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Short Communication

Isolation of cholesterol-lowering lactic acid bacteria from human intestine for probiotic use

Hyeong-Jun Lim[1], So-Young Kim[2], Wan-Kyu Lee[1,*]

[1]*College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea*
[2]*Department of Food Science and Technology, and Research Center for Bioresource and Health, Chungbuk National University, Cheongju 361-763, Korea*

Cholesterol-lowering effect of lactic acid bacteria (LAB: *Streptococcus, Lactobacillus* and *Bifidobacterium*) is well-known. Thus, we investigated LAB isolated from human intestine on the cholesterol-lowering effect *in vitro*. Seven *Streptococcus* (61.1%), 11 *Lactobacillus* (71.8%) and 7 *Bifidobacterium* (27.9%) were isolated as acid (pH 2.5 and 3.0) and bile (0.3% oxgall) tolerant strains. *Streptococcus* HJS-1, *Lactobacillus* HJL-37 and *Bifidobacterium* HJB-4 were finally selected as probiotic strains to use through the bile salt hydrolase (BSH) activity assay by using MRS media added taurodeoxycholic acid (TDCA) and the cholesterol-lowering test by using soluble cholesterol containing MRS broth. These studies suggested that the isolated LAB had an excellent hypocholesterolemic effect.

Key words: Lactic acid bacteria (LAB), probiotics, cholesterol, bile salt hydrolase (BSH)

Cardiovascular disease is the most important cause of death in the westernized countries and it is strongly associated with hypercholesterolemia [17]. Decreasing serum cholesterol is, therefore, very important to prevent cardiovascular disease. HDL-cholesterol has been known to prevent arteriosclerosis by removing cholesterol from blood stream, whereas LDL-cholesterol fastens arteriosclerosis by accumulating cholesterol in the blood vessel [16,17].

The plasma cholesterol concentration can be regulated by the biosynthesis of cholesterol from saturated fat, removal of cholesterol from the circulation, absorption of dietary cholesterol, and excretion of cholesterol via bile and feces. Cellular cholesterol homeostasis is very important for the prevention of cardiovascular disease, and numerous studies have been already reported that enzyme inhibitors for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acyl CoA: cholesterol acyltransferase (ACAT) have beneficial effects on hypercholesterolemia and arteriosclerosis [12].

Some natural microorganisms in human intestine are beneficial in terms of lowering serum cholesterol [5,7,18]. The lactic acid bacteria (LAB), *Lactobacillus* and *Bifidobacterium* spp. in particular, have the ability to metabolize cholesterol [3]. Blood cholesterol synthesis is decreased by the inhibition of HMG-CoA reductase that convert HMG-CoA to mevalonate and by organic acids in the fermented milk. Gilliland *et al*. reported that *Lactobacillus acidophilus* reduces blood cholesterol by direct breakdown of cholesterol and deconjugation of bile salt [9]. In particular, cholesterol metabolism is closely linked to the formation of bile salts, that is, the water-soluble excretory end-products of cholesterol. The bile salts may be transformed by enzyme activities of some intestinal bacteria during the enterohepatic circulation. Bile-salt hydrolase (BSH) is the enzyme responsible for deconjugation of bile acid, and it split glycine or taurine from the steroid moiety, resulting in free (deconjugated) bile salts. BSH activity is observed in some strains associated with the gastrointestinal tract (GIT), representing several species of *Lactobacillus*, *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Clostridium*, and *Bacteroides* [1].

This study was to investigate the effects of LAB isolated from human intestine on cholesterol lowering through the BSH activity assay by using MRS media added taurodeoxycholic acid (TDCA) and the cholesterol-lowering test by using soluble cholesterol MRS broth.

Fecal specimens were collected from seven healthy humans (3 adult males, 2 adult females and 2 male children) and inoculated into a tube containing 9 ml transport anaerobic media (BHI broth) [19,21] replaced by O_2-free CO_2 gas. Four plate media were used to isolate LAB, TATAC for *Streptococcus*, LBS for *Lactobacillus*, BS for *Bifidobacterium* and BL for the most part of LAB. Collected

*Corresponding author
Tel: 82-43-261-2960; Fax: 82-43-267-3150;
E-mail: wklee@cbu.ac.kr

