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Two Routes of Metabolic Cross-Feeding between *Bifidobacterium adolescentis* and Butyrate-Producing Anaerobes from the Human Gut

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Dietary carbohydrates have the potential to influence diverse functional groups of bacteria within the human large intestine. Of 12 *Bifidobacterium* strains of human gut origin from seven species tested, four grew in pure culture on starch and nine on fructo-oligosaccharides. The potential for metabolic cross-feeding between *Bifidobacterium adolescentis* and lactate-utilizing, butyrate-producing *Firmicute* bacteria related to *Eubacterium hallii* and *Anaerostipes caccae* was investigated in vitro. *E. hallii* L2-7 and *A. caccae* L1-92 failed to grow on starch in pure culture, but in coculture with *B. adolescentis* L2-32 butyrate was formed, indicating cross-feeding of metabolites to the lactate utilizers. Studies with [^{13}C]lactate confirmed carbon flow from lactate, via acetyl coenzyme A, to butyrate both in pure cultures of *E. hallii* and in cocultures with *B. adolescentis*. Similar results were obtained in cocultures involving *B. adolescentis* DSM 20083 with fructo-oligosaccharides as the substrate. Butyrate formation was also stimulated, however, in cocultures of *B. adolescentis* L2-32 grown on starch or fructo-oligosaccharides with *Roseburia* sp. strain A2-183, which produces butyrate but does not utilize lactate. This is probably a consequence of the release by *B. adolescentis* of oligosaccharides that are available to *Roseburia* sp. strain A2-183. We conclude that two distinct mechanisms of metabolic cross-feeding between *B. adolescentis* and butyrate-forming bacteria may operate in gut ecosystems, one due to consumption of fermentation end products (lactate and acetate) and the other due to cross-feeding of partial breakdown products from complex substrates.

Microbial growth and metabolism in the human large intestine depend to a large extent on the supply of dietary carbohydrates that resist digestion in the upper gut. The fermentation of these compounds, which include plant cell wall polysaccharides and some storage polysaccharides and oligosaccharides, has a major influence on health (9, 20, 43). Indeed, specific carbohydrates are now widely used as functional foods and as prebiotics, based on the concept that they stimulate particular gut bacteria that promote gut health (18) and, at the same time, reduce the populations of nonutilizing bacteria through competition. Inulin and fructo-oligosaccharides (FOS), for example, were originally proposed as prebiotics that selectively stimulate bifidobacteria. While there is evidence that this occurs (11, 19, 26, 45), other studies, using molecular techniques, have revealed that a variety of other bacterial groups, including clostridium-related species, also respond to inulin or FOS supplied as prebiotics in either fermentor experiments or animal models (13, 25).

Among the possible explanations for this diversity in response to prebiotics is that complex gut microbial communities involve extensive metabolic interactions (10, 46). Metabolic products produced from dietary prebiotics by one bacterial species may then provide substrates to support growth of other populations, and this is termed cross-feeding. Such cross-feeding can result in metabolic consequences that would not be predicted simply from the substrate preferences of isolated bacteria. For example, both resistant starch and FOS can be butyrogenic in vivo (23, 42, 43), although the main utilizers of such substrates so far identified have been lactic acid bacteria (31, 43). This may be due to compositional changes of bacterial communities within the colon following the reduction in pH that results from rapid carbohydrate fermentation (44) together with the fact that some butyrate producers are able to utilize those substrates (2, 37), but it is also possible that lactate (produced by bifidobacteria, for example) can be converted to butyrate by other species (24). The latter possibility is supported by the recent isolation from human feces of bacteria that convert lactate and acetate to butyrate (14) and by the observation that butyrate can be the main product formed from lactate by mixed human fecal bacteria (5).

This paper examines the potential role of metabolic cross-feeding between strains of *Bifidobacterium adolescentis* that are able to utilize starch or FOS as growth substrates and strains of butyrate-producing bacteria that cannot themselves utilize starch or FOS but can potentially utilize the lactate and acetate formed by *B. adolescentis*. Using isotopically labeled substrates, we confirmed that cross-feeding of lactate can occur in cocultures. These experiments also reveal a second mechanism of metabolic cross-feeding, however, that may boost butyrate formation by non-lactate-utilizing species found in the human colon.

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MATERIALS AND METHODS

Bacterial strains and maintenance. All bacterial strains included in this study were of human origin. *Anaerostipes caccae* L1-92 (DSM14662^T) (41), the *Eubac-*

terium hallii-like strain L2-7 (DSM 17630) (14), and *Roseburia* sp. strain A2-183 (DSM 16839) (2) are available from the Deutsche Sammlung von Mikroorganismen (DSMZ). *Bifidobacterium adolescentis* L2-32 was isolated from an infant (14), while strain 70/18, which shares 99% 16S rRNA sequence homology with *Bifidobacterium bifidum* (S. H. Duncan and H. J. Flint, unpublished data), was isolated from an adult human fecal sample. Other *Bifidobacterium* strains were obtained from the DSMZ (*B. adolescentis* DSM 20083 and DSM 20086, *B. angulatum* DSM 20098, *B. breve* DSM 20213, *B. longum* biotype *longum* DSM 20219 [40], *B. longum* biotype *infantis* DSM 20088 [40], *B. pseudocatenulatum* DSM 20438, and *B. bifidum* DSM 20456) or from the National Collection of Industrial and Marine Biology (NCIMB) (Aberdeen, United Kingdom) (*B. breve* NCIMB 8807 and *B. longum* biotype *longum* NCIMB 8809). All strains were routinely maintained in M2GSC broths and stored in medium containing 0.75% agar (35).

