

## *In vitro* assessment of probiotic properties and gamma-aminobutyric acid production of selected lactic acid bacteria strains

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### Abstract

This study aimed to perform an *in vitro* assessment of probiotic properties and to select lactic acid bacteria (LAB) strains that can produce the bioactive compound, gamma-aminobutyric acid (GABA). A total of seven LAB strains of various species were examined for probiotic properties. These strains produced antimicrobial substances to inhibit the growth of target pathogenic bacteria. All selected LAB strains could withstand GI tract conditions. The strains showed bile salt hydrolase (BSH) activities. An antibiotic susceptibility test revealed that all examined strains were susceptible to ampicillin, chloramphenicol, erythromycin, gentamycin, penicillin and tetracycline, whereas they were resistant to kanamycin, norfloxacin, streptomycin and vancomycin. All strains of LAB in the current study gave high adhesion to Caco-2 cells ranging from 23.32-75.48%. Moreover, all tested LAB cells could inhibit *Salmonella enterica* serovar Enteritidis invasion of Caco-2 cells. Additionally, the tested LAB cells and cell-free supernatants (CFS) showed low cytotoxicity to inhibit the proliferation of Caco-2 cells. Moreover, three LAB strains, *L. curvatus* FJNP12, *L. plantarum* SKKL1 and *W. cibaria* SFM5, clearly showed GABA production based on the TLC results. Additionally, these strains also yielded high amounts of GABA according to an HPLC method, 15.66, 11.76 and 5.62 g/L, respectively. The open reading frame (ORF) of the *gadB* gene of *L. plantarum* SKKL1 revealed that it was 1,407 bp in length, encoding a polypeptide of 469 amino acids, with a molecular weight of 53.56 kDa and an isoelectric point (pI) of 5.58 predicted using the EXPASY program. These strains could be used in starter cultures for value-added agricultural products.

## 1. Introduction

Probiotics are live microorganisms when consumed in adequate amounts confer health benefits (FAO/WHO, 2002). The potential advantages of probiotics include protection against diseases such as inflammatory bowel disease and cancer, mitigating lactose intolerance, lowering serum cholesterol levels, increased utilization of nutrients and decreased use of antibiotics (Guo *et al.*, 2010). Normally, probiotic bacteria at a level of 10<sup>6</sup> CFU/mL or g in food and feed have been recommended for health benefits (Fuller, 1989). The favourable probiotic properties of lactic acid bacteria (LAB) starter cultures are numerous. They have to be non-pathogenic, have the ability to produce antimicrobial substances to inhibit pathogenic bacteria, and survive in the gastrointestinal (GI) tract. Additionally, they should

produce bile salt hydrolase (BSH) to lower serum cholesterol levels and increase the utilization of nutrients. Moreover, they must be susceptible to antibiotics and enable decreased use of antibiotics (Guo *et al.*, 2010), while producing no blood haemolysis, but allowing for adhesion to intestinal epithelium cells to inhibit pathogens and modulate the host immune response, as well as exhibiting *in vitro* hydrophobicity (Fuller, 1989; Ratanaburee *et al.*, 2013a). Moreover, they should adhere to the intestinal mucus and epithelial cells to form a barrier to inhibit the colonization of pathogenic bacteria (Ehrmann *et al.*, 2002). Probiotic LAB can be used as a substitute for antibiotic growth promoters to improve livestock growth and prevent disease. LAB are generally recognized as safe (GRAS) and are frequently used as probiotics. *Lactobacillus* and *Bifidobacterium*

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strains have been used due to their beneficial effects on host health. LAB has an important role in food fermentation and preservation either as natural microflora or as starter cultures added under controlled conditions (Pumriw *et al.*, 2021). They can produce organic acids such as lactic acid, which lower pH and inhibit pathogenic bacteria (Halami *et al.*, 2000; Cleveland *et al.*, 2001). Gamma-aminobutyric acid (GABA) is a four-carbon free amino acid, a non-protein, that is widely distributed in microorganisms (bacteria, fungi, yeasts), plants, animals and vertebrates (Li *et al.*, 2008). It has well-known physiological functions such as its function in neurotransmission, induction of hypotension, diuretic tranquilizer effects and as an inhibitory neurotransmitter in the central nervous system (Watanabe *et al.*, 2011). Consequently, it is used for the treatment of sleeplessness, depression and autonomic disorders (Siragusa *et al.*, 2007; Di Cagno *et al.*, 2010). The use of GABA-producing LAB as starter cultures in fermentation processes could help to achieve the bioactivity of GABA. Currently, bio-synthetic production of GABA by LAB is done for the manufacture of fermented milk products, dairy products such as yoghurt, black raspberry juice, fermented soybeans, kimchi, Nham and cheese (Komatsuzaki *et al.*, 2005; Park and Oh, 2007; Dhakal *et al.*, 2012; Kim and Kim, 2012; Ratanaburee *et al.*, 2013b; Shan *et al.*, 2015). GABA-producing LAB has been reported. These include *Lactobacillus namurensis*, *Pediococcus pentosaceus* and *Levilactobacillus brevis* F064A isolated from fermented Thai foods (Ratanaburee *et al.*, 2013b; Kanklai *et al.*, 2021), *L. futsaii* isolated from Thai fermented shrimp (Sanchart *et al.*, 2016), *L. brevis* isolated from Kimchi (Park and Oh, 2007), fresh milk (Huang *et al.*, 2007), alcohol distillery lees (Yokoyama *et al.*, 2002), yogurt (Nomura *et al.*, 1999) and cheese (Siragusa *et al.*, 2007). *Lactococcus lactis* has been isolated from cheese (Nomura *et al.*, 1999; Park and Oh, 2007), *L. paracasei* from cheese (Siragusa *et al.*, 2007) and Japanese traditional fermented fish (Komatsuzaki *et al.*, 2005). *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* have been found in cheese (Siragusa *et al.*, 2007). GABA-producing ability varies widely among the LAB strains (Yokoyama *et al.*, 2002; Komatsuzaki *et al.*, 2005; Huang *et al.*, 2007; Park and Oh, 2007). However, the potential for using GABA-producing LAB probiotics has been of interest. LAB are microorganisms and thus attract more attention to GABA production (Li and Cao, 2010). Moreover, LAB can catalyze the  $\alpha$ -decarboxylation of monosodium glutamic acid (MSG) using glutamate decarboxylase (GAD), resulting in the release of GABA and CO<sub>2</sub> (Higuchi *et al.*, 1997). The production of GABA by LAB is safe and eco-friendly and this provides the possibility of the production of new

naturally fermented health-oriented products enriched in GABA for use as food and feed. Selection of LAB that produces high levels of GABA and evaluation of the probiotic properties of LAB strains to identify single and mix-strains that may have potential uses as probiotics in food or animal production is undertaken. Moreover, fermentation offers the possibility to use low-cost agricultural waste substrates as raw materials for GABA-producing LAB strains. An objective of the current study is to examine the fermentative production of high levels of GABA. This is of interest for food and feed applications to obtain potential probiotic products and take advantage of the functional properties of GABA. This study aimed to assess the probiotic properties of selected LAB strains that are able to produce the bioactive compound, GABA. It further aimed to characterize the glutamate decarboxylase gene and its role in starter cultures for food and feed supplements.

## 2. Materials and methods

### 2.1 *In vitro* assessment of probiotic properties of selected lactic acid bacteria strains

#### 2.1.1 Microorganisms and cultivation

A total of seven LAB strains were obtained from the culture collections of the Fermentation Research Center for Value Added Agricultural Products (FerVAAP) and the Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand (Table 1). All selected LAB strains were stored in 50% (v/v) glycerol at -80°C. Each strain was reactivated by streaking it on de Man Rogosa and Sharpe (MRS) agar containing 0.5% (w/v) CaCO<sub>3</sub>, followed by incubation at 37°C for 24 hrs. A fresh culture was sub-cultured into MRS broth.

