Identification and Characterization of *Lactobacillus salivarius* subsp. *salivarius* from Korean Feces

Hyoung-Churl Bae

(Division of Animal Science & Resources, Chungnam National University)

Identification and Characterization of *Lactobacillus salivarius* subsp. *salivarius* from Korean Feces

Bae, Hyoung-Churl

Division of Animal Science & Resources, Chungnam National University

ABSTRACT

This study was conducted to isolate lactobacilli having probiotic characteristics to be used as health adjuncts with fermented milk products. Acid tolerant strains were selected in Lactobacilli MRS broth adjusted to pH 4.0 from 80 healthy persons (infants, children and adults). And bile tolerant strains were examined in Lactobacilli MRS broth in which 1.0% bile salt was added. By estimation above characteristics, the strains No. 27, which was isolated from adult feces, was selected and identified as *Lactobacillus salivarius* subsp. *salivarius* based on carbohydrate fermentation and 16S rDNA sequencing.

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℃. 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℃. 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 hours 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.

Purified α-galactosidase was obtained by DEAE-Sephadex A-50 ion exchange chromatography, Mono-Q ion exchange chromatography and HPLC column chromatography from *L. salivarius* subsp. *salivarius* 27. The specific activity of the purified enzyme was 8,994 units/mg protein, representing an 17.09 folds purification of the original cell crude extract. The molecular weight of enzyme was identified about 53,000 dalton by 12% SDS-PAGE. Optimal temperature and pH for activity of this enzyme were 40℃ and 7.0 respectively. The enzyme was found to be stable between 25 and 50℃. α-galactosidase activity was lost rapidly below pH 5.0 and above pH 9.0. This enzyme was liberated galactose from melibiose, raffinose, and stachyose, and also the hydrolysis rate of substrate was compared by HPLC.

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*.

INTRODUCTION

Lactic acid bacteria (LAB) are sometimes termed probiotic and are used as health adjuncts in food to provide a wide variety of health benefits (Metchnikoff, 1908). These bacteria mainly lactobacilli and bifidobacteria, may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improved lactose utilization, and anticarcinogenic activity (Collin and Hall, 1984; Fernandes et al., 1987; Fuller, 1992a; Fuller, 1992b; Gilliland, 1979; Gilliland et al., 1985; Mitsuoka, 1990; Wood, 1992; Yuguchi et al., 1992; Singh et al., 2001).

In the development of probiotic foods intended for human consumption, strains of LAB such as *Lactobacillus, Bifidobacterium* and *Streptococcus* have been most commonly used, due primarily to the perception that they are desirable members of the intestinal microflora (Berg, 1998; Goldin and Gorba, 1992). Their colonization may be improved by host specific adherence properties (Tannock, 1990) Colonization has been shown to be important for the survival of probiotic strains in competition with other intestinal microbes (Saxelin, 1991). Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputhra et al., 1995). After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Lankaputhra et al., 1995). After traveling through this harsh environment, the organisms colonize the epithelium of the lower intestinal tract (Conway et al., 1987). Thus,

strains selected for using probiotic bacteria should be able to tolerate acid for 90 min, tolerate bile, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits.

The use of *Lactobacillus salivarius* as a dietary adjunct can provide several benefits to the digestive system (Lisa et al., 1999; Guarner and Schaafsma, 1998). The most frequently mentioned role for this organism is to control undesirable microorganisms in the intestinal tract. Its presence in milk is also beneficial to those who cannot digest lactose adequately. The bacterial cells serve as a source of an enzyme system for hydrolyzing lactose in the intestinal tract.

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; Mukai et al., 2002; Horie et al., 2002; Matar et al., 2001;Gill and Rutherfurd, 2001; Gupta et al., 2001 Singh and Bhat, 2001). 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 aerobic-tolerance from Korean feces in the 1st step. And in the 2nd step we examined in characteristics of *L. salivarius* subsp. *salivarius*.

MATERIALS AND METHODS

Isolation of bacterial strains and media

Salmonella sp. was grown in Trypticase Soy broth (BBL, Cockeysville, MD) for 18 h at 37℃. *E. coli* was grown in LB (Difco, Detroit, MI)) for 18h at 37℃. *Lactobacilli* sp. were isolated from feces of 80 healthy persons (infants, children and adults). Feces samples diluted in 0.1M

phosphate-buffered saline (PBS; 0.85% NaCl, pH 7.2) were plated on Lactobacillus Selection (LBS) agar and incubated for 48 h at 37℃.

Selection of acid-tolerant lactobacilli

Cells were harvested by centrifugation (at 4000×g for 10 min), washed with PBS three times, inoculated (2%) into Latobacilli MRS broth acidified with concentrated HCl to pH 4.0 and 5.0 and incubated at 37℃. For selection of acid-tolerant strains, bacterial growth was measured as cell density at 600nm by spectrophotometer after 24 h incubation.

Selection of bile-tolerant lactobacilli

Lactobacilli MRS broth was prepared with 0.3 and 1.0% of bile salts (Difco). Each strains isolated from feces was incubated for 8, 16, 24 h at 37℃. The growth of the isolates was measured as mentioned in acid-tolerant.

Selection of aerotolerant lactobacilli

The methods of selected aerotolerant lactobacilli were similar to that described by Heo and Yoon (1996). The growth of strains was examined in different atmospheric conditions to see whether they could grow in reasonable numbers without the aid of an anaerobic culture system. Anaerobic growth was achieved by growing cells in GasPak Jar (BBL, USA), and aerobic growth was done by growing cells in incubator.

Carbohydrate fermentation of lactobacilli

Carbohydrate fermentation tests were carried out using the relevant API strips according to the instructions of the manufacturer (bioMerieux SA, Marcy-l' Etoile, France). Identifications were performed by comparing the fermentation profiles with the available databases (version 3.3.3 of APILAB Plus; bioMerieux).

DNA amplification by PCR

Genomic DNA was isolated by the methods of Maniatis et al.(1982). Amplification of 16S rDNA was conducted by using two primers (Stackebrandt and Liesack, 1993), 5'-GAGTTTGATCCTGGCTCAG-3' (position 9 to 27, in *E. coli* 16 S rRNA numbering) and 5'-AGAAAGGAGGTGATCCAGCC-3' (position 1542 to 1525, in *E. coli* 16S rDNA numbering). Thermal conditions for the first round of PCR were 95℃ for 3 min, followed by 35 cycle of 1 min at 95℃, 62℃ for 1 min, 72℃ 1 min and final extension step at 72℃ for 10 min.

Restriction enzyme digestion of PCR products

Reaction and thermal conditions for restriction enzyme digestion of PCR product were as follows ; 10μl of PCR products were digested for 3 hr at 37℃ with 10 units of Alu I (Bioneer Co., Korea) in a total volume of 20μl.

16S rDNA sequencing of selected strains

A nucleotide sequenceof PCR products were determined by direct automated sequencing methods using ABI PRISMTM 377 DNA Sequencer (Perkin Elmer Co.). The purification of PCR product was performed with Qiaquick PCR purification Kit (QIAGEN, USA) and Quick Spin TM columns (Boehringer Mannheim, Germany).

Phylogenetic Analysis of selected strains

The 16S rDNA sequence of selected strains determined in this study was aligned by using CLUSTAL W software (Thompson el al., 1994). The sequence of representative species of the genus *L. salivarius* and related taxa were cited by using GenBank database. A phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987) from a distance matrix calculated.

Fermentation patterns of selected strains in skim milk

Yogurt preparation : The skim milk was pasteurized at 92°C for 10 min. After the pasteurized skim milk was cooled to 40℃, and inoculated (2.5%) with activated starter using selected lactobacilli and purchased standard strains. The inoculated milk was incubated in a incubator at 37℃.

Changes of viable cell counts : 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 HPLC system contained a Waters model Waters 600E Multisolvent Delivery System, and a Waters (Waters Associates, USA) model 2487 Dual λ Absorbance Detector fitted with 210 nm, and using a SUPELCOGELTM C-610H column (30 cm 7.8 mm i.d., Sigma-Aldrich Co., USA). A mobile phase was 0.1% phosphoric acid, at a flow rate of 0.5 ml/min., the elution time was completed for 30 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).

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 HPLC system contained a Waters model Waters 600E Multisolvent Delivery System, and using a SUPELCOGELTM C-610H column. A mobile phase was water (TEDIA Company Inc., USA) at a flow rate of 1 ml/min., the elution time was 10 min.

