การคัดแยกและการศึกษาคุณลักษณะของแบคทีเรียกรดแลคติกที่มีฤทธิ์เอนไซม์ไบล์ซอลท์ไฮโดรเลส

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บทคัดย่อ

ภาวะหลอดเลือดแดงแข็งตัว เป็นปัจจัยสำคัญที่ทำให้เกิดความผิดปกติของหลอดเลือด นำไปสู่ภาวะโรคหลอดเลือดอุดตัน และเป็นสาเหตุของการเสียชีวิต เช่น โรคหลอดเลือดหัวใจอุดตัน หรือ โรคหลอดเลือดสมองอุดตัน ซึ่งการมีระดับคอเลสเตอรอลในเลือดสูง เป็นหนึ่งในสาเหตุที่ทำให้เกิดภาวะหลอดเลือดแข็งตัว ปัจจุบันพบว่าแบคทีเรียกรดแลคติกหลายชนิดมีการสร้างเอนไซม์ไบล์ซอลท์ไฮโดรเลสและสามารถสลายพันธะของเกลือน้ำดีที่ผลิตจากคอเลสเตอรอล และเมื่อมีการสลายพันธะของเกลือน้ำดีมากขึ้น จึงมีการนำคอเลสเตอรอลออกมาใช้ในการสร้างเกลือน้ำดีมากขึ้น ทำให้ช่วยลดระดับคอเลสเตอรอลในร่างกายได้ จุดประสงค์ของงานวิจัยในครั้งนี้ เพื่อคัดแยกและคัดกรองคุณสมบัติของแบคทีเรียกรดแลคติกที่แยกได้จากอาหารหมักดองและผลไม้ไทยที่สามารถสร้างเอนไซม์ไบล์ซอลท์ไฮโดรเลสได้โดยวิธีอาการ์สปอตบนจานอาหารเลี้ยงเชื้อ สายพันธุ์ของแบคทีเรียกรดแลคติกได้นำมาทดสอบคุณสมบัติโพรไบโอติกในหลอดทดลอง ได้แก่ ความสามารถในการยึดเกาะกับเยื่อบุลำไส้และความสามารถในการทนต่อกรดและเกลือน้ำดี นอกจากนี้ สายพันธุ์ที่สร้างเอนไซม์ไบล์ซอลท์ไฮโดรเลสนำมาพิสูจน์สายพันธุ์ โดยอาศัยลักษณะทางฟีโนไทป์และจีโนไทป์ของสายพันธุ์ จากการศึกษาพบว่ามีแบคทีเรียกรดแลคติก 2 สายพันธุ์ จากที่แยกได้ 91 สายพันธุ์ คือ สายพันธุ์ F34-4 และสายพันธุ์ F35-5 ที่แยกจากแหนมหมูและตะขบป่า (*Flacourtia indica* (Burm. f.) Merr.) ตามลำดับ สามารถสร้างเอนไซม์ไบล์ซอลต์ไฮโดรเลส และทั้งสองสายพันธุ์มีความสามารถในการเกาะติดกับเยื่อบุทางเดินอาหารจากการทดสอบใน Caco-2 cell line และมีคุณสมบัติที่สามารถทนต่อกรดและน้ำดีได้ นอกจากนี้ ลักษณะทางจีโนไทป์ โดยการวิเคราะห์ลำดับของสารพันธุกรรมในช่วง 16S ribosomal RNA gene ของทั้งสองสายพันธุ์ได้ผลออกมาใกล้เคียงกับแบคทีเรียกรดแลคติกชนิด *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917 มีความเหมือนที่ 99.78% และ 99.43% ตามลำดับ แบคทีเรียกรดแลคติกสายพันธุ์ที่สามารถสร้างเอนไซม์ไบล์ซอลต์ไฮโดรเลสจะสามารถนำมาใช้เพื่อเป็นอาหารเสริมโพรไบโอติก เพื่อควบคุมระดับคอเลสเตอรอลในเลือดของผู้ป่วย รวมทั้งนำมาผลิตเป็นชีวภัณฑ์รักษาสำหรับผู้ป่วยที่มีภาวะระดับคอเลสเตอรอลในเลือดสูง หรือผู้ที่มีความเสี่ยงในการเกิดโรคหลอดเลือดอุดตันเพื่อทดแทนการใช้ยาที่ทำจากสารเคมีซึ่งมีผลข้างเคียงค่อนข้างมาก

