Viability of *Lactobacillus gasseri* and Its Cholesterol-Binding and Antimutagenic Activities During Subsequent Refrigerated Storage in Nonfermented Milk

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ABSTRACT

The effect storage at 4°C on the viability of *Lactobacillus gasseri* and its sodium taurocholate-deconjugating and cholesterol-binding abilities as well as desmutagenic activity was investigated. Unfermented milks containing *L. gasseri* strains SBT0274 and SBT0270 at 10^9 cfu/ml were prepared using 10% skim milk. Total and bile-tolerant lactobacilli for strains SBT0274 and SBT0270 generally decreased after 14 d of storage at 4°C; however, viable cells of these strains were still at 10^8 cfu/ml after 28 d of storage. The amounts of cholic acid released and of cholesterol bound by strains SBT0274 and SBT0270 declined over time, especially at 21 d of storage. Antimutagenic activity of unfermented milk made from both strains was attributed to the bacterial cells, and the activity was stable during storage at 4°C for 28 d.

(**Key words:** viability, sodium taurocholate deconjugation, cholesterol-binding ability, antimutagenicity)

Abbreviation key: Trp-P1 = 3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole.

INTRODUCTION

The *Lactobacillus acidophilus* group of lactic acid bacteria is widely used in fermented milks such as yogurt (1, 5, 6, 22) and sweet acidophilus milk (27, 28) as a probiotic because of its presumed beneficial effect on the health of the host (29, 35). One of the important beneficial health effects attributed to *L. acidophilus* is its ability to reduce serum cholesterol (5, 13, 14, 32). There are two proposed mechanisms for the hypocholesterolemic action of *L. acidophilus*. The first mechanism is by binding dietary cholesterol to the bacterial cells in the small intestine before cholesterol can be absorbed into the body (7, 19), and the second is by deconjugation of bile acids (3, 9, 12, 46).

Lactobacillus acidophilus and its fermented dairy products have been shown to have antimutagenic or anticarcinogenic properties or both. Oral administration of *L. acidophilus* cells suppresses the multiplication of Ehrlich ascites carcinoma by 20 to 40%; fermented milk with *L. acidophilus* shows 16 to 40% suppression (38). More recently, we reported (44) antimutagenicity of milk cultured with *L. acidophilus* strains against mutagenicity induced by heated tauco, a traditional fermented soybean in Indonesia.

Unfermented acidophilus milk, produced by adding *L. acidophilus* to cool pasteurized milk, is a milk product that can provide viable and active *L. acidophilus* cells to consumers, especially those who do not prefer the acidic taste of fermented products (27). Many reports (23, 24, 26, 27, 30, 37) have been published on the management of lactose maldigestion in humans by consumption of unfermented acidophilus milk; however, most of the results (26, 30, 37) showed that *L. acidophilus* is not effective in improving lactose digestion.

Few reports exist on the potential effect of milk containing *L. acidophilus* on the reduction of cholesterol. The only finding was reported by Brashears and Gilliland (2) who studied the ability of three strains of *L. acidophilus* to assimilate cholesterol during refrigerated storage. To our knowledge, no evidence is available on the antimutagenic activity of unfermented milk made from *L. acidophilus*. Unfermented milk and fermented milk, such as yogurt, are immediately stored under refrigeration until used (6, 8, 22, 36), suggesting the necessity to study the effect of refrigerated storage on the viability of the cultures. Of the six *L. acidophilus* groups, the B1 group, *Lactobacillus gasseri* of human origin, was used in the present study. We screened the ability of 28 strains of *L. gasseri* to bind cholesterol, deconjugate taurocholate, and grow in the presence of bile; we found that strains SBT0274 and SBT0270 were tolerant to bile and exhibited high cholesterol-binding and taurocholate-deconjugating activities among strains tested (45).

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The objectives of the study were 1) to evaluate the effect of refrigerated storage on the viability, sodium taurocholate deconjugation, and cholesterol-binding abilities of *L. gasseri* strains in milk and 2) to assay the antimutagenic activity of unfermented milk samples made from two strains of *L. gasseri* against the mutagenicity induced by 3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole (**Trp-P1**), one of the heterocyclic amines that usually is formed during cooking and processing of protein-rich foods.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Lactobacillus gasseri (subgroup B1 of the acidophilus group) strains SBT0270 and SBT0274 used in this study were obtained from Snow Brand Milk Products Co. Ltd. (Saitama, Japan). These two cultures were maintained by subculture in MRS broth with 1% inocula and 18 h of incubation at 37°C and were stored at 4°C between transfer. Each culture was subcultured twice in MRS broth prior to experimental use.

