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Effect of dilution rate and carbon availability on *Bifidobacterium breve* fermentation

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Abstract. *Bifidobacterium breve* NCFB 2257 was grown in glucose-limited and nitrogen (N)-limited chemostats at dilution rates (D) from 0.04 to 0.60 h^{-1}, to study the effect of nutrient availability on carbohydrate metabolism. The results showed that D had little effect on fermentation product formation, irrespective of the form of nutrient limitation. However, marked differences were observed in the distribution of fermentation products, that were attributable to glucose availability. In glucose-limited cultures, formate and acetate were the principal end-products of metabolism. Lactate was never detected under these growth conditions. In contrast, lactate and acetate were mainly formed when glucose was in excess, and formate was not produced. These results are explained by the metabolic fate of pyruvate, which can be dissimilated by either phosphoroclastic cleavage to acetyl phosphate and formate, or alternatively, it may be reduced to lactate. Enzymic studies were made to establish the mechanisms that regulated pyruvate metabolism. The data demonstrated that control was not exercised through regulation of the synthesis and activity of lactate dehydrogenase (LDH), phosphofructokinase or alcohol dehydrogenase. It is possible however, that there was competition for pyruvate by LDH and the phosphoroclastic enzyme, which would determine the levels of lactate and formate produced respectively. These results demonstrate the metabolic flexibility of *B. breve*, which preferentially uses lactate as an electron sink during N-limited growth, whereas under energy-limitation, carbon flow is directed towards acetyl phosphate to maximise ATP synthesis.

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Introduction

Bifidobacteria are Gram-positive, saccharolytic anaerobes. They are major components of the gut microfloras in man (Mitsuoka 1982; Hidaka et al. 1986) and many animals (Moore and Holdeman 1974; Bullen et al. 1976; Finegold et al. 1983). Lactic and acetic acids formed in a 2:3 ratio were originally thought to be the exclusive fermentation products of bifidobacteria (Rasic 1983). They are produced by the fructose-6-phosphate shunt (Scardovi and Trovatelli 1965; DeVries et al. 1967). Hexoses undergo a series of cleavage and isomerisation reactions that produce pentose phosphates, which are then metabolized to acetyl phosphate and glyceraldehyde-3-phosphate. The acetyl phosphate is used to generate ATP, whereas the glyceraldehyde-3-phosphate is converted to pyruvate by mechanisms similar to those found in the Embden-Meyerhof-Parnas pathway (Modler et al. 1990). It is now known however, that under certain growth conditions ethanol and formate can also be formed by bifidobacteria (DeVries and Stouthamer 1968). This is because pyruvate can be metabolized by two alternative routes: apart from its reduction by NADH to yield lactate, pyruvate can also be cleaved by a phosphoroclastic enzyme to formate and acetyl phosphate. Half of the resulting acetyl phosphate must then be reduced to ethanol in order to oxidize the NADH that was produced earlier in the metabolic pathway, whilst the remainder is available to produce extra ATP through the formation of acetate (DeVries and Stouthamer 1968). A simplified scheme of the reactions involved in carbohydrate breakdown by bifidobacteria is shown in Fig. 1.

Although the general metabolic steps involved in fermentation of sugars by these bacteria are now well understood, the factors that control the route of dissimilation of pyruvate are unclear. It has, however, been demonstrated that the amount of pyruvate converted by either pathway varies depending on the bacterial strain used, and that greater amounts of acetate and formate are produced during the exponential

