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Kinetic Analysis of Bifidobacterial Metabolism Reveals a Minor Role for Succinic Acid in the Regeneration of NAD^+ through Its Growth-Associated Production

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Several strains belonging to the genus *Bifidobacterium* were tested to determine their abilities to produce succinic acid. *Bifidobacterium longum* strain BB536 and *Bifidobacterium animalis* subsp. *lactis* strain Bb 12 were kinetically analyzed in detail using in vitro fermentations to obtain more insight into the metabolism and production of succinic acid by bifidobacteria. Changes in end product formation in strains of *Bifidobacterium* could be related to the specific rate of sugar consumption. When the specific sugar consumption rate increased, relatively more lactic acid and less acetic acid, formic acid, and ethanol were produced, and vice versa. All *Bifidobacterium* strains tested produced small amounts of succinic acid; the concentrations were not more than a few millimolar. Succinic acid production was found to be associated with growth and stopped when the energy source was depleted. The production of succinic acid contributed to regeneration of a small part of the NAD^+, in addition to the regeneration through the production of lactic acid and ethanol.

Bifidobacteria have been in the spotlight of scientific research since the 1990s. This attention has to do with the health-promoting effects of these organisms in humans; because of these effects bifidobacteria are often used in probiotic preparations or as target organisms for prebiotic substrates (9, 20, 41).

Bifidobacteria are gram-positive, nonsporulating, nonmotile, saccharoclastic, and usually catalase-negative rods with various shapes (35). They can dominate the intestinal microbiota of babies, but the amounts decrease over time and the bifidobacteria usually account for approximately 3% of the human colon microbiota of adults (10, 14, 18, 22). Bifidobacteria are considered key commensals in human-microbe interactions and contribute to the degradation of undigested polysaccharides in the human colon (1, 37). Because of their ability to break down and metabolize a variety of substrates, different polysaccharides can be used as prebiotics to specifically stimulate bifidobacteria in the human colon; this is the so-called bifidogenic effect (12). Of all the candidate prebiotics tested to date, inulin-type fructans are the most promising, and consequently, the consumption of fructans by bifidobacteria has been studied in detail both in vitro and in vivo (13, 15, 24, 31, 33, 39–41).

Bifidobacteria are nutritionally less fastidious than lactobacilli (32, 38). Carbohydrates are degraded exclusively and characteristically by the fructose-6-phosphate shunt, and acetic acid and lactic acid are major metabolites (6, 36, 44). Some researchers have proposed a theoretical molar ratio of acetic acid to lactic acid of 1.5, although other workers have proven that this ratio is not always obtained (1, 5, 19, 26, 29, 31, 34, 39). The variation is explained by the production of other sugar metabolites, such as formic acid and ethanol (5). This limits the production of lactic acid, which results in an increase in the theoretical ratio of acetic acid to lactic acid. Moreover, degradation of inulin-type fructans increases acetic acid production at the cost of lactic acid (39, 40). Detailed knowledge about the metabolism of bifidobacteria is very important because of the widespread use of these microorganisms as probiotics or as target organisms for prebiotic substrates.

Bifidobacteria are also able to produce small amounts of succinic acid, although this has never been studied in detail (35). Lauer and Kandler (19) have shown that small amounts of succinic acid are probably produced through a CO_2 fixation reaction. However, Chiappini (4) showed that although external CO_2 can be fixed by bifidobacteria and seems to stimulate growth, it is not incorporated during the conversion of phosphoenolpyruvate (PEP) to oxaloacetate. An examination of the genomes of *Bifidobacterium longum* NCC2705 (16, 37) (GenBank accession no. NC_004307) and *B. longum* DJO10A (GenBank accession no. NZ_AABM00000000) revealed that several genes that encode enzymes that may be involved in the production of succinic acid from carbohydrates, such as PEP carboxylase and fumarate reductase, are present. Although this metabolic trait has been known for a long time, qualitative or quantitative data are scarce (19, 44).

The aim of this study was to obtain detailed information about the production of succinic acid by bifidobacteria. An effort was made to quantify the succinic acid production by different species of *Bifidobacterium*. Moreover, the effects of different energy sources on the metabolism and production of succinic acid were examined with two strains in an effort to elucidate the function of succinic acid production in the general sugar metabolism of bifidobacteria.

MATERIALS AND METHODS

Microorganisms and media. *Bifidobacterium adolescentis* LMG 10502, *Bifidobacterium bifidum* LMG 11583, *Bifidobacterium breve* LMG 11084, *Bifidobacterium pseudolongum* subsp. *globosum* LMG 11614, *Bifidobacterium infantis* LMG

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11570, *Bifidobacterium angulatum* LMG 11568, *Bifidobacterium pseudocatenulatum* LMG 10505, and *Bifidobacterium dentium* LMG 10507 were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium). *B. longum* strain BB536 was obtained from Morinaga Industry Co. Ltd. (Tokyo, Japan). *Bifidobacterium animalis* subsp. *lactis* strain Bb 12 was obtained from Christian Hansen (Hørsholm, Denmark). All strains were stored at −80°C in de Man-Rogosa-Sharpe medium (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with 25% (vol/vol) glycerol.