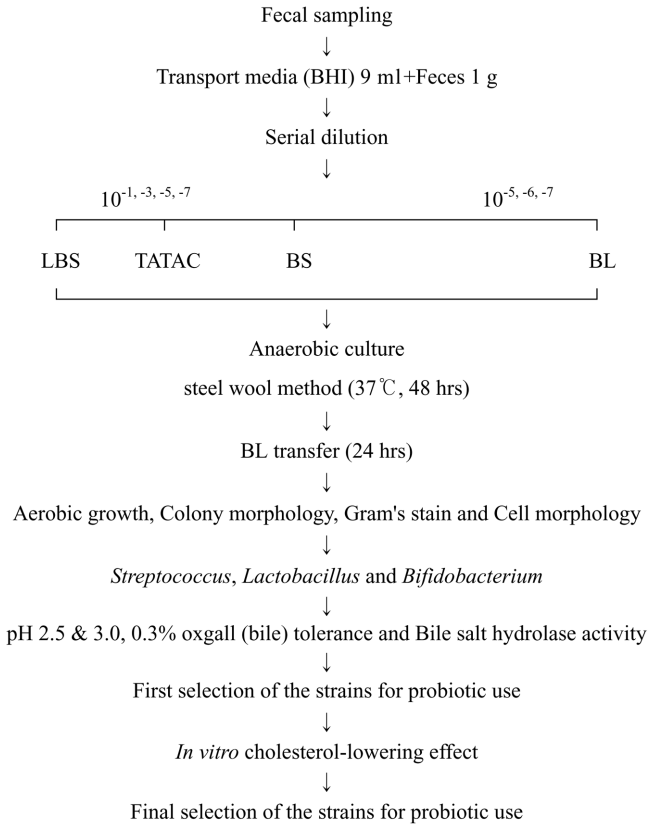

Fig. 1. Schematic diagrams for the isolation of human intestinal LAB for probiotic use.

feces were serially diluted with the Diluent A, and spread-plated as $10^{-1, -3, -5, -7}$ onto TATAC, LBS and BS media and as $10^{-5, -6, -7}$ onto BL media [15,21]. Plates were incubated at 37°C for 48 hrs in an anaerobic 'steel wool' jar filled with O_2-free CO_2 gas [20]. Then, typical colonies of LAB were isolated from the cultured media and were transferred onto BL media. They were incubated at 37°C for 24 hrs under anaerobic conditions, and regarded LAB as *Streptococcus*, *Lactobacillus* and *Bifidobacterium* by aerobic growth, Gram's stain and cell morphology. All isolates were maintained on BL agar plates in the anaerobic conditions and stored at 4°C [15,21]. The bacterial isolation procedure is schematically shown in Fig. 1.

To assess low pH tolerance, the first isolates, *Streptococcus*, *Lactobacillus* and *Bifidobacterium*, were incubated in MRS broth (Difco, USA) containing L-cysteine · HCl · H_2O (Junsei, Japan) as 0.05% concentration (w/v) at 37°C for 24 hrs under anaerobic conditions. MRS broth was adjusted to pH 2.5 (for *Streptococcus* and *Lactobacillus*) and pH 3.0 (for *Bifidobacterium*), respectively, by using 1 N HCl, and put into 3 ml per a 4 ml vial. *Streptococcus* and *Lactobacillus* were inoculated into MRS broth (pH 2.5) and *Bifidobacterium* was inoculated into MRS broth (pH 3.0) as 30 µl volume, then anaerobically incubated at 37°C for 3 hrs. Bacteria were

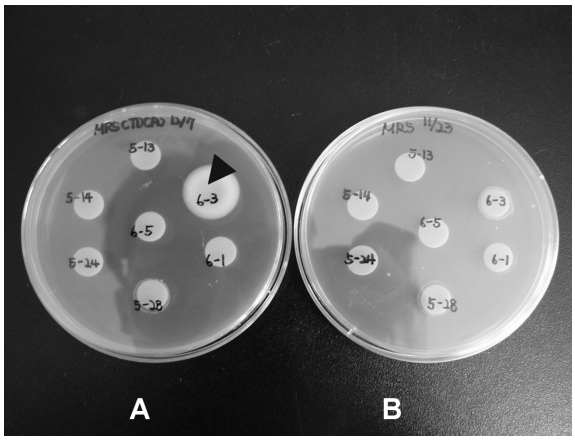

Fig. 2. Bile- salt hydrolase (BSH) activity as detected by the plate-assay method. A, MRS supplemented with 0.5% TDCA (sodium salt of taurodeoxycholic acid) and 0.37% g/l $CaCl_2$; B, MRS as control; Bile-salt hydrolysis positive was represented by black arrow.

spread onto BL media to discriminate the survival of bacteria and anaerobically incubated at 37°C for 48 hrs. If the colonies were formed on the BL media after 48hrs incubation, they were confirmed as the bacteria to have low pH tolerance [14].

