

Probiotic Characterization of Acid- and Bile-tolerant *Lactobacillus salivarius* subsp. *salivarius* from Korean Faeces

H. C. Bae, M. S. Nam* and J. Y. Lee¹

Division of Animal Science and Resources, Chungnam National University, Daejeon 305-764, Korea

ABSTRACT : This study was conducted to investigate *Lactobacillus salivarius* subsp. *salivarius* having probiotic properties to be used as the health adjuncts with fermented milk products. Acid- and bile-tolerant *Lactobacillus salivarius* subsp. *salivarius* was isolated with lactobacilli MRS broth from faeces of 80 healthy persons (infants, children and adults). It was used as a probiotic strain in fermented milk products. The pH of fermented milk decreased from pH 6.7 to 5.0 and titratable acidity increased from 0.3% to 1.0% by *L. salivarius* subsp. *salivarius* (isolation strain 20, 35, and 37), when incubated for 36 h at 37°C. The number of viable cell counts of fermented milk was maximized at this incubation condition. The SDS-PAGE evidenced no significant change of casein but distinct changes of whey protein were observed by isolated *L. salivarius* subsp. *salivarius* for titratable acidity being incubated by 0.9-1.0% at 37°C. All of the strains produced 83.43 to 131.96 mM of lactic acid and 5.39 to 26.85 mM of isobutyric acid in fermented products. The *in vitro* culture experiment was performed to evaluate ability to reduce cholesterol levels and antimicrobial activity in the growth medium. The selected *L. salivarius* subsp. *salivarius* reduced 23-38% of cholesterol content in lactobacilli MRS broth during bacterial growth for 24 h at 37°C. All of the isolated *L. salivarius* subsp. *salivarius* had an excellent antibacterial activity with 15-25 mm of inhibition zone to *E. coli* KCTC1039, *S. enteritidis* KCCM3313, *S. typhimurium* M-15, and *S. typhimurium* KCCM40253 when its pH had not been adjusted. Also, all of the isolated *L. salivarius* subsp. *salivarius* had partial inhibition zone to *E. coli* KCTC1039, *E. coli* KCTC0115 and *S. enteritidis* KCCM3313 when it had been adjusted to pH 5.7. The selected strains were determined to have resistances of twelve antibiotic. Strains 27 and 35 among the *L. salivarius* subsp. *salivarius* showed the highest resistance to the antibiotics. These results indicated that some of the *L. salivarius* subsp. *salivarius* (strain 27 and 35) are considered as effective probiotic strains with a potential for industrial applications, but the further study is needed to establish their use as probiotics *in vivo*. (*Asian-Aust. J. Anim. Sci.* 2002. Vol 15, No. 12 : 1798-1807)

Key Words : Probiotic, *Lactobacillus*, Fermented Milk

INTRODUCTION

Lactobacilli, the predominant bacteria in human intestinal microflora, are generally considered to benefit human health. Lactobacilli may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improved lactose utilization, anticarcinogenic activity, and stimulation of the immune system (Nagao et al., 2000; Usman and Hosono, 2000; Matar et al., 2001; Gill and Rutherford, 2001; Gupta et al., 2001; Singh and Bhat, 2001; Mukai et al., 2002; Horie et al., 2002). One of the interesting therapeutic functions is anticholesterol activity because high plasma cholesterol is associated with high risk of heart attacks (Gilliland, 1990). Usman and Hosono (2000), in an *in vitro* and *in vivo* study of lactobacilli on cholesterol, concluded to investigate the effects of dietary supplementation with fermented dairy products or lactic acid bacteria-containing dairy products in reducing serum cholesterol. Another important therapeutic function is antimicrobial activity (Mukai et al., 2002; Flynn et al.,

2002). Mukai and his coworkers (2002) has conducted that inhibition by selected *L. reuteri* strains help to prevent infection in an early stage of colonization in *H. pylori*. Also, Horie and his coworkers (2002) reported that *L. crispatus* inhibited the adhesion of enteric pathogens to a synthetic basement membrane.

Lactobacillus spp. used as probiotic adjuncts have the ability to resist the digestion process in the stomach and the intestinal tract (Suskovic et al., 2000; Chang et al., 2001). Therefore, we selected *L. salivarius* subsp. *salivarius* which having acid-tolerance, bile-tolerance and adhesion ability in intestinal track from Korean faeces in the 1st paper (Bae et al., 2001). This paper was examined in probiotic characteristics of *L. salivarius* subsp. *salivarius*.

MATERIALS AND METHODS

Microorganisms and media

The bacterial strains were maintained as frozen stocks at -70°C in 20% glycerol (Sigma Chemical Co., USA). Before experimental use, the bacteria were propagated twice in lactobacilli MRS broth at 37°C.

Fermentation patterns of selected strains in skim milk

Yogurt preparation : Raw milk was obtained from the

* Corresponding Author: M. S. Nam. Tel: +82-42-821-5782, Fax: +82-42-823-2766, E-mail: namsoo@cnu.ac.kr

¹ Department of Food Development and Marketing, Joongbu University, Geumsan 312-702, Korea.

Received April 15, 2002; Accepted August 24, 2002

Chungnam National University Animal Farm. The cream was separated from raw milk using the Disc Bowl Centrifuge (Armfield Technical Education Co. LTD. UK), based on the skim milk content determined by infrared milk analyzer (Milko Scan 104; A/S N. Foss Electric, Denmark). The fat content of the skim milk was determined 0.15%, protein 3.97%, lactose 4.93% and total solid 9.15%. The skim milk was pasteurized at 92°C for 10 min. After the pasteurized skim milk was cooled to 40°C, and inoculated (2.5%) with activated starter using selected *L. salivarius* subsp. *salivarius* and purchased standard strains. The inoculated milk was incubated in a CO₂ incubator at 37°C. Samples were drawn from each bottle at 0, 4, 8, 12, 24, 36, 48, 72 and 96 h.

Changes of pH, titratable acidity and viable cell counts : The pH values of culture samples were measured at 20°C using pH meter (420A; Orion Research Inc., USA). Titratable acidity was determined, according to the procedure of Dave and Shah (1998). Viable counts were done by serial dilution with sterile 0.85% saline and pour plating in triplicate using BCP plate count agar (Eiken Chemical Co. LTD., Japan).