คำสำคัญ: แบคทีเรียกรดแลคติก โพรไบโอติก ภาวะคอเลสเตอรอลในเลือดสูง ไบล์ซอลต์ไฮโดรเลส

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Isolation and characterization of lactic acid bacteria with bile salt hydrolase activity

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Abstract

Atherosclerosis is an important cause of atherosclerotic cardiovascular diseases and vascular death such as ischemic heart disease or ischemic stroke. Hypercholesterolemia is one of the major risk factors potentiated a prevalence of atherosclerosis. In probiotics aspect, several of lactic acid bacteria (LAB) can produce bile salt hydrolase (BSH) enzyme which promotes bile salt deconjugation leading to an increase in cholesterol utilization. The objective of this study was to isolate and screen BSH activity of lactic acid bacteria isolated from Thai fermented foods and fruits using agar spot method. These isolates were also tested in vitro for their probiotic properties such as adhesion to intestinal epithelial cell line and acid-bile tolerance. Moreover, the BSH-producing strains were identified by phenotypic and genotypic characteristics. Two of 91 different isolates, F34-4 and F35-5, were found to have BSH producing capability. These 2 isolates were isolated from sour pork *(naem)* and *Flacourtia indica* (Burm. f.) Merr. Fruit, respectively. Additionally, two selected isolates yielded a positive result for colonizing ability on intestinal cell lining by adhesion assay on Caco-2 cell line and tolerated acid and bile salt *in vitro*. Both of F34-4, and F35-5 isolates have their genotypic identification by 16S ribosomal RNA gene sequence analysis as *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917 with 99.78% and 99.43% similarity, respectively. The BSH-active LAB can be used as probiotic food supplements to control cholesterol level in patient and develop a potential biotherapeutic agent for hypercholesterolemia patients and who have a risk of vascular diseases instead of using chemical drugs which have many side effects.

Keywords: lactic acid bacteria, probiotics, hypercholesterolemia, bile salt hydrolase

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Introduction

Nowadays the incidence of deaths from atherosclerotic cardiovascular diseases (ACD) such as ischemic heart disease and ischemic stroke is the highest cause of death worldwide, estimated 17.7 million people per year.[1-2] The pathogenesis of atherosclerosis is complex but can be explained by response – to – injury hypothesis that is a chronic vascular inflammatory response due to endothelial injury. From the pathogenesis and evidence show that lowering of serum cholesterol by diet or drugs can slow the rate of progression of atherosclerosis, regress some plaques, and reduce the risk for cardiovascular diseases.[3]

In 2002, The joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Working Group's defined probiotics as "live microorganisms which when administered in adequate numbers confer a health benefit on the host".[4] Lactic acid bacteria are the major group of probiotics which express beneficial health effects. The beneficial effects of probiotics are introduced in many aspects not only treating gut diseases by maintaining intestinal microbial balance but also regulating the immune function like allergy or inflammation response and reducing incidence of the metabolic disorders such as hypercholesterolemia and type 2 diabetes mellitus.[5-9]

The lowering of serum cholesterol level by bile salt deconjugation is one of the most mentioned of the anti-metabolic disease properties of the probiotics. Bile salt hydrolase (BSH) is the enzyme that can be produced by many bacteria, especially probiotics lactic acid bacteria. This enzyme can hydrolyze the amide bond between amino acid; glycine/ tauline and unconjugated bile salt which is not absorbable by gut epithelial cell.[10-11] The mechanism of these probiotics is producing bile salt hydrolase in gut lumen and a higher number of intraluminal unconjugated bile salts decrease intestinal bile salt reabsorption of enterocyte to enterohepatic pathway and increase excretion of insoluble bile into feces. Moreover, the increase of unconjugated bile salts and bile salts excretion promote human cholesterol 7-alpha-hydroxylase (CYP7A1) activity to utilize the serum cholesterol as the precursor of bile salt to the primary bile salt in hepatocyte and finally reduce serum cholesterol.[11]

Objectives

1) To isolate lactic acid bacteria from Thai fermented foods and fruits.

2) To screen bile salt hydrolase activity of isolated lactic acid bacteria.

3) To determine probiotic properties, adherence activity and acid-bile tolerances.

4) To identify of BSH-active lactic acid bacteria by phenotype and genotype.