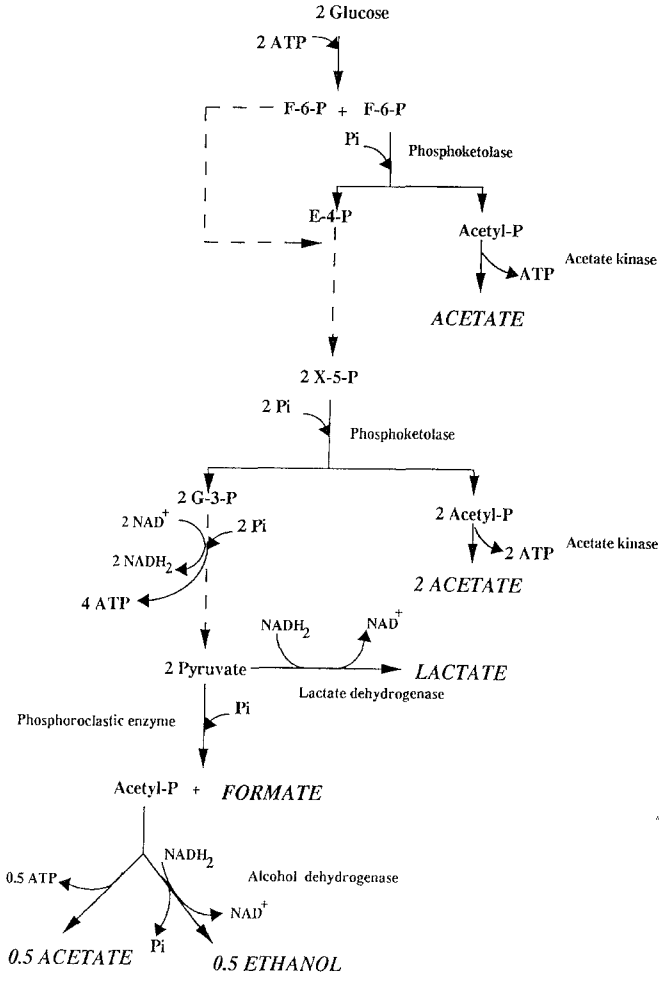

Fig. 1. Simplified scheme of the metabolism of glucose by bifidobacteria: F-6-P, fructose-6-phosphate; X-5-P, xylulose-5-phosphate; E-4-P, erythrose-4-phosphate; G-3-P, glyceraldehyde-3-phosphate; Pi, inorganic phosphate

phase of growth by *Bifidobacterium longum, B. bifidum* and *B. adolescentis* (Lauer and Kandler 1976). In this study, the relationship of carbon (C) availability to fermentation product formation was studied in *B. breve* using glucose-excess and glucose-limited cultures.

Materials and methods

Organism. B. breve NCFB 2257 was obtained from the National Collection of Food Bacteria, Reading, UK.

Growth of B. breve. The bacteria were grown by continuous culture in 0.28-l (working volume) glass fermentors on a basal medium consisting of ($g\,l^{-1}$): peptone water, 5.0; tryptone, 10; yeast extract, 5.0; Tween 80, 0.5; NaCl, 4.5; KCl, 0.5; $MgCl_2 \cdot 6H_2O$, 0.15; KH_2PO_4, 0.4; NH_4Cl, 1.0; cysteine, 1.2. Temperature (37° C) and pH (6.5) were maintained as described by Macfarlane and Englyst (1986), and anaerobic conditions were achieved by sparging the cultures with O_2-free CO_2 at a flow rate of $1.2\,l\,h^{-1}$. Both C-limited and nitrogen (N)-limited cultures were grown with glucose as the sole carbohydrate at concentrations of 2.0 and 17.0 $g\,l^{-1}$, respectively. The sugar was autoclaved separately and added to the cooled sterile basal medium to give the required concentration. After at least seven culture turnovers at each dilution rate, samples were taken for enumeration of the bacteria and dry weight determinations. At the same time, culture effluent (100 ml) was centrifuged at 23000 *g* (4° C for 30 min) to pellet the bacteria. The supernatant was kept frozen for subsequent analysis of residual carbohydrate and fermentation products. The bacteria were washed once in anaerobic potassium phosphate buffer (50 mM, pH 7.0) and after resuspension in the same buffer to give a final volume of 10 ml, the cells were disrupted by two passages through a French pressure cell (1.1×10^5 kPa) before measurements were made of enzyme activities and the RNA and DNA content of the bacteria.

