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RESEARCH ARTICLE

Effects of bifidogenic factors on growth of *Bifidobacterium bifidum* in cultured milk yoghurt

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Abstract: Two Bifidogenic factors, pantethine and proteose peptone (which contains pantethine) promote survival and growth of Bifidobacteria at low pH (3.8-4.0) in cultured milk yoghurt. Cultures of milk containing pantethine (1.25, 2.5, 5, 10, 100 and 500 µg/100 mL of milk) and proteose peptone (250,500, 1000 mg/100 mL milk; equivalent to 2.5, 5 and 10 µg pantethine) were inoculated with 5% (v/v) of *Bifidobacterium bifidum* strains β and B1, fermented at 37°C and monitored until fermentation was complete. When strain B1 was grown in milk without bifidogenic factors it had a shorter fermentation time and the drop in pH to 3.8 was rapid compared to β, a feature that is desirable in industry.Growth of B1 strain was observed when both pantethine and proteose pepetone were used, but the survival was better with pantethine. With proteose peptone, strain B1 showed rapid acidification of milk and growth.

Key words: Bifidobacteria, bifidogenic factors, cultured milk, pantethine, probiotic bacteria, proteose peptone, yoghurt

INTRODUCTION

The interest in Bifidobacteria is due to the potential health benefits of this micro-organism.[1-3] It is reported that bifidus milk fed to premature and new born babies created a bifidus microflora in these infants scarcely different from that of the breast fed child.[4] Bifidobacteria also produce organic acids which inhibit the growth of undesirable bacteria in the digestive tract and reduce the amount of toxic products in the faeces.[4]

A new infant formula fermented with *Bifidobacterium breve* and *Streptococcus thermophilus* has increased the bifidobacteria population in animals (mice with a human microflora), in healthy human volunteers and also in newborn babies.[5] A very recent study has shown that antipoliovirus response can be triggered by a fermented product that is able to favour intestinal bifidobacteria.[6]

Cultured milk products such as yoghurt are given to pregnant and lactating women, convalescents and the elderly, due to its improved digestibility.[7] Combination of the nutritional benefits of yoghurt and the health benefits of bifidobacteria would give a product that is more valuable than the traditional yoghurt made with *Lactobacillus delbrueckii* sub sp. *Bulgaricus* and *Streptococcus thermophilus* cultures.

Some of the problems of using bifidobacteria in cultured milk are its poor survival at pH 4.0 and below and the reduced rate of acid production. Rapid growth and acid production are desirable characteristics in a strain to be used in industry. Currently the yoghurt manufactured is at a pH range of 4.5 – 4.6. The survival and growth of bifidobacteria at this range is acceptable, but within this range a wide number of pathogens can grow and survive.[8] The difficulty of maintaining the viability of Bifidobacteria over long periods led to the use of bifidogenic or growth promoters. It has been reported that strains of *Bifidobacterium (B)* and *B. bifidus* var. *pennsylvanicus* are dependent on pantethine for growth because they cannot use pantothenic acid.[9] Two other studies have also reported on strains of bifidobacteria preferring pantethine to pantothenic acid.[10,11] Proteose peptone is a good source of pantethine and peptides that are required for the growth of Bifidobacteria and both are identified as new growth promoting factors.[8] *Bifidobacterium bifidum* was used in this study due to its presence in the digestive tract of infants and adults.[12]

The objective of this study was to evaluate the effects of pantethine and proteose peptone on the growth -and survival of *B. bifidum* at low pH of 4.00 and below.

METHODS AND MATERIALS

Cultures and yoghurt: Freeze dried cultures of *Bifidobacterium bifidum* β and B1, provided by the Dairy Microbiology Laboratory of the Reading University, U.K., were grown in sterile skimmed milk (12% total solids). From this 5% (v/v) was transferred to 100 mL sterile milk and incubated for 24h at 37°C to be used as the inoculum for every experiment in this study. The skim milk was autoclaved at 121°C for 15 min at 15 psi.