Growth rates of *Bifidobacterium* strains. The growth rates of 12 strains with either potato starch (BDH, Poole, United Kingdom) or FOS (Trouw International B.V., Holland) as a substrate were tested in anaerobically prepared yeast extract-Casitone-fatty acid (YCFA) medium (12) adjusted to pH 5.7. FOS (filter sterilized) or starch (sterilized by autoclaving) was added to give a final concentration of 0.2%. Specific growth rates (h^{-1}) were calculated from absorbance readings (optical density [OD] at 650 nm) during the exponential phase of growth at 37°C.

Coculture studies. Two types of coculture incubations were conducted. First, a known lactate producer, *B. adolescentis* L2-32 or DSM 20083 was incubated on medium containing either starch or FOS with a known lactate utilizer (either *A. caccae* or *E. hallii*) that is incapable of using either of the carbon substrates directly. Second, the use of nonlactate products of starch digestion from *B. adolescentis* metabolism was tested by coculture with another butyrate producer, *Roseburia* sp. strain A2-183, which lacks the ability to grow on lactate. In all cases, replicate tubes of anaerobic YCFA medium with the appropriate added carbon source (potato starch or FOS) were inoculated with each strain individually and with identical inocula of the two strains in combination. Cultures providing the inoculum were pregrown overnight in M2GSC medium (35). Duplicate experiments were performed in media that had been adjusted to two different initial pH values (5.7 ± 0.2 and 6.5 ± 0.2).

For the flux studies involving growth of *E. hallii* in monoculture, the strain was grown in the presence of acetate (33 mM) and lactate (45 mM), containing either [1-^{13}C]acetate or [U-^{13}C]lactate to give 10 molar percent excess (MPE). Replicate tubes were processed at 0, 3, 8, and 24 h to measure short-chain fatty acid (SCFA) and lactate concentrations and ^{13}C enrichments. For the flux studies involving cocultures, a filter-sterilized solution of [1-^{13}C]acetate or [3-^{13}C]lactate was added after 3 h of incubation to give 10 MPE. Samples were taken for estimation of SCFA and lactate concentrations and ^{13}C enrichments at the times indicated (see Results).

Analysis of short-chain fatty acids and [^{13}C]acetate, [^{13}C]butyrate, and [^{13}C]lactate enrichments. Replicate derivatized samples were routinely prepared for estimation of concentrations of SCFA and lactate by capillary gas chromatography (38). In experiments involving stable isotopes, lactate and SCFA enrichments and concentrations, estimated by isotope dilution, were measured by gas chromatography-mass spectrometry (GC-MS) analysis of the *tert*-butyldimethylsilyl derivatives. Procedures were as described previously (7, 15), except that 10 μl trypan blue was added to the initial sample to provide a visual aid in the transfer of the ether layer. GC-MS analyses were performed as described previously (15) with the following exceptions. The temperatures of the injector and the interface line were both 250°C. The GC separation was with an EC-1 capillary column (3 m by 0.25 mm by 0.25 μm) (Alltech, Carnforth, Lancs., United Kingdom) under the following conditions: 60°C for 3 min and then 25°C/min to 210°C for 4 min. Injections (1 μl) were made in the split mode with a 40:1 split and a 2-cm plug of silanized fused silica wool in the glass liner of the injector. The MS was operated under electron impact ionization conditions. For acetate, the ions M^+, M + 1, and M + 2 at mass/charge (m/z) ratios of 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 two to quantify butyrate formation from two [1-^{13}C]acetate and two [1,2-^{13}C]acetate molecules; while for lactate, M^+, M + 1, and M + 3 ion fragments (m/z 261, 262, and 264) were analyzed. In practice, the amounts of M + 2 or M + 4 labeled butyrate formed were close to those predicted by the laws of probability from precursor enrichments. For the concentration determinations, appropriate corrections were applied for the enrichments in the sample.

Kinetic modeling of SCFA and lactate metabolism. For simplicity, all units are expressed as C_2 units (thus, butyrate concentrations were doubled, while butyrate enrichments were halved [15]). Let C and E denote concentration (mM) and enrichment (MPE), respectively. Subscripts a, b, and l refer to acetate, butyrate, and lactate, respectively. Let i denote the interval between any two times t_0 and t_1, and let $F(i)$ denote the flow of labeled plus unlabeled material during i, while $F = \Sigma_i F(i)$ denotes the cumulative flow. Flows to pool y from pool x are denoted by F_{yx}. Flows of labeled material are denoted by f. $E(i)$ denotes the average enrichment during i. As the system was not in steady state, inflows to (subscript "in") and outflows from (subscript "out") the acetate and lactate pools were calculated separately. Therefore, at any time point, inflows and outflows may differ. Butyrate was assumed as an end product with inflow F_b. A schematic representation of the model is given in Fig. 1.