In order to assess bile salt tolerance of bacteria, the isolates of *Streptococcus*, *Lactobacillus* and *Bifidobacterium*, were incubated in MRS broth (pH 7.0) containing L-cysteine · HCl · H_2O as 0.05% concentration (w/v) at 37°C for 24 hrs under anaerobic conditions. MRS broth was supplemented with 0.3% (w/v) oxgall (Sigma, USA, pH 7.0). All bacteria were inoculated as 30 µl volume and incubated at 37°C for 3 hrs. Then, bacteria were spread onto BL agar plates to confirm the survival of bacteria and anaerobically incubated at 37°C for 48 hrs. If colonies were formed on the BL media, they were decided as the bacteria to have bile salt tolerance [2,14].

Isolates were screened by being impregnation around sterilized paper disks on the MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA, Sigma, USA) and 0.37 g/l $CaCl_2$ (Kanto, Japan) to confirm whether they have bile salt hydrolase (BSH) activity or not. Plates were anaerobically incubated at 37°C for 72 hrs, and the diameter of the precipitation zones around the disks was measured [2,3,4] (Fig. 2).

MRS broth (pH 7.0) containing L-cysteine · HCl · H_2O as 0.05% concentration (w/v) was prepared and autoclaved at 121°C for 15 min. Soluble cholesterol (polyoxyethanyl-cholesterol sebacate, Sigma, USA) was supplemented into the prepared MRS broth, and it was filtered through 0.45 µm Millipore. Inoculation volume was 15 µl provisional probiotic bacterial culture solution per 1 ml cholesterol-MRS broth, and that was anaerobically incubated at 37°C for 24 hrs with control. MRS broth without bacterial culture

Table 1. Number of acid- and bile-tolerant lactic acid bacteria isolated from the human intestine for probiotic use

	Acid[a]	Bile[b]	BSH[c]
Streptococcus	11/18 (61.1)[d]	17/18 (94.4)	7/18 (38.9)
Lactobacillus	28/39 (71.8)	37/39 (94.9)	5/39 (12.8)
Bifidobacterium	12/43 (27.9)	26/43 (60.5)	15/43 (34.9)
Total	51/100 (51.0)	80/100 (80.0)	27/100 (27.0)

[a]*Lactobacillus* and *Streptococcus* (pH 2.5), *Bifidobacterium* (pH 3.0)
[b]Bile (0.3% oxgall)
[c]Bile salt hydrolase activity in the MRS plate media containing taurodeoxycholic acid
[d]Number of resistant strains/Number of selected strains (%)

solution was also incubated at 37°C for 24 hrs for the control.

An Ektachem DT 60 analyzer (Johnson & Johnson, USA) was used to measure remaining volume of cholesterol in the cholesterol-MRS broth. The incubated cholesterol-MRS broth with isolates was centrifuged at 3000 rpm for 10 min. Ten micro liter supernatant was collected with a DT pipette. The supernatant was reacted with slide reagent on the DT slide. In order to measure cholesterol amount, dye layer is observed in 555 nm wave length.

Final isolates, *Streptococcus* and *Lactobacillus*, were aerobically incubated at 28°C for 24 hrs on MRS agar plates, and *Bifidobacterium* was anaerobically incubated at 35°C for 48 hrs on BL agar plate. Bacteria were preprocessed with reagents to extract fatty acid from the bacterial cell-wall and identified with microbial identification system (MIDI, Inc., USA).

Isolation of bacteria: One hundred strains of LAB were isolated from selective (TATAC, LBS and BS) and non-selective (BL) media; 18 *Streptococcus* strains, 39 *Lactobacillus* strains and 43 *Bifidobacterium* strains. The number of *Streptococcus* isolated from 7 volunteers was 10^2~10^7 CFU/ml, *Lactobacillus* was 10^3~10^8 CFU/ml and *Bifidobacterium* was 10^6~10^9 CFU/ml. In particular, the number of *Bifidobacterium* was higher than those of other LAB.

