Potential of Lactic Acid Bacteria Isolated from Dangke and Indonesian Beef as Hypcholesterolaemic Agent

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ABSTRACT

Lactobacillus fermentum strains were successfully isolated from dangke which was a fresh cheese-like product originating from Enrekang, South Sulawesi Province, Indonesia. In addition, Lactobacillus plantarum and Lactobacillus acidophilus were isolated from beef. This study aimed to investigate the ability of those 8 LAB strains from dangke and beef in lowering cholesterol level by using in vitro study. Strain of Lactic acid bacteria used were L. fermentum strains (A323L, B111K, B323K, C113L, C212L), L. plantarum strains (IIA-1A5 and IIA-2C12), and L. acidophilus IIA-2B4. Variables observed were identification of Bile Salt Hydrolase (BSH) gene by Polymerase Chain Reaction (PCR), BSH activity and cholesterol assimilation. Phylogenetic tree indicated homology of L. plantarum IIA-1A5 was 98% to BSH gene of L. plantarum Lp529 with access code of FJ439771 and FJ439775 obtained from GenBank. The results demonstrated that eight strains of LAB isolated from dangke and beef that potentially showed cholesterol-lowering effects were L. fermentum B111K and L. plantarum IIA-1A5. L. fermentum B111K was able to assimilate cholesterol by 4.10% with assimilated cholesterol of 0.13 mg in 10\textsuperscript{10} cells. In addition, L. plantarum IIA-1A5 had BSH gene and BSH activity, as well as the ability to assimilate cholesterol by 8.10% with assimilated cholesterol of 0.06 mg in 10\textsuperscript{10} cells. It is concluded that L. fermentum B111K and L. plantarum IIA-1A5 were strains that showed cholesterol-lowering effects.

Keywords: lactic acid bacteria, bile salt hydrolase, assimilation, cholesterol, dangke

ABSTRAK

Lactobacillus fermentum telah berhasil diisolasi dari dangke yang merupakan sejenis keju segar yang berasal dari Kabupaten Enrekang, Provinsi Sulawesi Selatan, Indonesia, dan L. plantarum dan L. acidophilus yang diisolasi dari daging sapi. Penelitian ini bertujuan untuk mengevaluasi kemampuan 8 strain BAL yang diisolasi dari dangke dan daging sapi dalam menurunkan kolesterol secara in vitro. Strain bakteri yang digunakan adalah L. fermentum (A323L, B111K, B323K, C113L, C212L), strain L. plantarum (IIA-1A5 dan IIA-2C12), dan L. acidophilus IIA-2B4. Peubah yang digunakan ialah uji keberadaan gen Bile Salt Hydrolase (BSH) yang diuji dengan Polymerase Chain Reaction (PCR), aktivitas BSH yang dianalisis secara deskriptif, dan asimilasi kolesterol yang dianalisis menggunakan ANOVA dengan 8 strain dan 3 ulangan. Hasil pohon filogenetik homologi gen BSH L. plantarum IIA-1A5 sebesar 98% dengan L. plantarum Lp529 FJ439771 dan FJ439775 dari GenBank. Hasil penelitian menunjukkan bahwa dari 8 strain BAL isolat dangke dan daging sapi yang diuji, yang berpotensi menurunkan kolesterol adalah L. fermentum B111K dan L. plantarum IIA-1A5. L. fermentum B111K mempunyai kemampuan mengasimilasi kolesterol sebesar 4.10% dengan jumlah kolesterol terasimilasi dalam 10\textsuperscript{10} sel sebanyak 0.13 mg. L. plantarum IIA-1A5 terbukti memiliki gen BSH yang positif memiliki aktivitas BSH serta mampu mengasimilasi kolesterol sebesar 8.10% dengan jumlah kolesterol terasimilasi dalam 10\textsuperscript{10} sel sebanyak 0.06 mg. Dapat disimpulkan bahwa strain bakteri yang berpotensi menurunkan kolesterol adalah L. fermentum B111K dan L. plantarum IIA-1A5.

