Rates of production and utilisation of lactate by microbial communities from the human colon

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Running title: Lactate metabolism by colonic microbiota

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Abstract

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

Introduction

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

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

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

bacteria (Bourriaud *et al*., 2005; Morrison *et al*., 2006), within the normal physiological pH range (Belenguer *et al*., 2007). At pH 5.2, however, lactate utilization was curtailed and this metabolite accumulated (Belenguer *et al*., 2007). This may explain high lactate concentrations in severe colitis (Vernia *et al*., 1988) where the colonic pH can approach that of the stomach (Fallingborg *et al*., 1993). The contribution of various bacterial species to lactate utilization remains ill-defined, however, but several are known to convert lactate to propionate or butyrate (Duncan *et al*., 2004b; Morrison *et al*., 2006; Falony *et al*., 2006). These include *Eubacterium hallii, Anaerostipes caccae* and an un-named species (Duncan *et al*., 2004b) that are butyrate-producing bacteria and belong to the dominant core group of species in the human intestinal microbiota (Tap *et al*., 2009; Walker *et al*., 2010).

Maintenance of low amounts of lactate within the colon represents a balance between utilization and production and imbalances in either can cause lactate accumulation. The current study uses two approaches, long-term (6 days) continuous fermentors and short term (24h) batch cultures to estimate rates of lactate production and utilization and determine if these link to certain bacterial groups. The pH of the culture media was shown to modify rates of lactate metabolism and stable isotope approaches were used to allow quantification of flow from starches to lactate and to end-product metabolites.

Material and methods

Collection of faecal samples

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

Continuous flow fermentor incubations

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

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

the infusion of labelled starch and lactate. The following day (day 6) both vessels received a bolus injection of L-[3-^{13}C]lactate (approximately 23 and 28 μmol in vessels 1 and 2, respectively) plus DL-lactate (approximately 230 and 275 μmol in vessels 1 and 2, respectively).

Daily samples were taken from each vessel to monitor SCFA and lactate concentrations. On the infusion day (day 5) samples were taken every 30 min for the first 3 h of the infusion and hourly thereafter until 10 h to measure SCFA and lactate concentrations and metabolite ^{13}C enrichments. On the injection day (day 6) samples were taken at 30 min intervals from just before until 4 h after the bolus injection, with lactate concentrations and metabolite ^{13}C enrichments measured.

Batch culture incubations

Fresh faecal samples from the same four volunteers (A, B, C and D) were collected weekly at 12 occasions over a three month period. Slurries of this material were used for batch culture incubations with an anaerobic medium similar to that used for the continuous flow fermentor incubations, based on Macfarlane *et al*. (1989) as modified by Walker *et al*. (2005). The carbohydrate sources present in the mixed substrate medium were potato starch (0.14% w/v) in addition to xylan, pectin, amylopectin, and arabinogalactan each at 0.015% (w/v). The total peptide concentrations (comprising equal amounts of casein hydrolysate and peptone water) were 0.2%. Samples were inoculated at two different pH values (mean ± standard deviation 5.9 ± 0.2 and 6.5 ± 0.2) and with either a carbohydrate mixture alone or with DL-lactate (approximately 35 mmol L^{-1} initial concentration) also present. SCFA were also added to the medium to give initial concentrations of approximately 33 mmol L^{-1} acetate, 9 mmol L^{-1} propionate, 5 mmol L^{-1} butyrate and 1 mmol L^{-1} each of valerate, iso-valerate, and iso-butyrate. The fermentor medium was dispensed into Hungate tubes under a stream of CO_2 (Miyazaki *et al*., 1997) and heat

sterilised at 121 ^{o}C (15 min). After cooling, heat-labile vitamins were added and the medium was inoculated with the faecal slurry under CO_2 and incubated at 37 ^{o}C. Faecal slurries (20%) were prepared within 2 h of collection in anaerobic phosphate buffer saline to give a final concentration of approximately 0.2%. Tubes were inoculated in duplicate and samples were taken at 24 h to measure SCFA and lactate concentrations. Samples of uninoculated medium were also taken to measure initial concentrations and initial pH values.