Preparation of Freeze-dried Bacterial Cells

Five hundred milliliters of MRS medium was inoculated with 2.5 ml of an active culture of each *L. gasseri* strain and was incubated at 37°C for 18 h. Whole cells were harvested by centrifugation at 2000 × *g* for 20 min and were washed twice with sterile distilled water. Cell suspensions were frozen at –80°C for 4 h and were dried under vacuum for 8 h in a chamber-type freeze-drier (Taitec UD-15, Taitec Co., Tokyo, Japan). Freeze-dried cells were kept in a refrigerator until further use.

Preparation of Unfermented Milk

Freeze-dried cells of *L. gasseri* strains SBT0270 and SBT0274 were resuspended in 10% NDM sterilized at 115°C for 15 min. Unfermented milks of *L. gasseri* were made by addition of this cell concentrate to 10% sterile NDM to titers of about 2×10^9 to 7×10^9 cfu/ml. Cell numbers were confirmed by plating anaerobically onto MRS agar plates with a GasPak hydrogen-carbon dioxide anaerobic system (BBL Becton Dickinson Microbiology System, Cockeysville, MD). Five bottles, each containing 200 ml of the unfermented milk, were stored at 4°C. A bottle was removed at d 0 (the day the unfermented milk was prepared), 7, 14, 21, and 28, and cultures were tested for ability to deconjugate sodium taurocholate and to bind cholesterol. The samples were also evaluated for the numbers of total and bile-tolerant lactobacilli. The unfermented milks at each storage interval were subjected to an antimutagenicity assay against the mutagenicity induced by Trp-P1.

Viability of the Cultures during Storage

Total and bile-tolerant counts of samples at each time interval were enumerated with MRS agar and MRSO agar (MRS agar supplemented with 0.3% oxgall powder), respectively. At 0, 7, 14, 21 and 28 d following refrigerated storage at 4°C, 1 ml of unfermented milk sample was diluted with 9 ml of 66 m*M* PBS, pH 6.8, and was mixed uniformly with a vortex mixer. Subsequent serial dilutions were made, and viable numbers were enumerated by pour-plate method. All plates were incubated anaerobically at 37°C for 48 h in a GasPak hydrogen-carbon dioxide anaerobic system. The experiments were repeated three times with duplicates each time. The means of data from the three trials are presented.

Assay for Acid Tolerance

Washed cell pellets of *L. gasseri* strains SBT0270 and SBT0274 were resuspended in sterile distilled water, and the absorbance (625 nm) was adjusted to 0.7 for each culture. Cell suspensions were inoculated (2%) into each of 10 ml of 2% NDM that had been adjusted to pH 1.5, 2.5, or 3.5 with concentrated HCl. The mixtures were incubated at 37°C for 0, 1, 2, and 3 h. Immediately after incubation, 1 ml of suspended cells was diluted with 9 ml of PBS and was mixed uniformly with a vortex mixer. Subsequent serial dilutions were made and plated by the pour-plate method with MRS agar. The plates were incubated at 37°C for 48 h under anaerobic conditions using a GasPak hydrogen-carbon dioxide anaerobic system before enumeration. The experiments were repeated three times with duplicates each time. The means of data from three trials were presented.

Assay for Deconjugation of Sodium Taurocholate

Deconjugation of sodium taurocholate was assayed in MRS-THIO broth supplemented with 0.2% sodium taurocholate. The broth, inoculated with 1% unfermented milk, was incubated at 37°C for 24 h. Analysis for free cholic acid released during deconjugation was carried out according to the method of Walker and Gilliland (46). All experiments were carried out with three replicates, and the results were expressed as micromoles of cholic acid per milliliter.

Assay for Cholesterol Uptake

Cholesterol-phosphatydilcholine micelles prepared according to the method described by Razin et al. (33)

were added to MRS broth containing 2% sodium thioglycholate and 0.3% oxgall (MRSO broth) to attain a concentration of cholesterol of 100 μg/ml. Ten milliliters of MRSO broth was distributed into sterile tubes and then inoculated individually with 100 μl of unfermented milk sample. After incubation at 37°C for 20 h, cells were removed by centrifugation at 12,000 × *g* for 10 min at 4°C. The spent broth was collected and analyzed for cholesterol with the method described by Rudel and Morris (34). Control broth that was not inoculated was assayed using the same procedure. Cholesterol-binding ability was estimated by the following formula:

$$A = 100 - [(B/C) \times 100]$$

where A = binding of cholesterol (%), B = cholesterol (μg) in the spent broth inoculated with unfermented milks containing *L. gasseri* strains, and C = cholesterol (μg) in the spent broth without inoculation.

