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An Influence of Levan on the Fermentation of Milk by a Probiotic ABT-Type Starter

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Abstract: The effects of high molecular weight (2000 kDa) levan from *Zymomonas mobilis* 13S on the fermentation of lactose in milk and lactose containing medium by dairy starter ABT-5 and constituent cultures (*Bifidobacterium lactis, Lactobacillus acidophilus, Streptococcus thermophilus*) were estimated in this study. An increase of biomass concentration (120-134% to control), sugar consumption and acidification power of starter association ABT-5 were observed in the presence of levan in milk and MRS medium. *Bifidobacterium lactis* Bb12 within the starter association exhibited the most pronounced response to levan in respect to cell growth (almost 2 times to control). The activity of constitutive β-fructanhydrolases was detected in cell free extract on sucrose or levan as a substrate. In the presence of inulin or levan as inductors the activities of extracellular β-fructanhidrolase were established for ABT-5 dairy starter and constituent cultures. The presence of more specific extracellular endo-fructanhydrolase was detected which might contribute to catabolism of levan. This study indicate certain prospects for application of polyfructans, particularly β-2.6 linked fructan polymers (levan) as a potent source of prebiotics to modify and improve the processes of lactose fermentation by ABT-5 type starter cultures.

Key words: Levan, milk, probiotics, prebiotics, dairy starters

INTRODUCTION

Current research in food biotechnology advance further development of functional food which delivers a benefit to health beyond that of strict nutritional value. Lactic acid bacteria and bifidobacteria as probiotics (live microorganisms that are added to food) and prebiotics (non-digestible food ingredients that stimulate the growth of beneficial bacteria in the host organism) should be considered as the guiding forces for the growing potential of functional food due both the food and therapeutic applications. Products obtained by lactic acid fermentation processes obviously are of special importance since the synbiotic (probiotic and prebiotic) properties could be obtained by an appropriate combination of probiotic lactic acid bacteria strains and prebiotics (Vuyst, 2000). The use of any prebiotic substance to enrich milk-based fermented products provides its delivery into human alimentary tract and hence, a stimulation of beneficial probiotic bacteria regardless of their origin (naturally inhabitants or delivered by functional products). In turn, the prophylactic and therapeutic qualities of fermented functional products obviously depend on the content of viable probiotic cells. Therefore it seems necessary to achive high cell count for probiotic bacteria (particularly of bifidobacteria and lactobacilli) at the stage of lactic acid fermentation of product by an addition of potent prebiotic substances. Fructose polymers and oligomers should be considered as appropriate additives for this purpose (Vuyst, 2000).

β-(2,1)-linked fructans of plant origin (inulin) and the related oligosaccharides (FOS) are generally acknowledged as efficient prebiotics (Roberfroid *et al*., 1998) whereas β-(2,6)-linked fructans of microbial origin (levan and FOS) remain less investigated and very few studies have demonstrated prebiotic properties of these substrates with regard to several strains of *Bifidobacteria* (Marx *et al*., 2000; Ehrmann *et al*., 2003; Bello *et al*., 2001) however bifidogenic effects were strongly affected by properties of microbial levan and different ability of bacteria to consume this polymer (Bello *et al*., 2001) and related fructan oligomers (Marx *et al*., 2000). We have shown recently (Semjonovs *et al*., 2004) that inulin appeared to be non-degradable whereas levan from *Zymomonas mobilis* 113 S was utilized in small amount to support the growth of *Bifidobacterium lactis* Bb-12 and fructan syrup (Bekers *et al*., 2004) consisting of levan, fructan oligomers, sucrose and reducing sugars ensured an efficient growth of this probiotic strain. *B. lactis* Bb-12 as a constituent goes into the functional dairy starter ABT-5 (Hansen Lab, 2004-2006) and starters of

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ABT-type find wide application in food industry (Vuyst, 2000). However, eventual prebiotic effects of β-(2,6)-linked fructans upon ABT-type starters as complex microbial system remain uninvestigated at present. A wide variety of potent physiological activities of levan should be considered as an advantage to achieve synbiotic qualities (Bekers *et al*., 2004; Ishihara, 1996; Liepa *et al*., 1993; Yamamoto *et al*., 1999; Sprudza *et al*., 2002; Yoo *et al*., 2004) of products. Besides favorable physico-chemical characteristics (Bekers *et al*., 2005) levan could improve rheological properties and texture of fermented products as reported for other microbial polysaccharides (Welman and Maddox, 2003). Here we report the effects of levan produced by *Zymomonas mobilis* 113S on the production of biomass, acidification power and assess the presence and activities of β-fructanhydrolases during lactic acid fermentation by probiotic dairy starter ABT-5 and its constituent cultures.

