

## Butyrate production from oligofructose fermentation by the human faecal flora: what is the contribution of extracellular acetate and lactate?

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Butyrate is an important substrate for maintenance of colonic health and oligofructose fermentation by human faecal bacteria can increase butyrate production *in vitro*. However, oligofructose appears to be fermented by mainly acetate and lactate-producing bacteria rather than butyrate-producing bacteria. Isotope labelling studies using [U-<sup>13</sup>C<sub>6</sub>]glucose were used to show that <sup>13</sup>C<sub>2</sub> and <sup>13</sup>C<sub>4</sub> were the major labelled butyrate species produced from glucose fermentation, via [<sup>13</sup>C<sub>2</sub>]acetate–acetyl CoA as intermediate. Bacterial interconversion reactions were quantified and acetate conversion to butyrate and lactate conversion to acetate, propionate and butyrate were observed. Addition of oligofructose to faecal batch cultures significantly increased butyrate production. Of the newly synthesised butyrate from oligofructose fermentation, 80% was derived from interconversion of extracellular acetate and lactate, with acetate being quantitatively more significant. Carbohydrates, such as oligofructose, have prebiotic properties. In addition, oligofructose selectively stimulates the bacterial conversion of acetate and lactate to butyrate. Carbohydrates with similar properties represent a refinement of the prebiotic definition, termed butyrogenic prebiotics, because of their additional functionality.

### Short-chain fatty acids: Butyrate: Stable isotopes: Oligofructose: Fermentation

During bacterial fermentation of non-digestible carbohydrates (NDC) in the large intestine, the SCFA anions acetate, propionate and butyrate are quantitatively the most important products (Cummings & Macfarlane, 1991). The rate, relative ratios and extent of SCFA production depend both upon the composition of the colonic microflora and the chemical properties of NDC available for fermentation (Laurentin & Edwards, 2004). There are important nutritional (in early life) and health (throughout life) benefits of colonic bacterial SCFA production. In neonates and in populations on marginal diets, acetate and lactate may be important sources of energy salvage (Parrett & Edwards, 1997) whilst butyrate exhibits anti-inflammatory properties (Luhrs *et al.* 2002; Vernia *et al.* 2003) and may confer protection against colon cancer (Lipkin *et al.* 1999; Nkondjock *et al.* 2003). If dietary strategies to optimise butyrate production and improve colonic health, by selecting specific NDC, are to succeed, a greater understanding of the mechanisms by which butyrate production can be promoted is required.

All dietary fermentable carbohydrates (NDC and unabsorbed monosaccharides) that reach the large intestine have the potential to produce butyrate. Therefore, optimising colonic butyrate production through the consumption of dietary NDC may confer protection against colonic inflammation and disease. However, not all NDC are equally butyrogenic. For example,

resistant starch (Champ, 2004) and oligofructose (OF; Kleessen *et al.* 2001) are associated with greater butyrate production, as a proportion of total SCFA production, than pectin (Berggren *et al.* 1995) or alginate (Michel *et al.* 1996). The chemical and physical properties of an NDC will influence which bacterial flora are involved in its fermentation and therefore SCFA production profiles. For example, OF is thought to be selectively fermented by bifidobacteria because they produce an inulinase ( $\beta$ -fructosidase) which cleaves the  $\beta$ (2-1) present in OF and inulin (Damian *et al.* 1999). Other factors influencing SCFA production are the presence of terminal electron acceptors (Allison & Macfarlane, 1988) and site of fermentation (Eckburg *et al.* 2005). In bacterial metabolism, butyrate production represents reduction and therefore electron disposal which, when coupled to the re-oxidation of reduced co-factors, can drive additional energy production (Miller & Jenesel, 1979). It is reasonable to assume, given the importance of butyrate to colonic epithelial metabolism, that butyrate-producing bacteria are well tolerated by the innate immune system. Previous reports have highlighted the biochemical complexity of butyrate production (Pryde *et al.* 2002). If manipulating colonic butyrate production through consumption of dietary NDC is to be an effective nutritional strategy to improve colonic health, quantification of the biochemical reactions that result in butyrate production from NDC is necessary.

