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ORIGINAL ARTICLE

Selection and evaluation of functional characteristics of autochthonous lactic acid bacteria isolated from traditional fermented stinky bean (*Sataw-Dong*)

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Abstract The aim of this study was to evaluate the technological and functional potential of lactic acid bacteria (LAB) isolated from fermented stinky bean (*Sataw-Dong*). Of the 114 LAB colonies isolated from spontaneously fermented stinky bean which showed inhibitory activity against two food-borne pathogens (*Staphylococcus aureus* DMST 4480 and *Escherichia coli* DMST 4212), the five isolates (KJ03, KJ15, KJ17, KJ22, KJ23) exhibiting excellent antagonistic activity were subjected to further study. These five strains showed titratable acidity as lactic acid in the range of 1.47–1.55 %, with strains KJ03 and KJ23 additionally exhibiting a high NaCl tolerance of >7 % (w/v). Using 16S rRNA gene sequence analysis, strains KJ03 and KJ23 were identified as *Lactobacillus plantarum* and *L. fermentum*, respectively, and further investigated for their functional properties in vitro. Both strains survived well in a simulated gastrointestinal tract environment with <1 log cell decrease over 8 h (>8 log CFU/ml). *Lactobacillus plantarum* KJ03 showed the best performance with respect to cholesterol removal (53 %), while *L. fermentum* KJ23 showed the highest cell-surface hydrophobicity (39.5 %). Neither of the two strains showed any hemolysis activity. Both strains hydrolyzed glycodeoxycholic and taurodeoxycholic acids. In terms of antibiotic susceptibility, *L. fermentum* KJ23 was not sensitive to tetracycline. Taking all of the results into account, *L. plantarum* KJ03 possessed desirable in vitro functional properties. This strain is therefore a good candidate for further investigation for use in *Sataw-Dong* fermentation to assess its technological performance as a potential probiotic starter.

Keywords Lactic acid bacteria · *Lactobacillus fermentum* · *Lactobacillus plantarum* · Probiotic · *Sataw-Dong*

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Introduction

Fermented foods are an important component of the human diet in many countries, especially at the household level. One of the advantages of fermentation is that it is an inexpensive process with the potential to preserve food, improve nutritional value, and enhance aroma and taste (Aloys and Angeline 2009). In addition, indigenous fermented foods are strongly linked to the culture and tradition of the country of their origin (Guo et al. 2015).

Foods which contain probiotics represent a group of health-promoting, functional foods of important commercial interest, and their share of the market is steadily increasing (Vuyst et al. 2008). Most probiotic bacteria belong to lactic acid bacteria (LAB) groups, especially the species of the genus *Lactobacillus*, which is one of the most fundamental of microbial groups. These LAB have been introduced into several of fermented food products. Many studies have reported that dairy products are the most commonly used food vehicles for the delivery of probiotics (Rubio et al. 2014). However, there has been a growing interest in developing non-dairy probiotics, and it is known that some traditional fermented foods may constitute a suitable base for the development of probiotic-type functional foods (Ruiz-Moyano et al. 2009). The potential to develop non-dairy probiotic products has

attracted the interest of individuals with lactose intolerance and consumers with cholesterol-restricted diets (Granato et al. 2010). Moreover, traditional fermented foods are a plentiful source of microorganisms, and some of them show probiotic properties (Rivera-Espinoza and Gallardo-Navorra 2010). Thus, non-dairy products made from fruits, vegetables, and cereals would appear to have a promising future (Martins et al. 2013).