Materials and Methods

Isolation of lactic acid bacteria from fermented foods and fruits

Eight samples of fermented foods and fruits including fermented cabbage, fermented lettuce, fermented bamboo sprouts, pickled

fish *(pla-som)*, pickled fish *(pla-jom)*, pickled shrimp *(kung-jom)*, sour pork *(naem)* and *Flacourtia indica* (Burm. f.) Merr. fruit *(bukben, takob-pa)*. The one-gram of each sample was activated in de Man Rogosa Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK) and incubated at 37°C for 48 hours under anaerobic condition in an anaerobic jar. The culture was then re-streaked for isolation onto MRS agar plates supplemented with 0.3% calcium carbonate and incubated at 37°C for 48 hours under anaerobic condition. The single pure colony that has different morphology produces clear zone around the colony was selected and tested for Gram's reaction. The pure cultures with Gram's positive cocci or bacilli were collected and stored in MRS broth with 20% glycerol at - 80°C for further studies.

Screening of bile salt hydrolase activity

Each lactic acid bacteria isolate was screened for BSH activity by using spot plate BSH assay as described in Moser SA[12] and Shehata MG protocol.[13] The concentrations of isolates were adjusted to 10^9 CFU/mL by spectrophotometer at wavelength of 600 nm. Ten µl (10^9 CFU/mL) of each isolate was spotted onto MRS agar plates with 0.5% Taurodeoxycholic acid (TDCA; conjugated bile salt, Sigma, USA) and 0.37 g/L Calcium chloride, then incubated at 37°C for 24-48 hours under anaerobic condition. The plate without TDCA were used as control. The BSH-active lactic acid bacteria hydrolyzed TDCA and showed precipitation zones of deoxycholic acid (unconjugated bile salt) around their colonies.

Determination of probiotic characteristics

Adhesion to intestinal epithelial cells

The adenocarcinoma cell lines (Caco-2) (ATCC, HTB-37) were used for adherence assay which modified from Maragkoudakis PA, et al.[14] The BSH-active isolates (F34-4 and F35-5) and positive control, *Lactobacillus rhamnosus* GG (LGG), were adjusted the concentration to 10^9 CFU/mL in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-invitrogen, USA) and doubly cultured on Caco-2 monolayer cells. After incubation for 1 hour, monolayer of Caco-2 cell lines were washed by phosphate buffered saline to wash out non-attached lactic acid bacteria. The adhered bacteria on Caco-2 cells were removed by 1 mL of 0.04% Polysorbate-80. The adhesion of isolates on Caco-2 cell line was calculated as percentage of viable lactic acid bacteria.

Tolerance to acid and bile salt

Tolerance test was modified form Ladda B.[15] The acid and bile tolerance test was done in BSH-active isolates. The isolates were adjusted the concentration to 10^9 CFU/mL. 100 µl of adjusted isolates were incubated in different levels of HCl-added MRS broth pH 2.0, 3.0, 4.0 and different concentrations of bovine bile salt (Sigma, USA) MRS broth (0.3%, 0.8%) at 37°C for 3 hours under anaerobic conditions. The number of total viable

colony forming units in 1 mL were counted at incubation time = 0 and 3 hours under 37°C anaerobic condition. The experiments were performed two times, in duplicate.

Phenotypic identification by physical and biochemical characteristics

The colony characteristics of each selected isolate were described, namely, shape, size, color, elevation, margin and shininess and microscopic characteristics including Gram's stain, size, shape and distribution. To identify their acid production from various forms of carbohydrate include Arabinose, Cellobiose, Fructose, Galactose, Glucose, Lactose, Maltose, Mannose, Mannitol, Melibiose, Raffinose, Rhamnose, Salicin, Sorbitol, Sucrose, Trehalose and Xylose, and determine their salt toleration at 1%, 6% and 8% NaCl and acid production from amino acids include esoulin, arginine. The cultured was incubated duplicate at 37°C for 24-48 hours under anaerobic condition using anaerobic jar.

Genotypic identification by 16S ribosomal RNA gene sequence analysis

Each selected isolate was cultured in MRS agar plates and incubated at 37°C for 24 hours and used for 16S ribosomal RNA gene sequence. The sequences of the PCR products using the prokaryotic 16S ribosomal DNA universal primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR template was mixed with PCR mixer 90 ul for 16S rRNA gene sequences and the 16S rDNA sequences coding region was amplified by PCR in a PCR thermal cycler. The PCR product was purified and analyzed for gene sequence analysis by Macrogen in Korea. The similarity of 16S rRNA gene sequences were determined using the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov) and EzTaxon bioinformatics software (https://www.ezbiocloud.net). The related species were identified by MEGA program version 7.0 with 1,000 neighbor-joining method of bootstrap analysis.

