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EFFECT OF VARIOUS SACCHARIDES ON MAIN PRODUCTS OF *BIFIDOBACTERIUM* FERMENTATION

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The products of fermentation of *Bifidobacterium* strains belonging to the 10 species were determined after incubation (37°C/48 h, anaerobic conditions) of the inoculated (10^6-10^7 cfu/mL) Garche's medium containing 1% (w/w) lactose, glucose or fructooligosaccharides (FOS) (Wako, Japan). The study confirmed the presence of acetic and lactic acids as the main products of *Bifidobacterium* fermentation, and small amounts of ethanol were additionally determined. Concentration of total acetic and lactic acids (A+L) ranged from 90.4 to 231.8 μmol/mL (av. 140.9 μmol/mL) for lactose, and from 93.7 to 159.3 μmol/mL (av. 115.0 μmol/mL) for glucose. Lactic acid prevailed in the majority of cultures containing lactose or glucose as substrates of fermentation. The A+L concentrations in the cultures with FOS were lower. They ranged from 64.5 to 200.6 μmol/mL (av. 94.8 μmol/mL). Using that substrate, nine strains produced preferably acetic acid whereas five strains produced higher amounts of lactic acid. The highest amounts of ethanol were produced as a result of FOS fermentation, ranging from 0.16 to 3.90 μmol/mL (av. 1.63 μmol/mL), lower from glucose - 0.67-3.55 μmol/mL (av. 1.46 μmol/mL), and the lowest from lactose – 0.33-2.12 μmol/mL (av. 0.84 μmol/mL). The pH level varied from 4.25-4.89 (av. 4.36), through 4.37-4.77 (av. 4.58) to 4.33-5.11 (av. 4.76) in cultures containing lactose, glucose or FOS, respectively.

The application of 4% Carbowax 20M liquid phase on Carbopack B-DA 80/120 (Supelco) for gas chromatography enabled the direct parallel analysis of both acids, which made it possible to evaluate not only the concentration but also the proportion of the main products of fermentation. The results indicate middle or strong negative correlation between the acidity and the total amount of acetic and lactic acid, which confirms the usefulness and reliability of the assumed method for the determination of *Bifidobacterium* fermentation products in liquid cultures.

INTRODUCTION

Bifidobacteria constitute one of the predominant groups of human intestinal microflora and fulfil numerous functions beneficial for human health [Mitsuoka, 1996; Gibson, 1998]. Their growth and activity in the gut depend on the presence of available substrates – some non-digestible saccharides, including fructooligosaccharides. Fermentation to acetic and lactic acids results in lowering of pH of the colon contents, inhibition of competing harmful bacteria (including pathogens), improvement of bioavailability of minerals, and the trophic effect on the intestinal epithelium. Due to the ideal chemical equation, in the so-called "bifid shunt", 3 moles of acetic acid and 2 moles of lactic acid are the products of 2 mole glucose fermentation [Bezkorovainy, 1989]. In fact, in the real fermentation processes the metabolites may differ in their amount and form, but scant information on these is available when it comes to bifidobacteria.

Another problem is parallel and direct determination of both acetic and lactic acids. They are mostly determined separately, for example lactic acid with enzymatic method and acetic acid with gas chromatography [Hove *et al*., 1994; Kontula *et al*., 1999; Franklin *et al*., 2002], or indirectly – both of them as their methyl esters or different extracts – with HPLC [Badoud & Pratz, 1986; Fernandez-Garcia & McGregor, 1994].

The aim of the study was to evaluate the capability of several *Bifidobacterium* species to ferment different saccharides as well as the influence of the substrates on the proportion of metabolites produced using gas chromatography for parallel determination of basic fermentation products.