Fermentations were performed in MCB (medium for colon bacteria), which supported the growth of different members of the human colon microbiota (40) and contained (per liter) 6.5 g bacteriological peptone (Oxoid), 5.0 g soy peptone (Oxoid), 2.5 g tryptone (Oxoid), 3.0 g yeast extract (VWR International, Darmstadt, Germany), 2.0 g KCl, 0.2 g $NaHCO_3$, 4.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.45 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MnSO_4 \cdot H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.005 g $ZnSO_4 \cdot 7H_2O$, 0.4 g cysteine-HCl, 0.005 g hemin, and 0.005 g menadione. The medium also contained 0.5 ml liter^{-1} H_3PO_4 and 2 ml liter^{-1} Tween 80. For small-scale fermentations (100 ml), glucose was used as the sole added energy source at a concentration of 15 g liter^{-1}, and the pH of the medium was adjusted to 6.50 before sterilization (210 kPa, 121°C, 20 min). For large-scale fermentations (1.5 liters), glucose, fructose, lactose, and oligofructose were used as the sole added energy sources at a concentration of 15 g liter^{-1}. Glucose, fructose, and lactose were sterilized (210 kPa, 121°C, 20 min) separately and added aseptically to the growth medium.

Oligofructose (RaftiloseP95) was kindly provided by ORAFTI N.V. (Tienen, Belgium). RaftiloseP95 is a commercial powder produced by enzymatic hydrolysis of chicory inulin. This powder contains oligofructose (≥93.2% [wt/wt]) and small amounts of glucose, fructose, and sucrose. The degree of polymerization of the oligofructose chains varies from 2 to 8, and the average degree of polymerization is 4. Oligofructose was filter sterilized using Sartolab P-20 filters (Sartorius AG, Goettingen, Germany) and was added aseptically to the growth medium.

Solid media were prepared by adding 1.5% (wt/vol) agar (Oxoid) to MCB with the appropriate energy source (MCB agar).

Fermentation experiments. The metabolism of all 10 *Bifidobacterium* strains was first examined using small-scale fermentations (100 ml) in MCB with glucose as the sole added energy source and without pH control. Initially, the strains were transferred from storage at −80°C to de Man-Rogosa-Sharpe medium and incubated anaerobically at 37°C in a modular atmosphere-controlled system (MG anaerobic work station; Don Withley Scientific, West Yorkshire, United Kingdom) that was continuously sparged with a mixture of 80% N_2, 10% CO_2, and 10% H_2 (Air Liquide, Paris, France). After this, the strains were propagated twice in MCB with glucose as the sole added energy source and finally added (5% [vol/vol]) to 100 ml of MCB with glucose as the sole added energy source. After 24 h of anaerobic incubation at 37°C, colony counts were obtained by plating the strains on MCB agar with glucose, and the final pH of the medium was determined. Samples were also removed for further analysis.

B. longum BB536 and *B. animalis* subsp. *lactis* Bb 12 were used for a detailed kinetic analysis. This analysis was performed by using 1.5-liter fermentations in a Biostat B-DCU fermentor (Sartorius AG). The inoculum build-up was the same as that described above (transfer volume, 5% [vol/vol]), using the same added energy source that was used later during the fermentation (glucose, fructose, lactose, or oligofructose). All fermentations were carried out anaerobically by sparging the medium with a mixture of 90% N_2 and 10% CO_2 (Air Liquide) at 37°C for 48 h. During fermentation, a linear pH profile, starting at pH 5.80 and ending at pH 6.80 after 48 h, was used to simulate the pH change during transit through the colon. The pH was controlled by automatic addition of 1.5 M NaOH. Gentle stirring (100 rpm) was used to keep the medium homogeneous. The temperature, pH, and agitation speed were controlled online (MFCS/win 2.1; Sartorius AG). Samples were removed at regular times for analysis.

All fermentations were carried out in duplicate. The results presented below are representative of both fermentations.

Analysis of growth. Colony counts (CFU per milliliter) were obtained by plating preparations on MCB agar with the appropriate energy source. The plates were incubated anaerobically (modular atmosphere-controlled system) at 37°C for 48 h. The biomass, expressed as cell dry mass (CDM), was determined by membrane filtration of a fixed volume of sample. The filters (0.45-μm cellulose nitrate filters; Sartorius AG) were dried at 105°C for 24 h and weighed.

Analysis of metabolites. The amounts of glucose, fructose, lactose, lactic acid, acetic acid, ethanol, and formic acid were determined by high-performance liquid chromatography with a Waters chromatograph (Waters Corp., Milford, MA) equipped with a 2414 differential refractometer, a 600S controller, a column oven, and a 717plus autosampler. An ICSep ICE ORH-801 column (Interchim, Montluçon, France) was used with 10 mN H_2SO_4 as the mobile phase at a flow rate of 0.4 ml min^{-1}. The column temperature was kept at 35°C. Samples were centrifuged (16,060 × *g* for 15 min), and an equal volume of 20% (vol/vol) trichloroacetic acid was added to remove proteins. After centrifugation (16,060 × *g* for 15 min) the supernatant was filtered (0.2-μm Minisart RC4 filters; Sartorius AG) before injection.

