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Bile salt hydrolase activity and cholesterol assimilation of lactic acid bacteria isolated from flowers

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ABSTRACT

A total of 16 isolates of lactic acid bacteria (LAB) from flowers were screened for the bile salt hydrolase activity on MRS (de Man, Rogosa and Sharpe; Difco) agar supplemented with 0.5% (w/v) taurodeoxycholic acid. The isolates were divided into two groups based on their phenotypic characteristics and 16S rRNA gene sequence analysis of the representative isolates. Group I isolates were cocci as the members of genus *Enterococcus*. Isolates FM1-1, FM1-2, FM12-1, and FM12-2 were identified as *Enterococcus durans* (100% similarity), isolate FM2-3 was identified as *Enterococcus gallinarum* (99.92% similarity), while the isolate FM11-2 was identified as *Enterococcus lactis* (99.77% similarity). Group II isolates were rods as the members of genus *Lactobacillus*. They were identified as *Lactobacillus plantarum* subsp. *plantarum* (the representative isolates, FM3-1 and FM16-2, showed 100% similarity). Eleven isolates, including FM1-1, FM1-2, FM2-3, FM3-1, FM4-2, FM11-2, FM12-1, FM12-2, FM14-1, FM14-2, and FM16-2, exhibited bile salt hydrolase activity. All LAB isolates showed the cholesterol assimilated ability ranged from 9.57% to 51.69%. The isolate FM11-2 efficiently assimilated the cholesterol with 51.69%.

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, acid-tolerant, non-sporulating, non-respiring, rod-shaped, or cocci that produce lactic acid as a major end product. Several genera of LAB, especially *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* involved with cholesterol-lowering effects in serum could produce bile salt hydrolase (BSH) (Ahn *et al*., 2003). LAB are isolated from various environments, including intestines, feces, fermented foods, dairy products, flowers, leaves, fruits (Abushelaibi *et al*., 2017; Itoi *et al*., 2009; Jarocki *et al*., 2014; Kawasaki *et al*., 2011; Kim *et al*., 2013; Xu *et al*., 2016), functional food materials, and probiotics (Foligne *et al*., 2013; Solieri *et al*., 2014). They are used as probiotics, live bacteria that are good for the host health. LAB play a beneficial role as a health promoter on their host. Nowadays, the interested ability of probiotic is BSH producing that has become the focus of attention on account of its influence on cholesterol metabolism. The bile salt hydrolase activity of probiotic bacterium residing in the gastrointestinal tract has often being associated with its cholesterol lowering effects. The aim of this study was to isolate and identify LAB obtained from the plant sources for the screening of their BSH activity and cholesterol assimilation.

MATERIALS AND METHODS

Sources and isolation

Eigth flower samples were collected from Nakhon Si Thammarat and Bangkok, Thailand (Table 1) and were kept in 4°C until the isolation. Approximately, 1 g of sample was inoculated in MRS under anaerobic conditions at 37°C for 48–72 hours (Prasirtsak *et al*., 2013). The aliquot was streaked on MRS agar plate containing

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Table 1. Source, location and identification based on 16S rRNA sequence of isolates.

Flower of	Isolate no.	Province	Group	Identification
Gardenia jasminoides	FM1-1, FM1-2	Nakhon Si Thammarat	I	*E. durans*
Hibiscus syriacus	FM12-1, FM12-2	Nakhon Si Thammarat	I	*E. durans*
Ixora lucida	FM2-3	Nakhon Si Thammarat	I	*E. gallinarum*
Solanum torvum	FM11-2	Nakhon Si Thammarat	I	*E. lactis*
	FM11-1, FM11-3		I	*Enterococcus* sp.
Wrightia religiosa	FM3-1,	Nakhon Si Thammarat	II	*L. plantarum*
	FM4-1, FM4-2		I	*Enterococcus* sp.
Leucaena leucocephala	FM13-1	Nakhon Si Thammarat	II	*L. plantarum*
Jatropha podagrica	FM14-1, FM14-2	Nakhon Si Thammarat	II	*L. plantarum*
Tabernaemontana divaricata	FM16-1, FM16-2	Bangkok	II	*L. plantarum*

0.3% $CaCO_3$ 37°C for 48 hours. The single colony of LAB, clear zone colony, was picked up and further purified on MRS agar plates at 37°C for 48 hours.