Quantification of bacteria in faecal and continuous fermentor samples by fluorescent in situ hybridization analysis

Samples were taken from faeces (0.5 g) and the fermentor incubations on day 5 (1 ml) for fluorescent in situ hybridization (FISH) analysis. Faecal samples were diluted with phosphate buffer (1:10), and all samples were fixed by mixing 1:3 in 4% (w/v) paraformaldehyde at 4°C for 16 h and stored at -20°C. FISH analysis was performed as described by Harmsen *et al*. (2002). Diluted cell suspensions were applied to gelatin-coated slides and the slides were hybridized overnight with the appropriate probes. 50 µl of Vectashield (Vector Laboratories, Burlingame, CA) was applied to each slide to prevent fading. Cells were counted automatically using image analysis software CellF (Olympus Soft Imaging Solutions GmbH, Germany) with an Olympus microscope, except when the number of cells was less than 10 per field of view, in which case the cells were counted manually. For each sample 30 microscopic fields were counted and the data averaged. All samples were assessed with the following probes: total bacteria (Eub338, Amann *et al*., 1990), *Bifidobacterium* spp. (Bif164, Langendijk *et al*., 1995), as lactate-producing bacteria, and the *Eubacterium hallii* (Ehal1469, Harmsen *et al*., 2002) and *Anaerostipes caccae* (Acac194, Hold *et al*., 2002) groups, as potential lactate utilisers.

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

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

Metabolic activities of pure cultures of bacteria

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

For both species, the number of bacteria in the respective incubations was determined by optical density (1 $OD_{600}=10^9$ cells; Lech *et al*., 1987).

Kinetic modelling

The model structure and fates of the various isotopes are shown in Fig. 1. Let q, Q, and E denote the labelled amount (µmol), the total (labelled plus unlabelled) amount (µmol) and enrichment (0.01 molar % excess, MPE) of either acetate, butyrate, propionate, lactate, or starch, denoted by subscripts 'a', 'b', 'p', 'l' and 's', respectively. Let i denote the interval between any two times t_0 and t_1, with $t_1 > t_0$, and let F(i) denote the flow of a metabolite (labelled plus unlabelled) during i. Eff(i) denotes the loss to the effluent during interval i, and E(i) denotes the average enrichment during i. Subscript 'in' refers to inflow (production) and subscript 'out' refers to use in further metabolic processes (e.g. acetate used to produce butyrate). For example, $F_{a.in}$ refers to acetate production, while $F_{a.out}$ stands for acetate outflow, etc. Flows to pool y from pool x are denoted by F_{yx}. Q, q, E and Eff were measured, whilst the F_{yx}, F_{in} and F_{out} were unknown.

Data are expressed in terms of two carbon (C_2) units, to allow for 'molar equivalent' transfers. To achieve this, the concentration of butyrate is multiplied by 2 and the enrichment divided by 2. The enrichments of propionate and lactate, in terms of C_2 units, are given as 0.01 (MPE(M+2) + MPE(M+3)). The concentrations of acetate, propionate and lactate and their M+1 enrichments are as measured directly.

Infusion day (day 5):

Calculations are based on time points during the continuous infusion of labelled starch and lactate between 4 and 10 h, after the prime doses of labelled acetate and propionate. Lactate formation ($F_{l.in}$) and utilisation ($F_{l.out}$) were obtained from the changes in labelled

(M+1) and total (labelled plus unlabelled) lactate as observed during the continuous infusion of [3-^{13}C]lactate:

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

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

It was assumed that butyrate was formed ($F_{b.in}$) via two pathways, either through the extracellular acetate pool (F_{ba}) or directly from lactate (F_{bl}). First, from the changes in the total butyrate concentration $F_{b.in}$ was obtained:

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

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

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

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

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

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

where it was assumed that propionate has no further metabolic fates, i.e. $F_{p.out} = 0$. Changes in labelled propionate derived from 3-^{13}C-lactate were modelled as:

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

which then provided an estimate for F_{pl}.