The amounts of mannitol, glycerol, and erythritol were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (Dionex, Sunnyvale, CA) using a CarboPac PA-10 column. The mobile phase (flow rate, 1.0 ml min^{-1}) consisted of ultrapure water (0.015 μS cm^{-1}) (eluent A) and 250 mM NaOH (eluent B), with the following gradient: zero time, 85% (vol/vol) eluent A and 15% (vol/vol) eluent B; 10.0 min, 85% (vol/vol) eluent A and 15% (vol/vol) eluent B; 20.0 min, 75% (vol/vol) eluent A and 25% (vol/vol) eluent B; 30.0 min, 65% (vol/vol) eluent A and 35% (vol/vol) eluent B; 50.0 min, 65% (vol/vol) eluent A and 35% (vol/vol) eluent B; 51.0 min, 100% (vol/vol) eluent B; 56.0 min, 100% (vol/vol) eluent B; 57.0 min, 85% (vol/vol) eluent A and 15% (vol/vol) eluent B; and 75.0 min, 85% (vol/vol) eluent A and 15% (vol/vol) eluent B. The supernatant was treated with trichloroacetic acid as described above. The samples were filtered (0.2-μm Minisart RC4) prior to injection.

The amount of succinic acid was determined using a Waters 2695 high-performance liquid chromatograph (Waters) coupled to a Quattro Micro mass spectrometer (Waters). The column (Atlantis; Waters) was kept at 35°C. The mobile phase (flow rate, 0.2 ml min^{-1}) was composed of ultrapure water (eluent A), acetonitrile (eluent B), and 10 mM ammonium acetate (pH 6.5) (eluent C). The gradient used was as follows: zero time, 85% (vol/vol) eluent A, 5% (vol/vol) eluent B, and 10% (vol/vol) eluent C; 15.0 min, 40% (vol/vol) eluent A, 50% (vol/vol) eluent B, and 10%(vol/vol) eluent C; 15.1 min, 10%(vol/vol) eluent A, 80% (vol/vol) eluent B, and 10% (vol/vol) eluent C; 23.0 min, 10% (vol/vol) eluent A, 80% (vol/vol) eluent B, and 10% (vol/vol) eluent C; 23.1 min, 85% (vol/vol) eluent A, 5% (vol/vol) eluent B, and 10% (vol/vol) eluent C; and 30.0 min, 85% (vol/vol) eluent A, 5% (vol/vol) eluent B, and 10%(vol/vol) eluent C. Samples were centrifuged (16,060 × *g* for 15 min), and 100 μl of an internal standard (3,4-dihydroxybenzoic acid) was added to 500 μl of supernatant. After this 600 μl of acetonitrile was added, and the samples were centrifuged again (16,060 × *g* for 15 min). The supernatant was filtered (0.2-μm Minisart RC4) and injected.

The amounts of short-chain fatty acids (SCFA) (acetic acid, propionic acid, butyric acid, valeric acid, and capronic acid) were determined by gas chromatography with an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5973N mass spectrometer (Agilent Technologies). A capillary column (DB-WAXetr; Agilent Technologies) was used together with the following oven temperature program: zero time, 90°C; 5.0 min, 90°C; 7.3 min, 125°C; 12.3 min, 125°C; 16.0 min, 180°C; 31.3 min, 180°C; 34.3 min, 230°C; and 47.3 min, 230°C. Helium (Air Liquide) was used as the carrier gas at a flow rate of 1.1 ml min^{-1}. The samples were centrifuged (16,060 × *g* for 15 min), and 100 μl of an internal standard (2,6-dimethylphenol) and 50 μl of H_2SO_4 were added to 500 μl of supernatant. After mixing for 15 s, 750 μl of diethyl ether was added to the sample and mixed thoroughly (30 min). After this, the organic phase was transferred into a vial. The extraction procedure with diethyl ether was performed twice, after which the samples were injected.

The amount of ethanol was also determined using the gas chromatography-mass spectrometry apparatus described above. The same column was used with the following temperature program: zero time, 40°C; 5.0 min, 40°C; 9.29 min, 100°C; 10.37 min, 230°C; and 15 min, 230°C. The procedure used for sample preparation was similar to the procedure used for SCFA, except that no H_2SO_4 was added, chloroform was used as the organic phase instead of diethyl ether, and methanol (0.5% [wt/vol] in ultrapure water) was used as the internal standard.

All samples were analyzed in triplicate, and the means ± standard deviations are given below. The level of carbon recovery (expressed as a percentage) was calculated by dividing the total amount of carbon recovered in the sugar metabolites by the total amount of carbon present in the added energy source. The amounts of acetic acid produced by conversion of acetyl-coenzyme A (acetyl-CoA) were calculated by subtracting the amount of acetic acid produced in the upper part of the metabolic pathway (3 mol of acetic acid are produced for every 2 mol of hexose consumed) from the total amount of acetic acid produced. NAD^+ recovery was calculated by taking into account the fact that 1 mol of $NADH + H^+$ was produced per mol of hexose consumed and the fact that 1 mol of NAD^+ was regenerated per mol of lactic acid, while 2 mol of NAD^+ was regenerated per mol of ethanol or succinic acid. The amount of acetyl-CoA converted was assumed to be equal to the sum of the amount of ethanol and the amount of acetic acid produced in the lower part of the pathway (1 mol of acetyl-CoA can be converted to 1 mol of ethanol or 1 mol of acetic acid). The

TABLE 1. Growth of and metabolite production by 10 *Bifidobacterium* strains grown in MCB at 37°C for 24 h