MATERIALS AND METHODS

Culture media and growth conditions: The commercial probiotic dairy starter ABT-5 (Chr. Hansens Applied Technological Laboratory, Denmark), containing *Lactobacillus acidophilus*, *Streptococcus thermophilus* and probiotic strain *Bifidobacterium lactis* Bb12, was stored in a dry state at -18°C and 0.03 g of dry biomass was used as a seed material for 100 mL media preparation. Fermentations were carried out in 100 mL flasks containing 50 mL growth media at 37°C for 10 or 12 h. In the case of individual constituent strains isolated from ABT-5 starter 2% of overnight culture in the MRS-lactose medium was used as an inoculum for trials.

For preparation of milk media reconstituted (75 g L^{-1}) dry skimmed milk (a/s Valmieras Piens) was supplemented with 0.3% yeast extract (Sigma, Germany) and the absence of inhibitors in milk was previously checked (Suhren and Heeschen, 1996). Milk media were supplemented by levan or inulin (10 or 20 g L^{-1}) before pasteurisation. The MRS growth medium (Man *et al*., 1960) was attuned according to the carbon source in milk by lactose (45 g L^{-1}) instead of glucose and correspondingly supplemented by levan.

B. lactis, *L. acidophilus* and *S. thermophilus* strains were isolated and selectively counted during growth of ABT-5 association by means of selective growth media. MRS agar with glucose 20 g L^{-1}) as a sole carbon source after autoclaving 121°C, 15 min) and pH adjusting to 6.2±0.2 was supplemented (g L^{-1}) by LiCl (3.000), nalidixic acid (0.015), neomycin-sulphate (0.100) and paromomycin-sulphate (0.125) and used for selective enumeration of *B. lactis* after anaerobic incubation (BBL Gas Pak 150™ System, USA) at 37°C for 72 h. Complete suppression of growth for *L. acidophilus* and *S. thermophilus* due to LiCl and antibiotics supplements was accompanied by noticeable reduction (Hansen Lab, 1994) of cfu for *B. lactis* itself and necessary multiplication factor equal to 1.4 was calculated from independent test series to correct the cfu data for *B. lactis*.

L. acidophilus cells were selectively enumerated on MRS agar containing maltose (20 g L^{-1}) as a sole carbon source under the same incubation conditions as for *B. lactis*. *S. thermophilus* did not grow on MRS-maltose medium under these conditions and present almost negligible amount of different very small-size colonies were attainted to *B. lactis* and eliminated. *S. thermophilus* cells were selectively enumerated on Lee's agar (Lee *et al*., 1974) after aerobic incubation at at 43°C for 48 h and both of this constituents of ABT-5 (*B. lactis*, *L. acidophilus*) did not exhibit any growth under these conditions. 200-400 cfu were counted for each variant with a standard deviation from the mean 5-7%.

Substrates and chemicals: High molecular weight levan (2×10^6 D) (Andersone *et al*., 2004) was obtained by the laboratory scale *Zymomonas mobilis* 113 S fermentation of sucrose and purified by reprecipitation by cold ethanol to eliminate oligomers and reducing sugars (Bekers *et al*., 2002). Inulin from dahlia tubers (Sigma, Germany) (5×10^3 Da) was used throughout this study as the reference substrate. Other chemicals and media components were obtained from Sigma-Aldrich Chemie GmbH (Germany).

Enzyme source preparation and fructan hydrolase assay: Probiotic association ABT-5 and constituent cultures (*B. lactis*, *L. acidophilus*, *S. thermophilus*) were cultivated in the MRS medium containing glucose, lactose or sucrose as a sole carbon source in the absence or presence of levan (20 g L^{-1}). Cells from the late logarithmic phase of growth (12 h) were separated by centrifugation (3000×g 15 min) from the culture liquid and washed two times with 50 mM acetate buffer (pH 5.0). The cell-free extract was obtained by exposing of cell suspension (20 g L^{-1}) to ultra-sonic oscillation (300 W cm^{-2}, 15 min) followed by centrifugation (10,000×g, 15 min) to remove all debris and assayed for the activity of intracellular fructan hidrolases. Suspension of intact cells (20 g L^{-1}) was incubated with sucrose (50 mM) and fructan polymers (inulin or levan) at concentration 17 g L^{-1} for 30 min at 30°C and cell-free incubation medium was assayed for the activity of secretory fructan hydrolases. Enzyme assays were performed in 50 mM acetate buffer pH 5.0 at 37°C with sucrose or levan (17 g L^{-1}) as the substrates and activity