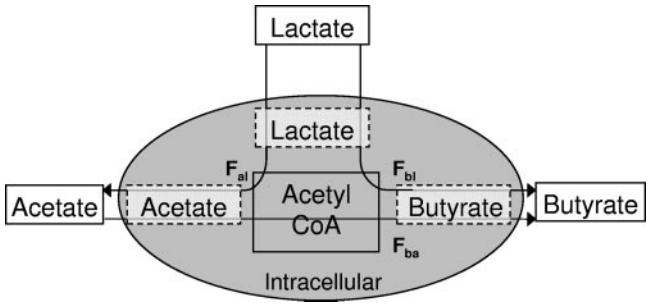

FIG. 1. Schematic representation of the model used for the C_2 flows. F_{bl}, flow of C_2 from lactate to butyrate via acetyl-CoA without exchange with exogenous acetate; F_{al}, flow of C_2 from lactate to acetate; F_{ba}, flow of C_2 from acetate to butyrate.

For the [1-^{13}C]acetate batch monocultures, $F_{a.\mathrm{out}}(i)$, $F_{a.\mathrm{in}}(i)$, $F_b(i)$, and $F_{ba}(i)$ are obtained from $E_a(t_1)C_a(t_1) = E_a(t_0)C_a(t_0) - E_a(i)F_{a.\mathrm{out}}(i)$, $C_a(t_1) = C_a(t_0) - F_{a.\mathrm{out}}(i) + F_{a.\mathrm{in}}(i)$, $C_b(t_1) = C_b(t_0) + F_b(i)$, and $E_b(t_1)C_b(t_1) = E_b(t_0)C_b(t_0) + E_a(i)F_{ba}(i)$.

Occasionally $F_{a.\mathrm{out}}(i)$ was less than $F_{ba}(i)$, and here $F_{a.\mathrm{out}}(i)$ was set equal to $F_{ba}(i)$. Let $p = F_{ba}/F_b$ and $q = F_{ba}/F_{a.\mathrm{out}}$, based on cumulative flows.

For the [U-^{13}C]lactate batch monocultures, $F_b(i)$ was obtained from $C_b(t_1) = C_b(t_0) + F_b(i)$, as given above. Assuming that the relative flows were similar for both the [U-^{13}C]lactate and [1-^{13}C]acetate studies, then $F_{ba}(i) = pF_b(i)$ and $F_{a.\mathrm{out}}(i) = F_{ba}(i)/q$. Furthermore, $f_b(i) = E_b(t_1)C_b(t_1) - E_b(t_0)C_b(t_0)$. Then, $f_{ba}(i) = pf_b(i)$, and $f_{a.\mathrm{out}}(i) = f_{ba}(i)/q$. Also, $f_{bl}(i) = f_b(i) - f_{ba}(i)$, and $f_{a.\mathrm{in}}(i) = E_a(t_1)C_a(t_1) - E_a(t_0)C_a(t_0) + f_{a.\mathrm{out}}(i)$. Assuming that $f_{al}(i) = f_{a.\mathrm{in}}(i)$, then $F_{bl}(i) = f_{bl}(i)/E_l(i)$ and $F_{al}(i) = f_{al}(i)/E_l(i)$.

For the batch cocultures, calculations based on [1-^{13}C]acetate data are identical to those for the monoculture, but those for the [U-^{13}C]lactate data are based on $E_l(t_1)C_l(t_1) = E_l(t_0)C_l(t_0) - E_l(i)F_{l.\mathrm{out}}(i)$ and $C_l(t_1) = C_l(t_0) + F_{l.\mathrm{in}}(i) - F_{l.\mathrm{out}}(i)$, giving $F_{l.\mathrm{out}}(i)$ and $F_{l.\mathrm{in}}(i)$. In contrast to the case for the monoculture, E_a and E_b were close to the detection limits, so lactate outflows to acetate (F_{al}) and butyrate (F_{bl}) could not be calculated directly. Therefore, using data from the [1-^{13}C]acetate coculture study, it was assumed that $F_{bl}(i) = F_b(i) - F_{ba}(i)$ and $F_{al}(i) = F_{l.\mathrm{out}}(i) - F_{bl}(i)$.

Results from the cocultures were analyzed by analysis of variance with substrate, pH, and their interaction as treatment effects, using Genstat release 8.1, 8th ed. (VSN International Ltd., Hemel Hempstead, Herts., United Kingdom).