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 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).

Antibacterial effects of selected strains

Antibacterial activity of selected strains was detected using the method described by Tagg and McGiven (1971). Six pathogenic strains were *E. coli, S. typhimurium, S. enteritidis.*

Antibiotic resistances of lactobacilli

The antibiotic resistances of selected Lactobacilli were detected using the 12 antibiotic agents. These 12 antibiotic agents (bioDiscs) were purchased in bioMerieu SA company (France), manufactures of actibiotic concentrations.

Statistical analysis

The analysis of significance test was analyzed by Duncan's multiple range test (DMRT). 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.

Enzyme activity patterns of lactobacilli

Enzyme activity patterns of the selected strains were examined by using API-ZYM enzyme system (bioMerieux SA, Marcy-l' Etoile, France).

Enzyme assay

Galactosidase activity was assayed by the method of Nelson(1944).

Purification of the intracellular -Galactosidase

Galactosidase purification was carried out 3 step by DEAE-ion exchange column chromatography, FPLC MONO-Q ion exchange column chromatography and HPLC C4 column chromatography.

RESULTS AND DISCUSSION

Selection of acid- and bile-tolerant LAB

For LAB to survive in gastrointestinal juice and bile, tolerant characteristics for acid and bile were required. Therefore, the strains having A600 value above 0.2 in Lactobacilli MRS broth adjusted to pH 4.0 were selected as acid-tolerant strains, and the strains having A600 value above 1.0 were selected as bile-tolerant strains (Table 1). Thirty strains were determined in the 1st selection.

Physiological characteristics and Carbohydrate fermentation patterns of selected strains

As shown in Table 2, all the isolated strains we studied were gram-positive, catalase negative and non-spore forming rods which grew at 45℃ but did not grow at 15℃, and did not produce gas from glucose. Also these bacteria could be grown both anaerobic and aerobic conditions. Fermentation types of these strains were found to homolactic fermentation by HPLC analysis.

As a result of carbohydrate fermentation patterns of the eight selected strains (Table 3), four strains

Table 1. Growth (A600) of LAB isolates from Korean feces at various pH and bile salt concentrations (%) for 24 h in incubator

Stain No.	Sources	pH			Bile salt (%)		
		4	5	7	0	0.3	1.0
1	infant feces	0.081	0.486	1.467	1.467	2.054	0.257
2	infant feces	0.087	1.030	1.385	1.385	2.213	0.262
3	infant feces	0.097	0.641	1.239	1.239	2.459	0.268
4	infant feces	0.094	0.499	1.257	1.257	2.203	0.119
5	infant feces	0.090	0.470	1.358	1.358	2.310	0.133
6	infant feces	0.094	0.470	1.326	1.326	2.267	0.101
7	infant feces	0.280	0.736	1.444	1.444	2.343	1.220
8	infant feces	0.130	0.699	1.472	1.472	2.245	0.914
9	infant feces	0.102	0.682	1.275	1.275	0.451	0.173
10	infant feces	0.202	1.117	1.489	1.489	2.301	2.219
11	infant feces	0.209	0.615	1.255	1.255	2.156	2.214
12	infant feces	0.290	1.020	1.445	1.445	2.356	2.302
13	infant feces	0.241	0.979	1.290	1.290	1.306	2.008
14	infant feces	0.105	1.112	1.398	1.398	2.504	2.241

Table 1. Continued.

Stain No.	Sources	pH			Bile salt (%)		
		4	5	7	0	0.3	1.0
15	infant feces	0.100	0.899	1.392	1.392	1.414	2.384
16	infant feces	0.230	0.838	1.233	1.243	2.094	1.502
17	infant feces	0.122	0.872	1.278	1.278	2.016	1.687
18	infant feces	0.120	0.882	1.189	1.189	2.402	2.297
19	infant feces	0.122	0.624	1.362	1.362	2.130	0.353
20	child feces	0.464	1.142	1.379	1.379	1.321	1.235
24	child feces	0.110	1.420	1.558	1.558	2.300	0.590
25	adult feces	0.294	1.208	1.345	1.345	2.223	2.517
27	adult feces	1.264	1.327	1.557	1.557	2.381	2.389
30	adult feces	0.584	1.327	1.398	1.398	2.227	1.341
31	child feces	0.282	1.188	1.386	1.386	1.894	1.585
33	adult feces	0.280	1.007	1.380	1.380	2.110	2.020
34	adult feces	0.162	1.136	1.715	1.715	2.280	0.349
35	adult feces	0.272	1.384	1.568	1.568	2.371	1.740
37	adult feces	0.275	1.204	1.790	1.790	2.160	2.428
38	adult feces	0.233	0.980	1.400	1.400	1.191	2.062
39	adult feces	0.205	1.230	1.395	1.395	2.469	2.181
40	adult feces	0.230	0.943	1.516	1.516	2.426	2.169
41	adult feces	0.205	1.144	1.597	1.597	2.125	2.095
42	adult feces	0.105	0.780	1.508	1.508	1.214	0.505
43	adult feces	0.200	1.021	1.326	1.326	2.394	1.701
44	adult feces	0.293	1.115	1.528	1.528	1.477	1.507
45	adult feces	0.217	1.084	1.497	1.497	2.064	1.641
46	child feces	0.096	0.862	1.294	1.294	2.260	2.312
51	adult feces	0.215	1.130	1.677	1.677	0.704	1.466
52	adult feces	0.108	1.053	1.468	1.468	1.331	0.483
56	adult feces	0.100	0.807	1.315	1.315	2.389	2.150
57	adult feces	0.120	0.907	1.218	1.218	2.412	2.194
61	adult feces	0.215	0.289	1.590	1.590	0.400	1.114
62	adult feces	0.212	1.182	1.462	1.462	1.822	1.769
63	adult feces	0.208	1.118	1.367	1.367	1.854	1.960
64	adult feces	0.233	1.008	1.460	1.460	2.236	1.650
65	adult feces	0.265	0.937	1.518	1.518	2.133	1.840
71	adult feces	0.102	1.089	1.293	1.293	1.980	2.558
72	adult feces	0.104	1.086	1.492	1.492	2.101	2.164
73	adult feces	0.098	1.093	1.370	1.370	1.900	2.442
74	adult feces	0.104	1.025	1.301	1.301	2.713	2.143
75	adult feces	0.102	1.079	1.349	1.346	1.908	2.088
76	adult feces	0.105	1.024	1.393	1.393	2.070	1.700
77	adult feces	0.102	1.029	1.286	1.286	2.144	1.644
78	adult feces	0.242	1.101	1.346	1.346	2.330	1.486
79	adult feces	0.220	1.097	1.313	1.313	1.994	1.903
80	adult feces	0.485	0.956	1.507	1.507	2.329	1.181

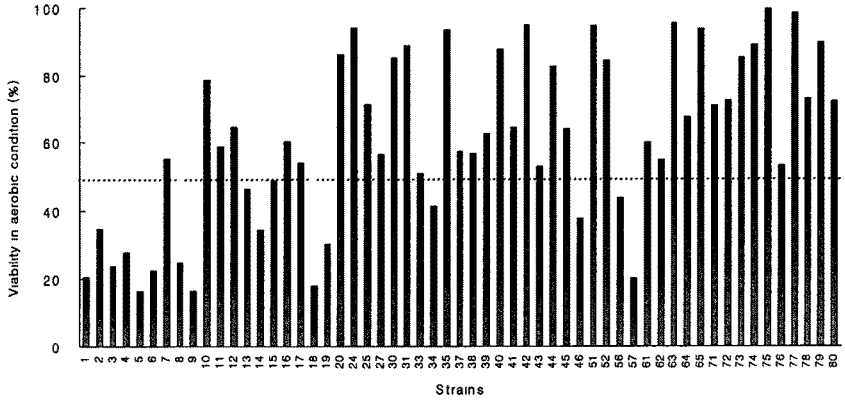


Fig. 1. Percent viability of LAB isolates from Korean feces at 37℃ for 2 days in aerobic condition.