**Abbreviations:** MPE, mole percent excess; NDC, non-digestible carbohydrates; OF, oligofructose.

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In the rumen, isotope tracer experiments have shown that up to 60% of butyrate is synthesised directly from extracellular acetate through interconversion reactions (Bergman *et al.* 1965; Linington *et al.* 1998). Extrapolating these observations to the human subject are unwise, however, because of nutritional, anatomical, physiological and microbiological differences. Other notable interconversion reactions were also observed in the rumen, including recycling of butyrate to acetate. Acetate, which is often regarded as an end product of fermentation, has been shown to be further metabolised by the human colonic microflora to produce butyrate (Duncan *et al.* 2004a). More recently, lactate has also been shown to act as a precursor for butyrate synthesis (Bourriaud *et al.* 2005) but the quantitative significance of these combined mechanisms to overall butyrate production from a butyrogenic NDC remains unclear. The aim of the present study therefore was to identify the major biochemical interconversions between SCFA in the human intestinal microflora and quantify the contribution of extracellular acetate and lactate to butyrate production from the butyrogenic NDC, OF.

## Experimental methods

### Subjects

Subjects were recruited to each study from the staff of the Division of Developmental Medicine, University of Glasgow, UK, and asked to provide a single faecal sample. None was receiving antibiotics or had gastrointestinal complaints during the 3 months prior to the study. The study was approved by the University of Glasgow ethical committee for non-clinical investigations in human volunteers.

### SCFA production from [ $U$ - $^{13}C_6$ ]glucose: experiment 1

Five healthy adults (three females and two males; age range 23–55 years) were recruited and asked to provide a single faecal sample. To identify the major SCFA isotopomers produced from an isotopically labelled carbohydrate, [ $^{13}C_6$ ]glucose (~20 mg; 99 atom %) was added to each batch fermenter (described later). The pattern of  $^{13}C$  incorporation into acetate, propionate and butyrate was determined by GC–MS analysis (described later) of the complete (carbon-containing) isotopomer envelopes of each SCFA. The enrichment of each isotopomer was determined after 6 h of glucose incubation and the data expressed in units of mole percent excess (MPE; Slater *et al.* 2001). The absolute quantity of each isotopomer ( $\mu$ mol) was calculated from the product of its enrichment and the pool size. From the abundance data, the relative proportions of each SCFA produced were also calculated.

### SCFA interconversion: experiment 2

Five healthy adults (four males and one female; age range 29–42 years; two males and one female were participants in experiment 1) were recruited and asked to provide a single faecal sample. Interconversion between acetate, propionate and butyrate was determined using  $^2H$ -labelled SCFA ([ $^2H_3$ ]acetate, [ $^2H_5$ ]propionate, [ $^2H_7$ ]butyrate) and

[ $^{13}C_3$ ]lactate. A single batch fermenter was prepared for each isotopically labelled species and each batch fermenter was prepared from the same stock faecal slurry. Each batch fermenter had an addition of isotopically labelled SCFA to attain an enrichment of ~50 MPE in the slurry SCFA pool. This was repeated for each of the five subjects. Due to the low concentration of lactate in the faecal slurries, each batch fermenter had an addition of a lactate blend of 50 MPE [ $^{13}C_3$ ]DL-lactate to a final concentration of 2 mmol/l. Samples were taken prior to addition of the labelled substrate and then at 6 h. Interconversion between SCFA was determined by GC–MS analysis of the complete isotopomer envelope of each SCFA. Increased enrichment of an isotopomer (above 1 MPE) was used as an indicator of interconversion.

### Contribution of acetate and lactate to butyrate production: experiment 3

The same five healthy adults recruited for experiment 2 were once again asked to provide a faecal sample. The contribution of acetate and lactate to butyrate production upon OF fermentation was investigated using [ $^2H_3$ ]acetate and [ $^{13}C_3$ ]lactate. Labelled acetate and lactate were added to separate batch fermenters at ~50 MPE (prepared from the same stock sample). Each batch fermenter also contained an addition of [ $^2H_7$ ]butyrate (~70  $\mu$ mol, ~50 MPE) to measure the rate of total butyrate production by isotope dilution. The experiment was designed to compare acetate and lactate conversion to butyrate in the faecal slurry without addition of substrate (–NDC) with a faecal slurry where fermentation was stimulated with 100 mg OF (+NDC; Rofitilose<sup>®</sup> P95; Orafiti Active Food Ingredients, Tienen, Belgium). Incorporation of added [ $^2H_3$ ]acetate and [ $^{13}C_3$ ]lactate into butyrate was determined by GC–MS. Increased enrichment of an isotopomer (above 1 MPE) was used as an indicator of interconversion. Each batch fermenter was sampled prior to addition of the labelled substrate and then at 3 and 6 h later.