Quantitative real-time PCR. The abundances of *B. adolescentis* L2-32 and *Roseburia* sp. strain A2-183 alone and in coculture were determined by quantitative real-time PCR. Equal volumes of cocultures grown in triplicate were combined and centrifuged at 10,000 × *g* for 5 min. For comparison, two sets of triplicate monocultures, grown for the same length of time, were combined and treated in the same way. Cell pellets were resuspended in 25 μl of sterile distilled H_2O and DNA extracted using the Fast DNA spin kit for soil (Qbiogene). DNA was diluted to 0.5 ng μl^{-1} in 5 μg ml^{-1} herring sperm DNA (Promega) and amplified with primers BifF (TCGCGTCYGGTGTGAAAG) (39) and g-Bifid-R (GGTGTTCTTCCCGATATCTACA) (34) for the quantification of *B. adolescentis* L2-32 and with primers Cclos99modF (TGAGTGGCGGACGGGTGAG, modified) (3) and CcmodRosR (TACCACCGGAGTTTTTCACAC, modified) (3) for the quantification of *Roseburia* sp. strain A2-183. Primers were checked for their specificity with the Probe Match function of the Ribosomal Database Project II (8). Standard template DNA was prepared from the 16S rRNA gene of *Roseburia* sp. strain A2-183 by amplification with primers 27F and RP2 and purification as described previously (30). Standard curves were prepared with five standard concentrations of 10^7 to 10^3 gene copies μl^{-1} in 5 μg ml^{-1} herring sperm DNA, with universal primers UniF (GTGSTGCAYGGYYGTCGTCA, modified) (33) and UniR (ACGTCRTCCMCNCCTTCCTC, modified) (33).

TABLE 1. Specific growth rates of *Bifidobacterium* strains in YCFA medium containing 0.2% potato starch or fructo-oligosaccharides

Strain	Specific growth rate (h^{-1})[a] on:	
	Starch	Fructo-oligosaccharides
B. adolescentis L2-32	0.40 ± 0.007	0.20 ± 0.012
B. adolescentis DSM 20083	—[b]	0.56 ± 0.045
B. adolescentis DSM 20086	—	0.42 ± 0.011
B. angulatum DSM 20098	0.41 ± 0.024	0.45 ± 0.085
B. bifidum 70/18	0.26 ± 0.090	—
B. breve DSM 20213	—	0.21 ± 0.006
B. longum (biotype longum) DSM 20219	—	0.30 ± 0.062
B. longum (biotype longum) NCIMB 8809	—	0.15 ± 0.026
B. longum (biotype infantis) 20088	—	0.54 ± 0.041
B. pseudocatenulatum DSM 20438	0.16 ± 0.053	0.45 ± 0.042

[a] Values are means of three replicates ± standard deviations.
[b] —, poor growth (final ΔOD of <0.13). *B. bifidum* DSM 20456 and *B. breve* NCIMB 8807 were also tested but gave final ΔODs of <0.1 on both substrates.

PCRs were performed in triplicate with iQ SYBR Green Supermix (Bio-Rad) in a total volume of 25 μl with primers at 500 nM in optical-grade 96-well plates sealed with optical sealing tape. Amplification was performed with an iCycler (Bio-Rad) with the following protocol: one cycle of 95°C for 3 min, 40 cycles of 95°C and 60°C for 30 s each, one cycle of 95°C for 1 min, one cycle of 55°C for 1 min, and a stepwise increase of the temperature from 55 to 95°C (at 10 s per 0.5°C) to obtain melt curve data. Data were analyzed using the iCycler IQ software version 3.1.

RESULTS

Growth and metabolism of *Bifidobacterium* strains with starch or FOS as a substrate. There was wide variation in the abilities of different *Bifidobacterium* strains isolated from the human gut to utilize potato starch and Trouw FOS for growth. Out of 12 strains that were tested, belonging to seven species, nine showed measurable rates of growth on FOS and four showed significant growth on starch (Table 1). Acetate, lactate, and formate were the major acid products formed. Lactate concentrations in growing cultures after 24 h ranged from 0.7 to 9 mM, accounting for 10 to 30% of the organic acids formed. In agreement with previous findings (22), the proportion of lactate tended to increase with increasing growth rate. Two strains of *B. adolescentis* were chosen for further study. These were *B. adolescentis* L2-32, which was used previously in cross-feeding experiments (14) and grows well on potato starch, and *B. adolescentis* DSM 20083, which showed the highest growth rate on FOS.

Coculture of *B. adolescentis* L2-32 and lactate-utilizing butyrate-producing *E. hallii* and *A. caccae* strains with starch as a substrate. Lactate accumulated in *B. adolescentis* L2-32 cultures grown on potato starch (Fig. 2). The butyrate-producing bacterium *E. hallii* L2-7 was unable to grow on starch in pure culture but can utilize lactate (14). In cocultures of *E. hallii* L2-7 and *B. adolescentis* L2-32, lactate concentrations decreased after the initial rise and there was a progressive increase in butyrate formation. This effect was seen both at pH 5.7 and 6.5, although utilization of lactate was less efficient at the lower pH (Fig. 2). Experiments with another lactate utilizer, *A. caccae* L1-92, gave similar results (not shown), except that lactate utilization was incomplete at pH 5.7 after 24 h.