Assay for Antimutagenic Activity

Salmonella typhimurium SD 510, a strain derived from *S. typhimurium* TA 98 (17) was used for an antimutagenicity assay. The tester strain SD 510 was grown in Oxoid nutrient broth number 2 (Unipath, Basington, United Kingdom) fortified with streptomycin at a final concentration of 20 mg/ml (SM20 broth). The culture grew overnight to an optical density of 1.3 at 660 nm (5×10^8 cfu/ml) in a shaking water bath at 37°C. The antimutagenicity assay was performed according to the plate incorporation methods of Maron and Ames (25) and Hosono et al. (16). Briefly, 30 μl of Trp-P1 (Wako Chemical Industries, Ltd., Osaka, Japan) solution (2 mg/ml in sterile distilled water) was mixed with 100 μl of unfermented milks or viable or dead cells (0.1, 0.5, 1.0, or 2.0 mg/100 μl of sterile distilled water) plus 300 μl of PBS and 100 μl of the tester culture (SD 510 strain) diluted 10^{-4} with PBS. After preincubation at 37°C for 30 min, 2 ml of soft agar (nutrient broth with 0.5% agar) maintained at 45°C was added, mixed by gently vortexing, and poured onto an Oxoid plate (nutrient broth with 1.5% agar). All plates were incubated at 37°C for 48 h, and the number of streptomycin-independent revertants was scored. The positive control contained test culture, distilled water instead of unfermented milk, and an aqueous solution of Trp-P1. The negative control consisted of test culture, unfermented milks of strains SBT0270 or SBT0274, and only sterile distilled water instead of Trp-P1. The antimutagenicity of each unfermented milk was estimated by measuring the decrease in mutations induced by Trp-P1 and was expressed as percentage inhibition, calculated by the formula

$$\text{inhibition (\%)} = 100 - \frac{\text{revertants in test sample plate}}{\text{revertants in control plate}} \times 100.$$

The experiments were repeated three times in triplicate each time. Means of the three trials are presented.

Statistical Analysis

Viability, sodium taurocholate deconjugation, cholesterol-binding ability, and antimutagenic activity were analyzed by the one-way ANOVA of SPSS (31). The least significant difference (Bonferroni *t*-test) was used to determine whether statistically significant differences occurred among means.

RESULTS

Total colony-forming units of *L. gasseri* strains SBT0274 and SBT0270 in milk samples decreased significantly ($P < 0.05$) during storage at 4°C. Total SBT0274 remained steady between 14 and 28 d; however, total counts of SBT0270 continued to decline through d 28 (Table 1). Bile-tolerant counts of SBT0274 in milk samples were stable during 7 d of storage at 4°C and decreased significantly after this period. The bile-tolerant lactobacilli in unfermented milk made from SBT0270 decreased continuously through 28 d of storage at 4°C. Acid tolerance of two *L. gasseri* strains is shown in Figure 1. At pH 2.5, the viability of strains SBT0274 and SBT0270 decreased by 1 to 2 log cycles after 1, 2, and 3 h. After incubation at pH 1.5 for 1 and 2 h, the viable counts decreased by about 4 to 5 log cycles. Incubation at pH 1.5 for 3 h reduced the viable counts of SBT0274 by about 5 cycles, whereas SBT0270 could not survive at the same conditions.

TABLE 1. Change in total counts and bile-tolerance of *Lactobacillus gasseri* in unfermented milk stored at 4°C.

	L. gasseri SBT0274		*L. gasseri* SBT0270	
Period	TC[1]	BTC[2]	TC	BTC
	(log 10 cfu/ml)			
0 d	$9.48^{a,x}$	$9.29^{a,x}$	$9.42^{a,x}$	$9.44^{a,x}$
7 d	$9.25^{ab,x}$	$9.26^{a,x}$	$9.30^{a,x}$	$8.87^{b,y}$
14 d	$9.04^{b,x}$	$8.80^{b,x}$	$8.91^{b,x}$	$8.89^{b,x}$
21 d	$9.19^{b,x}$	$8.57^{bc,y}$	$8.58^{c,x}$	$8.65^{bc,x}$
28 d	$8.94^{b,x}$	$8.50^{c,y}$	$8.55^{c,x}$	$8.30^{c,x}$

[a,b,c]Means in the same column with different superscript letters differ ($P < 0.05$).