Strain	pH	Concn (CFU/ml)	Concn (mM) of metabolites consumed or produced after 24 h[a]					
			Glucose	Lactic acid	Acetic acid	Ethanol	Formic acid	Succinic acid
Bb 12	3.87	8.00×10^8	−27.9 ± 0.2	24.3 ± 0.1	45.4 ± 0.2	2.2 ± 0.5	BQ[b]	0.25 ± 0.04
BB536	3.78	1.55×10^8	−35.5 ± 0.5	34.5 ± 0.6	52.7 ± 0.5	ND[c]	ND	0.64 ± 0.03
LMG 10502	3.63	3.80×10^8	−49.6 ± 0.3	49.3 ± 0.2	72.7 ± 0.5	ND	ND	0.41 ± 0.05
LMG 11583	4.35	8.00×10^6	−9.7 ± 0.2	9.5 ± 0.1	22.5 ± 0.2	2.0 ± 0.4	BQ	0.12 ± 0.03
LMG 11084	3.87	1.75×10^9	−29.2 ± 0.1	26.1 ± 0.1	46.2 ± 0.1	2.5 ± 0.5	BQ	0.37 ± 0.02
LMG 11614	4.06	6.60×10^8	−19.7 ± 1.8	14.5 ± 0.4	31.7 ± 0.2	3.5 ± 0.7	BQ	0.19 ± 0.01
LMG 11570	3.72	4.50×10^8	−42.0 ± 0.1	39.3 ± 0.3	59.6 ± 0.2	ND	ND	0.76 ± 0.10
LMG 11568	3.74	1.04×10^8	−42.3 ± 0.3	41.3 ± 0.2	62.8 ± 0.8	ND	ND	0.46 ± 0.05
LMG 10505	3.71	1.03×10^9	−39.7 ± 0.4	39.1 ± 0.3	65.4 ± 0.5	2.1 ± 0.4	BQ	0.33 ± 0.01
LMG 10507	3.68	1.75×10^9	−41.9 ± 0.3	44.9 ± 0.4	68.9 ± 0.6	ND	ND	0.54 ± 0.07

[a] Negative values indicate amounts consumed, and positive values indicate amounts produced.
[b] BQ, present but below the quantification limit (<10 mM for formic acid).
[c] ND, not detected.

specific sugar consumption rate was calculated by dividing the amount of hexose equivalents consumed by the time needed to consume this amount of sugar and by the biomass.

RESULTS

Production of succinic acid during pH free fermentation. The metabolism of 10 *Bifidobacterium* strains was studied in detail using in vitro fermentations in MCB (Table 1). All strains were able to grow well in the medium used, and the final pH values were 3.63 to 4.35 after 24 h of anaerobic incubation at 37°C. The total cell counts varied from 8.00×10^6 to 1.75×10^9 CFU ml^{-1}. The consumption of glucose resulted in production of mainly acetic acid (22.5 ± 0.2 to 72.7 ± 0.5 mM) and lactic acid (9.5 ± 0.1 to 49.3 ± 0.2 mM). The molar ratio of acetic acid to lactic acid varied from 1.5 to 2.4. *B. animalis* subsp. *lactis* Bb 12, *B. bifidum* LMG 11583, *B. breve* LMG 11084, *B. pseudolongum* subsp. *globosum* LMG 11614, and *B. pseudocatenulatum* LMG 10505 produced ethanol in addition to acetic acid and lactic acid. The highest ethanol concentration (3.5 ± 0.7 mM) was produced by *B. pseudolongum* subsp. *globosum* strain LMG 11614. All ethanol-producing strains produced formic acid as well, but the concentrations were too low (<10 mM) to quantify accurately by our high-performance liquid chromatography method. Other SCFA were not produced. Remarkably, all strains tested also produced succinic acid, although the concentrations were low (0.12 ± 0.03 to 0.76 ± 0.10 mM).

Kinetic analysis of the growth of and production of metabolites by *B. longum* BB536 on different energy sources. *B. longum* strain BB536 was able to metabolize glucose, fructose, lactose, and oligofructose (Table 2 and Fig. 1) within 48 h. The cell counts were always more than 1.0×10^9 CFU ml^{-1}. Glucose was consumed fastest, with a specific sugar consumption rate of 3.5 mmol hexose equivalents g CDM^{-1} h^{-1}. Oligofructose was consumed more slowly than all of the other substrates, with a specific sugar consumption rate of 1.3 mmol hexose equivalents g CDM^{-1} h^{-1}. Acetic acid was the major metabolite produced, and the concentrations ranged from 130 ± 7 mM for the fermentation with glucose to 161 ± 2 mM for the fermentation with oligofructose. Lactic acid, formic acid, and ethanol were also produced during all fermentations. In the fermentation with glucose, high concentrations of lactic acid were produced (74 ± 4 mM), and only traces of ethanol and formic acid were found. Larger amounts of acetic acid, formic acid, and ethanol and smaller amounts of lactic acid were produced during growth on fructose, lactose, or oligofructose than during the fermentation with glucose. In general, larger amounts of acetic acid corresponded with larger amounts of formic acid and ethanol and smaller amounts of lactic acid and vice versa. The molar ratio of acetic acid to lactic acid varied from 1.75 ± 0.14 (fermentation with glucose) to 7.06 ± 0.10 (fermentation with oligofructose). During all four fermentations, low concentrations of succinic acid were produced, and the concentrations ranged from 0.88 ± 0.01 mM for the fermentation with oligofructose to 2.05 ± 0.03 mM for the fermentation with lactose. Succinic acid production was always growth associated and stopped when the substrate was completely consumed. Although the production of lactic acid and ethanol accounted for most of the NAD^+ recuperation, the production of succinic acid still accounted for 2.0% (fermentation with oligofructose) to 4.6% (fermentation with lactose) of the total NAD^+ recuperation. It appeared that higher yields of succinic acid corresponded to a higher specific sugar consumption rate for this strain. We calculated that less than 50%