Statistical analysis

This study collected, analyzed and visualized data by Microsoft Excel 2016. The analytical data was used to comparison probiotic characteristics including adhesion assay, acid and bile tolerance test. In adhesion assay, one-way analysis of variance to comparison was used between two selected isolated and positive control. As for acid and bile tolerance test, independent t-test was used for analysis differentiation of mean total viable colony forming units between control group and testing groups in different conditions. All experiments were performed two times, in duplicate.

Results

Isolation of lactic acid bacteria

A Number of 91 lactic acid bacteria were isolated from eight samples, including fermented cabbage, fermented lettuce,

fermented bamboo sprouts, pickled fish *(pla-som)*, pickled fish *(pla-jom)*, pickled shrimp *(kung-jom)*, sour pork *(naem)* and *Flacourtia indica* (Burm. f.) Merr. fruit *(bukben, takob-pa)* as shown in Table 1. They were Gram positive cocci and bacilli with acid producing and catalase negative.

Table 1 The number of lactic acid bacteria isolates from all of samples in this study

Sample NO.	Source	Number of lactic acid bacteria isolates
F28	Fermented cabbage	14
F29	Fermented lettuce	17
F30	Fermented bamboo sprouts	16
F31	Pickled fish (*pla-som*)	5
F32	Pickled fish (*pla-jom*)	5
F33	Pickled shrimp (*kung-jom*)	8
F34	Sour pork (*naem*)	15
F35	*Flacourtia indica* (Burm. f.) Merr. fruit (*bukben, takob-pa*)	11
Total		91

Screening of bile salt hydrolase activity

Bile salt hydrolase activities of isolates were screened by spot plate BSH assay. The result indicated that two lactic acid bacteria isolates, F34-4 and F35-5, had BSH activities and revealed precipitation zones of unconjugated bile salt around their colonies (Figure 1).

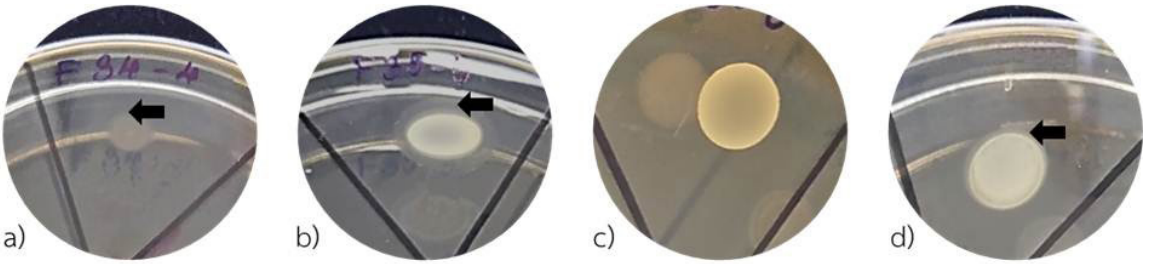

Figure 1 The spot plate BSH assay F34-4 isolate (a), F35-5 isolate (b), negative control (c), and positive control (d)

*black arrow indicates the precipitated zone

Screening of probiotic characteristics

Adhesion to intestinal epithelial cells

The lactic acid bacteria isolate with bile salt hydrolase activity were selected to evaluate the colonizing ability on intestinal cell lining by adhesion assay on Caco-2 cell line. F34-4 and F35-5 isolates exhibited ability to adhere at 3.15% and 3.12% respectively. Both isolates were as adhesive as Lactobacillus rhamnosus GG (LGG), positive control, with *p*-value from ANOVA test = 0.625. As shown in Table 2 and Figure 2.

Table 2 The percentage of ability of adhesion to intestinal cell lining of F34-4, F35-5 isolates. *Lactobacillus rhamnosus* GG (LGG) were used as positive control in this study.

Isolate	% Adhesion (Mean ± Standard Error)
F34-4	3.15 ± 0.66
F35-5	3.12 ± 0.81
Positive control (LGG)	3.91 ± 0