Analysis organic acid of fermented milk : The extraction methods based on the method of Dubey and Mistry (1996) were used. The culture samples of 5 g were transferred into test tubes and 1 ml of 12% (w/v) trichloroacetic acid (TCA) solution was added. Samples were mixed on a vortex mixer (37600 Mixer; Thermolyne, USA) for 30 seconds and then centrifuged in a high speed refrigerated centrifuge (Mega 17R; Hanil Science Industrial, Korea) at 11,000 rpm for 5 min. at 4°C. The supernatant was filtered through a 0.2 µm membrane filter (Sartorius AG, Germany) and used directly for HPLC analysis. The HPLC system contained a Waters model Waters 600E Multisolute Delivery System, a Rheodyne (Rheodyne, USA) model 7725i injector with a 20 µl sample loop, and a Waters (Waters Associates, USA) model 2487 Dual λ Absorbance Detector fitted with 210 nm, and using a SUPELCOGEL C-610H column (30 cm × 7.8 mm i.d., Sigma-Aldrich Co., USA), which was heated to 40°C by a Waters Column Heater Module (serial #F98CHM095M). A mobile phase was 0.1% phosphoric acid, at a flow rate of 0.5 ml/min., the elution time was completed for 10 min. Detector output was recorded on a Autochro-WIN 2.0 plus of software package (Young Lin Instrument Co., LTD., Korea).

Electrophoresis of proteins in fermented milk : Preparation of protein and SDS-PAGE determination was the same as Juan (1989). The culture samples of 5 g were transferred into test tubes and 5 ml of distilled water was added. Casein was separated from fermented milk by isoelectric precipitation at pH 4.6 with 0.1 N NaOH or 0.1 N HCl. After centrifugation at 5,000 rpm for 10 min. at

4°C, the whey supernatant was thrice dialyzed with distilled water for 3 days at 4°C using Dialysis Tubing (cellulose membrane M.W. 12,400; Sigma Chemical Co., USA), and stored lyophilized at -20°C. The casein pellet was washed thrice with distilled water, adjusted to pH 6.8 with 1 N NaOH, and stored lyophilized at -20°C. Fermented milk protein typing according to isolate LAB was carried out on 10×8 cm, 1.5 mm thick, 10 well, 15% separating gel containing acrylamide and bisacrylamide using a Mighty Small Mini-Vertical Electrophoresis system (Hofer Scientific Instruments, USA). The lyophilized samples were dissolved in sample buffer. The protein components were bound to SDS-PAGE by heating to 100°C for 4 min. The electrode chamber buffer was 0.025 M Tris-base, 0.192 M glycine, pH 8.3. After polymerizing the stacking gel for 1 h, 10 µl of sample were loaded into each well. Standards in the range of 14,400 to 97,400 Da (Bio-Rad Laboratories, USA) were used for identification. Fifteen microliters were used for electrophoresis. The gels were run 40 V (constant voltage) until each sample entered the running gel, after which the voltage was increased to 80 V. This voltage was maintained for 45 min. until the dye front reached the anode end of the gel.

Analysis carbohydrate of fermented milk : Lactose concentration was analyzed by HPLC (Waters Associates, USA). The extraction methods were based on the method of Jeon and his coworkers (1984). The culture samples of 5 g were transferred into test tubes, and protein of sample added 1 ml 12% TCA solution was deposited, and then centrifuged in a high speed refrigerated centrifuge at 11,000 rpm for 5 min. at 4°C. The supernatant was filtered through a 0.2 µm membrane filter and used directly for HPLC analysis. The HPLC system contained a Waters model Waters 600E Multisolute Delivery System, a Rheodyne model 7725i injector with a 20 µl sample loop, and a Waters model 2410 Refractive Index Detector, and using a SUPELCOGEL C-610H column (30 cm×7.8 mm i.d., Sigma-Aldrich Co., USA), which was heated to 40°C by a Waters Column Heater Module (serial #F98CHM095M). A mobile phase was water (TEDIA Company Inc., USA) at a flow rate of 1 ml/min., the elution time was 10 min. Detector output was recorded on a Autochro-WIN 2.0 plus of software package (Young Lin Instrument Co., LTD., Korea).

Changes of viable cell counts during the storage at 4°C : The titratable acidity of culture was measured at 0.9 to 1.0% intervals, each culture was stored in a low temperature incubator (BI-1000M; Jeio Tech Co. LTD., Korea) at 4°C. Viable numbers of each culture was determined after 0, 5, 10, 20 and 30 days of storage. Viable counts were done by serial dilution with sterile 0.85% saline and pour plating in triplicate using BCP plate count agar.

Reduction of cholesterol content by the selected strains

Reduction of cholesterol by the selected strains was performed according to the methods described by Gilliland and his coworkers (1985). Each prepared a culture of selected strains and was inoculated (1%) into 10 ml of MRS broth supplemented with 0.2% bile extract (Sigma Chemical Co., USA) and 1 ml of soluble cholesterol (Sigma Chemical Co., USA) solution (100 µg/ml) filtered through a sterile 0.45 µm membrane filter (Sartorius AG Germany) prior to use. The tubes were incubated in CO₂ incubator at 37°C for 24 h. After incubation, cells were removed by centrifugation for 10 min. at 12,000 rpm and 4°C. Then 0.5 ml of the supernatant, 2 ml of 50% (w/v) KOH, and 3 ml of 95% ethanol were placed in a capped tube, mixed thoroughly, and incubated in a 60°C water bath for 10 min. After the mixture cooled to room temperature (ca. 25°C), 5 ml of hexane was added. Three milliliters of distilled water were added, and the mixture was shaken for 1 min. to ensure complete mixing. 2.5 ml of the hexane layer was pipetted into a clean test tubes, and the solvent was evaporated to dryness at 60°C under a flow of nitrogen gas. 4 ml of a 0.05% (w/v) o-phthalaldehyde reagent were added to each tube, and the solution was mixed until the sample was completely dissolved. After 10 min., 2 ml of concentrated H₂SO₄ was added; the solutions were immediately mixed on a tube, and absorbance at 550nm was measured with a spectrophotometer (Pharmacia Biotech., England). The control solution was assayed using the same procedure but without cells.