Measurement of culture dry weights. A representative sample (1.0 ml) of culture taken from the fermentors was centrifuged at room temperature for 10 min at 15000 *g* in an Eppendorf bench centrifuge. The supernatant was discarded and a further 1.0 ml added and centrifuged. This was continued until bacteria from a total of 5.0 ml culture had been collected. The bacterial pellet was then washed with distilled water before being dried to constant weight at 130° C.

Enumeration of viable bacteria. A suspension of bacteria (1.0 ml) from the fermentors was thoroughly mixed with sterile, half-strength Wilkins Chalgren Anaerobe broth (9.0 ml) to form a one in ten dilution series (10^{-1} to 10^{-9}). Samples (0.1 ml) were then spread in duplicate on Wilkins Chalgren agar plates. All manipulations and incubations (37° C) were carried out in an anaerobic chamber containing an atmosphere of CO_2, H_2 and N_2 (10:10:80). The plates were preincubated in the chamber for 48 h before use, and then left in the system for a further 48 h before the colonies were counted.

RNA assay. Perchloric acid (2.5 M) was added to cell-free extract of *B. breve* (0.9 ml) to give a final concentration of 0.25 M. The acidified sample was then placed on ice for 30 min before being centrifuged at 15000 *g*. The precipitate was resuspended in 4.0 ml 0.5 M perchloric acid and heated at 70° C for 15 min with frequent mixing. The suspension was then centrifuged as before and 1.0 ml of 0.1 M HCl added to 1.0 ml supernatant. After mixing, 4.0 ml orcinol reagent was added and the tubes placed in a 90° C water bath for 30 min. The orcinol reagent was freshly prepared by adding together equal volumes of ferric chloride solution (100 mg in 100 ml concentrated HCl) and ethanolic orcinol (6.0 g added to 100 ml absolute ethanol in a darkened container). The absorbance at 665 nm (A_{665}) was then measured and the concentration of RNA ($\mu g\,ml^{-1}$) determined from a standard curve.

DNA assay. Samples (1.0 ml) of *B. breve* cell-free extract were placed in a screw-capped tube and 1.0 ml of 20% (w/v) perchloric acid added, followed by 2.0 ml glacial acetic acid containing 4% (w/v) diphenylamine and 0.2 ml 0.16% (w/v) acetaldehyde. After mixing thoroughly, the suspension was incubated overnight at 30° C and then A_{595} minus A_{700} was determined. This value was converted to $\mu g\,ml^{-1}$ DNA using a standard curve.

Carbohydrate measurements. Residual carbohydrate in spent culture media was determined using DNS reagent containing ($g\,l^{-1}$): dinitrosalicylic acid, 10; NaOH, 16; sodium potassium tartrate, 300. Equal volumes (1.0 ml) of DNS and cell-free supernatant from the cultures were mixed together and placed in a 100° C water bath for 10 min. The tubes were then cooled before adding 5 ml distilled water. The A_{530} of the resulting solution was measured spectrophotometrically and converted to $mg\,ml^{-1}$ glucose using a standard curve.

Measurement of fermentation products. The method of Holdeman et al. (1977) was used to determine the amounts of short-chain fatty acids and lactate produced by the bacteria. Ethanol was determined by gas chromatography as described by Macfarlane et al. (1992). Formate was measured by HPLC using a HPX-87H ion exclusion column (Bio-rad). Analyses were carried out at

35° C using 0.004 M H_2SO_4 as the eluant, at a flow rate of 0.6 ml min^{-1}. The products were detected at 210 nm using an LKB 2151 variable wavelength monitor.