Table 2. The physiological characteristics of the isolated strains from Korean feces

Characteristics	Isolated strains							
Gram reaction	12	20	Nam 27	30	35	37	62	80
Morphology	+	+	+	+	+	+	+	+
Spore formation	rods	rods	rods	rods	rods	rods	rods	rods
Aerobic growth	−	−	−	−	−	−	−	−
Anaerobic growth	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+
Catalase test	−	−	−	−	−	−	−	−
Growth at 15℃	−	−	−	−	−	−	−	−
Growth at 45℃	−	−	−	−	−	−	−	−
Fermentation type	+	+	+	+	+	+	+	+
	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo

Table 3. Identification of the isolated strains by carbohydrate fermentation patterns

Strains	Species Identification	Similarity(%)	
11	*L. rhamnosus*	96.6	acceptable identification
12	*L. rhamnosus*	99.9	good identification
20	*L. salivarius*	99.2	acceptable identification
27	*L. salivarius*	95.6	good identification
30	*L. rhamnosus*	99.9	good identification
35	*L. salivarius*	98.7	good identification
37	*L. salivarius*	98.9	acceptable identification
61	*L. rhamnosus*	−	unacceptable profile
62	*L. rhamnosus*	99.9	acceptable identification
63	*L. rhamnosus*	99.9	acceptable identification
64	*L. rhamnosus*	−	unacceptable profile
65	*L. rhamnosus*	−	unacceptable profile
78	*L. rhamnosus*	−	unacceptable profile
79	*L. rhamnosus*	−	unacceptable profile
80	*L. rhamnosus*	99.9	acceptable identification

Table 4. Cellular fatty acid composition of isolated strains from Korean feces

Fatty acid and its derivatives	Content (%) of fatty acid and its derivatives							
	12	20	27	30	35	37	62	80
12:0[a]	0.39	0.37	–	–	–	–	–	–
14:0	6.09	5.74	3.52	6.19	7.83	2.47	7.28	5.06
15:0	0.55	–	–	–	0.66	–	–	–
16:1 w7c	12.96	9.46	2.95	10.07	14.84	3.10	14.52	9.46
15:0 iso	–	3.00	1.99	–	–	1.43	–	–
16:0	12.03	34.29	44.52	15.89	15.84	29.45	14.23	15.13
15:0 30H	1.33	–	–	–	–	–	–	0.88
17:1 w8c	0.77	0.45	–	–	–	0.35	–	0.70
17:0 cyc	0.41	0.71	–	2.09	0.71	–	0.60	–
18:2 w6,9c	1.43	0.83	0.50		2.40	0.50	1.68	0.75
18:1 w9c	25.59	20.48	21.37	23.77	21.29	31.81	23.92	21.74
18:0	1.05	0.63	2.05	1.77	1.04	1.12	1.23	1.38
19:0 iso	0.86	–	–	–	0.84	–	0.78	0.80
19:0 cyc	36.54	24.04	23.10	40.22	34.55	29.77	35.76	44.10
Named(%)	95.55	95.04	96.62	96.27	96.83	97.68	93.99	97.58
Group of fatty acid pattern	A	B	B	A	A	C	A	A
Similarity	*L. casei*	*L salivarius*	*L salivarius*	*L brevis*	*L spp*	*L salivarius*	*L rhamnosus*	*L brevis*
(%)	56.2	46.7	22.7	20.6	11.5	80.5	22.4	48.9

[a] Number on the left of colon is the number of carbon atoms and that on the right is the number of double bond.

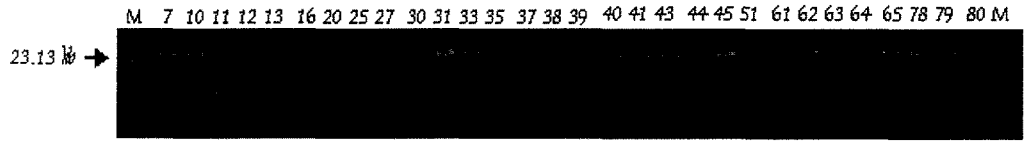


Fig. 2. Electrophoretic patterns of genomic DNA purified from isolated strains. Genomic M : DNA size marker, lanes 7 ~ 80; isolated strains from Korean feces.

belonged to *L. salivarius*, other four strains belonged to *L. rhamnsus*. Of *L. salivarius*, two subspecies have been recognized by Bergey's Manual *L. salivarius* subsp. *salivarius* that ferments rhamnose but not salicin and esculin, and *L. salivarius* subsp. *salicinus* that does not ferment rhamnose. Of the *L. salivarius* strains isolated in this work, four strains No. 20, 27, 35 and 37 were coincided with *L. salivarius* subsp. *salivarius* except salicine fermentation.

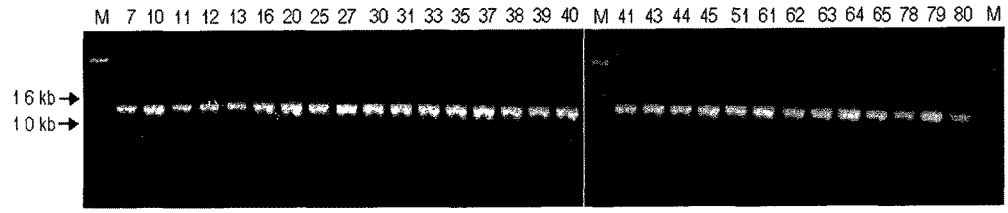
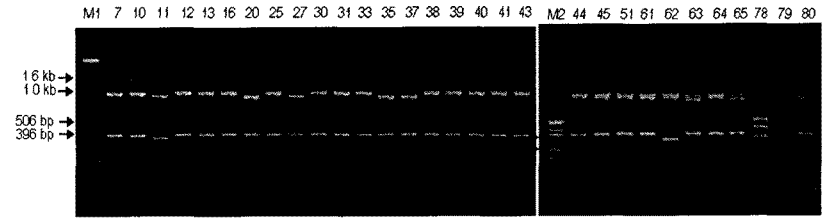
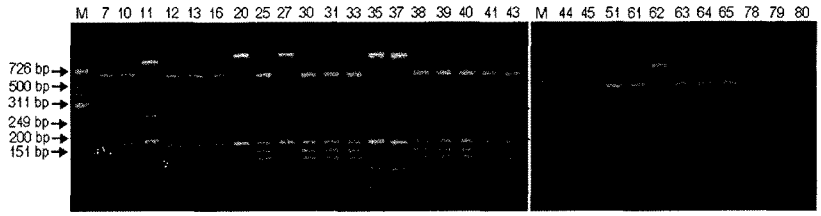


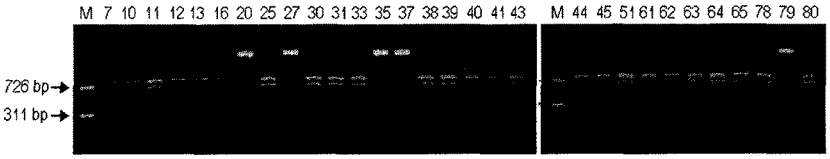
Fig. 3. The electrophoretic patterns of amplified PCR products.



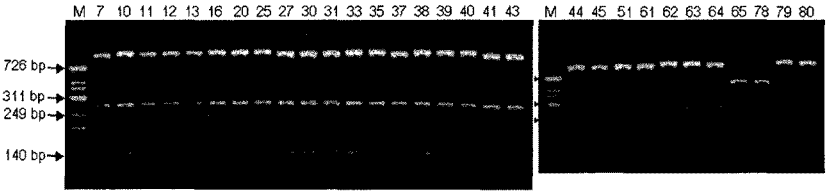
(A) *Hinf* I



(B) *Alu* I



(C) *EcoR* I



(D) *Rsa* I

Fig. 4. Restriction enzyme patterns of PCR products from the selected strains. The restriction enzymes are *Hinf* I (A), *Alu* I (B), *EcoR* I (C), *Rsa* I (D) for the analysis of sample. The restriction fragments were resolved on a 9% polyacrylamide gel.

Restriction enzyme digestion patterns of 16S rDNA from the selected lactobacilli

Fig. 4 shows AluI digestion patterns of 16S rDNA, which is obtained by PCR (Fig. 3), from the eight selected strains. Two types of patterns are shown: one group is No. 20, 27, 35 and 37:

another group is No. 12, 30, 62 and 80. These results were coincided with a carbohydrate fermentation pattern in Table 3. Thus, those strains of No. 20, 27, 35, and 37 identified as *L. salivarius* by carbohydrate fermentation test showed the same restriction enzyme pattern by AluI. And those strains of No. 12, 30, 62, 80 identified as *L. rhamnosus* also showed the same pattern, suggesting that restriction enzyme digestion pattern of 16S rDNA from lactobacilli could be used as a quick and simple method for identification of lactobacilli.