Stink bean (*Parkia speciose*), a Southeast Asian plant of the genus *Parkia* in the family Fabaceae, grows wild in tropical forests and is often cultivated in Southern Thailand. The beans are flattened and elliptical in shape with a nutty and firm texture. They are believed to contain medicinal compounds which exhibit potential biological activity, such as antibacterial (Sakunpak and Panichayupakaranant 2012), antiangiogenic (Aisha et al. 2012), anticancer (Ali et al. 2006), antioxidant (Aisha et al. 2012) and hypoglycemic activities (Jamaluddin and Mohamad 1993). Stink beans preserved in brine for several days undergo fermentation, with the production of lactic acid; the fermentation product is called fermented stink bean or *Sataw-Dong* (in the Thai language) and consumed as a raw pickle. While there are many similar LA fermentation products, to date cucumber, cabbage and olives are the only vegetables that are fermented in large volumes for human consumption (Montet et al. 2006).

However, all natural fermentation processes have a common problem in that they are relatively uncontrollable, resulting in variations in product stability and quality attributes (Parkouda et al. 2010). It has been reported that these problems can be overcome by using a starter culture, controlling the microflora, accelerating the ripening time, inhibiting the growth of pathogenic and spoilage bacteria and alleviating variations in organoleptic quality problems in fermented foods, thereby improving the overall quality of fermented vegetable products (Font de Valdez et al. 1990; Caplice and Fitzgerald 1999).

The aim of the study reported here was to: (1) characterize LAB isolated from traditionally fermented stink bean; (2) select the most suitable strains according to their technological characteristics, including antagonistic activity against foodborne pathogens; (3) investigate a number of the functional properties the isolates may have.

Materials and Methods

Sataw-Dong preparation

Sataw-Dong was prepared according to the traditional recipe. Whole pods were blanched and added to a brine prepared by boiling clean water containing 3–4 % NaCl, 5 % sugars, and 0.5 % Malabar tamarind (*Garcinia cambogia*). The beans together with the brine were transferred to 12 glass jars (100 g), which were covered with lids and allowed to ferment spontaneously at room temperature (27–32 °C) for 10 days. Samples obtained at 0, 1, 2, 4, 6, 8, and 10 days of fermentation were used to isolate LAB.

Isolation of LAB showing antagonistic activity against foodborne pathogens

Samples (25 g) of *Sataw-Dong* were added to 0.1 % (w/v) peptone water (225 ml) and placed in a stomacher for 2 min. Ten-fold serial dilutions were made in peptone water, and the appropriate dilutions were spread onto MRS agar (Lab M Ltd., Heywoord, UK) supplemented with bromocresol purple (0.02 %, w/v), and NaCl (3 %, w/v), and the plates were incubated at 37 °C for 24 h for the isolation of presumptive LAB. After 24 h, the plates of LAB were individually overlaid with Tryptone Soy Agar (TSA; Hi Media Laboratories, Mumbai, India) (0.75 % agar, w/v) seeded with *Staphylococcus aureus* DMST 8840 and *Escherichia coli* DMST 4212 [approx. 10^6 colony-forming units (CFU)/ml]. The plates were then incubated at 37 °C for 24 h. Inhibition zones were detected by a clear zone around the tested strains (Schillinger and Lücke 1989).

Bacterial colonies exhibiting an inhibition zone were individually picked and streaked on MRS agar two to three times to purify the isolates. All Gram-positive, catalase-negative isolates were defined as LAB (Hwanhlem et al. 2011), and these isolates were maintained in MRS broth containing glycerol (60 %) at −20 °C. For routine analysis, strains were subcultured twice in MRS broth at 37 °C for 24 h.

Confirmation of the antagonistic activity

Antagonistic activity against bacterial indicators was investigated using the agar spot test and agar well diffusion assay as described by Jones et al. (2008) and Schillinger and Lücke (1989), respectively. The agar spot test experiment was conducted by spotting each overnight LAB culture (5 μl) onto the surface of a MRS agar plate and incubating the plate at 37 °C for 24 h. These plates were then overlaid with 10 ml of TSA (0.75 % agar, w/v) seeded with 100 μl of each tested indicator strain. After an overnight incubation at 37 °C, the plates were examined for zones of inhibition.