[x,y]Means within a row within the same strain with different superscript letters differ ($P < 0.05$).

[1]Total counts (TC) were enumerated by using MRS agar. All values are the mean of three trials.

[2]Bile-tolerant counts (BTC) were enumerated by using MRS agar containing 0.3% oxgall. All values are the mean of three trials.

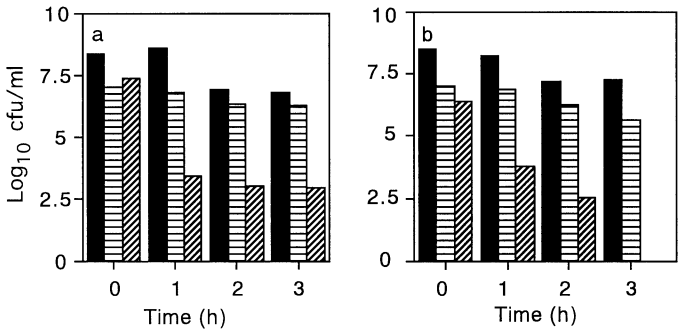

Figure 1. Acid tolerance of *Lactobacillus gasseri* strains SBT0274 (a) and SBT0270 (b) at pH 3.5 (solid), 2.5 (horizontal stripe), and 1.5 (diagonal stripe).

The sodium taurocholate-deconjugating ability of *L. gasseri* strains stored at 4°C in unfermented milk is shown in Table 2. A significant decrease of cholic acid release was observed by strain SBT0274 only after 28 d. Deconjugation of sodium taurocholate by SBT0270 significantly decreased after 14 d and continued to decline until the end of the 28-d storage period. The ability of the two strains stored at 4°C in milk to bind cholesterol decreased as storage period increased (Table 3). The amount of cholesterol bound by both strains significantly decreased after 21 d of storage at 4°C. Especially after this storage period, the cholesterol-binding activity of SBT0270 was significantly less than that of SBT0274.

We evaluated antimutagenic activity against Trp-P1 of unfermented milk samples containing strains SBT0274 and SBT0270 (Table 4). No significant decrease in the antimutagenicity of either unfermented milk sample was observed during storage at 4°C for 28 d. Antimutagenic activity was mainly attributed to cells of *L. gasseri* that were present in milk samples, and antimutagenicity increased as number of cells increased (Figure 2). Antimutagenic activity was not significantly affected by the number of viable cells in milk but depended on the total cells in sample regardless of whether the cells were alive or dead (Figure 3).

TABLE 2. Deconjugation of sodium taurocholate by *Lactobacillus gasseri* in unfermented milk stored at 4°C.[1]

Period	Cholic acid released	
	L. gasseri SBT0274	*L. gasseri* SBT0270
	(μmol/ml)	
0 d	1.47^{a,x}	1.41^{a,x}
7 d	1.35^{ab,x}	1.21^{ab,x}
14 d	1.17^{ab,x}	1.17^{ab,x}
21 d	1.39^{ab,x}	1.03^{b,x}
28 d	1.05^{b,x}	0.28^{c,y}

^{a,b,c}Means in the same column with different superscript letters differ ($P < 0.05$).

^{x,y}Means in the same row with different superscript letters differ ($P < 0.05$).

[1]Cultures were grown for 24 h in MRS-THIO broth containing 0.2% sodium taurocholate. All values are the mean of three trials.

TABLE 3. Binding of cholesterol by *Lactobacillus gasseri* in unfermented milk stored at 4°C.[1]

Period	Binding of cholesterol	
	L. gasseri SBT0274	*L. gasseri* SBT0270
	(%)	
0 d	46.95^{a,x}	35.89^{a,x}
7 d	44.87^{a,x}	34.95^{ab,x}
14 d	37.00^{ab,x}	30.19^{ab,x}
21 d	35.03^{bc,x}	17.18^{bc,y}
28 d	25.99^{c,x}	9.34^{c,y}

^{a,b,c}Means in the same column with different superscript letters differ ($P < 0.05$).

^{x,y}Means in the same row with different superscript letters differ ($P < 0.05$).

[1]Cultures were grown in MRS-THIO broth containing 0.3% oxgall and 100 μg of cholesterol-phosphatidylcholine/ml for 20 h. All values are the mean of three trials.