16S rDNA sequencing and phylogenetic analysis

Sequence of 16S rDNA was analyzed to determine which species would be matched with the strain No. 27, which was selected as the best one among observations about acid- and bile -tolerance, at the highest homology among LAB cited in GenBank. Sequence data (GenBank accession No. AF335475, the full-length 16S rDNA sequence of the strain No. 27 consists of 1517 bp) were aligned to construct the phylogenetic tree. Phylogenetic position of the strain No. 27 was compared with some of the LAB and related taxa in the dendrogram. In the phylogenetic tree, the strain 27 was the nearest one with *L. salivarius* subsp. *salivarius* ATCC1174T (Fig. 6).

In Fig. 6, the sequence of the strain No. 27 was the most identical with that of *L. salivarius* subsp. *salivarius* (99.9%). Regarding the carbohydrate fermentation profile and the 16S rDNA sequence, the strain 27 could be assigned to a strain of *L. salivarius* subsp. *salivarius.* The strains No. 20, 35 and 37 were all identified as *L. salivarius* subsp. *salivarius* from the partial sequencing results (about 1.1 Kb) of 16S rDNA.

```
   1 GACGAACGCT GGCGGCGTGC CTAATACATG  CAAGTCGAAC  GAAACTTTCT  TACACCGAAT  GCTTGCATTC
  71 ACCGTAAGAA GTTGAGTGGC GGACGGGTGA  GTAACACGTG  GGTAACCTGC  CTAAAAGAAG  GGGATAACAC
 141 TTGGAAACAG GTGCTAATAC  CGTATATCTC  TAAGGATCGC  ATGATCCTTA  GATGAAAGAT  GGTTCTGCTA
 211 TCGCTTTTAG ATGGACCCGC  GGCGTATTAA  CTAGTTGGTG  GGGTAACGGC  CTACCAAGGT  GATGATACGT
 281 AGCCGAACTG AGAGGTTGAT  CGGCCACATT  GGGACTGAGA  CACGGCCCAA  ACTCCTACGG  GAGGCAGCAG
 351 TAGGGAATCT TCCACAATGG  ACGCAAGTCT  GATGGAGCAA  CGCCGCGTGA  GTGAAGAAGG  TCTTCGGATC
 421 GTAAAACTCT GTTGTTAGAG  AAGAACACGA  GTGAGAGTAA  CTGTTCATTC  GATGACGGTA  TCTAACCAGC
 491 AAGTCACGGC TAACTACGTG  CCAGCAGCCG  CGGTAATACG  TAGGTGGCAA  GCGTTGTCCG  GATTTATTGG
 561 GCGTAAAGGG AACGCAGGCG  GTCTTTTAAG  TCTGATGTGA  AAGCCTTCGG  CTTAACCGGA  GTAGTGCATT
 631 GGAAACTGGA AGACTTGAGT  GCAGAAGAGG  AGAGTGGAAC  TCCATGTGTA  GCGGTGAAAT  GCGTAGATAT
 701 ATGGAAGAAC ACCAGTGGCG  AAAGCGGCTC  TCTGGTCTGT  AACTGACGCT  GAGGTTCGAA  AGCGTGGGTA
 771 GCAAACAGGA TTAGATACCC  TGGTAGTCCA  CGCCGTAAAC  GATGAATGCT  AGGTGTTGGA  GGGTTTCCGC
 841 CCTTCAGTGC CGCAGCTAAC  GCAATAAGCA  TTCCGCCTGG  GGAGTACGAC  CGCAAGGTTG  AAACTCAAAG
 911 GAATTGACGG GGGCCCGCAC  AAGCGGTGGA  GCATGTGGTT  TAATTCGAAG  CAACGCGAAG  AACCTTACCA
 981 GGTCTTGACA TCCTTTGACC  ACCTAAGAGA  TTAGGCTTTC  CCTTCGGGGA  CAAAGTGACA  GGTGGTGCAT
1051 GGCTGTCGTC AGCTCGTGTC  GTGAGATGTT  GGGTTAAGTC  CCGCAACGAG  CGCAACCCTT  GTTGTCAGTT
1121 GCCAGCATTA AGTTGGGCAC  TCTGGCGAGA  CTGCCGGTGA  CAAACCGGAG  GAAGGTGGGG  ACGACGTCAA
1191 GTCATCATGC CCCTTATGAC  CTGGGCTACA  CACGTGCTAC  AATGGACGGT  ACAACGAGTC  GCGAGACCGC
1261 GAGGTTTAGC TAATCTCTTA  AAGCCGTTCT  CAGTTCGGAT  TGTAGGCTGC  AACTCGCCTA  CATGAAGTCG
1331 GAATCGCTAG TAATCGCGAA  TCAGCATGTC  GCGGTGAATA  CGTTCCCGGG  CCTTGTACAC  ACCGCCCGTC
1401 ACACCATGAG AGTTTGTAAC  ACCCAAAGCC  GGTGGGGTAA  CCGCAAGGAG  CCAGCCGTCT  AAGGTGGGAC
1471 AGATGATTGG GGTGAAGTCG  TAACAAGGTA  GCCGTAGGAG  AACCTGC
```

Fig. 5. 16S rDNA sequence of the strain number 27.

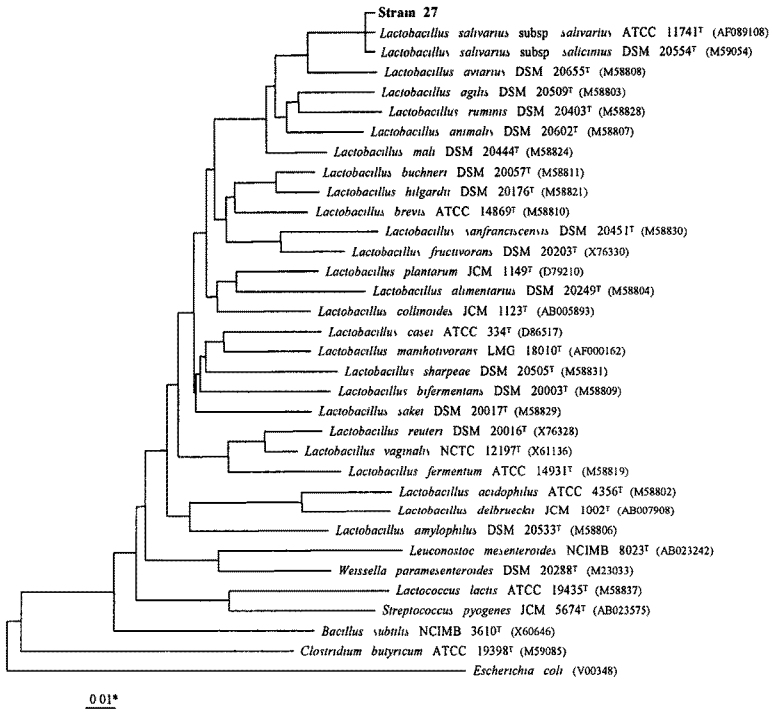


Fig. 6. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain 27, the type strains of some *Lactobacillus* species and the representatives of some others related taxa.