The cholesterol-binding ability of cells was estimated by the following formula: $A=100-[(B/C)\times 100]$, where A= the cells of cholesterol-binding (percentage), B= cholesterol (micrograms) in the supernatant containing the cells, and C=cholesterol (micrograms) in the supernatant containing no cells (control).

Antibacterial effects of selected strains

Antibacterial activity of selected strains was detected using the method described by Tagg and McGiven (1971). Six pathogenic strains (*E. coli*, *S. typhimurium*, *S. enteritidis*) were incubated in each medium at 37°C for 24 to 48 h. The titratable acidity of culture got into 0.9 to 1.0% intervals, and cells were centrifuged in high speed refrigerated centrifuge at 12,000 rpm for 10 min. at 4°C. The supernatant was collected, and one the pH was adjusted to 6.5, another pH was not adjusted. The supernatant was filtered through a 0.2 µm membrane filter. Sterile paper discs (ø8×1.5 mm; Toyo Roshi Kaisha, LTD., Japan) were placed in this supernatant for 10 sec. Discs saturated with the supernatant were placed on the plate agar which contained a confluent lawn of the pathogenic strain. Plates

were incubated at 37°C for 48 h. Inhibition zones around the disc were measured after the incubation.

Antibiotic resistances of lactobacilli

The antibiotic resistances of selected *L. salivarius* subsp. *salivarius* were detected using the 12 antibiotic agents. These 12 antibiotic agents (bioDiscs) were purchased in bioMerieu SA company (France). 12 antibiotic agents and concentration were the same as these: amikacin (30 µg), mpicillin (10 µg), cephaixin (30 µg), colistin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), neomycin (30 µg), nofloxacin (10 µg), penicillin G (10 units), spiramycin (100 µg), tetracycline (30 µg). These bioDiscs with the antibiotics were placed on the plate lactobacilli MRS agar which contained a confluent lawn of the isolated LAB strain. Plates were incubated at 37°C for 48 h. Inhibition zones around the disc were measured after the incubation.

Statistical analysis

The analysis of significance test was analyzed by Duncan's multiple range test (DMRT). Data were summarized using descriptive statistics such as the mean and standard error of the mean. Statistical comparisons of the treatment versus control group were analyzed by student's t test using MYSTAT statistical analysis system (MYSTAT 2.0, Korea). $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Fermentation patterns

Acid production : Figure 1 shows the changes of pH, titratable acidity and viable cell counts during the fermentation periods by the selected *L. salivarius* subsp. *salivarius*. Fermentation characteristics of selected strain 27 in skim milk determined a very slow decrease in pH during the fermentation periods, titratable acidity appeared to be deficient, also numbers of viable cells decreased faster than other strains, but strains 20, 35 and 37 were consistently more than 10¹⁰ cfu/ml at 24-36 h. The strain 27 had strong acid-tolerant ability in MRS broth at pH 4.0 but deficient fermentation ability in skim milk. And strains 20, 35 and 37 have a trait that was useful for milk fermentation and may play a role in its ability to survive in dairy products as a probiotic agent. Also, as shown in Figure 1, in the case of *L. acidophilus* KCTC3145 (LE), after fermentation in the skim milk produce 1.0% titratable acidity in 20 h. fermentation patterns of standard strain LA (*L. acidophilus* KCTC3150) and LE (*L. acidophilus* KCTC3145) excellently produced more acid than other strains. Chou and Bart (1999) reported that acid productive ability of *L.*