The agar well diffusion assay was performed to characterize the type of antimicrobial compounds. Cell-free supernatants were collected by centrifugation (8000 *g*, 10 min, 4 °C) from overnight cultures. Two samples of supernatant taken from each strain were used in trials as follows: (1) the pH was adjusted to 6.5 and the supernatant heated at 90 °C for 10 min, and (2) the supernatant without adjustment was used as control. Sterile culture plates containing 20 ml TSB and 1.0 % (w/v) agar were then prepared and subsequently seeded with each bacterial indicator. Wells (diameter 7 mm) were

punched into the agar layer, and cell-free supernatants (50 μl) were placed into each well; the plates were then incubated at 37 °C for 24 h. The inhibition activity of each supernatant was assessed based on the diameter of the inhibition zone.

Determination of pH value and total titratable acidity

All LAB cultures were diluted to an absorbance of 0.5 (10^6 CFU/ml). Each LAB suspension (1 %) was inoculated into MRS broth and the cultures incubated at 37 °C for 24 h, following which aliquots (20 ml) were taken for the measurements. The pH was directly measured using a pH meter (OHAUS, Shanghai, China). A cell-free supernatant was used to determine the total titratable activity by titration against 0.1 N NaOH using phenolphthalein (0.1 % w/v in 95 % ethanol) as an indicator.

Determination of NaCl tolerance of LAB

The NaCl concentration in MRS broth was adjusted to 0, 3, 5, 7, 9, and 12 % (w/v), following which 1 % (v/v) of active LAB was inoculated into MRS broth containing the different concentrations of NaCl and cultivated at 37 °C for 24 h. The survival of LAB was determined by the drop plate method on MRS agar and reported as colony-forming units per milliliter.

Molecular identification and sequencing of 16S rRNA gene of selected LAB

DNA of the selected LAB was extracted using an extraction kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol and stored at −20 °C. 16S rRNA gene amplification was performed in a thermocycler (Techne, Abingdon, UK) with the following universal primers: 27 F (5′-AGAG TTTGATCCTGG CTCAG-3′) and 1492R (5′-GGTT ACCTTGTTACGACTT-3′) according to the protocol described by Cai et al. (1999). The PCR products were purified using a PCR purification kit (QIAgen) and sequenced (Ward Medic Ltd., Selangor, Malaysia). The basic local alignment search tool (BLAST) was used to compare the obtained sequences against the public data library of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The nucleotide sequences of all the bacterial isolates were deposited in the GenBank database (accession numbers LC016617 and LC037354).

Determination of functional properties in vitro

Resistance to lysozyme Lysozyme resistance to assess the in vitro ability of the strains to survive transit through the oral cavity was performed as described by Turchi et al. (2013), with slight modifications. LAB grown overnight were harvested by centrifugation (8000 *g*, 10 min) and resuspended in 2 ml of phosphate buffer saline (PBS) in the presence of lysozyme (100 mg/L) (Sigma-Aldrich, St. Louis, MO). Bacterial suspensions without lysozyme were used as controls. Samples were incubated at 37 °C, and viable cell counts after 10 and 20 min were enumerated on MRS agar by the drop plate method. Survival rates were calculated as a percentage of growth.

Survival of LAB under simulated gastric and intestinal juices Cells of the LAB isolates were collected by centrifugation (8000 *g*, 10 min) and washed twice with PBS before being resuspended in PBS solution at pH 2.5 containing pepsin (3 mg/ml; Sigma-Aldrich) as simulated gastric juice (Maragkoudakis et al. 2006). Viable counts were carried out after incubation at 37 °C for 2 h. After this step, the cultures were centrifuged and washed twice with PBS solution before being transferred to PBS solution, pH 8.0, containing pancreatin (1 mg/ml) and ox bile salts (3 mg/ml) (Sigma-Aldrich) as simulated intestinal fluid. The samples were incubated at 37 °C in for 6 h. Survival of LAB was enumerated after 3 h and 6 h of incubation.