Table 5. Result in identification of selected LAB according to several methods

Strain No.	Methods of identification (similarity %)			
	Carbohydrates utilization pattern	Cellular fatty acid composition	PCR-RFLP	16S rDNA sequence
12	*L. rhamnosus* (99.9)	*L. casei* (56.2)	A	*L sakei* (99.5)
20	*L. salivarius* (99.2)	*L. salivarius* (46.7)	B	*L. salivarius* subsp. *salivarius* (99.7)
27	*L. salivarius* (95.6)	*L. salivarius* (22.7)	B	*L. salivarius* subsp. *salivarius* (99.9)
30	*L. rhamnosus* (99.9)	*L. brevis* (20.6)	A	*L. brevis* (97.3)
35	*L. salivarius* (98.7)	-	B	*L. salivarius* subsp. *salivarius* (99.7)
37	*L salivarius* (98.9)	*L. salivarius* (80.5)	B	*L. salivarius* subsp. *salivarius* (99.7)
62	*L. rhamnosus* (99.9)	*L rhamnosus* (22.4)	C	-
80	*L rhamnosus* (99.9)	*L. brevis* (48.9)	D	*L brevis* (98.4)

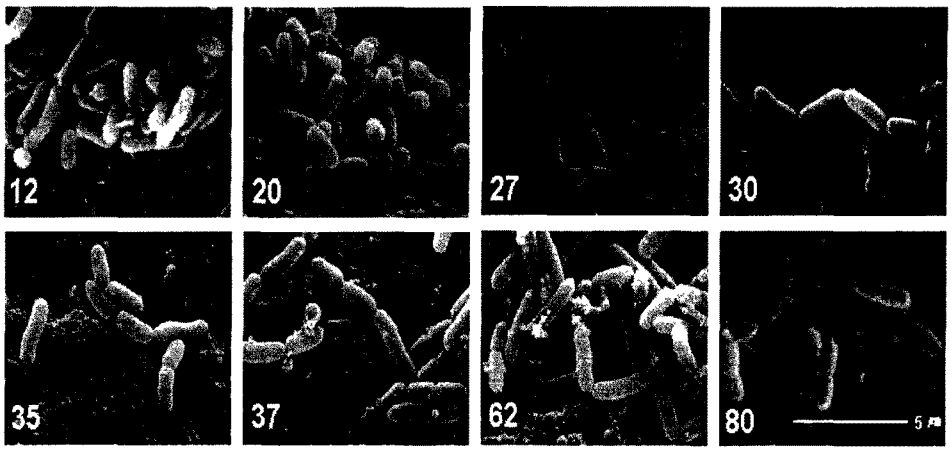


Fig. 7. Scanning electron micrograph showing cell morphology of the isolated LAB. (magnification × 5,000).

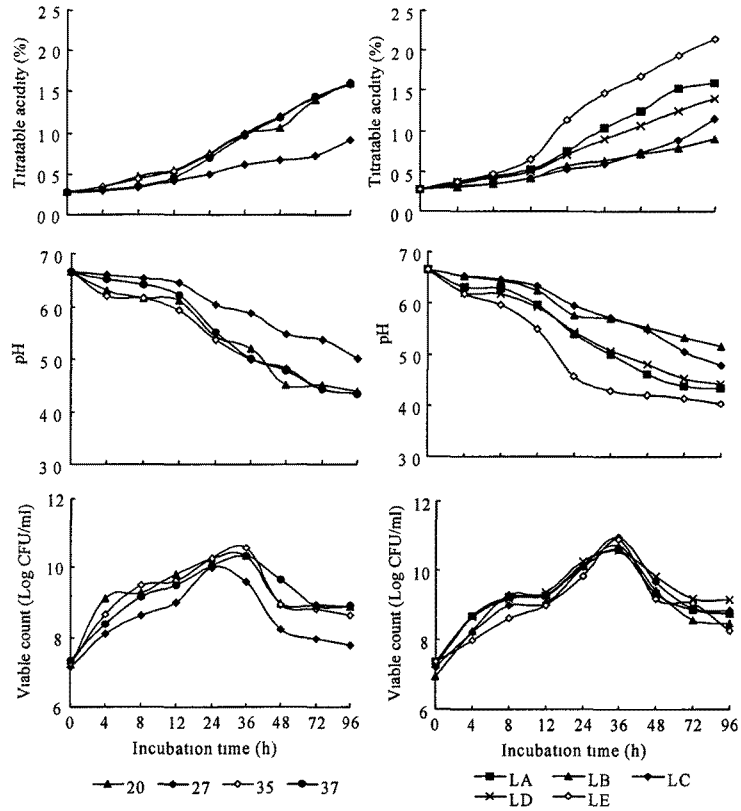


Fig. 8. Changes of titratable acidity, pH and viable cell counts during fermentation by the selected *L. salivarius* subsp. *salivarius* in skim milk at 37°C.

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.

Fermentation patterns

Acid production : Fig. 8 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 10^{10} 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 Fig. 8, in the case of *L acidophillus* 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 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*

Table 6. Contents of organic acids in fermented milk by LAB

Strains	Organic acid[1)] (mM)							
	Oxalic acid	Citric acid	Tartaric acid	Malic acid	Lactic acid	Formic acid	Acetic acid	Isobutylic acid
Control	–	7.63	32.84	2.39	12.94	1.13	22.10	–
12	0.33	7.45	25.01	1.27	126.08	3.66	22.79	9.13
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
30	0.07	6.13	22.35	2.11	119.07	3.58	29.81	10.86
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
62	0.10	6.31	23.23	1.92	124.08	3.43	28.99	12.58
80	0.09	5.67	24.28	3.47	128.71	3.24	35.72	–
LA	0.08	5.98	24.61	3.15	130.18	2.69	31.56	10.68
LB	0.07	7.10	26.39	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.

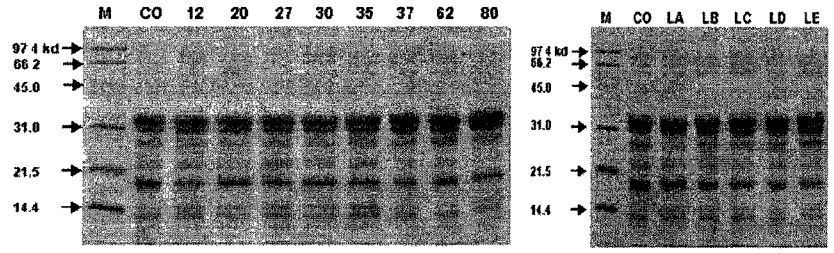
*Correlation coefficients between amount and area in standard calibration of organic acids by HPLC.

Control : skim milk.

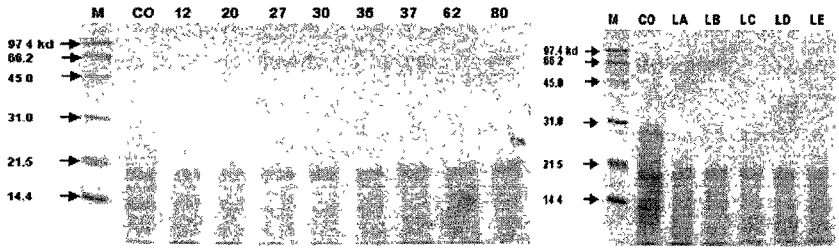
KCTC3619) in the acid productivity and growth ability in the skim milk.

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 6. All of the strains produced fermentation products such as a typical lactic acid fermentation. All of the selected *L. salivarius* subsp. *slaivarius* and standard strains produced fermentation products showed isobutylic acid. Butyric acid is a main source of energy of human intestine epithelia, which inhibit the growth of enteropathogenic *Clostridium perfringens*, and butric 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).

Proteolysis : Fig. 9(A) shows SDS-electrophoresis patterns for casein of raw milk and fermented milk by the isolated strains. Changes in the casein patterns did not be seem to be significant or different from each other; However, changes in the whey proteins (Fig. 9(B)) 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).



(A) Casein



(B) Whey protein

Fig. 9. Polyacrylamide gel electrophoretic (PAGE) patterns for casein(A) and whey protein(B) of raw milk and milk fermented by isolated strains. CO: raw milk.

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 hydrolized by the proteolytic enzyme.

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 7. 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

Table 7. 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.

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 Fig. 10, 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℃. Coliforms were not detected in any samples during the storage period. The strains 20, 35, and 37 tended to decrease slowly from 10^{10} cfu/ml to 10^{9} cfu/ml, but changes in the viable counts of strain 27 decreased significantly from 10^{8} cfu/ml to 10^{5} cfu/ml during storage; the substantial reduction took place after the 20 days of storage. The viability during storage is very useful for fermentation milk product and may play a role in its ability to survive in dairy products for delivery as a probiotic strain.

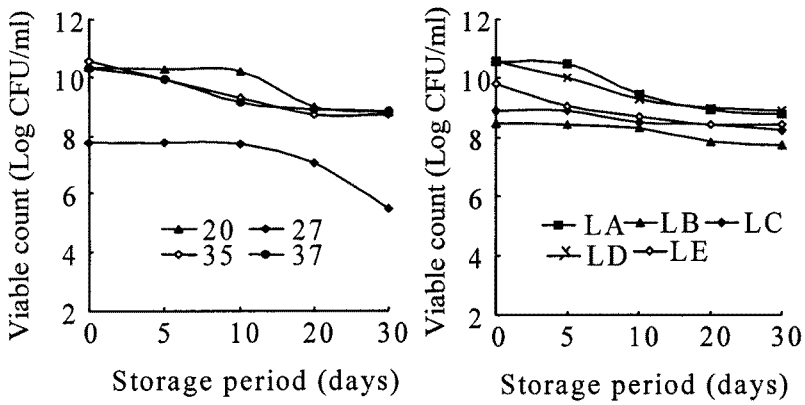


Fig. 10. Comparison of the viability of LAB during storage at 4C. 20, 27, 35, and 37 were selected *L. salivarius* subsp. *salivarius*.