Conversion of acetate and lactate to butyrate by *E. hallii*. The mechanism proposed previously for the cross-feeding phenomenon illustrated in Fig. 2 is that L-lactate and acetate produced by *B. adolescentis* drive butyrate formation by *E. hallii* (14). To clarify the carbon flows involved, *E. hallii* was first grown in pure culture in the presence of unlabeled acetate plus [U-^{13}C]lactate, or in the presence of [1-^{13}C]acetate plus unlabeled lactate, in duplicate experiments at two initial pH values (Table 2). The carbon flows through lactate, acetate, and butyrate pools were estimated by kinetic modeling (see Materials and Methods) (Fig. 1). As found previously for related *Roseburia* species, *E. hallii* showed active interchange between internal and external C_2 pools (15). The lactate was initially converted intracellularly by *E. hallii* to acetyl coenzyme A (acetyl-CoA), which then rapidly interconverted with exogenous acetate. Consequently, a high proportion of butyrate carbon was derived through that acetate pool (endogenous plus exogenous). Overall, lactate contributed between 57 to 62% to butyrate carbon, with the majority (95 and 80% at pH 5.7 and 6.5, respectively) via the acetate pool (Table 2).

Carbon flow of lactate and acetate to butyrate in cocultures. Carbon flow was next studied in coculture experiments involving either *B. adolescentis* L2-32 and *E. hallii* L2-7 grown on starch or *B. adolescentis* DSM 20083 and *E. hallii* L2-7 grown on FOS. Each experiment was performed at two initial pH values, 5.7 and 6.5. [3-^{13}C]lactate or [1-^{13}C]acetate was added as a tracer after 3 h of growth, and their incorporation into acetate and butyrate was followed (as shown for the experiment involving *B. adolescentis* L2-32 and *E. hallii* L2-7, at initial

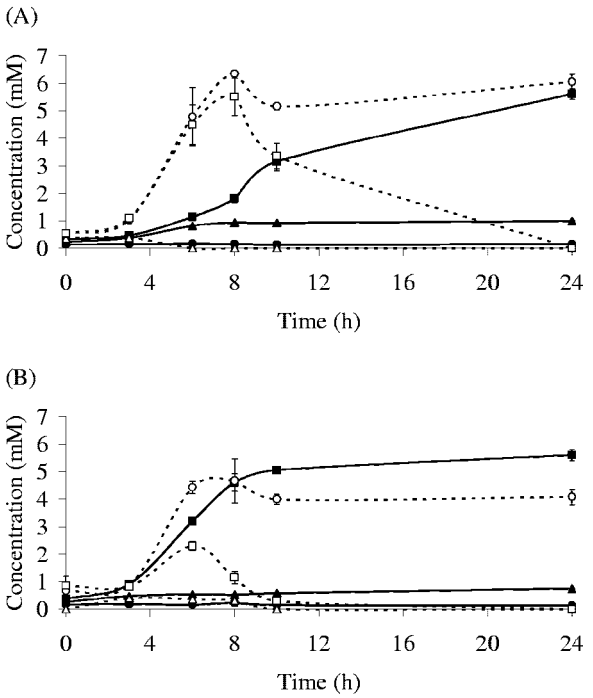

FIG. 2. Changes in butyrate (closed symbols) and lactate (open symbols) concentrations during incubation of monocultures of *B. adolescentis* L2-32 (circles), *E. hallii* L2-7 (triangles), and their cocultures (squares) on potato starch at either pH 5.7 (A) or 6.5 (B).

TABLE 2. Conversion of lactate and acetate to butyrate by *Eubacterium hallii* L2-7 incubated in YCFA in the presence of acetate (33 mM) and lactate (45 mM) and with the addition of [1-^{13}C]acetate or [U-^{13}C]lactate

Parameter[a]	Value with the following addition and initial pH:			
	[^{13}C]acetate		[^{13}C]lactate	
	5.7	6.5	5.7	6.5
Acetate outflow ($F_{a.\mathrm{out}}$)				
Total	25.95	44.48	26.27	52.00
To butyrate (F_{ba})	22.99	40.16	23.27	46.63
Acetate production ($F_{a.\mathrm{in}}$)	15.45	25.26	16.06	28.44
p_1 (%)	88	74		
Lactate outflow				
To butyrate (F_{bl})			0.77	7.57
To acetate (F_{al})			16.30	34.06
p_2 (%)			57	62
Butyrate production (F_b)	26.27	53.96	26.59	62.67

[a] p_1, percentage of butyrate carbon (C) coming from acetate C, estimated as F_{ba}/F_b; p_2, percentage of butyrate C coming from lactate C (either directly or indirectly via conversion to acetate), calculated as $[(q \times F_{al}) + F_{bl}]/F_b$, with q being the proportion of acetate C going to butyrate C ($F_{ba}/F_{a.\mathrm{out}}$). All flows are expressed in terms of C_2 units (mmol/liter per 24 h).

pH 5.7, in Fig. 3). The flows of carbon in the two coculture experiments are presented in Table 3. In the experiment involving *B. adolescentis* L2-32 with starch as a substrate, lactate production was approximately 30% of the value for acetate production at pH 5.7 but was only 10% at pH 6.5, due mainly to a 70% decline in lactate formation. All lactate formed was metabolized, however, with most entering the exogenous acetate pool. Only 11 to 21% was converted to butyrate without exchange with exogenous acetate. In the experiment performed with *B. adolescentis* DSM 20083 and FOS as the substrate, lactate production was slightly lower at pH 6.5 than at pH 5.7. Again all the lactate produced was metabolized, with the majority (61 to 77%) entering the exogenous acetate pool. In both experiments, the estimated contribution of lactate to butyrate carbon (p_2 in Tables 2 and 3) was somewhat lower in the cocultures (44 to 48%) than in the pure culture of *E. hallii* grown on lactate and acetate (57%) at pH 5.7 but was markedly lower at pH 6.5 (25 to 28%, compared with 62% in the pure culture).