Table 1. Various species of lactic acid bacteria were used in this study

LAB strain	Source
<i>Lactobacillus plantarum</i> SKKL1	Fermented meat (Mum)
<i>L. curvatus</i> FJNP12	Fermented fish (Pla-som)
<i>L. pentosus</i> SKKP2	Fermented meat (Mum)
<i>Pediococcus pentosaceus</i> SFM1	Local Thai sausage
<i>Weissella cibaria</i> SFM5	Local Thai sausage
<i>W. halotolerans</i> FFJSS5	Fermented fish (Pla-som)
<i>Enterococcus</i> sp. FeHuE3	Baby feces

#### 2.1.2 Antagonistic activity

An antagonistic activity test was performed using an agar well diffusion method. The pathogenic bacteria, *Bacillus cereus* TISTR 678, *Staphylococcus aureus* TISTR 1466, *Enterococcus faecalis* TISTR 379, *Salmonella enterica* serovar Typhimurium TISTR 292, *S. enterica* ser. Enteritidis, *Enterobacter aerogenes* TISTR 1540, *Escherichia coli* TISTR 780 and *Pseudomonas aeruginosa* TISTR 1467 were used as

target organisms. All of the targeted strains were grown in tryptic soy agar (TSA) at 37°C for 24 hrs. One loopful of each culture was inoculated into tryptic soy broth (TSB) followed by incubation for 24 hrs. Each culture broth was adjusted to approximately  $1 \times 10^6$  cells/mL for swabbing onto TSA plates.

The selected LAB strains were separately grown in MRS broth and incubated at 37°C for 24 hrs. The pH values of the culture broths were measured. Cell-free supernatants (CFS) were obtained by centrifugation at 10,000 rpm for 10 mins and subsequent filtration through a 0.45 µm filter. A volume of 140 µL of each CFS was added to each well of the pathogen target seeded TSA plates. All plates were incubated at 37°C for 24 hrs before measuring inhibition zone diameters (mm). The results are reported indicating the degree of inhibition.

### 2.1.3 Acid, bile salt and fresh chicken bile tolerance

The method described by Vinderola and Reinheimer (2003) was used. Briefly, each strain grown in MRS broth at 37°C for 24 hrs was harvested by centrifugation at 10,000 rpm for 10 mins. The bacterial cells were washed twice and re-suspended in normal saline (0.85% (w/v) NaCl). Then, cells were tested for their ability to grow in MRS broth media at an acidic pH (pH 2.5) and in the presence of 0.3% (w/v) bile salts at 37°C for 3 and 24 hrs, to test their acid and bile tolerance, respectively. The tolerance of LAB to these factors was assessed in terms of viable colony counts on MRS agar containing 0.5% (w/v) CaCO<sub>3</sub>.

The fresh chicken bile tolerance method described by Gilliland *et al.* (1984) was used. An overnight grown culture of each LAB strain was inoculated into an MRS broth containing 3% (w/v) fresh chicken bile. Subsequently, the cultures were incubated at 37°C for 3 hrs. Growth under both a control treatment (without bile) and 3% (w/v) fresh chicken bile was determined through viable cell counts of LAB on MRS agar containing 0.5% (w/v) CaCO<sub>3</sub>.

### 2.1.4 Cell surface hydrophobicity

A test of adhesion between bacterial cells and hydrocarbon solvents, chloroform (a monopolar acidic solvent), xylene (nonpolar neutral solvent) and hexadecane (nonpolar solvent) was modified from Guo *et al.* (2010) and Pringsulaka *et al.* (2015). Briefly, each strain of LAB was grown in MRS broth at 37°C for 24 hrs and washed with sterile phosphate-buffered saline solution (PBS, pH 7), harvested and re-suspended in the same buffer. The suspension was then adjusted to approximately  $1 \times 10^8$  CFU/mL ( $OD_{640nm} = H_1$ ). Aliquots (3 mL) of each bacterial suspension were placed into 1 mL of each of the hydrocarbon solvents. The optical

density of the aqueous phase ( $H_2$ ) that resulted from thoroughly mixing the bacterial suspension with each of the solvents by vortexing for 2 mins was determined spectrophotometrically at 640 nm. The percentage of hydrophobicity was calculated using the following equation:

$$\text{Hydrophobicity (\%)} = [(H_1 - H_2)/H_1] \times 100 \quad (1)$$

LAB strains with greater than 70% hydrophobicity were classified as high, 36-70% were classified as moderate, and 0-36% were classified as having low hydrophobicity.

### 2.1.5 Mucin adhesion

The protocol for determining mucin adhesion followed Tallon *et al.* (2007) with modification. Briefly, mucin was dissolved in PBS (pH 7) to a final concentration of 10 mg/mL. 100 µL aliquots of this solution were immobilized in microtiter plate wells by overnight incubation at 4°C. Wells were washed twice with 200 µL of PBS and saturated with a 2% (w/v) bovine serum albumin (BSA) solution for 4 hrs at 4°C. Last, the wells were washed twice with 200 µL of PBS. A minimum of four replicates was used to estimate the adhesion of each strain.

LAB strains were grown separately in MRS broth at 37°C for 18 hrs and centrifuged at 10,000 rpm for 10 mins. The cell pellets were washed twice with sterile PBS, resuspended in PBS and adjusted to an optical density at 600 nm ( $OD_{600nm}$ ) = 0.1. Approximately, 100 µL of the bacterial suspension was added to each well. Then, the plates were incubated at 37°C for 1 hr. The wells were washed 12 times with 200 µL of sterile PBS to remove unbound bacteria. Each of the wells was then treated with 200 µL of a 0.5% (v/v) Triton X-100 solution to desorb the bound bacteria. Plates were then incubated at room temperature under orbital agitation for 2 hrs. Approximately 100 µL of the contents of each well were removed, diluted in PBS and plated onto MRS agar plates. The per cent adhesion was determined using Equation (2).

$$\text{Adhesion (\%)} = (A_1/A_0) \times 100 \quad (2)$$

Where,  $A_0$  = log number of free cells before adhesion to mucin per microtiter plate well (CFU/mL), and  $A_1$  = log number of free cells in wells after adhesion to mucin per microtiter plate well (CFU/mL).

### 2.1.6 Haemolytic activity

The haemolytic activity of the selected LAB strains was evaluated by growing the cells in MRS broth and incubating them at 37°C for 24 hrs. Then, the cells were transferred onto blood agar plates. The plates were incubated at 37°C for 48 hrs and observed for subsequent

haemolytic reactions noted. Partial hydrolysis of red blood cells showed greening zones around colonies ( $\alpha$ -haemolysis). A clear zone around bacterial growth showed haemolytic action ( $\beta$ -haemolysis). No reaction indicated  $\gamma$ -haemolysis.

### 2.1.7 Bile salt hydrolase activity

Semi-qualitative bile salt hydrolase (BSH) activity of the cultures was determined with a procedure modified from Du Toit *et al.* (1998) and Guo *et al.* (2010). Briefly, 20  $\mu$ L of cultures of each LAB strain grown overnight were spotted onto MRS agar plates supplemented with 0.37 (g/L)  $\text{CaCl}_2$  and 0.5% (w/v) of human bile salts as the sodium salts of taurocholic acid (TC) and taurodeoxycholic acid (TDC). Plates were incubated at 37°C for 48-72 hrs. The presence of precipitated bile acid around the colonies (opaque or halo) was considered indicative of a positive result.

### 2.1.8 Autoaggregation assay

A test for autoaggregation was performed with a method modified from Del Re *et al.* (2000). Briefly, a 0.1 mL cell suspension was mixed with 1.9 mL of PBS to prepare a 2 mL system containing approximately  $1 \times 10^9$  CFU/mL. It was vortexed for 10 s and incubated at 37°C for 24 hrs. 0.1 mL of the upper layer suspension was carefully collected, mixed with 0.9 mL of PBS and its optical density at 600 nm ( $\text{OD}_{600\text{nm}}$ ) was measured. Autoaggregation is expressed as  $1 - (\text{OD upper suspension} / \text{OD total bacterial suspension}) \times 100$ .

### 2.1.9 Coaggregation assay

Coaggregation between each LAB and pathogenic bacteria strain was determined following the method of Savedboworn *et al.* (2014). Briefly, equal volumes (2 mL) of each LAB and pathogen cell suspension were mixed in pairs by vortexing for 10 secs. Control tubes were set up at the same time, containing 4 mL of each bacterial suspension. The optical density (A) at 600 nm ( $\text{OD}_{600\text{nm}}$ ) of the suspensions was measured after incubation at 37°C for 5 hrs. Samples were taken using the same procedure as in the autoaggregation assay. The percentage of coaggregation was calculated as:

$$\text{Coaggregation (\%)} = \frac{\left(\frac{Ax + Ay}{2}\right) - A(x + y)}{\left(\frac{Ax + Ay}{2}\right)} \times 100 \quad (3)$$

Where, x and y represent LAB and pathogenic bacteria, respectively and (x+y) represents the mixture of LAB and each pathogenic bacteria.