Effect of micro-aerobic and anaerobic conditions on growth of LAB LAB strains (1 %, v/v) were inoculated into MRS broth (micro-aerobic condition) and MRS broth supplemented with L-cysteine (0.5 mg/ml) covered with liquid paraffin (anaerobic condition) (Talwalkar et al. 2001). The samples were incubated at 37 °C for 24 h. For micro-aerobic condition, cells were enumerated on MRS agar and incubated at 37 °C for 24 h. On the other hand, cells were dropped on MRS agar supplemented with L-cysteine and overlaid with agar, followed by incubation at 37 °C for 24 h in an anaerobic jar.

Cell-surface hydrophobicity assay Cell-surface hydrophobicity was determined by a microbial adhesion to hydrocarbon test as described by Otero et al. (2004). LAB were grown in MRS broth at 37 °C for 24 h and harvested by centrifugation (8,000 *g*, 10 min). The pellets were washed twice with PBS (pH 7.4) and resuspended in the same buffer to an absorbance of 0.8–1.0 at 600 nm (OD_{600}). Equal volumes of cell suspension and *n*-hexadecane were mixed in duplicate and vortexed thoroughly for 2 min. The tubes were allowed to separate into two phases for 30 min. The aqueous phase was then measured in a spectrophotometer at 600 nm. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (% H).

Blood hemolytic activity Fresh overnight LAB cultures were streaked in triplicate on TSA plates supplemented with 5 % (v/v) human blood (obtained from Songklanagarind Hospital, Songkhla, Thailand) and incubated at 37 °C for 48 h.

Hemolytic activities of the bacterial culture were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green zones around colonies), or γ-haemolysis (no clear zones around colonies) (Hargrove and Alford 1978).

Bile salt hydrolase activity Qualitative bile salt hydrolase (BSH) activity was evaluated as described by Wang et al. (2012). The MRS agar plate was prepared by adding 0.5 % (w/v) of bile salt (Sigma-Aldrich) as follows: glycocholic acid, taurocholic acid, glycodeoxycholic acid (GDC) and taurodeoxycholic acids (TDC). Overnight cultures of each LAB strain (10 μl) were spotted onto the agar plates and incubated anaerobically at 37 °C for 24–72 h. The presence of precipitated bile acid around the colonies (opaque halo) was considered to be a positive result. MRS agar plate without supplemented bile acid was used as control.

Cholesterol-lowering property The cholesterol-lowering property was determined according to Wang et al. (2014) with some modification. The MRS broth was supplemented with 0.3 % (w/v) Ox gall (Hi-Media). Water-soluble cholesterol (polyoxyethylene cholesteryl sebacate; Sigma-Aldrich) was filter-sterilized and added into the broth at a final concentration of 100 μg/ml, inoculated with each LAB strain, and incubated at 37 °C for 24 h. After the incubation, cells were removed by centrifugation, and residual cholesterol concentration in the broth was determined using a modified colorimetric method. Briefly, 1 ml of the broth was added to 1 ml of KOH (33 %, w/v) and 2 ml of absolute ethanol, mixed for 1 min, then heated at 70 °C for 30 min. After cooling, 5 ml of hexane was added to the tube and the tube vortexed for 1 min. The hexane layer (4 ml) was transferred to a clean glass tube and evaporated. The residue was dissolved in 2 ml of *O*-phthalaldehyde reagent. After mixing, 1 ml of concentrated sulfuric acid was added and the mixture vortexed for 1 min. Absorbance was read at 540 nm. All experiments were performed in duplicate.

Determination of antibiotic susceptibility The LAB strains were tested for resistance to antibiotics by the broth microdilution method. The minimum inhibitory concentration (MIC) values were determined in the LAB susceptibility test medium (LSM) broth formulation described by Federici et al. (2014) using ampicillin, vancomycin, chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline, clindamycin, and ciprofloxacin at concentrations of 0.065–1024 mg/L. The individual inoculum was adjusted to an absorbance of 0.2–0.3 (600 nm), equivalent to 10^5 CFU/ml. In brief, 100 μl of bacterial suspension was mixed with the antibiotic solution (100 μl) to be tested in 96-well plates and incubated overnight at 37 °C. Growth was determined visually after incubation. Susceptible and resistant strains were distinguished according to the breakpoints (cutoff values) reported by European Food Safety Authority (2012). Accordingly, strains showing MICs higher than the respective cutoff values were considered to be resistant.