Yoghurt was prepared according to Robinson.[13] Reconstituted skim milk (100 mL) containing 12% total solids was placed in 150 mL bottles and heat-treated at 85°C for 30 min in a water bath, cooled to 42°C, and inoculated with 5% (v/v) of the 24 h liquid culture.

Media: Bifidobacteria from fermented milk were isolated and enumerated using Rogosa's Modified agar and broth (RM), and Rogosa's Modofied Selective agar and broth (RMS)

Incubation conditions: Plates were incubated anaerobically at 37°C for 48 h. Anaerobic conditions were achieved by using the Oxoid gas generating kit (Oxoid Ltd. Basing Stoke, Hampshire, U.K.) in poly carbonate jars of 3 L capacity. The sachets produce 1800 mL hydrogen and 350 ml carbon dioxide.

Analytical methods

Bacterial growth: Samples were collected aseptically at 6 h intervals for 24 h. Bifidobacteria were enumerated by the pour method of Harrigan and McCance[14] at dilutions of 10^{-1} – $10.^{-8}$ A laser counter, model 500- A (Spiral Instruments Inc.) was used to record the counts. Colonies were selected and gram stained according to Harrigan and MacCance[14] except that safranin replaced the dilute carbol fuchsin solution.

Acid production: Samples were collected at 6 h intervals for 24 h, mixed thoroughly and the pH was measured (Fisher Accumet model 620, Fisher Scientific Company, Pittsburgh, PA, U.S.A.).

Biochemical tests

Enzyme Activity: API ZYM™- semi-quantitative micro method. A single colony each from RM and RMS agar of B1 strain were isolated and grown in RM broth at 37°C for 3 d anaerobically. The broth culture was centrifuged and a suspension was prepared with turbidity between a McFarland No. 5 and 6 standards. Two drops of this suspension were transferred with a Pasteur pipette to each cupule of the API ZYM™ (Oxoid Ltd. Basing Stoke, Hampshire) test strip and incubated anaerobically for 4 h at 37°C and recorded results.

Carbohydrate metabolism: API 50 CH™ (Oxoid Ltd. Basing Stoke, Hampshire; U.K.) test strip. This strip allows the study of carbohydrate metabolism in microorganisms. A single colony of B1 strain was isolated from the RM agar and grown anaerobically. The broth culture was centrifuged and a suspension was prepared with the API 10 solution provided, to a turbidity of McFarland No. 2 standard. A drop of this suspension was transferred with a Pasteur pipette to each cupule of the test strip and incubated anaerobically. Results were recorded at 24 h and 48 h intervals.

Bifidogenic factors

Chemicals: Pantethine-bis[N-Pantothenyl amido ethyl]disulphide analar grade (BDH Inc., France), Proteose peptone (Oxoid Ltd Basing Stoke, Hampshire, U.K.).

Pantethine: 66.6 mg was dissolved in 20 mL of distilled water and filtered through a Nylon Acro disc 13 (13 mm HPLC certified 0.2 µm filter) into sterile bottles. The stock was used to prepare solutions at ranges of 1.25, 2.5, 5, 10, 100 and 500 µg per 100 mL of milk.

Proteose peptone: stock (2g in 20 mL distilled water) was prepared and autoclaved at 15 psi for 15 min at 121°C. The concentrations of peptone solutions (250, 500 and 1000 mg) corresponded to 2.5, 5 and 10 µg of pantethine respectively. According to Rasic and Kurmann[7] 100 mg of peptone contains 1 µg pantethine. György and Rose[9] have reported using 0.5 µg per 10 mL of skimmed milk. The reason for targeting the concentrations of pantethine above and below 5 µg per 100 mL of skimmed milk both in the pure form and as a component in proteose peptone.

Preliminary experiments: Identity of B1 strain as *B. bifidum* was confirmed by the API ZYM™ and API 50 CH™ tests. Growth and acid production by the strains β and B1 of *B. bifidum* were established by monitoring bacterial growth and acid production on RM and RMS. Gram staining was carried out on both strains. Another preliminary experiment was done to determine the level of pantethine that could be used without affecting the gel properties of yoghurt. Pantethine solutions containing 500 and 1000 µg was added to 100 mL of milk inoculated with 5% (v/v) of the 24 h B1 culture and incubated at 37°C until the gel was formed.

