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Original Article

Cholesterol-lowering activity and functional characterization of lactic acid bacteria isolated from traditional Thai foods for their potential used as probiotics

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Abstract

Hypercholesterolemia is one of the major risk factors related to global health problems. Probiotics with cholesterol-lowering activity can improve cholesterol metabolism without any adverse effects. In this study, seventeen lactic acid bacteria (LAB) isolated from traditional Thai foods exhibited the ability to produce bile salt hydrolase (BSH) and assimilate cholesterol. From these isolates, *Lactobacillus reuteri* TF-7, *Enterococcus faecium* TF-18, and *Bifidobacterium animalis* TA-1 were selected for cholesterol-lowering LAB candidates due to their robust cholesterol reduction activity with different genera. Furthermore, these strains had proven to be safe and expressed different characteristics. *L. reuteri* TF-7 could produce gamma-aminobutyric acid (GABA), whereas *B. animalis* TA-1 was the most potent antioxidant strain. Moreover, all three LAB strains could tolerate under the acid and bile salt conditions, and adhered to the intestinal epithelial cells. According to these findings, three LAB strains are potential cholesterol-lowering probiotics, which can be applied as probiotic supplements.

Keywords: cholesterol-lowering activity, bile salt hydrolase, functional characteristics, lactic acid bacteria, probiotics

1. Introduction

Hypercholesterolemia is one of the major risk factors for the development of cardiovascular disease (CVD) and associated metabolic syndromes, which contributes to the serious complications and eventual death of people worldwide (Akashi *et al.*, 2017). Although changing food consumption behavior and administration of HMG-CoA reductase inhibitors have been used successfully in the treatment of patients with hypercholesterolemia (Lei & Yang, 2020), long-term administration of these drugs causes the severe adverse effects, such as myopathy and rhabdomyolysis (0.2% of patients treated with statin) (Bellosta, Paoletti, & Corsini, 2004). Therefore, dietary supplements with health benefits, especially probiotics, have been attracted a lot of attention to use as alternative treatments.

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Probiotics are an essential constituent of the gastrointestinal microbiota, which develop as symbiotic microorganisms for providing health-promoting effects. Thus, probiotics are defined as "living-microorganisms which when administered in adequate amounts confer health benefits to the host" (Food and Agriculture Organization, World Health Organization, 2002). Lactic acid bacteria (LAB) are beneficial microorganisms that possess probiotic properties according to Generally Recognized as Safe Guidelines (Thomas, 2018). Various studies have reported that several genera of LAB, especially *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* are able to reduce cholesterol by bile salt hydrolase (BSH) enzyme activity and cholesterol assimilation (Ding *et al.*, 2017). Since BSH is a potential enzyme secreting from LAB that plays a significant role in biodegradation of bile salts, leading to a reduction of cholesterol levels. Cholesterol assimilation, a direct mechanism of cholesterol reduction, utilizes cholesterol to incorporate into plasma membrane (Bustos, Font de Valdez, Fadda, & Taranto, 2018; Lye, Rahmat-Ali, & Liong, 2010). Moreover, the diversity of LAB can synergize their health-promoting effects. Thus, prior to alternative treatment development, it is crucial to select different genera of cholesterol-lowering LAB to enhance the hypocholesterolemic effect because cholesterol-lowering activity is strain dependence which differs within the similar genera (Al-Muzafar & Amin, 2017). However, the safety and specific properties of probiotics should also be characterized in cholesterol-lowering LAB in addition to cholesterol reduction. Moreover, adherence ability and gastrointestinal tolerance to gastric acid and bile salt are essential characteristics of probiotics for establishment as the gastrointestinal microbiota when they are used as orally supplements (Ladda, Theparee, Chimchang, Tanasupawat, & Taweechotipatr, 2015; Mainville, Arcand, & Farnworth, 2005).

Therefore, the purposes of this study were to determine the cholesterol-lowering effect by BSH activity and cholesterol assimilation of LAB isolated from traditional Thai foods. The different genera of LAB with the highest ability to reduce cholesterol were selected as cholesterol-lowering LAB candidates after genotypic identification by 16S ribosomal RNA (16S rRNA) gene sequencing to evaluate *in vitro* probiotic characteristics, including safety, gamma-aminobutyric acid (GABA) biosynthesis, antioxidant activity, acid and bile salt tolerance, as well as adherence to the intestinal epithelial cells.

2. Materials and Methods

2.1 Isolation of LAB from traditional Thai foods

Fifty samples of traditional foods were taken from the crown property market, Chachoengsao, Thailand. Two grams of each sample were added in 5 mL of De Man, Rogosa, and Sharpe (MRS) broth (HiMedia, India), followed by anaerobic incubation (10% (v/v) CO_2, 10% (v/v) H_2, and 80% (v/v) N_2) at 37 ℃ for 48 h. After that, bacterial suspension was spread on MRS agar plates supplemented with 0.3% (w/v) $CaCO_3$ and incubated under the same conditions. A single, pure colony with the clear zone (lactic acid production) was selected for Gram's staining and catalase test. Based on results, each bacterial isolate with Gram-positive and catalase-negative was stored in MRS broth supplemented with 40% (v/v) glycerol at -80 ℃ for further use.