### Preparation of faecal batch fermenters

All unlabelled chemicals were purchased from Sigma-Aldrich (Gillingham, UK). Isotopically labelled substrates were purchased from Cambridge Isotopes Ltd through CK Gas Products Ltd (Hook, UK). Fresh faecal samples were processed immediately after collection, to reduce exposure to oxygen. Each sample was homogenised in degassed PBS (0.16 M-sodium chloride, 0.003 M-potassium chloride, 0.008 M-disodium hydrogen phosphate, 0.001 M-potassium dihydrogen phosphate; pH 7.3) to produce a 10% (w/v) slurry. The process was undertaken using a standard kitchen blender contained within a Class II Microbiological safety cabinet. Each batch fermenter consisted of a crimp-top, gas-tight glass vessel of 100 ml total volume, which included an indwelling luer-lock tap system for access and sampling. Following addition of 50 ml faecal slurry, the head-space of each vessel was sparged with oxygen-free nitrogen to reduce the levels of oxygen and thereafter incubated at 37°C in a waterbath under gentle agitation (60 rpm).

### GC-MS analysis

SCFA concentration and enrichment was measured by GC-MS as previously described (Morrison *et al.* 2004). Briefly, to 300  $\mu$ l aqueous faecal sample, 3-methyl valeric acid (1200 nmol) was added as internal chemical standard to determine SCFA concentrations. Samples were acidified with 6 M hydrochloric acid and protonated SCFA were extracted with diethyl ether. The ether phase (100  $\mu$ l) was transferred to a new vial and 100  $\mu$ l acetonitrile (containing 100 nmol hexanoic acid as a second standard to quantify blank contributions) and 20  $\mu$ l methyltertbutylsilyl trifluoroacetamide were added. Vials were heated at 60°C for 60 min to form the *tert*-butyldimethylsilyl esters of SCFA for MS quantification. SCFA *tert*-butyldimethylsilyl esters fragment under electron impact ionisation to form a prominent  $[M-57]^+$  ion, which retains the isotopic signature. Thus, the complete isotopomer envelope for each SCFA was analysed in selective ion recording mode. The enrichment of each isotopomer was calculated and included a correction for blanks and subtraction of the natural abundance to calculate the isotopomer ratio to its base peak and its enrichment in MPE. The isotopically labelled SCFA used were only labelled with one isotope (i.e. either  $^{13}\text{C}$  or  $^2\text{H}$ ) although at multiple sites. Therefore the ions monitored were  $[M]^+-[M+3]^+$  for acetate,  $[M]^+-[M+5]^+$  for propionate,  $[M]^+-[M+7]^+$  for butyrate and  $[M]^+-[M+3]^+$  for lactate. The concentration of each SCFA was determined from the sum of their isotopomer ratios to the internal standard (to include the contribution of the tracer to the overall pool size) and was expressed in mmol/l. The absolute abundance of each isotopomer ( $\mu$ mol) was calculated from the product of enrichment (MPE/100), concentration (mmol/l) and pool size (50 ml).

### Calculations

The rate of butyrate production from free acetate was determined using a fractional synthetic rate approach using a linear model (Slater *et al.* 1995). An examination of the use of non-linear modelling showed that in the early part of the experiment the model approximated to a linear model (data not shown). Thus all data were calculated from the earliest time points (0–3 h). The rate of net butyrate production ( $R_b$ ) was calculated by isotope dilution of  $[^2\text{H}_7]$ butyrate using a linear model. The change in enrichment over the time interval and the pool size were taken to calculate total butyrate production, thus:

$$R_b = \left[ \frac{1 - \left( \frac{d_7 B_{ii}}{d_7 B_{i0}} \right)}{t_i - t_0} \right] \times C_{b_{ii}} \times \text{Vol}$$

where  $d_7 B_{ii}$  =  $[^2\text{H}_7]$ butyrate enrichment at time  $i$  (or time 0) in MPE;  $C_{b_{ii}}$  = concentration of the butyrate pool at time  $i$ , determined by isotope dilution (mmol/l); Vol = volume of the faecal slurry (litres).

