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Yoghurt fermented by *Lactobacillus delbrueckii* subsp. *bulgaricus* H^+-ATPase-defective mutants exhibits enhanced viability of *Bifidobacterium breve* during storage

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Abstract

Persistent acid production by *Lactobacillus delbrueckii* subsp. *bulgaricus* during refrigerated storage is a major cause of reduced viability of probiotic strains such as *Bifidobacterium breve* in yoghurt. It was established that H^+-ATPase-defective mutants of lactic acid bacteria have reduced growth and metabolism in low pH environments. Therefore, the aim of this study was to evaluate inhibition of post-acidification and maintenance of *B. breve* viability in yoghurt fermented by *L. delbrueckii* subsp. *bulgaricus* mutants with reduced membrane-bound H^+-ATPase activity during refrigerated storage. Spontaneous neomycin mutants of *L. delbrueckii* subsp. *bulgaricus* that had a significantly ($P \leq 0.05$) reduced H^+-ATPase activity were successfully isolated. Yoghurt fermented using *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant) starter culture had markedly reduced post-acidification and maintained viability ($\geq 10^8$ CFU/ml) of both *Bifidobacteruim breve* JCM 1192^T and *Bifidobacteruim breve* JCM 7017 during storage at 10 °C for 21 days. These results clearly showed that yoghurt fermented by mutants of *L. delbrueckii* subsp. *bulgaricus* with reduced membrane-bound H^+-ATPase activity has reduced post-acidification that prolongs viability of *B. breve* in yoghurt during refrigerated storage.
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Keywords: H^+-ATPase; *Bifidobacterium breve*; *Lactobacillus delbrueckii* subsp. *Bulgaricus*; Post-acidification

1. Introduction

Fermented dairy products, such as yoghurt, have been widely accepted as vehicles for transmission of probiotics to consumers (Adhikari et al., 2003). In yoghurt production, milk is fermented using a co-culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* at 42 °C up to pH 4.5. After fermentation, yoghurt is stored at 4–10 °C either in retail outlets during marketing or in home refrigerators awaiting consumption for a period ranging from 1 to 2 weeks depending on the yoghurt shelf life declared by the manufacturer. The viability of probiotic bacteria in yoghurt depends on the strains used, interaction between species present, culture conditions, production of hydrogen perioxide due to bacterial metabolism, final acidity of the product, dissolved oxygen and the concentrations of lactic, and acetic acids (Dave and Shah, 1997; Shah, 2000). However, low medium pH has been identified as the most important factor hindering viability of lactic acid bacteria (Shah and Jellen, 1990). Furthermore, *L. delbrueckii* subsp. *bulgaricus* produces lactic acid during refrigerated storage; the latter process is known as 'post-acidification' (Shah, 2000). The survival of *Bifidobacterium breve* in yoghurt is quite low because of the low pH of this product (4.2–4.6) and reduced viability of probiotic bacteria in yoghurt has been extensively reported (Shah et al., 1995; Vinderola et al., 2000; Lucas et al., 2004). In comparison to other strains of lactic acid bacteria, strains of *Bifidobacterium*

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are more sensitive to low pH; which makes maintaining of strains of *Bifidobacterium* in fermented milks products very difficult. It is essential that products sold with any health claims meet the criterion of a minimum 10^6 CFU/ml probiotic bacteria at the expiry date; because the minimum therapeutic dose per day is suggested to be 10^8–10^9 cells (Kurman and Rasic, 1991). In Japan, a standard has been developed by the fermented milks and lactic acid beverages association; 10^7/ml of viable bifidobacteria cells has been judged the minimum count for fresh dairy products (Ishibashi and Shimamura, 1993). To guarantee this, one of the important variables to be considered is that numbers of probiotic cells survive throughout the shelf life of the product (Heller, 2001). Determination of the methodology to increase the numbers of probiotic organisms that grow during the manufacture of a cultured product such as yoghurt and to improve stability of the product is needed to provide the consumer with adequate numbers of the organisms (Gilliland et al., 2002). Attempts to ferment yoghurt using *S. thermophilus* only or with other strains such as *Lactobacillus acidophilus* has been less successful because of the mild (less acidic), but non-aromatic products (Mollet, 1999).

