Isolation and Identification of Acid- and Bile-Tolerant *Lactobacillus salivarius* subsp. *salivarius* from Human Faeces

Hyoung Churl Bae, Seong Hyun Choi[1] and Myoung Soo Nam*

Division of Animal Science & Resources, College of Agriculture, Chungnam National University, Daejon 305-764, Korea

ABSTRACT : This study was conducted to isolate lactobacilli having 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 human faeces. Bile tolerant strains were examined in Lactobacilli MRS broth in which 1.0% bile salt was added. Microhemagglutination tests using swine erythrocytes were performed to select lactobacilli having adherence properties to survive in the intestinal tract. By examination of these characteristics the strain Nam 27, which was isolated from adult faeces, was selected and identified as *Lactobacillus salivarius* subsp. *salivarius* based on carbohydrate fermentation and 16S rDNA sequencing. ***(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 8 : 1170-1178)***

Key Words : Acid-, Bile-tolerant, Microhemagglutination, Probiotic, *Lactobacillus salivarius*

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 use as probiotic bacteria should be able to tolerate acid for 90 min, and tolerate bile, attach to the epithelium, and grow in the lower intestinal 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.

The aim of the present study was to isolate and develop a new probiotic strain. We isolated lactobacilli from human faeces, and selected acid- and bile-tolerable LAB in the 1st step. In a 2nd step, microhemagglutination test with swine erythrocyte was performed as an *in vitro* test to obtain lactobacilli attachable to epithelium in the intestinal tract. The best selected strain was identified as *L. salivarius* subsp. *salivarius*.

MATERIALS AND METHODS

Bacterial strains and media

Salmonella sp. was grown in Trypticase Soy Broth (BBL, Cockeysville, MD) for 18 h at 37°C. *E. coli* was grown in LB (Difco, Detroit, MI)) for 18 h at 37°C. *Lactobacilli* were isolated from faeces of 60 healthy persons (infants, children and adults). Faeces samples diluted in 0.1 M phosphate-buffered saline (PBS; 0.85% NaCl, pH 7.2) were plated on *Lactobacillus* Selection (LBS) agar and incubated for 48 h at 37°C in a carbon dioxide enriched atmosphere (the LBS agar was prepared according to the manufacturer's direction). Isolated colonies were inoculated into Lactobacilli MRS Broth (Difco) and incubated for 18 h

* Address reprint request to Myoung Soo Nam. Tel: +82-42-821-5782, Fax: +82-42-823-2766, E-mail: namsoo@cnu.ac.kr.

[1] Department of Food Science & Technology, College of Agriculture, Chungnam National University, Daejon 305-764, Korea.

Received January 27, 2001; Accepted March 19, 2001

at 37°C. Stock cultures were prepared by growing the strains for 16 to 24 h and inoculating 0.3 ml into 1.2 ml of 12% skim milk solution containing 2% glycerol. Each vial was frozen immediately and was stored at -70°C for further use.

Selection of acid-tolerant isolates

Each strain isolated from the faeces was cultured twice in Lactobacilli MRS Broth for 16 to 24 h at 37°C. 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°C. For selection of acid-tolerant strains, bacterial growth was measured as cell density at 600 nm by spectrophotometer after 24 h incubation.

Selection of bile-tolerant isolates

Lactobacilli MRS Broth was prepared with 0.3 and 1.0% of bile salts (Difco), and was dispensed in 10 ml volumes and sterilized by autoclaving at 121°C for 15 min. Each strain isolated from faeces was incubated for 8, 16, 24 h at 37°C. The growth of the isolates was measured as mentioned in acid-tolerance above.

Microhemagglutination test

Swine blood was collected in vacuum tubes (Becton Dickinson vacutainer system) containing 0.5 M EDTA as anticoagulant. The erythrocytes were collected from the blood by centrifuging at 3,000×g for 15 min and diluted with 0.1 M PBS to make a 2% erythrocyte cell suspension of packed cells. Bacterial cells were harvested with PBS from cultures grown on Lactobacilli MRS agar for 24 h at 37°C and adjusted to approximately 2.0 of observance at 640 nm. Serial two-fold dilutions of each bacterial suspension (50 μl) were prepared in the wells of microtiter plates (Falcon 3911, Becton Dickinson Co. USA) and equal volumes of 2% erythrocyte suspension were added. Microtiter plates were rotated at 150 rpm for 20 min and kept at 4°C for 3～4 h until the degree of agglutination was determined visually (Choi et al., 1996; Shin et al., 2000).