### 2.1.10 Antibiotic susceptibility

An agar disc diffusion method was used to test the antibiotic resistance of LAB strains with the following

antibiotics: ampicillin (10  $\mu$ g), chloramphenicol (30  $\mu$ g), erythromycin (15  $\mu$ g), gentamycin (10  $\mu$ g), kanamycin (30  $\mu$ g), norfloxacin (10  $\mu$ g), penicillin G (10 U), streptomycin (10  $\mu$ g), tetracycline (30  $\mu$ g) and vancomycin (30  $\mu$ g). An overnight culture of each LAB strain was prepared to obtain a cell suspension of approximately  $1 \times 10^6$  CFU/mL. The selected LAB suspension was swabbed onto MRS agar plates. Later discs onto which various antibiotics had been absorbed were placed on the agar and incubated at 37°C for 24 hrs. The diameters of the inhibition zones were measured after incubation and compared with those in the interpretative standard chart of the Clinical and Laboratory Standards Institute (2014). The results were reported as resistant (R), intermediate (I) and susceptible (S).

### 2.1.11 Coexistence test

Coexistence among seven LAB strains was determined using a cross-streak method (modified from Guo *et al.* (2010) and Pringsulaka *et al.* (2015)). Briefly, each of the four LAB strains was streaked perpendicularly from other streaked strains on MRS agar containing 0.5% (w/v)  $\text{CaCO}_3$ . The plates were incubated at 37°C for 48 hrs to observe the antagonism of bacteria against one another.

### 2.1.12 Adhesion of selected lactic acid bacteria to Caco-2 cells

Cell lines and culture conditions: Cells of a human intestinal epithelial cell line (Caco-2) are routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FBS) and 1% (v/v) penicillin-streptomycin (10,000 IU/mL). The cells were incubated at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . Intestinal epithelial cells were seeded with 1 mL of a culture medium containing  $1 \times 10^5$  cells/mL in 24-well tissue culture plates. The cell culture medium was changed every 2 days, and the cells were subcultured at 80% confluence. The intestinal epithelial cells were used at 14 days post-confluence after becoming fully differentiated. A medium of non-supplemented DMEM was replaced at least 1 hr before these assays.

Preparation of bacterial cells and cell-free culture supernatants: LAB strains were grown in MRS broth at 37°C for 24 hrs. Cell-free culture supernatant (CFS) was collected by centrifugation at 10,000 rpm for 10 mins. It was then filter-sterilized using a 0.45  $\mu$ m pore size filter. The pelleted cells at concentrations of  $1 \times 10^8$  CFU/mL were resuspended in an equal volume of serum-free DMEM.

Adhesion of selected LAB to Caco-2 cells: The LAB

cultures in MRS broth were harvested by centrifugation at 10,000 rpm for 10 mins, washed twice with PBS and diluted to a concentration of approximately  $1 \times 10^8$  CFU/mL in serum-free DMEM (with no antibiotics). Concurrently, Caco-2 cells were also washed twice with PBS to remove antibiotics before any bacterial suspension was added. One millilitre of each LAB at a specified bacterial concentration was added to each well of a 24-well plate. The plates were incubated at 37°C for 1 hr under 5% CO<sub>2</sub>. Then, the supernatants were removed and wells were gently washed three times with PBS to remove unattached bacteria. Cells were lysed with 0.1% (v/v) Triton-X 100 for 5 mins. The adhering bacterial cells were placed in MRS agar and then incubated at 37°C for 48 hrs. Adhesion data were expressed as the percentage of bacteria adhering compared to the total of bacteria added (CFU bacteria adhered/CFU bacteria added).

### 2.1.13 Infection inhibition activity of selected lactic acid bacteria against *Salmonella enterica* serovar Enteritidis invasion of Caco-2 cells

The methods of Hudault *et al.* (1997) and Thirabunyanon and Hongwittayakorn (2013) were used with minor modifications. The enteropathogenic bacterium, *S. enterica* ser. Enteritidis and LAB strains were cultured overnight in TSB or MRS broth. Bacteria were harvested by centrifugation at 10,000 rpm for 10 mins at 4°C, washed twice with PBS and diluted to a concentration of  $1 \times 10^8$  CFU/mL in DMEM (with no antibiotics). After that, non-supplemented DMEM was replaced at least 1 hr before the invasion assay. The intestinal epithelial cells were washed twice with PBS. Approximately, 1 mL of *S. enterica* ser. Enteritidis at approximately  $1 \times 10^8$  CFU/mL was loaded into the intestinal epithelial cell culture monolayers in 24-well tissue culture plates. Then, one mL of each LAB culture in DMEM was added at a concentration of approximately  $1 \times 10^8$  CFU/mL. These were mixed into the wells and incubated at 37°C for 2 hrs under 5% CO<sub>2</sub>. After that, the supernatants were removed and wells were gently washed three times with PBS to remove non-attached bacteria. Intestinal epithelial cells were lysed with 0.1% (v/v) Triton X-100. The adhering bacteria were counted on xylose lysine deoxycholate agar (XLD agar) plates. The viability of *S. enterica* ser. Enteritidis was used to estimate the invasion rates. Adhesion data were expressed as the percentage of bacteria adhered compared to the total bacteria added (CFU bacteria adhered/CFU bacteria added).

### 2.1.14 Cytotoxicity assay

The activity of LAB on the antiproliferation of Caco-2 cells was evaluated using MTT assays, as previously

described by Thirabunyanon and Hongwittayakorn (2013). The MTT assay used in this experiment employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT). Caco-2 cells were prepared in 100 µL of a suspension at a density of  $10^5$  cells/well in a 96-well plate. The cell suspensions were incubated in a CO<sub>2</sub> incubator at 37°C for 24 hrs. After incubation, 100 µL of CFS was added to each well and incubation continued for another 24 hrs under the same conditions. Cells were then washed twice with PBS. Next, 10 µL of MTT solution with 0.5 mg/mL dimethyl sulfoxide (DMSO) was added to each well and incubated at 37°C for 4 hrs. The precipitate of formazan was then solubilized by adding 100 µL of DMSO with continued incubation at 37°C for 5 mins. Optical density was measured at 595 nm (OD<sub>595nm</sub>) using a microplate reader for the cell viability calculation, while the absorbance of the control group, which used MRS broth, was taken as 100% cell viability. The percentage of cell viability = (sample OD/control OD) × 100.

## 2.2 Selection of Gamma-aminobutyric acid-producing lactic acid bacteria

### 2.2.1 Determination of Gamma-aminobutyric acid-producing lactic acid bacteria

Each strain of LAB was inoculated into an MRS broth supplemented with 5% (w/v) monosodium glutamate (MSG). LAB strains were incubated at 37°C for 24 hrs and then GABA production of each LAB culture broth was determined using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Choi *et al.*, 2006; Thwe *et al.*, 2011; Ratanaburee *et al.*, 2013a).

### 2.2.2 Cloning of the *gadB* gene from *Lactobacillus*

LAB that produced high amounts of GABA were selected for further study of the *gadB* gene. LAB genes were amplified using a primer set based on *gadB* from *L. plantarum* *gadB* LP pET26b, *gadB*\_F (5'-CATATGGCAATGTTATACGGTAA-3') and *gadB*\_R (5' GTCGACGTGTGTGAATCCGTATTCT-3'). Amplified fragments were ligated with a pT7blue vector. *E. coli* DH5α cells were transformed with the ligation mixture by a standard protocol (Sambrook *et al.*, 1989). DNA sequences were analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The molecular mass and the predicted isoelectric point (pI) of the corresponding GAD protein were obtained using the ExpASY program.

## 2.3 Statistical analysis

The data were expressed as a mean ± standard deviation. The experimental data were analyzed using a one-way analysis of variance (ANOVA) with SPSS.

Significant differences were accepted at  $P < 0.05$  using Duncan's multiple range test (DMRT).