The best approach to limit post-acidification and still produce the yoghurt flavour is to regulate growth and maintenance of *L. delbrueckii* subsp. *bulgaricus* by controlling its energy metabolism (Mollet, 1999). One of the key enzymes involved in energy metabolism of lactic acid bacteria (LAB) is the proton translocating (F_0F_1) ATPase. The structures of the H^+-ATPase complexes from different sources are similar and consist of two parts: a membrane integral part, F_0, which forms a proton channel and a soluble part, F_1, which contains the catalytic site for ATP hydrolysis (Koebmann et al., 2000). It has been proposed that the primary function of the H^+-ATPase in anaerobic bacteria is the regulation of the cytoplasmic pH through extrusion of protons driven by ATP hydrolysis to maintain intracellur pH and to generate the proton motive force required for transport systems (Kobayashi, 1985). In LAB, maintenance of a higher internal pH is vital for metabolism and survival under acidic pH conditions (Kashket, 1987). Selection for resistance to the antibiotic neomycin has been used in the isolation of strains defective in ATPase activity due to the inability of these mutants to generate sufficient energy to concentrate the antibiotic (Kanner and Gutnick, 1971; Rosen, 1973). Mutants of *Lactococcus lactis* (Yokota et al., 1995); *Oenococcus oeni* (Tordot-Maréchal et al., 1999), *Streptococcus bovis*, (Miwa et al., 2000) and *Lactobacillus rhamnosus* GG, (Corcoran et al., 2005) with reduced membrane-bound H^+-ATPase activity have been isolated as neomycin-resistant mutants and show increased sensitivity in low pH environments. A single point mutation in the γ-subunit has been attributed to prevention of F_1-ATPase assembly leading to a defective proton pathway (Sekine et al., 2001). However, the relationship between the H^+-ATPase activity of *L. delbrueckii* subsp. *bulgaricus* and yoghurt post-acidification has not yet been explored. Therefore, our aim was to study the inhibition of post-acidification and maintenance of *B. breve* viability in yoghurt fermented by *L. delbrueckii* subsp. *bulgaricus* mutants with reduced membrane-bound H^+-ATPase activity during 21 days of storage at 10 °C. An additional objective was to determine the sensory attributes of yoghurt fermented by *L. delbrueckii* subsp. *bulgaricus* H^+-ATPase-defective mutants.

2. Materials and methods

2.1. Strains and growth conditions

L. delbrueckii subsp. *bulgaricus* SBT0164 No. 20-1 was kindly provided by Snow Brand Milk Products Co. Ltd., Sapporo research laboratory, Sapporo, Japan, and *S. thermophilus* 21702 was obtained from Hokkaido Food Processing Research Center, Ebetsu, Japan. *B. breve* JCM 1192^T and *B. breve* JCM 7017 were obtained from Japan Collection of Microorganisms, Saitama, Japan. All strains were kept in 10% glycerol at −80 °C. *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 and the mutants derived from *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 were routinely grown on half strength (27.5 g/L) de Man Rogosa Sharpe (MRS) broth (Becton, Dickinson Co., Sparks, Md., USA). Strains of *B. breve* were grown on Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical, Tokyo, Japan). *S. thermophilus* 21702 was grown on M17 broth (Becton, Dickinson Co., Sparks, Md., USA). All strains were cultured under anaerobic conditions consisting of N_2, CO_2 and H_2 in the ratio of 8:1:1 respectively at 37 °C. Growth was determined as Optical Density (OD) at 660 nm using spectrophotometer (Milton Roy).

2.2. Derivation of H^+-ATPase-defective mutants from L. delbrueckii subsp. bulgaricus SBT0164 No. 20-1

Cells of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 at the stationary growth phase were harvested by centrifugation at 3000 ×*g*, for 10 min at 4 °C. The cell pellet was washed twice in sterile saline (0.85% NaCl) solution and suspended in saline to an OD of about 0.450. Suspended cells (0.1 ml) were inoculated into MRS agar plates containing neomycin sulfate (50 μg/ml) and incubated at 37 °C for 72 h in an air tight jar (AnaeroPack Rectangular Jar™) containing an O_2 absorbing and CO_2 releasing pack (AnaeroPack™, Mitsubishi Gas Chemical Co., Tokyo, Japan). Single colonies were picked and streaked on fresh MRS agar plates containing neomycin and incubated as described above. This procedure was repeated at least two times in order to purify the colonies. Growth of the mutants were checked and only mutants that had a peak OD less than 0.7 and lowered the pH of the MRS broth not lower than 4.8 after 48 h cultivation were selected and kept as glycerol stocks at −80 °C. Two mutants identified as *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 that met the selection criteria were used for further trials.

2.3. Growth and medium acidification characteristics of H^+-ATPase-defective mutants

After two successive transfers of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus*

SBT0164 No. 20-1 in MRS broth at pH 6.5, the cell culture (30 µl) was inoculated into 3 ml of MRS broth and cultivated at 37 °C anaerobically for 48 h. Growth was determined after every 1 h for 10 h and then after 12, 24 and 48 h of cultivation meanwhile the culture medium acidification by *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 was monitored by checking medium pH after 12, 24, and 48 h of cultivation.

2.4. Membrane H^+-ATPase assay

2.4.1. Preparation of crude enzyme

Strains were cultured as described above for 18 h. Cells were harvested by centrifugation at 5000×*g* for 10 min at 4 °C; washed twice using Tris–HCl buffer (pH 7.5) at 4 °C and kept at −20 °C for a maximum period of 2 weeks. Membranes were extracted as described by Sakamoto et al. (2002) with some modifications. Wet cells (0.5 g) suspended in 5 ml of Tris–HCl buffer were lysed at 37 °C by treatment for 1.5 h with 1 mg of lysozyme (Sigma, Missouri, USA) and 50 µg mutanolysin (Sigma-aldrich) in the presence of a cocktail of proteinase inhibitors (Sigma-aldrich). After the addition of DNase I (50 µg/ml) and RNase (1 µg/ml), the cells were further broken by sonication (duty cycle 50%, out put control 4.5 for 10 min) at temperatures less than 2 °C. The sonication process was repeated three times. Permeabilized cells were then centrifuged at 20,000 ×*g* for 10 min at 4 °C and the pellet was discarded. The supernatant was further purified by ultra-centrifugation at 160,000 ×*g* for 1 h at 4 °C. The pellet obtained (crude membrane extract) was washed three times with Tris–HCl buffer and suspended in 1 ml of Tris–HCl buffer which was then used for both H^+-ATPase and protein assays.