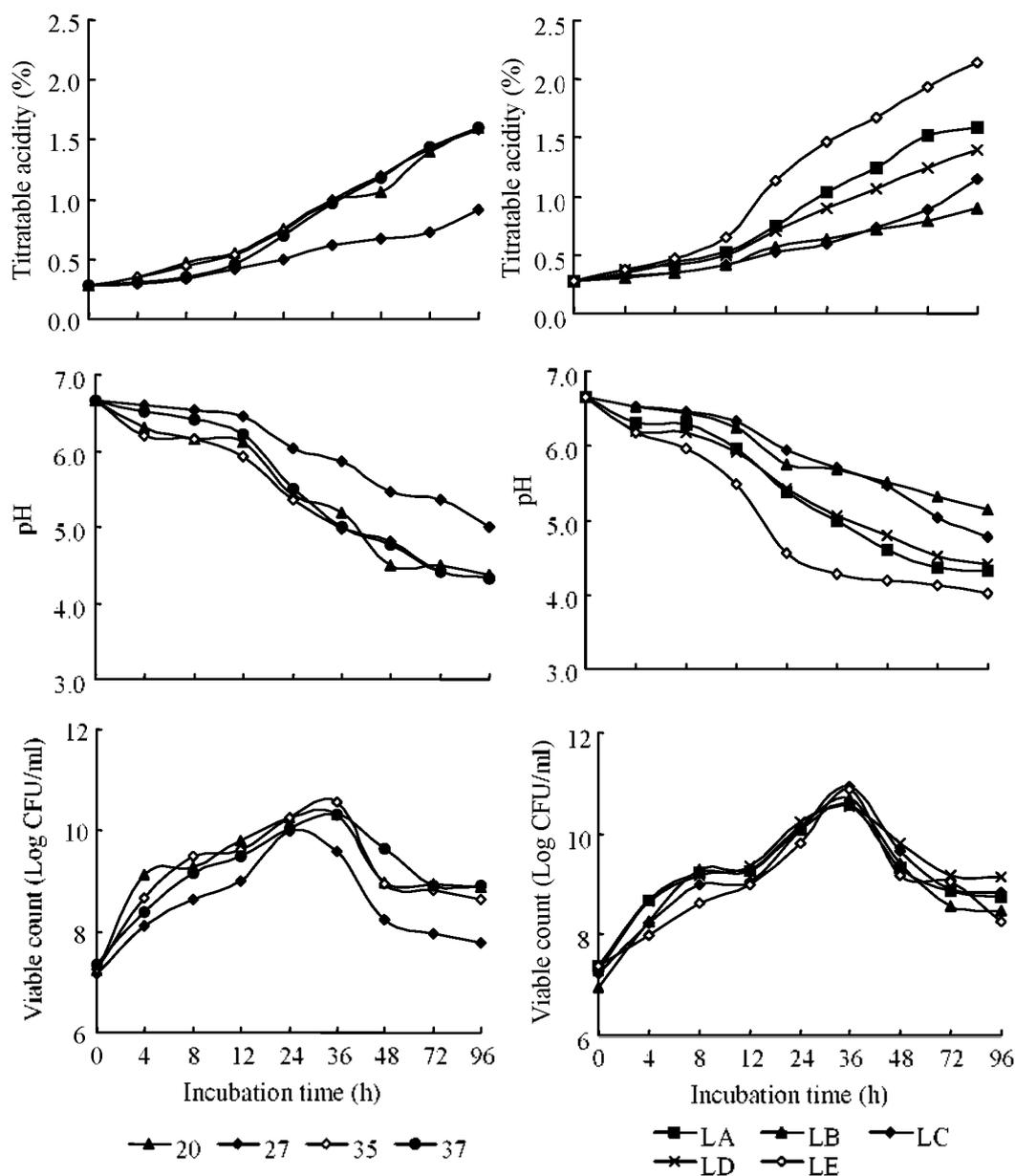


Figure 1. Changes of titratable acidity, pH and viable cell counts during fermentation by the selected *L. salivarius* subsp. *salivarius* in skim milk at 37°C.

20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgaricus* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145.

acidophilus shown more excellent tendency than other *lactobacillus* genus. Also, selected strains 20, 35 and 37 are displaying resemblant tendency with standard strains LB (*L. casei* KCTC3189), LC (*L. delbrueckii* subsp. *bulgaricus* KCTC3188) and LD (*L. lactis* subsp. *cremoris* KCTC3619) in the acid productivity and growth ability in the skim milk.

It was considered when there were many viable cell counts of culture, and taken for sampling when pH and titratable acidity reached 4.5-5.0 and 0.9-1.0%, respectively. Organic acid production of the culture shown in Table 1. All of the strains produced fermentation products such as a typical lactic acid fermentation. All of the selected *L.*

salivarius subsp. *salivarius* and standard strains produced fermentation products showed isobutyric acid. Butyric acid is a main source of energy of human intestine epithelia, which inhibit the growth of enteropathogenic *Clostridium perfringens*, and butyric acid inhibited propagation of tumor cells (Kwag et al., 1989).

Organic acids such as acetic and lactic acid which were produced by lactic acid bacteria (LAB) had inhibited the growth of many bacteria, especially pathogenic gram-negative types (Daly et al., 1972). As it is well known, Lactic acid is the main metabolic product of all homo- and hetero-fermentative species of *Lactobacillus*. In the case of

Table 1. Contents of organic acids in fermented milk by the LAB

Strains	Organic acid ¹⁾ (mM)							
	Oxalic	Citric	Tartaric	Malic	Lactic	Formic	Acetic	Isobutylic
20	0.32	6.36	24.84	1.29	122.29	2.48	24.35	10.92
27	0.07	7.53	10.46	-	83.43	1.98	8.32	26.85
35	0.26	6.49	23.29	1.68	119.12	3.44	29.02	11.54
37	0.10	5.68	24.56	3.68	131.96	3.11	34.23	11.17
LA	0.08	5.98	24.61	3.15	130.18	2.69	31.56	10.68
LB	0.07	7.10	26.69	3.87	91.23	2.79	21.01	7.88
LC	0.07	2.77	22.20	3.35	112.96	1.77	46.39	16.72
LD	0.07	4.65	23.41	1.98	111.72	3.18	34.91	10.52
LE	0.04	8.29	24.63	4.58	115.82	3.23	29.99	5.39
*R ²	0.999571	0.999863	0.962798	0.992022	0.999045	0.994868	0.974382	0.971657

¹⁾ Means concentration of organic acids in the fermented milk by LAB.

* Correlation coefficients between amount and area in standard calibration of organic acids by HPLC.

20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *Bulgarius* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145.

hetero-fermentative species, acetic acid and CO₂ are also produced.

Proteolysis : Figure 2 shows SDS-electrophoresis patterns for casein of raw milk and fermented milk by the isolated strains. Changes in the casein patterns did not seem to be significant or different from each other; however, changes in the whey proteins (Figure 3) were shown by the disappearance of the band in the region of 14,000 daltons. The whey protein of region about 14,000 daltons was considering α -lactalbumin. According to the strains, change of whey protein was not shown. Alm (1982b) reported that SDS-electrophoresis patterns for casein in fermented milk using *Lactobacillus* spp. were shown in few and small changes. In this study, SDS-electrophoresis patterns for

casein were similar to that described by Alm (1982b).

Lactic acid is the main metabolic product of all species of *Lactobacillus*. It is considered not only favourable for the development of certain sensoric properties and better preservation of the product, but also for the improvement of digestibility of casein. Lactic acid affects the colloidal suspension of calcium-phospho-caseinate. This is due to the decrease of the pH by an increase of the lactate during fermentation. The calcium-phosphate in the casein micelle is desintegrated leading finally to a precipitation of casein. In its decalcified form, at the pH of yogurt which is normally between 3.9-4.2, the casein reaches the pH of gastric action faster and promotes a finer flocculation which is more easily hydrolyzed by the proteolytic enzyme.

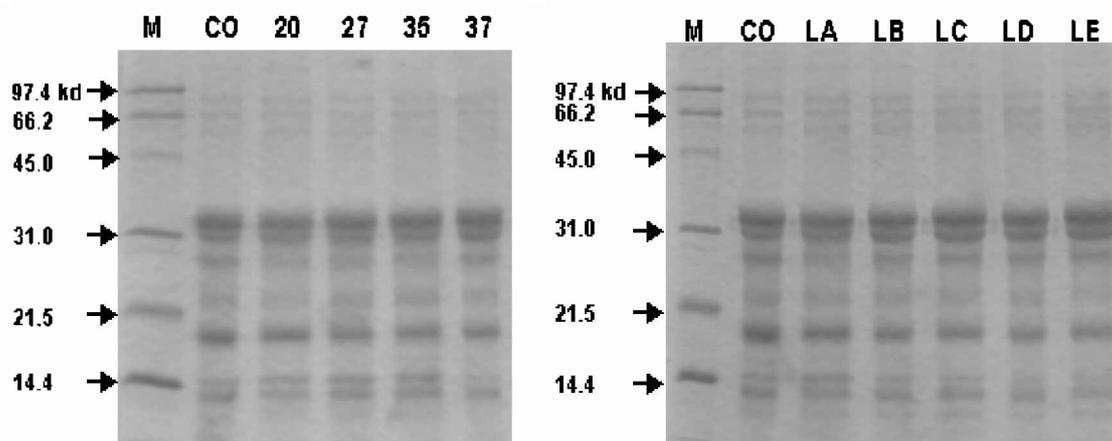


Figure 2. Polyacrylamide gel electrophoretic (PAGE) patterns for casein of raw milk and milk fermented by isolated strains.