of fructan hydrolases expressed in arbitrary units (a) as an amount of liberated reducing sugars (μg min^{-1}) per mg of dry biomass during incubation (30 min) assuming an average protein content to be 55% of dry biomass; (b) as an amount of enzymatically hydrolysed levan (μg min^{-1}) per mg of dry biomass during incubation (120 min) by monitoring (Shimadzu UV-vis, Japan) the decrease of Absorbance at 340 nm (A_{340}) using the calibration curve previously obtained for levan standard solutions of varied concentration. Enzyme assays were performed at least twice with three replicates to each point and fitted using a Hill plot for sigmoidal kinetic relationships (Kurganov, 1982) to obtain the apparent values of kinetic parameters with a standard deviation within 5% of mean values.

Other analytical determinations: The growth of ABT-5 and individual cultures in MRS medium was monitored by the online spectrophotometric (Shimadzu UV-vis, Japan) measurements of biomass concentration at 550 nm using the calibration curves obtained for each culture as described elsewhere (Zikmanis *et al*., 1997). Method of decimal dilutions (IDF Standard 117A: 1988) was used for preliminary evaluation of cell biomass in fermented milk, except that a diluent (peptone water) was replaced by a milk medium. The total biomass concentration in fermented milk was estimated turbidimetrically at 600 nm after solubilization of the casein micelles at low temperature with sodium hydroxide and EDTA (Kanasaki *et al*., 1975). Content of total carbohydrates was measured by the anthron method (Morris, 1948). Titrable acidity was monitored by titrating 100 mL of sample thinned with 2 parks of distilled water, with 0.1 N NaOH using phenolphthalein (pH 8.4) as the indicator and expressed as Therner degrees (°Th) in accordance with Latvian State Standard LVS 288: 2000 (Semjonovs *et al*., 2004). The concentration of acetic acid was measured by means of HPLC equipped with the Pinacle Amino column (250×4.6 mm) (Bekers *et al*., 2001). Reducing sugars were determined by Dinitrosalicilic acid (DNS) method (Miller, 1959). Protein in cell-free extract was assayed by the dye-binding procedure (Sedmak and Grossberg, 1977).

RESULTS AND DISCUSSION

A supplementation of milk by levan (20 g L^{-1}) provided somewhat shortened time of clot formation (0.5-1.5 h difference as compared to control), higher level of acidification (110-114% to control) as well as suggested an increase of cell count for starter ABT-5 (near to order of magnitude as estimated by decimal dilutions) during 10-12 h of fermentation. These preliminary observations were further confirmed by comparative measures of differential acidification rate (Fig. 1) cell count, total carbohydrate consumption and acidification power in the absence and in the presence of levan or inulin during fermentation of milk by ABT-5 (Fig. 1, 2 and Table 1) and individual cultures previously isolated from this association (Table 2). The addition of levan exhibited more pronounced effects (Fig. 1 and Table 1) as compared to inulin during fermentation by ABT-5 association, however differently affected (Fig. 2) the growth of their individual constituents. Thus, a distinct bifidogenic effect of levan was observed concomitantly with significantly higher cell count for *S. thermophilus* within the association supplemented by inulin and slight stimulation of *L. acidophilus* growth appeared in the presence of both polyfructans (Fig. 2).

Time courses of biomass formation in the lactose-containing MRS media displayed the same pattern of differences between stimulatory effect of levan and inulin during the logarithmic phase of growth (Fig. 3, Table 3). These effects resulted in significantly shortened duration times (lag) to enter the logarithmic phase increased

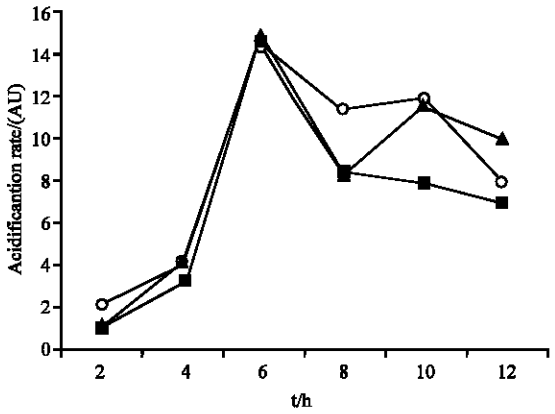

Fig. 1: The differential acidification rate during fermentation of milk by ABT-5, in the absence (■) and in the presence of levan (○) and inulin (▲) expressed as Arbitrary Units (AU). 1 AU is attributed to the increase of titrable acidity (°Th) per 1 h of fermentation

