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Potential of Lactic Acid Bacteria Isolated from Dangke and Indonesian Beef as Hypocholesterolaemic 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 acidophillus* **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. acidophillus* **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-IA5 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^{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^{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

L. 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. acidophillu***s 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. acidophillus* **IIA-2B4. Peubah yang digunakan ialah uji keberadaan gen** *Bile Salt Hydrolas***e (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-IA5 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. plantaru***m IIA-1A5.** *L. fermentum* **B111K mempunyai kemampuan mengasimilasi kolesterol sebesar 4.10% dengan jumlah kolesterol terasimilasi dalam 10^{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^{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

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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-cholesterolaemic 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 hypocholesterolaemic 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 bile salts hydrolase (BSH) by removing the amino acid residues and form de-conjugated bile acids (cholic acid) (Kumar *et al*., 2012).

Several studies have demonstrated that some LAB can reduce cholesterol *in vitr*o and *in vivo* (Begley *et al*., 2006; Kimoto-Nira *et al*., 2007; Lye *et al*., 2010). In hyperlipidemic subjects, the consumption of probiotics could attenuate their cholesterol level, whereas decrease in triglyceride level was observed in normal subjects (Lye *et al*., 2010).

LAB is naturally found in food, and its use in the fermentation process is safe and beneficial to health, which in turn make it as GRAS (Generally Recognized as Safe) for human consumption (FAO/WHO, 2002). LAB strains isolated from dangke which was a fresh cheese-like product originating from Enrekang were *L. fermentum* A323L, *L. fermentum* B111K, *L. fermentum* B323K, *L. fermentum* C113L, and *L. fermentum* C212L (Syah *et al*., 2017). Meanwhile, Arief *et al*. (2015a) reported that LAB strains isolated from beef were *L. plantarum* IIA-2C12, *L. plantarum* IIA-1A5, and *L. acidophillus* IIA-2B4. Two LAB strains (*L plantarum* IIA- 2C12 and *L plantarum* IIA-1A5) have been reported to produce plantaricin exhibiting inhibitory effects against pathogen bacteria (Ariel *et al*., 2012; Arief *et al*., 2013; Arief *et al*., 2015). LAB contained in food may show different effects on suppression of cholesterol level. Nuraida *et al.* (2011) reported that a total of 13 LAB isolates from breast milk showed various abilities to assimilate cholesterol. Only *Pediococcus pentosaceus* I-A31, *P. pentosaceus* 2-B2, and *P. pentosaceus* 2-A16 that were able to exert the highest assimilation level. As previously mentioned, some LAB have the ability to decrease cholesterol levels. The capability and properties of each strain are various, thus selection of BSH presence in isolates from dangke (a fresh cheese like from the district of Enrekang, South Sulawesi) and beef is required. The cholesterol-lowering properties of lactic acid bacteria isolates from dangke and beef were evaluated according to their ability to assimilate cholesterol and the activity of enzymes. The study was aimed to select 8 strains of LAB from livestock products that have the potential to lower cholesterol. 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. *acidophillus* 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 Arief *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 dH_2O), 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'-AGTCCATATGT

GCACTGGTGTCCGTTTCTCC-3'), BSH R 951-931 (5'-AGTCAAGCTTCAAT CGGCGGTGATCAG CTCG-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/). Electropherogram of DNA sequences were analyzed by MEGA 4 sofware 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 $CaCl_2$ (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 $CaCl_2$ and bile salt, because the cholic acid would react with $CaCl_2$ to form precipitated salts (Sedlacova *et al.,* 2015).

Assimilation of Cholesterol by *in vitro*

Cholesterol assimilation test was performed using produres 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 H_2O 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 mixed with 250 μL of H_2SO_4. 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)= $[\text{Cholesterol}(\mu g/mL)]_{0hour} - [\text{Cholesterol } (\mu g/mL)]_{24hour}$

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. acidophillus* 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).