Low pH tolerance: Low pH tolerance of isolated LAB was assessed in pH 2.5 (*Streptococcus* and *Lactobacillus*) and in pH 3.0 (*Bifidobacterium*). As shown in Table 1, 61.1% in *Streptococcus* strains (11/18 strains) and 71.8% in *Lactobacillus* (28/39 strains) were also tolerant in pH 2.5. In case of *Bifidobacterium*, 27.9% (12/43 strains) were tolerant in pH 3.0. In addition, *Bifidobacterium* was the weakest in low pH conditions among isolated LAB.

Bile salt tolerance: Results of bile salt tolerance were shown in Table 1. Among LAB, 94.4% in *Streptococcus* (17/18 strains), 94.9% in *Lactobacillus* (37/39 stains) and 60.5% in *Bifidobacterium* (26/43 strains) were tolerable to bile salt. Most bacteria were tolerable to bile salt but *Bifidobacterium* was relatively weak compared to other LAB in bile salt tolerance assay.

Bile salt hydrolase (BSH) activity assay: As shown in Table 1, 38.9% (7/18 strains) and 34.9% (15/43 strains) in *Streptococcus* and *Bifidobacterium*, respectively, had BSH activity (Table 1 and Fig. 2). *Streptococcus* HJS-1 produced large precipitation zone (19 mm). But only 5 strains (12.8%) among *Lactobacillus* had BSH activity.

***In vitro* cholesterol-lowering test:** Among isolated 100 strains of LAB, 7 of *Streptococcus* strains, 11 of *Lactobacillus* strains and 7 of *Bifidobacterium* strains were selected as provisional probiotic strains due to their superiority on low pH and bile salt tolerance and BSH activity assay (Table 2).

Among *Streptococcus* strains, the *Streptococcus* HJS-1 decreased cholesterol concentration from 203.3 mg/dl to 87.5 mg/dl (57.0%) in the MRS broth without bile salt and to 102.9 mg/dl (49.4%) in the 0.3% bile salt MRS broth. Eight strains of Lactobacilli decreased cholesterol by more than 50% in the MRS broth regardless to the presence of bile salt. *Bifidobacterium* HJB-4 and *Bifidobacterium* HJB-25 reduced cholesterol by about 50% in the MRS broth regardless the presence of bile salt. These results showed that many LAB have a great cholesterol-lowering activity in the MRS broth regardless to the presence of bile salt (0.3% oxgall) (Table 2).

Identification of final isolates: As considering bacterial preparation such as low pH and bile salt tolerance, BSH activity, *in vitro* cholesterol-lowering activity and so on, we selected LAB strains finally from human intestine for probiotic use. The final selected probiotic bacteria were *Streptococcus* HJS-1, *Lactobacillus* HJL-37, and *Bifidobacterium* HJB-4.

Finally selected probiotic bacteria were identified using the microbial identification system (MIDI, HP Inc., USA). *Streptococcus*-HJS 1, *Lactobacillus*-HJL 37, and *Bifidobacterium*-HJB 4 were identified as *Enterococcus faecium* (Similarity index (SI): 0.601), *Lactobacillus delbrueckii* (SI: 0.521), and *Bifidobacterium longum* (SI: 0.467), respectively.

Cardiovascular disease is the most important cause of death in Korea and in the western countries. In the United States, 10 million people suffer from ischemic coronary arterial diseases, and spend 115 billion dollars per year to treat it. According to NHANES (the third national health and nation examination survey) data and NCEP (national

Table 2. Change in cholesterol level after in *in vitro* incubation with selected LAB[a]

Strain No.	Oxgall concentration [mg/dl] (Reduction rates of cholesterol [%])	
	0%	0.3%
Control	203.3 (0)	203.3 (0)
Streptococcus HJS-1	87.5 (57.0)	102.9 (49.4)
Streptococcus HJS-3	149.5 (26.5)	166.1 (18.3)
Streptococcus HJS-6	152.6 (24.9)	169.5 (16.6)
Streptococcus HJS-9	157.4 (22.6)	174.9 (14.0)
Streptococcus HJS-11	150.1 (26.2)	176.6 (13.1)
Streptococcus HJS-12	203.5 (0)	203.9 (0)
Streptococcus HJS-17	168.3 (17.2)	182.9(10.0)
Lactobacillus HJL-4	86.1 (57.6)	92.6 (54.5)
Lactobacillus HJL-20	125.1 (38.5)	139.3 (31.5)
Lactobacillus HJL-24	117.6 (42.2)	130.7 (35.7)
Lactobacillus HJL-25	107.0 (47.4)	121.6 (40.2)
Lactobacillus HJL-30	71.1 (60.0)	88.9 (56.3)
Lactobacillus HJL-32	83.2 (59.1)	104.9 (48.4)
Lactobacillus HJL-33	92.6 (54.5)	101.8 (49.9)
Lactobacillus HJL-34	69.4 (65.9)	86.7 (57.4)
Lactobacillus HJL-37	72.4 (64.4)	85.2 (58.1)
Lactobacillus HJL-38	67.5 (66.8)	84.4 (58.5)
Lactobacillus HJL-39	85.3 (58.0)	100.3 (50.7)
Bifidobacterium HJB-4	84.2 (58.6)	105.3 (48.2)
Bifidobacterium HJB-23	172.7 (15.1)	191.9 (5.6)
Bifidobacterium HJB-25	83.1 (59.1)	103.9 (48.9)
Bifidobacterium HJB-33	173.0 (14.9)	192.2 (5.5)
Bifidobacterium HJB-34	151.3 (25.6)	178.0 (12.4)
Bifidobacterium HJB-35	143.2 (29.6)	168.5 (17.1)
Bifidobacterium HJB-39	201.0 (1.1)	201.1 (1.1)

