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Characterization of natural isolate *Lactobacillus acidophilus* BGRA43 useful for acidophilus milk production

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Lactobacillus acidophilus BGRA43 was selected from a set of human origin isolates of *Lact. acidophilus* strains for the highest growth rates and antagonistic effect against both Gram-positive and Gram-negative bacteria. The strain BGRA43 also exhibited an inhibitory effect on the growth of *Clostridium sporogenes.* Inhibition of this strain seems to be due to lactic acid production rather than hydrogen peroxide or bacteriocin. Growth of *Lact. acidophilus* BGRA43 in non-fat skim milk for 6 h at 37 °C resulted in a lowering of the pH value to 4·53. Besides the fast acidification, this strain generated a high viscosity of skim milk. These characteristics make the strain BGRA43 attractive for acidophilus milk production. *Lactobacillus acidophilu*s BGRA43 produces an extracellular proteinase. Whole cells efficiently degraded casein for 3 h at 37 °C especially α- and β-casein fractions. Total DNA isolated from the strain BGRA43 did not show any hybridization with lactococcal proteinase probes indicating that this strain produces a distinctive proteinase.

INTRODUCTION

In recent years new fermented milks containing viable bacteria of human origin, including lactobacilli and bifidobacteria, have been developed. An increasing interest exists for dairy products containing specific bacterial species with potential health improving properties (Portier *et al.* 1993). The fermentation of milk especially with intestinal species of *Lactobacillus acidophilus* and/or *Bifidobacterium bifidum* is being applied for a number of products (Driessen and Boer 1989). These species are increasingly connected with health promoting effects (as probiotics) in the human and animal intestinal tract. Their probiotic effects are thought to be the inhibition of activity of pathogenic bacterial species, reduction in the risk of colon cancer, increase in the immune response, decrease in plasma cholesterol and release of the active β-galactosidase (Gilliland and Speck 1977; Kim and Gilliland 1983; Gilliland 1990; Havenaar *et al.* 1992; Salminen *et al.* 1996). In addition, for some particular *Lact. acidophilus* strains, probiotic properties and clinical effects have been documented (Lee and Salminen 1995).

Milk fermented with strains of *Lact. acidophilus* has been known for many years as acidophilus milk. Moreover, different *Lact. acidophilus* strains are used in the processing of dairy products such as acidophilus yoghurt and sweet acidophilus milk. The nutritional and therapeutic benefits derived through consumption of dairy products containing viable *Lact. acidophilus* as a food or feed supplement have been the focus of studies for the last two decades (Salminen *et al.* 1996). However, milk products based on *Lact. acidophilus* have met with many problems. Major difficulties for market expansion throughout the world are: the slow growth of *Lact. acidophilus* in milk especially without growth promoters; maintenance of bacterial viability during storage; relatively high acidity; and the unattractive flavour and consistency of the product (Brasheras and Gilliland 1995).

In this report, we present the characterization of a natural fast-growing isolate of *Lact. acidophilus* BGRA43. Besides the fast acidification, this strain generated a high viscosity of skim milk and showed very high bacterial viability during storage. These characteristics make the strain *Lact. acidophilus*

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BGRA43 attractive for acidophilus milk production as well as for other fermented products.

MATERIALS AND METHODS

Bacterial strains and media

Lactobacillus acidophilus BGRA43 from the human intestinal tract was characterized by using the Gram-stain reaction, catalase test and ability to grow at 15 °C, 30 °C and 45 °C. In addition, the carbohydrate fermentation pattern was determined by API 50 CH (API System S.A, Montelieu, Vercieu, France). The culture was maintained by propagation in sterile 10% non-fat milk solids (10%, w/v) and stored at 4 °C. Strain *Lact. acidophilus* NCDO1748 was chosen as a reference strain. Overnight cultures of *Lact. acidophilus* BGRA43 and *Lact. acidophilus* NCDO1748 were obtained by inoculation of MRS broth (Difco, Detroit, MI, USA) and incubation at 37 °C. *Escherichia coli* and *Clostridium sporogenes* were grown at 37 °C in LB broth and sulphite broth (TORLAK, Belgrade, Yugoslavia), respectively. Specific growth rates (k) of the *Lact. acidophilus* strains BGRA43 and NCDO1748 were determined in MRS broth and non-fat skim milk (10%) from the exponential portion of growth curves at temperatures indicated by using the equation given by Lee and Collins (1975).