Detection of the presence of virulence genes To evaluate the safety of the selected LAB for their application in fermentation processes, we performed PCR assays to detect the presence of genes encoding potential virulence genes using genomic DNA obtained as described in section Molecular identification and sequencing of 16S rRNA gene of selected LAB. The specific primers are described in Table 1 (Vankerckhoven

Table 1 PCR primers and products for the detection of virulence determinants

Target genes[a]	Sequence (5′ to 3′)	T_m (°C)	Product size (bp)	References
ace	5′-GAATTGAGCAAAAGTTCAATCG-3′ 5′-GTCTGTCTTTTCACTTGTTTC-3′	56	1008	Ben Omar et al. (2004)
*asa*1	5′-GCACGCTATTACGAACTATGA-3′ 5′-TAAGAAAGAACATCACCACGA-3′	56	375	Vankerckhoven et al. (2004)
*cyl*A	5′-ACTCGGGGATTGATAGGC-3′ 5′-GCTGCTAAAGCTGCGCTT-3′	58	688	Vankerckhoven et al. (2004)
*cly*B	5′-AAGTACACTAGTAGAACTAAGGGA-3′ 5′-ACAGTGAACGATATAACTCGCTATT-3′	52	843	Semedo et al. (2003)
efaAfs	5′- GACAGACCCTCACGAATA-3′ 5′- AGTTCATCATGCTGTAGTA −3′	54	705	Eaton and Gasson (2001)
esp	5′-AGATTTCATCTTTGATTCTTGG-3′ 5′-AATTGATTCTTTAGCATCTGG-3′	56	510	Vankerckhoven et al. (2004)
*gel*E	5′-ACCCCGTATCATTGGTTT-3′ 5′-ACGCATTGCTTTTCCATC-3′	52	419	Eaton and Gasson (2001)

T_m, Melting temperature

[a] *asa*1, Aggregation substance gene; *Cyl*A/B, cytolysin A/B genes; *efaAfs*, cell-wall adhesion gene; *esp*, enterococcal surface protein gene; *gel*E, gelatinase gene

et al. 2004). The target genes were *ace* (adhesin of collagen protein), *asa*1 (aggregation substance), *Cyl*A/B (cytolysins), *efaAfs* (cell-wall adhesion), *esp* (enterococcal surface protein) and *gel*E (gelatinase). Of the strains tested, *Enterococcus feacalis* Van B tested positive for the *ace*, *asa*1, *Cyl*A/B, *efaAfs*, and *gelE* genes and *Enterococcus feacalis* 13–5 tested positive for the *esp* gene.

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance with Duncan's post hoc test. Significance was set at $p < 0.05$. All data are expressed as the mean ± standard deviation.

Results and Discussion

Isolation of LAB with primary antagonistic activity

Lactic acid bacteria originally isolated from vegetables are probably the most suitable candidates for improving the microbiological safety of fermented vegetable products because they are well-adapted to the conditions of the matrices used in the fermentation process and should therefore be more competitive than LAB obtained from other sources (Ponce et al. 2008). A total of 114 colonies which showed clear zones against *S. aureus* DMST 8840 and *E. coli* DMST 4212. All of those isolates were identified as LAB based on two criteria: Gram-positive and catalase-negative (data not shown).

Screening for antagonistic activity

Antagonistic activity of LAB strains may contribute to the improvement in the quality of fermented foods, which is achieved through the control of spoilage and pathogenic bacteria, thus extending shelf-life and improving sensory quality (Begonović et al. 2014). All 114 LAB strains identified were tested for antagonistic activity against *Staphylococcus aureus* DMST 8840 and *Escherichia coli* DMST 4212 according to the safety criteria of Thai Community Product Standard (317/2004). However, only five strains (KJ03, KJ15, KJ17, KJ22, KJ23) showed excellent inhibition zones (diameter > 15 mm), with strain KJ03 having the highest inhibition zone (Table 2).