[a]Amounts of cholesterol were measured after 36 hrs incubation at 37°C with acid (*Streptococcus* and *Lactobacillus*; pH 2.5, *Bifidobacterium*; pH 3.0) and bile (0.3% oxgall) tolerant LAB isolated human intestine in MRS broth containing cholesterol with (0.3%) or without oxgall.

cholesterol education program) guide, half million people have died of ischemic cardiac disease. Fifty two million US people are recommended to eat a diet to decrease their serum cholesterol, and 13 million people need a pharmacotherapy to treat cardiac diseases. Recently, considerable researches on the favorable health effects of probiotics have been recently reported. The probiotics have been known to mitigate the risk of arteriosclerosis associated with dyslipoproteinemia, obesity, and diabetes. [1,8,11,22,23]

It was hypothesized that deconjugation of bile salts may contribute to lower cholesterol levels as free bile salts may be excreted more likely from the GIT than conjugated bile salts [6]. However, the hypothesis is disputable and incompatible with current knowledge with regard to the passive absorption kinetics of free bile salts in the GIT. Fecal loss of bile salts may indeed result in an increased requirement for cholesterol for maintaining serum cholesterol levels. Klaver and van der Meer suggested that *in vitro* cholesterol reduction by some *Lactobacillus* spp. results from its coprecipitation with deconjugated bile salts [13]. De Smet *et al*. suggested that highly BSH-active *Lactobacillus* spp. may reduce serum cholesterol levels [2], and they hypothesized that BSH activity may be an important factor for bile tolerance [3]. BSH-active lactobacilli may thus have an advantage to survive and colonize in the lower small intestine where the enterohepatic circulation takes place.

In general, probiotic bacteria must colonize in GIT of host, have acid- and bile salt-tolerance and be antagonist against putrefactive bacteria in GIT [4,9]. In this study, *Streptococcus*, *Lactobacillus* and *Bifidobacterium* from human intestine were selected as optimal probiotic bacteria. Low pH- and bile salt-tolerance were assessed for 100 probiotic isolates. Eleven *Streptococcus* strains (61.1%), 28 *Lactobacillus* strains (71.8%), and 12 *Bifidobacterium* strains (27.9%), were selected as provisional probiotic strains. As considered with BSH activity, and *in vitro* cholesterol-lowering test, the final probiotic strains were 7 in *Streptococcus* strains, 11 in *Lactobacillus* strains and 7 in *Bifidobacterium* strains. In this study, *Streptococcus* HJS-1, *Lactobacillus* HJL-37 and *Bifidobacterium* HJB-4 had the best hypocholesterolemic effects (57.0%, 64.4% and 58.6%, respectively) in the MRS broth with soluble cholesterol containing 0.3% oxgall. From present results, it was suggested that the finally isolated LAB had an excellent hypocholesterolemic effect. They will be use as a probiotics to prevent hypercholesterolemia for human health. However, the mechanisms of regulating serum cholesterol and the effect on the serum cholesterol level *in vivo* animal experiment needs further extensive investigations.