2.4.2. Measurement of H^+-ATPase activity

H^+-ATPase assay was performed as described by Sekine et al. (2001). The reaction mixture consisted of 20 mM bis–tris propane (pH 6.5), 2.5 mM Na_2ATP, 1 mM $MgCl_2$, 10 µg bovine serum albumin (BSA), 50 µl ethanol (final concentration; 8.3% v/v), 50 µl crude enzyme, in a total volume of 600 µl. The reaction mixture without Na_2ATP was incubated at 37 °C for 20 min and then Na_2ATP was added to start the reaction. After 15 min at 37 °C, 300 µl of 0.1 N HCl was added to stop the reaction, and the mixture was immediately cooled on ice. The mixture was centrifuged at 5000 ×*g* at room temperature for 10 min, and the amount of phosphate in the supernatant was determined. Determination of the amount of phosphate was carried out by mixing 0.7 ml of the supernatant with 1.63 ml of coloring agent, followed by incubation at 18 °C for 15 min, and immediate measurement of optical density at 660 nm. The coloring agent was prepared by mixing 1 part of 5 N H_2SO_4, 1 part of 25 g/l ammonium molybdate, 1 part mixture of 10 g/l *p*-(methylamino) phenol sulfate and 30 g/l $NaHSO_3$, and 4 parts water just prior to use. When necessary, *N*,*N′*-dicyclohexylcarbodiimide (DCCD) was added to the reaction mixture. DCCD was included in the 50 µl ethanol portion of the reaction mixture to make a final concentration of 0.2 mM.

2.4.3. Protein assay

The protein concentration of the crude enzyme was determined using DC Protein assay kit (Bio-Rad, California, USA) with BSA as the standard. The crude enzyme (270 µl) was mixed with 15 µl of 10% SDS and 15 µl of 10 N NaOH, and boiled for 10 min. After cooling, the amount of protein contained in the *L. delbrueckii* subsp. *bulgaricus* membrane extracts was determined as per the manufacturer's instructions. The specific activity was defined as nanomol of phosphate formed per min per mg of crude enzyme.

2.5. Preparation of fermented milk and yoghurt

2.5.1. Skim milk preparation

Skim milk powder was reconstituted (12.6% w/v) by dissolving in sterilized and distilled water. Reconstituted skim milk was pasteurized at 90 °C for 10 min.

2.5.2. Preparation of starter cultures

L. delbrueckii subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 and *S. thermophilus* 21702 were cultured as described above for 18 h at 37 °C. Cells were harvested by centrifugation at 5000 ×*g* for 10 min at 4 °C; washed twice in sterile saline solution; suspended in sterilized skim milk and kept at −80 °C. This was used as starter cultures for fermentation. For *B. breve* JCM 1192^T and *B. breve* JCM 7017, −80 °C stock cultures were cultivated in GAM broth at 37 °C for 18 h and inoculated (1% v/v) into fresh GAM broth, cultivated, harvested, washed and suspended in sterilized skim milk as described above. Suspended *B. breve* JCM 1192^T and *B. breve* JCM 7017 cultures in sterilized skim milk were kept overnight at 4 °C.

2.5.3. Inoculation of starters and fermentation

Two methods were used to ferment milk. In the first method, milk was fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* only. Three different batches of pasteurized skim milk coded A1, B1 and C1 were inoculated with ca. 5.0×10^6 CFU/ml of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 respectively. Milk was fermented at 37 °C up to pH 5.0 cooled and stored at 4, 10 and 20 °C for 21 days. The pH of fermented milk was checked after 0, 7, 15 and 21 days of storage.

In the second method, milk was fermented using starter cultures consisting of *S. thermophillus* and *L. delbrueckii* subsp. *bulgaricus*. *B. breve* was added prior to fermentation. Four different batches of yoghurt coded A2, B2, C2 and D2 were made. Batches A2 and B2 were inoculated with *B. breve* JCM 1192^T whereas batches C2 and D2 were inoculated with *B. breve* JCM 7017. Both strains of *B. breve* were added at ca. 8.0×10^8 CFU/ml. *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant) was inoculated to batches A2 and C2 meanwhile *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (parent) was inoculated to batches B2 and D2. *L. delbrueckii*

subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 were inoculated at ca. 5.0 × CFU/ml. *S. thermophillus* 21702 (5.0×10^6 CFU/ml) was inoculated to all yoghurt batches. The inoculated skim milk was incubated at 42 °C until pH of 5.0. After fermentation the yoghurt was immediately cooled, and stored at 10 °C for 21 days. Viable counts of *B. breve* JCM 1192^T and *B. breve* JCM 7017 in yoghurt were determined. The pH was determined using Shindengen ISFET pH meter (model No. KS723).