MATERIAL AND METHODS

Main saccharide fermentation products of twenty three strains of *Bifidobacterium* belonging to ten species were evaluated. The substrates for fermentation were lactose, glucose or fructooligosaccharides (FOS, Wako Pure Chemical Industries, Japan), the latter being a 93.0% mixture of 1-kestose, nystose and 1-fructofuranosyl-D-nystose. The modified liquid Garche's medium [Rasic, 1990] (with bacto-casitone replaced by Peptobak, BTL, Łódź, Poland and without lithium chloride), containing saccharides as the only source of carbon and energy, was inoculated with *Bifidobacterium* cultures in the amount of 10^6-10^7 cfu/mL and incubated at 37°C/48 h under anaerobic conditions (under pyrogallol plug). After incubation, the acidity of medium was measured using an HI 9025 microcomputer pH-meter (Hanna Instruments), and the contents of acetic and lactic acids, and ethanol were determined with gas chromatography. The application of 4% Carbowax 20M liquid phase on Carbopack B-DA 80/120 (Supelco) was supposed to enable the direct analysis of both acids together.

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The analysis proceeded as follows: 1 mL of 25% metaphosphoric acid was added to 5 mL of 48-hour-culture, followed by (after 10 min) the addition of 0.25 mL of formic acid; the sample was centrifuged using a centrifuge type 310" (Unipan, Poland) at 7500 g for 10 min; the supernatant obtained was mixed with 0.3 mol/L oxalic acid (9:1 v/v), tightly closed and stored at 5°C until insertion on the column. A mixture of 0.25% (v/v) lactic acid, 0.25% (v/v) acetic acid and 0.01% (v/v) ethanol in bacterial medium (as above), combined with 0.3 mol/L oxalic acid (9:1 v/v) was used as a standard solution. The standard was stored under analogous conditions as the samples. It was injected on the column before every batch of 3-6 samples.

The concentrations of metabolites were determined in supernatant using a PU4600 gas chromatograph with a glass column (1 m x 4 mm i.d.) and an FID detector. Argon was used as the carrier gas with a flow rate of 40 mL/min. The temperatures of an oven, a detector and an injector were 185°C, 250°C and 225°C, respectively. The column was injected with 1 µL of 0.03 mol/L oxalic acid in water, followed by 100 ppm of each analyte. The samples were examined in duplicates.

Peak areas obtained on chromatographs for adequate metabolites were converted into their concentration, as mg/mL of culture, on the basis of peak area of standards, and next converted into µmol/mL. The applied manner of calculations enabled a quantitative comparison of different metabolites, as well as determination of the proportion of acetate to lactate (A:L) and the total amount of acids (A+L).

RESULTS

The method confirmed the presence of acetic and lactic acids as the main metabolites of *Bifidobacterium* (Figure 1). Small amounts of ethanol were additionally identified. As examples, the chromatographs of the acid standard and main products of *B. catenulatum* KD14 following FOS utilisation are presented. Retention times for ethanol (peak 1), acetate (peak 2) and lactate (peak 3) were respectively 0.772, 1.884, and 14.500 min for the standard, and 0.788, 1.916, and 14.531 min for the sample (Figure 1).

In the media containing lactose and glucose, the concentration of total acetic and lactic acids (A+L) ranged from 90.4 to 231.8 µmol/mL (av. 140.9 µmol/mL) for lactose (Table 1), and from 93.7 to 159.3 µmol/mL (av. 115.0 µmol/mL) for glucose (Table 2). Lactic acid, ranging from 44.4 to 173.7 µmol/mL or from 24.9 to 99.2 µmol/mL, prevailed in the majority of cultures containing respectively lactose or glucose, as substrates of fermentation. The concentration of acetic acid varied from 40.4 to 73.4 µmol/mL and from 39.9 to 68.8 µmol/mL, respectively.

The A+L concentrations in the cultures with FOS ranged from 64.5 to 200.6 µmol/mL (av. 94.8 µmol/mL) and were lower than in the cultures containing lactose or glucose (Table 3). Using that substrate, nine strains produced preferably acetic acid whereas five strains produced higher amounts of lactic acid.

The highest amounts of the total acids (A+L), ranging from 160.0 to 231 µmol/mL, were produced by the strains of *B. pseudocatenulatum* ATCC 27919 and KD15, *B. breve* ATCC 15700, *B. pseudolongum* PS36, and *B. adolescentis* ATCC 15703 in the medium containing lactose and by the strain *B. pseudocatenulatum* ATCC 27919 in the medium containing FOS. All those strains produced also the highest amounts of lactic acid, ranging from 101.1 to 173.7 µmol/mL. On the contrary, the lowest amounts of the total acids were produced by the strains of *B. bifidum* (90.4-99.0 µmol/mL) in the medium containing lactose, and by the strains of *B. animalis*, *B. longum*, *B. globosum* and *B. pseudolongum* (63.5-81.9 µmol/mL) in the medium containing FOS.