2.2 Cholesterol-lowering assays

2.2.1 Qualitative investigation of BSH activity

BSH activity was determined according to the previous method of Allain *et al.* (2018). MRS agar plates containing either 0.5% (w/v) sodium salt of tauro-deoxycholic acid (TDCA) or 0.5% (w/v) sodium salt of glyco-deoxycholic acid (GDCA, Sigma, USA) and 0.037% (w/v) $CaCl_2$ were prepared to test BSH activity. Briefly, LAB isolates were grown in MRS broth under anaerobic conditions at 37 ℃ for 24 h. Bacterial culture was washed twice and re-suspended in phosphate buffered saline (PBS, 0.1 M, pH 7.2) to $1x10^9$ CFU/mL, then 10 µL of bacterial suspension was spotted on modified MRS agar plates. After anaerobic incubation at 37 ℃ for 72 h, the diameter of precipitation zone (deconjugated bile salt precipitates) was measured.

2.2.2 Cholesterol assimilation

Cholesterol assimilation of LAB isolates was measured with slight modifications, based on the previous procedure (Choi & Chang, 2015). Bacterial cells ($1x10^9$ CFU/mL) were cultured in MRS broth containing 0.3% (w/v) bovine bile (Sigma, USA) and 0.1 g/L sterile filtered water-soluble cholesterol (Cholesterol–PEG 600, Sigma, USA), followed by anaerobic incubation at 37 ℃ for 24 h. The uninoculated medium was used as a control. Subsequently, the supernatant was obtained by centrifugation at 5,000×g, 4 ℃ for 10 min. The reaction mixture of *O*-phthalaldehyde method was prepared with the addition of 0.5 mL of supernatant, 1.5 mL of 95% (v/v) ethanol, and 1 mL of 33% (w/v) KOH. Then, the mixture was shaken and incubated at 60 ℃ for 10 min, followed by addition of 2.5 mL of hexane and 0.5 mL of distilled water after cooling down. Following incubation at 25 ℃ for 10 min, 1.5 mL of hexane phase was collected and evaporated with flowing nitrogen gas. Two milliliters of *O*-phthalaldehyde solution (0.5 mg of *O*-phthalaldehyde/mL of acetic acid, Sigma, USA) were added, then the solution was incubated at 25 ℃ for 10 min before adding with 1 mL of H_2SO_4. The optical absorbance of sample (A_s) and control (A_c) was measured at a wavelength of 550 nm. Cholesterol assimilation was calculated as percentage according to the equation:

$$\% \text{ cholesterol assimilation} = ((A_c - A_s) / A_c) \times 100$$

2.3 Genotypic identification using 16S rRNA gene sequencing

16S rRNA gene was a target sequence of polymerase chain reaction (PCR) using forward primer (27F, 5'-AGAGTTTGATCCTGGCTCAG-3') and reward primer (1492R, 5'-GGTTACCTTGTTACGACTT-3'), following to the previous method (Lillo *et al.*, 2006). Briefly, single colony of LAB isolates with the cholesterol-lowering ability was extracted DNA using HiPurA™ Bacterial Genomic DNA Purification Kit (HiMedia, India) according to the manufacturer's instruction. PCR Master mix was prepared

with One PCR™ *Plus* pre-mixed solution (GeneDireX, USA) and then the 16S rRNA gene amplification was performed by PCR reaction using C1000™ Thermal Cycler (Bio-Rad, USA). After that, PCR products were purified using PCR Clean-Up & Gel Extraction Kit (GeneDirex, USA).

The nucleotide sequences of 16S rRNA gene were carried out by Macrogen Inc. (Seoul, Korea). Subsequently, the 16S rRNA gene-based nucleotide sequences were analyzed to identify bacterial species 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 closest relative species (≥ 99%) of the 16s rRNA gene sequences were used as the criterion for identification. The different genera of LAB with the most robust BSH activity and cholesterol assimilation were selected as cholesterol-lowering LAB candidates for further study.

2.4 Evaluation of safety

2.4.1 Hemolytic activity

Overnight cholesterol-lowering LAB culture was washed twice and re-suspended with PBS to $1x10^9$ CFU/mL, then 10 µL of bacterial suspension was spotted on brain heart infusion (BHI) agar (HiMedia, India) plates supplemented with 5% (v/v) sheep blood and anaerobically incubated at 37 °C for 48 h. Hemolytic zone surrounding the colony was observed, compared with *Streptococcus pyogenase* DMST 4478 (positive control) and *E. faecalis* DMST 4737 (negative control).