TABLE 2. Specific sugar consumption rates and metabolite production for *B. longum* BB536 after growth in MCB containing different energy sources

Energy source	Specific sugar consumption rate (mmol hexose equivalents/g CDM/h)	Yield (mol/mol hexose equivalents consumed)					Carbon recovery (%)	NAD^+ recovery (%)
		Lactic acid	Acetic acid	Formic acid	Ethanol	Succinic acid		
Glucose	3.5	0.92	1.61	0.07	0.04	0.02	103 ± 4	104 ± 6
Fructose	2.5	0.37	1.85	0.57	0.30	0.02	101 ± 2	101 ± 3
Lactose	2.4	0.49	1.74	0.46	0.23	0.02	100 ± 7	101 ± 6
Oligofructose	1.3	0.27	1.91	0.72	0.37	0.01	102 ± 3	103 ± 3

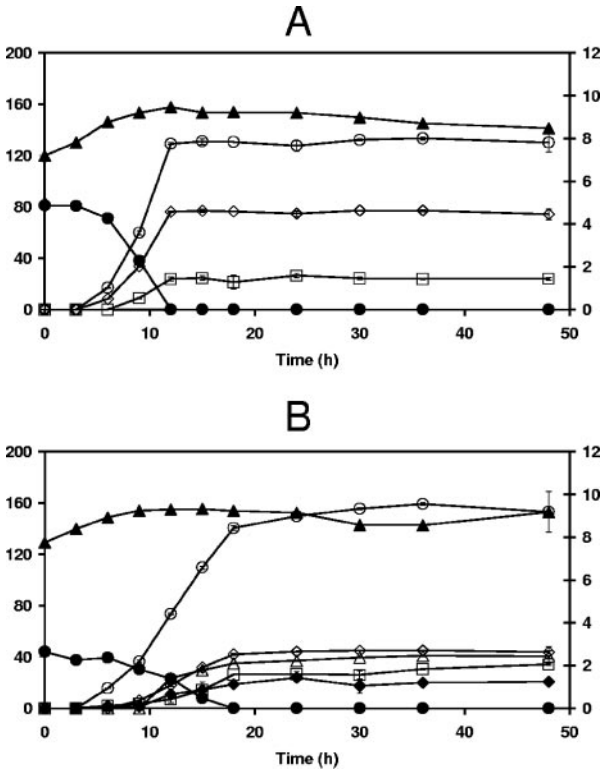

FIG. 1. *B. longum* BB536 fermentation in MCB with 15 g liter^{-1} glucose (A) or 15 g liter^{-1} lactose (B). The left axis indicates the glucose concentration (mM) (A) or the lactose concentration (mM) (B) (●), the acetic acid concentration (mM) (○), the lactic acid concentration (mM) (◇), the ethanol concentration (mM) (◆), and the formic acid concentration (mM) (△). The right axis indicates the concentration of the organism (log CFU ml^{-1}) (▲) and the succinic acid concentration (mM) (□). The data for fructose or oligofructose (RaftiloseP95) as the sole added energy source are from reference 40.

of the acetyl-CoA was converted into ethanol and that more than 50% of the acetyl-CoA was converted into acetic acid. Other possible electron sinks, such as mannitol, glycerol, and erythritol, were not produced. Performing the fermentation using glucose as the sole added energy source and only nitrogen instead of the N_2/CO_2 mixture to sparge the medium had no influence on the production of succinic acid as similar concentrations were obtained, indicating that external CO_2 is not necessary for succinic acid production (data not shown).

Kinetic analysis of the growth of and production of metabolites by *B. animalis* subsp. *lactis* Bb 12 on different energy sources. In contrast to *B. longum* strain BB536, *B. animalis* subsp. *lactis* Bb 12 was not able to grow on fructose. Therefore, only results for growth on glucose, lactose, and oligofructose are shown in Table 3 and Fig. 2. The cell counts were always more than 1.0×10^9 CFU ml^{-1}. Lactose was consumed faster than glucose or oligofructose, and the accompanying specific sugar consumption rates were 3.9, 2.0, and 0.5 mmol hexose equivalents g CDM^{-1} h^{-1}, respectively. The total amount of acetic acid produced was greatest for the fermentation with glucose (160 ± 4 mM), although the molar yield of acetic acid (i.e., moles of acetic acid per mole of hexose equivalents consumed) was greatest for the fermentation with oligofructose. As not all oligofructose was consumed within 48 h, the total amount of acetic acid produced was less than the amounts observed during the other fermentations. The concentration of lactic acid produced was highest for the fermentation with lactose (75 ± 2 mM) and lowest for the fermentation with oligofructose (10 ± 1 mM). Lower rates of specific sugar consumption corresponded well with larger amounts of acetic acid, formic acid, and ethanol and with smaller amounts of lactic acid (and vice versa), as was the case for *B. longum* BB536 (Fig. 3A). The molar ratio of acetic acid to lactic acid varied between 1.92 ± 0.06 (fermentation with lactose) and 8.97 ± 1.30 (fermentation with oligofructose). Growth-associated production of succinic acid was observed during all fermentations. Although the production of lactic acid and the production of ethanol were the main metabolic routes used to regenerate NAD^+, the production of succinic acid still accounted for 2.1% (fermentation with lactose) to 3.3% (fermentation with oligofructose) of the total NAD^+ recuperation. Higher yields of succinic acid seemed to correspond with a lower specific sugar consumption rate for this strain, which was the opposite of what was found for the *B. longum* BB536 strain (Fig. 3B). However, less than 50% of the acetyl-CoA was metabolized to ethanol, and more than 50% of the acetyl-CoA was metabolized to acetic acid. Mannitol, glycerol, and erythritol were not produced. This strain also produced similar amounts of succinic acid during fermentation with glucose as the sole added energy source and when pure nitrogen instead of the N_2/CO_2 mixture was used to sparge the medium (data not shown).