CO: raw milk, 20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgarius* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145, M: Molecular marker.

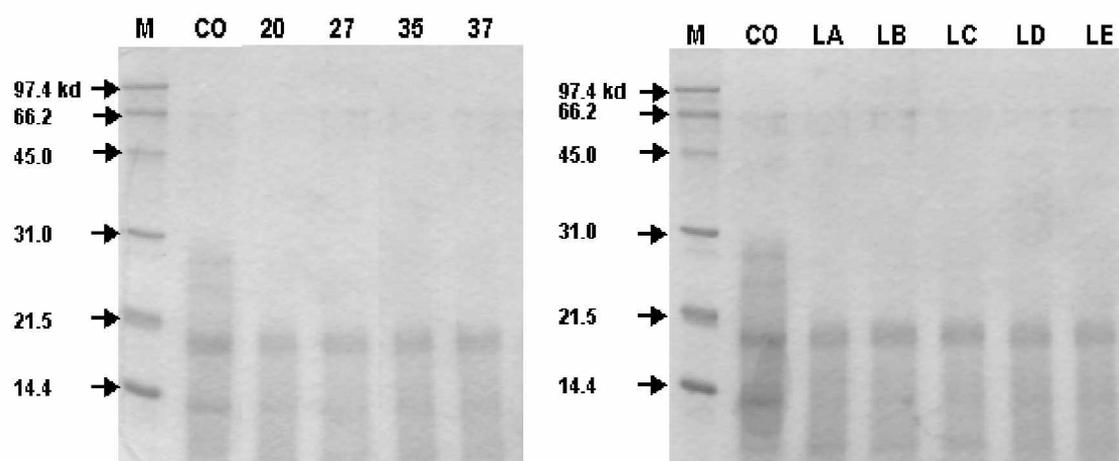


Figure 3. Polyacrylamide gel electrophoretic (PAGE) patterns for whey protein of raw milk, commercial yogurt and fermented milk with isolated strains.

CO: raw milk, 20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgaricus* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145, M: Molecular marker.

In the industrial production of fermented milks, heat treatment of the raw milk within the range of 80-100°C and a holding time of up to 15-20 min. is usually applied. As far as microbiological aspects are concerned, less heat would also be good. However, in order to improve the viscosity of the products like in the manufacture of yogurt, an extensive denaturation of whey proteins is necessary which can only be achieved by high heat treatment of the milk prior to fermentation.

Carbohydrate patterns : Common to all fermented milks is the lactic acid fermentation in which a part of the lactose is transformed into lactic acid. Depending on the micro-organisms involved, the amount of lactic acid produced varies between 0.6 and 1.5%. At the same time the lactose content is reduced from 4.6-4.8% to 3.8-2.8%, i.e. up to 40% (Puhan, 1985). In this study, Contents of lactose in the culture by LAB were shown in Table 2. The standard strains LA (*L. acidophilus* KCTC3150), LB (*L. casei* KCTC3189), LC (*L. delbrueckii* subsp. *bulgaricus* KCTC3188), LD (*L. lactis* subsp. *cremoris* KCTC3619), LE (*L. acidophilus* KCTC3145) were reduced low ratio of 10-14% in generally. *L. salivarius* subsp. *salivarius* (stains 20, 27, 35, 37) were reducing contents of lactose over 21.61%. No free glucose and galactose were detected during the fermentation. Bouzar and coworkers (1997) reported that content of glucose in fermented milk using *Lactobacillus* spp. was not detected during the fermentation. In this study, content of glucose was similar to that described by them.

Viable cell during storage : Carbohydrate consumption of lactobacilli has the potential to aid lactose digestion (Kim and Gilliland, 1983), to aid in controlling serum cholesterol (Gilliland et al., 1985), to control intestinal infections

(Perdigon, 1990), and to exert antitumor activity (Kato et al., 1994). For most of these benefits, adequate numbers of viable cells of lactobacilli need to be taken. Thus, it is important that the lactobacilli remain viable during storage of products containing them.

As shown in Figure 4, the cultures were sampled after titratable acid was detected in 0.9 to 1.0%, showing the viability of the isolated strains for 30 days at 4°C. Coliforms were not detected in any samples during the storage period. The strains 20, 35 and 37 tended to decrease slowly from 10¹⁰ cfu/ml to 10⁹ cfu/ml, but changes in the viable counts of strain 27 decreased significantly from 10⁸ cfu/ml to 10⁵ cfu/ml during storage; the substantial

Table 2. Changes of carbohydrates during the fermentation of milk by LAB

Strains	Carbohydrates ¹⁾ (%)	
	Lactose	Reduce of lactose
20	3.96	24.28
27	3.95	24.47
35	4.10	21.61
37	3.89	25.62
LA	4.49	14.15
LB	4.69	10.33
LC	4.56	12.81
LD	4.56	12.24
LE	4.49	14.15
*R ²	0.999448	

¹⁾ Means of carbohydrates concentration in the fermented milk by LAB.

* Correlation coefficients between amount and area in standard calibration of organic acids by HPLC.

20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgaricus* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145.

reduction took place after the 20 days of storage. The viability during storage is very useful for fermentation milk

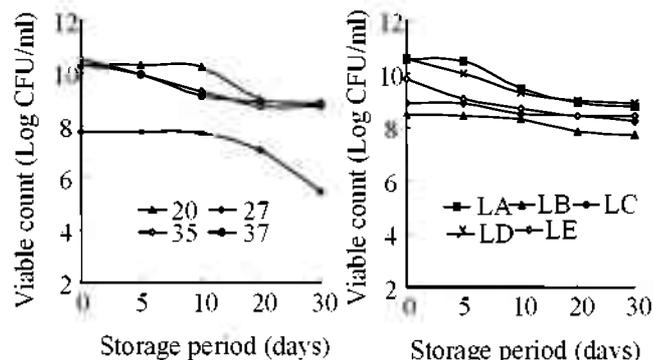


Figure 4. Comparison of the viability of LAB during storage at 4°C.