2.4.2 Antibiotic susceptibility

Eight different antibiotic disks (Thermo Fisher Scientific, USA) were selected according to the clinical importance, including ampicillin (AMP, 10 µg), chloramphenicol (CHL, 30 µg), gentamycin (GEN, 10 µg), nalidixic acid (NAL, 30 µg), penicillin G (PEN, 10 U), erythromycin (ERY, 15 µg), tetracycline (TET, 30 µg), and vancomycin (VAN, 30 µg). Overnight bacterial culture was adjusted with PBS to $1x10^8$ CFU/mL, and the bacterial suspension was swabbed on Mueller-Hinton (MH) agar (HiMedia, India) plates. Antibiotic disks were then placed on MH agar plates containing bacterial cells. After anaerobic incubation at 37 °C for 24 h, the diameter of the inhibition zone was measured to interpret the antibiotic susceptibility (Haghshenas *et al.*, 2017).

2.5 GABA biosynthesis

Glutamic acid and monosodium glutamate (Sigma, USA) were used as substrates to determine GABA production. Cholesterol-lowering LAB were cultured in MRS broth supplemented with either 5% (w/v) glutamic acid or 5% (w/v) monosodium glutamate at an initial concentration of $1x10^9$ CFU/mL and anaerobically incubated at 37 °C for 24 h. The supernatant was obtained by centrifugation at 5,000×g, 25 °C for 10 min. Thin-layer chromatography (TLC) was used to determine GABA. Briefly, 0.2 µL of the supernatant was spotted onto Silica gel TLC plates (Merck, Germany) and placed in the mobile phase containing 50% (v/v) n-butanol, 30% (v/v) acetic acid, and 20% (v/v) distilled water. Finally, TLC plates were dried and sprayed with 0.5% (w/v) ninhydrin. GABA production was compared with GABA standard, supernatant of *L. brevis* ST-69 (source: kimchi, Bangkok, Thailand) (positive control), and MRS broth (negative control), respectively.

2.6 Antioxidant assays

2.6.1 2,2–diphenyl–1–picrylhydrazyl radicals scavenging activity

The scavenging of 2,2–diphenyl–1–picrylhydrazyl (DPPH, Sigma, USA) radicals was performed according to previously described by Lin *et al.* (2018). First, 0.04 mM DPPH solution was prepared in 95% (v/v) ethanol. Then, 0.5 mL of ethanolic DPPH solution was added with 1.0 mL of intact bacterial cells ($1x10^9$ CFU/mL) and mixed vigorously. The control solution was prepared with deionized water instead of intact cells. After incubation at 25 °C in the dark for 30 min, the supernatant was obtained by centrifugation at 5,000×g, 25 °C for 10 min. The optical absorbance of sample (A_s) and control (A_c) solution was measured at a wavelength of 517 nm with ethanol as a blank (A_b). Then, 1% (w/v) ascorbic acid (Sigma, USA) was used as a positive control. The percentage of scavenging activity was calculated as following equation:

$$\% \text{ scavenging activity} = (1 - (A_s - A_b / A_c)) \times 100$$

2.6.2 Hydroxyl radicals scavenging activity

The scavenging of hydroxyl radicals was measured with the Fenton reaction (Wang, Zhao, Yang, Zhao, & Yang, 2015). The mixture was prepared by adding together of 0.5 mL of intact bacterial cells ($1x10^9$ CFU/mL), 1.0 mL of 0.5 mM $FeSO_4$, 0.5 mL of 0.435 mM brilliant green, and 0.75 mL of 3% (w/v) H_2O_2. The control solution was prepared with deionized water instead of intact cells. The mixture was incubated at 37 °C for 1 h with constant shaking, then the supernatant was collected by centrifugation at 5,000×g, 25 °C for 10 min. The optical absorbance of sample (A_s) and control (A_c) mixture was measured at a wavelength of 624 nm with brilliant green as a blank (A_b). A positive control was 1% (w/v) ascorbic acid. The percentage of scavenging activity was calculated according to the equation:

$$\% \text{ scavenging activity} = ((A_s - A_c) / (A_b - A_c)) \times 100$$

2.7 Acid and bile salt tolerance assay

Gastrointestinal tolerance of cholesterol-lowering LAB was determined under the acid and bile salt conditions, according to the previous method of Ladda *et al.* (2015) with minor modifications. For acid conditions, MRS broth was adjusted with 1 M HCl to either pH 2.0, 3.0, or 4.0. For bile salt conditions, MRS broth was supplemented with bovine bile at 0.3 or 0.8% (w/v). Unadjusted MRS broth was used as a control. Overnight bacterial culture was inoculated into each acid and bile salt conditions at an initial concentration of $1x10^9$ CFU/mL. After anaerobic incubation at 37 °C for 3 h, bacterial cells were serially diluted 10-fold with PBS and

spread on MRS agar plates, followed by anaerobic incubation at 37 °C for 48 h. After that, the number of survival bacteria was analyzed as $\log_{10}$ CFU/mL. *L. rhamnosus* GG (LMG 18243) was used as a reference strain.

