Enzymatic Activity in Fermented Milk Products Containing Bifidobacteria

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Abstract

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Streptococcus thermophilus, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Bifidobacterium animalis* strains were tested for α-galactosidase, β-galactosidase and α-glucosidase activities. Commercially available yoghurts with bifidobacteria were also tested. While bifidobacteria produced all the enzymes mentioned above, lactobacilli and streptococci exhibited only β-galactosidase activity. In yoghurts, only β-galactosidase was detected, while practically no α-galactosidase, and in one product only little α-glucosidase activities were exhibited. It could be concluded that the consumption of bifidobacteria via yoghurt has probably no substantial effect on the digestion of saccharides in the gut.

Keywords: fermented milk products; enzymatic activity; bifidobacteria

Fermented milk products find favour with public. Yoghurt production is the result of an arranged development of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, microorganisms that remain vital throughout the maintenance period (Torriani *et al.* 1997). Lactic acid production by the two genera combined is clearly superior to that by a single component. The growth of *Streptococcus thermophilus* is, in fact, stimulated by amino acids and peptides produced by *Lactobacillus bulgaricus* from milk proteins (Torriani *et al.* 1997). On the other hand, the growth of *Lactobacillus bulgaricus* is stimulated by the compounds produced by *Streptococcus thermophilus*, such as carbon dioxide and formic acid (Rosado *et al.* 1992).

Fermented milk products represent a rich source of nutrients and may improve lactose digestion through splitting lactose into glucose and galactose by bacterial enzymes (Kolars *et al.* 1984). The transformation of lactose into lactic acid is the most significant phenomenon taking place during the fermentative process and is responsible for almost all of the fermentation products (Marshall & Tamime 1997).

Fermented milk products also help enhance the immune system via modulation of the cellular immune response through bioactive peptides whose

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activity may extend beyond the immune functions by some mechanisms still unclear. Proof now exists that fermentation products (Molin 2001), fermented milks (Heller 2002), and probiotics used for fermentative purposes (Cross *et al.* 2001; Heller 2001) may all contribute to health benefits, but clear study designs are needed to clarify the roles and specific domains for these activities.

Lately, probiotics are added into yoghurts. A "probiotic", by the generally accepted definition, is a "live microbial food and feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1997). "Pro-biotic" means "for life", which already indicates the most essential property of probiotic cultures. This term was first used in the 1970s to describe naturally occurring microorganisms that could be beneficial to health when regularly consumed. Most probiotics are either lactobacilli or bifidobacteria.

Bifidobacteria are Gram-positive, non-spore-forming, non-motile, anaerobic, irregular rods. The typical habitat of bifidobacteria is human, warm-blooded animals and honeybee intestinal tracts (Scardovi 1986). The genus *Bifidobacterium* constitutes a significant proportion of the probiotic cultures used in the food industry (Saavedra *et al.* 1998; Langhendries *et al.* 1995). The employment of strains belonging to *B. animalis*, *B. longum*, *B. bifidum* and *B. infantis* as probiotic starter cultures is due to their important role played in the gut (Modler *et al.* 1990; Gibson & Roberfroid 1995). They suppress harmful bacteria by controlling pH of the large intestine through the production of lactic and acetic acids (Gibson *et al.* 1997). Bifidobacteria have antitumoral activity (Reddy & Rivenson 1993; Rastall & Gibson 2002) as well as anticholesterolemic (Pereira & Gibson 2002) and immune system activation effects (Mitsuoka 1992). Other effects that have been ascribed to this genus are the alleviation of lactose intolerance, and vitamin production (Hughes & Hoover 1995; Fooks *et al.* 1999).

In the fermentation process, lactose is transported via permease into the cell, where β-galactosidase hydrolyses the disaccharide into glucose and galactose. Galactose is not fermented and is discharged out of the cell. Glucose, on the other hand, is rapidly phosphorylated and, by intervention of aldolase, is converted to pyruvic acid in accordance with the glycolytic pathway. Pyruvic acid is then converted to lactic acid by lactate dehydrogenase (Marshall & Cole 1983).

The aims of this work were to assess the counts of bacteria in fermented milk products (total bacteria counts, bifidobacteria, streptococci and lactobacilli) and to observe the enzymic activities, especially those of glycosidases (α-galactosidase, α-glucosidase, and β-galactosidase).

MATERIAL AND METHODS

Six commercial fermented milk products with probiotic cultures – Bi-Fi, Activia, Kefir ABT, Revital Active, Cultured milk, and Yogurt were tested.