Table 1: Influence of fructans on the growth of ABT-5 starter in milk, during 12 h cultivation

Medium	Cell biomass/(g L^{-1})	Titrable acidity/(°Th)	Acetic acid mM^{-1}	Carbohydrate consumption/(g/L/h)
Control	1.580	108	9.26	0.35
Levan, 20 g L^{-1}	2.117	122	28.44	0.74
Inulin, 20 g L^{-1}	1.706	116	24.69	0.66

Table 2: Influence of levan on the growth of ABT-5 constituents, in milk during 12 h cultivation

	Control (milk without additives)			Levan (20.0 g L^{-1})		
Culture	Cell count/ (log cfu mL^{-1})	Titrable acidity/(°Th)	Acetic acid mM^{-1}	Cell count/ (log cfu mL^{-1})	Titrable acidity/(°Th)	Acetic acid mM^{-1}
B. lactis	5.496	65	78.9	5.796	104	126.9
L. acidophilus	7.716	138	31.5	7.788	159	46.5
S. thermophilus	8.988	109	21.4	9.042	131	40.0

Table 3: The influence of fructans on the productivity of biomass (Qx) and duration of lag phase for ABT-5 and their constituents

	ABT-5		*B. lactis*		*L. acidophilus*		*S. thermophilus*	
Carbohydrate source	Qx/(g/L/h)	Lag min^{-1}	Qx/(g/L/h)	Lag min^{-1}	Qx/(g/L/h)	Lag min^{-1}	Qx/(g/L/h)	Lag min^{-1}
Control (lactose 45.0 g L^{-1})	0.013	310	0.023	220	0.037	350	0.098	190
Levan (20.0 g L^{-1})	0.025	210	0.026	60	0.046	230	0.116	90
Inulin (20.0 g L^{-1})	0.019	230	0.025	180	0.044	240	0.142	160

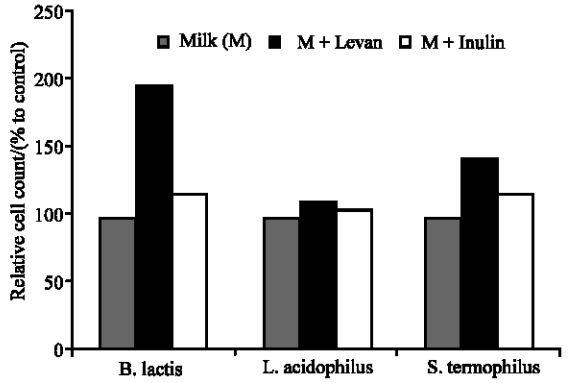

Fig. 2: Relative changes in cell count for individual constituents of ABT-5 on selective media during 12 h cultivation in milk supplemented by fructans. Cell count values in controls: $5.55\pm0.56\times10^{6}$ (*B. lactis*); $6.30\pm0.65\times10^{7}$ (*L. acidophilus*); $1.31\pm0.12\times10^{9}$ (*S. thermophilus*)

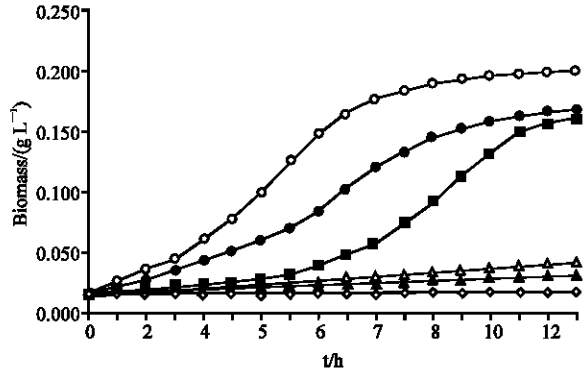

Fig. 3: Time courses of ABT-5 biomass formation in the MRS-lactose medium in absence (■) and in the presence of inulin (●) or levan (○) supplement. Controls: Levan (Δ) and inulin (▲) as sole carbon sources, medium without carbohydrate supplements (◊)

productivity (Qx) of biomass (Table 3). Like in the fermentation of milk (Table 1 and 2) more pronounced effects of levan were observed, particularly in respect to Qx for ABT association and lag time for *B. lactis* as an individual constituent culture (Table 3). However, in both cases an influence of polyfructans on the development of ABT-5 appeared more efficiently (Table 1 and 3, Fig. 3) in comparison to the individual growth of constituent cultures (Table 2 and 3). It could be explained by good conformity between strains within this complex microbial system and, hence, by possible synergetic or system effects due to different proteolytic properties, oxygen-binding capacity etc. as previously reported for L. acidophilus and *B. lactis* (Gomes *et al*., 1998; Gomes and Malcata, 1999) and *S. thermophilus* and *Bifidobacteria* (1) mutual relationships.