Bile tolerance assay

To test bile tolerance, MRS broth lacking or containing 0·3% and 0·6% Oxgall as bile salts (BBL Microbiology Systems, Becton Dickinson, MD, USA) was inoculated (1%) with overnight culture (16 h). Samples were incubated at 37 °C in a water bath without shaking and the growth of cells was followed by measuring the optical density of culture ($O.D._{650\ nm}$).

Antimicrobial activity test

Antagonistic substances produced by *Lact. acidophilus* BGRA43 were detected by the spot-on-lawn method. Aliquots (50 μl) of supernatant fluid from non-fat skim milk cultures (24 h) of the strains BGRA43 and NCDO1748 were spotted on the surface of a freshly prepared lawn containing about 10^7 cfu ml^{-1} of indicator strains in MRS soft agar (0·7%, w/v). Either *L. cremoris* NS1, *L. lactis* NCDO712, *Lact. casei* NCDO393, *Lact. plantarum* A112, *Staphylococcus aureus, E. coli* C600, *B. mycoides* or *Pseudomonas* spp. were used as indicator strain. Plates were incubated overnight at 37 °C and the appearance of an inhibition zone around the spotted culture was taken as an indication that strains produced some inhibitory substances. To eliminate the effect of hydrogen peroxide, samples of cultures were pretreated with catalase. To test the ability of *Lact. acidophilus* BGRA43 to inhibit the growth of *Cl. sporogenes*, sulphite agar was inoculated with 1% of clostridium culture (48 h). At the top of the sulphite agar 4 ml of 0·7% MRS top agar with and without overnight culture of *Lact. acidophilus* BGRA43 (1%) was applied and incubated for 48 h at 37 °C.

To test for possible production of bacteriocin, MRS plates were overlaid by 3 ml of MRS soft agar (0·7%, w/v) containing 0·1 ml of 10^{-2} diluted fresh culture of the indicator strain. Wells were made in the lawn of hardened soft agar. Aliquots (50 μl) of supernatant fluid collected from overnight culture of the BGRA43 strain were poured into the wells. Crystal of pronase E was placed close to the edge of the well. Plates were incubated overnight at 37 °C.

Assay of proteolytic activity

Proteolytic activity of *Lact. acidophilus* BGRA43 was assayed as described previously (Kojic *et al.* 1991). For enzymatic assays, the BGRA43 strain was grown on milk-citrate agar (MCA) plates containing 4·4% reconstituted non-fat skim milk (RSM), 0·8% Na-citrate, 0·1% yeast extract, 0·5% glucose and 1·5% agar (w/v) for 48 h at 37 °C and in MRS broth for 10 h at 37 °C before cell collection. Collected cells (5 mg) were resuspended in 100 mmol l^{-1} Na-phosphate buffer (pH 6 · 5) to an approximate density of 10^{10} cells ml^{-1}. The cell suspension was mixed with substrate dissolved in the same buffer at a 1:1 volume ratio. The resulting mixtures were incubated at 30 °C, 37 °C and 42 °C for 3 h. As a substrate, total casein (12 mg ml^{-1}) (Sigma Chemie GmbH, Deisenhofen, Germany) was used in the test. Casein hydrolysis was analysed by SDK-PAGE by loading 12% (w/v) acrylamide gel with prepared samples. Gels were run on vertical slab electrophoresis cells (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD, USA) for 20 h at 20 mA constant current and stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany).