Carbohydrate fermentation and enzyme activity patterns

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). Enzyme activity patterns of the selected strains were examined by using API-ZYM enzyme system (bioMerieux SA, Marcy-l' Etoile, France). Further identification of *Lactobacillus salivarius* Nam 27 was confirmed by 16S rDNA sequence.

DNA amplification by PCR

Lactobacilli cells were centrifuged at 3,000×g for 15 min, diluted with TES buffer, and were mixed completely with microbial pellets by softly vortexing. 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'-GAGTTTGA-TCCTGGCTCAG-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). PCR was performed in a final reaction volume of 100 μl which contained 0.5 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 μM $MgCl_2$, 0.01% (w/v) gelatin and 2.5 U of Taq DNA polymerase in a DNA thermal cycle (Model 2400, Perkin-Elmer Co., USA). Thermal conditions for the first round of PCR were 95°C for 3 min, followed by 35 cycle of 1 min at 95°C, 62°C for 1 min, 72℃ 1 min and final extension step at 72°C for 10 min.

Restriction enzyme digestion of PCR products

Reaction and thermal conditions for restriction enzyme digestion of PCR product were as follows ; 10 μl of PCR products were digested for 3 h at 37°C with 10 units of *Alu* I (Bioneer Co., Korea) in a total volume of 20 μl. Reaction mixture of PCR products was resolved by 3% agarose gel electrophoresis (Hoefer Scientific Instruments, USA) at 120 V for 1 h and λDNA digested with *Hind*Ⅲ (Sigma, USA) was used as a DNA standard marker.

16S rDNA sequencing of the strain Nam 27

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

The sequencing was made up of 50 ng/μl of DNA template, 3.2 pmol of primer and 8 μl of terminator reaction mix (Bigdye terminator, Perkin Elmer) in a total volume of 20 μl. Thermal conditions were 25 cycles at 96℃ for 10 sec, 50°C for 5 sec, 60°C for 4 min. Reaction products were precipitated with an ethanol treatment. The loading samples containing cocktail (formamide, 50 mM EDTA, 50 mg/ml Blue dextran) were heated to 95°C and resolved by 5% polyacrylamide gel electrophoresis. Sequences of PCR products after electrophoresis were analysed by DNA sequencing analysis software (Perkin Elmer Co.). The 16S rDNA sequence of the strain Nam 27 determined in the present study has been deposited in the GenBank, NCBI

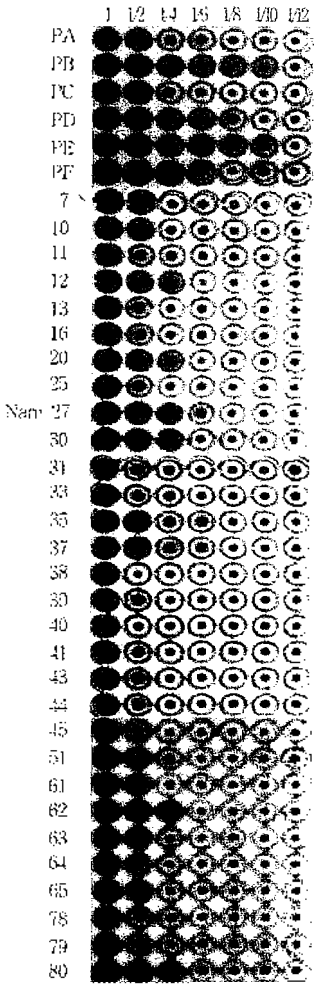

Figure 1. Results of microhemagglutination with swine erythrocyte of isolated strains
PA, *E. coli* KCTC 1039; PD : *S. typhimurium* M-15
PB, *E. coli* KCTC 1021; PE : *S. typhimurium* KCCM 40253
PC, *E. coli* KCTC 0115; PF : *S. enteritidis* KCCM 3313
1～80 : LAB isolates

data library under accession number AF335475.