DISCUSSION

Although bifidobacterial metabolism was discovered in the 1960s, it is often misinterpreted. For instance, it is often reported that only acetic acid and lactic acid are produced by these bacteria at a molar ratio of 3:2. However, researchers have described other features of bifidobacterial metabolism, such as the variation of the ratio of acetic acid to lactic acid and the production of other metabolites, such as formic acid and ethanol (1, 5, 19, 26, 29, 31, 39). It is clear from our study that the specific rate of sugar consumption plays an important role

TABLE 3. Specific sugar consumption rate and metabolite production for *B. animalis* subsp. *lactis* Bb 12 after growth in MCB containing different energy sources

Energy source[a]	Specific sugar consumption rate (mmol hexose equivalents/g CDM/h)	Yield (mol/mol hexose equivalents consumed)					Carbon recovery (%)	NAD^+ recovery (%)
		Lactic acid	Acetic acid	Formic acid	Ethanol	Succinic acid		
Glucose	2.0	0.43	1.86	0.70	0.24	0.01	104 ± 2	94 ± 3
Lactose	3.9	0.81	1.56	0.17	0.06	0.01	98 ± 3	96 ± 3
Oligofructose	0.5	0.21	1.92	0.75	0.37	0.02	101 ± 10	100 ± 7

[a] There was no growth on fructose.

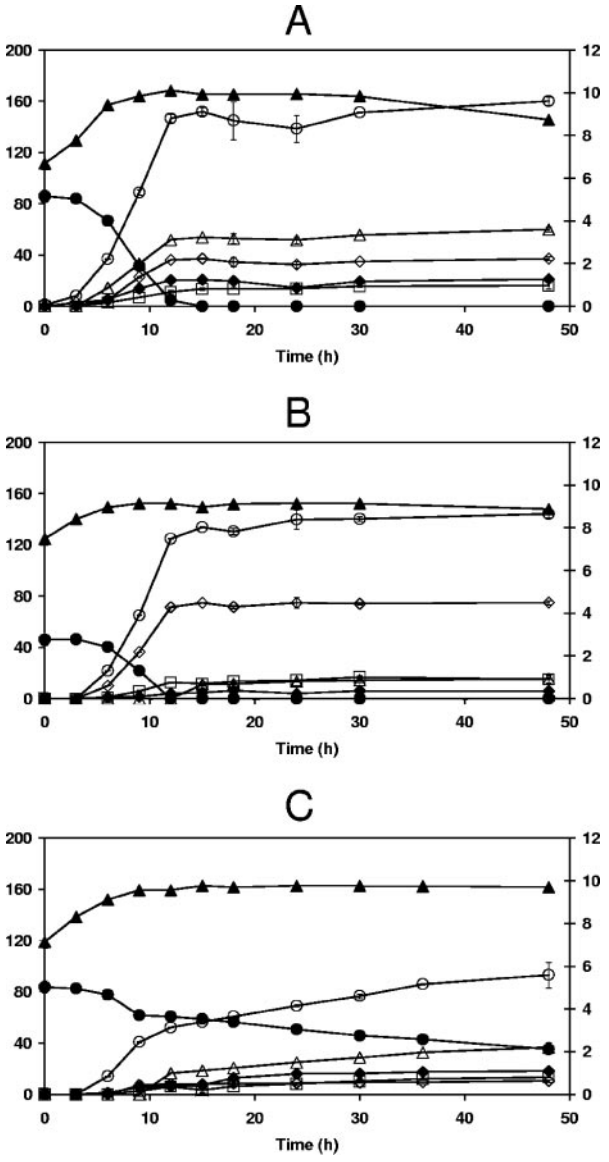

FIG. 2. *B. animalis* subsp. *lactis* Bb 12 fermentation in MCB with 15 g liter^{-1} glucose (A), 15 g liter^{-1} lactose (B), or 15 g liter^{-1} oligofructose (RaftiloseP95) (C). The left axis indicates the glucose concentration (mM) (A), lactose concentration (mM) (B), or oligofructose concentration (expressed as mM fructose) (C) (●), acetic acid concentration (mM) (○), lactic acid concentration (mM) (◇), ethanol concentration (mM) (◆), and formic acid concentration (mM) (△). The right axis indicates the concentration of the organism (log CFU ml^{-1}) (▲) and the succinic acid concentration (mM) (□).