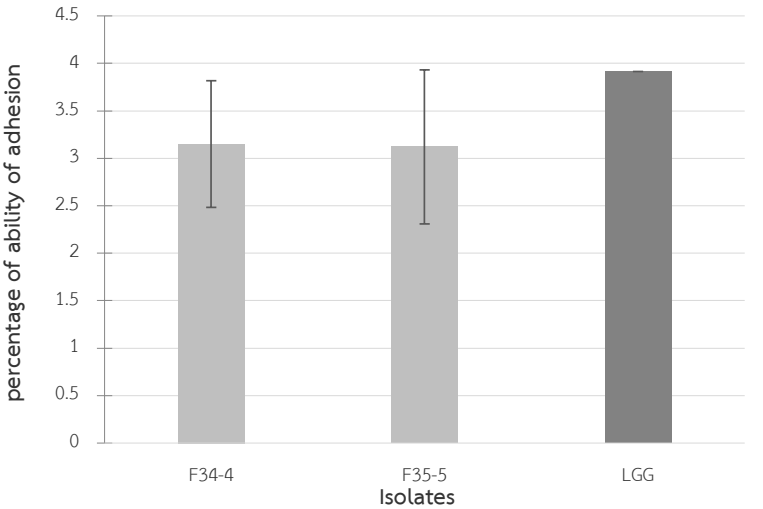

Figure 2 The percentage of ability of adhesion to intestinal cell lining

Tolerance to acid

Two candidate isolates were tested for gastrointestinal condition by acid tolerance test. The result revealed the log number of total viable count of both F34-4 and F35-5 which were insignificantly different at pH 4.0 and pH 3.0 from that of control MRS broth at pH 6.5. But at pH 2.0, both log number of total viable colony forming units significantly decreased as shown in Table 3 and Figure 3 - 4. This finding indicated two lactic acid bacteria, F34-4 and F35-5 were able to survive in acidic condition.

Table 3 The viability of F34-4 and F35-5 isolates in different level of acidic to evaluate acid tolerance ability

Isolate	0 hour (CFU/mL)	3 hours (CFU/mL)			
		Control pH 6.5	pH 4.0	pH 3.0	pH 2.0
F34-4	5.85×10^8	7.90×10^8	6.60×10^8	5.30×10^8	55.00×10^5
F35-5	8.05×10^8	10.15×10^8	21.60×10^8	12.00×10^8	5.00×10^5

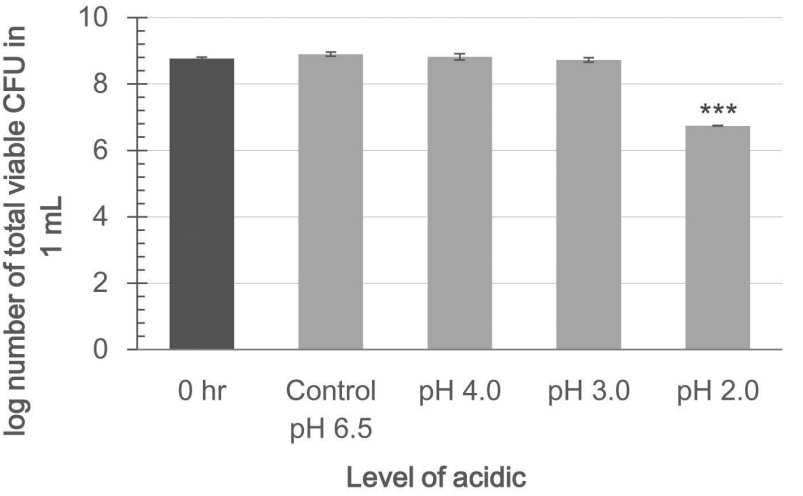

Figure 3 Log number of total viable colony forming units in 1 mL of F34-4 isolate in various pH conditions compared with control (* *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001)

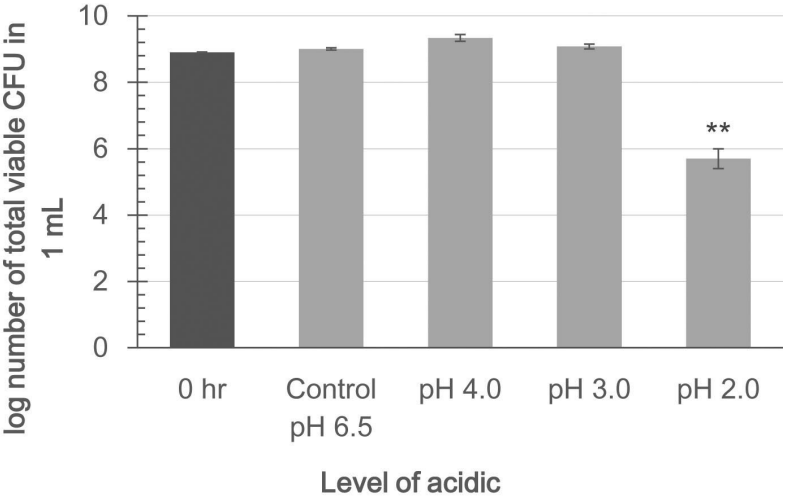

Figure 4 Log number of total viable colony forming units in 1 mL of F35-5 isolate in various pH conditions compared with control (* *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001)

Tolerance to bile salt

Two candidate isolates, F34-4 and F35-5 were tested for their bile tolerance. The viability of each isolate in 0.3% and 0.8% of bile salt in MRS broth did not show a significant difference between log number of colonies in MRS control, 0.3% and 0.8% bile group as shown in Table 4 and Figure 5-6. The result indicated two isolates were able to tolerate in bile salt, which represent the intestinal environment.