Phylogenetic Analysis of the strain Nam 27

The 16S rDNA sequence of the strain Nam 27 determined in this study was aligned by using CLUSTAL W software (Thompson et al., 1994). The sequence of representative species of the genus *L. salivarius* and related taxa were cited by using GenBank database. The values of 16S rDNA similarity were calculated from the alignment and the evolution any distances were calculated by using Kimura two-parameter correction. A phylogenetic tree was constructed by using the neighbor-joining method (Saitou and Nei, 1987) from a distance matrix calculated.

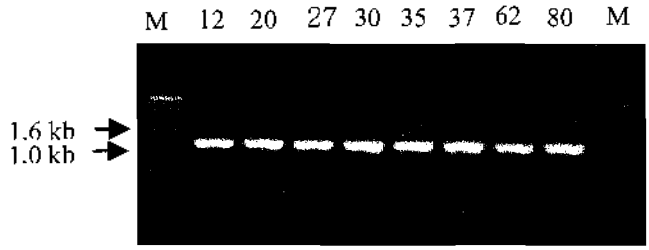

Figure 2. The electrophoresis of amplified PCR products (M is DNA size marker; 1 kb DNA Ladder)

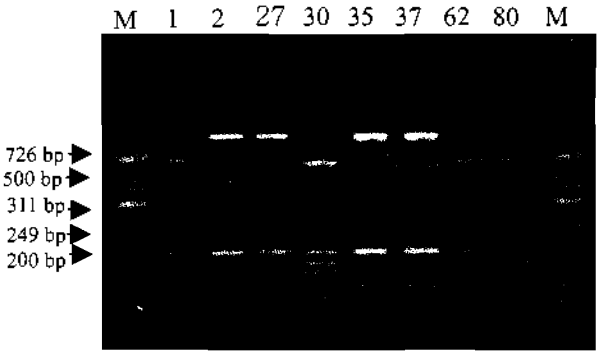

Figure 3. Restriction enzyme patterns of PCR products from the selected strains. The restriction enzyme is *Alu* I for the analysis of sample. The restriction fragments were resolved on a 9% polyacrylamide gel (M is DNA size marker; ΦX174 DNA/*Hinf* I Markers).

RESULTS

Selection of acid- and bile-tolerant LAB

For LAB to survive in gastrointestinal juice and bile, tolerance characteristics for acid and bile were required. Therefore, the strains having A_{600} value above 0.2 in Lactobacilli MRS Broth adjusted to pH 4.0 were selected as acid-tolerant strains, and the strains having A_{600} value above 1.0 were selected as bile-tolerant strains (table 1). Thirty strains were determined in the 1st selection.

Microhemagglutination test

Swine erythrocytes were used for microhemagglutination test (figure 1). The strain Nam 27 shows the best result among isolated lactobacilli and shows positive reaction by one sixth of bacterial dilution. Adhesion ability of microorganisms in intestinal track could be estimated by the microhemagglutination test using swine erythrocyte (Shin et al., 2000). Adhesion ability of isolated lactobacilli, pathogenic strains of *Salmonella* sp., and *E. coli* was tested as references. *Salmonella* sp., which is causative of enteric fever and enterocolitis, having flagella and shows as excellent an adhesion ability as swine erythrocytes by one tenth of bacterial dilution in microtiter plate.

Table 1. Growth (A_{600}) of LAB isolates from human faeces at various pH and bile salt concentrations (%) for 24 h in CO_2 incubator