### 3. Results and discussion

#### 3.1 In vitro assessment of probiotic properties of selected lactic acid bacteria strains

##### 3.1.1 Antagonistic activity

Selected LAB strains of various species (*Lactobacillus plantarum* SKKL1, *L. curvatus* FJNP12, *L. pentosus* SKKP2, *Pediococcus pentosaceus* SFM1, *Weissella cibaria* SFM5, *W. halotolerans* FFJSS5 and *Enterococcus* sp. FeHuE3) were tested for inhibitory action against pathogenic bacteria using an agar well diffusion assay. All LAB strains produced culture pH values in the range of 3.6-3.7. Antagonistic activities of their culture-free supernatants (CFS) are presented in Table 2. Interestingly, all of the selected LAB showed growth inhibition of all target microorganisms (*B. cereus* TISTR 678, *S. aureus* TISTR 1466, *E. faecalis* TISTR 379, *S. enterica* ser. Typhimurium TISTR 292, *S. enterica* ser. Enteritidis, *E. aerogenes* TISTR 1540, *E. coli* TISTR 780 and *P. aeruginosa* TISTR 1467). *Salmonella enterica* serovar Enteritidis was the most sensitive strain (inhibition zone  $>20$  mm), while *E. faecalis*, *E. aerogenes*, *P. aeruginosa* and *B. cereus* were the most tolerant strains (inhibition zone  $<10$  mm). Additionally, *E. coli* and *S. aureus* exhibited moderate tolerance (inhibition zone  $>15$  mm). It has been recognized that some LAB produces antimicrobial substances, for instance, bacteriocins, diacetyl, hydrogen peroxide and short-chain fatty acids (Cleveland *et al.*, 2001; Foulquié Moreno *et al.*, 2006; Kantachote *et al.*, 2010). These antimicrobial substances can inhibit the growth of other microorganisms. Accordingly, these LAB were subjected to antibacterial activity assays against representative intestinal pathogens. As expected, all LAB strains exhibited strong inhibitory activity against each of the tested pathogenic bacteria.

Antibacterial activity against pathogenic bacteria is an attractive property of starter cultures because it could help to achieve safe fermented foods (Kantachote *et al.*,

2010). All LAB strains in these tests had final pH values below 4. They all could inhibit the target microorganisms, whereas culture filtrates at pH 6.5 of these strains did not inhibit pathogens. This indicates pH and organic acids might be the sole origin of antibacterial activity in culture filtrates. Therefore, it would be interesting to use these strains as starter cultures, as doing so could help to reduce the post-acidification of products such as fermented ground pork.

##### 3.1.2 Acidic pH, bile salt and fresh chicken bile tolerance

The results of acidic pH, bile salt and fresh chicken bile tolerance of selected challenge LAB strains are shown in Figure 1. All of the selected LAB strains could survive in the highly acidic condition (pH 2.5) in the range of 88.47-96.72%, and tolerate up to 0.3% (w/v) bile salt in the range of 67.42-78.74%. These LAB could tolerate 3% (w/v) fresh chicken bile in the range of 89.15 -97.28%. Evaluation at an acidic condition of pH 2.5 showed that almost all isolates had high survival and tolerance under these circumstances. Additionally, all isolates were shown to have good survival and tolerance in 0.3% (w/v) bile salt and 3% (w/v) fresh chicken bile in

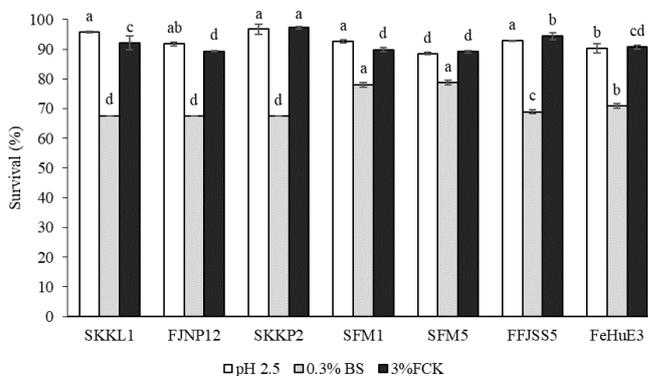


Figure 1. Tolerance of selected LAB strains in the presence of acidic acid (pH 2.5), 0.3% bile salt (BS) and 3% fresh chick bile (FCK). Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

Table 2. Antagonistic activities of selected LAB strains against pathogenic bacteria

Strains	pH of CFS	Degree of inhibition								
		SE	EC	ST	SA	EF	EA	PA	BC	
<i>L. plantarum</i> SKKL1	3.7±0.1	+++	++	+	++	+	+	+	+	
<i>L. curvatus</i> FJNP12	3.7±0.1	+++	++	+	++	+	+	+	+	
<i>L. pentosus</i> SKKP2	3.6±0.1	+++	+	+	+	+	+	+	+	
<i>P. pentosaceus</i> SFM1	3.7±0.1	++	+	+	+	+	+	+	+	
<i>W. cibaria</i> SFM5	3.7±0.1	++	+	+	+	+	+	+	+	
<i>W. halotolerans</i> FFJSS5	3.7±0.1	++	++	+	+	+	+	+	+	
<i>Enterococcus</i> sp. FeHuE3	3.7±0.1	++	++	+	+	+	+	+	+	

Note: CFS: cell free supernatant, SE: *S. enterica* ser. Enteritidis, BC: *B. cereus*, EC: *E. coli*, ST: *S. enterica* ser. Typhimurium, SA: *S. aureus*, EF: *E. faecalis*, EA: *E. aerogenes*, PA: *P. aeruginosa*

Degree of inhibition; - = no clear zone, 10-15 mm = + (fair),  $>15-20$  mm = ++ (good),  $>20$  mm = +++ (very good)

the GI tract model.

The tests used for *in vitro* functionality screening were a determination of survival under conditions resembling the pH and digestive enzymes of the human oral cavity and GI tract (Saarela *et al.*, 2000). LAB strains should survive in harsh GI tract environments of gastric juice at pH 2.5 (Kimoto *et al.*, 2000) and bile (Begley *et al.*, 2006) secreted into the human GI tract. Other beneficial functional aspects include adherence to an intestinal mucin layer, production of antimicrobial compounds and health-promoting enzymes such as  $\beta$ -galactosidase and BSH activity (Collado *et al.*, 2008; Pithva *et al.*, 2014). It is a precondition for a strain to become a part of the GI microflora to extend health benefits to a host (Shekh *et al.*, 2016). Based on our results, all of the isolates survived under acidic conditions and high concentrations of bile salt treatments. The acidity (pH 2.5 to 3.5) of gastric secretions and bile salts destroys the majority of bacteria that enter the GI tract (Holzapfel *et al.*, 1998). However, many LAB showed an ability to survive in simulated GI tract fluid, which was not unexpected. These bacteria are well-known indigenous flora of the GI tracts of humans and animals. In this ecological niche, these LAB encounter bile salts (Bhardwaj *et al.*, 2010).

### 3.1.3 Cell surface hydrophobicity

The percentage of cells adhering to chloroform (monopolar acidic solvent), xylene (nonpolar neutral solvent) and hexadecane (nonpolar solvent) demonstrated hydrophobic cell surface properties, as shown in Figure 2. LAB isolates with greater than 70% adhesion were classified as highly hydrophobic, 36-70% were classified as moderately hydrophobic, and 0-36% were classified as having low hydrophobicity. Results of the hydrophobicity tests revealed that the percentages of hydrophobicity of each solvent were chloroform at 4.88-95.93%, xylene at 0.76-44.72%, and hexadecane at 5.99-47.57%. It was demonstrated that high hydrophobicity was found only in chloroform.

High hydrophobicity (>70%) of cell surfaces was found in all strains of *Lactobacillus* (SKKL1, FJNP12 and SKKP2) when tested with chloroform. These strains showed moderate and low hydrophobicity when tested with xylene and hexadecane, respectively. The strain, *P. pentosaceus* SFM1, showed moderate hydrophobicity in all tested solvents, while the strains, *W. cibaria* SFM5 and *Enterococcus* sp. FeHuE3, exhibited low hydrophobicity in all tested solvents. *W. halotolerans* FFJSS5 also showed low hydrophobicity in the tested solvents, except in chloroform where moderate hydrophobicity was obtained. Interestingly, the tested strains showed similar hydrophobicity results in xylene

and hexadecane. Bacterial adhesion to hydrocarbons indicates cell surface hydrophobicity plays a key role in non-specific interactions between microbial cells and mucus or epithelial cells (Osmanagaoglu *et al.*, 2010). Hydrophobicity is generally thought to be correlated with bacterial adhesion to the intestinal mucosa. It has been suggested that bacterial cells with a high hydrophobicity usually form strong interactions with mucosal cells or adhere strongly to epithelial cells or mucus. Mechanisms of adherence to an epithelial surface involve receptor-specific binding, charge and hydrophobic interactions. These differences in cell surface hydrophobicity could be due to variations in the levels of expression of cell surface proteins among strains of a species, as well as due to environmental conditions that could affect the expression of surface proteins (Sayedboworn *et al.*, 2014). Bacterial adhesion to a host is the initial step for their colonization and can subsequently lead to commensalism or infectious diseases. This adhesion to host cell surfaces initially involves nonspecific binding such as hydrophobic interactions (Collado *et al.*, 2007; Piwat *et al.*, 2015), followed by specific interactions between bacterial surface components, such as lipoteichoic acid and surface layer proteins as well as their receptors on the host epithelial cells (Vélez *et al.*, 2007; Zhang *et al.*, 2013).