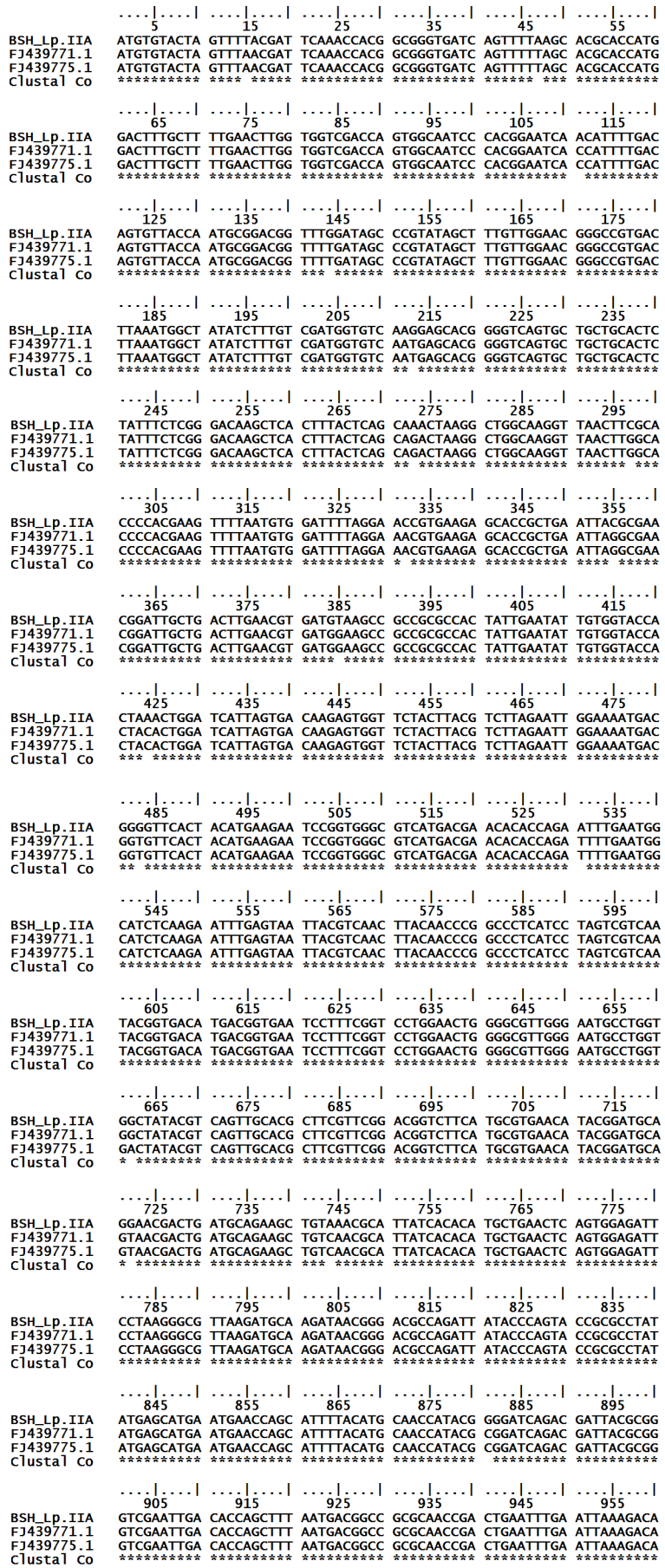

Figure 2. Aligment of BSH *L. plantarum* IIA-1A5 with BSH *L. plantarum* Lp529 bsh FJ439771 and BSH FJ439775 from GenBank (Continued on the next page).

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                    ....|....| ....|....| ....|..
                         965        975        985
BSH_Lp.IIA          ACCCAACAGT TCTGGTTAGC AACCTAA
FJ439771.1          ACCCAACAGT TCCGGTTAGC AAACTAA
FJ439775.1          ACCCAACAGT TCCGGTTAGC AAACTAA
Clustal Co          ********** ** ******* ** ****
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Figure 1. Aligment of BSH gene *L. plantarum* IIA-1A5 with BSH *L. plantarum* Lp529 bsh FJ439771 and bsh FJ439775 from GenBank

Phylogenetic tree was constructed using MEGA6 software, neighbor joining method with boothstrap 1000x, and presented in Figure 2. The dendogram indicated that the homology of *L. plantarum* IIA-IA5 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-IA5 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.

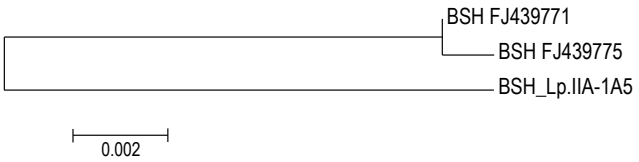

Figure 2. Phylogenetic tree of BSH *L. plantarum* IIA-1A5 with gen BSH *L. plantarum* Lp529 bsh FJ439771 and bsh FJ439775 from GenBank.

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. acidophillus* IIA-2B4	N
8	*L. plantarum* IIA-2C12	N

Note: N: Negative; P: Positive.

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. acidophillus* 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 in MRS 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 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 ± 0.36^{a}	0.13 ± 0.03^{a}
3	*L. fermentum* B323K	-	-
4	*L. fermentum* C113L	-	-
5	*L. fermentum* C222L	-	-
6	*L. plantarum* IIA-1A5	8.10 ± 0.65^{b}	$0.06^{+}0.01^{b}$
7	*L. acidophillus* 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-1A5 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 hydrolase, 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-1A5 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-1A5. 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 peptidogly can abundance in the 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-1A5. *L. fermentum* B111K was able to assimilate cholesterol, while *L. plantarum* IIA-1A5 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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