Results of the preliminary experiments led to the choice of B1 for further studies of bifidogenic factors. All experimental cultures were started by inoculation with 5% (v/v) of the 24 h B1 culture followed by incubation at 37°C for 24 h. The cultured milk without bifidogenic factors was used as the control.

Statistical Analysis

Data were analysed using Minitab for windows (release 10 Xtra). Analysis of variance (ANOVA) and Tukeys pairwise comparison were carried out on the bacterial growth and acid production data at the end of the 24 h period to detect the difference between treatments. All experiments were done in duplicate.

RESULTS AND DISCUSSION

Growth and morphological characteristics of β and B1 strains

The first preliminary experiment indicated that β and B1 behaved differently with strain B1 showing a shorter fermentation than β (Table 1). The dilutions showing colony counts between 30-300 were selected.[14] Average colony counts for β and B1 strain in the RM agar were similar, β (2.91×10^8 n=4), B1 (2.84×10^8 n=4). The growth of B1 was observed only at a dilution of 10^{-5} in RMS and a rod shaped structure was observed after gram staining (Table 1).

The external appearances of both types of colonies B1 and β in RM and RMS agar were similar. They were creamy white, opaque, circular, convex and smooth.

Gram staining of β in RM and RMS agar showed a branched morphology typical of bifidobacteria. The B1 strain in RM and RMS agar had an unbranched morphology (spherical or short rods in chains). This unusual behaviour of B1 indicated either contamination with streptpcocci, and therefore the structure being spherical or short rod shaped chains, or that it belonged to another species of bifidobacteria or to another biotype or mutant of the same species. Further tests were done to determine its identity.

Identification of the B1 strain

β-galactosidase was the only enzyme detected in the API ZYM™ test in B1 strain. According to Bergeys Manual[15], to assign to the genus *Streptococcus* an organism must be positive for α and β -galactosidase and also β-glucuronidase. Hence the conclusion from the API ZYM™ test was that the organism was not a streptoccal bacterium. Further with the API 50-CH™ test, showed that glucose, fructose and galactose were fermented, while adonitol, dulcitol, eryhtritol, glycerol, rhamnose and sorbose were not. These results led to the conclusion that strain B1 belongs to the genus *Bifidobacterium.* According to literature [7,16,17] an organism must be able to ferment lactose and cellobiose: for assignment to the species *bifidum.* This too was observed. Hence B1 was concluded as a strain of *B. bifidum.* According to Brown and Townsley[4], when a culture of *B. bifidum* changes morphology from branched to unbranched there is an accompanying change in acid production. They observed that 3 branched strains of *B. bifidum,* changed to pleomorphic or bent rods with an increase in lactic acid production. Increases in lactic acid production between 60 – 225% were seen in some strains. Thus, a possible explanation of the different behaviour of β and B1 strains is that sub cultured B1 changed from branched to unbranched pleomorphic form and therefore could produce acid at a faster rate (Table 1).

Effect of Pantethine

The preliminary study revealed that the maximum amount of pantethine that could be added without

Table 1: Time taken for the two strains of *Bifidobacterium bifidum* β and B1 to drop to a pH of 4.0 or below when grown in skim milk containing 12% total solids

Organism	Initial pH[a]	Final pH[a]	Time (h)	cfu (x 10^6) mL[b] 24 h culture (RMS)	cfu (x 10^5) mL[b] 24 h culture (RMS)
β	6.35 ±0.07	4.13±0.04	50	291	>300
B1	6.38 ±0.04	3.81±0.02	21	284	11

a – Average and Standard deviation of the pH in duplicate analyses

b – Average of the cfu/mL in (n=4) anlyses.

affecting gel properties was 500 µg per 100 mL of skimmed milk.