The highest amounts of ethanol were produced as a result of FOS fermentation, ranging from 0.16 to 3.90 µmol/mL (av. 1.63 µmol/mL), lower from glucose – 0.67-3.55 µmol/mL (av. 1.46 µmol/mL), and the lowest from lactose – 0.33-2.12 µmol/mL (av. 0.84 µmol/mL) (Tables 1, 2, 3). The strains producing the highest amounts of ethanol were as follows: *B. animalis* J38; *B. longum* KNA1, KN4, KN29.1;

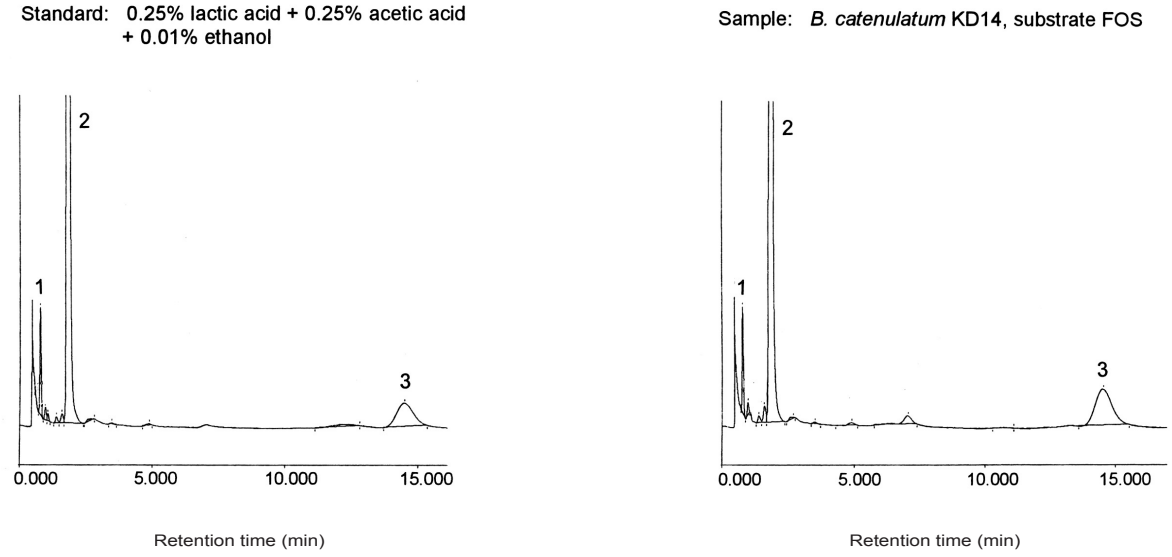

FIGURE 1. Main products of *Bifidobacterium* fermentation – chromatographs of the standard and one of the tested strains.

TABLE 1. The amount and proportion of basic metabolites produced by bifidobacteria following lactose fermentation (A – acetic acid, L – lactic acid, E – ethanol, μmol/mL).