Identification methods

Phenotypic characterization

The cells cultivated on MRS agar plate containing 0.3% $CaCO_3$ after incubated at 37°C for 48 hours were determined for Gram staining, cell morphology, and catalase activity. Phenotypic characteristics, including aesculin hydrolysis, nitrate reduction, arginine hydrolysis, growth in 0%, 4%, 6%, and 8% (w/v) NaCl concentrations, and growth at the temperature 15°C, 30°C, 37°C, and 45°C and at pH 2.0, 4.0, and 9.0, and acid formation on various carbon sources, were determined as previously described (Tanasupawat *et al*., 1998). Hierarchical cluster analysis was conducted by using SPSS for Windows version 22.0 based on the phenotypic characteristics of the isolates.

Genotypic characterization

16S rRNA gene of each represented groups was amplified using polymerase chain reaction (PCR) with two primers as 20F (5′-AGTTTGATCCTGGCTC-3′) and 1530R (5′-AAGGAGGTGATCC AGCC-3′). Agarose gel electrophoresis was performed to validate the quality of PCR fragments. The purified PCR products were sequenced with the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The amplified 16S rRNA gene sequence was analyzed by Macrogen, Korea. The almost-complete 16S rRNA gene sequence was obtained and was aligned manually against the sequences which were obtained from the GenBank/EMBL/DDBJ database using BioEdit software (Ibis Biosciences). The sequences of strains were aligned with the selected sequences obtained from GenBank using CLUSTAL_W algorithm (Clustal *et al*., 1994). The values for sequence similarity among the closest strains were analyzed using the EzTaxon-e server (Kim *et al*., 2012). The alignment was edited manually to remove gaps and ambiguous nucleotides before the construction of phylogenetic trees. The confidence values of individual branches in the neighbor-joining phylogenetic tree were reconstructed using MEGA version 7.0 (Kumar *et al*., 2016; Saitou and Nei, 1987). Topologies of phylogenetic trees were evaluated via bootstrap analysis based on 1,000 replicates (Felsenstein, 1985).

Screening of acid and bile tolerance

Acid and bile tolerance were performed according to a modification of the previously described method (Thamacharoensuk *et al*., 2013). Briefly, 10^8 CFU/ml of each LAB culture was inoculated into MRS broth adjusting to pH 2 for acid tolerance and containing 0.3% bile salt for bile tolerance, and then incubated at 37°C. Cell viability was screened on MRS agar plate using the dropping on MRS plate at 0 and 3 hours in duplicate. *Lactobacillus rhamnosus* LMG 18243^T (strain Gorbach–Goldin, GG) was a positive control.

Screening of bile salt hydrolase activity

All LAB strains were tested for bile salt hydrolase activity by qualitative direct plate assay (Ahn *et al*., 2003; Dashkevicz and Feighner, 1989; Sedláčková *et al*., 2015). Briefly, 10 µl (10^9 cell/ml) of bacteria cell were spotted on MRS agar plates supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma, USA) and 0.37 g/l of calcium chloride ($CaCl_2$) incubated under anaerobic condition at 37˚C for 48–72 hours. MRS agar medium plates without the supplementation of TDCA were used as a negative control. The presence of precipitated bile acid around colonies (called as opaque halo) was considered as a positive reaction. The experiment was performed in triplicate.

Screening of cholesterol assimilation

All LAB were investigated to assimilate cholesterol in MRS broth containing 100 µg/ml of cholesterol-polyethylene glycol (PEG) 600 (Sigma, India). Each inoculum (1%, v/v) was added into MRS-cholesterol-PEG 600 and was incubated at 37˚C for 24 hours. The cholesterol in the spent broths was first extracted by the procedure described by Tomaro-Duchesneau *et al*. (2014). The total cholesterol content of the evaporated residues was then determined using a protocol modified from the previous experiment (Rudel and Morris, 1973). A standard curve of absorbance related to cholesterol concentrations was generated using the cholesterol concentrations: 0, 6.25, 12.5, 25, 50, 100, and 200 µg/ml cholesterol in MRS.