Measurement of phosphofructokinase (PFK) activity. Levels of PFK in cell-free extracts of *B. breve* were assayed by coupling the formation of fructose-1,6-bisphosphate (F16BP) to its conversion to dihydroxyacetone phosphate (DHAP) and glyceraldehde-3-phosphate, which was catalysed by aldolase. The reduction of DHAP by the action of α-glycerophosphate dehydrogenase was then monitored by following the decrease in A_{340}. Cell-free extract (0.1 ml) was added to the following reaction mixture, which had been preincubated at 37° C: 0.5 ml TRIS buffer (0.2 M, pH 8.0); 0.3 ml of 0.02 M ATP, pH 7.0; 0.2 ml of 0.01 M fructose-6-phosphate, pH 7.0; 0.3 ml of 0.01 M $MgCl_2 \cdot 6H_2O$; 0.1 ml of 0.2 M cysteine HCl, pH 7.0; 0.25 ml of 0.006 M NADH; 1.2 ml distilled water; 4.8 units aldolase (where 1 unit converts 1.0 μmol F16BP to DHAP and glyceraldehyde-3-phosphate min^{-1} at pH 7.4, 25° C); 4.0 units α-glycerophosphate dehydrogenase (where 1 unit converts 1.0 μmol of DHAP to α-glycerophosphate min^{-1} at pH 7.4, 25° C); 2.0 units triose phosphate isomerase (where 1 unit converts 1.0 μmol of glyceraldehyde-3-phosphate to DHAP min^{-1} at pH 7.6, 25° C). A control assay omitting cell-free extract was also carried out.

Assay of lactate dehydrogenase (LDH) activity. Levels of LDH were measured by following the decrease in A_{340} as pyruvate was reduced to lactate using NADH. The assay was carried out in the presence and absence of F16BP to determine its influence on activity. A stock of TRIS buffer (0.2 M, pH 7.0) containing 0.29 mM NADH, 0.15 mM pyruvate and 0.73 mM F16BP was incubated at 37° C, before 3.0 ml was removed and 0.1 ml cell-free extract added to it. The decrease in A_{340} was then continuously measured spectrophotometrically. A control reaction was done in the absence of sample.

Alcohol dehydrogenase (ADH) assay. This enzyme was assayed by adding 0.1 ml cell-free extract to a reaction mixture preincubated at 37° C containing the following components: sodium pyrophosphate, 0.06 M; semicarbazide hydrochloride, 0.06 M; glycine, 0.017 M; NaOH, 0.053 M; NAD, 0.002 M; ethanol, 0.1 M (pH adjusted to 8.7 with perchloric acid). The assay was followed by measuring the increase in A_{340} and a control reaction without the addition of sample was carried out.

Phosphoroclastic enzyme. Measurements of this enzyme were made according to the methods of Rabinowitz (1972).

Chemicals. Formulated bacteriological media were obtained from Oxoid. Unless otherwise stated, all other chemicals were purchased from Sigma.

Results

Relationship between dilution rate and nutrient availability on growth of B. breve in continuous culture

Under both glucose-limited and N-limited conditions, viable bacterial counts increased concomitantly with dilution rate until maxima were reached at $D = 0.18\ h^{-1}$ and $D = 0.31\ h^{-1}$ respectively (Fig. 2a and b). At growth rates above these values, cell population densities decreased rapidly as the bacteria washed out. Culture dry weights also increased with growth rate (Fig. 2a and b); however, in glucose-excess vessels, a plateau was reached after $D = 0.31\ h^{-1}$. A significant difference was seen between the dry weight