Kata kunci: bakteri asam laktat, bile salt hydrolase, asimilasi, kolesterol, dangke
INTRODUCTION

World Health Organization (WHO) estimates that cardiovascular disease (CVD) will still be a main contributor of death in the world in 2030 (FAO/WHO, 2012). Elevated total cholesterol, specifically low density lipoprotein (LDL) cholesterol that exceeds normal limits may contribute to hypercholesterolemia. Consumption of diets containing high cholesterol is strongly associated with hypercholesterolemia. Consequently, LDL cholesterol level is excessive while good cholesterol HDL (High Density Lipoprotein) is insufficient to neutralize cholesterol.

To cope with hypercholesterolaemic condition, consumption of functional foods and anti-cholesterolaeic drugs has been considered. Unfortunately, these drugs were reported to have deleterious side effects on human health. In addition, the use of probiotic from specific strain has been currently developed to promote hypo-cholesterolaemic conditions. The cholesterol-lowering effect of lactobacilli is linked with the presence of bile salt hydrolase (BSH) that conjugates bile salts and assimilates cholesterol in the small intestine.

One of probiotic properties of Lactic Acid Bacteria (LAB) is its resistance to acid and bile salts (Liévin-Le Moal & Servin, 2014; Arief et al., 2015; Syah et al., 2016). The ability of gut microbes to promote de-conjugation of bile acids was considered as a probiotic activity. Bile acids were synthesized in the liver from cholesterol, and secreted as conjugates of glycine and taurine into the duodenum, and would facilitate the absorption of fat and the enterohepatic circulation. During circulation in the gastrointestinal tract, bile salts could be modified by the intestinal microbiota through de-conjugation of bile salts by the activity of enzymes. The specific objectives of this study were to analyze and identify the presence of BSH genes, BSH activity, and the ability of LAB isolates from dangke and beef to assimilate cholesterol.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

LAB strains isolated from dangke were L. fermentum A323L, L. fermentum B111K, L. fermentum B323K, L. fermentum C113L, and L. fermentum C212L (Syah et al 2016). Dangke was taken from 3 different units of traditional dangke processing in South Sulawesi, produced from cow milk. LAB strains isolated from fresh beef were L. plantarum IIA-2C12, L. plantarum IIA-1A5, and L. acidophilus IIA-2B4 (Ariel et al., 2015a). LAB was maintained in MRS broth. Cultures were kept at -20 °C, and incubated at 37 °C before being used.

Identification of BSH Gene using Polymerase Chain Reaction (PCR)

DNA extraction was modified from the method prescribed by Ariel et al. (2015a). LAB cultures incubated for 24 h at 37 °C were centrifuged at 10000 × g (at 4°C) for 1 min, and the supernatant was discarded. The precipitate was added to 200 mL of solution I (25 mM Tris-HCl buffer pH 8.0, 10 mM EDTA pH 8.0, 50 mM glucose), and then resuspended using pipette, and incubated for 5 min at room temperature. A total of 400 mL of solution II (1.2 N NaOH and 1% SDS) were mixed gently and incubated with ice for 5 min, and mixed with 300 mL of solution III (60 mL of 5 M potassium acetate, 11.5 mL of acetic acid, 28.5 mL dH2O), incubated at 5 °C and centrifuged at 10000 × g for 1 min at 4 °C. The supernatant was transferred into a new tube, and added with 1 μL of 1 mg mL-1 RNaseA, mixed gently and incubated at room temperature for 15 min, then mixed with 2-propanol and centrifuged at 10000 × g for 10 min at 4 °C. The supernatant was discarded. A total of 500 μL of 70% ethanol were mixed gently and centrifuged for 1 min, and the supernatant was discarded and then dried. The solution was dissolved with Tris EDTA buffer (100 μL) prior to PCR analysis.

Amplification of PCR BSH gene selection was performed by PCR with universal primers used by Kim et al. (2004), BSH F 1-24 (5'-AGTCCCATATG...
GC ACTGTGTCGTTTCTCC-3'), BSH R 951-931 (5'-AGCTAGCTTCAATCGGCGTGATCAGCTCC-3'). The reaction mixture (45 µL) which consisted of DNA templates (3 µL), primer forward (0.3 µL), primer reverse (0.3 µL), deoxyribonucleic triphosphate (dNTPs) (0.9 µL), MgCl2 (2.5 µL), Phire Hot Start II DNA Polymerase (0.2 µL), Phire Buffer Reaction 5X (9 µL), and double-distilled H2O (28.8 µL). PCR amplification was performed at the initial temperature of 95 °C for 15 min, followed by 35 cycles consisting of denaturation step at 94 °C for 1 min, annealing at 55 °C for 30 s. Extension phase was performed at 72 °C for 1 min. Post extension was applied for 10 min at 72 °C. PCR products were taken and stored at 4 °C for next analysis using 1% agarose electrophoresis and for DNA sequencing.