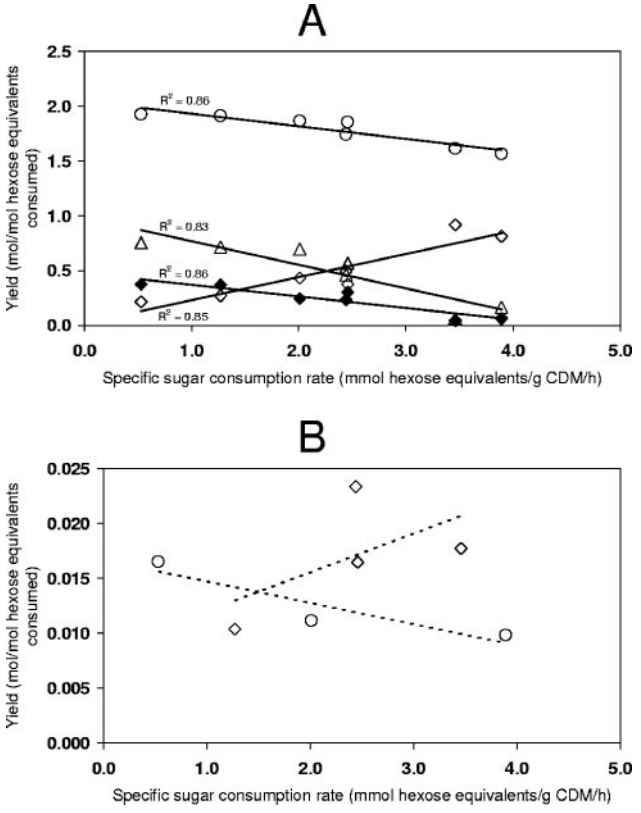

FIG. 3. (A) Relationship between the specific sugar consumption rate and the yields of acetic acid (○), lactic acid (◇), formic acid (△), and ethanol (◆) for *B. longum* BB536 and *B. animalis* subsp. *lactis* Bb 12. (B) Relationship between the specific sugar consumption rate and the yield of succinic acid for *B. longum* BB536 (◇) and *B. animalis* subsp. *lactis* Bb 12 (○). The yields are expressed in mol produced per mol of hexose equivalents consumed. The correlation coefficients (R^2) are indicated.

in the ratio of the final metabolites produced, as has been shown previously for a strain of the lactic acid bacterium *Lactococcus lactis* (11). If bifidobacteria consume the energy source fast, larger amounts of lactic acid and relatively smaller amounts of acetic acid, formic acid, and ethanol are produced. If the energy source is consumed slowly (e.g., the oligofructose used in this study), less lactic acid is produced and more acetic acid, formic acid, and ethanol are produced. This is clear proof of the production of formic acid, acetic acid, and ethanol by bifidobacteria through dissipation of pyruvate (Fig. 4). Several factors related to the specific sugar consumption rate, such as the sugar uptake systems and the conversions necessary to incorporate a specific sugar into the fructose-6-phosphate pathway, can have great effects on which metabolic route is used (3, 17). It has also been reported that in *L. lactis* the NADH + H^+/NAD^+ ratio (11) or the pool of ADP and ATP (30) can have an effect on changes in end product formation. However, the fact that *B. longum* BB536 had almost the same specific rate of sugar consumption on fructose and on lactose, although the amounts and ratios of the end products differed, proved that the specific sugar consumption rate was not the only determining factor. Other factors can also be important, such as the amount or the regulation of the production of certain enzymes, as has been reported for *L. lactis* (23). Although it is still not clear how sugar metabolism is controlled in bifidobacteria and lactic acid bacteria, this metabolism is certainly regulated by a complex interaction of different factors (27).

If less lactic acid is produced, ethanol production is the main pathway for regenerating NAD^+. As 2 mol of NAD^+ is regenerated per mol of ethanol produced (Fig. 4), only part of the acetyl-CoA (theoretically 50%) has to be metabolized in this pathway. The remaining part can be used for additional ATP production through conversion into acetic acid. This results in a more efficient use of the energy source, as was observed for a bile-adapted strain of *B. animalis* by Ruas-Madiedo et al.

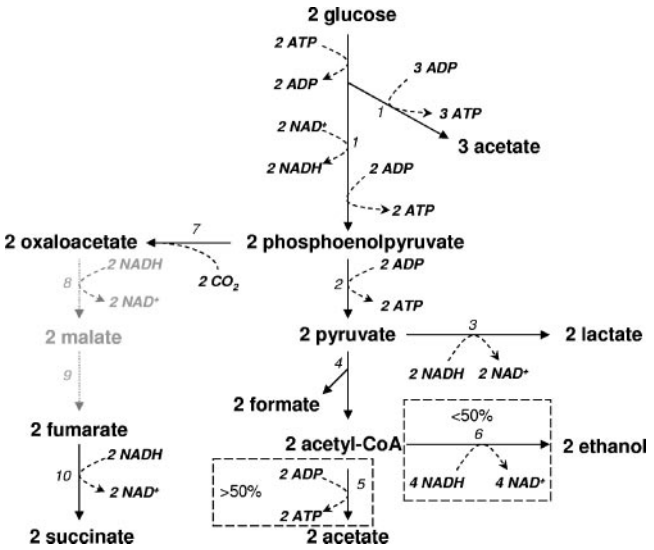

FIG. 4. Schematic diagram of bifidobacterial sugar metabolism. 1, Enzymes of the bifidobacterial fructose-6-phosphate shunt; 2, pyruvate kinase (*pyk*); 3, lactate dehydrogenase (*ldh*); 4, pyruvate formate lyase (*pfl*); 5, phosphotransacetylase (*pta*) and acetate kinase (*ack*); 6, acetaldehyde dehydrogenase (*adh*) and alcohol dehydrogenase (*adh*); 7, phosphoenolpyruvate carboxylase (*ppc*); 8, malate dehydrogenase (coding sequence not found in either *B. longum* genome); 9, fumarase (coding sequence not found in either *B. longum* genome); 10, succinate dehydrogenase (*sdh*). The values in boxes are the percentages of conversion of acetyl-CoA.