2.6. Enumeration of B. breve in yoghurt

Samples were serially diluted in sterile PBS (pH, 7.4) and were spread plated on TOS (transoligosaccharide) propionate agar (Yakult Honsha Co. Ltd, Japan) with the following composition (per litre): trypticase medium (BBL) 10 g; yeast extract (Becton, Dickinson Co., Sparks, Md., USA) 1.0 g; KH_2HPO_4 3.0 g; Ammonium sulfate 3.0 g; $MgSO_4\cdot7H_2O$ 0.2 g; l-cysteine·HCl·H_2O 0.5 g; TOS (Yakult Honsha Co. Ltd, Japan) 10 g; Agar (Becton, Dickinson Co., Sparks, Md., USA) 15 g, was used for enumeration of *B. breve*. Inoculated TOS propionate agar plates were incubated anaerobically at 37 °C for 48 h.

2.7. Sensory evaluation

Eighteen panelists were asked to evaluate the sensory attributes of yoghurt. The 9 point hedonic scale was used; a score of 9 means like extremely and a score of 1 means dislike extremely. Yoghurt sensory parameters evaluated were; general taste, acidity, odour and overall impression. To minimize bias all samples were three digit coded. The yoghurt was served to panelists after 7 days of storage at 10 °C in order cater for any residual fermentation that might occur during storage. Yoghurt serving temperature was 8 °C ± 2. Sensory parameters analyzed were general taste, acidity and odour. General taste refers to taste perceptions other than acidity such as bitterness. Acidity was perceived as the degree of sourness with a score of 9 implying less sour and a score of 1 implying very sour.

2.8. Statistical analysis

Each experiment was independently replicated three times in a completely randomized design. All data were analyzed using one way ANOVA by means of statistical computer software (Cohort 6.0, Monterey, California, USA). Least significant difference was determined at $P \leq 0.05$.

3. Results

3.1. Membrane-bound H^+-ATPase activity of mutant strains

Neomycin uptake by bacterial cells is an energy depended process (Kanner and Gutnick, 1971). Therefore mutants with reduced membrane H^+-ATPase activity do not generate sufficient energy to concentrate neomycin and are resistant to neomycin. *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 did not grow in MRS broth containing 25 μg/ml of neomycin whereas *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 were able to grow in MRS broth containing 100 μg/ml of neomycin (data not shown). Furthermore it was suggested that H^+-ATPase plays a role in glucose uptake in *L. delbrueckii* subsp. *bulgaricus* (Savoy de Giori et al., 2002). Glucose uptake of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 was 7.3, 4.5 and 64.6% respectively after 48 h of cultivation in MRS broth (data not shown). These results clearly indicated that the reduction in H^+-ATPase activity did not only result in greatly reduced neomycin uptake but also significantly reduced glucose metabolism. Proton-ATPase activity of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 was 38 and 68% respectively less than that of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (Fig. 1). The H^+-ATPase activities of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 were significantly ($P \leq 0.05$) but not completely inhibited by DCCD, a known H^+-ATPase inhibitor. The inhibition of H^+-ATPase activity by DCCD confirms that H^+-ATPase is the major form of ATPase in the membranes of *L. delbrueckii* subsp. *bulgaricus*. Therefore the determined ATPase activity corresponds to the H^+-ATPase activity but not other types of ATPases. Furthermore these results established that the selected neomycin-resistant mutants (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8) had reduced membrane-bound H^+-ATPase activity.

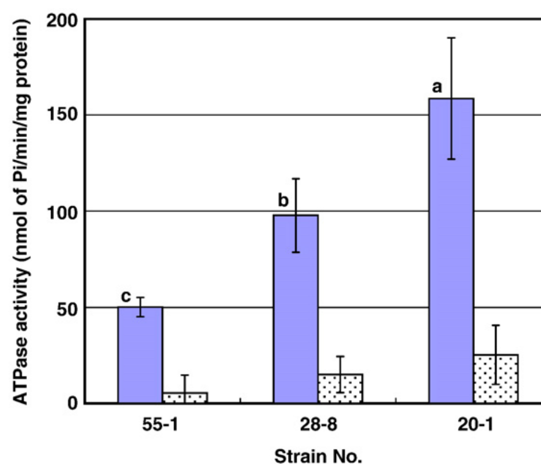

Fig. 1. Membrane-bound H^+-ATPase activity of *L. delbrueckii* subsp. *bulgaricus* SBT0164 strains in reaction mixtures without DCCD (grey bars) and in reaction mixtures containing 0.2 mM DCCD (dotted bars). Reaction mixture contained 20 mM bis–tris propane (pH 6.5), 2.5 mM Na_2ATP, 1 mM $MgCl_2$, 10 μg bovine serum albumin, 50 μl ethanol (final concentration; 8.3% v/v), and 50 μl crude enzyme in a total volume of 600 μl. The enzyme reaction was performed for 15 min at 37 °C. The ATPase activity is expressed as the amount of inorganic phosphate (Pi) released per minute per milligram protein. The values are means of three independent determinations.