The incorporation of lactate into acetate (F_{al}) is obtained from assuming that lactate may be utilised only to produce acetate, butyrate and propionate: $F_{l.out} = F_{al} + F_{bl} + F_{pl}$. Furthermore, the M+1 acetate movements yield an estimate for $F_{a.out}$, based on:

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

$F_{a.in}$ follows from

$$Q_a(t_1) = Q_a(t_0) + F_{a.in}(i) - F_{a.out}(i) - Eff_a(i) \quad (8)$$

Incorporation of starch into acetate (F_{as}), lactate (F_{ls}) and propionate (F_{ps}) was obtained from the changes in labelled metabolites that were produced from the infused [U-^{13}C] starch. For F_{ls}:

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

Here it is assumed that both M+2 and M+3 lactate were formed from [U-^{13}C]starch (i.e. $E_{l.m+2}$ = 0.01 $MPE_{lactate}$(M+2) + 0.01 $MPE_{lactate}$(M+3)). Making similar assumptions for propionate, F_{ps} follows from:

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

Incorporation of starch into acetate follows from:

$$q_{a.m+2}(t_1) = q_{a.m+2}(t_0) + E_{l.m+2}(i)\ F_{al}(i) + E_s\ F_{as}(i) - E_{a.m+2}(i)\ F_{a.out}(i) - E_{a.m+2}(i)\ Eff_a(i) \quad (11)$$

Bolus injection of labelled lactate (day 6):

Except in the vessel at the lower pH (5.5) inoculated with a faecal suspension from volunteer A, no lactate was detected so that lactate enrichments $E_{l.m+1}$ and $E_{l.m+2}$ could not be determined. Estimates of lactate formation were based, instead, on samples collected following the bolus injection of [3-^{13}C]lactate on day 6. Lactate utilisation ($F_{l.out}$) on day 6 was obtained from the changes in labelled (M+1) lactate (based on equation (1), with 'Infusion' set equal to zero). This was then used to obtain $F_{l.in}$, based on changes in total (labelled plus unlabelled) lactate (equation (2), with 'Infusion' set to zero). The remaining calculations are based on the day 5 measurements, as follows. It was assumed that $F_{l.in}$ was the same on days 5 and 6 and this was substituted in equation (2) to derive $F_{l.out}$ on day 5. Subsequently, an estimate of the lactate M+1 enrichment on day 5, denoted by $E^*_{l.m+1}$, was obtained from $E^*_{l.m+1}$ = Infusion rate / ($F_{l.in}$ + Infusion rate), assuming that the infusate was

fully labelled. In subsequent calculations, $E^{*}_{l.m+1}$ replaced $E_{l.m+1}$, so that F_{bl}, F_{ba}, F_{pl}, F_{al}, $F_{a.out}$ and $F_{a.in}$ could be calculated as before, based on equations (4), (6)-(8). To estimate the fates of starch, it was assumed that 40% of the lactate came from starch, so that the lactate M+2 enrichment was assumed to be 0.4 of the starch enrichment. This estimate of the lactate M+2 enrichments, denoted as $E^{*}_{l.m+2}$, then replaced $E_{l.m+2}$ in equations (9) – (11) to provide estimates for F_{ls}, F_{ps} and F_{as}. Note that $F_{b.in}$ and $F_{p.in}$ (equations (3) and (5) respectively) are unaffected by any of the assumptions.