Statistical analysis

Statistical difference was analyzed by using the one-way analysis of variance (ANOVA) of the Tukey method (SPSS 22.0). A probability of p value <0.05 was considered as significant.

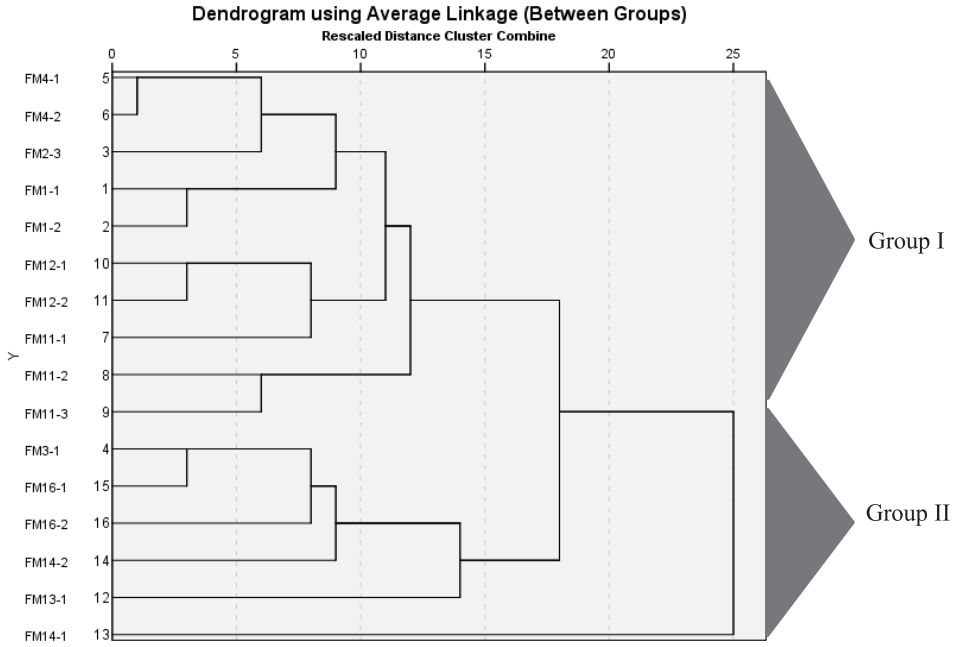

Figure 1. Dendrogram using average linkage (between groups) presenting the hierarchical cluster of LAB isolates based on thephenotypic characteristics.