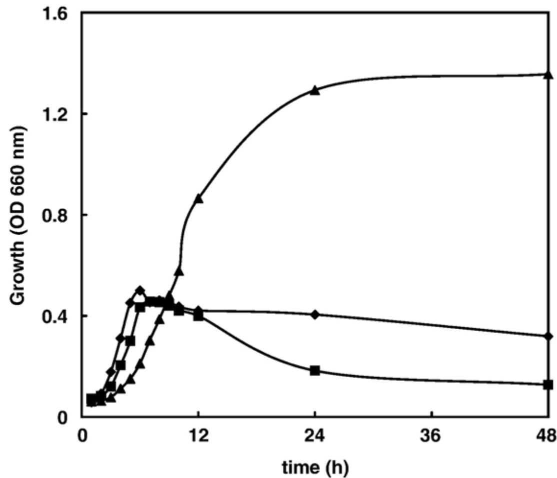

Fig. 2. Growth of *L. delbrueckii* subsp. *bulgaricus* SBT0164 strains; mutant No. 55-1 (■), mutant No. 28-8 (◆) and parent No. 20-1 (▲) in MRS broth when cultured at 37 °C under anaerobic conditions consisting of N_2, $C0_2$ and H_2 in the ratio of 8:1:1 respectively. Growth was determined after every 1 h for 10 h and then after 12, 24 and 48 h of cultivation. The values are means of three independent determinations.

3.2. Acid sensitivity of L. delbrueckii subsp. bulgaricus mutants with reduced membrane-bound H^+-ATPase activity

Growth and acid production characteristics of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No 28-8 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 were compared at 37 °C in MRS broth (Fig. 2). *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 exhibited a lower growth profile than the parent strain (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1). The cell density (OD 660 nm) of the parent strain culture was about 3 fold higher than the mutant strains and the of pH parent strain culture reached 3.7 but the pH of mutant strain cultures was 5.2 after 48 cultivation. (Fig. 3). When the initial pH of MRS broth was adjusted to 4.5, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 (mutant strains) did not decrease the pH of the cultures but *L. delbrueckii* subsp. *bulgaricus* SBT0164 20-1 (parent) produced more acid up to pH 3.7 after 48 h of cultivation at 37 °C (data not shown). For *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and, *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 the critical pH limiting growth and acid production was around pH 5.2. Reduction in acid production and growth under low pH conditions indicates that mutants with reduced membrane-bound H^+-ATPase activity (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8) are more acid sensitive that parent strain (*L. delbrueckii* subsp. *bulgaricus* SBT0164 20-1) and this characteristic could be of importance in minimizing post-acidification during yoghurt storage.

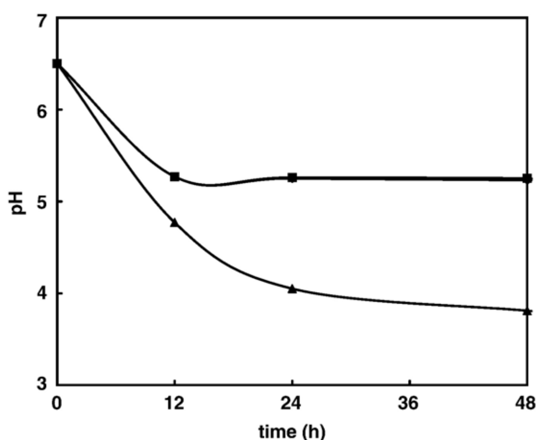

Fig. 3. Decrease in pH of cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (■), *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 (◆), and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (▲) when cultured at 37 °C under anaerobic conditions consisting of N_2, $C0_2$ and H_2 in the ratio of 8:1:1 respectively. The pH of the medium was checked after 12, 24, and 48 h of cultivation. *Lactobacillus delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant) and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 (mutant) lowered the pH of the cultures exactly in the same manner. The values are means of three independent determinations.

3.3. Post-acidification of fermented milk during storage

Post-acidification of fermented milk was assessed during 21 days storage at 4, 10, and 20 °C (Fig. 4A–C). *L. delbrueckii* subsp. *bulgaricus* No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* No. 28-8 did not greatly reduce the pH of fermented milk during storage at 4 and 10 °C. These results signify that mutants of *L. delbrueckii* subsp. *bulgaricus* with reduced H^+ATPase activity have reduced post-acidification rates compared to the parental strains.

In the second experiment milk was fermented using both *L. delbrueckii* subsp. *bulgaricus* and S. *thermophillus* as described in the materials and methods section. *L. delbrueckii* subsp. *bulgaricus* No. 28-8 was not used because it showed a relatively high rate of post-acidification at 10 °C storage. Fermentation time for yoghurts A2, C2 and D2 was 5.5 h and for yoghurt B2 was 4 h. There was a significant ($P \leq 0.05$) difference between final pH of yoghurts fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (H^+-ATPase-defective mutant) and yoghurts fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (parent) after 21 days of storage at 10 °C (Table 1). These results further supported the notion that yoghurt fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (H^+-ATPase-defective mutant) has greatly reduced post-acidification rates during fermentation and storage. Even if the yoghurt culture contains; *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilllus* and *B. breve*, post-acidification seems to be mainly attributed to *L. delbrueckii* subsp. *bulgaricus.*

3.4. Viability of B. breve JCM 1192^T and B. breve JCM 7017 in yoghurt during storage

Changes in viability of *B. breve* JCM 1192 and *B. breve* JCM 7017 was monitored during storage at 10 °C for 21 days.