Plasmid isolation

Analysis of plasmid content of *Lact. acidophilus* BGRA43 was done by isolation of plasmids from the strain and running them on agarose gel (1%) electrophoresis. Plasmid isolation was done as follows. A logarithmic culture was prepared in 4 ml of MRS broth. Cells were pelleted by centrifugation (1 min at 12 000 *g*), resuspended in 1·5 ml TEN buffer (50 mmol l^{-1} TRIS -HCl, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} NaCl, pH 8) and pelleted again by the same centrifugation. Cells were resuspended in 100 ml PP buffer (0·5 mol l^{-1} sucrose, 40 mmol l^{-1} NH_4-acetate, 1 mmol l^{-1} Mg-acetate, pH 8) to which 4 mg ml^{-1} of lysozyme was added. This suspension was incubated for 15 min at 37 °C. After incubation, 200 μl of 1% SDS in TE-1 buffer (100 mmol l^{-1} TRIS-HCl,

10 mmol l^{-1} EDTA, pH 12) was added and the microcentrifuge tubes were gently inverted five to six times. Glacial acetic acid (100 μl) was added and the contents mixed gently by inversion followed by addition of 120 μl NaCl (5 mol l^{-1}). The mixture obtained was again gently inverted twice and centrifuged (15 min at 12 000 *g*). The clear supernatant fluid (approximately 400 μl) was transferred into a new microcentrifuge tube to which 2 volumes of cold 100% ethanol was added. After immediate centrifugation (10 min at 12 000 *g*), the resulting pellet was washed twice in 1 ml 70% ethanol, dried and resuspended in 20 μl H_2O. Isolation of plasmids was also performed following the procedure for isolation of large plasmids (O'Sullivan and Klaenhammer 1993).

DNA/DNA hybridization

The proteinase gene probes (Q1, Q6 and Q92), originating from *L. lactis* ssp. *cremoris* Wg2, were kindly provided by Dr J. Kok. Labelling of probes and hybridization experiments were carried out essentially as described previously (Kojic *et al.* 1991). Hybridization was performed at 68 °C and 45 °C.

RESULTS

Characteristics of the *Lactobacillus acidophilus* strains

Both cultures being tested, *Lact. acidophilus* NCDO1748 and BGRA43, were Gram-positive, catalase-negative, rod-shaped bacteria. Neither of them grew at 15 °C, but grew at 37 °C and 42 °C in MRS broth. Considering these characteristics and fermentation patterns both strains were confirmed to be *Lact. acidophilus*. The only difference between these strains was that BGRA43 did not ferment fructose whereas the strain NCDO1748 did. Neither produced gas from glucose and both were resistant to NaCl (2%, w/v). It appeared that both strains were sensitive to Oxgall (0·3%, w/v), since their growth was completely prevented at this concentration of bile salts.

Influence of temperature on growth

Growth rates and generation times of *Lact. acidophilus* BGRA43 and NCDO1748 were determined in MRS broth for each of the strains at 30 °C, 37 °C and 42 °C (Fig. 1). Optimal growth temperature of both strains in MRS was found to be 37 °C. However, the strain *Lact. acidophilus* BGRA43 showed faster growth in comparison to the growth rate of the strain *Lact. acidophilus* NCDO1748 during the first 10 h of incubation. Moreover, besides a shorter lag phase (2 h), the strain BGRA43 reached $O.D._{650\ nm}$ 0·8 for 10 h of growth. For the same period of incubation the strain NCDO1748 showed lower turbidity ($O.D._{650\ nm} = 0{\cdot}6$). Consequently, generation times at 37 °C were 1·72 h and 2·78 h for the strains BGRA43 and NCDO1748, respectively. There was no difference in the growth rate between these strains at 42 °C, but their growth was lower than at 37 °C.