Those five LAB strains were subsequently analyzed for their production of antibacterial compounds by the agar well diffusion method. Supernatants obtained from the five strains did not exhibit the inhibition zones after pH adjustment to 6.5 (data not shown), suggesting that the antimicrobial activity of these five LAB strains may come from organic acids. In fact, the drop in pH arising from the production of lactic acid can be sufficient to inhibit certain bacteria as the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell which causes a collapse in the electrochemical proton gradient in sensitive bacteria, thereby resulting in a bacteriostatic or bactericidal effect (González et al. 2007). The antibacterial activity of LAB strains selected from vegetables has been confirmed in several previous studies; i.e., from fermented cucumber, organic leafy vegetables (Ponce et al. 2008) and from fermented Himalayan vegetables (Dewan and Tamang 2007).

Lactic acid production and pH reduction ability

Lactic acid produced by LAB is an essential compound for food preservation because it maintains the acidity conditions of the fermented foods and it is antagonistic against food spoilage and poisoning bacteria (Hwanhlem et al. 2011). LAB ferment sugars via a number of different pathways, resulting in homo-, or mixed acid fermentation. Homofermentation produces lactic acid as the sole end product of glucose metabolism when the Embden–Meyerhof–Parnas pathway is used (Hofvendahl and Hahn–Hägerdal 2000).

We therefore measured lactic acid production and pH reduction in these five LAB isolates (KJ03, KJ15, KJ17, KJ22,

Table 2 Inhibition zone diameter of lactic acid bacteria isolated from *Sataw-Dong* on agar in the presence of indicator bacterial species *Staphylococcus aureus* DMST8840 and *Escherichia coli* DMST4212

LAB isolate no.	Inhibition zone (mm)	
	Staphylococcus aureus DMST8840	*Escherichia coli* DMST4212
KJ03	29.7 ± 0.6[a]	23.0 ± 1.0
KJ15	26.7 ± 0.6	17.7 ± 1.2
KJ17	27.7 ± 0.5	18.3 ± 0.6
KJ22	25.3 ± 0.7	19.0 ± 1.0
KJ23	29.3 ± 1.2	21.7 ± 0.6

LAB, Lactic acid bacteria

[a] Data are presented as the mean ±standard deviation (SD) from triplicate determinations

KJ23) exhibiting excellent antagonistic activity against *S. aureus* DMST 8840 and *E. coli* DMST 4212. After 24 h of incubation, strain KJ23 was the highest producer of lactic acid at 1.55 % (in % equivalents lactic acid), and strain KJ15 was the lowest lactic acid producer at 1.47 %. The remaining three strains showed no significant lactic acid production, with the exception of strain KJ15. The pH values were correlated with these results in the range of 3.86–4.00 (Table 3).

Effect of NaCl on growth of isolates

The cell viability of the five LAB isolates after incubation in MRS broth supplemented with NaCl is shown in Fig. 1 reports. NaCl supplemented at a concentration of 3–5 % did not significantly affect the growth of these LAB strains as they grew over 9 log CFU/ml. In addition, the growth of three isolates (KJ03, KJ22, KJ23) appeared to be slightly stimulated with the addition of 3 % NaCl to the incubation broth. As predicted, increasing NaCl concentration had a negative effect on the growth of all isolates, with a subsequent decrease in cell viability (Fig. 1). In particular, LAB viability was strongly affected by 7 % NaCl. Isolates KJ03 and KJ23 best tolerated the highest NaCl concentrations (>8 log CFU/ml), and we therefore selected these two strains for further investigations. In MRS broth supplemented with 9–12 % NaCl, these two LAB strains were not able to grow throughout the 24-h incubation period, although cell counts remained constant (approx. 5–6 log CFU/ml) for the entire incubation time. The tolerance to salt concentrations of 2–10 % is a limiting factor affecting the persistence, competitiveness, and metabolism of the starter culture over the entire fermentation due to its water binding and ionic characteristics (Ammor and Mayo 2007). The growth of LAB is sometimes enhanced in the presence of low concentration of NaCl (1–2 %, w/v) (Leroy and de Vuyst 1999). Consequently, a salt tolerance of at least 7 % is considered to be necessary for potential starter cultures in *Sataw-Dong* fermentation because the presence of 5–7 % NaCl is normally found in many recipes of *Sataw-Dong*.