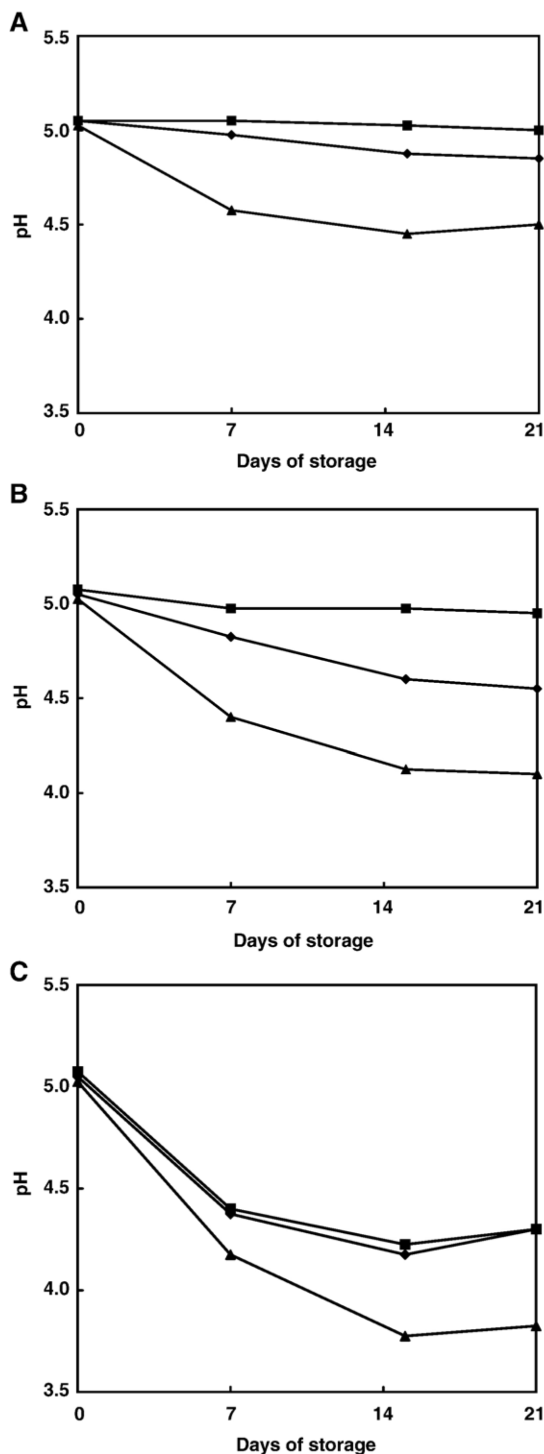

Fig. 4. Decrease in pH of three different batches of fermented milk coded A1 (■), B1 (◆), and C1 (▲) during storage at 4 °C (A), 10 °C (B) and 20 °C (C) for 21 days. Batches A1, B1 and C1 were fermented with *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant), *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 (mutant) and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (parent) respectively. Each batch of milk was fermented at 37 °C up to pH 5.0 and cooled before storage. The pH of fermented milk was checked after 0, 7, 15 and 21 days of storage. The values are means of three independent determinations.

Table 1
Changes in pH of yoghurt during storage at 10 °C

Time (days)	Yoghurt codes[a]			
	A2	B2	C2	D2
1	4.9±0.2a	4.9±0.1a	5.0±0.1a	4.9±0.1a
7	4.8±0.2ab	4.3±0.2b	4.9±0.0ab	4.5±0.0b
15	4.7±0.1b	4.2±0.1b	4.9±0.1b	4.3±0.0b
21	4.7±0.2b	4.1±0.1b	4.8±0.0b	4.1±0.0c

Mean (±standard deviation) of results from three separate experiments. Means with different lowercase letters were significantly different ($P<0.05$). Comparisons were made only within the same column.

[a]Yoghurt batches coded A2 and B2 were inoculated with *B. breve* JCM1192^T whereas batches C2 and D2 were inoculated with *B. breve* JCM 7017. Both strains of *B. breve* were added at ca. 8.0×10^8 CFU/ml. *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant) was inoculated to batches A2 and C2 meanwhile *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 was inoculated to batches B2 and D2. Both *L. delbrueckii* subsp. *bulgaricus* strains were inoculated at ca. 5×10^6 CFU/ml. *S. thermophillus* 21702 was inoculated to all yoghurt batches at ca. 5×10^6 CFU/ml.

There was a slight increase (0.2 to 0.4 log CFU/ml) in viable counts of *B. breve* JCM 1192^T during fermentation (Table 2). Viable counts of both *B. breve* JCM 1192^T and *B. breve* JCM 7017 in yoghurt fermented using H^+-ATPase-defective mutant (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1) did not decrease below 10^8 CFU/ml after 21 days of storage at 10 °C. Reduction in viable counts of yoghurts fermented using H^+-ATPase-defective mutant (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1) and parent (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1) after 21 days of storage at 10 °C ranged from 9.0 to 4.8% and 16.7 to 33.7%, respectively. The results demonstrate that it was possible to maintain viability of both *B. breve* JCM 1192^T and *B. breve* JCM 7017 at a level greater than

Table 2
Viability of *B. breve* JCM 1192^T and *B. breve* JCM 7017 in yoghurt (log CFU/ml)

Time[a]	Yoghurt codes[b]			
	A2	B2	C2	D2
0 h	8.5±0.2abc	8.2±0.1a	8.4±0.4a	8.4±0.4a
4–5.5 h	8.9±0.3a	8.6±0.3a	8.4±0.3a	8.4±0.0a
7 d	8.6±0.3ab	6.8±0.8b	8.2±0.6a	8.1±0.2b
15 d	8.4±0.4bc	6.6±0.1b	8.1±0.1a	7.2±0.1b
21 d	8.1±0.6c	5.7±1.5b	8.0±0.0a	7.3±0.1b
% decrease	9.0	33.7	4.8	16.7

Mean (±standard deviation) of results from three separate experiments. Means with different lowercase letters were significantly different ($P<0.05$). Comparisons were made only within the same column.