(34). In this study, we found that both *B. longum* BB536 and *B. animalis* subsp. *lactis* Bb 12 converted more than 50% of acetyl-CoA into acetic acid during all fermentations. As this percentage is higher than the percentage that is theoretically possible, there must be other ways to regenerate NAD^+; otherwise, the redox balance would remain out of equilibrium. As observed for other lactic acid bacteria, the reduction of fructose to mannitol is a possible pathway (43), but mannitol was not produced (detection limit, 50 μM). The production of erythritol or glycerol can also be an alternative pathway for regenerating NAD^+ (42), but neither of these compounds was found (detection limits, 80 μM and 100 μM, respectively). However, production of succinic acid can be an alternative way to equilibrate the redox balance (Fig. 4).

The production of succinic acid by *Bifidobacterium* strains was observed by Lauer and Kandler (19) and Wolin et al. (44). All 10 *Bifidobacterium* strains tested in our study, which belonged to different species, produced small amounts of succinic acid (<1 mM during pH free fermentations). The two *Bifidobacterium* strains that were studied in more detail produced succinic acid during growth on all carbohydrate sources tested, and we observed that the production of succinic acid was growth associated and stopped when the energy source was depleted. The production of succinic acid probably occurred through the conversion of PEP into oxaloacetate with the incorporation of CO_2, due to the action of a PEP carboxylase. The gene coding for PEP carboxylase has recently been found in the genomes of two *B. longum* strains (37) (GenBank accession no. NC_004307 and NZ_AABM00000000). However, external CO_2 is not incorporated during the conversion of PEP into oxaloacetate, as has been observed by Chiappini (4). Indeed, we found no differences in succinic acid production between fermentations that were sparged with external CO_2 and fermentations that were not sparged with external CO_2. When we examined the genome information, only fumarate reductase, which is responsible for the conversion of fumarate into succinate, was found, but both malate dehydrogenase and fumarase, which are responsible for the conversion of oxaloacetate into fumarate, were not found. However, minor activity of the malate dehydrogenase enzyme in bifidobacteria was found (data not shown), as reported previously (4). Furthermore, similarity-based tools may not successfully annotate many genes, thereby creating gaps in certain metabolic pathways (28). Thus, it is possible that these genes are indeed present but are not detected due to low degrees of homology with known genes. Sequencing more *Bifidobacterium* genomes or a search on the molecular level for these enzymes may provide an answer to this question.

The reason why small amounts of succinic acid are produced is still unclear. As the conversion of PEP into oxaloacetate is mediated through the PEP carboxylase, as it is in *Escherichia coli* (25), the energy conserved in PEP is dissipated when the molecule is converted into succinic acid. In this way, energy is lost, which may explain why only small amounts of succinic acid are produced. Bacteria that produce large amounts of succinic acid (e.g., the colon bacterium *Bacteroides*) use a PEP carboxykinase which conserves the energy of PEP through production of ATP during conversion of PEP into oxaloacetate (21). Nevertheless, succinic acid is always produced by bifidobacteria, which was clear from our study. The metabolic route shown in Fig. 4 indicates that 2 mol of NAD^+ is regenerated when PEP is converted into succinic acid. When theoretically calculating the $NADH + H^+/NAD^+$ balance, we noticed that a small percentage of NAD^+ recuperation occurs through this pathway, which may explain why succinic acid is produced. However, the question is why not all NAD^+ is regenerated through the production of ethanol, since this pathway involves the conversion of PEP into pyruvate, enabling the production of ATP, which is not the case during the conversion of PEP into oxaloacetate in bifidobacteria. A clear relationship between the yield of succinic acid and the specific sugar consumption rate could not be detected, since it was observed that in *B. longum* strain BB536 higher yields of succinic acid corresponded with a higher specific sugar consumption rate, whereas the opposite was observed for *B. animalis* subsp. *lactis* Bb 12 strain. Detailed kinetic analysis of more *Bifidobacterium* strains may reveal a possible relationship.

The low specific rate of sugar consumption during growth of the *Bifidobacterium* strains on the prebiotic oligofructose may indicate that mainly acetic acid and to a lesser extent formic acid, ethanol, and lactic acid are produced upon degradation of this prebiotic in vivo. Moreover, we have previously shown that bifidobacteria preferentially use the short fractions of oligofructose rapidly, resulting in production of acetic acid and lactic acid during growth on these shorter fractions (39, 40). Therefore, acetic acid and lactic acid are the main metabolites produced by bifidobacteria upon prebiotic stimulation with oligofructose, because bifidobacteria probably profit from the degradation of the longer oligofructose fractions by other colon bacteria (40). In this way, ingestion of oligofructose may indirectly stimulate the production of butyric acid in the colon through cross-feeding of other colon bacteria on the bifidobacterial metabolites (2, 7, 8).

In conclusion, we found that the changes in end product formation in strains of *Bifidobacterium* can be related to the specific rate of sugar consumption. When the latter increased, relatively more lactic acid and less acetic acid, formic acid, and ethanol were produced, and vice versa. Furthermore, we found that succinic acid production occurs in all *Bifidobacterium* strains tested and that this production is growth associated. In general, the concentrations of succinic acid produced were low, usually 1 mM to a few millimolar. Although only small amounts of succinic acid were produced, succinic acid production contributed to the regeneration of NAD^+, which in *Bifidobacterium* strains occurs mainly through the production of lactic acid or ethanol.

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