The fractional synthesis rate of butyrate from acetate ( $R_{ba}$ ) was determined from the change in product enrichment of the  $[M+3]^+$  isotopomer of butyrate in relation to the change of the precursor acetate pool enrichment, thus:

$$R_{ba} = \left[ \frac{d_3 B_{ii} - d_3 B_{i0}}{d_3 A \times (t_i - t_0)} \right] \times C_{b_{ii}} \times \text{Vol}$$

where  $d_3 B_{ii}$  =  $[^2\text{H}_3]$ butyrate enrichment at time  $i$  (or time 0) (MPE);  $d_3 A$  = mean  $[^2\text{H}_3]$ acetate enrichment between time  $i$  and 0 (MPE);  $C_{b_{ii}}$  = concentration of the butyrate pool at time  $i$ , determined by isotope dilution (mmol/l); Vol = volume of the faecal slurry (litres).

Similarly the rate of production of butyrate from lactate ( $R_{bl}$ ) was calculated from fractional synthesis using the appearance of  $[^{13}\text{C}_2]$ butyrate and the enrichment of the precursor lactate pool, thus:

$$R_{bl} = \left[ \frac{^{13}\text{C}_2 B_{ii} - ^{13}\text{C}_2 B_{i0}}{^{13}\text{C}_3 L \times (t_i - t_0)} \right] \times C_{b_{ii}} \times \text{Vol}$$

where  $^{13}\text{C}_2 B_{ii}$  =  $[^{13}\text{C}_2]$ butyrate enrichment at time  $i$  (or time 0) (MPE);  $^{13}\text{C}_3 L$  = mean  $[^{13}\text{C}_3]$ lactate enrichment between time  $i$  and 0 (MPE);  $C_{b_{ii}}$  = concentration of the butyrate pool at time  $i$ , determined by isotope dilution (mmol/l); Vol = volume of the faecal slurry (litres).

The proportion of total butyrate formed from the free acetate pool ( $P_{ba}$ ) was calculated as follows:

$$P_{ba} = \frac{R_{ba}}{R_b}$$

The fractional synthesis of butyrate from acetate was expressed as a percentage (i.e.  $P_{ba} \times 100$ ).

A similar approach was used to calculate the proportion of total butyrate formed from free lactate ( $P_{bl}$ ) and the fractional synthesis of butyrate from lactate ( $P_{bl} \times 100$ ).

The overall contribution of free acetate and lactate to total butyrate production ( $P_{bal}$ ) was calculated as

$$P_{bal} = P_{ba} + P_{bl}$$

### Statistical methods

Calculated rates from isotope data were pooled for the subject group and means analysed by ANOVA with Tukey's *post hoc* analysis (SPSS version 11.0; SPSS Inc., Chicago, IL, USA) to determine significant differences.

## Results

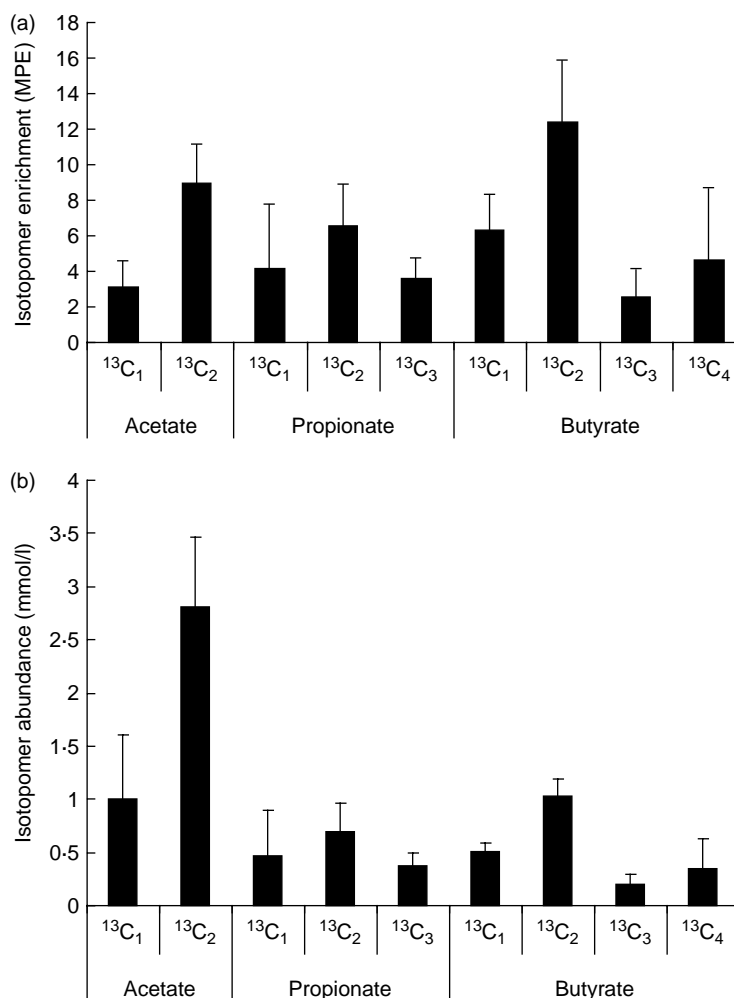
### SCFA production from $U\text{-}^{13}\text{C}_6$ (universally) labelled glucose: experiment 1