Evidence for a second mechanism of cross-feeding. Coculture studies of *B. adolescentis* L2-32 with *E. hallii* L2-7 or *A. caccae* L1-92 were also conducted with Trouw FOS as the substrate. As noted above, *B. adolescentis* L2-32 grew poorly on this substrate (Table 1). Although only low concentrations of lactate were detected with the pure culture of *B. adolescentis* L2-32 on FOS, the coculture with *E. hallii* L2-7 or *A. caccae* L1-92 nevertheless gave rise to substantial butyrate (Fig. 4). This observation suggested that another mechanism apart from lactate cross-feeding might be responsible for the stimulation of butyrate in this case. In order to explore this possibility further, we chose to examine cocultures involving a butyrate producer, *Roseburia* sp. strain A2-183, which cannot utilize lactate. Table 4 shows that butyrate formation was also observed when *Roseburia* sp. strain A2-183 was cocultured with L2-32 on FOS or starch, although A2-183 was unable to grow significantly on FOS or starch in monoculture. The relative abundances of *B. adolescentis* L2-32 and *Roseburia* sp. strain A2-183 were estimated by 16S rRNA-based real-time PCR in these cocultures and compared with results for mixtures of the control pure cultures incubated for the same period of time (see Materials and Methods). This revealed significant stimulation of the *Roseburia* rRNA gene copy number in the cocultures on FOS at an initial pH of 6.5, the pH that produced the greatest butyrate formation, and on starch at both initial pH values (Table 4). The presence of the *B. adolescentis* L2-32 therefore appeared to stimulate growth and butyrate production by the *Roseburia* strain. This effect must be due to a mechanism that is independent of lactate utilization, and it is probably the result of cross-feeding of partially degraded carbohydrate substrate. This mechanism is assumed to account also for most of the butyrate formation seen in cocultures of *B. adolescentis* L2-32 and *A. caccae* L1-92 (Fig. 4) or *E. hallii* L2-7 on medium containing FOS.

DISCUSSION

There is much interest in the impact of nondigestible but fermentable dietary carbohydrates, including prebiotics (18), on gut metabolism and health in humans (43). The effects of resistant starch and FOS on microbial metabolism and bacterial populations have been studied in humans and in animal models (4, 16, 17, 29), and several studies have reported the

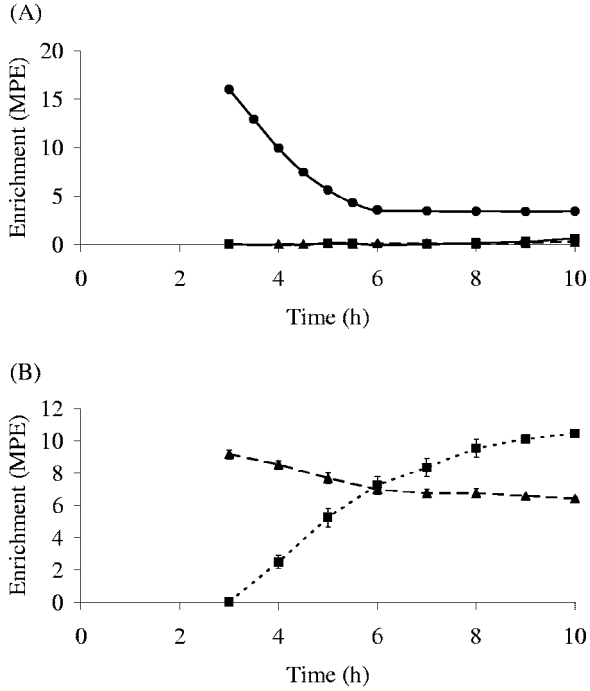

FIG. 3. Enrichments of lactate (circles), acetate (triangles), and butyrate (squares) in cocultures of *Bifidobacterium adolescentis* L2-32 and *Eubacterium hallii* L2-7 on starch at pH 5.7 following [3-^{13}C]lactate (A) or [1-^{13}C]acetate (B) injection.