The results of the one way ANOVA indicated that there was a significant difference ($p<0.05$) only between 100 µg and the 2.5 µg pantethine additions. However, there was no significant difference between the control and pantethine at 100 µg (Table 2). The growth curve of pantethine at different concentrations is shown in Figure 1. As indicated in the figure for the numbers to be higher than the control in the death phase the growth of strain B1 was greater at these concentrations. Since there was no significant difference between the lowest and the highest concentration (1.25 and 500 µg) the lower concentration would be preferable for economic reasons. There was no significant difference ($p>0.05$) for pH between the different pantethine concentrations and the control (Table 2 and Figure 2). It must be noted that the pH did drop to 4.00 with pantethine, which is desirable to industry.

Effect of proteose peptone

The effect of concentrations of peptone 1 and peptone 2 were significantly different ($p<0.05$) from peptone 3 and the control for the bacterial counts (Table 2). The growth curve (Figure 3) reveals that the drop in cfu/mL for peptone 1 and 2 are greater than peptone 3 or the control, even though the cfu/mL for peptone 1 and 2 were higher in the growth phase. This indicates that survival in peptone 1 and 2 in the death phase are significantly lower than in peptone 3 and the control. The drop in pH were not significantly different between peptone 2 and 3 but they were different ($p<0.05$) from peptone 1 and the control (Table 2 and Figure 4). The drop in pH for all the concentrations of peptone was less than pH 4.0 However, the survival of B1 strain at the concentrations of peptone 1 and 2 were lower.

Table 2: Effect of pantethine and proteose peptone on counts of *Bifidobacterium bifidum* B1 and pH in yoghurt after 24 h of fermentation

Bifidogenic Factors	Cfu/mL	pH
Pantethine(1.25µg)	179^{ab}	4.03^{A}
Pantethine(2.5µg)	232^{b}	4.00^{A}
Pantethine(5µg)	148^{ab}	4.05^{A}
Pantethine(10µg)	165^{ab}	4.03^{A}
Pantethine(100µg)	134^{a}	4.03^{A}
Pantethine(500µg)	152^{ab}	4.00^{A}
Peptone 1	96^{c}	3.72^{B}
Peptone 2	86^{c}	3.81^{C}
Peptone 3	145^{d}	3.81^{C}
Control	133^{acd}	3.98^{A}

^{a,b,c,d}, Means (n=4) in the cfu/mL column with no common superscripts within bifidobacteria fators and control are significantly different ($p<0.05$).

^{A,B,C}, Means (n=2) in the pH column with no common superscripts within bifidogenic factors and control are significantly different ($p<0.05$).

*Values for counts are given as cfu (x 10^6) per mL culture.