20, 27, 35, and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgaricus* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145.

product and may play a role in its ability to survive in dairy products for delivery as a probiotic strain.

Reduction of cholesterol concentration

As shown in Figure 5, there were the effects on reducing cholesterol concentration over 23% in all isolated strains. The isolated strains 27 and 37 showed that reduction effect more than 30%. Also, *L. acidophilus* species (LA and LE) of standard strains were shown cholesterol reduction effect more than 30% compared with other standard strains. These results were similar to that described by Gilliland (1987). These results of *L. salivarius* subsp. *salivarius* (strains 27 and 37) were considered that cholesterol reduction effect appeared more than in the standard strains LA, LB, LC, LD and LE. Although the result obtained from

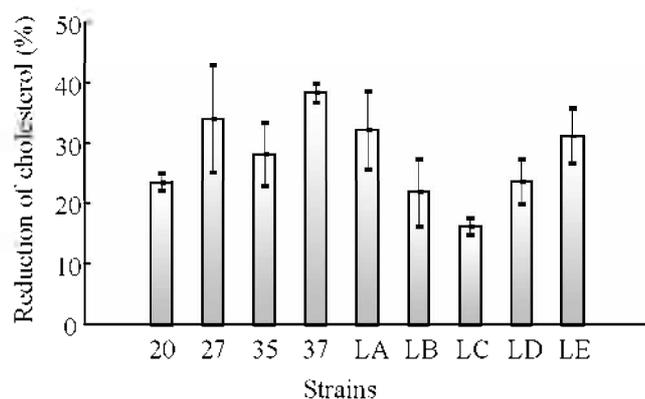


Figure 5. Reduction of cholesterol concentration by LAB in MRS broth.

20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LA: *L. acidophilus* KCTC3150, LB: *L. casei* KCTC3189, LC: *L. delbrueckii* subsp. *bulgaricus* KCTC3188, LD: *L. lactis* subsp. *cremoris* KCTC3619, LE: *L. acidophilus* KCTC3145, Error bars indicate standard errors.

in vitro examination has not yet been tested in an animal model, but it does have the potential of helping control serum cholesterol levels.

Noh and his coworkers (1997) indicated that cholesterol removed from laboratory media during the growth of *L. acidophilus* was assimilated by the culture. Klaver and van der Meer (1993), however, suggested that the cholesterol merely coprecipitated with free bile salts that were released through deconjugation of the conjugated bile salts in the growth medium. This conclusion was based largely on their observation that no cholesterol was removed when the growth medium was maintained at pH 6.0, which would prevent the precipitation of free bile salts.

In this study, *L. salivarius* subsp. *salivarius* (strain 27 and 37) showed a cholesterol reducible effect. Also, the case of *L. acidophilus* (strain LA and LE) showed a cholesterol reducible effect, and was similar to that described in Noh et al. (1997).

Antibacterial activity and antibiotic resistance of lactobacilli

As shown in Table 3, the selected strains had excellent antibacterial activity in the inhibition zone 15-25 mm to PA, PD, PE, and PF of 4 pathogenic strains when it had not been adjusted pH (pH 4.5-5.0). Also, All of the selected strains had partial inhibition zone to PA, PC, and PD of 3 pathogenic strains when it had been adjusted to pH 5.7. These antibacterial activities were considered by the bacteriocin which produced probiotic strains. Flynn et al. (2002) and Virginia et al. (1999) reported that *L. salivarius* had excellent antibacterial effect by produced bacteriocin. These results of this study were similar to that described by them.

The antibiotic resistances of the isolated LAB to 12 antibiotic agents was determined by the agar diffusion method for the stable production of yogurt. As shown in Table 4, the isolated strains 20 (*L. salivarius* subsp. *salivarius*) to the β -lactam spectrum, such as ampicillin and Penicillin G showed the highest resistance compared to the standard strain LE (*L. acidophilus*). Also, the isolated strains 37 (*L. salivarius* subsp. *salivarius*) to the aminoglycoside spectrum, such as amikacin, gentamycin and neomycin, showed the highest resistance. The *L. salivarius* subsp. *salivarius* (isolated strain 27 and 35) showed the highest resistance to ciprofloxacin and norfloxacin among the Quinolone spectrum. Kim and his coworkers (1995) reported that LAB was usually sensitive to penicillin G and Gram-positive spectrum antibiotic. In this study, the selected strains appeared most sensitive to penicillin G among the antibiotics. Consequently, *L. salivarius* subsp. *salivarius* of the isolated strains 27 and 35 showed the highest resistance.

In conclusion, the probiotic potential of *L. salivarius*

Table 3. Antimicrobial activity¹⁾ of the selected LAB from Korean faeces

Strains	PA		PB		PC		PD		PE		PF	
	R ²⁾	S ³⁾	R	S	R	S	R	S	R	S	R	S
20	++	P ⁴⁾	++	-	+	-	++	P	++	-	++	P
27	++	P	+	-	+	-	++	P	++	-	++	P
35	++	P	++	-	++	P	++	P	++	-	+++	P
37	++	P	+	-	++	P	++	P	++	-	++	P
LA	++	P	++	-	++	P	++	P	++	-	+++	P
LB	++	P	-	-	+	P	+	P	++	-	++	P
LC	++	P	++	-	+	P	++	P	++	-	++	P
LD	++	P	+	-	++	P	++	P	++	-	++	P
LE	++	P	+	-	+	P	++	P	++	-	++	P

PA. *E. coli* KCTC1039; PB. *E. coli* KCTC1021; PC. *E. coli* KCTC0115; PD. *S. typhimurium* M-15; PE. *S. typhimurium* KCCM40253; PF. *S. enteritidis* KCCM3313.

¹⁾ Estimation by the agar diffusion method, inhibition zone in omm around the DISC (-, no inhibition zone; +, 9-14 mm inhibition zone; ++, 15-25 mm inhibition zone; +++-, 26-35 mm inhibition zone).

R²⁾: pH4.5-5.0 of supernatants was not adjusted.

S³⁾: pH of supernatants was adjusted to 5.7±0.1.