Table 4 The viability of F34-4 and F35-5 isolates in different concentration of bile salt to evaluate bile tolerance ability

Isolate	0 hr (CFU/mL)	3 hours (CFU/mL)		
		Control	0.3% bile	0.8% bile
F34-4	4.75×10^8	4.90×10^8	4.86×10^8	4.30×10^8
F35-5	2.36×10^8	2.13×10^8	2.11×10^8	2.89×10^8

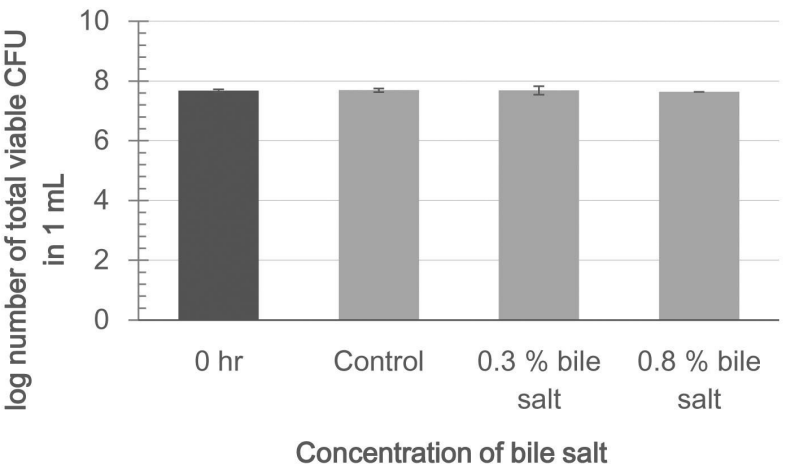

Figure 5 Log number of total viable colony forming units in 1 mL of F34-4 isolate in 0.3%, 0.8% bile salt compared with control (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001)

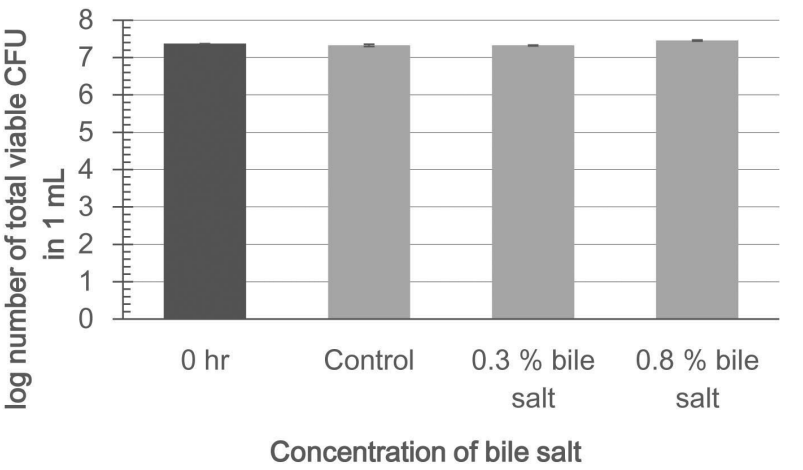

Figure 6 Log number of total viable colony forming units in 1 mL of F35-5 isolate in 0.3%, 0.8% bile salt compared with control (* *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001)

Phenotypic identification

The BSH-producing lactic acid bacteria appeared to be gram positive bacilli and did not show distinct differences in the characteristics of colony as shown in Table 5 and Figure 7. The F34-4 and F35-5 isolates could produce acid from large variety of carbohydrates fermentation and exhibited salt tolerance and amino acid utilization as shown in Table 6-7.