Strain No.	Sources	pH			bile salt (%)		
		4	5	7	0	0.3	1.0
1	infant faeces	0.081	0.486	1.467	1.467	2.054	0.257
2	infant faeces	0.087	1.030	1.385	1.385	2.213	0.262
3	infant faeces	0.097	0.641	1.239	1.239	2.459	0.268
4	infant faeces	0.094	0.499	1.257	1.257	2.203	0.119
5	infant faeces	0.090	0.470	1.358	1.358	2.310	0.133
6	infant faeces	0.094	0.470	1.326	1.326	2.267	0.101
7	infant faeces	0.280	0.736	1.444	1.444	2.343	1.220
8	infant faeces	0.130	0.699	1.472	1.472	2.245	0.914
9	infant faeces	0.102	0.682	1.275	1.275	0.451	0.173
10	infant faeces	0.202	1.117	1.489	1.489	2.301	2.219
11	infant faeces	0.209	0.615	1.255	1.255	2.156	2.214
12	infant faeces	0.290	1.020	1.445	1.445	2.356	2.302
13	infant faeces	0.241	0.979	1.290	1.290	1.306	2.008
14	infant faeces	0.105	1.112	1.398	1.398	2.504	2.241
15	infant faeces	0.100	0.899	1.392	1.392	1.414	2.384
16	infant faeces	0.230	0.838	1.233	1.243	2.094	1.502
17	infant faeces	0.122	0.872	1.278	1.278	2.016	1.687
18	infant faeces	0.120	0.882	1.189	1.189	2.402	2.297
19	infant faeces	0.122	0.624	1.362	1.362	2.130	0.353
20	child faeces	0.464	1.142	1.379	1.379	1.321	1.235
24	child faeces	0.110	1.420	1.558	1.558	2.300	0.590
25	adult faeces	0.294	1.208	1.345	1.345	2.223	2.517
Nam 27	adult faeces	1.264	1.327	1.557	1.557	2.381	2.389
30	adult faeces	0.584	1.327	1.398	1.398	2.227	1.341
31	child faeces	0.282	1.188	1.386	1.386	1.894	1.585
33	adult faeces	0.280	1.007	1.380	1.380	2.110	2.020
34	adult faeces	0.162	1.136	1.715	1.715	2.280	0.349
35	adult faeces	0.272	1.384	1.568	1.568	2.371	1.740
37	adult faeces	0.275	1.204	1.790	1.790	2.160	2.428
38	adult faeces	0.233	0.980	1.400	1.400	1.191	2.062
39	adult faeces	0.205	1.230	1.395	1.395	2.469	2.181
40	adult faeces	0.230	0.943	1.516	1.516	2.426	2.169
41	adult faeces	0.205	1.144	1.597	1.597	2.125	2.095
42	adult faeces	0.105	0.780	1.508	1.508	1.214	0.505
43	adult faeces	0.200	1.021	1.326	1.326	2.394	1.701
44	adult faeces	0.293	1.115	1.528	1.528	1.477	1.507
45	adult faeces	0.217	1.084	1.497	1.497	2.064	1.641
46	child faeces	0.096	0.862	1.294	1.294	2.260	2.312
51	adult faeces	0.215	1.130	1.677	1.677	0.704	1.466
52	adult faeces	0.108	1.053	1.468	1.468	1.331	0.483
56	adult faeces	0.100	0.807	1.315	1.315	2.389	2.150
57	adult faeces	0.120	0.907	1.218	1.218	2.412	2.194
61	adult faeces	0.215	0.289	1.590	1.590	0.400	1.114
62	adult faeces	0.212	1.182	1.462	1.462	1.822	1.769
63	adult faeces	0.208	1.118	1.367	1.367	1.854	1.960
64	adult faeces	0.233	1.008	1.460	1.460	2.236	1.650
65	adult faeces	0.265	0.937	1.518	1.518	2.133	1.840
71	adult faeces	0.102	1.089	1.293	1.293	1.980	2.558
72	adult faeces	0.104	1.086	1.492	1.492	2.101	2.164
73	adult faeces	0.098	1.093	1.370	1.370	1.900	2.442
74	adult faeces	0.104	1.025	1.301	1.301	2.713	2.143
75	adult faeces	0.102	1.079	1.349	1.346	1.908	2.088
76	adult faeces	0.105	1.024	1.393	1.393	2.070	1.700
77	adult faeces	0.102	1.029	1.286	1.286	2.144	1.644
78	adult faeces	0.242	1.101	1.346	1.346	2.330	1.486
79	adult faeces	0.220	1.097	1.313	1.313	1.994	1.903
80	adult faeces	0.485	0.956	1.507	1.507	2.329	1.181