Enzyme assays on sucrose, levan or inulin as a substrate revealed the capability of Cell-free Extracts (CE) from cells cultured on MRS-glucose medium (Fig. 4 and Table 4) to liberate Reducing Sugars (RS) thus confirming the presence of constitutive hydrolases of wide substrate specifity (Takahari *et al*., 1985; Burne *et al*., 1987; Warchol *et al*., 2002) within the cells of ABT-5 association and constituent cultures. The almost negligible activity MRS-glucose grown *S. thermophilus* CE on sucrose as a substrate (Table 4) suggests the presence of inducible β-fructofuranosidase (invertase) as previously observed (Somkuti and Steinberg, 1991) for this genera since can be detected only in MRS-sucrose grown cells (data not shown). Observed activity of fructan hydrolases in principle could be responsible for the eventual contribution of levan and inulin as a co-substrate in the process of lactose fermentation. However, any catabolism of fructose polymers obviously requires the presence of extracellular, i.e. secretory fructan hydrolases.

Incubation of separated ABT-5 cells with sucrose, levan or inulin after growth in MRS-glucose or

Table 4: The activity of β-fructanhydrolase in the cell free extracts of ABT-5 and constituent cultures

	Enzyme activity/(RS μg/mL/min /(mg dry mass)		
	Reaction substrate		
Culture	Sucrose	Levan	Inulin
ABT-5	1.50	4.42	23.13
B. lactis	60.58	16.77	70.83
L. acidophilus	2.11	5.61	0.23
S. thermophilus	0.01	8.58	19.54

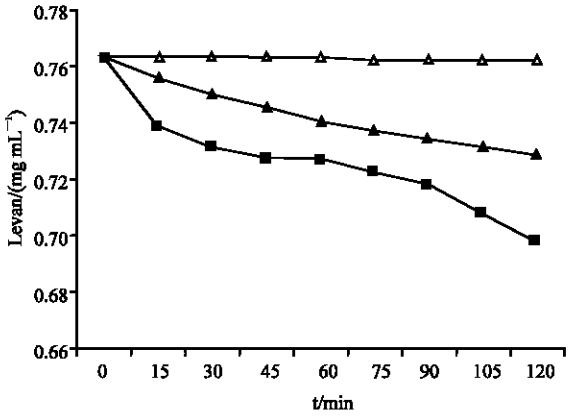

Fig. 4: Changes of levan (▲) and inulin (■) concentration in the presence and in the absence (Δ) of extracellular fructan hydrolases from the cultivation medium supplemented by levan

MRS-lactose media resulted in the secretion of β-fructofuranosidases (invertases) since the cell free incubation medium exhibited the capability to liberate reducing sugar from sucrose, levan and inulin as a substrate (Fig. 5, Table 5 and 6). Inulin was found to be the better inducer and cells cultivated in the MRS-lactose medium exhibited somewhat reduced extracellular enzyme activity during incubation (Table 5). However in both cases inulin appeared as more efficient inducer for concentration-dependent secretion of fructan hydrolases into the medium. Thus, two-fold increase of inulin concentration (8.6 and 17.2 g L^{-1}) was accompanied by an increase of enzyme activity (0.35 and 3.90 μg min^{-1}, respectively) in proportion just as for levan in somewhat higher level of concentration. In a similar way the presence of inulin in the incubation medium resulted in the release of ß-fructanhydrolase activity for all constituent cultures of ABT-5 starter (Table 7). Besides, the incubation medium displayed noticeable endo-fructanhydrolase activity being completely absent in the CE of incubated cells (Table 3).