P⁴⁾: partial inhibition.

Table 4. Antibiotic resistances¹⁾ of LAB depending upon various antibiotics

Antibiotics	Concentration	Strains					
		12	27	35	37	LE	HC
		Inhibition zone (mm)					
Amikacin	30 µg	11 ^{abc}	11 ^{abc}	12 ^{ab}	11 ^{abc}	13 ^a	13 ^a
Ampicillin	10 µg	26 ^{cd}	30 ^{ab}	30 ^{ab}	27 ^{bcd}	30 ^{ab}	29 ^{abc}
Cephaixin	30 µg	12 ^d	18 ^b	26 ^a	12 ^d	15 ^{bed}	17 ^{bc}
Colistin	10 µg	8 ^a	8 ^a	8 ^a	8 ^a	9 ^a	8 ^a
Ciprofloxacin	5 µg	17 ^a	8 ^b	8 ^b	16 ^a	17 ^a	15 ^a
Erythromycin	15 µg	32 ^a	25 ^c	23 ^c	32 ^a	29 ^{ab}	32 ^a
Gentamycin	10 µg	9 ^c	13 ^{ab}	11 ^{abc}	9 ^c	14 ^a	14 ^a
Neomycin	30 µg	10 ^{bc}	13 ^{ab}	13 ^{ab}	9 ^c	13 ^{ab}	14 ^a
Nofloxacin	10 µg	15 ^a	8 ^b	8 ^b	13 ^a	14 ^a	14 ^a
Penicillin G	10 units	31 ^{bc}	32 ^{abc}	30 ^c	34 ^{ab}	35 ^a	35 ^a
Spiramycin	100 µg	25 ^{ab}	21 ^c	20 ^c	25 ^{ab}	26 ^{ab}	27 ^a
Tetracycline	30 µg	32 ^a	23 ^b	24 ^b	31 ^a	31 ^a	33 ^a

¹⁾ Estimation by the agar diffusion method, inhibition zone in 0 mm around the paper disc (ø 8 mm).

^{ab,c,d} In a row, means followed by a common letter are not significantly different at the 1% level by DMRT.

20, 27, 35 and 37: selected *L. salivarius* subsp. *salivarius*, LE: *L. acidophilus* KCTC3145, HC: *L. casei* YA-70.

subsp. *salivarius*, which selected acid-tolerant (pH 4.0) and bile-tolerant (1.0% bile salt) strains with the lactobacilli MRS broth in the 1st paper (Bae et al., 2001), showed various probiotic features such as fermentation characteristic in skim milk, reduction of cholesterol contents, antibacterial effect, etc. These results indicated that strain 27 and 35 among selected *L. salivarius* subsp. *salivarius* have potential benefits for industrial application.

REFERENCES

Ahn, L. 1982a. Effect of fermentation on lactose, glucose, and galactose content in milk and suitability of fermented milk products for lactose intolerant individuals. *J. Dairy Sci.* 65:346-352.
 Ahn, L. 1982b. Effect of fermentation on proteins of Swedish fermented milk products. *J. Dairy Sci.* 65:1696-1704.
 Bae, H. C., S. H. Choi and M. S. Nam. 2001. Isolation and

Identification of Acid- and Bile-Tolerant *Lactobacillus salivarius* subsp. *salivarius* from Human Faeces. *Asian-Aust. J. Anim. Sci.* 14(8):1170-1178.
 Baird-Parker, A. C. 1980. Organic acids. In: *Microbial ecology of foods*, vol. 1. Intern. Comm. on Microbiol. Spec. Foods. Academic Press, New York. 126-159.
 Barth, C. A. and M. D. Vrese. 1984. D-lactate in the metabolism of man-foreign substance or physiological metabolite, *Kieler Milchw. Forsch. Ber.* 36:155-161.
 Bemardeau, M., J. P. Vemoux and M. Gueguen. 2001. Probiotic properties of two *Lactobacillus* strains *in vitro*. *Milchwissenschaft.* 56(12):663-667.
 Blanc, B. 1984. In "Fermented Milks", *Int. Dairy Fed., Doc.* 179.
 Bouzar, F., J. Cerning and M. Desmazeaud. 1997. Exopolysaccharide production and Texture-promoting abilities of mixed-strain starter cultures in yogurt production. *J. Dairy Sci.* 80:2310-2317.
 Broussalian, J. and D. Westhoff. 1983. Influence of lactose concentration of milk and yogurt on growth rate of rats. *J.*