Fig. 1 shows that each SCFA isotopomer became enriched over the experimental period, indicating the utilisation of the glucose carbon skeleton for production of these SCFA. The dominant isotopomer in each SCFA pool was  $^{13}\text{C}_2$ -labelled. Glucose fermentation produced more acetate than either propionate or butyrate with most of the carbon skeleton incorporated into  $[^{13}\text{C}_2]$ acetate. The most abundant isotopomer of butyrate was  $^{13}\text{C}_2$ -labelled and not  $^{13}\text{C}_4$ -labelled. The mean proportions ( $n$  5) of each SCFA produced, calculated by adding the absolute isotopomer abundance in each SCFA pool, were 0.51 (SD 0.11), 0.21 (SD 0.08) and 0.28 (SD 0.06) for acetate, propionate and butyrate, respectively.

### SCFA interconversion: experiment 2

The interconversion reactions observed are summarised in Table 1. These observations were replicated in all faecal

SCFA production from oligofructose



**Fig. 1.** Production of  $^{13}\text{C}$ -labelled acetate, propionate and butyrate isotopomers through the fermentation of  $[\text{U-}^{13}\text{C}_6]\text{glucose}$  measured after 6 h ( $n$  5). For details of procedures, see pp. 571–572. Values are means with their standard deviations depicted by vertical bars. (a), Measured isotopomer enrichment (mole percent excess, MPE); (b), absolute abundance of each isotopomer.

samples. Incorporation of  $[\text{}^2\text{H}_3]\text{acetate}$  into butyrate was quantitatively the most significant SCFA interconversion observed with 0.28 (SD 0.2) mol/mol labelled acetate incorporated into butyrate. Lactate was also utilised producing  $[\text{}^{13}\text{C}_2]\text{acetate}$

and  $[\text{}^{13}\text{C}_2]\text{butyrate}$  and  $[\text{}^{13}\text{C}_3]\text{propionate}$ . There was no evidence for the incorporation of  $[\text{}^2\text{H}_3]\text{acetate}$  into propionate or the incorporation of propionate or butyrate into the other SCFA.

**Table 1.** SCFA interconversions in the human faecal flora\* (Mean values and standard deviations)

Precursor	Product†					
	Acetate		Propionate	Butyrate		Lactate
	Mean	SD		Mean	SD	
Acetate	–		X	11.6	3.0	X
Propionate	X		–	X		X
Butyrate	X		X	–		X
Lactate	67.9	45.4	15.3‡	4.0	3.2	–

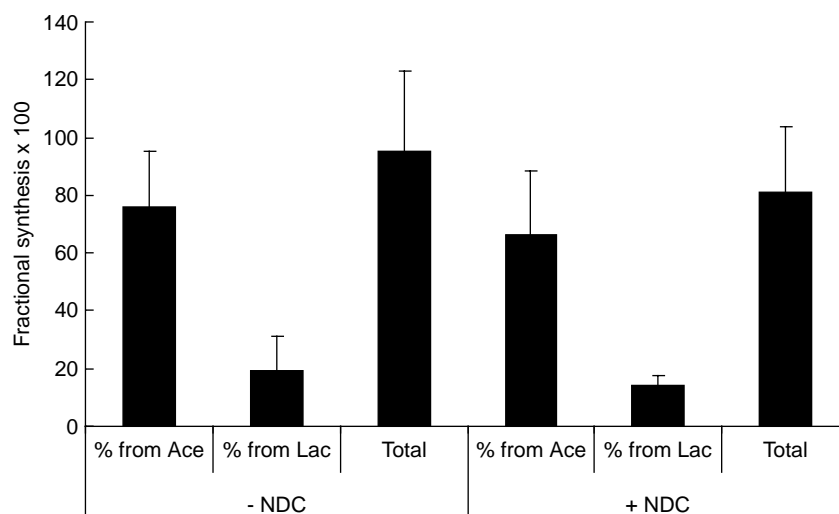
\* For details of procedures, see pp. 571–572.  $[\text{}^2\text{H}_3]\text{Acetate}$ ,  $[\text{}^2\text{H}_5]\text{propionate}$ ,  $[\text{}^2\text{H}_7]\text{butyrate}$  and  $[\text{}^{13}\text{C}_3]\text{lactate}$  were incubated separately.