A total of 1 µL of loading dye were prepared in paraffin, then mixed with PCR product (4 µL). The solution was then poured into the well using a micropipette, and added markers (3 µL). Electrophoresis instrument was run at 100 V for 30 min. The PCR product of observable DNA band was then sequenced to identify a gene. Sequencing of PCR products of BSH gene was conducted by using sequencer machine (ABI Prims 3100-Avant Genetic Analyzer) by First Base, Malaysia. Sequence identification is meaningful to determine gene identity by comparing international LAB isolate sequence data deposited in Genbank. The length of DNA product of L. plantarum IIA-1A5 BSH gene was 982 bp. Similarity test was carried out using online program BLAST NCBI (http://www.ncbi.nlm.nih.gov/). Electrophorogram of DNA sequences were analyzed by MEGA 4 software to construct phylogenetic tree, by using neighbor joining method bootstrap 1000x.

Activity of Bile Salt Hydrolase

Enzymatic activity of BSH test was tested using procedures by Sedlackova et al. (2015) with some modifications. MRS agar at pH 5.6 (MRS broth, Bacteriology Agar, bile salt 0.3% w/v) and CaCl2 (0.375 g/L). The petri dish was incubated anaerobically at 37 °C for 48 h. LAB was inoculated on MRSA by inoculating 80 µL of culture in wells and test medium, and incubated for 72 h at 37 °C. BSH activity was characterized by the formation of bile salt precipitation zone (sediment) around colonies on agar medium containing CaCl2 and bile salt, because the cholic acid would react with CaCl2 to form precipitated salts (Sedlacova et al., 2015).

Assimilation of Cholesterol by in vitro

Cholesterol assimilation test was performed using procedures by Tomaro-Duchesneau et al. (2014). Cholesterol PEG-600 (Sigma-Aldrich 250 mg) was incorporated into MRSB to reach final concentration of 100 µg/mL. A total of 1% (v/v) inoculum of LAB was refreshed for 24 h at 37 °C. After 24 h of incubation, bacterial viability was measured by plate count method. For the analysis of cholesterol, LAB suspension was centrifuged at 4000 rpm for 10 min at 4 °C to obtain the supernatant. The obtained supernatant was transferred into a new tube of 500 µL, then added 500 µL of 33% KOH and 1 mL ethanol. The solution was stirred for 1 min and incubated at 37 °C for 15 min at room temperature. For separation phase, the solution was added 1 mL H2O and 1.5 mL of hexane. The top layer of solution was transferred into 500 µL tube for evaporation using nitrogen, and added with 1 mL of o-phthalaldehyde 50 mg/dL dissolved with acetic acid, and then mixed with the sample. The mixture was then incubated for 20 min at room temperature, and the absorbance was measured using spectrophotometer UV at wavelength of 570 nm.

Standard curve of cholesterol concentration-absorbance was made with cholesterol concentrations of 0; 3.91; 7.81; 15.6; 31.25; 62.5; 125; and 250 ppm; and cholesterol in MRSB (R2= 0.9875). The assimilation of cholesterol by LAB probiotic strains were determined as follows:

1. Cholesterol assimilation (µg/mL)= [Cholesterol(µg/mL)_{0hour} - [Cholesterol (µg/mL)]_{24hour}

2. Percentage of assimilation= [Cholesterol assimilation (µg/mL)/Cholesterol (µg/mL)_{0hour}] x 100%

3. Cholesterol assimilation in 10^{10} cell= Cholesterol assimilation (mg/mL)/[Cell viability (cfu/mL) x 10^{10}]

Statistical Analysis

Identification of Bile Salt Hydrolase (BSH) gene was conducted by Polymerase Chain Reaction (PCR) with 10 replications and BSH activity with three replications used descriptive analysis. Cholesterol assimilation data were statistically evaluated by analysis of variance (Anova) with three replications and if the differences were found among treatments, Duncan test will be used as post-hoc test (Mattjik & Sumertajaya, 2013). Treatment was different strain of lactic acid bacteria (L. fermentum (A323L, B111K, B323K, C113L, C212L), L. plantarum (IIA-1A5 and IIA-2C12), and L. acidophilus IIA-2B4)

RESULTS

Detection of BSH Genes

Amplification of L. plantarum IIA-1A5 fragments was 900-1000 bp. Previous studies reported that L. plantarum had BSH gene, located in 800-1000 bp (Bin & Jiang, 2011; Kim et al., 2004).