2.8 Adherence assay

Adenocarcinoma cell line (Caco-2 cells, ATCC HTB-37) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin (Gibco, USA) at 37 °C with 5% CO_2 for the adherence assay (Kook, Chung, Lee, Lee, & Kim, 2019). Next, overnight bacterial culture was washed twice with PBS and diluted with antibiotic free DMEM to $1x10^9$ CFU/mL. One milliliter of bacterial suspension was inoculated onto the Caco-2 cells. After incubation at 37 °C with 5% CO_2 for 1 h, each well was washed thrice with PBS to remain only adhered bacterial cells and then Caco-2 cells were lysed with 0.5% (v/v) Triton® X-100 (Merck, Germany). Finally, the number of bacteria was analyzed as $\log_{10}$ CFU/mL by direct plate counting as explained above. *L. rhamnosus* GG was used as a reference strain.

2.9 Statistical analysis

All experiments were performed three times independently. The results were expressed as means and standard deviation (SD). Analysis of variance (ANOVA) with Tukey's multiple comparison test was used to test the statistical significance when values of $p < 0.05$ (GraphPad Prism software, version 8.0).

3. Results and Discussion

3.1 Isolation and cholesterol-lowering activity of LAB

Fifty traditional Thai food samples were cultured in MRS agar plates to isolate LAB colony. One hundred and twenty-four bacterial isolates exhibited clear zone in MRS containing $CaCO_3$ (lactic acid production). Gram's staining and catalase reaction revealed 74 LAB isolates with desired characteristics, such as Gram-positive and catalase-negative. Cholesterol-lowering activity was also examined in these isolates, and 17 out of 74 isolates expressed both BSH activity and cholesterol assimilation (Table 1). The most robust BSH activity and the highest levels of cholesterol assimilation were observed in TF-7 and TF-18 isolates (58.97±1.60% and 57.98±4.35%). Whereas, TF-6, TF-14, and TA-2 isolates showed moderate cholesterol assimilation activity. In addition, the range of cholesterol assimilation activity in these 17 isolates was from 14.00±1.13% to 58.97±1.60% (Table 1).

Our results showed the difference in such ability to produce BSH among 17 isolates. A previous study reported that LAB isolated from several environments exhibited different BSH activity due to the influence of disparate environments on the expression of *bsh* gene (Begley, Hill, & Gahan, 2006). This factor may be a cause of why each LAB isolate expressed different BSH activity. As an enzyme, BSH recognizes taurine, glycine, or both groups to hydrolyze amide bond between steroid nucleus and amino acid residues of conjugated bile salts (Allain *et al.*, 2018). It converts conjugated bile salts into deconjugated bile salts leading to impairments of lipid emulsification and absorption in the intestine. Moreover, de novo biosynthesis of bile salts in the liver, which is stimulated by bile salt biodegradation can eliminate stored cholesterol (Bustos *et al.*, 2018). Cholesterol residues in the intestine resulting from the defective lipid emulsification by BSH activity can be absorbed by LAB to incorporate in the bacterial plasma membrane during their growth, indicating that BSH activity may promote the cholesterol assimilation, as found in TF-7 and TF-18 isolates (Lye *et al.*, 2010). Although some isolates (TF-6, TF-14, and TA-2) showed moderate cholesterol assimilation, weak BSH activities were observed because the cholesterol assimilation depended on the absorption capability and growth rate of each strain (Lye *et al.*, 2010).

Table 1. Cholesterol-lowering activity of LAB isolates

Isolates	Sources	Cholesterol-lowering activity		
		BSH activity		Cholesterol assimilation (%)
		TDCA	GDCA	
TF-1	Dried mango paste	++	+	$25.24±1.70^{fghj}$
TF-3	Pickled salak	-	+	$21.88±1.27^{hk}$
TF-5	Pickled mustard	+	++	$14.91±1.63^{k}$
TF-6	Pickled bamboo shoot	+	+	$43.96±4.39^{b}$
TF-7	Pickled olives	+++	++	$58.97±1.60^{a}$
TF-9	Raw pork meat	+++	-	$33.42±3.58^{cdef}$
TF-12	Salted plum	++	+	$17.62±5.80^{ijk}$
TF-14	Agasta	++	-	$41.89±3.14^{bc}$
TF-16	Pickled garlic	+	++	$26.76±2.53^{fghi}$
TF-17	Pickled santol	++	+	$32.24±2.51^{cdeg}$
TF-18	Sweet fermented rice	+++	+++	$57.98±4.35^{a}$
TA-1	Raw cow milk	+	++	$36.91±2.01^{be}$
TA-2	Sticky rice	+	+	$40.83±3.98^{bd}$
TA-6	Pickled grape	-	++	$18.89±5.24^{ijk}$
TA-7	Sticky rice	++	+	$30.25±2.10^{eh}$
TA-10	Pickled bamboo shoot	+	-	$14.00±1.13^{k}$
TA-12	Pickled crab	+	++	$18.80±3.44^{ijk}$

BSH activity was interpreted based on the diameter of precipitation zone (mm): -, no zone; +, ≤ 8.0 mm (weak); ++, 8.1 – 13.0 mm (moderate); +++, ≥ 13.1 mm (strong). $^{a-k}$ Letters indicate statistically significant differences ($p < 0.05$) (n=3, means±SD).