[a]0 and 4–5.5 h refer to *B. breve* viability before and after fermentation respectively; 7 d, 15 d, and 21 d refer to *B. breve* viability after 7, 15 and 21 days of storage at 10 °C.

[b]Yoghurt batches coded A2 and B2 were inoculated with *B. breve* JCM1192^T whereas batches C2 and D2 were inoculated with *B. breve* JCM 7017. Both strains of *B. breve* were added at ca. 8.0×10^8 CFU/ml. *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 was inoculated to batches A2 and C2 meanwhile *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 was inoculated to batches B2 and D2. Both *L. delbrueckii* subsp. *bulgaricus* strains were inoculated ca. 5×10^6 CFU/ml. *S. thermophilus* 21702 was inoculated to all yoghurt batches at ca. 5×10^6 CFU/ml.

Table 3
Sensory analysis scores

Yoghurt code[a]	General taste	Acidity	Odour	Overall impression
A2	5.4a	5.7a	5.5a	5.4a
B2	4.8a	4.5ab	5.4a	4.5a
C2	4.9a	4.3ab	6.0a	4.9a
D2	4.3a	4.0b	5.6a	4.1a

Mean (±standard deviation) of results from three separate experiments.
Means with lowercase letters were significantly different ($P<0.05$). Comparisons were made only with the same column.
[a]Yoghurt batches coded A2 and C2 were fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 whereas yoghurt batches B2 and D2 were fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* No. 20-1. Yoghurt batches coded A2 and B2 contained *B. breve* JCM1192^T meanwhile batches C2 and D2 contained *B. breve* JCM 7017. *S. thermophilus 21702* was added to all yoghurt batches.

10^8 CFU/ml in yoghurt fermented using mutants with reduced H^+-ATPase activity (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1) No. 55-1 during 21 days of storage at 10 °C.

3.5. Sensory analysis

Sensory analysis was conducted to determine whether yoghurt fermented by the H^+-ATPase-defective mutant was acceptable to consumers. H^+-ATPase-defective mutants tend to have reduced acid production which can affect yoghurt taste. Yoghurt coded A2 that contained a mutant with reduced H^+-ATPase activity (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1) was the most preferred by panelists. On the contrary yoghurt coded D2 that contained the parent strain (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1) was the least preferred (Table 3). Odour was the most highly ranked sensory attribute indicating that addition of the *B. breve* strains together with the starter cultures did not result in production of foul yoghurt smell. There were no significant differences ($P \leq 0.05$) in all attributes tested except acidity. Sensory score for acidity of yoghurt coded A2 was significantly ($P \leq 0.05$) high. Demonstrating that yoghurt fermented using the H^+-ATPase-defective mutant had a less acidic taste which is preferred by Japanese consumers.

4. Discussions

In this study a mutant of *L. delbrueckii* subsp. *bulgaricus* with reduced H^+-ATPase activity was successfully isolated and applied in yoghurt production. Previously it was demonstrated that H^+-ATPase mutants could be selected as neomycin-resistant mutants in case of Gram-positive bacterium such as *L. lactis* subsp. *lactis* (Yokota et al., 1995). Our results showed that growth and acid production of mutants with reduced H^+-ATPase activity (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* No. 28-8) stopped at pH below 5.2 when cultured in MRS broth. Koebmann et al. (2000) demonstrated that H^+-ATPase is essential for growth of *L. lactis*, presumably because it is essential for maintaining the proton gradient necessary for solute transport and maintenance of cytoplasmic pH at an acceptable level. Furthermore, Savoy de Giori et al. (2002), indicated that in *L. delbrueckii* subsp. *bulgaricus*, all forms of glucose and also glutamate and aspartate are transported by an ATP-dependent mechanism. It was also found out that glutamate uptake was totally inhibited and glucose uptake was partially inhibited by DCCD and the involvement of membrane-bound H^+-ATPase in coupling glycolysis to the transport activity was suggested (Savoy de Giori et al., 2002). It is possible that reduction in the H^+-ATPase activity of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 and *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 28-8 might have attributed to reduced growth and solute transport under low pH conditions. Reduction in growth and metabolism under low pH conditions can therefore be of importance in prevention of yoghurt post-acidification during refrigerated storage.