Enzymatic activity of pure cultures and fermented milk products was determined using API ZYM kit (BioMérieux, France). Yoghurt samples (3 ml) were suspended in the suspension medium (5 ml). After 24 h cultivation, 10 ml of pure bifidobacterial cultures were centrifuged for 10 min at 6000 rpm and the sediment was dissolved in 2 ml of the suspension medium. The solution was inoculated (90 µl to each cup) onto an API ZYM strip. The inoculated strips were incubated 2 h at 37°C. The metabolic end products produced during the incubation period were detected by means of coloured reactions effectuated by the addition of reagents. The reactions were read according to the Reading Table.

Total counts of bacteria were determined using Wilkins-Chalgren agar (Oxoid, England), bifidobacteria counts using Tripticase-phytone-yeast extract (Sharlau, Spain) with the addition of mupirocin (100 mg/l) according to Rada and Koc (2000), streptococci counts using M17 agar (Oxoid, England), and lactobacilli counts using Rogosa agar (Oxoid, England). The colonies of streptococci were counted and isolated after 24 h of cultivation at 37°C, and those of bifidobacteria, lactobacilli, and total bacteria after 48 h of cultivation.

Pure cultures of *Streptococcus thermophilus* CCDM 55 and *Lactobacillus delbrueckii* ssp. *bulgaricus* CCDM 40 were obtained from the Culture collection of dairy microorganisms (MILCOM, a. s., Prague). *Bifidobacterium animalis* was isolated from yoghurt (Activia). The method of the F6PPK detection described by Scardovi (1986) and modified by Orban and Patterson (2000) was used for the identification of bifidobacterial isolate at the genus level. The isolate was cultivated anaerobically in an anaerobic chamber in 10 ml TPY

broth at 37°C for 24 h. The cells were harvested by centrifugation at 6000 rpm for 10 min. The pellet was washed twice with a phosphate buffer solution 1 (0.05M phosphate buffer, pH 6.5, plus cysteine 500 mg/l) and the cells were suspended in 1 ml of buffer. The cell membranes were disrupted using the pretreatment with 0.4 ml CTAB detergent in the solution containing 0.45 mg CTAB in 1 ml of distilled water. After the pretreatment, 0.25 ml of aqueous solutions containing sodium fluoride (NaF, 3 mg/ml) and potassium or sodium iodoacetate (5 mg/ml) were added. To that, 0.25 ml sodium fructose-6-phosphate (80 mg/ml in H_2O) was added, the solution was vortexed and incubated at 37°C for 30 min. Following the incubation, 1.5 ml of hydroxylamine HCl (13 mg/100 ml) was added, the mixture was vortexed and allowed to incubate at room temperature for 10 min. One ml of TCA (15% w/v), 1.0 ml of 4N HCl and 1.0 ml of ferric chloride ($FeCl \times 6H_2O$, 5% w/v in 0.1N HCl) were added, and the mixture was vortexed. The development of a reddish-violet colour immediately after shaking the tube indicated the presence of fructoso-6-phosphate phosphoketolase (Vlková *et al.* 2002). Finally, the strain was identified at the species level using API 50 CHL and rapid ID 32 A tests (BioMérieux, France) followed by computer analysis (http://kounou.lille.inra.fr/bifidAppl.html). PCR by 16S rRNA gene sequence species-specific primers was used to confirm these results. The genomic DNA of the strain was extracted by heating at 100°C for 10 min

Table 1. Enzymatic activity[+] of fermented milk products and pure cultures

Fermented milk products	Type of product	Days to expiration date	α-galactosidase	β-galactosidase	α-glucosidase
Bi-Fi	yoghurt	21	1	3	0
		21	0	3	0
		21	0	3	0
Activia	yoghurt	19	1	3	1
		19	0	3	1
		16	0	1	1
Kefir ABT	kefir milk	12	0	1	0
		12	0	1	0
		6	0	1	0
Revital Active	yoghurt	14	0	2	0
		18	0	3	0
		18	0	4	0
Cultured milk	cultured milk	6	1	1	0
		6	0	1	0
		10	0	1	0
Yogurt	yoghurt	15	0	2	0
		15	0	2	0
		15	0	2	0
*B. animalis**	pure culture		5	4	5
Lactobacillus delbrueckii subsp. *bulgaricus* CCDM 40**	pure culture		0	5	0
Streptococcus thermophillus CCDM 55**	pure culture		0	4	0

*isolated from fermented milk products; **received from culture collection MILCOM, a. s.; [+]0 – negative reaction, 5 – maximum activity