DISCUSSION

Unfermented milk was prepared with cells of *L. acidophilus* strains harvested from stationary growth phase, because the cells tend to survive better than those harvested from the late log phase during storage at refrigerated or frozen temperature (2). It has been suggested that a slimy and viscous material present on cells of some lactic acid bacteria is responsible for the better survival of the cells harvested in this phase. The stability of *L. acidophilus* during subsequent refrigerated storage was strain-dependent. Brashears and Gilliland (2) reported that among three strains of *L. acidophilus*, strain 223 is relatively stable during 28 d of storage at 7°C, but strain 606 is slightly affected, and

TABLE 4. Desmutagenicity[1] of unfermented milk containing *Lactobacillus gasseri* stored at 4°C against the mutagenicity induced by 3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole.

Period	Inhibition	
	L. gasseri SBT0274	*L. gasseri* SBT0274
	(%)	
0 d	19.48	14.39
7 d	15.95	11.46
14 d	16.70	12.63
21 d	19.06	11.92
28 d	18.05	12.99

[1]Desmutagenicity assay was performed by using *Salmonella typhimurium* SD 510 as a test culture. All values are the mean of three trials.

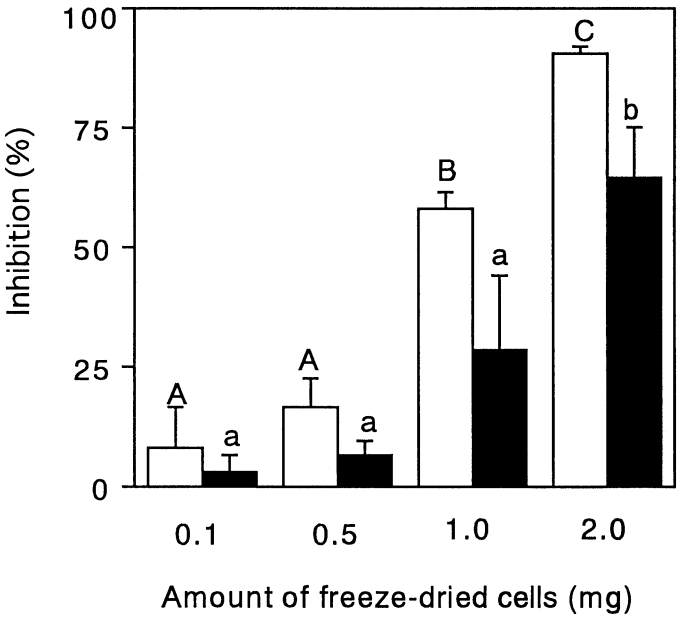

Figure 2. Dose response of antimutagenicity of *Lactobacillus gasseri* strains SBT0274 (□) and SBT0270 (■) against the mutagenicity induced by 3-amino-1,4-dimethyl-5*H*-pyrido [4,3-*b*]indole. Values with different letters are significantly different ($P < 0.05$).

strain 107 exhibits much greater loss in viability during storage. In the present study, we also observed that storage at 4°C for 28 d affected the viability of strains SBT0274 and SBT0270. However, the number of *L. gasseri* in milk samples remained at 10^8 cfu/ml after 28 d of storage. To become implanted in the intestinal tract, suggested daily consumption is 10^8 to 10^9 viable cells of *L. acidophilus* (39). Also bacteria will be in contact with pH ranging from 2.0 to 8.0 in the stomach (15). Thus, probiotic cultures must survive in an environment with gastric acid when viable cells go through the gastrointestinal tract. Survival at pH 3 for 2 h and growing in medium containing 1000 ppm of bile acids are considered standards for acid- and bile-tolerance of probiotic cultures (11, 21). Results from the present study showed that strains SBT0274 and SBT0270 were bile and acid tolerant; thus, they may survive the high acidity in the stomach and high concentrations of bile compounds in the intestine when consumed.