Bifidobacterium sp.	Strain	A	L	E	pH	A+L	A:L
B. catenulatum	KD14	54.0	79.3	0.54	4.27	133.3	0.68
	ATCC 27539	52.7	84.5	0.39	4.36	137.2	0.62
B. longum	KNA1	55.2	70.9	0.42	4.30	126.1	0.78
	KN29.1	50.6	83.8	0.66	4.26	134.4	0.60
	KN4	66.1	65.6	0.53	4.25	131.7	1.01
B. animalis	11	53.6	89.2	0.48	4.25	142.8	0.60
	30	50.3	82.1	0.29	4.26	132.4	0.61
	J38	56.6	93.6	0.52	4.25	150.2	0.60
B. bifidum	KD6	46.0	44.4	0.44	4.68	90.4	1.04
	KD7	46.3	52.7	1.46	4.54	99.0	0.88
	ATCC 29521	47.5	43.0	1.62	4.89	90.5	1.10
B. pseudocatenulatum	KD15	68.5	103.2	1.30	4.25	171.7	0.66
	ATCC 27919	59.1	172.7	0.33	4.29	231.8	0.34
B. globosum	PS11	58.7	82.9	1.54	4.26	141.6	0.71
	KSIb2	53.5	63.5	2.12	4.37	117.0	0.84
	DSMZ 20092	66.9	53.7	1.13	4.39	120.6	1.25
B. pseudolongum	PS36	58.9	101.1	0.54	4.37	160.0	0.58
	KSI9	61.7	63.3	1.33	4.44	125.0	0.97
	DSMZ 20099	73.4	64.5	0.43	4.38	137.9	1.14
B. infantis	ATCC 15697	51.2	91.3	0.33	4.30	142.5	0.56
B. breve	ATCC 15700	48.3	173.7	0.36	4.30	222.0	0.28
B. adolescentis	ATCC 15703	40.4	121.8	1.95	4.30	162.2	0.33
B. sp.	KP9	57.1	82.5	0.66	4.34	139.6	0.69
	average	55.5	85.4	0.84	4.36	140.9	0.73
	median	54.0	82.5	0.54	4.30	137.2	0.68

TABLE 2. The amount and proportion of basic metabolites produced by bifidobacteria following glucose fermentation (A – acetic acid, L – lactic acid, E – ethanol, μmol/mL).

Bifidobacterium sp.	Strain	A	L	E	pH	A+L	A:L
B. catenulatum	KD14	52.7	43.0	2.09	4.46	95.7	1.23
B. longum	KNA1	60.1	99.2	0.30	4.37	159.3	0.61
B. animalis	30	68.8	24.9	3.55	4.59	93.7	2.76
B. bifidum	KD6	50.5	54.9	0.67	4.67	105.4	0.92
	KD7	39.9	58.3	1.08	4.77	98.2	0.68
	ATCC 29521	50.6	86.8	1.07	4.60	137.4	0.58
	average	53.8	61.2	1.46	4.58	115.0	1.13
	median	52.7	58.3	1.08	4.60	105.4	0.92

and *B. breve* ATCC 15700 – in the medium containing FOS (1.93-2.76 μmol/mL); *B. animalis* 30 and *B. catenulatum* KD14 – in the medium containing glucose (3.55 and 2.09 μmol/mL, respectively); *B. globosum* KSIb2 and *B. adolescentis* ATTC 15703 – in the medium containing lactose (2.12 and 1.95 μmol/mL, respectively).

The pH level varied from 4.25-4.89 (av. 4.36), through 4.37-4.77 (av. 4.58), to 4.33-5.11 (av. 4.76) in the cultures containing lactose, glucose or FOS, respectively (Tables 1, 2, 3).

DISCUSSION

Scardovi [1986] noticed, that although the theoretical ratio of acetate to lactate is 3:2, it is scarcely ever found in growing cultures of bifidobacteria. Different bifidobacterial species

TABLE 3. The amount and proportion of basic metabolites produced by bifidobacteria following FOS fermentation (A – acetic acid, L – lactic acid, E – ethanol, μmol/mL).

Bifidobacterium sp.	Strain	A	L	E	pH	A+L	A:L
B. catenulatum	KD14	48.9	43.8	1.45	4.62	92.7	1.12
	ATCC 27539	54.9	68.2	0.44	4.35	123.1	0.80
B. longum	KNA1	42.8	34.9	2.02	4.81	77.7	1.22
	KN29.1	48.9	27.0	1.90	4.77	75.9	1.81
	KN4	55.2	26.7	1.97	4.76	81.9	2.07
B. animalis	11	46.8	17.7	1.40	5.01	64.5	2.64
	30	45.1	26.3	1.03	4.97	71.4	1.71
	J38	45.7	17.8	2.76	4.98	63.5	2.57
B. pseudocatenulatum	ATCC 27919	65.9	134.7	0.16	4.33	200.6	0.49
B. globosum	DSMZ 20092	43.7	37.8	1.44	4.82	81.5	1.16
B. pseudolongum	DSMZ 20099	44.4	36.8	1.31	4.91	81.2	1.21
B. breve	ATCC 15700	46.2	56.1	1.93	4.69	102.3	0.82
B. adolescentis	ATCC 15703	41.4	66.8	1.13	4.47	108.2	0.62
	average	45.0	45.7	1.46	4.73	94.2	1.40
	median	46.2	36.8	1.44	4.77	81.5	1.21