Table 2. Counts of streptococci, lactobacilli, bifidobacteria

	Bacterial counts (log CFU/1 ml)		
	streptococci	lactobacilli	bifidobacteria
Bi-Fi	9.15 ± 0.27*	5.61 ± 0.66	5.81 ± 0.02
Activia	8.09 ± 0.18**	6.36 ± 0.32	8.50 ± 0.10*
Kefir ABT	7.20 ± 0.07***	6.25 ± 0.06	5.43 ± 0.41
Revital Active	8.29 ± 0.06**	6.40 ± 0.15	5.41 ± 0.18
Cultured milk	7.16 ± 0.43***	6.34 ± 0.30	6.03 ± 0.77
Yogurt	6.76 ± 0.14***	6.54 ± 0.21	5.59 ± 0.68

*, **, ***values in columns with different asterisks significantly differ ($P < 0.05$); all values are means from triplicate determination ± S.D. of log CFU/ml

in 1% Triton X-100 (Sigma) (Wang *et al*. 1996). Amplifications were performed with a thermal cycler (Techne, Techgene, UK) as described by Matsuki *et al*. (1999). PCR amplified products were analysed using 1% agarose gel electrophoresis at a constant voltage of 7 V/cm and visualised with ethidium bromide (0.5 µl) under UV light (wavelength 320 nm).

RESULTS AND DISCUSSION

The bifidobacterial isolate from Activia yoghurt was identified as *Bifidobacterium animalis*. The enzymatic activity of the fermented milk products and pure bacterial cultures are shown in Table 1. The API ZYM strip is composed of 20 cupules, specially designed for the study of enzymatic reactions. Three of them seem to be related to the presence of bifidobacteria: α-galactosidase, α-glucosidase, and β-galactosidase (Chevalier *et al.* 1990; Rada 1997). Indeed, the pure culture of *Bifidobacterium animalis* had high activities of α-galactosidase, β-galactosidase, and α-glucosidase – enzymes characteristic for bifidobacteria. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* had high β-galactosidase activities – characteristic enzyme for yoghurt culture.

Fermented milk products with probiotic cultures which had high β-galactosidase activities and low α-galactosidase activities were detected only in one case from three in Bi-Fi, Activia, Cultured milk. Only Activia yoghurt showed moderate α-glucosidase activity (Table 1). Low or no α-galactosidase and α-glucosidase activities signify that bifidobacteria occurring in commercial products are not fully metabolically active and probably have no effects on lactose digestion in the gut. Probiotic fermented milk products tested in this study may improve lactose ingestion as the result of the presence of viable yoghurt bacteria (*Streptococcus thermophilus, Lactobacillus delbrueckii* sp. *bulgaricus*). Medicoscientific criteria for probiotic bacteria include the resistance to gastric acid and bile, epithelial adhesion, growth and enzymatic activity in the gut, coaggregation ability, and antimicrobial activity (Charteris *et al.* 1998; Hassan & Frank 2001). It seems that bifidobacterial strains currently used in milk industry have been selected rather according to the technological selection criteria which include rapid growth in inexpensive media, good survival during the product shelf life, and susceptibility to freezing or freeze drying with cryoprotection (Marshall & Tamine 1997). The use of bifidobacteria in the form of lyophilised powder for the production of fermented milk product could be the reason for the low enzymatic activity of these bacteria in this environment. Even high bifidobacterial counts in yoghurt (Activia) did not result in α-galactosidase and α-glucosidase occurence in the product. It seems that bifidobacteria were viable but not metabolically active.

Bacterial counts determined in commercial fermented milk products are shown in Table 2. Bifidobacteria counts are between 5.15 and 8.62 log CFU/ml. Official announcement No. 77/2003 (Milk and dairy products) set bifidobacterial counts to 10^6 CFU/ml in product. These values were observed only in Activia, cultured milk (in two cases from three) and Yogurt (in one case from three).

References

Charteris W., Kelly P., Morelli L., Collins J. (1998): Ingredient selection criteria for probiotic microorganisms in functional dairy foods. International Dairy Technology, **51**: 123–136.

Chevalier P., Roy D., Ward P. (1990): Detection of *Bifidobacterium* species by enzymatic methods. Journal of Applied Bacteriology, **68**: 619–624.

Cross M.L., Stevenson L., Gill H.S. (2001): Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? International Immunopharmacology, **1**: 891–901.

Fooks L.J., Fuller R., Gibson G.R. (1999): Prebiotics, probiotics and human gut microbiology. International Dairy Journal, **9**: 53–61.

Fuller R. (1997): Introduction. In: Fuller R. (ed.): Probiotics 2. Applications and Practical Aspects. Chapman and Hall, London: 1–9.

Gibson G.R., Roberfroid M.B. (1995): Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition, **125**: 1410–1412.

Gibson G.R., Saaverda J.M., MacFarlane S., MacFarlane G.T. (1997): Probiotics and intestinal infections. In: Fuller R. (ed.): Probiotics 2: Application and Practical Aspects. Chapman and Hall, London: 10–31.

