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; \textit{Streptococcus}, \textit{Lactobacillus} and \textit{Bifidobacterium}) is well-known. Thus, we investigated LAB isolated from human intestine on the cholesterol-lowering effect in vitro. Seven \textit{Streptococcus} (61.1%), 11 \textit{Lactobacillus} (71.8%) and 7 \textit{Bifidobacterium} (27.9%) were isolated as acid (pH 2.5 and 3.0) and bile (0.3% oxgall) tolerant strains. \textit{Streptococcus} HJS-1, \textit{Lactobacillus} HJL-37 and \textit{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. LDL-cholesterol has been known to prevent arteriosclerosis by removing cholesterol from blood stream, whereas HDL-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), \textit{Lactobacillus} and \textit{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 \textit{et al.} reported that \textit{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 glycyne 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 \textit{Lactobacillus}, \textit{Enterococcus}, \textit{Peptostreptococcus}, \textit{Bifidobacterium}, \textit{Clostridium}, and \textit{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 \textit{Streptococcus}, LBS for \textit{Lactobacillus}, BS for \textit{Bifidobacterium} and BL for the most part of LAB. Collected
feces were serially diluted with the Diluent A, and spread-plated as $10^{-1}$, $10^{-3}$, $10^{-5}$, $10^{-7}$ onto TA TAC, LBS and BS media and as $10^{-5}$, $10^{-6}$, $10^{-7}$ 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.

![Fig. 1. Schematic diagrams for the isolation of human intestinal LAB for probiotic use.](image)

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 (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