A significant difference in growth of the strains BGRA43 and NCDO1748 in non-fat skim milk (10%) was observed at 42 °C. The strain BGRA43 showed much faster growth in comparison with the strain NCDO1748 (Fig. 2). Within 6 h of growth the strain BGRA43 culture contained 3×10^8 cfu ml^{-1} and stationary phase was reached after 10 h of growth. In contrast, within the same time (6 h) the NCDO1748 culture contained only 1×10^7 cfu ml^{-1} and the stationary phase of growth was not reached even after 28 h of incubation. The fast growth of the strain BGRA43 in non-fat skim milk was also observed at 37 °C resulting in the lowering of the pH value to 4·53 as well as in very high viscosity of the milk. However, it was not possible to detect viscosity of the strain NCDO1748 non-fat skim milk culture under the same conditions of cultivation.

Antimicrobial activity of *Lact. acidophilus* BGRA43 and NCDO1748

To test the inhibitory activity of *Lact. acidophilus* BGRA43 and NCDO1748, cultures (24 h) were prepared in non-fat skim milk. *Lactobacillus acidophilus* BGRA43 showed an inhibitory effect on the growth of some *Lactococcus* and *Lactobacillus* strains as well as *Staph. aureus*, *E. coli*, *B. mycoides* and *Pseudomonas* sp. *Lactobacillus acidophilus* NCDO1748 exhibited inhibition of the same indicator strains, but its efficiency of inhibition seemed to be less pronounced than that of strain BGRA43 (Table 1). However, inhibition of indicator strain growth by *Lact. acidophilus* BGRA43 and NCDO1748 seems not to be due to bacteriocin production, since inhibition zones were not affected by the addition of pronase E (data not shown).

Lactobacillus acidophilus BGRA43 also exhibited an inhibitory effect on the growth of *Cl. sporogenes.* Results showed that inhibition of this strain seemed to be due to lactic acid production (pH of the MRS culture used in the test was 5) rather than hydrogen peroxide or bacteriocin (Fig. 3).

Proteolytic activity

To test the ability of *Lact. acidophilus* BGRA43 and NCDO1748 to hydrolyse total casein, MRS cultures grown for 10 h were prepared. In parallel, cells of these two strains were collected from MCA agar plates after incubation for 48 h at 37 °C. Whole cells were incubated in the presence of total casein for 3 h at 30 °C, 37 °C and 42 °C. Results showed that *Lact. acidophilus* BGRA43 completely hydrolysed α- and