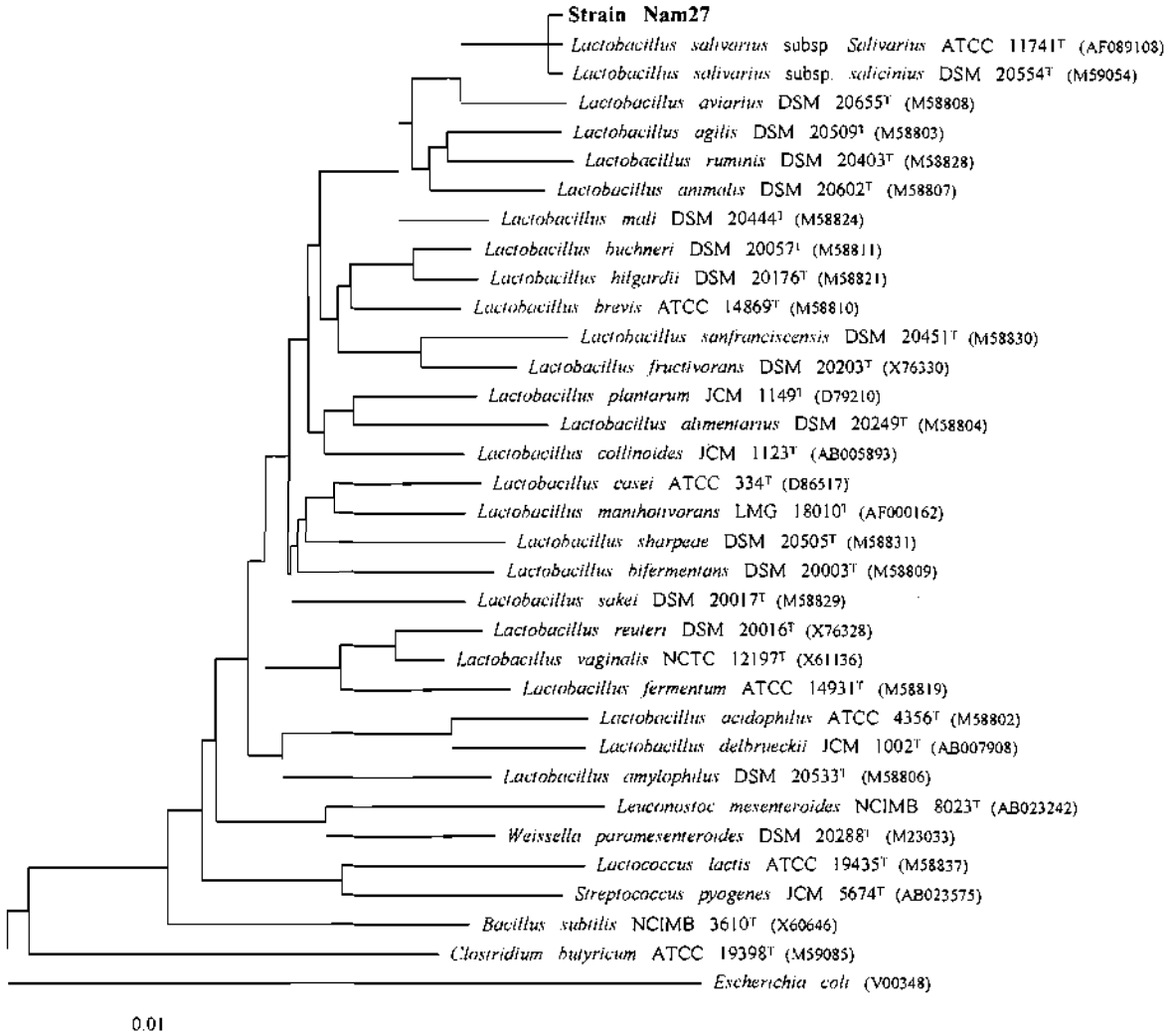

Figure 4. Phylogenetic tree based on 16S rDNA sequences showing the positions of strain Nam 27, the type strains of some *Lactobacillus* species and the representatives of some other related taxa. Scale bar represents 0.01 substitution per nucleotide position

Table 2. The physiological characteristics of the isolated strains from human faeces

Characteristics	Isolated strains							
	12	20	Nam 27	30	35	37	62	80
Gram reaction	+	+	+	+	+	+	+	+
Morphology	rods	rods	rods	rods	rods	rods	rods	rods
Spore formation	−	−	−	−	−	−	−	−
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

Adhesion ability of *E. coli* KCTC 1021, one of *E. coli* strains examined was also excellent and equal to *Salmonella typhimurium* KCCM 40253. Of Lactobacilli belonging to enteric microorganisms, *L. salivarius* could survive in the human intestinal tract for a long time (Pirkka et al., 1998). Lisa M. et al. (1999) describes the ability of *L. salivarius* strains to survive through the Balb/c mice murine gastrointestinal tract, and this bacterium is shed in high numbers in faeces. Also Virginia et al. (1999) reported that *L. salivarius* subsp. *salivarius* could be used for the design of a probiotic to prevent human urogenital infection.