3.2 Genotypic identification of 16S rRNA gene-based nucleotide sequences

The genotypic identification of 17 LAB isolates belonged to four genera, including *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Bifidobacterium* (Table 2). Considering the results of genotypic identification combined with cholesterol-lowering activity, TF-7, TF-18, and TA-1 isolates were selected for cholesterol-lowering LAB candidates, based on the most robust cholesterol-lowering

Table 2. Genotypic identification of LAB isolates by 16S rRNA gene-based nucleotide sequences

Isolates	Closest relative microorganisms	GenBank No.	Identity (%)
TF-1	*Lactobacillus mucosae* S32	AF126738.1	99.09
TF-3	*Streptococcus gallolyticus* subsp. *macedonicus* NCTC 13767	UHFM01000006.1	99.51
TF-5	*Lactobacillus fermentum* E4	MF179548.1	99.79
TF-6	*Lactobacillus fermentum* E1	MF179545.1	100.00
TF-7	*Lactobacillus reuteri* JCM 1112	AP007281.1	99.79
TF-9	*Lactobacillus mucosae* S32	AF126738.1	99.74
TF-12	*Enterococcus durans* NBRC 100479	BCQB01000108.1	99.64
TF-14	*Lactobacillus agilis* DSM 20509	AYYP01000002.1	99.44
TF-16	*Lactobacillus fermentum* C8.1	MF179543.1	99.66
TF-17	*Lactobacillus animalis* KCTC 3501	AEOF01000010.1	99.65
TF-18	*Enterococcus faecium* LMG 11423	AJ301830.1	99.51
TA-1	*Bifidobacterium animalis* subsp. *lactis* DSM 10140	CP001606.1	99.57
TA-2	*Lactobacillus gasseri* DMBCT6	KM056281.1	99.51
TA-6	*Lactobacillus reuteri* LGM7-1	KU612253.1	99.35
TA-7	*Lactobacillus johnsonii* ATCC 33200	ACGR01000047.1	99.79
TA-10	*Lactobacillus amylovorus* DSM 20531	AZCM01000082.1	84.20
TA-12	*Enterococcus faecium* LAC7.2	CP045012.1	99.64

activity when compared with the same genera. These candidates were defined according to the closest homology with strains deposited in the NCBI GenBank database under the strain names as *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* TA-1, respectively (Figure 1).

From our results, *Lactobacillus*, facultative anaerobes, was the major genera identified from cholesterol-lowering LAB isolated from traditional Thai foods. This finding was in agreement with previous study reported that the relatively high percentage of *Lactobacillus* could be found in nature among other genera due to its ability to persist under harsh environments (Nuraida, 2015). In contrast, *Bifidobacterium* was obligate anaerobes with tardy propagation. However, it was selected because it is commonly used probiotics in human that is compatible with the host's physiological activity (O'Callaghan & Sinderen, 2016).

3.3 Evaluation of safety

The hemolytic activity and antibiotic susceptibility were assessed to ensure the safety of using cholesterol-lowering LAB as probiotic supplements. Three cholesterol-lowering LAB exhibited γ-hemolysis, indicating that these strains did not exert the pathological effect to produce lysis on erythrocytes (Table 3). Three LAB strains were susceptible to antibiotics, including CHL, NAL, ERY, and TET. However, these strains were resistant to PEN. Intermediate susceptibility to AMP and GEN was observed in *E. faecium* TF-18, whereas *L. reuteri* TF-7 exhibited intermediate susceptibility to GEN only. Resistant to VAN was observed in *E. faecium* TF-18 and *B. animalis* TA-1 (Table 3). These results were in agreement with the previous study, in which some LAB strains were resistant to few antibiotics (Haghshenas *et al.*, 2017). Probiotics in the gastrointestinal microbiota have been reported that they were resistant to some antibiotics because several species of *Enterobacteriaceae* were able to disseminate antibiotic-resistant genes to the gastrointestinal microbiota (Zheng *et al.*, 2017). It is possible that these LAB strains may receive some genes contributing to antibiotic resistance. However, these results indicated that three cholesterol-lowering LAB were relatively safe to be used as probiotics.

3.4 GABA biosynthesis

GABA production was determined in three cholesterol-lowering LAB to indicate their metabolic activity of glutamic acid decarboxylase enzyme, which is another specific property of probiotics (Ohmori, Tahara, & Ohshima, 2018). As shown in Figure 2a and b, GABA production from glutamic acid and monosodium glutamate was detected only *L. reuteri* TF-7 in this condition. Therefore, these results suggested that *L. reuteri* TF-7 can utilize glutamic acid and monosodium glutamate from digested molecules of food to produce GABA after oral administration.