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References

1. **Canzi E, Zanchi R, Camaschella P, Cresci A, Greppi GF, Orpianesi C, Serrantoni M, Ferrari A.** Modulation by lactic acid bacteria of the intestinal ecosystem and plasma cholesterol in rabbits fed a casein diet. Nutr Res 2000, **20**, 1329-1340.
2. **De Smet I, van Hoorde L, De Saeyer N, vande Woestyne M, Verstraete W.** *In vitro* study of bile-salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. Micro Ecol Health Dis 1994, **7**, 315-329.
3. **De Smet I, van Hoorde L, vande Woestyne M, Christiaens H, Verstraete W.** Significance of bile-salt hydrolytic

activities of lactobacilli. J Appl Bacteriol 1995, **79**, 292-301.
4. **Du Toit M, Franz CMAP, Dicks LMT, Schillinger U, Haberer P, Warlies B, Ahrens F, Holzapfel WH.** Characterization and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. Int J Food Microbiol 1998, **40**, 93-104.
5. **Fernandes CF, Shahani KM, Amer MA.** Therapeutic role of dietary lactobacilli and *Lactobacillus* fermented dairy products. FEMS Microbiol Rev 1987, **46**, 343-356.
6. **Fukushima M, Fujii S, Yoshimura Y, Endo T, Nakano M.** Effect of rice bran on intraintestinal fermentation and cholesterol metabolism in cecectomized rats. Nutr Res 1999, **19**, 235-245.
7. **Fukushima M, Yamada A, Endo T, Nakano M.** Effects of a mixture of organisms, *Lactobacillus acidophilus* or *Streptococcus faecalis* on delta6-desaturase activity in the livers of rats fed a fat- and cholesterol-enriched diet. Nutrition 1999, **15**, 373-378.
8. **Gallo LL.** Cholesterol and other sterols: absorption, metabolism, roles in atherosclerosis. In: Watson RR (ed.). Nutrition and heart disease. pp. 83-109, Churchill Livingstone, London, 1983.
9. **Gilliland SE, Nelson CR, Maxwell C.** Assimilation of cholesterol by *Lactobacillus acidophilus*. Appl Environ Microbiol 1985, **49**, 377-381.
10. **Gilliland SE.** Health and nutrition benefits from lactic acid bacteria. FEMS Microbiol Rev 1990, **7**, 175-188.
11. **Gilliland SE, Walker DK.** Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. J Dairy Sci 1990, **73**, 905-911.
12. **Hay JM, Yu WM, Ashraf T.** Pharmacoeconomics of lipids-lowering agents for primary and secondary prevention of coronary artery disease. Pharmacoeconomics 1999, **15**, 47-74.
13. **Klaver FAM, van der Meer R.** The assumed assimilation of cholesterol by *Lactobacillus* and *Bifidobacterium bifidum* is due to their bile salt-deconjugation activity. Appl Environ Microbiol 1993, **59**, 1120-1124.
14. **Kimoto H, Kurisaki J, Tsuji NM, Ohmomo S, Okamoto T.** Lactococci as probiotic strains: adhesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile. Lett Appl Microbiol 1999, **29**, 313-316.
15. **Lee WK, Lee SM, Bae HS, Y. J. Baek YJ.** Effect of *Bifidobacterium longum* HY8001 administration on human fecal bacterial enzyme and microflora. Korean J Appl Microbiol Biotechnol 1999, **27**, 267-272.
16. **Lee YW.** Effect of fermented milk on the blood cholesterol level of Korean. J Food Safety 1997, **12**, 83-95.
17. **Lee YW, Roh WS, Kim JG.** Benefits of fermented milk in rats fed by hypercholesterolemic diet (II). Korean J Food Hygiene 1992, **7**, 123-135.
18. **Mann G.V, Spoerry A.** Studies of a surfactant and cholesterolemia in the Massai. Am J Clin Nutr 1974, **27**, 464-469.
19. **Oh CY, Lee WK** Cholesterol lowering effect of lactic acid bacteria isolated from the human intestine. Korean J Vet Public Health 2000, **24**, 181-188.
20. **Parker CA.** Anaerobiosis with iron wool. Aust J Exp Biol Med Sci 1955, **33**, 33-37.
21. **Mitsuoka T.** The World Anaerobic Bacteria. pp. 13-65. Sobun Press. Tokyo, 1980.
22. **Mutai M, Tanaka R.** Ecology of *Bifidobacterium* in the human intestinal flora. Bifidobacteria Microflora 1987, **6**: 33-41.
23. **Park SY, Bok SH, Jeon SM, Park YB, Lee SJ, Jeong TS, Choi MS.** Effect of rutin and tannic acid supplements on cholesterol metabolism in rats. Nutr Res 2002, **22**: 283-295