In a similar way the addition of levan (10 g L^{-1}) as a co-substrate to MRS-glucose resulted in the release of extracellular endo-fructanhydrolases into the cell free growth medium (Table 3) in combination with relatively

Table 5: The activity of β-fructanhydrolase in the cell free incubation medium of ABT-5 grown on different substrates

	Enzyme activity/(RS μg/mL/min/(mg dry mass)		
	Incubation substrate (17.0 g L^{-1})		
Growth substrate	Sucrose	Levan	Inulin
Glucose	0.45	0.56	3.96
Lactose	0.57	0.16	1.55

Table 6: The activity of β-fructanhydrolase in the cell free incubation medium of ABT-5

		Enzyme activity/(RS μg/mL/min/(mg dry mass)		
		Reaction substrate		
Growth substrate	Incubation substrate	Sucrose	Levan	Inulin
Glucose	Inulin	3.96	1.93	1.53

Table 7: The activity of β-fructanhydrolase in the cell free incubation medium of ABT-5 constituent cultures grown on glucose

Culture	Incubation substrate	Reaction substrate	Enzyme activity/(RS μg/mL /min/(mg dry mass)
B. lactis	Inulin	Sucrose	1.75
L. acidophilus			1.37
S. thermophilus			1.02

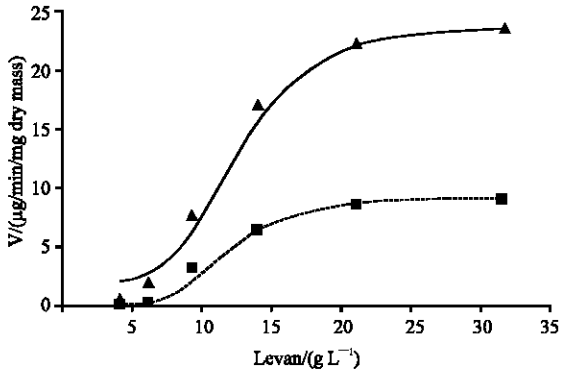

Fig. 5: Kinetics of ABT-5 fructanhydrolases-catalysed reactions upon concentration of levan as substrate: Liberation of reducing sugars in the presence of cell-free extract (■) and decrease of levan concentration in the presence of cell free growth medium (▲)

small (0.018-0.02 μg/min/mg dry mass) liberation of RS, i.e., low exo-fructanhydrolase activity. Alike simultaneous presence of various exo- and endo-fructanhydrolases of low substrate-specifity in microbial cells has been extensively reported (Takahashi *et al*., 1985; Burne *et al*., 1987; Warchol *et al*., 2002; Somkuti and Steinberg, 1991). Due to it observed sigmoidal responses of the reaction rate to the concentration of sucrose or levan and elevated values of the Hill coefficients (Table 3 and 8) more likely could reflect the positive interaction of various binding sites in the complex of fructan hydrolases presented in constituent cultures of ABT-5 association. However,

Table 8: Kinetic parameters of ABT-5 fructanhydrolases upon different substrates and enzyme sources

Carbohydrate source in growth medium	Enzyme source	Substrate	Kinetic parameters: Concentration of substrate at ½ Vmax, [$S_{0.5}$]/(μg mL^{-1})	Kinetic parameters: Maximum velocity, Vmax/(μg/mL/min/ (mg dry mass)	Kinetic parameters: Hill koefficient N_H
Glucose	Cell-free extract	Sucrose	7.31	1.90	1.82
Glucose	Cell-free extract	Levan	11.87	9.00	5.40
Glucose + Levan	Cell-free growth medium	Levan[a]	11.16	23.70	4.17
Glucose + Levan	Cell-free growth medium	Levan[b]	3.06	0.02	1.61

a) Decrease of levan concentration (A_{330}); b) Liberation of RS

co-existence of multiple binding sites, perhaps for the operation of levan endo-hydrolase (Table 3 and 8), can not be excluded and remain to be elucidated at the level of individual enzyme.

Marked positive cooperativity, the values of V_{max} and affinity ($[S]_{0.5}$) to levan as a substrate (Table 8) could provide sufficiently high velocities and pronounced responses to substrate concentration (Kurganov, 1982) of fructan hydrolase-catalysed reactions to contribute to processes of lactose fermentation. It should be noted that observed positive influence of fructan polymers, particularly levan, on the growth of ABT-5 and constituent cultures might involve direct stimulation due to supplement of fructose from hydrolysed polyfructans as well as more complex indirect effects. Thus, almost doubled consumption of total carbohydrates (Table 1) during fermentation of milk in the presence of fructose polymer can not be attributed entirely to the fermentation of additional fructose since the operation of fructan hydrolase even at the highest velocities (Table 8) could cover only the minor part of observed surplus in sugar consumption. An increase of lactose consumption in the presence of polyfructan could be suggested as reasonable consequence and thereby should be considered as an advantage for these compounds as prebiotic supplements due to widespread lactose intolerance within adult consumers of dairy products (Roberfroid, 2000), however requires extensive further research effort. In turn, observed shift towards heterofermentative process in the presence of levan or β-(2,6) linked oligosaccharides (Marx *et al*., 2000) could affect taste and aroma qualities of product due to increased acetic acid concentration and, hence, acceptance of consumers (Gomes and Malcata, 1999). This shift most probably reflects the connection with increased cell growth due to additional amount of ATP in the pathway of acetate formation (Macfarlane and Macfarlane, 2002). On the other hand, the synthesis of short-Chain Fatty Acids (CFA) in lactic acid bacteria depends on the variety of factors such as properties of strains, fermentation conditions and medium composition, including size and linkage-type of added fructans (Marx *et al*., 2000; Macfarlane and Macfarlane, 2002; Perrin *et al*., 2002) and appropriate combination of them might improve the actual SFA ratio. Thus, a cultivation of *B. lactis* in the medium containing a mixture of β-(2,6) linked oligosaccharides and same amount of glucose and fructose (fructan syrup)favored to increasing lactic acid concentration in contrast to effects of β-(2,1)-linked fructan oligosaccharides (Semjonovs *et al*., 2004).