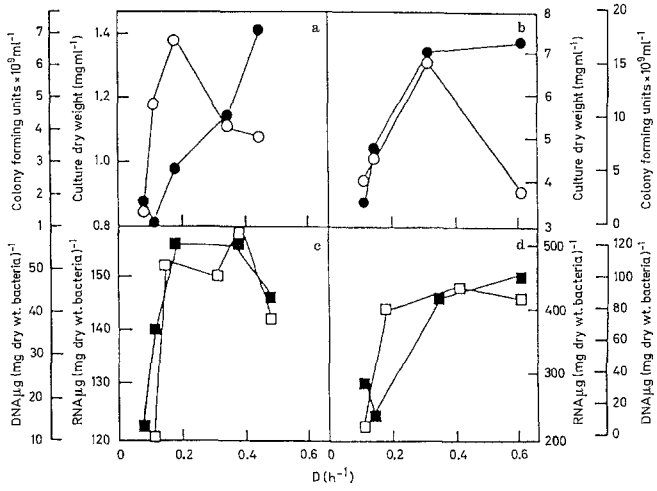

Fig. 2a–d. Influence of dilution rate (D) on culture dry weights (●), viable counts (○) and DNA (□) and RNA (■) content of *Bifidobacterium breve* grown in glucose-limited (**a, c**) and nitrogen-limited (**b, d**) chemostats

measurements of each culture, with an approximate sevenfold difference observed at the highest growth rates (Fig. 2a and b), which was not paralleled in the viable counts. The RNA and DNA contents of *B. breve* were also proportionally related to D in the cultures up to $D = 0.4\ h^{-1}$. At each growth rate, there was significantly more RNA than DNA, and although DNA concentrations were generally comparable between the cultures, more RNA was detected during growth with excess C (Fig. 2c and d).

Influence of dilution rate on glucose fermentation by B. breve

Results in Table 1 show that C availability rather than dilution rate per se had the greatest influence on fermentation product formation by chemostat cultures of *B. breve*. Acetate and formate were the major metabolites during glucose-limited growth, with low amounts of ethanol and no lactate being detected. In contrast, lactate and acetate were produced under N-limitation, and significantly, no formate was detected. The relative molar ratios of these metabolites were generally unaffected by D, although acetate and ethanol production did decrease slightly with increasing growth rates in the nitrogen-limited chemostats (Table 1). Determination of C balances showed acceptable recoveries for growth in the glucose-limited chemostats with values ranging between 101 and 111%, but results obtained from the N-limited vessels were more variable and ranged from 77 to 226% (Table 1).

Effect of dilution rate on activities of metabolic enzymes in cell-free extracts of B. breve

LDH, ADH and PFK activities were recorded in both glucose-limited and N-limited cultures at each dilution

Table 1. Influence of carbon availability and dilution rate on fermentation product formation by *Bifidobacterium breve* grown in continuous culture

D (h^{-1})	Culture conditions	Concentration (mM)				Carbon recovery (%)
		Lactate	Acetate	Ethanol	Formate	
0.10	Glucose-limited	ND	23.8 (59)	3.1 (8)	13.0 (32)	102
0.14		ND	23.8 (59)	3.3 (8)	12.5 (31)	101
0.27		ND	16.7 (61)	2.3 (9)	7.3 (28)	111
0.45		ND	22.9 (60)	3.8 (10)	10.9 (29)	101
0.04	Nitrogen-limited	21.8 (6)	278 (82)	33.9 (10)	ND	129
0.09		73.6 (25)	208 (70)	9.8 (3)	ND	129
0.31		36.8 (27)	96 (71)	1.9 (1)	ND	77
0.60		41.3 (25)	110 (68)	9.6 (6)	ND	226

Results are representative of data obtained from three separate experiments. Molar ratios are given in parentheses. D, Dilution rate; ND, not detected

Table 2. Influence of carbon availability and dilution rate on enzyme activities in *B. breve* grown in continuous culture

D (h^{-1})	Culture conditions	Enzyme activity			
		LDH+F16BP	LDH−F16BP	ADH	PFK
0.10	Glucose-limited	105	45	47	28
0.14		29	15	95	10
0.27		83	52	8.6	20
0.45		111	66	2.0	14
0.04	Nitrogen-limited	68	48	44	52
0.09		30	13	4.8	18
0.31		70	31	3.2	32
0.60		63	33	ND	38