L. delbrueckii subsp. *bulgaricus* SBT0164 No. 28-8 was not used in yoghurt fermentation because it had a higher H^+-ATPase activity and post-acidification rates relative to *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1. Nevertheless our results showed that pH of yoghurt (coded A2 and C2) fermented using a mutant with reduced H^+-ATPase activity (*L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1) slightly decreased during 21 days storage at 10 °C. This indicates that fermenting yoghurt with *L. delbrueckii* subsp. *bulgaricus* strains with reduced H^+-ATPase activity results to greatly reduced post-acidification during refrigerated storage. In this study *B. breve* JCM 1192^T and *B. breve* JCM 7017 was added to yoghurt prior to fermentation at a level ranging from 8.2 to 8.5 log CFU/ml to ensure that at the end of fermentation and throughout the designated shelf life the viability of *B. breve* was maintained higher than 10^7 CFU/ml. Further more, Kailasapathy and Chin (2000), hinted that the cell count at the end of incubation must be sufficiently high to allow up to 90% mortality of probiotic bacteria during storage and yet still leave their numbers above the desired minimum level. In case of *B. breve* JCM 1192^T there was 0.4 log CFU/ml increase in viability at the end of fermentation. A similar increase in viability (0.2–0.3 log cycles) during yoghurt manufacture has been reported (Picot and Lacroix, 2004). Viability of *B. breve* in yoghurt was conducted at 10 °C for 21 days to mimic adverse storage conditions. Dave and Shah (1997), reported that under the commercial practices, the temperature of 3–4 °C may not represent the actual temperature of storage of yoghurt in the supermarkets; on occasions, the temperature may rise to 10 °C. Additionally they stated that the temperature of storage adversely affected the viability of bifidobacteria due to the slightly lower pH and high acidity in yoghurts stored at 10 °C. In this study, *B. breve* JCM 1192^T and *B. breve* JCM 7017 viable counts could be maintained above 8 log CFU/ml throughout the designated shelf life in yoghurt fermented using starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1. The loss in viability ranged from 0.4 to 0.8 log CFU/ml in yoghurt fermented using starters cultures of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 (mutant).Whereas in yoghurt fermented using starter cultures of *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 20-1 (parent) the loss in viability ranged from 1.1 to 2.9 log CFU/ml. Vinderola et al. (2000), reported viability loss of *B. bifidum* ranging from 1.6 to 4.0 log CFU/ml orders and found that the

loss in cell viability depended on the yoghurt type and the lactic starter used. In addition, Maus and Ingham (2003) hypothesized that the yoghurt fermentation process itself could be considered an acid adaptation treatment (pH drops from 6.5 to 4.7 in about 5 h) and found that the yoghurt environment enhanced the acid tolerance of *Bifidobacteruim lactis*. Similarly, Gilliland et al. (2002) also suggested that stopping fermentation at higher pH could influence the survival of beneficial bacteria during storage. Therefore addition of *B. breve* to yoghurt prior to fermentation, stopping the fermentation at pH 5.0 coupled with the low yoghurt post-acidification rates might have provided a mildly acidic environment that enhanced viability of *B. breve* JCM 1192 and *B.* breve JCM 7017. More so, viable counts of total LAB in yoghurt fermented by mutant was assessed during 21 days of storage at 10 °C and found to range from 7.6 to 8.5 log CFU/ml (data not shown). This is above the minimum level of 7.0 log CFU/ml recommended by Japan fermented milks and lactic acid bacterial beverages association (Anonymous, 2004). However, the counts of *L. delbrueckii* subsp. *bulgaricus* in yoghurt decreased from 2.6×10^8 to 1.5×10^6 CFU/ml (data not shown) during 2 weeks of storage at 4 °C. Lower counts of *L. delbrueckii* subsp. *bulgaricus* have been reported to be advantageous for the viability of probiotic organisms (Dave and Shah, 1997).

Gilliland (1991), reported that the primary function of both *L. delbrueckii* subsp*. bulgaricus* and *S. thermophillus* is to produce the lactic acid required for the production of the coagulum and a combination of lactic acid and volatile components is responsible for the typical flavour and texture of cultured yoghurt. Changes in the yoghurt manufacturing process such as curtailing the fermentation at pH above 5.0, can ensure viability but can result in a product without the characteristic aroma (Adhikari et al., 2000). In addition, a pleasant taste and attractive texture are essential for all dairy products, regardless of the “health message” of the product (Saxelin et al., 1999). Our results showed that there were no significant ($P \leq 0.05$) differences in the sensory attributes evaluated apart from acidity and further revealed that yoghurt fermented with starter cultures containing *L. delbrueckii* subsp. *bulgaricus* SBT0164 No. 55-1 had a less sour taste that was favourable to Japanese consumers. Besides, both *B. breve* JCM 1192^T and *B. breve* JCM 7017 did not produce foul smell or any other undesirable sensory attributes when added to yoghurt at the beginning of fermentation.

Before a probiotic can benefit human health, it must fulfill several criteria. It must have good technological properties so that it can be manufactured and incorporated in food products without loosing viability and functionality or creating unpleasant flavours (Mattila-Sandolm et al., 2002). Our results show that yoghurt fermented with starter cultures containing *L. delbrueckii* subsp. *bulgaricus* strain with reduced membrane-bound H^+-ATPase activity had greatly reduced post-acidification and could enhance viability of *B. breve* during refrigerated storage. Consumer preference tests indicate that yoghurt fermented using the mutant strain had the desired sour taste and is acceptable for consumption. Currently there is no data describing the H^+-ATPase activity of the various *L. delbrueckii* subsp. *bulgaricus* strains, particularly under low pH conditions. There is a possibility that strains of *L. delbrueckii* subsp. *bulgaricus* strains with reduced H^+-ATPase activity exist naturally. Application of *L. delbrueckii* subsp. *bulgaricus* strains with reduced H^+-ATPase activity in yoghurt fermentation could then be one of the avenues of minimizing yoghurt post-acidification during refrigerated storage.

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