Table 3. Carbohydrate fermentation patterns of the isolated strains from human faeces

	Carbohydrates	Isolated strains							
		12	20	Nam 27	30	35	37	62	80
0	Control	-	-	-	-	-	-	-	-
1	Glycerol	-	-	-	-	-	-	-	-
2	Erythritol	-	-	-	-	-	-	-	-
3	D-Arabinose	-	-	-	-	-	-	-	-
4	L-Arabinose	+	+	+	+	+	±	+	+
5	Ribose	+	+	+	+	+	+	+	+
6	D-Xylose	-	-	-	-	-	-	-	-
7	L-Xylose	-	-	-	-	-	-	-	-
8	Adonitol	-	-	-	-	-	-	-	-
9	β Methyl-xyloside	-	-	-	-	-	-	-	-
10	Galactose	+	+	+	+	+	+	+	+
11	D-Glucose	+	+	+	+	+	+	+	+
12	D-Frutose	+	+	+	+	+	+	+	+
13	D-Mannose	+	+	+	+	+	+	+	+
14	L-Sorbose	+	-	-	+	-	-	+	+
15	Rhamnose	+	±	+	+	+	±	+	+
16	Dulcitol	±	-	-	±	-	-	+	±
17	Inositol	-	-	-	-	-	-	-	-
18	Mannitol	+	+	+	+	+	+	+	+
19	Sorbitol	+	+	+	+	+	+	+	+
20	α Methyl-D -mannoside	-	-	-	-	-	-	-	-
21	α Methyl-D-glucoside	±	-	-	±	-	-	±	±
22	N Acetyl glucosamine	+	+	+	+	+	+	+	+
23	Amygdaline	+	-	-	±	-	-	±	±
24	Arbutine	+	±	+	+	±	±	+	+
25	Esculine	-	-	-	-	-	-	-	-
26	Salicine	+	±	+	+	+	+	+	+
27	Cellobiose	+	+	+	+	+	+	+	+
28	Maltose	+	+	+	+	+	+	+	+
29	Lactose	+	+	+	+	+	+	+	+
30	Melibiose	+	+	+	+	+	+	+	+
31	Saccharose	+	+	+	+	+	+	+	+
32	Trehalose	+	+	+	+	+	+	+	+
33	Inuline	-	-	-	-	-	-	-	-
34	Melezitose	+	+	+	+	+	+	+	+
35	D-Raffinose	-	+	+	+	+	+	+	+
36	Amidon	±	-	-	-	-	-	±	±
37	Glycogene	-	-	-	-	-	-	-	-
38	Xylitol	-	-	-	-	-	-	-	-
39	β Gentiobiose	+	-	-	+	-	-	+	+
40	D-Turanose	+	±	±	+	±	±	+	+
41	D-Lyxose	+	-	-	+	-	-	+	+
42	D-Tagatose	+	±	±	+	±	±	+	+
43	D-Fucose	-	-	-	-	-	-	-	-
44	L-Fucose	-	-	-	-	-	-	-	-
45	D-Arabitol	-	-	-	-	-	-	-	-
46	L-Arabitol	-	-	-	-	-	-	-	-
47	Gluconate	+	±	±	+	±	±	+	+
48	2 keto-gluconate	-	-	-	-	-	-	-	-
49	5 keto-gluconate	-	-	-	-	-	-	-	-
	Similarity strain	*L. rhamnosus*	*L. salivarius*	*L. salivarius*	*L. rhamnosus*	*L. salivarius*	*L. salivarius*	*L. rhamnosus*	*L. rhamnosus*
		99.9	99.2	95.6	99.9	98.7	98.9	99.9	99.9
	Identification (%)	good identification	acceptable identification	good identification	good identification	good identification	acceptable identification	good identification	good identification

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-sporeforming rods which grew at 45℃ but did not grow at 15℃, and did not produce gas from glucose. Also these bacteria could be grown in both anaerobic and aerobic conditions. Fermentation types of these strains were found to be homolactic fermentation by HPLC analysis.