- Dairy Sci. 66:438-443.
- Chadwick, R. W., S. E. George and L. R. Claxton. 1992. Role of gastrointestinal mucosa and microflora in the bioactivation of dietary and environmental mutagens or carcinogens. *Drug Metab. Res.* 24:425-492.
- Chang, Y. H., J. K. Kim, H. J. Kim, W. Y. Kim, Y. B. Kim, Y. H. Park, Y. H. Chang, J. K. Kim, H. J. Kim, W. Y. Kim, Y. B. Kim and Y. H. Park. 2001. Selection of a potential probiotic *Lactobacillus* strain and subsequent in vivo studies. *Antonie van Leeuwenhoek.* 80(2):193-199.
- Chassy, B. M. 1986. Gene transfer and advances in the molecular genetics of lactic acid bacteria. The 6th international symposium on lactic acid bacteria and human health. pp. 245-273.
- Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol.* 43:164-168.
- Daly, C., W. E. Sandine and P. R. Elliker. 1972. Interaction of food starter cultures and food-borne pathogens: *Streptococcus diacetilactis* versus food pathogens. *J. Milk Food Technol.* 35:349-357.
- Dave, R. I. and N. P. Shah. 1998. Ingredient supplementation effects on viability of probiotic bacteria in yogurt. *J. Dairy Sci.* 81:2804-2816.
- Dubey, U. K. and V. V. Mistry. 1996. Growth characteristics of bifidobacteria in infant formulas. *J. Dairy Sci.* 79:1146-1155.
- Eyssen, H. 1973. Role of the gut microflora in metabolism of lipids and sterols. *Proc. Nutr. Soc.* 32:59-64.
- Flynn, S., D. V. Sinderen, G. M. Thornton, H. Holo, I. F. Nes, J. K. Collins and D. V. Sinderen. 2002. Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium *Lactobacillus salivarius* subsp. *salivarius* UCC118. *Microbiology Reading.* 148(4):973-984.
- Gill, H. S. and K. J. Rutherford. 2001. Immune enhancement conferred by oral delivery of *Lactobacillus rhamnosus* HN001 in different milk-based substrates. *Journal of Dairy Research.* 68(4):611-616.
- Gilliland, S. E. 1990. Health and nutritional benefits from lactic acid bacteria. *FEMS Microbiol. Rev.* 87:175-188.
- Gilliland, S. E. 1987. Benefits of dietary lactobacilli in controlling levels of serum cholesterol. The 5th international symposium on lactic acid bacteria and human health, pp. 199-209.
- Gilliland, S. E., C. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* 49:377-381.
- Goodenough, E. R. and D. H. Kleyn. 1976. Influence of viable yogurt microflora on digestion of lactose by the rat. *J. Dairy Sci.* 59:601-606.
- Gupta, P. K., R. S. Chauhan, G. K. Singh and D. K. Agrawal. 2001. *Lactobacillus acidophilus* as a potential probiotic. Advances in immunology and immunopathology. In: Proceedings of a national symposium on immunomodulation in health and disease. Society for Immunology & Immunopathology, Pantnagar, Indiapp. pp. 66-69.
- Horie, M., A. Ishiyama, Y. Fujihira-Ueki, J. Sillanpaa, T. K. Korhonen and T. Toba. 2002. Inhibition of the adherence of *Escherichia coli* strains to basement membrane by *Lactobacillus crispatus* expressing an S-layer. *Journal of Applied Microbiology.* 92(3):396-403.
- Jeon, I. J., S. J. Galitzer and K. J. Hennessy. 1984. Rapid determination of lactose and its hydrolyzates in whey and whey permeate by high performance liquid chromatography. *J. Dairy Sci.* 67:884-887.
- Juan, F. M. and S. Linda. 1989. Milk protein typing of bovine mammary gland tissue used to generate a complementary deoxyribonucleic acid library. *J. Dairy Sci.* 72:3190-3196.
- Kato, I., K. Endo and T. Yokokura. 1994. Effects of oral administration of *Lactobacillus casei* on antitumor responses induced by tumor resection in mice. *Int. J. Immunopharmacol.* 16:29-34.
- Kilara, A. and K. M. Shahani. 1976. Lactase activity of cultured and acidified dairy products. *J. Dairy Sci.* 61:2031-2035.
- Kim, H. S. and S. E. Gilliland. 1983. *Lactobacillus acidophilus* as a dietary adjunct for milk to aid lactose digestion in humans. *J. Dairy Sci.* 66:959-964.
- Klaver, F. A. M. and R. van der Meer. 1993. The assumed assimilation of cholesterol by *Lactobacillus* and *Bifidobacterium bifidum* is due to their bile salt-deconjugation activity. *Appl. Environ. Microbiol.* 59:1120-1124.
- Kwang, J. H., J. C. Lee, T. H. Kim, P. K. Chung and K. K. Lee. 1989. Isolation and characterization of a butyric acid bacterium from infant feces. *Kor. J. Appl. Microbiol. Bioeng.* 17:56-62.
- Lisa, M., D. Colum, K. Barry, S. Fergus, C. O. Gerald and J. K. Collins. 1999. *In vivo* assessment of potential probiotic *Lactobacillus salivarius* strains: evaluation of their establishment, persistence, and localization in the murine gastrointestinal tract. *Microbial ecology in health and disease.* Scandinavian Uni. Press. pp. 149-157.
- Matar, C., J. C. Valdez, M. Medina, M. Rachid and G. Perdigon. 2001. Immunomodulating effects of milks fermented by *Lactobacillus helveticus* and its non-proteolytic variant. *Journal of Dairy Research.* 68(4):601-609.
- Mukai, T., T. Asasaka, E. Sato, K. Mori, M. Matsumoto and H. Ohori. 2002. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. *FEMS Immunology and Medical Microbiology.* 32(2):105-110.
- Nagao, F., M. Nakayama, T. Muto and K. Okumura. 2000. Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the immune system in healthy human subjects. *Bioscience Biotechnology and Biochemistry.* 64(12):2706-2708.
- Noh, D. O., S. H. Kim and S. E. Gilliland. 1997. Incorporation of cholesterol into cellular membrane of *Lactobacillus acidophilus* ATCC 43121. *J. Dairy Sci.* 80:3107-3113.
- Perdigon, G., M. E. N. Demacias, S. Alvarez, G. Oliver and A. A. D. Ruiz. 1990. Prevention of gastrointestinal infection using immunobiological methods with milk fermented with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. *J. Dairy Res.* 57:255-259.
- Pubin, H. E. and F. Vaughan. 1979. Elucidation of the inhibitory factors of yogurt. *J. Dairy Sci.* 1873-1879.
- Puhan, Z. 1985. The Role of Fermented Milks in Nutrition. The 4th International Seminar on Lactic Acid Bacteria and Human Health. pp. 160-173.
- Singh, B. and T. K. Bhat. 2001. Exploiting gastrointestinal microbes for livestock and industrial development. - Review -. *Asian-Aust. J. Anim. Sci.* 14:567-586.

- Speck, M. L. and K. R. Ltz. 1980. ACDPI status paper: nutritive and health values of cultured dairy foods. *Cultured Dairy Products J.* 15:10-12.
- Suskovic, J., B. Kos, S. Matosic and V. Besendorfer. 2000. The effect of bile salts on survival and morphology of a potential probiotic strain *Lactobacillus acidophilus* M92. *World Journal of Microbiology and Biotechnology.* 16(7):673-678.
- Tagg, J. R. and A. R. McGiven. 1971. Assay systems for bacteriocins. *App. Microbiol.* 21:943-945.
- Usman and A. Hosono. 2000. Cholesterol-lowering activity of *Lactobacillus gasseri*: *in vitro* and *in vivo* results. *Recent Research Developments in Agricultural and Biological Chemistry.* 4:1-34.
- Virginia, S. O., A. P. Aida and M. E. Nader-Macias. 1999. Characterization of a bacteriocin-like substance produced by a vaginal *Lacobacillus salivarius* strain. *Appl. Environ. Microbiol.* 65:5631-5635.