Results are representative of data obtained from three separate experiments. LDH, Lactate dehydrogenase ± fructose −1,6-biphosphate (nmol NADH oxidized mg protein^{-1} min^{-1}); ADH, alcohol dehydrogenase (nmol NAD reduced mg protein^{-1} min^{-1}); PFK, phosphofructokinase (nmol NADH oxidized mg protein^{-1} min^{-1})

rate tested, but the phosphoroclastic enzyme was never detected (Table 2). LDH activity was unaffected by dilution rate, but was influenced by F16BP availability. Assays carried out without addition of exogenous F16BP showed an approximate 50% reduction in activity of this enzyme compared to reactions in its presence. ADH activity in cell-free extracts was inversely related to dilution rate, whereas levels of PFK were relatively unaffected by variations in this parameter (Table 2).

Discussion

Apart from the different types and amounts of fermentation products formed by glucose-limited and N-limited cultures of *B. breve* (Table 1), a significant difference was also seen in the physical appearance of the cultures in each chemostat. A white flocculant was produced by the organisms, especially at high growth rates, when they were grown under conditions of N-limitation, but not in the C-limited vessels. Formation of this substance accounted for the large differences observed between the dry weights of the two cultures (Fig. 2a and b) and suggested that an extracellular polysaccharide was synthesised by the bacterium. The glucose-excess values do not therefore provide an accurate assessment of cell production.

DNA concentrations increased rapidly with D in both glucose-limited and N-limited chemostats until a maximum was reached at approximately $D = 0.2\ h^{-1}$. Above this value the rate of increase in the concentration of DNA slowed down, which correlated with the viable counts. RNA levels were also proportionally related to dilution rate which has been observed during growth of C-limited cultures of *Bacteroides thetaiotaomicron* (Salyers et al. 1981). From that study it was concluded that since most bacterial RNA is ribosomal RNA, the number of ribosomes per cell increased with growth rate and hence protein synthesis was also increased at the higher growth rates. The fact that RNA concentrations were greater in the N-limited vessels suggests a higher level of protein synthesis under these conditions compared to glucose-limited growth.

Results in Table 1 showed that pyruvate metabolism was greatly influenced by C availability in *B. breve*. When glucose availability, and hence ATP synthesis were limited, pyruvate was preferentially cleaved to

acetyl phosphate and formate instead of being reduced to lactate (Table 1). This enabled the bacteria to maximise formation of ATP while still allowing them to oxidize NADH that was formed earlier in the metabolic pathway during the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. Half of the acetyl phosphate formed in these reactions must be reduced to ethanol in order to regenerate NAD^+ (DeVries and Stouthamer 1968; Bezkorovainy 1989); however, the remainder is available to synthesise extra ATP through the formation of acetate (see Fig. 1). In contrast, as the flow of C through the cells was increased during growth in the N-limited chemostats, production of energy was not a problem for the bacteria and there was less need for the generation of extra energy. Therefore, oxidation of NADH was achieved without production of ATP, through the reduction of pyruvate to lactate. The formation of this electron sink product is not an energy-generating reaction in *B. breve* but serves as an uncoupling mechanism allowing the flow of large quantities of C through the cells without concomitant generation of energy.

Significant levels of ethanol were detected in N-limited chemostats (Table 1) and, since this metabolite is ultimately formed by the reduction of acetyl phosphate derived from the cleavage of pyruvate, formate would also be expected to be present in these chemostats. However no formate was found in these vessels. The most likely explanation for this is that because the carbohydrate load was so high in these cultures, acetyl phosphate formed by cleavage of fructose-6-phosphate and xylulose-5-phosphate was also reduced to ethanol instead of being used to generate ATP. This would also explain why the ratios of acetate to lactate were not closer to the expected value of 3:2 in these vessels. If acetyl phosphate generated by the action of phosphoketolase enzymes was being reduced to ethanol, there would be less requirement to reduce pyruvate to lactate, and hence lactate synthesis would be lower than predicted.