will yield different relative amounts of acetate, lactate, ethanol, and formate under the same conditions [Bezkorovainy, 1989]. For example the acetate to lactate ratio following glucose fermentation of various bifidobacterial species and strains ranged from 0.5 to 16.8 [de Vries & Stouthamer, 1967, cited after Bezkorovainy, 1989]. Moreover, varying conditions of growth, such as the type and quantity of the carbon source, may also result in the production of varying quantities of the fermentation products [Bezkorovainy, 1989]. Dubey and Mistry [1996] showed different amounts of acetic and lactic acid of *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* growing on different infant formula and non-fat milk, with molar ratios of 0.84-2.5, 0.93-1.1, 1.07-2.04, and 0.77-1.0, respectively.

The results showed that the amount and proportion of acids produced by bifidobacteria is highly dependent on the substrate metabolised. Much lower amounts of total acids were produced when the substrate was FOS than glucose or lactose, the highest being on the latter. The concentration of acetic acid was comparable, influenced by neither the substrate nor the strain. Thus the fermentation of FOS resulted in the prevalence of acetic acid, whereas lactic acid was the main metabolite of lactose fermentation. The studied strains produced also little amounts of ethanol, higher in the medium containing FOS than lactose. Summarising, the kind of substrate affected mostly the level of lactic acid, of which the lowest amounts were determined in the medium with FOS and the highest in the medium with lactose. Contrary to the acetic acid, the examined strains and species differed by the amounts of the produced lactic acid. The data suggest that generation of acetic acid by bifidobacteria is much more stable than that of lactic acid, though the concentration of the latter may be higher, especially when the substrate is easily and well metabolised.

In fact, saccharide metabolism in bifidobacteria seems to be a more complex problem. Degnan and Macfarlane [1994] studying the impact of source of carbon on *B. breve* NCFB 2257 fermentation showed that at limited glucose availability the cultures produced mainly acetic and formic acid, whereas in the excess of glucose – lactic and acetic acid. In the opinion of Macfarlane and Gibson [1995] lactate frequently appears following rapid fermentation of carbohydrate, and acts as an electron sink to dispose of excess reducing power, when substrate is plentiful. The possible explanation of the mechanism, given earlier by Scardovi [1986] and confirmed by Degnan and Macfarlane [1994], is metabolic fate of pyruvate which may be reduced to lactate, or alternatively, it can be dissimilated by phosphoroclastic cleavage to formic acid and acetyl phosphate followed by the reduction of acetyl phosphate to ethanol. The latter pathway can often alter the fermentation balance in favour of the production of acetate and some formic acid and ethanol. Degnan and Macfarlane [1994] indicated the metabolic flexibility of the tested strain of *B. breve*, which preferentially used lactate as an electron sink during nitrogen-limited growth, whereas under energy-limitation, carbon flow was directed towards acetyl phosphate to maximise ATP synthesis.

CONCLUSIONS

The application of 4% Carbowax 20M liquid phase on Carbopack B-DA 80/120 (Supelco) and Column Gas Chromatography enabled the direct parallel determination of lactic and acetic acids, which made it possible to evaluate not only the concentration but also the proportion of the main products of fermentation. The total amount of acetic and lactic acids produced by bifidobacteria was the highest in the medium containing lactose, on the same or lower level in the medium containing glucose, and the lowest in the medium containing FOS. The majority of the examined strains produced higher amounts of lactic than acetic acid in the medium with lactose as

well as glucose, whereas the major product of FOS fermentation was acetic acid. All strains of *Bifidobacterium* produced small amounts of ethanol – the higher in the medium containing FOS and glucose than lactose. The results indicate middle or strong negative correlation between the acidity and the total amount of acetic and lactic acid, which confirms the usefulness and reliability of the assumed method for the determination of *Bifidobacterium* fermentation products in liquid cultures.

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