As a result of carbohydrate fermentation patterns of the eight selected strains (table 3), four strains belonged to *L. salivarius*, and the other four strains to *L. rhamnosus*. 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, Nam 27, No. 35 and No. 37 coincided with *L. salivarius* subsp. *salivarius* except for salicine fermentation.

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 4). All eight strains possesed β-galcatosidase, and No. 20 and Nam 27 possesed α-galcatosidase strongly. All eight strains did not possess β-Glucuronidase. β-Glucuronidase could invert benzopyrene to carcinogenic substance, therefore it is called a carcinogenic enzyme (Nanno et al., 1986). The strains producing leucine arylamidase were considered as acid producers (Desjardins et al., 1990). All of the eight selected strains showed positive results for leucine arylamidase, suggesting that all should be acid producers.

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

Figure 3 shows *Alu*I digestion patterns of 16S rDNA, which is obtained by PCR (figure 2), from the eight selected strains. Two types of patterns are shown: one group is No. 20, Nam 27, No. 35 and No. 37; another group is No. 12, No. 30, No. 62 and No. 80. These results coincided with a carbohydrate fermentation pattern in table 3. Thus, those strains of No. 20, Nam 27, No. 35, and No. 37 identified as *L. salivarius* by carbohydrate fermentation test showed the same restriction enzyme pattern by *Alu*I. And those strains of No. 12, No. 30, No. 62, No. 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 Nam 27, which was selected as the best one among observations about acid- and bile -tolerance and microhemagglutination test, at the highest homology among LAB cited in GenBank. Sequence data (GenBank accession No. AF335475, the full-length 16S rDNA sequence of the strain Nam 27 consists of 1517 bp) were aligned to construct the phylogenetic tree. Phylogenetic position of the strain Nam 27 was compared with some of the LAB and related taxa in the dendrogram. In the phylogenetic tree, the strain Nam 27 was the nearest one with *L. salivarius* subsp. *salivarius* ATCC1174T (figure 4). In table 5, the sequence of the strain Nam 27 was the most identical with that of *L. salivarius* subsp. *salivarius* (99.9%). Regarding the carbohydrate fermentation profile

Table 4. Enzyme profiles of the selected strains from human faeces

	Enzyme	selected strains							
		12	20	Nam 27	30	35	37	62	80
1	Control	0	0	0	0	0	0	0	0
2	Alkaline phosphatase	5	3	3	5	5	5	5	5
3	Esterase(C4)	5	5	5	5	5	5	5	5
4	Esterase Lipase (C8)	5	5	4	5	5	5	5	5
5	Lipase(C14)	3	3	3	3	3	2	2	3
6	Leucine arylamidase	5	5	5	5	5	5	5	5
7	Valine arylamidase	5	5	5	5	5	5	5	5
8	Crystine arylamidase	4	3	3	4	4	4	3	3
9	Trypsin	1	0	0	1	0	0	1	0
10	α-chymotrypsin	4	3	3	5	5	3	5	5
11	Acid phospatase	5	5	5	5	5	5	5	5
12	Naphtol-AS-B1-phosphohy drolase	5	5	5	4	5	5	5	5
13	α-galactosidase	0	5	5	0	0	0	0	0
14	β-galactosidase	5	5	5	5	5	5	5	5
15	β-glucuronidase	0	0	0	0	0	0	0	0
16	α-glucosidase	5	5	5	5	5	5	5	5
17	β-glucosidase	5	4	2	5	4	5	5	4
18	N-acetyl-β-glucosaminidase	0	0	0	0	0	0	0	0
19	α-mannosidase	0	0	0	0	0	0	0	0
20	α-fucosidase	1	1	0	1	1	1	1	1

A value ranging from 0 to 5 is assigned to the color standard. 0 represents a negative reaction; 5 represents a maximum intensity reaction. Values 1-4 represent intermediate reactions. 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.