Glutamic acid and monosodium glutamate are catalyzed to synthesize GABA by glutamic acid decarboxylase enzyme, in which the GABA molecules are transported to the extracellular environment via the vesicles. GABA produced from probiotics is a microbial derived neurotransmitter, which plays a significant role in the microbiota-gut-brain axis. Previous study has reported that GABA-producing LAB could improve neurological function and gut barrier by communicating through the enteric nervous system (Sarkar *et al.*, 2016). Moreover, GABA and topiramate could also inhibit the formation of macrophage-derived foam cells via suppressing LOX-1, CD36, and SR-A expression. The consequences were the reduction of cholesterol accumulation and amelioration of atherosclerosis (Yang *et al.*, 2014).

3.5 Antioxidant activity

Antioxidant assay of three cholesterol-lowering LAB was performed using DPPH and hydroxyl radicals to indicate their ability to scavenge oxidizing agents. Antioxidant activities of these strains were displayed in values ranging from 18.38±3.60% to 44.93±1.19%. Among three LAB strains, *B. animalis* TA-1 exhibited the highest scavenging activity against DDPH and hydroxyl radicals with statistically

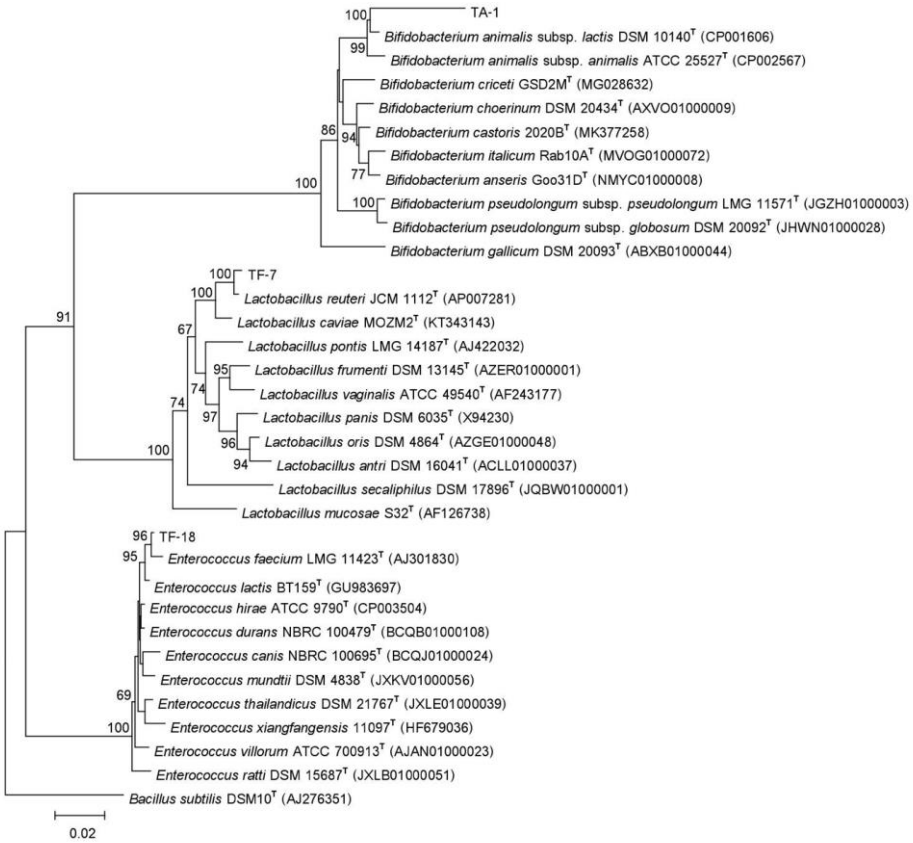

Figure 1. Phylogenetic tree of three genera of cholesterol-lowering LAB with their closest relation of 16S rRNA gene-based nucleotide sequences among the genus *Lactobacillus*, *Enterococcus*, and *Bifidobacterium*. Phylogenetic tree was constructed using the neighbour-joining method with 1000 bootstrap values. Bar, 0.02 substitutions per nucleotide position

Table 3. Safety evaluation of cholesterol-lowering LAB

Strains	Hemolytic activity	Antibiotic susceptibility							
		AMP	CHL	GEN	NAL	PEN	ERY	TET	VAN
TF-7	γ	25.67±0.58 (S)	31.67±0.58 (S)	8.33±0.58 (I)	30.67±1.15 (S)	11.33±1.15 (R)	25.33±0.58 (S)	31.00±1.00 (S)	21.67±2.08 (S)
TF-18	γ	16.00±1.73 (I)	34.00±1.00 (S)	8.67±0.58 (I)	31.67±0.58 (S)	7.67±1.15 (R)	26.33±0.58 (S)	34.67±0.58 (S)	0.00±0.00 (R)
TA-1	γ	22.67±0.58 (S)	26.33±0.58 (S)	30.33±0.58 (S)	31.33±1.15 (S)	9.00±1.73 (R)	26.00±1.00 (S)	29.33±1.15 (S)	8.33±1.53 (R)