The result of PCR product and DNA sequences showed that BSH gene of L. plantarum IIA-1A5 demonstrated a similarity to L. plantarum Lp529 with access code of FJ439771 and FJ439775 (Figure 1). Furthermore, the alignment result indicated that base sequence of BSH gene of L. plantarum IIA-1A5 was well-aligned, and possessed fixed or similar base formation to the results of ClustalW analysis (Figure 1).
Figure 2. Alignment of BSH *L. plantarum* IIA-1A5 with BSH *L. plantarum* Lp529 bsh FJ439771 and BSH FJ439775 from GenBank (Continued on the next page).
Phylogenetic tree was constructed using MEGA6 software, neighbor joining method with bootstrap 1000x, and presented in Figure 2. The dendogram indicated that the homology of L. plantarum IIA-1A5 was 98% to BSH gene of L. plantarum Lp529 with access code of FJ439771 and FJ439775 obtained from GenBank. The similarity of L. plantarum IIA-1A5 and L. plantarum Lp529 BSH FJ439771 and FJ439775 had bootstrap value of 95%. This value denotes that topology at that branch is considered to be very accurate or consistent, even though it was tested by other methods of construction of phylogenetic tree (Horiike et al., 2009).

Activity of BSH (Bile Salt Hydrolase)

BSH activity by in vitro on 8 strains of LAB is presented in Table 1. L. plantarum IIA-1A5 was confirmed to have BSH activity indicated by the white precipitate formed, and the white precipitate was salt. The condition indicated deconjugation activity against bile salts (Sedlackova et al., 2015). Detection of BSH activity is due to the production of BSH by L. plantarum IIA-1A5. The result was augmented by the presence of the BSH gene in L. plantarum IIA-1A5. This finding was in accordance with the report of Sedlackova et al. (2015), that Lactobacillus have BSH activity except Lactobacillus strains isolated from raw cow’s milk, cheese and the colostrum.

Cholesterol Assimilation

Assimilation of cholesterol on 8 strains isolated from dangke and beef is presented in Table 2. Differences in the assimilation ability were confirmed by previous study of Tomaro-Duchesneau et al. (2014) each strains was L. reuteri NCIMB 11951, L. reuteri NCIMB 701359, L. reuteri NCIMB 702655, L. reuteri NCIMB 701089, L. reuteri NCIMB 705626, L. fermentum NCIMB 5221, L. fermentum NCIMB 8829, L. fermentum NCIMB 2797, L. rhamnosus ATCC 53103 GG, L. acidophilus ATCC 314, and L. plantarum ATCC 14917. L. fermentum B111K and L. plantarum IIA-1A5 showed assimilation level of 4.10% and 8.10%, respectively. Table 2 shows that both strains indicate significant difference in the percentage of the assimilated cholesterol (P<0.05). L. plantarum IIA-1A5 exhibited a significantly higher percentage of cholesterol assimilation. Assimilated cholesterol, in terms of mg cholesterol assimilated per 10^10 cell is presented in Table 2. When bacterial cell counts were taken into account, the results obtained were different from those previously described. Cholesterol assimilation of L. fermentum B111K and L. plantarum IIA-1A5 was significantly different, 0.13 mg of cholesterol assimilated per 10^10 cfu and 0.06 mg/10^10 cfu, respectively.

DISCUSSION

BSH in bacteria provides a special advantage for probiotic bacteria which grow in a highly competi-

Table 1. The result of bile salt hydrolase activity

| No. | Strain     | BSH activity |
|-----|------------|--------------|
| 1   | L. fermentum A323L | N            |
| 2   | L. fermentum B111K | N            |
| 3   | L. fermentum B323K | N            |
| 4   | L. fermentum C113L | N            |
| 5   | L. fermentum C222L | N            |
| 6   | L. plantarum IIA-1A5 | P           |
| 7   | L. acidophilus IIA-2B4 | N         |
| 8   | L. plantarum IIA-2C12 | N         |

Note: N: Negative; P: Positive.

