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

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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 glycerin 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 O2-free CO2 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
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 O₂-free CO₂ 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 inoculated in MRS broth (Difco, USA) containing L-cysteine · HCl · H₂O (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 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 inoculated in MRS broth (pH 7.0) containing L-cysteine · HCl · H₂O 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 CaCl₂ (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 · H₂O as 0.05% concentration (w/v) was prepared and autoclaved at 121°C for 15 min. Soluble cholesterol (polyoxyethylencyl-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
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 10^10–10^11 CFU/ml, *Lactobacillus* was 10^7–10^8 CFU/ml and *Bifidobacterium* was 10^7–10^9 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 strains) 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 decreased 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

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

|          | Acid³ | Bile³ | BSH³ |
|----------|-------|-------|------|
| *Streptococcus* | 11/18 (61.1)% | 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) |

³ *Lactobacillus and Streptococcus* (pH 2.5). *Bifidobacterium* (pH 3.0)
³ Bile (0.3% oxgall)
³ Number of resistant strains/Number of selected strains (%)
Table 2. Change in cholesterol level after in vitro incubation with selected LAB

| 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)                         |

*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%) 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 hypcholesterolemic 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 hypcholesterolemic 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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