Hemolytic activity was presented as γ (non-hemolysis). Antibiotic susceptibility was interpreted based on the diameter of inhibition zone (mm), an antibiotic disk with a diameter of 6.5 mm (n=3, means±SD).
GEN: (R), ≤ 8.0 mm (resistant); (I), 8.1 – 10.0 mm (intermediate); (S), ≥ 10.1 mm (susceptible)
ERY: (R), ≤ 13.0 mm (resistant); (I), 13.1 – 23.0 mm (intermediate); (S), ≥ 23.1 mm (susceptible)
VAN: (R), ≤ 12.0 mm (resistant); (I), 12.1 – 13.0 mm (intermediate); (S), ≥ 13.1 mm (susceptible)
Other antibiotics: (R), ≤ 12.4 mm (resistant); (I), 12.5 – 17.4 mm (intermediate); (S), ≥ 17.5 mm (susceptible)

significant ($p < 0.05$), compared with *L. reuteri* TF-7 and *E. faecium* TF-18 (Figure 3a and b). These findings were in agreement with previous studies, in which several LAB strains including *B. bifidum* WBIN03, *L. plantarum* R315, and *E. faecium* BDU7 exhibited scavenging activity against DPPH, hydroxyl, and superoxide radicals (Abdhul *et al.*, 2014; Li *et al.*, 2014).

Oxidative stress has been reported to be a causative factor of lipid peroxidation associated with hyper cholesterolemia and dyslipidemia. The study in rabbits and rats revealed that oxidizable lipid molecules were the increasing by-products of lipid peroxidation. Furthermore, lipid peroxidation contributed to the accumulation of cholesterol generating by mevalonate pathway to repair lipoprotein and plasma membrane deterioration. These adverse effects of lipid peroxidation resulted in the hypercholesterolemia (Balkan, Doğru-Abbasoğlu, Aykaç-Toker, & Uysal, 2004; Lin *et al.*, 2018). Based on these reports, antioxidant activity is another important property of cholesterol-lowering LAB because it may enhance the hypocholesterolemic effect by preventing the hyper cholesterolemia induced by oxidative stress.

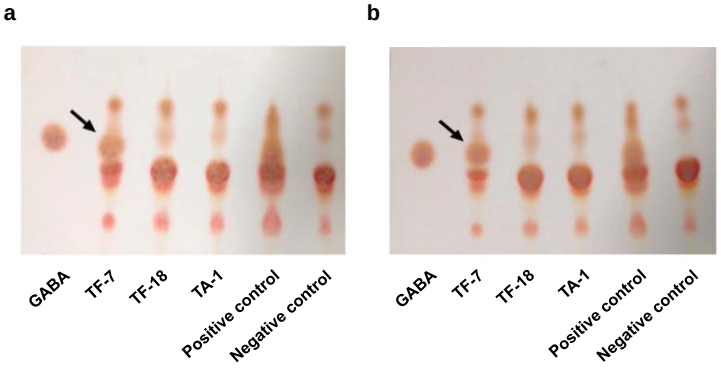

Figure 2. GABA biosynthesis of cholesterol-lowering LAB in MRS broth supplemented with 5% (w/v) glutamic acid (a) and 5% (w/v) monosodium glutamate (b). Arrow indicates the GABA production, compared with GABA standard, positive, and negative control.

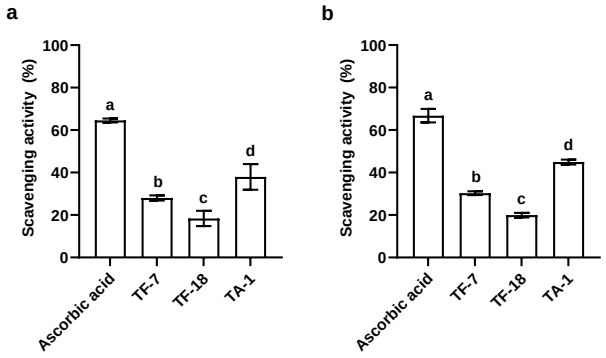

Figure 3. Antioxidant activity of cholesterol-lowering LAB on DDPH radicals (a) and hydroxyl radicals (b). Ascorbic acid was used as a positive control. [a-d] Letters indicate statistically significant differences ($p < 0.05$) (n=3).

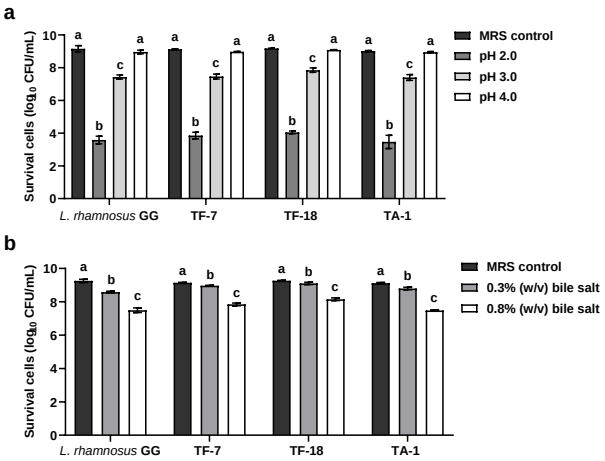

Figure 4. Survival of cholesterol-lowering LAB under the different acid conditions (a) and bile salt conditions (b). *L. rhamnosus* GG was used as a reference strain. [a-c] Letters indicate statistically significant differences ($p < 0.05$) (n=3).

