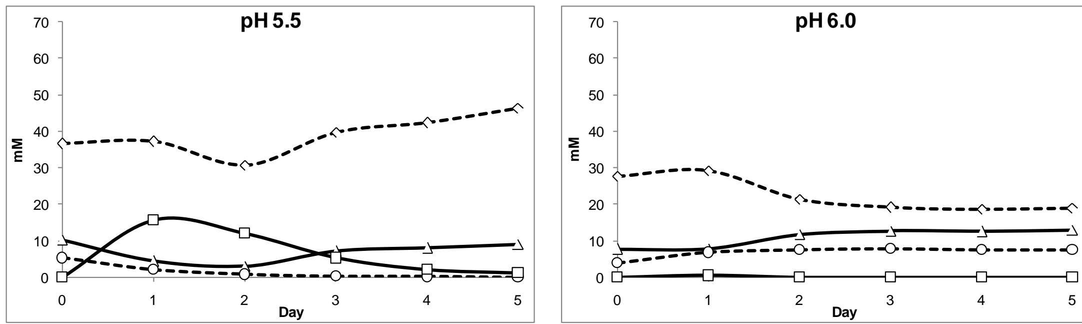
723

724

725

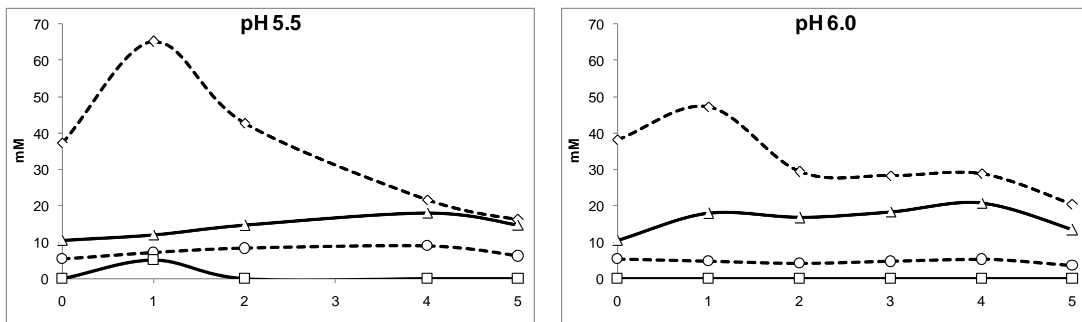
726 **Fig. 2.**

727 **A.**



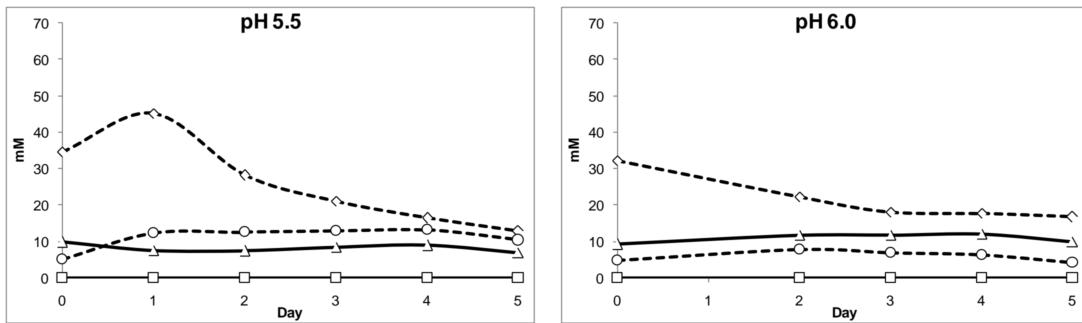
728

729 **B.**



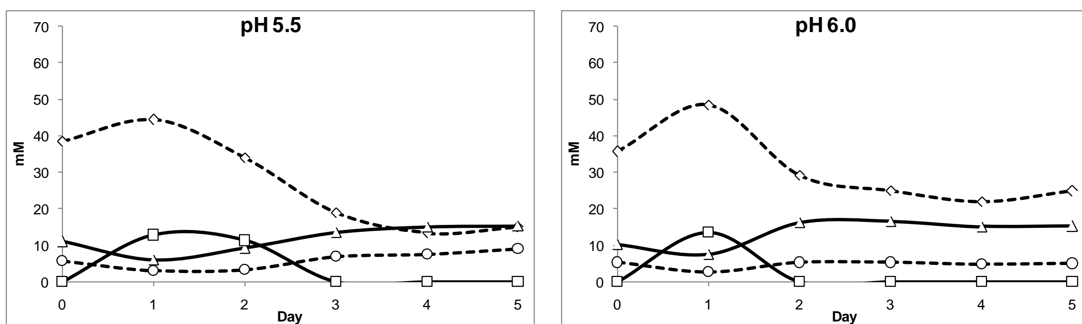
730

731 **C.**



732

733 **D.**



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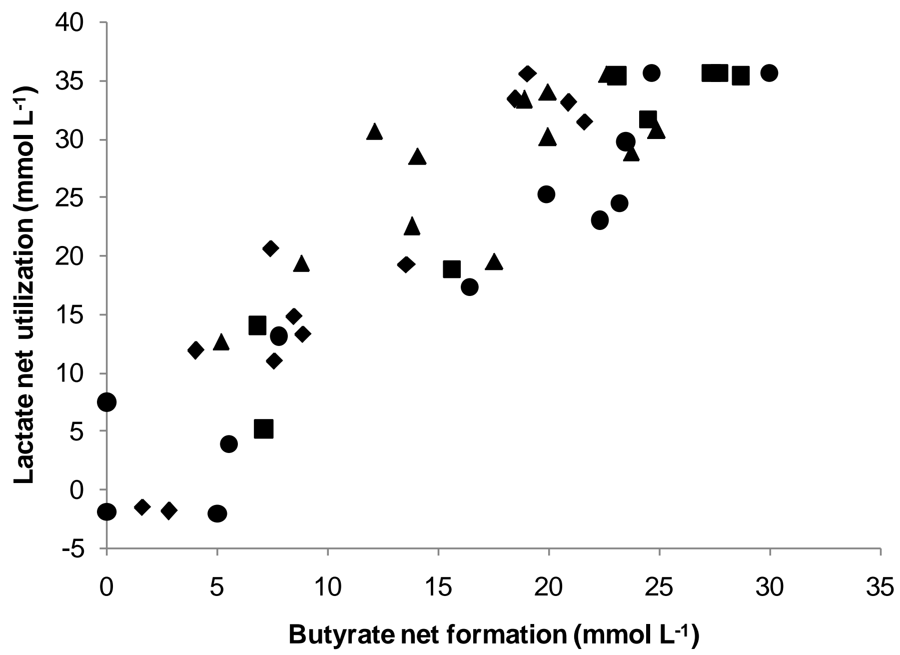
735

736

737 **Fig. 3.**

738



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