Table 5 The physical characteristics and microscopic characteristics of F34-4 and F35-5 isolates

Isolate	Colony characteristic	Microscopic characteristic
F34-4	Large, cream-colored, convex, shiny, and entire margin colonies	Gram-positive, thick short rod
F35-5	Small, white, convex, shiny, and entire margin colonies	Gram-positive, medium rod

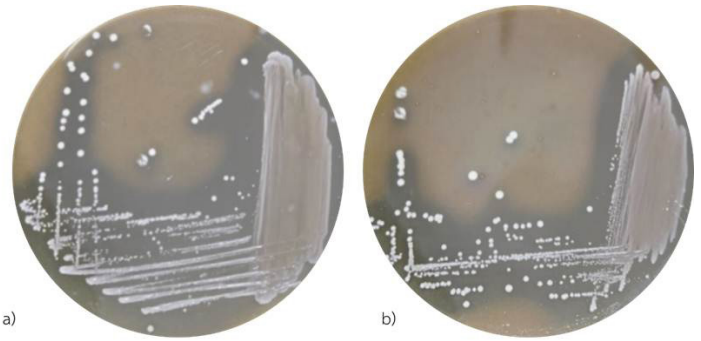

Figure 7 Colony characteristics of F34-4 isolate (a) and F35-5 isolate (b)

Table 6 The characteristics of F34-4 and F35-5 isolates in fermentation of several carbohydrates

Isolate	Carbohydrate																
	Arabinose	Cellobiose	Fructose	Galactose	Glucose	Lactose	Maltose	Mannose	Mannitol	Melibiose	Raffinose	Rhamnose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose
F34-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F35-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+ is indicated that there is acid production, but – means that there is not acid production)

Table 7 The characteristics of salt tolerance and amino acid utilization of F34-4 and F35-5 isolates

Isolate	NaCl			Amino acid	
	1%	6%	8%	Esculin	Arginine
F34-4	+	-	-	+	-
F35-5	+	-	-	+	-

(+ is indicated that there is survival ability, but – means that these bacteria do not survive)

Genotypic identification by 16S ribosomal RNA gene sequences

From sequence analysis of 16S ribosomal RNA gene of F34-4 and F35-5 isolates, 1,286 bases from F34-4 gene and 1,549 bases of F35-5 gene were shown to have the similarity with those of Lactobacillus *plantarum* subsp. plantarum ATCC 14917 at 99.78% and 99.43%, respectively. The phylogenetic tree of F34-4 and F35-5 indicated the relationship between each species of Lactobacillus as shown in Figure 8.