† Mean rates of production of product from precursor were calculated using fractional synthesis rates and absolute pool size to derive absolute synthesis rates in  $\mu\text{mol/h}$  ( $n$  5). X indicates no interconversion measured at 6 h.

‡ Enrichment in the propionate pool at  $[\text{M} + 3]$  was only observed in two subjects.

*Contribution of acetate and lactate to butyrate production: experiment 3*

The molar fraction of  $[\text{}^2\text{H}_7]\text{butyrate}$  remained constant in batch cultures derived from the faeces of the five subjects (data not shown), allowing the use of this tracer to measure net butyrate production by isotope dilution. The production rate of butyrate measured by isotope dilution,  $R_b$ , occurred at a significantly faster rate (measured at 3 h) of 33.1 (SD 6.5) v. 16.9 (SD 2.3)  $\mu\text{mol/h}$  ( $P=0.01$ ) when OF was added. This represented 8.2% of the added OF converted to butyrate (as C3 equivalents; one fructose unit =  $2 \times \text{C3}$  equivalents). Fig. 2 illustrates that in the absence of added NDC (– NDC) all butyrate was synthesised from acetate and lactate and that upon addition of OF (+ NDC), a similar proportion of newly synthesised butyrate was produced from the conversion of acetate and lactate to



**Fig. 2.** The contribution of acetate ( $P_{ba} \times 100$ ) and lactate ( $P_{bl} \times 100$ ) and their sum total contribution ( $P_{bat} \times 100$ ) to total butyrate synthesis from oligofructose fermentation determined using the appearance of isotope-labelled [ $^2\text{H}$ ]butyrate from [ $^2\text{H}_3$ ]acetate (Ace) and [ $^{13}\text{C}$ ]butyrate from [ $^{13}\text{C}_2$ ]lactate (Lac) in the butyrate pool. For details of procedures, see pp. 571–572. Values are means with their standard deviations depicted by vertical bars. +NDC, addition of 100 mg oligofructose; –NDC, no addition of oligofructose.

butyrate. A significant increase was observed in the rate of acetate conversion to butyrate,  $R_{ba}$  ( $P=0.044$ ). When lactate data were pooled there was no significant difference in lactate conversion to butyrate ( $R_{bl}$ ,  $P=0.99$ ) when OF was added. However there was considerable variation in the net increase of lactate to butyrate flux within the subject group upon addition of OF; two subjects exhibited no net change, two subjects exhibited a small net change and one subject a large (6-fold) net change in lactate to butyrate flux during OF fermentation. The transient nature of lactate was apparent when sampled at 24 h where lactate concentrations had returned to pre-OF fermentation levels. There appeared to be non-uniform utilisation of labelled acetate into C1 and C2 v. C3 and C4 of butyrate as determined by [M + 1] v. [M + 3] butyrate (from [ $^2\text{H}_3$ ]acetate). The mean enrichment of [M + 1] butyrate was only 0.8 (SD 0.1) of the enrichment measured in [M + 3] butyrate, irrespective of whether OF was added or not.

## Discussion

The production of butyric acid by the bacterial flora in the human large bowel has received much attention in recent years because of the anti-inflammatory (Luhrs *et al.* 2002; Cavaglieri *et al.* 2003) and anti-neoplastic properties (Lupton, 2004; Scheppach & Weiler, 2004) of butyrate on cell tissue cultures *in vitro*. This has sharpened interest in the protective and therapeutic potential of butyrate in inflammatory bowel disease and colorectal cancer. However, little quantitative information exists to explain why bacterial fermentation of certain carbohydrates, such as resistant starch and OF, selectively increase the proportion of butyrate produced (relative to total SCFA) and others do not. The current study illustrates the complexity of butyrate production and shows that butyrate production, from OF fermentation, can be largely accounted for by interconversion reactions from primarily acetate, and lactate.