Reduction of cholesterol concentration

As shown in Fig. 11, 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 *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

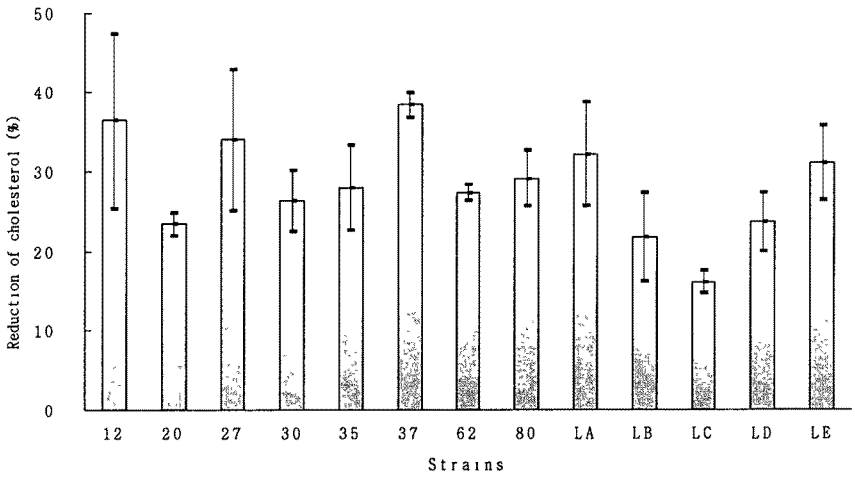


Fig. 11. Reduction of cholesterol concentration by LAB in MRS broth.

(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 8, 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 poduced 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 9, 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. acidophillus*). 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.

Table 8. Antimicrobial activity[1] of the selected LAB from Korean feces

Strains	PA		PB		PC		PD		PE		PF	
	R[2]	S[3]	R[2]	S[3]	R[2]	S[3]	R[2]	S[3]	R[2]	S[3]	R[2]	S[3]
12	++	P[4]	++	–	++	P	++	P	++	–	++	P
20	++	P	++	–	+	–	++	P	++	–	++	P
27	++	P	+	–	+	–	++	P	++	–	++	P
30	++	P	++	–	+	P	++	P	++	–	++	P
35	++	P	++	–	++	P	++	P	++	–	+++	P
37	++	P	+	–	++	P	++	P	++	–	++	P
62	++	P	+	–	++	P	++	P	++	–	++	P
80	++	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 ømm around the DISC (–, no inhibition zone; +, 9~14 mm inhibition zone; ++, 15~25 mm inhibition zone, +++, 26~35 mm inhibition zone; ++++, 36~45 mm inhibition zone).

R[2]: pH 4.5~5.0 of supernatants was not adjusted, S[3]: pH of supernatants was adjusted to 5.7±0.1 P[4]: partial inhibition

Table 9. Antibiotic resistances[1] of LAB depending upon various antibiotics

Antibiotics	Concentration	Strains									
		12	20	27	30	35	37	62	80	LE	HC
		---------- Inhibition zone (mm) ----------									
Amikacin	30 μg	10^{abc}	11^{abc}	11^{abc}	9^{bc}	12^{ab}	11^{abc}	10^{abc}	8^{c}	13^{a}	13^{a}
Ampicillin	10 μg	24^{d}	26^{cd}	30^{ab}	27^{bcd}	30^{ab}	27^{bcd}	25^{d}	32^{a}	30^{ab}	29^{abc}
Cephaiexin	30 μg	12^{d}	12^{d}	18^{b}	13^{d}	26^{a}	12^{d}	14^{cd}	18^{b}	15^{bcd}	17^{bc}
Colistin	10 μg	8^{a}	8^{a}	8^{a}	8^{a}	8^{a}	8^{a}	8^{a}	8^{a}	9^{a}	8^{a}
Ciprofloxacine	5 μg	15^{a}	17^{a}	8^{b}	16^{a}	8^{b}	16^{a}	17^{a}	8^{b}	17^{a}	15^{a}
Erythromycin	15 μg	30^{a}	32^{a}	25^{c}	30^{a}	23^{c}	32^{a}	30^{a}	26^{bc}	29^{ab}	32^{a}
Gentamycin	10 μg	10^{bc}	9^{c}	13^{ab}	10^{bc}	11^{abc}	9^{c}	10^{bc}	10^{bc}	14^{a}	14^{a}
Neomycin	30 μg	11^{abc}	10^{bc}	13^{ab}	10^{bc}	13^{ab}	9^{c}	11^{abc}	10^{bc}	13^{ab}	14^{a}
Nofloxacin	10 μg	13^{a}	15^{a}	8^{b}	13^{a}	8^{b}	13^{a}	14^{a}	8^{b}	14^{a}	14^{a}
Penicillin G	10 units	26^{d}	31^{bc}	32^{abc}	30^{c}	30^{c}	34^{ab}	30^{c}	34^{ab}	35^{a}	35^{a}
Spiramycin	100 μg	25^{ab}	25^{ab}	21^{c}	25^{ab}	20^{c}	25^{ab}	26^{ab}	23^{bc}	26^{ab}	27^{a}
Tetracycline	30 μg	33^{a}	32^{a}	23^{b}	31^{a}	24^{b}	31^{a}	33^{a}	21^{b}	31^{a}	33^{a}

[1] Estimation by the agar diffusion method, inhibition zone in ømm around the paper disc (ø8mm).

[a b c, d] In a row, means follwed by a common letter are not significantly different at the 1% level by DMRT.

12~80 : LAB isolates, LE : *L acidophilus* KCTC3145, HC : *L. casei* YA-70.

salivarius (isolated strain 27 and 35) showed the highest resistance to ciprofloxacin and nofloxacin 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.

Enzyme activity patterns of the selected lactobacilli

Enzyme activity patterns of eight strains selected in the 2nd step were examined by using API-ZYM enzyme system (Table 10). All of eight strains possess β-galactosidase, and No. 20 and 27 possess α-galcatosidase strongly. All of eight strain do not possess β-Glucuronidase. β -Glucuronidase could invert benzopyrene to carcinogenic substance, therefore it is called carcinogenic enzyme (Nanno et al. 1986). The strains producing leucine arylamidase were considered as acid producer (Desjardins et al., 1990). All of the eight selected strains showed the positive results for leucine arylamidase, suggesting that the eight strains all should be acid producers.

Table 10. Enzyme profiles of the selected strains from Korean feces

No.	Enzyme	Strains														
		11	12	20	27	30	35	37	61	62	63	64	65	78	79	80
1	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	Alkaline phosphatase	3	5	3	3	5	5	5	3	5	5	5	4	5	5	5
3	Esterase(C4)	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
4	Esterase Lipase(C8)	4	5	5	4	5	5	5	5	5	5	5	4	5	5	5
5	Lipase(C14)	3	3	3	3	3	3	2	3	2	2	2	3	3	3	3
6	Leucine arylamidase	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
7	Valine arylamidase	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
8	Crystine arylamidase	3	4	3	3	4	4	4	3	3	3	3	4	3	3	3
9	Trypsin	3	1	0	0	1	0	0	1	1	1	1	0	0	1	0
10	α-chymotrypsin	5	4	3	3	5	5	3	5	5	5	5	5	5	5	5
11	Acid phospatase	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
12	Naphtol-AS-B1-phosphohy drolase	5	5	5	5	4	5	5	5	5	5	4	5	5	5	5
13	α-galactosidase	0	0	5	5	0	0	0	0	0	0	0	0	0	0	0
14	β-galactosidase	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
15	β-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	α-glucosidase	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
17	β-glucosidase	5	5	4	2	5	4	5	5	5	5	4	4	4	4	4
18	N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	α-mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	α-fucosidase	2	1	1	0	1	1	1	1	1	1	1	1	1	1	1

1: A value ranging from 0 to 5 is assigned to the color standard. 0 represent a negative reaction; 5 represent a maximum intensity reaction. Values 1~4 represent intermediate reaction. The approximate activity may be estimated from color strength; 1 corresponds to 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles, 5 to 40 nanomoles.