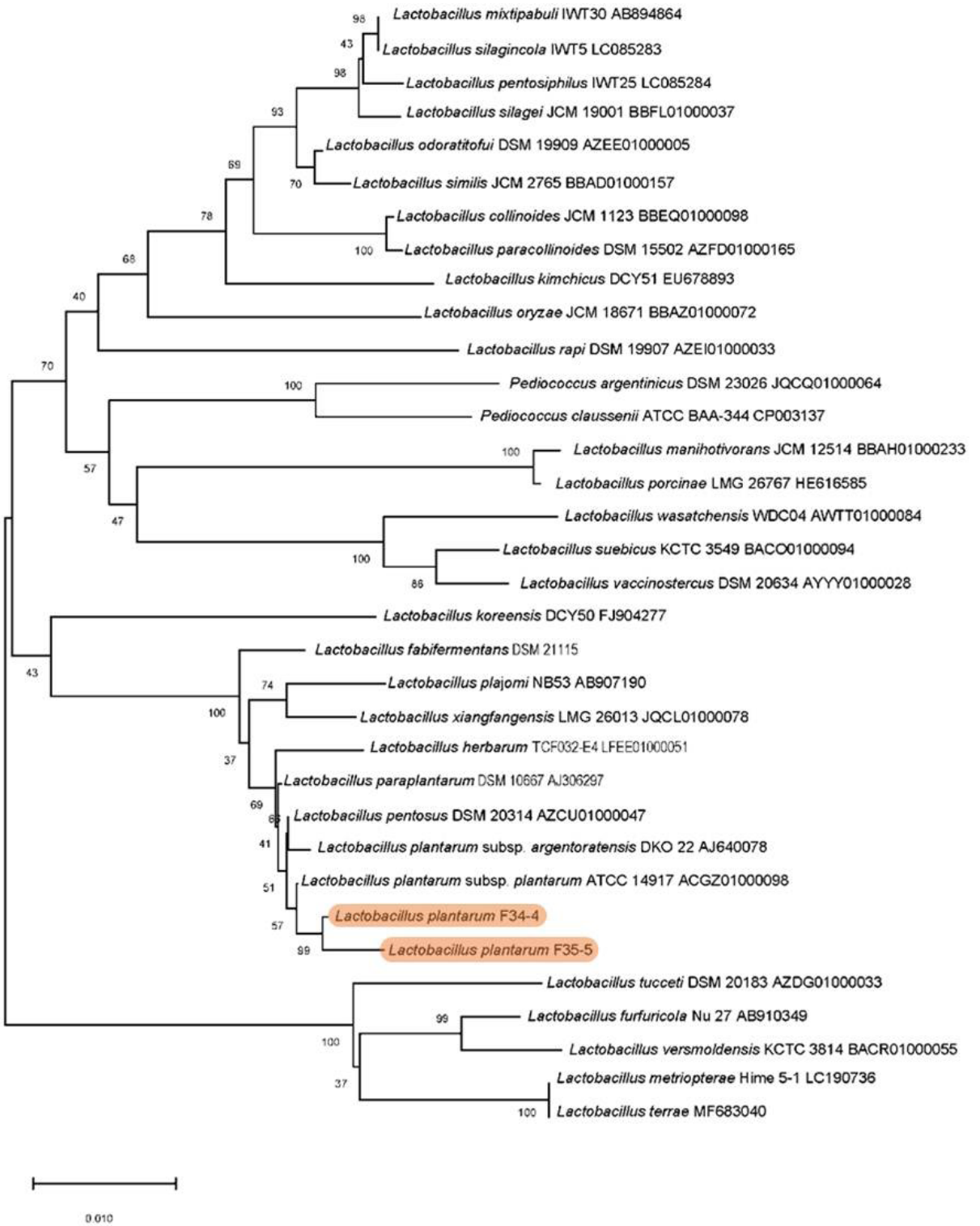

Figure 8 Phylogenetic tree of F34-4 and F35-5 isolates

Discussion

Probiotics have been recently researched in many aspects of benefits shown in meta-analysis such as antihypertensive effects, and the ability of lowering BMI, blood glucose including LDL-cholesterol.[16-17] Especially, the anti-hypercholesterolemia effect which is now widely interesting due to the potential risk of high level of cholesterol of cardiovascular consequences.

One of the most widely used probiotics, *Lactobacillus plantarum* show varieties of beneficent properties. Focusing on an effective cholesterol lowering effect, three hypothesized pathways have been formulated. Bile salt hydrolase deconjugation was promising in *Lactobacillus plantarum* species[18] and consistent with result from our data of *Lactobacillus plantarum* F34-4 and *Lactobacillus plantarum* F35-5.

Survival and colonization of probiotics in gastrointestinal mucosa depend on the adhesion ability, acid tolerance, and bile salt tolerance. Adherence to intestinal epithelial cells and bile salt tolerance of F34-4 and F35-5 isolates were not shown any significant difference from those of control. On the contrary, with the acid toleration test, colonies survival was significantly decreasing in pH 2.0. Our data is consistent with those from other studies; one from Indian fermented food indicated acid tolerance ability,[19] and one from kefir (Malaysian fermented food) was able to withstand moderate level of acidity, pH 3.0 and pH 4.0 while all isolates did not survive in pH 2.0 circumstances[20]. Suggestion of further use of F34-4 and F35-5 isolates might have to avoid contact to harsh acidic surrounding such as gastric content.

Genotypic identification of F34-4 and F35-5 isolates from 16s ribosomal RNA gene appeared that both isolates were *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917 with 99.78% and 99.43% similarity respectively.

The BSH-active LAB can be used as probiotic food supplements to control cholesterol level[17] in patient and development of a potential biotherapeutic agent for hypercholesterolemia patients and who have a risk of vascular diseases instead of using chemical drugs which have many side effects.

Conclusion

In this study, lactic acid bacteria have the ability to hydrolyze conjugated bile salt to the unabsorbable unconjugated bile salts which tend to increase bile salt excretion and indirectly lower cholesterol by utilization to integrate new bile salts. The study of two BSH-active LABs, *Lactobacillus plantarum* F34-4 isolated from Sour pork *(naem)* and *Lactobacillus plantarum* F35-5 isolated from fruit of *Flacourtia indica* (Burm. f.) Merr. *(bukben, takob-pa)*, showed that both isolates could hydrolyze TDCA in agar plate and colonize on the intestinal Caco-2 cells. In addition, these two isolates could tolerate to pH 3.0-4.0 and 0.3%-0.8% bile salt which infer that these LAB can survive in gut environment. From the phenotypic and genotypic studies, the isolates showed the most similarity to

those of *Lactobacillus plantarum* subsp. *plantarum* ATCC 14917.

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