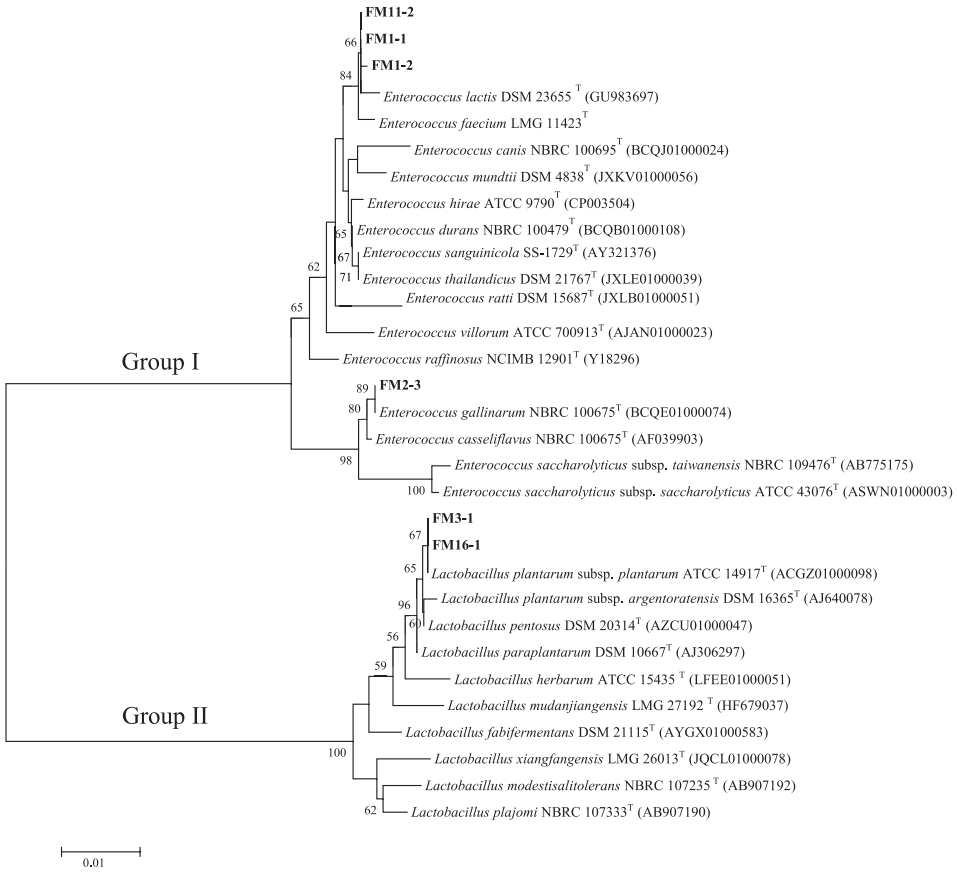

Figure 2. Neighbor-joining tree of the representative isolates based on the 16S rRNA gene sequences. Bootstrap values are showed as percentages of 1,000 replications; only values >50% are indicated. Bar 0.01 substitutions per nucleotide position.

Table 2. Differential phenotypic characteristics of isolates and BSH activity.

Characteristics	Group I	Group II
No. of isolate	**10**	**6**
Growth temp (°C)	15–45	15–37
Bile salt tolerance (0.3%)	+ (−1)	+ (−1)
Aesculin hydrolysis	-	+
Arginine hydrolysis	− (+3)	+ (−2)
Nitrate reduction	− (+3)	-
Acid production from		
D-Galactose	+ (−1)	+
Sorbitol	− (+1)	+ (−1)
Sucrose	+	+ (−1)
D-Xylose	+ (w 2)	+
BSH activity positive	FM1-1, FM1-2, FM2-3, FM4-2, FM11-2, FM12-1, FM12-2	FM3-1, FM14-2, FM14-1, FM16-2

+: positive reaction; w: weak reaction; −: negative reaction. Numbers in the parentheses indicate the number of isolates shows positive, weakly and negative reaction.

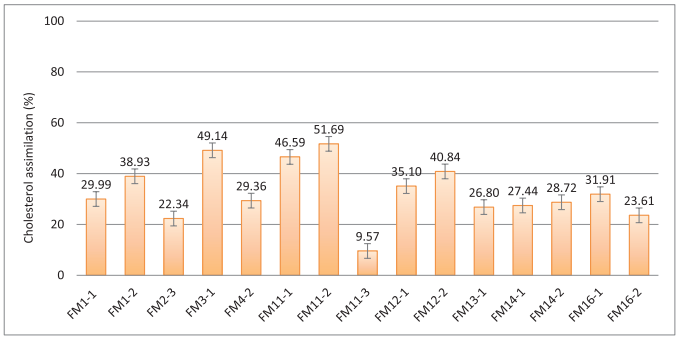

Figure 3. Cholesterol assimilation of isolates in MRS broth supplemented 100 µg/ml of cholesterol, incubated for 48 hours (n = 3). MRS broth supplemented cholesterol without bacterial cells was used as controls. Statistical analysis: ANOVA, Tukey's homogenous subsets generated from pair wise comparison.

RESULTS AND DISCUSSION

Identification of isolates

Sixteen LAB strains were isolated from nine flower samples collected from Nakhon Si Thammarat and Bangkok, Thailand (Table 1). All strains were Gram-positive, including 10 cocci (FM1-1, FM1-2, FM2-3, FM4-1, FM4-2, FM11-1, FM11-2, FM11-3, FM12-1, and FM12-2) and six rod-shaped bacteria (FM3-1, FM13-1, FM14-1, FM14-2, FM16-1, and FM16-2). They were facultative anaerobic and catalase negative. Most of them grew at pH 2.0 and tolerated in 0.3% bile salt and in 4%–8% NaCl. All did not produce gas from the glucose. They produced acid from L-arabinose, D-cellobiose, D-fructose, glucose, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, ribose, salicin, trehalose, and D-xylose. They were divided into two groups as the members of genus *Enterococcus* and *Lactobacillus* based on their phenotypic characteristics and 16S rRNA gene sequence similarity of the representative strains (Figs. 1 and 2). Their variable characteristics are shown in Table 2.