Table 5. Levels of 16S rDNA similarity for strain Nam 27, the type strains of some *Lactobacillus* species and representatives of some related taxa

Strains	% Similarity in																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Strain Nam 27																	
2 *Lactobacillus salivarius* subsp. *salivarius* ATCC 11741T	99.9																
3 *Lactobacillus salivarius* subsp. *salicinius* DSM 20554T	99.8	99.5															
4 *Lactobacillus aviarius* DSM 20655^T	95.5	94.9	95.5														
5 *Lactobacillus agilis* DSM 20509^T	95.1	94.9	94.8	94.2													
6 *Lactobacillus mali* DSM 20444^T	94.7	94.2	94.7	94.0	94.6												
7 *Lactobacillus brevis* ATCC 14869^T	91.5	91.1	90.9	90.5	92.0	93.1											
8 *Lactobacillus plantarum* JCM 1149^T	90.9	90.8	90.4	89.9	92.2	92.9	94.2										
9 *Lactobacillus casei* ATCC 334^T	91.4	91.3	91.0	90.8	91.3	92.3	91.7	91.8									
10 *Lactobacillus sakei* DSM 20017^T	91.4	91.4	91.2	90.8	90.7	92.8	92.1	93.3	92.9								
11 *Lactobacillus fermentum* ATCC 14931^T	89.9	89.5	89.6	90.4	90.4	90.2	91.6	91.7	90.0	90.3							
12 *Lactobacillus delbrueckii* JCM 1002^T	88.3	88.4	88.0	87.4	87.1	87.3	88.3	88.2	88.4	87.7	88.9						
13 *Leuconostoc mesenteroides* NCIMB 8023^T	86.3	86.2	86.1	86.1	86.8	86.7	86.0	86.5	86.9	87.5	87.3	86.1					
14 *Weissella paramesenteroides* DSM 20288^T	88.9	88.8	89.0	89.0	89.2	89.9	89.3	89.4	89.5	89.7	88.7	86.3	90.3				
15 *Lactococcus lactis* ATCC 19435^T	85.9	85.5	86.1	85.7	85.7	87.8	86.6	86.7	86.3	87.6	85.7	85.6	83.8	86.0			
16 *Bacillus subtilis* NCIMB 3610^T	87.8	87.8	87.9	87.2	87.7	89.2	87.8	88.2	87.4	89.0	86.9	87.1	85.0	86.8	85.0		
17 *Clostridium butyricum* ATCC 19398^T	84.0	83.9	83.9	83.1	83.9	83.8	82.4	83.0	83.5	83.9	82.7	82.4	80.8	82.5	82.6	84.6	
18 *Escherichia coli*	78.1	78.0	77.5	78.2	77.5	77.2	76.7	77.6	77.6	77.4	77.5	77.9	78.4	77.2	77.2	79.4	77.8

and the 16S rDNA sequence, the strain Nam 27 could be assigned to a strain of *L. salivarius* subsp. *salivarius*. The strains No. 20, No. 35 and No. 37 were all identified as *L. salivarius* subsp. *salivarius* from the partial sequencing results (about 1.1 Kb) of 16S rDNA (data not shown).

In summary, this study describes the isolation and identification of acid- and bile-tolerant *L. salivarius* subsp. *salivarius* from human faeces. The isolated *L. salivarius* strain may include the ability to survive in human gastric acid and to grow at physiological concentrations of human bile. Future study involving an application test in fermented milk products may reveal the true potential of this strain for prevention of intestinal disease, possibly through modification of the gastrointestinal tract microbial populations.

REFERENCES

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

Choi, S. H., M. J. Oh and C. Sung. 1996. Hemagglutination and colony hybridization for the identification of enterotoxigenic *Escherichia coli* isolated from healthy pig. Asian-Aus. J. Anim. Sci. 9(6):671-677.

Collin, E. B. and B. J. Hall. 1984. Growth of bifidobacteria in milk and preparation of *Bifdobacterium infantis* for 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.

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.

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.

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

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

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., C. R. Nelson and C. Maxwell, 1985. Assimilation of cholesterol by *lactobacillus acidophilus*. Appl. Environ. Microbiol. 49:337-381.

Goldin, B. R. and S. L. Gorbah. 1992. Probiotics for humans. In: Ed. Probiotics: (Ed. R. Fuller). The Scientific Basis. Chapman and Hall, London. pp. 355-376.

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

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.

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.

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.

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.

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., 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.

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

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.

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.

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.