Characterization of α-galactosidase from *L. salivarius* subsp. *salivarius* 27

Lactobacillus salivarius subsp. *salivarius* 27 possess a high level of α-galactosidase activity. In this study the α-galactosidase (α-D-galactoside-galactohydrolase, EC 3.2.122) has been purified and characterized from the soluble intracellular fraction of *L. salivarius* subsp. *salivarius*. Purified α -galactosidase was obtained after sonication of harvested cell pellet followed by DEAE-Sephadex A-50 ion exchange chromatography (Fig. 13) and Mono Q anion exchange chromatography (Fig. 14). The specific activity of the purified enzyme, tested with *p*-nitrophenyl-α-galactopyranoside as substrate, was 8,994 units/mg protein, representing an 17.09 folds purification of the original cell crude extract (Table 11). The molecular weight of native enzyme was about 53,000 dalton and

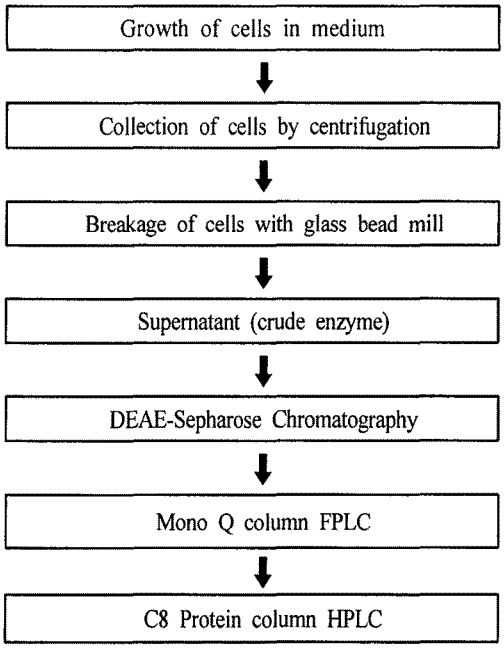


Fig. 12. Schematic diagram for purification of α-galactosidase from *Lactobacillus salivarius* subsp. *salivarius* 27.

Table 11. Effect of different steps in the purification of α-galactosidase

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (Unit/ml)	Specific activity (Unit/mg)	Total enzyme activity (Unit)	Fold Purification	Yield (%)
Cell extract	200	3.70	740.0	1,947	526	389,472	1.00	100
DEAE chromatography	60	0.38	23.0	700	1,825	41,975	3.47	10.8
Mono Q chromatography	4.5	0.20	0.9	1,799	8,994	8,095	17.09	2.1

the enzyme consisted of monomer in 12% SDS-PAGE gels (Fig. 16). Optimal temperature and pH for activity of this enzyme were 40℃ and 7.0 respectively. The enzyme was found to be stable between 25 and 50℃ (Fig. 17). α-galactosidase activity was lost rapidly below pH5.0 and above pH9.0. The enzyme liberated galactose from melibiose, raffinose, and stachyose, and also the hydrolysis rate of substrate was compared by HPLC (Fig. 18).

In conclusion, the probiotic potential of *L. salivarius* subsp. *salivarius*, which slected acid-tolerant (pH 4.0) and bile-tolerant (1.0% bile salt) strains with the lactobacilli MRS broth in the 1st step, showed various probiotic features such as fermentation characteristic in skim milk, reduction of cholesterol contents, antibacterial effect, enzyme characteristic, etc in the 2nd step. These results indicated that strain 27 and 35 among selected *L. salivarius* subsp. *salivarius* have potential benefits for industrial application.

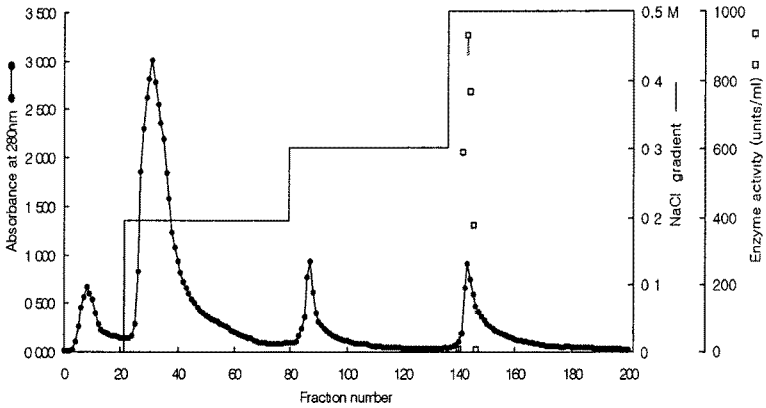


Fig. 13. DEAE-sepharose anion exchange chromatogram of α-galactosidase of *Lactobacillus salivarius* subsp. *salivarius* 27.

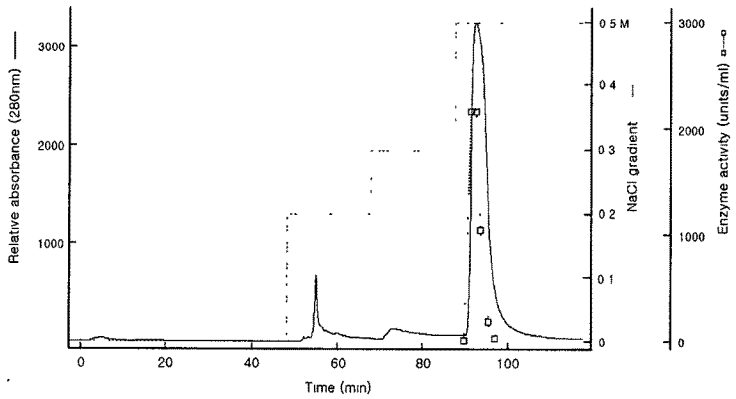


Fig. 14. Mono Q anion exchange chromatography of α-galactosidase from *Lactobacillus salivarius* subsp. *salivarius* 27.

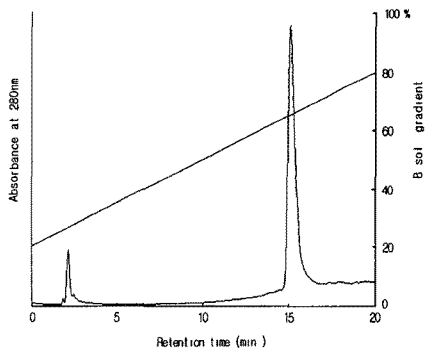


Fig. 15. HPLC pattern of the purified α-galactosidase

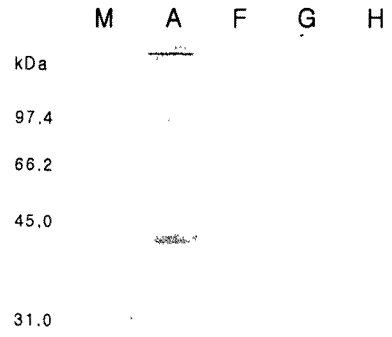


Fig. 16. SDS-PAGE of different steps in the purification of α-galactosidase.