SCFA have often been regarded as end-products of microbial fermentation that allow bacteria to dispose of electrons in an environment limited in oxygen to act as a terminal electron acceptor. Many of the dominant fermentation pathways were observed upon fermentation of [U- $^{13}\text{C}_6$ ]glucose producing [ $^{13}\text{C}_2$ ]acetate through the Embden Meyerhof Parnas pathway (Miller & Wolin, 1996) and  $^{13}\text{C}_1$  through the Wood–Ljungdahl pathway (Drake, 1994). Propionate was produced from glucose via the symmetrical succinate intermediate that can be randomly decarboxylated yielding either  $^{13}\text{C}_2$ - or  $^{13}\text{C}_3$ -labelled propionate. [ $^{13}\text{C}_1$ ]Propionate probably reflects carboxylation of unlabelled phosphoenolpyruvate with  $^{13}\text{CO}_2$  followed by decarboxylation of the unlabelled carbonyl carbon atom, via succinate. Lactate can also produce [ $^{13}\text{C}_3$ ]propionate without alteration of the carbon skeleton through acrylate as intermediate. The major isotopomer of butyrate produced was  $^{13}\text{C}_2$ -labelled and  $^{13}\text{C}$  incorporation was observed in all butyrate isotopomers. Butyrate is formed from  $2 \times$  acetyl-CoA and there are therefore a number of possible explanations for the dominance of [ $^{13}\text{C}_2$ ]butyrate. Intracellular (unlabelled) acetyl-CoA flux will be rapid and from many sources resulting in only [ $^{13}\text{C}_2$ ]butyrate when carbon from [U- $^{13}\text{C}_6$ ]glucose enters the cell as [ $^{13}\text{C}_2$ ]acetyl-CoA. Undoubtedly the intracellular acetyl-CoA pool becomes enriched with time and this will account for  $^{13}\text{C}_4$ -labelled butyrate. An alternative route is the utilisation of  $^{13}\text{C}_2$ -labelled extracellular acetate as a precursor of butyrate synthesis. The observation of  $^{13}\text{C}_1$  (combination of  $^{13}\text{C}_1$  and unlabelled acetyl-CoA) and  $^{13}\text{C}_3$  (combination of  $^{13}\text{C}_1$  and  $^{13}\text{C}_2$  acetyl-CoA) labelled butyrate isotopomers suggests this route to be quantitatively significant (Duncan *et al.* 2004a).

Increased butyrate production from OF has previously been reported (Berggren *et al.* 1995; Khan & Edwards, 2006). This observation appears somewhat counterintuitive because OF has often been reported to be mainly fermented by bifidobacteria and to a lesser extent lactobacilli, which are predominantly

acetate and lactate producers and not butyrate producers (Kolida *et al.* 2002). Stimulation of other bacterial groups through OF fermentation has also been observed, including butyrate-producing bacteria (Berggren *et al.* 1995). There are two distinct pathways of intracellular butyrate production in bacteria, via butyrate kinase (Miller & Jenesele, 1979) or butyryl CoA:acetyl CoA transferase (Duncan *et al.* 2002). The functional significance of these pathways has been established and the butyryl CoA:acetyl CoA transferase pathway appears to be more dominant in the human bacterial flora. The increased flux of extracellular acetate to butyrate, upon OF fermentation, observed in this current study is in agreement with butyryl CoA:acetyl CoA transferase being the dominant butyrate-producing pathway.

The initial high enrichment of precursor SCFA is necessary to achieve sufficient enrichment in the product isotopomer envelope to be amenable to analysis by quadrupole mass spectrometry. Such increases in precursor pool size may alter substrate flux and partitioning of precursor through product pathways. Alternative MS techniques, such as an isotope ratio MS, may obviate the need for initial high and subsequent product enrichment, facilitating the use of near true tracer (< 1 MPE) enrichment. The contribution of extracellular acetate to butyrate production (67% in the presence of OF) was lower than previously reported for chicory inulin (87%) and similar to that reported for dahlia inulin (61%) (Duncan *et al.* 2004a). The difference observed may be explained by the fact that OF is a purified, low-molecular weight fraction of inulin and may have different fermentation characteristics to whole chicory or dahlia inulins. In the current study, the major isotopomer of butyrate produced from [<sup>2</sup>H<sub>3</sub>]acetate was [<sup>2</sup>H<sub>3</sub>]butyrate, suggesting that this extracellular acetate was being incorporated into butyrate with retention of the deuterated methyl group. The incorporation of [<sup>2</sup>H<sub>3</sub>]acetate into butyrate results in either [M + 3] butyrate (retention of methyl deuterium in methyl group of butyrate) and [M + 1] butyrate produced via the unsaturated intermediate, crotonyl CoA. The enrichment ratio [M + 3]:[M + 1] was not unity (0.8), reflecting non-uniform sequestration of acetate into the carbon skeleton of butyrate. Uptake of extracellular acetate has been observed in bacteria and the regulation of acetyl-CoA synthetase, which commits acetate to further intracellular metabolism, may be sensitive to extracellular acetate concentrations (Kumari *et al.* 2000). This is likely to be a widely available non-specific mechanism in colonic bacteria. The second mechanism committing acetate via the butyryl CoA:acetyl CoA transferase pathway will promote acetate utilisation concomitant to butyrate production. It appears that this pathway is selectively activated upon fermentation of specific NDC, such as OF; otherwise all acetate-producing NDC would enhance butyrate production.