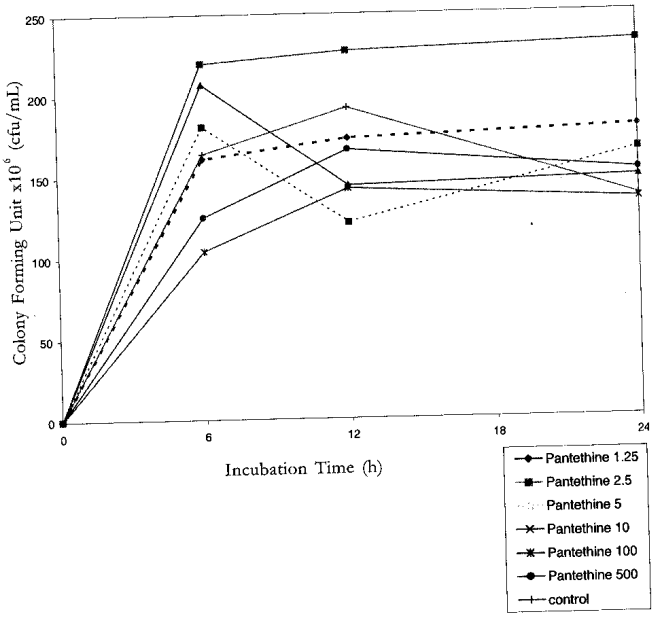

Figure 1: Growth curve for strain B1 in RM agar with Pantethine

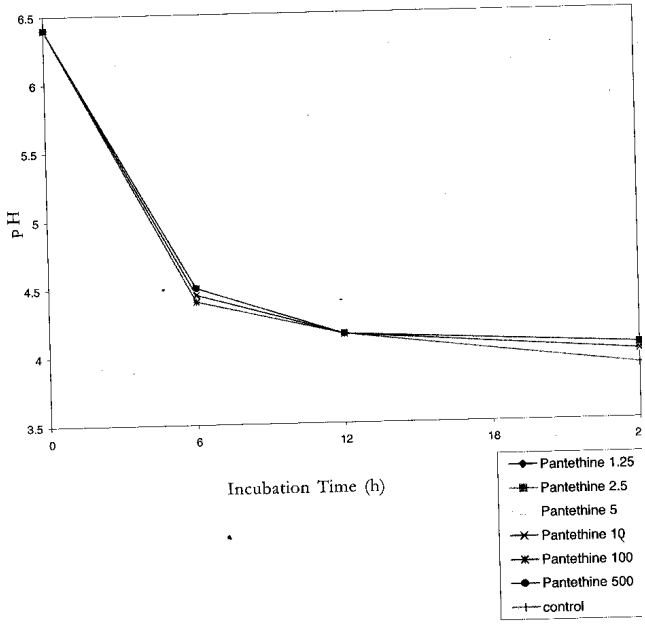

Figure 2: Drop in pH during the 24 h fermentation period with Pantethine

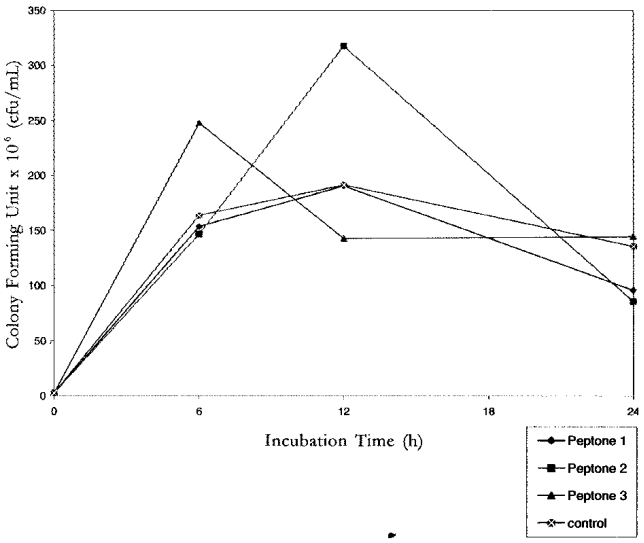

Figure 3: Growth curve for strain B1 in RM agar with proteose peptone

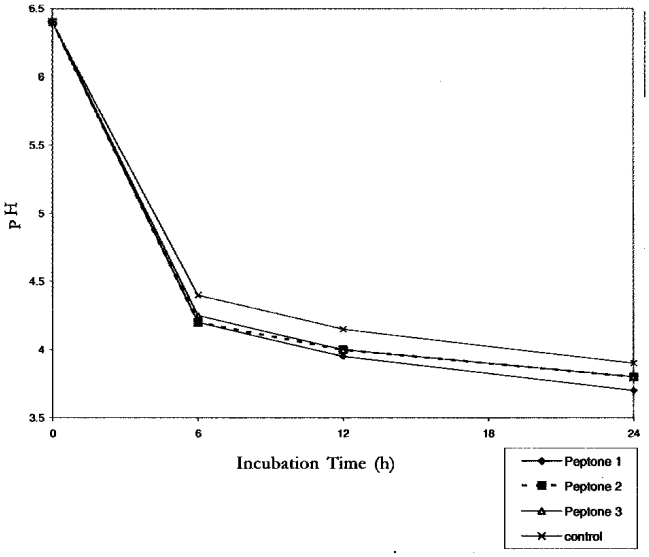

Figure 4: Drop in pH during the 24h fermentation period with proteose peptone

According to previous studies[7], good cultured milk should contain $10^6 - 10^8$ cfu/mL at the completion of yoghurt fermentation. The bacterial counts in all the concentrations of pantethine and peptone were 10^8 cfu/mL after 24 hours of fermentation except with peptone 1 and 2, where the count was 10^7 cfu/mL.