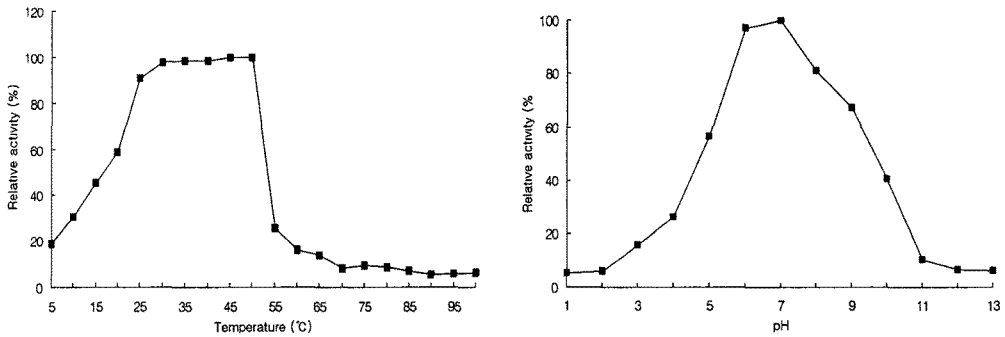
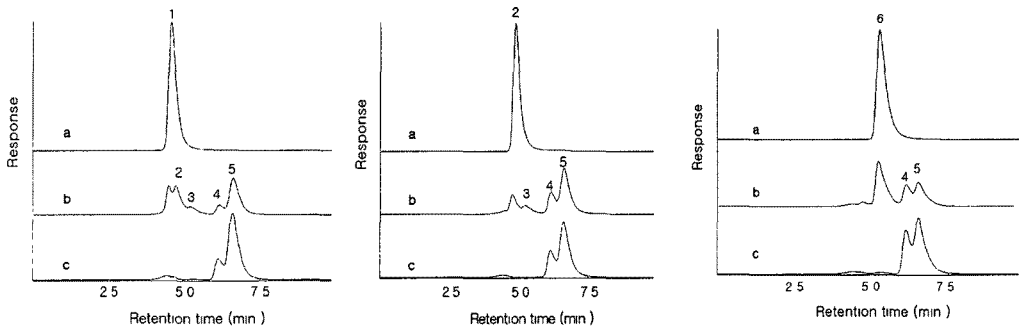


Fig. 17. Optimum temperature and pH of α-galactosidase from *Lactobacillus salivarius* subsp. *salivarius* 27.



(A) Hydrolysis of Stachyose (B) Hydrolysis of Raffinose (C) Hydrolysis of Melibiose

Fig. 18. Hydrolysis patterns of stachyose (A), raffinose (B) and melibiose (C) by α-galactosidase from *Lactobacillus salivarius* subsp. *salivarius* CNU27. a is o hour hydrolysis, b is 6 hour hydrolysis, c is 24 hour hydrolysis. 1 is stachyrose, 2 is raffinose, 3 is sucrose, 4 is glucose, 5 is galactose, 6 is melibiose.

REFERENCES

Alm, L. 1982a. Effect of fermentation on lactose, glucose, and galactose content in milk and suitability of fermentd milk products for lactose intolerant individuals. J. Dairy Sci. 65:346-352.

Alm, 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.

Bae, H. C., M. S. Nam and J. Y. Lee. 2002. Probiotic Characterization of Acid- and Bile-tolerant *Lactobacillus salivarius* subsp. *salivarius* from Korean Faeces. Asian-Aust. J. Anim. Sci. 15(12):1798-1807.

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.

Berg, R. D. 1998. Probiotics. prebiotics or "conbiotics". Trends Microbiol. 6:89-92.

Bernardeau, M., J. P. Vernoux 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. Georgeand 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 latobacilli. The 6th international symposium on lactic acid bacteria and human health. pp. 245-273.

Collin, E. B. and B. J. Hall. 1984. Growth of bifidobacteria in milk and preparation of Bifdobacterium infantisfor dairy adjunct. J. Dairy Sci. 67:1376-1380.

Conway, P. L., S. L. Gorbach and B. R. Golden. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70:1-12.

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 yohurt. J. Dairy Sci. 81:2804-2816.

Desjardins, M. L., D. Roy, C. Toupin and J. goulet. 1990. Uncoupling of growth and acids production in *Bifidobacterium* spp. J. Dairy Sci. 73:1478-1484.

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.

Fernandes, C. F., K. M. Shahani and M. A. Amer. 1987. Therapeutic role of dietary lactobacilli and lactobacilli fermented dairy products. FEMS Microbiol. Rev. 46:343-356.

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.

Fuller, R. 1992a. A review: probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Fuller, R. 1992b. Probiotics In: The Scientific Basis. (Ed. Chapman & Hall). NY.

Gao, Z. and A. A. Schaffer. 1999. A Novel alkaline α-galactosidase from melon fruit with a substrate preference for raffinose. Plant Physiology. 119:979-987.

Gill, H. S. and K. J. Rutherfurd. 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. 1979. Beneficial interrelationships between certain microorganisms and human: candidate microorganisms for use as dietary adjuncts. J. Food Prot. 42:164-167.

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. 1990. Health and nutritional benefits from lactic acid bacteria. FEMS Microbiol. Rev. 87:175-188.

Gilliland, S. E., C. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 49:377-381.

Goldin, B. R. and S. L. Gorbah. 1992. Probiotics for humans. In: Fuller R. ed. Probiotics the scientific basis. London: Chapman and Hall, pp.355-376.

Goodenough, E. R. and D. H. Kleyn. 1976. Influence of viable yogury microflora on digestion of lactose by the rat. J. Dairy Sci. 59:601-606.

Guarner, F. and G. J. Schaafsma. 1998. Probiotics. Int. J. Food Microbiol. 39:237-238.

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. Gillilacd. 1983. *Lactobacillus acidophilus* as a dietary adujunct 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.

Kwag, J. H., J. C. Lee, T. H. Kim, P. K. Chung and K. K. Lee. 1989. Isolation and characteriaztion of a butyric acid bacterium from infant feces, Kor. J. Appl. Microbiol. Bioeng. 17:56-62.

Lankaputhra W. E. V. and N. P. shah. 1995. Survival of *Lactobacillus acidophilus* and *Bifidobacterum* spp. in the presence of acid and bile salts. Cult. Dairy Prod. J. 30:2-7.

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.

Maniatis, t., e. F. Fritsch and J. Sambrook. 1982. Molecular Cloning, a Laboratory Manual. Cold

Spring Harbor Laboratory. NY.

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.

Metchnikoff, E. 1908. The Prolongation of Life. (Ed. G. P. Putnams & Sons). NY.

Mitsuoka, T. 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol. 6:263-268.

Montelongo, J. L. 1995. Alpha-galactosidase from *lactobacillus salivarius*: isolation, purification, and biochemical characterization. Ph. D. thesis, Illinois Univ., Urbana, U.S.A. pp. 216.

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.

Nanno, M., M. Morotomi, H. Takayama, T. Kuroshima, R. Tanaka and M. Mutai. 1986. Mutagenic activation of biliary metabolites of benzo(a) Pyrene by β-glucuronidase-positive bacteria in human faeces. J. Med. Bacteriol. 22:351-355.

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. Alverez, G. Oliver and A. A. D. Ruiz. 1990. Prevention of gastrointestinal infection using immounobiological methods with milk fermented with milk fermented with *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Dairy Res. 57:255-259.

Pirkka, V. K., C. O. Arthur, I. Erika and J. S. Seppo. 1998. The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiology Letter. 167:185-189.

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.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Saxelin, M. 1991. Dose response colonisation of faeces after oral administration of *Lactobacillus casei* strain GG. Microb. Ecol. Health Dis. 4:209-214.

Shin, S. Y., J. H. Lee, S. H. Huh, Y. S. Park, J. M. Kim and I. S. Kong. 2000. Overexpression and characterization of *Vibrio mimicus* Metalloprotease. J. Microbiol. Biotchnol. 10(5):612-619.

Singh, B. and T. K. Bhat. 2001. Exploiting gastrointestinal microbes for livestock and industrial

development. - Review -. Asian-Aust. J. Anim. Sci. 14:567-586.

Singh, B., T. K. Bhat and B. Singh. 2001. Exploiting Gastrointestinal Microbes for Livestock and Industrial Development - Review -. Asian-Aust. J. Anim. Sci. 14(4):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.

Stackebrandt, E. and W. Liesack. 1993. Nucleic acids and classification. In: Handbook of new bacterial systematics, (Ed. M. Good fellow & A. G. O'Donnell). Academic Press, London, pp. 152-189.

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.

Tannock, G. W. 1990. The microecology of *lactobacilli inhabiting* the gastrointestinal tract. Adv. Microb. Ecol. 11:147-171.

Thompson, J. D., D. G. Higgins and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.

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(12):5631-5635.

Wood, B. J. B. 1992. The Lactic Acid Bacteria in Health and Disease. Vol. 1. Elsevier Applied Sci., London, England.

Yeo, N. I., Lee, S. K. and G. E. Ji. 1993. Characterization of α-galactosidase from *Bifidobacterium* sp. Int-57. Korean J. Food Sci. Technol. 25(6):689-693.

Yuguchi, H., T. Goto and S. Okonogi. 1992. Fermented milks, lactic drinks and intestinal microflora. In: Functions of Fermented Milk; Challenges for the Health Sciences. (Ed. Y. Nakazawa and A. Hosono). Elsevier Applied Sci., NY. pp. 247-272.