After demonstrating that C availability had a major effect on pyruvate metabolism, experiments were carried out to determine how the reactions were controlled (Table 2). Three hypotheses have been proposed to explain the regulation of pyruvate dissimilation (DeVries and Stouthamer 1968). Firstly, it has been shown that in some bacteria LDH has an absolute requirement for F16BP for its activity (DeVries et al. 1967) and therefore, by regulating the amounts of this molecule in the cell, perhaps by controlling the activity of PFK (the enzyme involved in its synthesis), it would be possible to indirectly influence the production of lactate. Secondly, the relative amounts of LDH and the phosphoroclastic enzyme within the cell may vary under different growth conditions. Thirdly, these two enzymes may have different affinities towards pyruvate. A series of enzyme assays were therefore carried out to measure the activity of LDH in the presence and absence of F16BP, together with the levels of ADH, PFK and the phosphoroclastic enzyme.

Attempts to measure the phosphoroclastic enzyme were consistently unsuccessful, even though it must have been present in samples obtained from the glucose-limited cultures, as evidenced by the absence of lactate and the production of formate in these vessels (Table 1). DeVries and Stouthamer (1968) were also unable to demonstrate the presence of this enzyme in cell-free extracts of six strains of *B. bifidum*, or in later studies with *Lactobacillus casei* (DeVries et al. 1970). The phosphoroclastic enzymes of *L. casei* (DeVries et al. 1970) and *Enterococcus faecalis* (Lindmark et al. 1969) have been found to be extremely sensitive to O_2 and do not function under aerobic conditions. Therefore, although every attempt was made to ensure anaerobic conditions during preparation of the enzymes and their assay in the present study, this may explain why we could not detect phosphoroclastic activity.

Lactate was only produced by the bacteria in the N-limited chemostats, but LDH activity was detected in bacteria from both C- and N-limited vessels, indicating that the enzyme was constitutively produced. This led to the possibility that the levels of F16BP in the C-limited cultures may not have been sufficient to activate the LDH. This has been proposed by DeVries et al. (1970), who demonstrated large quantities of LDH in extracts of *L. casei* at low dilution rates, without concomitant production of lactate. However, levels of F16BP do not appear to be the controlling factor with *B. breve*. Subsequent experiments showed that although LDH was stimulated by the presence of F16BP, enzyme activity could still be detected without addition of exogenous F16BP, demonstrating that when the cells were harvested, intracellular levels were sufficient for activation of the enzyme. This was also confirmed by the observations that PFK activity was present in all samples examined.

ADH activity decreased with increasing dilution rate, a trend that was also seen with ethanol measurements made on supernatants obtained from the N-limited cultures (Table 2). This pattern of enzyme activity also correlates with the hypothesis that as the flow of C through the cells is increased, there is less cleavage of pyruvate and hence less requirement for the reduction of acetyl phosphate.

Therefore, of the three proposals put forward by DeVries and Stouthamer (1968) to explain how pyruvate metabolism is controlled in bifidobacteria, the possibility that the choice of pathway taken is regulated by the levels of F16BP within the cell or due to the repression of LDH synthesis have been eliminated. LDH was constitutively produced by *B. breve*, and in all samples assayed, there was sufficient PFK activity to maintain concentrations of F16BP at levels needed for activation. This would suggest that there is either competition between LDH and the phosphoroclastic enzyme for pyruvate, the outcome of which is determined either by the relative amounts of each enzyme or their relative affinities towards the substrate (DeVries and Stouthamer 1968), or alternatively, that there is a specific activator of the phosphoroclastic protein, the levels of which vary with different growth conditions. A reliable assay for this bifidobacterial enzyme

is required to conclusively distinguish between these possibilities.

Overall, the results reported in this paper demonstrate that cleavage of pyruvate to acetyl phosphate and formate in *B. breve* is strongly dependent on growth conditions. Under certain circumstances it may be the predominant reaction, contradicting the original observations that lactate and acetate were the exclusive metabolites of bifidobacteria. Our data suggest that this may only be the case in areas where fermentable C is present in relative abundance.

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