TABLE 3. Conversion of lactate and acetate to butyrate in the cocultures between *Bifidobacterium* strains and *Eubacterium hallii* L2-7 with potato starch or fructo-oligosaccharides as the substrate

Parameter[a]	Value with the following substrate and initial pH:				SEM[d]	*P* value[d]		
	Starch[b]		FOS[c]					
	5.7	6.5	5.7	6.5		Substrate	pH	Substrate · pH
Lactate production ($F_{l.in}$)	6.11	1.78	4.79	3.19	0.145	0.761	<0.001	<0.001
Lactate outflow ($F_{l.out}$)	7.67	2.96	6.01	4.12	0.162	0.203	<0.001	<0.001
To acetate (F_{al})	6.07	2.61	4.63	2.53				
To butyrate (F_{bl})	1.60	0.34	1.37	1.60				
Acetate production ($F_{a.in}$)	21.81	17.53	21.59	20.02	0.843	0.248	0.026	0.184
Acetate outflow ($F_{a.out}$)								
Total	15.69	12.25	13.32	15.36	0.790	0.660	0.427	0.026
To butyrate (F_{ba})	8.67	7.00	7.97	10.21	0.307	0.015	0.397	0.003
Butyrate production (F_b)	10.26	7.34	9.34	11.81	0.276	<0.001	0.433	<0.001
p_1	89	90	85	87	0.431	0.002	0.086	0.192
p_2	48	25	44	28				

[a] p_1, percentage of butyrate carbon (C) coming from acetate C, estimated from ^{13}C acetate studies as F_{ba}/F_b; p_2, percentage of butyrate C coming from lactate C (either directly or indirectly via conversion to acetate), calculated as $[(q \times F_{al}) + F_{bl}]/F_b$, with q being the proportion of acetate C going to butyrate C ($F_{ba}/F_{a.out}$). All flows are expressed in terms of C_2 units (mmol/liter per 21 h).

[b] Incubation with *B. adolescentis* L2-32.

[c] Incubation with *B. adolescentis* DSM 20083.

[d] From analysis of variance with substrate, pH, and their interaction (substrate · pH) as treatment effects. Values are based on 8 observations (4 residual df), except for F_b, which is based on 16 observations (12 residual df). F_{al}, F_{bl}, and p_2 were calculated from combinations of mean values obtained from [^{13}C]acetate and [^{13}C]lactate studies, which did not allow for statistical analysis.

stimulation of human fecal bifidobacteria by FOS or inulin (11, 19, 26). Previous work has also indicated varied capability among bifidobacterial strains to use FOS and starch (22). In the present study, of 12 *Bifidobacterium* strains of human gut origin examined, four grew well on potato starch and nine on Trouw FOS, although growth rates varied. This suggests that prebiotic stimulation of bifidobacterial populations might prove to be both strain and substrate specific. *B. adolescentis* was selected for these studies because it is one of the most abundant species of bifidobacteria in the human colon (1) and thus has the potential to play a significant role in diet utilization and colonic health.

It has been proposed that cross-feeding of lactate produced by bifidobacteria can stimulate the formation of butyrate by other bacteria within the gut community (14, 24). This proposal arose from the observation that the same substrates that probably promote bifidobacterial populations in vivo can also be butyrogenic (28). The recent isolation of butyrate-producing species such as *E. hallii* and *A. caccae* that are able to utilize lactate (14) offered the chance to investigate the potential significance of lactate cross-feeding in defined cocultures. The stable-isotope experiments showed that *E. hallii* L2-7 converts L-lactate to acetyl-CoA, and this is rapidly exchanged with exogenous acetate, thus providing precursors for butyrate synthesis. The fate of labeled lactate was entirely consistent with conversion of lactate to pyruvate via lactate dehydrogenase, as proposed previously (14). No evidence was found for the conversion of lactate to butyrate through a distinct pathway. In cocultures involving *B. adolescentis* L2-32 on starch or *B. adolescentis* DSM 20083 on FOS, the *Bifidobacterium* strain was shown to produce lactate in the presence of *E. hallii*, with the latter organism being responsible for conversion of the lactate into butyrate. The pH of the proximal colonic lumen is reported to fall below pH 6.0 as a result of active microbial fermentation of certain dietary substrates (6, 36). The ability of *E. hallii* to utilize lactate in cocultures with *B. adolescentis* both at pH 5.7 and at pH 6.5 could therefore have important implications for the supply of butyrate to various regions of the colon.

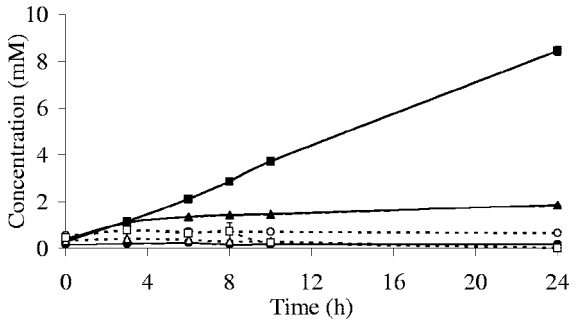

FIG. 4. Changes in butyrate (closed symbols) and lactate (open symbols) concentrations during incubation of monocultures of *B. adolescentis* L2-32 (circles), *A. caccae* L1-92 (triangles), and their cocultures (squares) on fructo-oligosaccharides at pH 5.7.