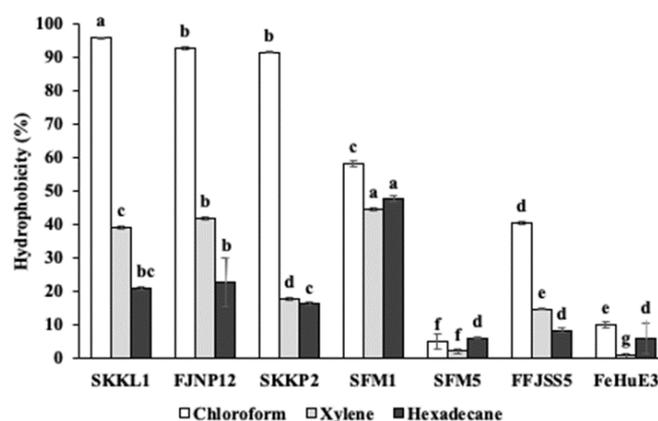


Figure 2. Adhesion capability to cell surface hydrophobicity of selected LAB strains (>70% high, 36-70% moderate, 0-36% low hydrophobic). Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, FFJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.1.4 Mucin adhesion

The results of mucin adhesion testing are presented in Figure 3. All tested LAB strains showed high mucin adhesion, in the range of 73.17-84.89%. *W. halotolerans* FFJSS5 gave the highest value of 84.89%, followed by *L. plantarum* SKKL1 (78.69%), *L. pentosus* SKKP2

(76.71%), *Enterococcus* sp. FeHuE3 (76.34%), *L. curvatus* FJNP12 (75.87%), *P. pentosaceus* SFM1 (75.50%) and *W. cibaria* SFM5 (73.17%). The epithelial cells of the GI tract are covered by a layer of mucus that protects the epithelium from damage and pathogens. It also provides habitat and nutrients for the intestinal microflora (Tuomola *et al.*, 1999). Mucus is the first barrier that ingested microorganisms confront in the gut. Thus, adhesion to this matrix is considered a prerequisite for colonization of the gut (Ouweland *et al.*, 2001). The major components of mucus are polymeric glycoproteins called mucins. They have been widely used for *in vitro* adhesion tests of probiotics (Tuomola *et al.*, 2000; Matsumoto *et al.*, 2002). Mucus is continuously renewed. Therefore bacteria that are unable to bind to the epithelium are eliminated.

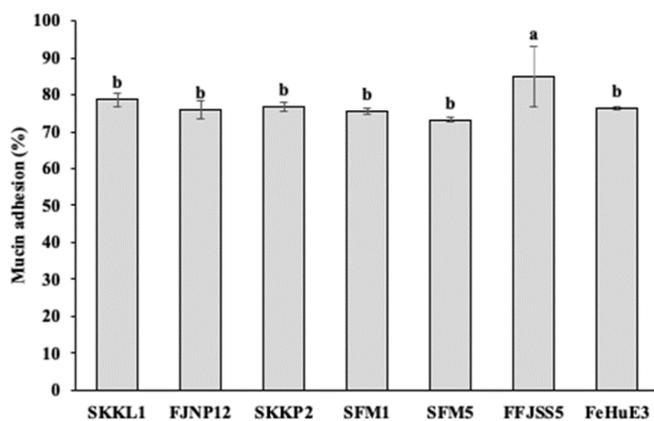


Figure 3. Adhesion capability to mucin of selected LAB strains. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.1.5 Blood haemolysis

Results showed that all LAB strains showed  $\gamma$ -haemolysis. None displayed  $\alpha$  or  $\beta$  blood haemolysis. These LAB are not harmful to human red blood cells since they produce no haemolysis. This is a further sign of their potential utility for use as probiotic microorganisms.

### 3.1.6 Bile salts hydrolase activity

The results of BSH activities are shown that all LAB strains exhibited the same BSH activity. Almost all deconjugated taurocholic acid (TC) and taurodeoxycholic acid (TDC). Only *W. cibaria* SFM5 gave a negative result for TDC. One of the reasons that the tested LAB strains could survive in such conditions was their ability to hydrolyze various bile salts (TC and TDC) via BSH, decreasing the solubility of these salts. It is well-recognized that undissociated forms of bile salts can act as antimicrobial agents, such as organic acids. In

this way, microbial cell membranes are altered by bile salts (Ratanaburee *et al.*, 2013a). The above results suggest that all tested LAB strains might be able to compete in the human intestine. The selected LAB strains showed BSH activity in all strains tested. Guo *et al.* (2010) suggested that microbial BSH functions in the detoxification of bile salts as well as increasing the intestinal survival and persistence of the producing strains (Begley *et al.*, 2006). Beneficial effects of BSH-positive LAB strains (such as lowering serum cholesterol levels) *in vivo* were also reported (Du Toit *et al.*, 1998).

### 3.1.7 Autoaggregation ability

The autoaggregation ability of selected LAB strains was in the range of 69.07-84.81%, as shown in Figure 4. *W. halotolerans* FFJSS5 and *Enterococcus* sp. FeHuE3 gave the highest aggregation value, 84.81%, followed by *W. cibaria* SFM5 (80.00%), *L. pentosus* SKKP2 (78.12%), *L. curvatus* FJNP12 (74.59%), *P. pentosaceus* SFM1 (69.27%) and *L. plantarum* SKKL1 (69.07%). In the current study, all tested LAB strains showed high autoaggregation. As shown in Figure 4, most isolates exhibited strong autoaggregation after 24 hrs of incubation. Autoaggregation of probiotic strains refers to the clumping of bacterial cells from the same strain (Lee *et al.*, 2014). This phenomenon appears necessary for adhesion to intestinal epithelial cells. Furthermore, autoaggregation could be useful in forming biofilms during GI tract colonization, which is a way to form a barrier against colonization by pathogens (Ramirez-Chavarin *et al.*, 2013; Savedboworn *et al.*, 2014). Aggregation and cell surface hydrophobicity was used to preliminarily screen for probiotic properties. This procedure has been proposed as an indirect method for the evaluation of the adhesion ability of LAB.

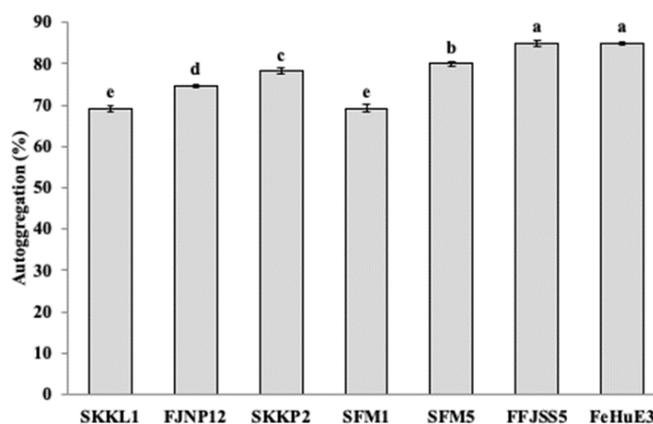


Figure 4. Autoaggregation ability of selected LAB strains. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.1.8 Coaggregation ability

The coaggregation between LAB strains and pathogens showed great variation. All LAB strains could coaggregate to all pathogens, event at the low percentages, as shown in Figure 5. Clearly, all LAB strains could coaggregate with *S. aureus* TISTR 1466 better than *S. enterica* ser. Typhimurium TISTR 292 and *E. coli* TISTR 780 (in the range of 21.99-26.70%, 7.71-12.37% and 5.84-9.67%, respectively). Interestingly, *L. curvatus* FJNP12 gave the highest coaggregation to all pathogenic bacteria, while the lowest coaggregation to all pathogens was found in *L. plantarum* SKKL1. These results were similar to Lapsiri *et al.* (2011) who reported that 13 strains of *L. plantarum* exhibited low coaggregation to *E. coli* O157:H7 DMST 12743 (3.68-19.17%), *S. enterica* ser. Typhimurium ATCC 13311 (3.85-12.16%). Alternatively, Savedboworn *et al.* (2014) revealed that 23 strains of LAB isolated from fermented vegetables showed coaggregation ability with *E. coli* (1.92-53.12%), and *S. enterica* ser. Typhimurium ATCC 13311 (3.25-53.28%) that were higher than in the current study. It has been suggested that probiotic microorganisms that have the ability to coaggregate with

pathogens may be better able to kill undesirable bacteria. This is because they can produce antimicrobial substances in very close proximity to their target (Savedboworn *et al.*, 2014). Coaggregation mechanisms between probiotic and pathogenic bacteria could be involved in a reduction in pathogen adhesion to mucus. Additionally, it has been suggested that the coaggregation of probiotic bacteria may enable the formation of a barrier to prevent colonization by pathogens.