Regardless of some limitations obviously requiring further research effort, the above results indicate certain prospects for application of polyfructans, particularly β-(2,6) linked fructan polymers (levan) as a potent source of prebiotics to modify and improve the processes of lactose fermentation by ABT-5 type starter cultures.

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REFERENCES

Andersone, L., L. Auzina, A. Vigants, O. Mutere and P. Zikmanis, 2004. Formation of levan from Raffinose by levansucrase of *Zymomonas mobilis*. Eng. Life Sci., 1: 56-59.

Bekers, M., J. Laukevics, A. Karsakevich, E. Ventina, E. Kaminska, D. Upite, I. Vina, R. Linde and R. Scherbaka, 2001. Levan-ethanol biosynthesis using *Zymomonas mobilis* cells immobilized by attachment and entrapment. Proc. Biochem., 36: 979-986.

Bekers, M., J. Laukevics, D. Upite, E. Kaminska, A. Vigants, U. Viesturs, L. Pankova and A. Danilevics, 2002. Fructooligosaccharide and levan producing activity of *Zymomonas mobilis* extracellular levansucrase, Proc. Biochem., 38: 701-706.

Bekers, M., M. Marauska, M. Grube, D. Karklina and M. Duma, 2004. New prebiotics for functional food. Acta Aliment, 33: 31-37.

Bekers, M., D. Upite, E. Kaminska, J. Laukevics, M. Grube, A. Vigants and R. Linde, 2005. Stability of levan produced by *Zymomonas mobilis*. Proc. Biochem., 40: 1535-1539.

Burne, R.A., K. Shilling, W.H. Bowen and R.E. Yasbin, 1987. Expression, purification and characterization of exo-beta-D-fructosidase of *Streptococcus mutans*. J. Bacteriol., 169: 4507-4517.

Dal Bello, F., J. Walter, C. Hertel and W.P. Hammes, 2001. *In vitro* study of Prebiotic Properties of Levan-type Exopolysaccharides from Lactobacilli and Non-digestible Carbohydrates Using Denaturing Gradient Gel Electrophoresis. Sys. Applied Microbiol., 24: 232-237.

De Man, J.C., M. Rogosa and M.E. Sharpe, 1960. A medium for the cultivation of lactobacilli. J. Applied Bacteriol., 23: 130-135.

De Vuyst, L., 2000. Technology Aspects Related to the Application of Functional Starter Cultures. Food Tech. Biotech., 38: 105-112.

Ehrmann, M.A., M. Korakli and R.F. Vogel, 2003. Identification of gene for β-fructofuranosidase of *Bifidobacterium lactis* DSM10140^T and Characterization of the Enzyme Expressed in *Escherichia coli*. Curr. Microbiol., 46: 391-397.

Gomes, A.M.P., F.X. Malcata and F.A.M. Klaver, 1998. Growth enhancement of *Bifidobacterium lactis* B0 and *Lactobacillus acidophilus* Ki by Milk Hydrolysates. J. Dairy Sci., 81: 2817-2825.

Gomes, A.M.P. and F.X. Malcata, 1999. *Bifidobacterium* sp. and *Lactobacillus acidophilus*: Biological, biochemical, technological and therapeutical properties relevant for use as probiotics. Trends Food Sci. Tech., 10: 139-157.

Hansen Lab, Chr., 1994. Method of analysis for identification of *L. acidophilus* and *Bifidobacteria*, Denmark.

Hansen Lab, Chr., 2004-2006. Product Range, Dairy Cultures, Denmark.