The assumption of $F_{l.in}$ on day 5 being equal to that on day 6 was supported by data from donor A at pH 5.5 (0.29 and 0.28 µmol mL^{-1} h^{-1} respectively. Data from the same volunteer showed that approximately 40% of the lactate came from starch and this value was applied to the other volunteers. Based on the model adopted, this assumption should only influence the calculation of F_{ls}, F_{ps} and F_{as}. In practice, sensitivity analysis with the proportion of lactate from starch varied from 1 to 99% only impacted seriously on F_{as}, F_{ps} and F_{px}, with the coefficient of variation lower than 36%, except for F_{px} that showed flows lower than 0.15 µmol mL^{-1} h^{-1}.

Statistical analysis

Where SCFA data were replicated the average values were used. The daily SCFA data from the continuous fermentors were analysed as repeated measures, with volunteer and time point nested within volunteer as random effects, while time point, pH and their interaction were taken as fixed effects. The weekly lactate data from the batch culture incubations were analysed using the same random structure, but with fixed effects now consisting of time point, pH, substrate and their interactions. To account for dependency on previous time points, a suitable covariance structure (compound symmetry) was fitted on the basis of Schwarz's Bayesian information model fit criterion**.** Quantities (such as carbon flows, numbers of bacteria) obtained from the day 5 (or day 6) data in the continuous

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

Results

Concentrations of SCFA and lactate over time in continous flow fermenters

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

Rates of lactate formation and utilisation

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

calculated based on formation determined on day 6 (Table 1) plus metabolite masses and enrichments from day 5. These parameters of endogenous lactate metabolism are presented in Table 2.

Lactate formation was consistently greater at the lower pH ($P < 0.05$; Tables 1 and 2) and a similar trend ($P = 0.053$) was also seen for endogenous lactate utilization (Table 2). As expected, rates of production and utilization were closely matched to maintain constant lactate concentrations, even below the limit of detection.

Carbon flows between starch, lactate and SCFA in continous fermentors

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

The proportions of lactate carbon converted to acetate, propionate and butyrate were 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

complete in most incubations at pH 6.5 and was always greater ($P < 0.001$) than at pH 5.9 (Table 5). In the absence of lactate, acetate was the main end product, whereas butyrate accumulated ($P < 0.001$) when lactate was present. The presence of lactate also decreased net production of acetate ($P < 0.001$) but increased net formation of propionate ($P < 0.001$). Furthermore, net production of all three of these SCFA was enhanced at the higher pH ($P < 0.005$), although for propionate and butyrate this effect was more pronounced with the mixture plus lactate than the mixture alone (interaction of substrate x pH, $P < 0.001$). Net lactate utilization was also greater at the higher pH, but again this occurred mainly in the presence of lactate (interaction of substrate x pH, $P < 0.001$). At the lower pH (5.9) and with the mixture plus lactate cultures, a linear relationship was observed between net lactate utilisation and butyrate formation ($P < 0.001$; adjusted $r^2 = 0.79$; Fig. 3).

Responses varied between volunteers and weeks. For example, net lactate utilisation and butyrate production were lower for volunteers C and D than A and B, at the lower pH, and net formation of propionate and butyrate differed between weeks.

Activity of pure cultures

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

Discussion

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

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

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

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

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652 that increases dietary starch in the human colon. *Appl Environ Microbiol* **65**: 2807-
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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

Table 2. Estimated carbon flows (μmol C_2 mL^{-1} h^{-1}) between starch, lactate, acetate, propionate and butyrate estimated from the continuous infusion of labelled [$^{13}C_6$]-starch and [3-^{13}C]-lactate and the bolus injection of [1-^{13}C]-acetate, [1,2-^{13}C]-acetate and [1-^{13}C]-propionate in single stage continuous fermentors inoculated with faecal suspensions from 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

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

SED, standard error of the difference.

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

Fig. 1. Tracer and tracee flows. Assumed to be in C_2 units. Black: tracee flow; Orange: M+2 and tracee flows; Red: M+2, M+1 and tracee flows. All pools also have loss of material via the effluent, but this has been omitted from the schematic below.