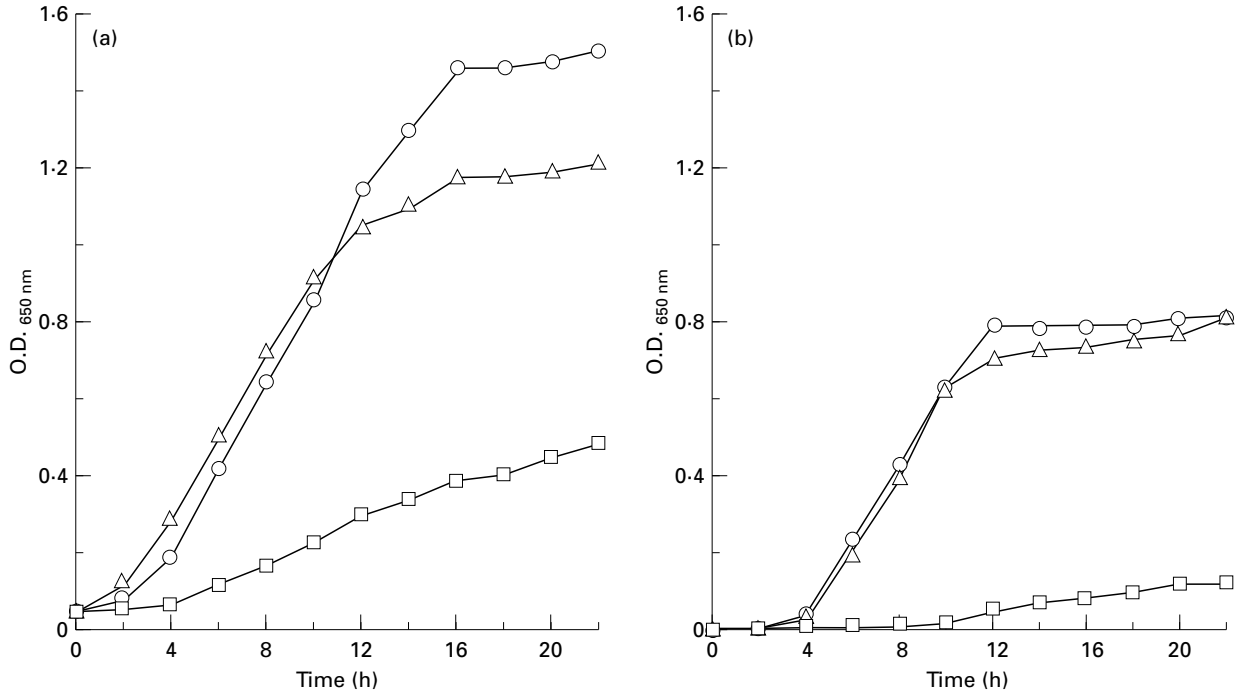

Fig. 1 Growth rates of *Lactobacillus acidophilus* (a) BGRA43 and (b) NCDO1748 determined in MRS broth. ■, 30 °C; ◆, 37 °C; ●, 42 °C