### 3.1.9 Antibiotic susceptibility

All tested strains were susceptible to ampicillin, chloramphenicol, erythromycin gentamycin penicillin G and tetracycline. They were resistant to four antibiotics (kanamycin, norfloxacin, vancomycin and streptomycin) as reported in Table 3. Some strains (*L. pentosus* SKKP2, *P. pentosaceus* SFM1 and *W. halotolerans* FFJSS5) showed an intermediate level of streptomycin susceptibility. The susceptibility and resistance to antibiotics of various strains were variables depending on the species. LAB must show an ability to resist various antibiotics and subsequently exhibit beneficial effects on the health of the host to be used as probiotics (Lim and Im, 2009). Antibiotics are utilized by the medical and pharmacological industries to fight pathogenic bacteria. Resistance of probiotic strains to some antibiotics could be useful for both preventive and therapeutic purposes in controlling intestinal infections.

### 3.1.10 Compatibility assay

All LAB strains (*L. plantarum* SKKL1, *L. curvatus* FJNP12, *L. pentosus* SKKP2, *P. pentosaceus* SFM1, *W. cibaria* SFM5, *W. halotolerans* FFJSS5 and *Enterococcus* sp. FeHuE3) showed no antagonism against each other on MRS agar. Thus, these seven LAB strains can be combined as a probiotic mixture.

### 3.1.11 Adhesion to intestinal epithelial cells

The adhesion of seven LAB isolates (*L. plantarum*

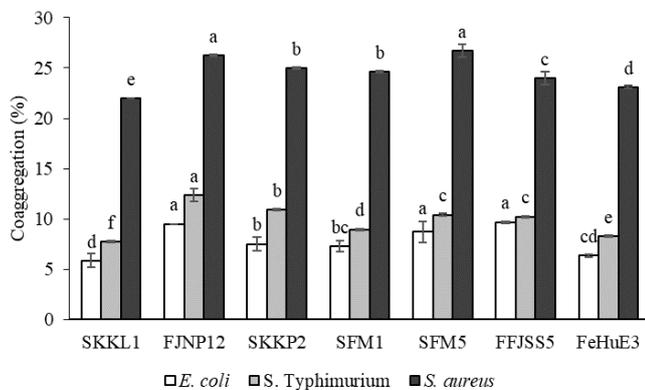


Figure 5. Coaggregation ability of selected LAB. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

Table 3. Antibiotic susceptibility of selected LAB strains

LAB	Antibiotics									
	AMP	CHL	ERY	GEN	KAN	NOR	PEN	STR	TET	VAN
SKKL1	S	S	S	S	R	R	S	R	S	R
FJNP12	S	S	S	S	R	R	S	R	S	R
SKKP2	S	S	S	S	R	R	S	I	S	R
SFM1	S	S	S	S	I	R	S	I	S	R
SFM5	S	S	S	S	R	R	S	R	S	R
FFJSS5	S	S	S	S	R	R	S	I	S	R
FeHuE3	S	S	S	S	R	R	S	S	S	R

Note: Resistant (R), Intermediate (I), Susceptibility (S), ampicillin (AMP), chloramphenicol (CHL), erythromycin (ERY), gentamycin (GEN), kanamycin (KAN), norfloxacin (NOR), penicillin (PEN), streptomycin (STR), tetracycline (TET), vancomycin (VAN); (SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3)

SKKL1, *L. curvatus* FJNP12, *L. pentosus* SKKP2, *P. pentosaceus* SFM1, *W. cibaria* SFM5, *W. halotolerans* FFJSS5 and *Enterococcus* sp. FeHuE3) to intestinal epithelial cells is shown in Figure 6. There was great variation in their adhesion. All LAB strains showed adhesion to Caco-2 cells, ranging from 23.32-75.48%. *W. halotolerans* FFJSS5 had the highest adhesion (75.48%), followed by *Enterococcus* sp. FeHuE3 (50.00%), *P. pentosaceus* SFM1 (36.67%), *L. plantarum* SKKL1 (35.09%) and *L. pentosus* SKKP2 (32.89%). *L. curvatus* FJNP12 and *W. halotolerans* FFJSS5 showed low adhesion, 26.36 and 23.32%, respectively. Adhesion to intestinal epithelia is the main criterion for the selection of probiotic strains. Additionally, when probiotic cells adhere, they have beneficial effects on their host, such as when they are involved in preventing colonization of the gut by pathogens and maintaining gut mucosal immunity (Tancrede, 1992). In our study, all LAB exhibited an excellent ability to adhere to Caco-2 cells. The high adhesion of these strain cells indicates their ability to colonize the intestinal epithelium and represents a barrier that protects intestinal tissue from pathogens (Pringsulaka et al., 2015).

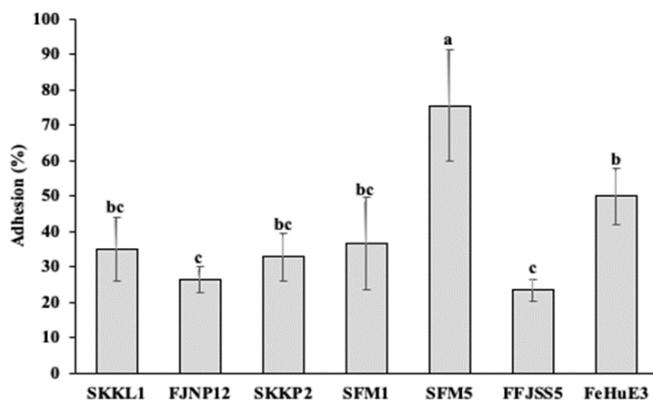


Figure 6. Adhesion of selected LAB strains on Caco-2 cells. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.1.12 Competition assay

The activity of seven LAB cells against *S. enterica* ser. Enteritidis invasion of Caco-2 cells showed that the strains were effective, in the range of 20.44-50.72%. *L. pentosus* SKKP2 showed the highest invasion rate (50.72%), followed by *W. halotolerans* FFJSS5 (37.53%), *P. pentosaceus* SFM1 (35.12%), *L. plantarum* SKKL1 (35.10%), *W. cibaria* SFM5 (27.73%), *L. curvatus* FJNP12 (26.01%), and *Enterococcus* sp. FeHuE3 (20.44%) (Figure 7) to this intestinal epithelial cell line. All tested LAB cells could inhibit *S. enterica* ser. Enteritidis invasion of Caco-2 cells. Moreover, these

strains had a protective activity against *S. enterica* ser. Enteritidis infection and they were able to competitively exclude it from the gastrointestinal tract, which is the origin of foodborne illnesses.