Ishihara, K., 1996. Antihyperlipidemic and antiobesity agent comprising levan or hydrolysis products thereof obtained from Streptococcus salivarius. US. Patent, 5: 527-784.

Kanasaki, M., S. Breheny, A.J. Hiller and G.R. Jago, 1975. A rapid method for the estimation of bacterial populations in milk. Aus. J. Dairy Tech., 30: 142-144.

Kurganov, B., 1982. Allosteric Enzymes, Kinetics Behaviour, John Willey and Sons Inc., New York, pp: 27-53.

Lee, S.Y., E.R. Vedamuthu and C.J. Washam, 1974. An agar medium for the differential enumeration of youghurt starter bacteria. J. Milk Food Tech., 37: 272.

Liepa, V., G. Zakenfelds, E. Valpe, Z. Koronova, R. Lapsa, M. Laivenieks, M. Bekers and I. Pospishil, 1993. Levan prolongs the life span of tumor-beering or irradiated mice and enhance the antitumor activity of leucocyts. Proc. Latv. Acad Sci., 5B: 59-64.

Macfarlane, S. and G.T. Macfarlane, 2000. Regulation of short-chain fatty acid production. Proc. Nutr. Soc., 62: 67-72.

Marx, S.P., S. Winkler and W. Hartmeier, 2000. Metabolisation of β-(2,6)-linked fructose-oligosaccharides by different bifidobacteria. FEMS. Microbiol. Lett., 182: 163-169.

Miller, G.L., 1959. Use of dinitrosalicilic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Morris, D.L., 1948. Quantitative detection of carbohydrates with Deywood`s anthrone reagent. Science, 107: 254-255.

Perrin, S., C. Fougnies, J.P. Grill, H. Jacobs and F. Schneider, 2002. Fermentation of chicory fructo oligosaccharides in mixtures of different degrees of polymerisation by three strains of bifidobacteria. Can. J. Microbiol., 48: 759-763.

Roberfroid, M.B., J.A.E. Van Loo and G.R. Gibson, 1998. The Bifidogenic Nature of Chicory Inulin and Its Hydrolysis Products. J. Nutr., 128: 11-19.

Roberfroid, M.B., 2000. Prebiotics and probiotics: Are they functional foods?. Am. J. Clinic. Nutr., 71: 1682-1687.

Sedmak, J.J. and S.E. Grossberg, 1977. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue g 250. Anal. Biochem., 79: 544-552.

Semjonovs, P., M. Marauska, R. Linde, M. Grube, P. Zikmanis and M. Bekers, 2004. Development of *Bifidobacterium lactis* Bb12 on β-(2,6)-linked Fructan-containing substrate. Eng. Life Sci., 5: 433-437.

Somkuti, G.A. and D.H. Steinberg, 1991. β-fructofuranosidase activity in disaccharide transport mutants of *Streptococcus thermophilus*. Biotech. Lett., 13: 809-814.

Sprudza, D., M. Marauska, A. Antonovica and V. Gordjusina, 2002. Fructan syrup reduces the blood cholesterol level and stimulates the development of *Bifidobacterium*. Proc. Latv. Acad Sci., 56: 243-247.

Suhren, G. and W. Heeschen, 1996. Detection of inhibitors in milk by microbial tests. A Review. Nahrung, 40: 1-7.

Takahashi, N., F. Mizuno and K. Takamori, 1985. Purification and preliminary characterization of Exo-β-D-Fructosidase in *Streptococcus salivarius* KTA-19. Infect. Immun., 47: 271-276.

Warchol, M., S. Perrin, J.P. Grill and F. Schneider, 2002. Characterisation of a purified β-fructofuranosidase from *Bifidobacterium infantis* ATCC 15697. Lett. Applied Microbiol., 35: 462-467.

Welman, A.D. and I.S. Maddox, 2003. Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. Trends Biotech., 21: 269-274.

Yamamoto, Y., Y. Takahashi, M. Kawano, M. Yizuka, T. Matsumoto, S. Saeki and H. Yamaguchi, 1999. *In vitro* digestibility and fermentability of levan and its hypocholesterolemic effects in rats. J. Nutr. Biochem., 10: 131-138.

Yoo, S.H., E.J. Yoon, J. Cha and H.G. Lee, 2004. Antitumor activity of levan polysaccharides from selected microorganisms. Int. J. Biol. Macromol., 34: 37-41.

Zikmanis, P., R. Kruce and L. Auzina, 1997. An elevation of the molar growth of *Zymomonas mobilis* during aerobic exponential growth. Arch. Microbiol., 167: 167-171.