Low lactate concentrations are normally observed in human faeces except when the large bowel is diseased, such as in ulcerative colitis (Hove *et al.* 1994; Vernia *et al.* 1988) or absent, such as in short bowel syndrome (Kaneko *et al.* 1997). This could be due to reduced absorption, increased production, reduced metabolism or a combination. Observations of increased lactate production from OF fermentation compared with other NDC are inconsistent in the literature (summarised in Nyman, 2002). Two explanations for these observations are: (1) the frequency of lactate measurements

as it is a transient product of fermentation; (2) intra-individual differences in lactate production (Bourriaud *et al.* 2005) linked to bacterial activity which are manifest by the design of human faecal-rat-associated microflora studies (which often use a single faecal sample to inoculate all animals). Lactate production from longer-chain inulins appears to increase in a dose-dependent manner (Levrat *et al.* 1991). In the present study, the major isotopomer of butyrate produced from [<sup>13</sup>C<sub>3</sub>]lactate was [<sup>13</sup>C<sub>2</sub>]butyrate, highlighting that butyrate was produced via acetyl-CoA. This mechanism was observed in all experiments, illustrating the ability of all subjects' microflora to utilise lactate. However, addition of OF did not appear to stimulate this pathway in all subjects, suggesting selectivity through OF fermentation in different subjects. Such intra-individual differences have been observed previously (Bourriaud *et al.* 2005). The study of Bourriaud *et al.* (2005) was conducted at a lower pH and recent data have illustrated that pH does influence metabolite production (Walker *et al.* 2005). This trend in data suggests an increased production of butyrate with decreasing pH and a lower starting pH in the current study may have exhibited greater proportional conversion of, particularly, lactate to butyrate. Lactate-utilising bacteria from the human flora have been previously identified as belonging to the *Clostridia* cluster XIVa, based on their 16S rRNA sequences (Duncan *et al.* 2004b). Fermentation produces both isomeric forms of lactate and there appears to be a large capacity for bacteria to utilise both isomers and for the rapid racemisation of lactate (Bourriaud *et al.* 2005; Hove *et al.* 1994). There appears to be more favourable absorption of L-lactate across the colonic epithelium (Ritzhaupt *et al.* 1998). Conversion of DL-lactate, produced from rapid fermentation, to more reduced products will favour electron disposal in lactate-utilising bacterial species. This mechanism of, especially D-lactate disposal, may also explain why low lactate concentrations are found routinely in human faeces even though the absorption of D-lactate from the colon may be limited.

The findings of the present study raise the question: can supplementing the diet with OF stimulate butyrate production *in vivo*? Selectively increasing butyrate production will depend on the regional differences throughout the colon of bacteria expressing butyryl CoA:acetyl CoA transferase and the regional differences in lactate-utilising, butyrate-producing bacteria. The propensity for the growth of these organisms, requiring low oxygen potentials and a highly reducing environment, is likely to be towards the distal colon. The OF used in the present study is of relatively short chain length and is rapidly fermentable which may suggest that *in vivo* its site of fermentation may be more proximal towards the caecum and therefore butyrate production may be limited. Until a method to measure colonic butyrate production *in vivo* becomes available, it is impossible to conclusively resolve this question. One requirement therefore for selectively increasing butyrate production through dietary NDC is selective stimulation of acetate- and lactate-utilising, butyrate-producing bacteria. This selectivity is determined by the physicochemical properties of the NDC and therefore butyrate-enhancing NDC may represent a refinement of the definition of prebiotics to include this sub-group of butyrogenic prebiotics. Dietary supplementation with bacteria, which convert acetate or lactate to butyrate, may also be considered to be probiotic.

The present study highlights the potential for nutritional intervention with specific NDC to selectively increase butyrate production, which may have health benefits for the population in general and also have therapeutic potential for the management of colonic disease.

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