3.6 Acid and bile salt tolerance

Cholesterol-lowering LAB candidates were investigated their tolerant ability to various conditions of gastric acid and bile salt. Since probiotics must be able to survive after exposure to the harsh environments within the stomach and small intestine (Bezkorovainy, 2001). At pH 4.0, viabilities of all three LAB were comparable to the control. However, their survivals significantly decreased by 16.91±2.11% and 58.45±2.93% when the pH was 3.0 and 2.0, respectively ($p < 0.05$) (Figure 4a). Similarly, the study of Ladda *et al.* (2015) showed the survival of *L. paracasei* MSMC39-1 decreased when exposed to severe gastric conditions at pH 3.0 and 2.0. However, in the physiological condition, pH level increases to 4.2 during food ingestion in the stomach.

Moreover, the survivals of three LAB strains also significantly decreased when the concentrations of bile salt increased, compared with control ($p < 0.05$). However, they were able to survive to a great extent with survival reduction by 2.44±0.93% and 14.72±2.99% in both concentrations of bile salt at 0.3 and 0.8% (w/v), respectively (Figure 4b). These results suggested that bile salt conditions had relatively low effect on bacterial survival. In addition, *L. reuteri* TF-7 and *E. faecium* TF-18 showed slightly more bile salt tolerance over *B. animalis* TA-1. The enhancement of bile salt tolerance might be due to potent BSH activity produced from LAB. This enzyme converts conjugated bile salts into deconjugated form, which is less toxic and eliminated through feces (Bustos *et al.*, 2018).

3.7 Adherence property

Next, we investigated the colonization potential of cholesterol-lowering LAB on the intestinal mucosa using *in vitro* adherence assay, which is an important characteristic of probiotics within the gastrointestinal microbiota. The numbers of adhered cells of *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* TA-1 were 8.16±0.04 $\log_{10}$ CFU/mL, 7.33±0.07 $\log_{10}$ CFU/mL, and 6.53±0.21 $\log_{10}$ CFU/mL, respectively, from the similar initial cell numbers about 8.97±0.07 $\log_{10}$ CFU/mL (Figure 5). Interestingly, *L. reuteri* TF-7 and *E. faecium* TF-18 exhibited stronger cell adhesion than *L. rhamnosus* GG (7.08±0.17 $\log_{10}$ CFU/mL), but this value had no statistically significant difference, which was consistent with previous study (Kook *et al.*, 2019). Furthermore, all three LAB strains exhibited the adherence levels above 72.79%, which were considered sufficient for colonization.

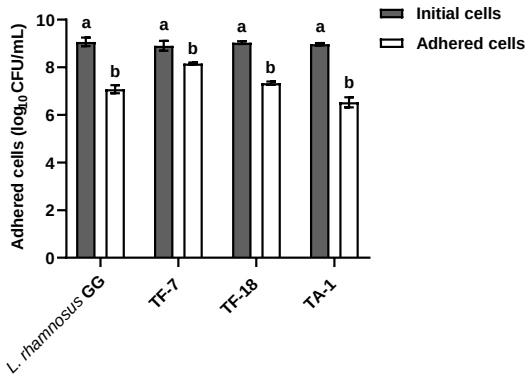

Figure 5. Adherence ability of cholesterol-lowering LAB to Caco-2 cells. *L. rhamnosus* GG was used as a reference strain. [a-b] Letters indicate statistically significant differences ($p < 0.05$) (n=3).

These results suggested that adherence ability varied among different strains. The difference in such ability could be due to the differences in cell surface hydrophobicity and auto-aggregation, which have been reported to be strain dependence (Xu, Jeong, Lee, & Ahn, 2009).

4. Conclusions

In this study, we found 17 LAB isolates with ability to produce BSH and cholesterol assimilation from traditional Thai foods. *L. reuteri* TF-7, *E. faecium* TF-18, and *B. animalis* TA-1 with the most robust cholesterol-lowering activity among the same genera were selected for cholesterol-lowering LAB candidates. Each strain was relatively safe and strikingly expressed probiotic characteristics, in particular, *L. reuteri* TF-7, which exhibited the most potent GABA biosynthesis. In addition, the prominent antioxidant effect was observed in *B. animalis* TA-1. This study also proved that all strains relatively tolerated in the acid and bile salt conditions and adhered to the intestinal epithelium. On the whole, this study indicated that three cholesterol-lowering LAB are potential probiotics, which can be used as mixed probiotics for biotherapeutics of hypercholesterolemia and exerting health benefits with synergistic effect when administered simultaneously. Further study is necessary to prove *in vivo* hypocholesterolemic effect prior to development as the probiotic supplements.

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