Fig. 2. Time course of the concentrations of acetate (diamond), propionate (triangle), butyrate (circle) and lactate (square) in single stage-fermentor systems at two different pH values (5.5 and 6.0) using four different volunteers (A, B, C, and D).

Fig. 3. Relationship between 24h lactate utilisation (mmol L^{-1}) and butyrate formation (mmol L^{-1}) in batch cultures inoculated with faecal samples from 4 volunteers (different symbols for each volunteer), with a mixture of carbohydrates and DL-lactate (35.6 mmol L^{-1}) as substrates at pH 5.9. ($P < 0.001$ and adjusted $r^2 = 0.79$)

720 **Fig. 1.**

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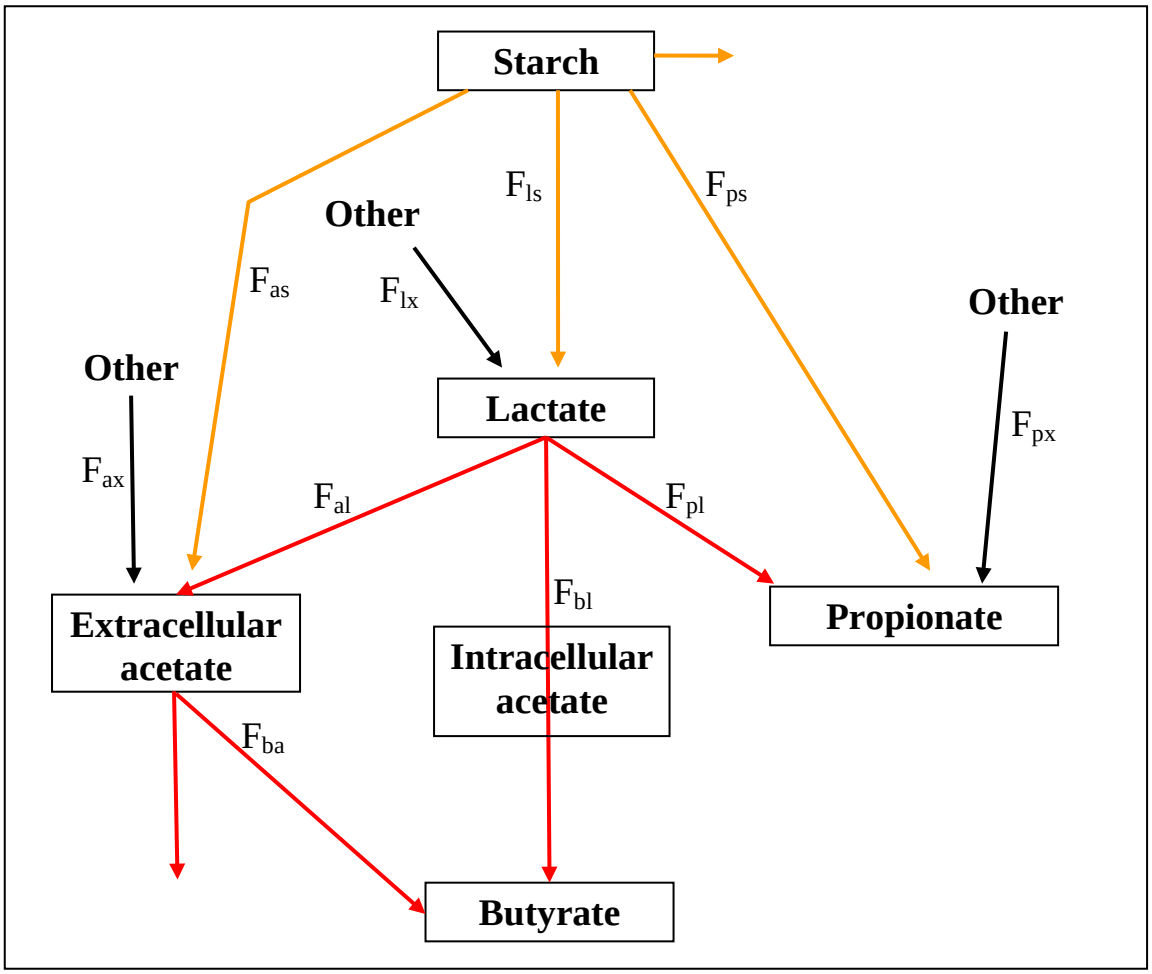