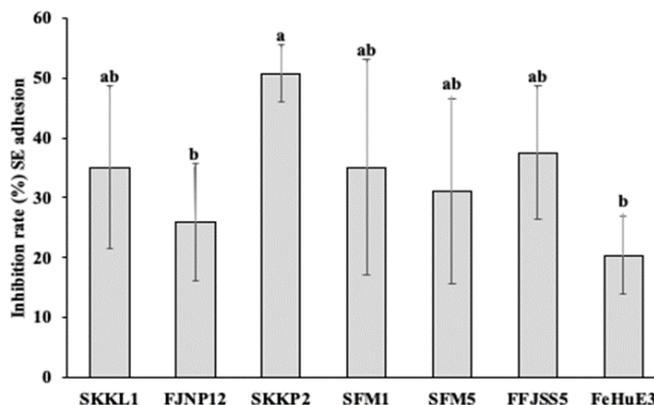


Figure 7. Inhibition rates of selected LAB stains against *S. enterica* ser. Enteritidis on Caco-2 cells. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.1.13 Antiproliferation activity (Cytotoxicity)

The potential actions of cells and cell-free supernatant (CFS) were correlated with the antiproliferation activity of Caco-2 cells by MTT assays. CFSs and cells gave similar results for antiproliferation activity. CFS activities of all strains to inhibit proliferation of Caco-2 cells were found significant ( $P < 0.05$ ) in each active isolate. The observed values ranged from 29.50-43.62% (Figure 8) with a low cytotoxicity. The highest antiproliferation activity was exhibited by *L. plantarum* SKKL1 (43.62%) followed by *W. cibaria* SFM5 (39.56%), *L. curvatus* FJNP12 (37.22%), *P. pentosaceus* SFM1 (36.03%), *W. halotolerans* FFJSS5 (36.00%) and *Enterococcus* sp. FeHuE3 (33.10%) ( $P < 0.05$ ). *L. pentosus* SKKP2 showed the lowest rate of cytotoxicity, 29.50%. Alternatively, no significant ( $P > 0.05$ ) antiproliferation activity was observed in cells of LAB strains. The observed values ranged from 23.60-33.15%. Higher rates were exhibited by *L. plantarum* SKKL1 (33.15%), followed by *W. cibaria* SFM5 (28.20%), *L. curvatus* FJNP12 (27.93%), *W. halotolerans* FFJSS5 (27.57%), *P. pentosaceus* SFM1 (26.04%), *Enterococcus* sp. FeHuE3 (25.41%) and *L. pentosus* SKKP2 (23.60%). The implication is that LAB strains may have anticancer actions, such as the antiproliferation of colon cancer cells. This may be important in interrupting the process of colon cancer cell formation. These probiotic LAB strains might be useful in colon cancer prevention and treatment. The mechanism for proliferative inhibition of colon cancer cells may be induced when probiotic LAB adhere

directly to these colon cancer cells and/or induce bioproduction of antimicrobial substances such as bacteriocins, reuterin, hydrogen peroxide, organic acids, and biosurfactants (Thirabunyanon and Thongwittaya, 2012; Thirabunyanon and Hongwittayakorn, 2013). This growth inhibition of enteric pathogens suggests that these LAB isolates might have a protective role against the initiation of colon cancer.

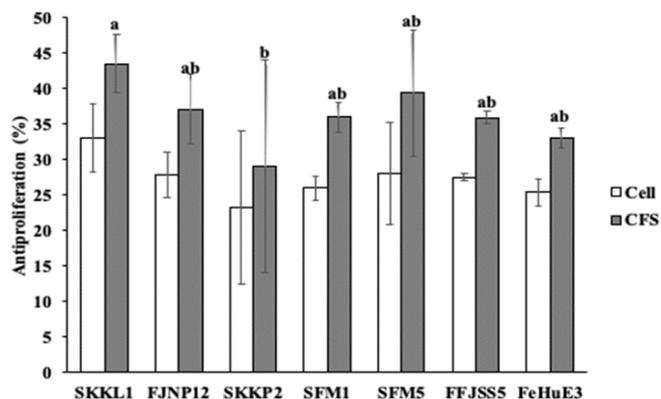


Figure 8. Antiproliferation of selected LAB strains. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

### 3.2 Selection of Gamma-aminobutyric acid-producing lactic acid bacteria

#### 3.2.1 Gamma-aminobutyric acid production by selected lactic acid bacteria

Only three LAB strains showed clear GABA production based on the TLC chromatogram results. They included *L. plantarum* SKKL1, *L. curvatus* FJNP12 and *W. cibaria* SFM5 (Figure 9). The cell-free supernatants of *P. pentosaceus* SFM1, *W. halotolerans* FFJSS5, *L. pentosus* SKKP2, and *Enterococcus* sp. FeHuE3 strains did not show clear chromatograms indicating the presence of GABA. The amount of GABA after cultivation of LAB strains in MRS broth supplement with 5% (w/v) MSG detected by HPLC was statistically significant ( $P < 0.05$ ). The results of GABA production by selected LAB strains are shown in Figure 10. *L. curvatus* FJNP12 was the best GABA producer (15.66 g/L) followed by *L. plantarum* SKKL1 (11.76 g/L) and *W. cibaria* SFM5 (5.62 g/L). The four poorest GABA-producing strains produced this substance in concentrations ranging from 0.30-0.99 g/L including *Enterococcus* sp. FeHuE3 (0.99 g/L), *L. pentosus* SKKP2 (0.41 g/L), *W. halotolerans* FFJSS5 (0.38 g/L), and *P. pentosaceus* SFM1 (0.30 g/L). These results were similar to those of other research groups (Park and Oh, 2007; Kim et al., 2009; Ratanaburee et al., 2013a), who previously reported that *Lactobacillus* and *Lactococcus* spp. produced high amounts of GABA. Since

extracellular GABA production was observed in LAB culture broth, it may be possible to use these GABA-producing LAB as starter cultures in fermented foods. All GABA-producing LAB strains can potentially be used as starter cultures.

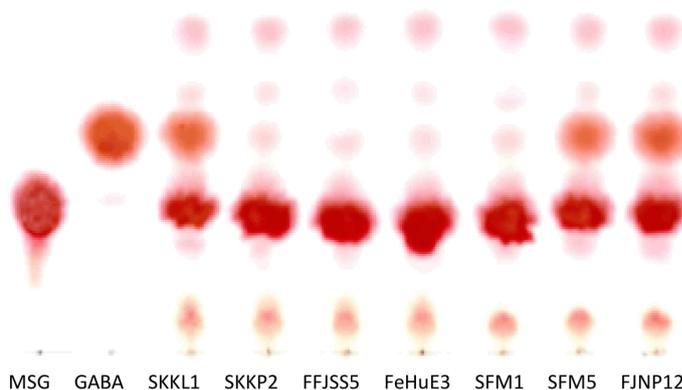


Figure 9. TLC chromatogram of GABA production by selected LAB. (Lanes: 1, MSG; 2, GABA standard; 3-9, LAB isolates tested; *L. plantarum* SKKL1, *L. curvatus* FJNP12, *L. pentosus* SKKP2, *P. pentosaceus* SFM1, *W. cibaria* SFM5, *W. halotolerans* FFJSS5 and *Enterococcus* sp. FeHuE3)

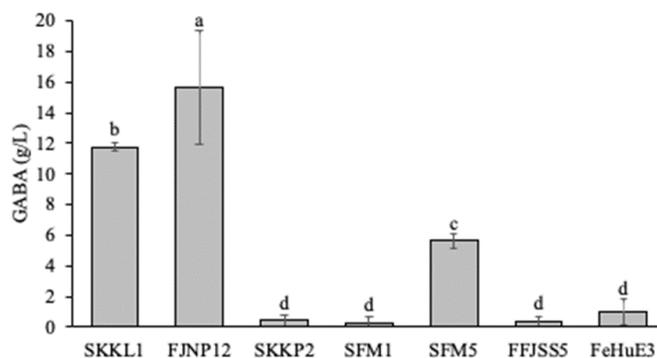


Figure 10. The amount of GABA production by isolated LAB in MRS broth containing 5% (w/v) MSG by HPLC. Bars with different alphabet notations are significantly different at  $P < 0.05$ . SKKL1 = *L. plantarum* SKKL1, FJNP12 = *L. curvatus* FJNP12, SKKP2 = *L. pentosus* SKKP2, SFM1 = *P. pentosaceus* SFM1, SFM5 = *W. cibaria* SFM5, SSJSS5 = *W. halotolerans* FFJSS5 and FeHuE3 = *Enterococcus* sp. FeHuE3

#### 3.2.2 Nucleotide and deduced amino acid sequences of *gadB*

*Lactobacillus plantarum* SKKL1 and *L. curvatus* FJNP12, which produced high amounts of GABA, were selected to further study the *gadB* gene. *gadB* genes in LAB strains have been previously reported (Park et al., 2014). The *gadB* gene was only found in *L. plantarum* SKKL1. Sequence analysis revealed that a cloned fragment of the *gadB* gene from *L. plantarum* SKKL1 contained a complete open reading frame (ORF) of 1,407 nucleotides, encoding a protein of 469 amino acids (Figure 11) with a predicted molecular weight of 53.56 kDa and an isoelectric point (pI) of 5.58, as estimated using the ExPASy program. Moreover, the deduced amino acid sequence corresponding to the *L. plantarum* SKKL1 *gad* gene belongs to the *gadB* family of

pyridoxal dependent decarboxylases. Alignment of the deduced amino acid sequence with other GAD sequences revealed a highly conserved catalytic domain that belonged to the pyridoxal 5-phosphate (PLP)-dependent decarboxylase superfamily. The deduced amino acid sequence of the *gadB* gene from *L. plantarum* SKKL1 revealed a conserved lysine residue (Lysine280) that is known to be crucial for the binding of PLP, as well as the proposed active site residues (Threonine215 and Aspartate247) that promote decarboxylation. Additionally, the motif HVDAASGG, which is highly conserved in PLP-dependent decarboxylases, was found in the *L. plantarum* SKKL1 *gadB* gene sequence (Figure 11).