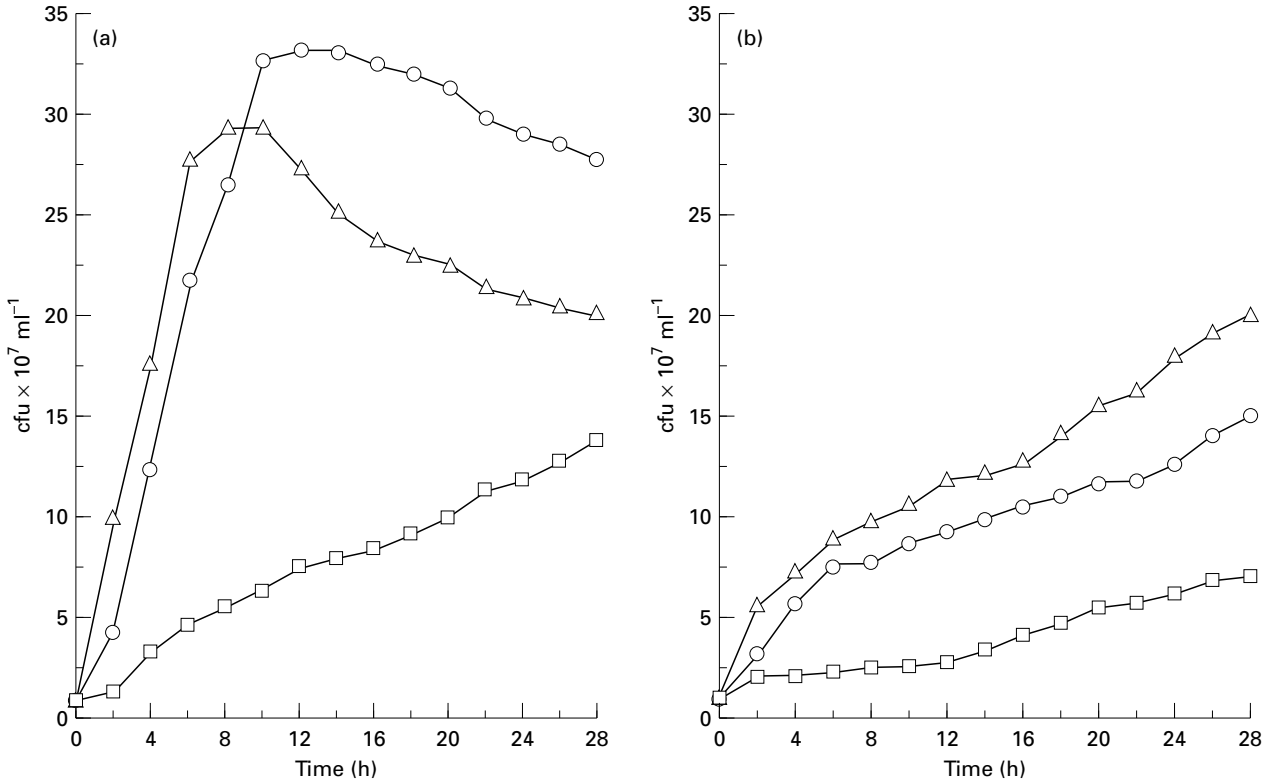

Fig. 2 Growth rates of *Lactobacillus acidophilus* (a) BGRA43 and (b) NCDO1748 determined in 10% non-fat skim milk. ■, 30 °C; ◆, 37 °C; ●, 42 °C

Table 1 Antimicrobial activity of *Lactobacillus acidophilus* BGRA43 and NCDO1748

Indicator strain	BGRA43	NCDO1748
Lactococcus cremoris NS1	+	+
Lactococcus lactis NCDO712	+	+
Lactococcus casei NCDO393	+	+
Lactococcus plantarum A112	+	+
Staphylococcus aureus	+ + +	+ + +
Escherichia coli C600	+ + +	+ +
Bacillus mycoides	+ + +	+ +
Pseudomonas spp.	+ + +	+ +

+, + + and + + +, Diameter of inhibition zone up to 2 mm, 4 mm and over 4 mm, respectively.

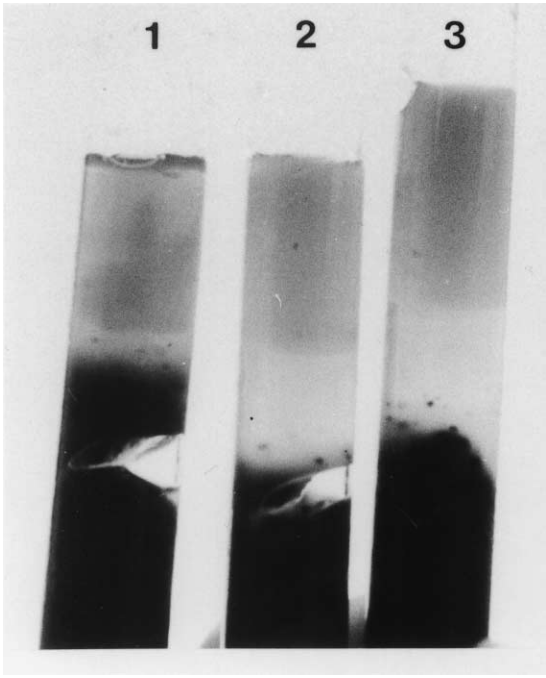

Fig. 3 Inhibition of *Clostridium sporongenes* growth. 1, Sulphite agar containing clostridium overlaid with MRS top agar ; 2, sulphite agar containing clostridium overlaid with MRS top agar containing BGRA43 culture ; 3, sulphite agar containing clostridium overlaid with MRS top agar containing BGRA43 culture pre-treated with catalase

β-casein fractions at all three temperatures regardless of the source of cells (MRS broth or MCA agar plates). Hydrolysis of κ-casein appeared to be partial, especially when the cells were grown in MRS broth. In contrast, there is no visible casein degradation of any casein fraction by *Lact. acidophilus* NCDO1748 under the same experimental conditions (Fig. 4).

The similarity of the proteinase gene sequence of *Lact. acidophilus* with that of known proteinases was tested. Hybridization of total DNA isolated from *Lact. acidophilus* with lactococcal proteinase gene probes (Q1, Q6 and Q92) did not give any signal regardless of the conditions of hybridization. Therefore, it is most probable that the strain BGRA43 produces a distinct proteinase.

Plasmid profile

Analysis of plasmid contents of *Lact. acidophilus* BGRA43 and NCDO1748, regardless of the procedure used for plasmid isolation, revealed that only the former strain contained one plasmid of 2·4 kb. This plasmid appeared to be very stable since 10 successive subcultivations of the strain BGRA43 did not result in plasmid loss. Results also showed that this plasmid was not responsible for proteinase production because proteinase non-producing derivatives of the strain BGRA43 contained the same plasmid (data not shown).