Group I consisted of 10 isolates, which were FM1-1, FM1-2, FM2-3, FM4-1, FM4-2, FM11-1, FM11-2, FM11-3, FM12-1, and FM12-2. They were cocci in the genus *Enterococcus*. The 16S rRNA gene sequence results indicated that FM1-1, FM1-2, FM12-1, and FM12-2 were closely related to *Enterococcus durans* NBRC 100479^T (99.71% and 99.78% similarity) and were identified as *E. durans* (Collins *et al*., 1984). Isolates FM2-3 and FM11-2 showed 99.92% and 99.78% similarity to *Enterococcus gallinarum* NBRC 100675^T and *Enterococcus lactis* BT159^T; therefore, they were identified as *E. gallinarum* and *E. lactis*, respectively (Collins *et al*., 1984; Morandi *et al*., 1992).

Group II consisted of six isolates, which were FM3-1, FM13-1, FM14-1, FM14-2, FM16-1, and FM16-2. They were rods in the genus *Latobacillus*. Their variable characteristics are shown in Table 2. The 16S rRNA gene sequence of representative strains in this group (FM3-1 and FM16-1) showed 100% similarity to *Lactobacillus plantarum* ATCC 14917^T, therefore, they were identified as *L. plantarum* (Bringel *et al*., 2005).

From these results, LAB isolated from different species of flowers showed unique properties, such as the ability to ferment many kinds of carbohydrates, including fructose that they are considered to have fructophilic properties. Their distribution is correlated with the major carbohydrate sources in a specific habitat (Endo, 2012; Kuda *et al*., 2016).

Screening of acid and bile tolerance

LAB isolates could tolerate in the medium at pH 2 except the isolates FM1-2 and FM12-1 in Group I and FM14-2 in Group II, while all isolates could tolerate in the medium containing 0.3% bile except the isolate FM14-2 in Group I and FM 14-1 in Group II.

Screening of bile salt hydrolase activity

Enterococcus isolates FM1-1, FM1-2, FM2-3, FM4-2, FM11-2, FM12-1, FM12-2, *L. plantarum* isolates FM3-1, FM14-1, FM14-2, and FM16-2 (Table 2) exhibited BSH activity, while *E. durans* V18 and *Leuconostoc mesenteroides* V12 and V21 from sichuan kimchi, *E. faecium* B20, B21 isolated from stinky soybean, *Pediococcus ethanolidurans* D13 and *L. plantarum* D24 and D25 from dongbei kimchi and (Xu *et al*., 2016), *Leuc. lactis* KC117496 from idli batter (Saravanan *et al*., 2016), *L. plantarum* Lp3 from fermented yak milk (Ding *et al*., 2017), and *L. plantarum* RC from raw cheese (Shekh *et al*., 2016) were also reported to have BSH activity.

Screening of cholesterol assimilation

The isolates exhibited cholesterol assimilation ability ranged from 9.57% to 51.69%. The isolate *E. lactis* FM11-2 could efficiently assimilate and showed the highest cholesterol assimilation with 51.69% (Fig. 3). According to the probiotics, LAB are Generally Recognized as Safe (Rolfe, 2000) are most commonly used because of their significant role in resistance to the disease. Our LAB isolates could exhibit BSH activity that may be useful to reduce serum cholesterol levels in the patient with hypercholesterolemia and also prevent hypercholesterolemia in normal people (Chae *et al*., 2013). The other probiotic properties of the isolates should be done for further study.

CONCLUSION

In this study, *Enterococcus* strains were isolated from the flowers of *Gardenia jasminoides*, *Hibiscus syriacus*, *Ixora*

lucida, and *Solanum torvum*; while *Lactobacillus* strains were from *Wrightia religiosa*, *Leucaena leucocephala*, *Jatropha podagrica*, and *Tabernaemontana divaricate*. Seven *Enterococcus* isolates and four *L. plantarum* isolates exhibited BSH activity. All isolates exhibited cholesterol assimilation, but isolate FM11-2 showed the highest activity that may be useful as probiotics for the reduction of cholesterol levels.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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