1	ATGCCAATGTTATACGTTAAACCAATCATGAAGCTGAAGATACTTGGAAACAGCTTTTGGTGGGCTTCTGAACACATGATCTTCCT
1M	A M L Y G K H N H E A E E Y L E P V F G A P S E Q H D L P
91	AAGTATCGGTACCAAGCAATTCATTAATCCCTCGAAGACCGATCGCTTGTAGTATGATGATTAATAGATGAAGGCAATCACGACTG
31K	Y R L P K H S L S P R E A D R L V R D E L L D E G N S R L
181	AACCTGCGACCTTTTGTGACACTATATGGAACCGAAGCGTIGAAITGATGAAGGATACGCTGGCTAAGAATGCCATCGACAAATCT
61N	L A T F C Q T Y M E P E A V E L M K D T L A K N A I D K S
271	GAGTACCCCGCAGCGGAGATTGAAAATCGTGTGTGACCAITATGCCAATCTGTGGCAGCGCATGATGACGAACACTTACCGGT
91E	Y P R T A E I E N R C V N I I A N L W H A P D D E H F T G
361	ACCTCTAGGATGGCTCCTCTGAAGCTTGTATGTTAGCGGTTTAGCAATGAATTCGCTGGCTAAGCGCTCAAGCGCGCAGGTTTA
121T	S T I G S S E A C M L G G L A M K F A W R K R A Q A A G L
451	GATCTGAATGCCATCGACCTAACCTCGTATTTTCGGCTGGCTATCAAGTTTGTCTGGGAAAAGTTTGTGTCTACTGGGACGTTGACATG
151D	L N A H R P N L V I S A G Y Q V C W E K F C V Y W D V D M
541	CACGTGGTCCCAATGATGAGCAACACATGGCCCTTGCAGTTAACCAGCTTCTAGACTAGCTGGCAATACACAATTTGATCGTGGT
181H	V P M D E Q H M A L D V N H V L D Y V D E Y T I G I V G
631	ATCATGGGATCACTTATACCGGTCAATATGACGACTAGCGCGCTGATTAAGTGTCTACTCACTCAATCATCAGCATCCCAATTA
211I	M G I (Y) T G Q Y D D L A A L D K V V T H Y N H Q H P K L
721	CCAGTCTACATTCAGCTCGACCGCAGCTGCTTATACCGCTTATGAGCGCACTCATCTGGGACTTCGGTTGGCTAAC
241P	V Y I (H V D) A A S G G (F) Y T P F I E P Q L I W D F R L A N
811	GTGCTTTCGATCAAGCTCCGSSCAGCAAGTTCAGTTTATCCCGSSGTCGGCTSSGTTGGCTGGTATGCTGATTTTACCG
271V	(S I N A S S H K) Y G L V Y P S V G N V V N R (D) R Q F L P
901	CCAGAAATAGCTTCAAAGTTAGTATTAATAGTGGGAGTGGCGAATCACTTCTCACTAGTGGCAGCCAGCTCATTGGA
301P	E L V F K V S Y L G G E L P T M A I N F S H S A A Q L I G
991	CAATACTATAATTTCAITCGCTTTGGTATGACGCGTTCACCGGAGAITCAACCAAGACTCACGATGTTGGCCGCTACTGGCAGCGCT
331Q	Y Y N F I R F G M D G Y R E I Q T K T H D V A R Y L A A A
1081	CTGGAATAAGTGGTGAATTAAGATGATCAATAAGCGACCAACTCCCTGATTTGTTAACAACCTAGCTGGCGAAGATCGTGAA
361L	D K V G E F K M I N N G H Q L P L I C Y Q L A S R E D R E
1171	TGGACCTTTATGATTTATCGGCTTATTAATGAACGTTGGCAATACCAAGCTATCTTACTGCTAATCTGGAACCAACGACT
391W	T L Y D L S D R L L M N G W Q V P T Y P L P A N L E Q Q V
1261	ATCCACAGATCGTGGTGGCTGACTTGGCATGAATATGGCCAGGATTCATGGATGACCTGACCAAGGCTGTCCATGACTTAAC
421I	Q R I V V R A D F G M N M A H D F M D D L T K A V H D L N
1351	CAAGCCCACTTGTCTATCATCATGACGCGGCACTAAGAATAACGGATTACACAC
451Q	A H I V Y H H D A A P K K Y G F T H

Figure 11. Nucleotide and deduced amino acid sequence of *gadB* gene from *L. plantarum* SKKL1. The box represents the amino acid residues HVDAASGG motif, which is highly conserved in PLP-dependent decarboxylases. The presumed catalytic amino acid residues are Threonine215, Aspartate247 and Lysine280. The amino acid residues in the dashes box are the PLP binding domain.

Lin et al. (2013) and Sanchart et al. (2016) reported that GABA synthesis was related to bacterial GAD, which plays a major role in the acid-resistance mechanism. Cytoplasmic decarboxylation resulted in the consumption of an intracellular proton after the uptake of glutamate by its specific transporter. The reaction product, GABA, was exported from cells by an antiporter, and the net result was an increase in the pH of the cytoplasm, owing to the removal of hydrogen ions, and a slight increase in the extracellular pH. This was due to the exchange of extracellular glutamate for the more alkaline GABA (Cho et al., 2014). Of the three acid resistance systems known in *E. coli*, the *gad* system is by far the most effective in conferring acid resistance to bacteria in the stationary phase. It gives them a

survival capacity for at least 2 hrs in a strongly acidic environment (pH<2.5), such as that of the stomach (Capitani et al., 2003). It is hence conceivable that the *gad* system plays a similar role in lactobacilli. The presence of *gad* genes in LAB has been shown in several previous reports (Park and Oh, 2007; Fan et al., 2012; Sanchart et al., 2016). Its presence suggests an increased ability to produce GABA, since GAD must be active to convert glutamate into GABA. We cloned a DNA fragment from *L. plantarum* using primers for conserved regions of published *gad* genes. These results might provide useful information for the potential expression of a *Lactobacillus gadB* gene. The *gadB* gene characterization of *L. plantarum* SKKL1 should be useful in future work with this enzyme.

#### 4. Conclusion

A total of seven LAB strains of various species were selected to determine their probiotic properties and production of the bioactive compound, GABA. They should be considered for further use as novel probiotic starters, either alone or in combination for food or feed production. All selected LAB strains studied contained probiotic properties such as inhibiting the growth of pathogenic bacteria, tolerance to GI tract properties, bile salt hydrolase (BSH) activities, none of haemolysis and adhesion to Caco-2 cells. Additionally, three strains, *L. curvatus* FJNP12, *L. plantarum* SKKL1 and *W. cibaria* SFM5, showed clear GABA production measured using a TLC method. Consequently, HPLC indicated that these strains produce high amounts of GABA, 15.66, 11.76 and 5.62 g/L, respectively. One of these strains, *L. plantarum* SKKL1, contained *gadB* gene. The ORF of the GAD gene was 1,407 bp in length, encoding a polypeptide of 469 amino acids with a predicted molecular weight of 53.56 kDa and an isoelectric point (pI) of 5.58. All selected LAB strains were found to have probiotic properties. Three strains produced high amounts of GABA and only one lactobacillus, which contained the *gadB* gene, would be further used as a starter culture for value-added agricultural products. The use of these three strains as probiotics in food is under investigation.

#### Conflict of interest

The authors declare no conflict of interest.

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