DISCUSSION

From an industrial point of view, *Lact. acidophilus* strains should have a short lag growth phase and proper acid formation. Many *Lact. acidophilus* strains grow poorly in milk without additives or when grown alone. The selected strains should have the ability to acidify milk to below pH 5 within 24 h at 37 °C. Improvement of the growth rate of *Lact. acidophilus* can be achieved by growing in symbiosis with lactic acid bacteria such as *Streptococcus thermophilus* (Montes *et al.* 1995). *Lactobacillus acidophilus* was generally found to grow more easily in the presence of the other organisms (Hunger and Peitersen 1993). Comparing with these data, the analysed strain BGRA43 grew very fast in milk (6 h, pH 4·5) as well as in MRS ($A_{600\ nm}$; 0·858, 10 h). The generation time in MRS was shorter at both temperatures regarding that of the strain NCDO1748. In addition, this strain is aciduric at pH 4·1–3·8 in the coagulated milk after 5 d of storage (10^7 cfu ml^{-1}) and the fermented product has an acid flavour and high viscosity. It appeared that *Lact. acidophilus* BGRA43 also influenced other important properties of the product, such as taste, aroma and viscosity.

In the mixture with *Strep. thermophilus* (3:1), BGRA43 produces less acid and, after 6 h at 37 °C, the pH of milk was 4·80 (data not shown). The fermented milk product has a mild acid flavour and also high viscosity. The production of exopolysaccharide (EPS) by lactic acid bacteria (LAB) is thought to be responsible for the viscosity of milk (Cerning 1990; Kojic *et al.* 1992). Other reports suggested that a slimy and viscous material is present only on cells of some LAB harvested later in the stationary phase (Aguilera and Kessler 1989; Amice-Quemeneur *et al.* 1995). The high viscosity of the fermented milk produced by *Lact. acidophilus* BGRA43