Hassan A.N., Frank J.F. (2001): Starter cultures and their use. In: Marth E.H., Steele J.L. (eds): Applied Dairy Microbiology. Marcel Dekker, Inc., New York: 151–206.

Heller K.J. (2001): Probiotics bacteria in fermented foods: product characteristics and starter organisms. American Journal of Clinical Nutrition, **73** (Supplement): 374S–379S.

Heller K.J. (2002): Will living starter bacteria no longer be an argument for the quality of fermented products? Nahrung, **46**: 307.

Hughes B.B., Hoover D.G. (1995): Viability and enzymatic activity of bifidobacteria in milk. Journal of Dairy Science, **78**: 268–276.

Kolars J.C., Levitt M.D., Aouji M., Savaiano D.A. (1984): Yogurt – an autodigesting source of lactose. New England Journal of Medicine, **310**: 1–3.

Langhendries J.P., Detry J., Van Hees J., Lamboray J.M. (1995): Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. Journal of Pediatric Gastroenterology and Nutrition, **21**: 177–181.

Marshall V., Cole W.M. (1983): Threonine aldolase and alcohol dehydrogenase activities in *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* and their contribution to flavour production in fermented milk. Journal of Dairy Research, **50**: 375–379.

Marshall V.M.E., Tamime A.Y. (1997): Physiology and biochemistry of fermented milks. In: Low B.A. (ed.): Microbiology and Biochemistry of Cheese and Fermented Milk. Elsevier Applied Science Publishers, London and New York: 153–192.

Matsuki T., Watanabe K., Tanaka R., Fukuda M., Oyaizu H. (1999): Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Applied and Environmental Microbiology, **65**: 4506–4512.

Mitsuoka T. (1992): The human gastrointestinal tract. In: Wood B.J.B. (ed.): The Lactic Acid Bacteria in Health and Disease. Elsevier Applied Science, London: 69–114.

Modler H.W., McKellar R.C., Yaguchi M. (1990): Bifidobacteria and bifidogenic factors. Canadian Institute of Food Science & Technology Journal, **23**: 29–41.

Molin G. (2001): Probiotics in foods not containing milk or milk constituents, with special reference to *Lactobacillus plantarum* 299v. American Journal of Clinical Nutrition, **73** (Supplement): 380S–385S.

Orban J.I., Patterson, J.A (2000): Modification of the phosphoketolase assay for rapid identification of bifidobacteria. Journal of Microbiological Methods, **40**, 221–224.

Pereira D.I.A., Gibson G.R. (2002): Cholesterol assimilation by lactic acid bacteria and bifidobacteria isolated from the human gut. Applied and Environmental Microbiology., **98**: 4689–4693.

Rada V. (1997): Detection of *Bifidobacterium* species by enzymatic methods and antimicrobial susceptibility testing. Biotechnology Techniques, **11**: 909–912.

Rada V., Koc J. (2000): The use of mupirocin for selective enumeration of bifidobacteria in fermented milk products. Milchwissenschaft, **55**: 65–67.

Rastall R.A., Gibson G.R. (2002): Prebiotic oligosaccharides: evaluation of biological activities and potential future developments. In: Tannock G.W. (ed.): Probiotics and Prebiotics Norfolk. Caister Academic Press, Norfolk: 107–148.

Reddy B.S., Rivenson A. (1993): Inhibitory effect of *Bifidobacterium longum* on collon, mammary and liver carcinogenesis induced by 2-amino-3-methylimidazo(4,5-f) quinoline, a food mutagen. Cancer Research, **53**: 3914–3918.

Rosado J.L., Solomons N.W., Allen L.H. (1992): Lactose digestion from unmodified, low-fat and lactose-hydrolysed yogurt in adult lactose-maldigesters. European Journal of Clinical Nutrition, **46**: 61–67.

Saavedra J.M., Abi-Hanna A., Moore N., Yolken R. (1998): Effect of long term consumption of infant formulas with bifidobacteria and *S. thermophilus* on stool pattern and diaper rash in infants. Journal of Pediatric Gastroenterology and Nutrition, **27**: 483–486.

Scardovi V. (1986): Genus *Bifidobacterium*. In: Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore: 1418–1434.

Torriani S., Vescovo M., Dicks L.M.T. (1997): *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*: a review. Annali di microbiologia ed enzimologia, **47**: 29–52.

Vlková E., Medková J., Rada V. (2002): Comparison of four methods for identification of bifidobacteria to the genus level. Czech Journal of Food Sciences, **20**: 171–174.

Wang R., Cao W.W., Cerniglia C.E. (1996): PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Applied and Environmental Microbiology, **62**: 1242–1247.

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