722

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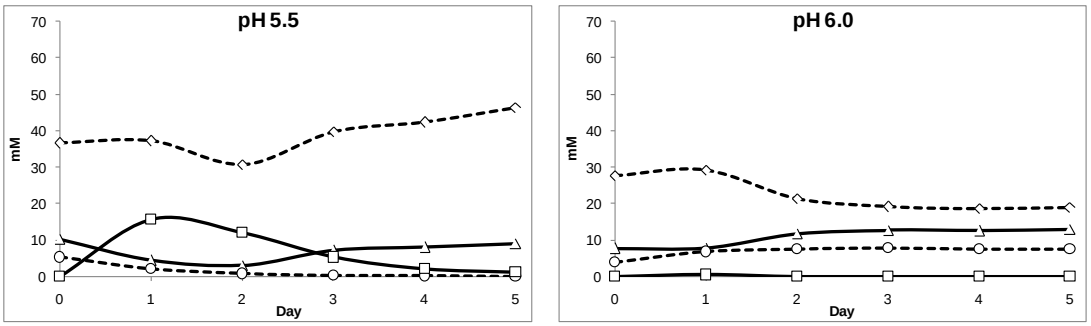
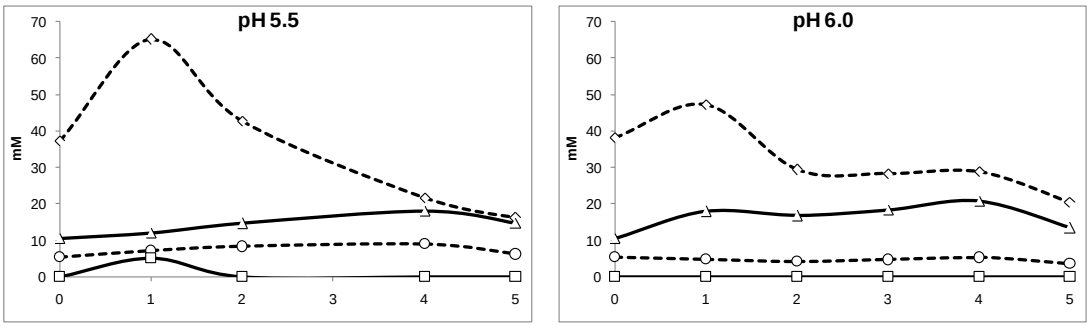
Fig. 2.

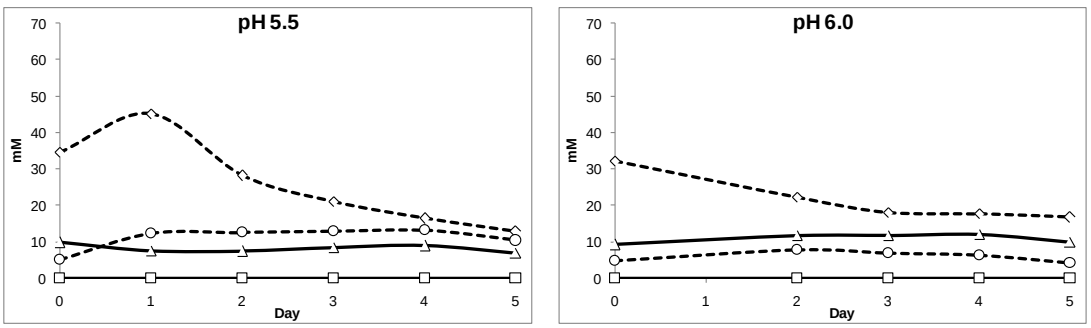
A.