Table 2. Percent of cholesterol assimilation containing 100 µg/mL of cholesterol PEG-600 for 24 hours and the amount of cholesterol assimilation expected in a probiotic dose containing 10^10 cell

| No. | Strain     | Cholesterol assimilated (%) | Cholesterol assimilated (mg/10^10 cfu) |
|-----|------------|-----------------------------|--------------------------------------|
| 1   | L. fermentum A323L | -                           | -                                    |
| 2   | L. fermentum B111K | 4.10±0.36ᵃ                  | 0.13±0.03ᵃ                           |
| 3   | L. fermentum B323K | -                           | -                                    |
| 4   | L. fermentum C113L | -                           | -                                    |
| 5   | L. fermentum C222L | -                           | -                                    |
| 6   | L. plantarum IIA-1A5 | 8.10±0.65ᵇ                 | 0.06±0.01ᵇ                           |
| 7   | L. acidophilus IIA-2B4 | -                           | -                                    |
| 8   | L. plantarum IIA-2C12 | -                           | -                                    |

Note: Means in the same column with different superscripts differ significantly (P<0.05).
tive environment such as the gastrointestinal tract by providing a better resistance to bile salts. The presence of this enzyme was useful for reduction of cholesterol (Begley et al., 2006). Cholesterol-lowering effect by BSH in vitro was revealed by BSH activity of LAB.

In this experiment, L. plantarum IIA-IA5 was confirmed to show BSH activity in 8 isolates tested. Several studies showed that Lactobacillus strains had different BSH activities (Hae-Keun et al., 2008; Mahrous, 2011). Liong & Shah (2005) and Lye et al. (2010) observed the quantitative activity of BSH by measuring the levels of amino acids (glycine/taurine) produced from conjugated bile salts. BSH activity (U/mL) was defined as the amount of enzyme to form 1 μmol of free amino acids per minute from the substrate. Their experiment indicated that the activity of BSH in L. acidophilus, Lactobacillus casei, Lactobacillus bulgaricus ranged from 0.25-1.81 U/mL.

The plausible mechanism of cholesterol reduction by BSH activity was attributed to the increase in the secretion of bile salt hydrolysis, resulting in de-conjugated bile acids in the form of free cholic acid. The substance was difficult to be reabsorbed through the enterohepatic circulation and, therefore, it was secreted through the feces. This condition led to the increase in cholesterol demand in the body, and consequently blood cholesterol level was reduced (Surono, 2004).

The level of cholesterol assimilation was affected by the number of bacteria cultured for 24 h in MRS media. The presence of the bacteria was positively correlated with the degree of assimilation. Percentage of cholesterol assimilation by L. plantarum IIA-IA5 was higher than that of L. fermentum B111K, while in assimilated cholesterol of 10^10 cells, the value of L. fermentum B111K was higher than L. plantarum IIA-IA5. The similar result was reported by Tomaro-Duchesneau et al. (2014) that percentage of cholesterol assimilation of Lactobacillus reuteri NCIMB 702656 was higher compared to L. plantarum ATCC 14917, while in assimilated cholesterol of 1010 cells, L. plantarum ATCC 14917 showed the higher value than L. reuteri NCIMB 702656.

Cholesterol assimilation was also influenced by the ability of LAB to bind cholesterol. The cholesterol binding by LAB was due to the peptidoglycan abundance in cell wall. The cell walls were able to bind or absorb cholesterol into the cell and used for the formation of cell membranes (Kumar et al., 2012).

In addition, cholesterol absorption by LAB activity in the gastrointestinal tract provides desirable effects. This absorption promotes the reduction of cholesterol availability, leading to a lower concentration of cholesterol in blood vessels. Ultimately, this action is useful to reduce the risk of hypercholesterolemia (Liong & Shah, 2005; Lye et al., 2010).

CONCLUSION

The strains of lactic acid bacteria that exhibited cholesterol-lowering effect were L. fermentum B111K and L. plantarum IIA-IA5. L. fermentum B111K was able to assimilate cholesterol, while L. plantarum IIA-IA5 had BSH gene, BSH activity, and showed cholesterol assimilation. The homology of L. plantarum IIA-IA5 was 98% to BSH gene of L. plantarum Lp529 FJ439771 and FJ439775 obtained from GenBank.

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