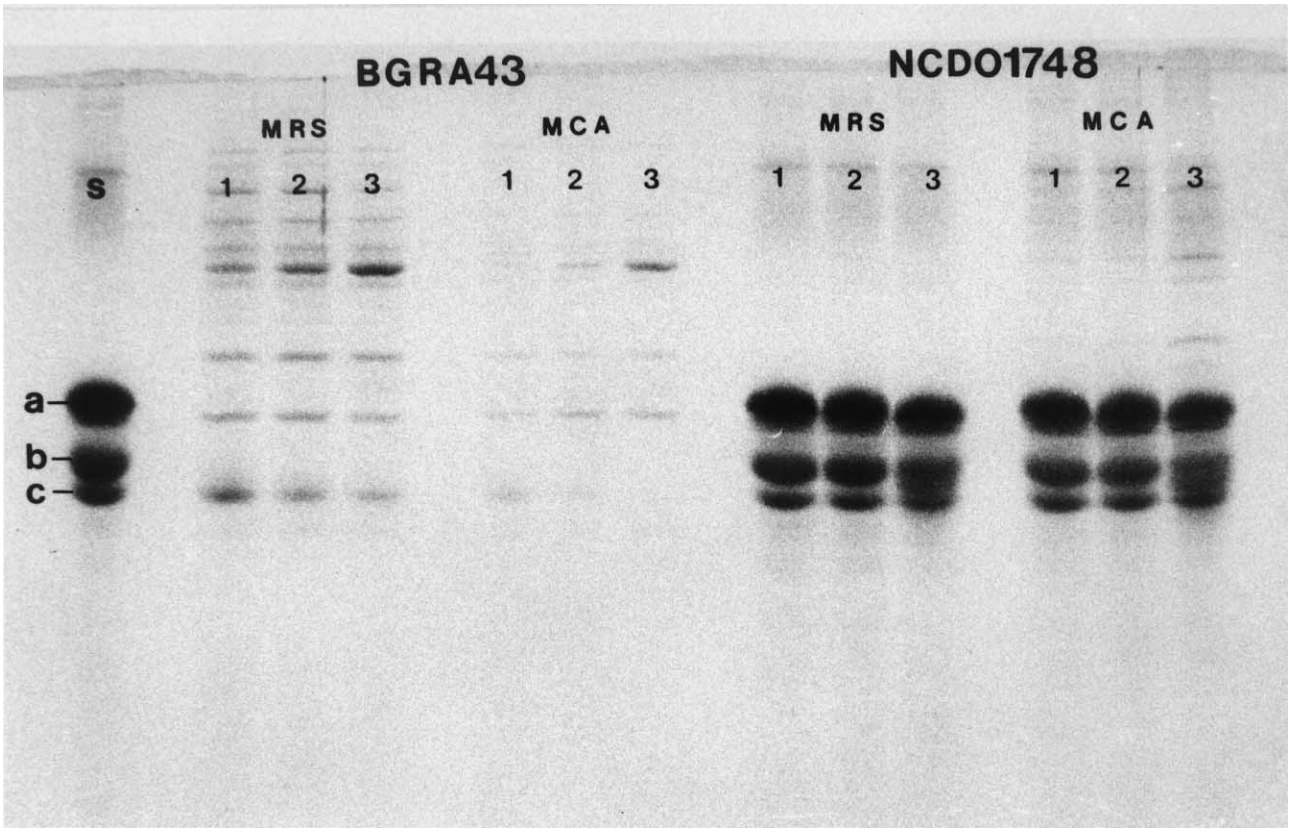

Fig. 4 Total casein hydrolysis by *Lactobacillus acidophilus* BGRA43 and NCDO1748. Lane : s, substrate—total casein ; 1, 2 and 3, samples incubated at 30 °C, 37 °C and 42 °C, respectively. a, α-casein ; b, β-casein ; c, κ-casein. MRS, MRS broth ; MCA, milk-citrate agar

appeared to be due to casein particle formation rather than EPS production. The viscosity of the fermented milk product based on *Lact. acidophilus* BGRA43 has decreased after mechanical treatment. A similar conclusion was drawn from a study of stirred yoghurt rheology (Ramaswamy and Basak 1991).

Lactobacillus acidophilus BGRA43 does not produce bacteriocins but inhibits the growth of various bacterial strains (Table 1). It is well known that LAB could influence the growth of various species of micro-organisms by different mechanisms (Fernandes *et al.* 1987). The major antagonistic activity (besides bacteriocin production) in milk is attainment of a rapid decrease in pH when organic acids are present (Kleter *et al.* 1982). Besides other species the strain BGRA43 also inhibited the growth of *Cl. perfringens.* In view of the fast growth, this strain rapidly reached a high level of acidity in the initial phase of the fermentation process, which indicated that a complete inhibition of the activity of clostridia and other micro-organisms should be easy to achieve. Moreover, it has been shown that the MIC undiss-acid value for *Cl. tyobutyricum* strains was 4·6–9·6 mmol l^{-1} of lactic acid (Jonson 1989).

It is very important for the good quality of the fermented product to use strains that are capable of producing enough lactic acid in a short time, e.g. strains that grow fast. Milk is a specific environment containing protein as a significant component. The ability to degrade protein is one of the most important features of LAB that influence their growth rates (Pritchard and Coolbear 1993). *Lactobacillus acidophilus* BGRA43 produces a very efficient proteinase in contrast to *Lact. acidophilus* NCDO1748. As a consequence, the growth of the strain BGRA43 in milk was much more efficient than that of the strain NCDO1748.

In conclusion, the high growth rate of *Lact. acidophilus* BGRA43 in milk, most probably due to synthesis of efficient proteinase, resulted in high production of lactic acid, which is known to have a probiotic effect. Therefore, the application of *Lact. acidophilus* BGRA43 as an intestinal micro-organism in traditional milk products such as cheese, ice cream and in

other foods, for example sausages, beverages, sour dough, etc., is worthy of study.

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