One of the beneficial health effects attributed to *L. acidophilus* as a probiotic is its ability to reduce serum cholesterol, possibly through binding of the dietary cholesterol by the bacterial cells in the small intestine or deconjugation of bile salts (3, 5, 9, 12, 13, 14, 19, 32, 45, 46). Deconjugation of bile acids may help reduce serum cholesterol in humans because deconjugated bile acids are excreted more rapidly than are conjugated forms (4). Sodium taurocholate is a major bile salt in humans and carnivores (43); therefore, it was used to study the bile salt-deconjugating abilities of strains SBT0274 and SBT0270. The amounts of cholic acid that were released by these strains decreased with increased storage. The decline of sodium taurocholate deconjugation was probably due to declines in viability of the cells. We reported earlier (45) that maximal deconjugation of sodium taurocholate by SBT0274 and SBT0270 was reached after 12 h of incubation at the early stationary growth phase. In that experiment, freshly prepared cultures were inoculated into the MRS-THIO broth. However, in the present study, the unfermented milk samples that were prepared by addition of freeze-dried cells were inoculated into the same medium. We found that incubation for 12 h resulted in much less cholic acid released by the two strains (data not shown), and maximal deconjugation was achieved only after incubation for 24 h. This finding might have been due to the longer lag phase needed by the cultures to grow as the result of prior freeze-dry or refrigerated storage treatments. The same phenomenon was observed in the amounts of cholesterol bound by strains SBT0274 and SBT0270. The cholesterol-binding ability of these two strains decreased with increased storage and was related to the number of viable cells. Brashears and Gilliland (2) suggested that the decrease in viable cells during storage would result in apparent reduced binding of cholesterol because a fixed incubation time was applied in the assays. They also suggested that the cells might not

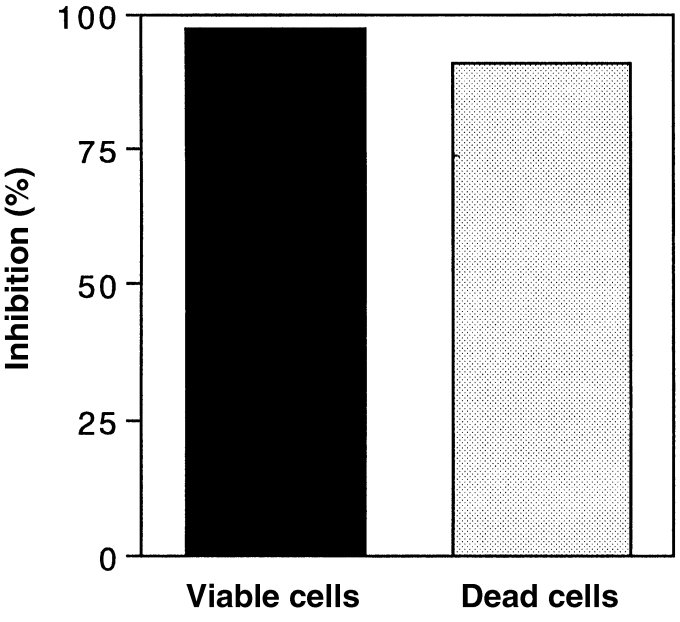

Figure 3. Antimutagenicity of viable and dead cells of *Lactobacillus gasseri* SBT0274 against the mutagenicity induced by 3-amino-1,4-dimethyl- 5*H*-pyrido [4,3-*b*]indole. Two milligrams per plate of viable or dead cells (autoclaved at 121°C for 5 min) was used for the assay.

grow as well in the broth used in th assays as in the medium used to enumerate viability of the cells.

Lactic acid bacteria and their fermented dairy products have been shown to produce antimutagenic or anticarcinogenic activities or both. Antimutagenic properties of lactic acid bacteria and their fermented milk products against some heterocyclic amines, N-nitroso compounds, fecal mutagens from various mammals, and mutagenic heated foods have been well studied by Hosono and coworkers (16, 17, 18, 20, 41, 42). The result from the present study also showed antimutagenic activity of unfermented milk samples made from strains SBT0274 and SBT0270 against the mutagenicity induced by Trp-P1. Antimutagenic activity of milk samples was stable during storage at 4°C for 28 d and was attributed to the bacterial cells. Also the cells exhibited a dose-dependent inhibition of the mutagenicity of Trp-P1. These results are consistent with work reported earlier (40, 44). Unfermented milk was prepared and distributed into several bottles with the same volume. Thus, each bottle contained relatively the same amount of cells, viable and dead, at each period of storage. These might cause stability of antimutagenic activity of unfermented milk samples during subsequent storage at 4°C for 28 d.

In summary, viability of strains SBT0274 and SBT0270 decreased as storage time increased; however, viable cells remained at 10^8 cfu/ml, the number required to perform their beneficial health activities. Sodium taurocholate-deconjugating and cholesterol-binding abilities of these two strains declined as the viability declined, but their antimutagenic activities remained stable even until 28 d of storage at 4°C. The present study suggests that consumption of unfermented milk made from these *L. gasseri* strains will be useful in reducing the human serum cholesterol level and protecting from the occurrence of mutagenesis of cells caused by food-borne mutagens like Trp-P1. To generalize our conclusion, in vivo animal model experiments are necessary.

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