pH 5.5
pH 6.0
mM
Day
0
10
20
30
40
50
60
70
0
1
2
3
4
5

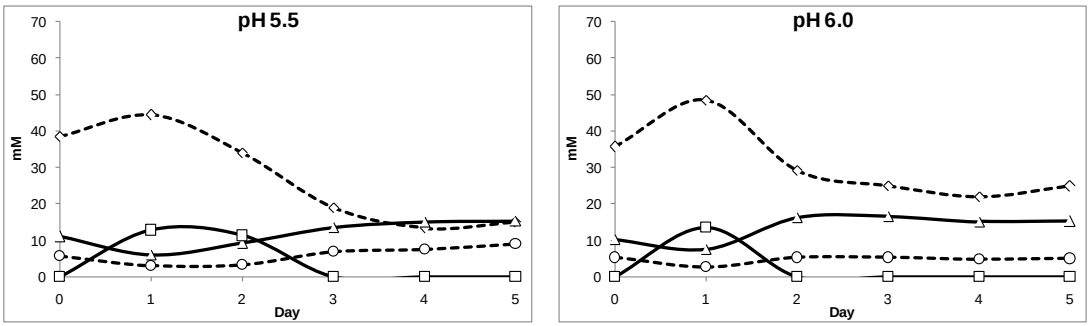
B.


pH 5.5
pH 6.0
mM
Day
0
10
20
30
40
50
60
70
0
1
2
3
4
5

C.


pH 5.5
pH 6.0
mM
Day
0
10
20
30
40
50
60
70
0
1
2
3
4
5

D.


pH 5.5
pH 6.0
mM
Day
0
10
20
30
40
50
60
70
0
1
2
3
4
5

737 **Fig. 3.**

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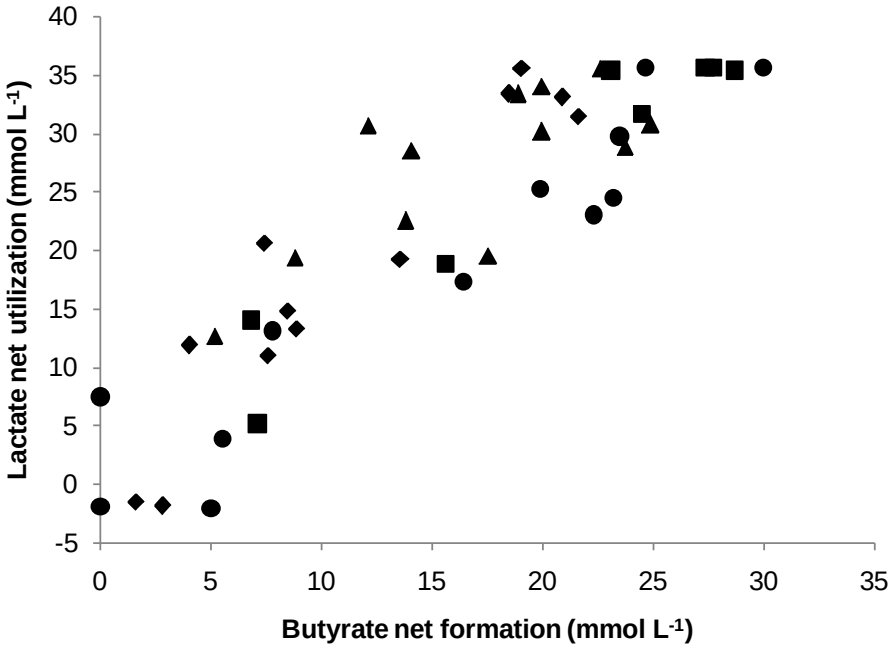

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740

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