E. hallii and its relatives can account for 4% of human bacteria (21), and such bacteria may play a significant role in preventing lactate accumulation in vivo. Indeed, a recent study examined the fate of [^{13}C]lactate in human fecal slurries, maintained at pH 5.8, and found that for two out of the three donors lactate was mainly converted to butyrate via acetyl-CoA (5). *Bifidobacterium* spp. can account for up to 15% of fecal

TABLE 4. Influence of pH on SCFA concentrations and relative proportions of each strain in monocultures of *B. adolescentis* L2-32 and *Roseburia* sp. strain A2-183 and their cocultures when incubated in YCFA medium containing 0.2% potato starch or fructo-oligosaccharides

SCFA	Concn change (mM) with the following substrate, pH, and inoculum[a]											
	Starch						FOS					
	5.7			6.5			5.7			6.5		
	B	R	R + B	B	R	R + B	B	R	R + B	B	R	R + B
Acetate	15.2 ± 2.2	−0.8 ± 0.6	0.02 ± 3.2	18.0 ± 1.4	2.4 ± 1.4	5.3 ± 1.2	6.3 ± 2.1	−1.3 ± 0.7	7.3 ± 1.9	12.1 ± 2.4	−1.1 ± 1.4	11.5 ± 3.1
Butyrate		2.5 ± 0.1	8.1 ± 1.8		2.6 ± 0.2	6.1 ± 0.9		1.5 ± 0.1	5.1 ± 0.4		1.5 ± 0.1	9.9 ± 2.1
Lactate	6.5 ± 0.6		1.9 ± 0.7	3.2 ± 0.2	0.4 ± 0.1	1.4 ± 0.1	1.1 ± 0.1		1.3 ± 0.2	0.7 ± 0.1		1.8 ± 0.7
R/B[b]	0.60		2.07	0.25		1.34	0.31		0.41	0.41		1.69

[a] Values are means of three replicates ± standard deviations. B, *B. adolescentis* L2-32; R, *Roseburia* sp. strain A2-183; R + B, coculture.
[b] R/B, ratio of abundances of *Roseburia* sp. strain A2-183 and *B. adolescentis* L2-32 in pellets obtained from the combination of equal volumes of the cocultures or the monocultures of both strains.

bacteria (27) and therefore make a potentially important contribution to lactate production in vivo. Further work is needed, however, to determine rates of lactate formation and disposal in the complete gut community under conditions that operate within the colon in vivo. The molar proportion of lactate in pure cultures of bifidobacteria was found here to range from 30% down to 10% of total SCFA, and lactate production by *B. breve* is known to decrease under carbon limitation (32). On the other hand, bacteria other than *Bifidobacterium* spp. also have the potential to be major producers of lactate in vivo.

A second form of cross-feeding was also inferred from the increased production of butyrate by *Roseburia* sp. strain A2-183 when in coculture with *B. adolescentis* L2-32. In pure culture *Roseburia* sp. strain A2-183 is unable to utilize lactate or to grow on potato starch or Trouw FOS. The butyrate production observed in these cocultures is probably due to cross-feeding of products released by partial hydrolysis of FOS or starch by enzymes from *B. adolescentis*, most likely in the form of small fructo-oligosaccharides or malto-oligosaccharides. Indeed, the ability of *Roseburia* sp. strain A2-183 to survive in fermentor systems inoculated with mixed human fecal bacteria and supplied with different polysaccharide substrates was previously attributed to this type of cross-feeding (13). This mechanism probably operated in combination with lactate utilization to account for the butyrate formation in the cocultures involving *A. caccae* and *E. hallii* strains; indeed, this is a likely explanation for the observation (Tables 2 and 3) that lactate contributed less to butyrate carbon in the coculture experiments than in the pure-culture experiments with *E. hallii*. Since the majority of butyrate producers in the human gut are not lactate utilizers (2), such "substrate spillover" in fact represents a more generic mechanism of metabolic cross-feeding with the ability to also stimulate butyrate production. Cross-feeding of breakdown products between primary polysaccharide-degrading and oligosaccharide-utilizing gut bacteria has been recognized as a wide-ranging phenomenon in gut microbial ecosystems (10).

In conclusion, several mechanisms may contribute to the butyrogenic effects of dietary substrates such as FOS and starch. First, active fermentation tends to decrease the pH of the colonic lumen (6). This may have the effect of reducing competition for carbohydrate substrates from nonbutyrogenic species such as *Bacteroides* when the pH is decreased from 6.7 to 5.7, as suggested by a recent study in vitro (44). Butyrate-producing bacteria that are able to directly utilize FOS and starch (13) therefore may be expected to compete better for these substrates and to contribute to increased butyrate production at the lower pH (44). Second, the current data demonstrate two potential indirect mechanisms that involve metabolic cross-feeding. The importance of specific cross-feeding in vivo via lactate needs to be assessed further by determining the rate of lactate production and utilization in the complete ecosystem. This may depend partly on the abundances of lactate utilizers in different individuals. In a wider context, cross-feeding of polysaccharide breakdown products released by bifidobacteria has the potential to stimulate butyrate production regardless of the ability of butyrate producers to utilize lactate.

The relative importance of these various mechanisms has yet to be established in vivo but will probably vary between individuals and between different dietary regimens. In particular, the pH of the colonic lumen is likely to be a key factor in determining both the competition between different groups of polysaccharide-utilizing bacteria and the nature and extent of metabolic cross-feeding.

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