CONCLUSION

Pantethine and peptone have a positive effect as growth promoting factors on *B. bifidum* strain B1. The pH drop to 4.0 in the case of pantethine and to below 4.0 for peptone is in the range which is ideal for industrial production of yoghurt. Rapid acidification with peptone was observed. The survival of strain B1 was better when pantethine was used as a bifidogenic factor than proteose peptone. Further research should be conducted to evaluate the sensory properties of the resulting products.

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References

1. Robinson R.K. (1989). Special yoghurts: the potential health benefits. *Dairy Industries International* **54**(7):24-25.
2. Kailasapathy K. & Rybka S. (1997). *Lactobacillus acidophilus* & *Bifidobacterium* spp: their therapeutic potential and survival in yoghurt. *Australian Journal of Dairy Technology* **52**:28-35.
3. Abd El-Gawad I.A., El-Sayed E.M., Hafez S.A., El-Seini H.M. & Saleh F.A. (2005). The hypocholesterolaemic effect of milk yoghurt and soy-yoghurt containing bifidobacteria in rats fed on cholesterol-enriched diet. *International Dairy Journal* **15**(1): 37-44.
4. Brown C.D. & Townsley P.M. (1970). Fermentation of milk by *Lactobacillus bifidus*. *Canadian Institute of Food Technology Journal* **3**: 121-128.
5. Romond M.B., Ais A., Guillemot F., Bounouader R., Cortot A. & Romond C. (1998) Cell-free whey from milk fermented with *Bifidobacterium breve* C50 used to modify the colonic microflora of healthy subjects. *Journal of Dairy Science* **81**:1229-35.
6. Mulliè C., Yazourh A. & Thibault H. (2004). Increased poliovirus-specific intestinal actibody response coincides with promotion of *Bifidobacterium longum-infantis* and *Bifidobacterium breve* in infants: a randomised, double-blind, placebo-controlled trial. *Pediatric Research* **56**:1-6.
7. Rasic J. L. & Kurmann J. A. (1983). *Bifidobacteria and Their Role*. Birkhauser Verlag, Basel, Switzerland.
8. Samona A., Robinson R.K. & Marakis S. (1996). Acid production of bifidobacteria & yoghurt bacteria during fermentation and storage of milk. *Food Microbiology* **13**(4):275-280.
9. György P. & Rose C.S. (1955). Further observations on the metabolic requirements of *Lactobacillus bifidus* var *Pennsylvanicus*. *Journal of Bacteriology* **69**: 483-490.
10. Craig J.A. & Snell E.E. (1951). The comparative activities of pantethine, pantothenic acid and Coenzyme A for various microorganisms. *Journal of Bacteriology* **61**: 283-291.
11. McRorie R.A. & Williams W.L. (1951). Studies on the relationship between the *Lactobacillus bulgaricus factor* (LBF) & pantothenic acid. *Journal of Bacteriology* **61**: 737-745.
12. Modler H.W., McKellar R.C. & Yaguchi M. (1990). Bifidobacteria & bifidogenic factors. *Canadian Institute of Food Science & Technology Journal* **23**:29-41.
13. Robinson R.K. (1988). Cultures for yoghurt their selection and uses. *Dairy Industries International* **53**(7):9-15.
14. Harrigan W.F. & McCance M.E. (1976). *Laboratory methods in food and dairy microbiology*. Academic Press, London.
15. Bergey's Manual of Systematic Bacteriology (1984). Williams and Wilkins Co. Baltimore, USA.
16. Resnick I.G. & Levin M.A. (1981). Quantitative procedure for enumeration of bifidobacteria. *Applied & Environmental Microbiology* **42**(3): 427-432.
17. Roy D. & Ward P. (1990). Evaluation of rapid methods for differentiation of bifidobacterium species. *Journal of Applied Bacteriology* **69**:739-749.