Identification of LAB isolates

The 16S rRNA gene sequences of strains KJ03 and KJ23 were compared with those other bacterial strains in GenBank database and identified and subsequently confirmed as *Lactobacillus plantarum* (99 % similarity) and *L. fermentum* (99 % similarity), respectively. The 16S rRNA gene sequences of strains KJ03 and KJ23 were deposited in the DDBJ/EMBL/Genbank with accession numbers LC016617 and LC037354, respectively. These strains were found to be Gram-positive, non-spore-forming rods; they were catalase-negative and form off-white cream colonies (Table 4). The fermentation of vegetables is mainly carried out by *L. plantarum* and *L. fermentum* strains for improving their nutritional and sensory features, and these strains are also considered to be the best candidates for starters with probiotic properties (Swain et al. 2014).

Functional properties of selected LAB in vitro

An important step towards the selection of potential probiotic LAB is to evaluate their resistance and survival through the human gastrointestinal tract (GIT). *Lactobacillus plantarum* subsp. *plantarum* strain JCM1149, originally isolated from pickled cabbage, is a commercial type strain with probiotic properties (Zago et al. 2011; Nazzaro et al. 2012) and was also used in this study to compare properties among our isolates. The results of our study on the functional properties of our two isolated *Lactobacillus* strains (KJ03 and KJ23) are shown in Table 5.

Resistance to lysozyme The resistance of *L. plantarum* KJ03 and *L. fermentum* KJ23 to lysozyme, expressed as percentage of survival, ranged from a minimum mean value of 99.73 % to a maximum mean value of 100 %. These isolates revealed a high lysozyme resistance after both 10 and 20 min incubation, with a survival rate >98 % (Table 5). Lysozyme present in human saliva is the first challenge to survival. Resistance to

Table 3 pH values and titratable acidity of cell-free supernatants of the five lactic acid bacterial strains isolated from *Sataw-Dong* after 3, 6, 12, and 24 h of incubation

Isolated no.	pH				Titratable acidity (%)			
	3 h	6 h	12 h	24 h	3 h	6 h	12 h	24 h
KJ03	6.93	4.91	3.89	3.86	0.07 ± 0.01^{a}	0.44 ± 0.01^{a}	1.35 ± 0.01^{a}	1.53 ± 0.01^{a}
KJ15	6.96	4.91	3.94	3.88	0.05 ± 0.01^{a}	0.44 ± 0.01^{a}	1.29 ± 0.01^{b}	1.52 ± 0.01^{a}
KJ17	6.93	5.05	3.97	3.90	0.07 ± 0.03^{a}	0.40 ± 0.03^{b}	1.36 ± 0.01^{a}	1.47 ± 0.02^{b}
KJ22	6.90	5.35	4.01	4.00	0.05 ± 0.01^{a}	0.31 ± 0.02^{c}	1.35 ± 0.00^{a}	1.53 ± 0.02^{a}
KJ23	6.94	5.14	3.99	3.97	0.06 ± 0.01^{a}	0.46 ± 0.01^{a}	1.27 ± 0.01^{b}	1.55 ± 0.01^{a}

Data on titratable acidity are presented as the mean ± SD from triplicate determinations. Mean values followed by different superscript lowercase letter in the same column are significantly different at $p \leq 0.05$

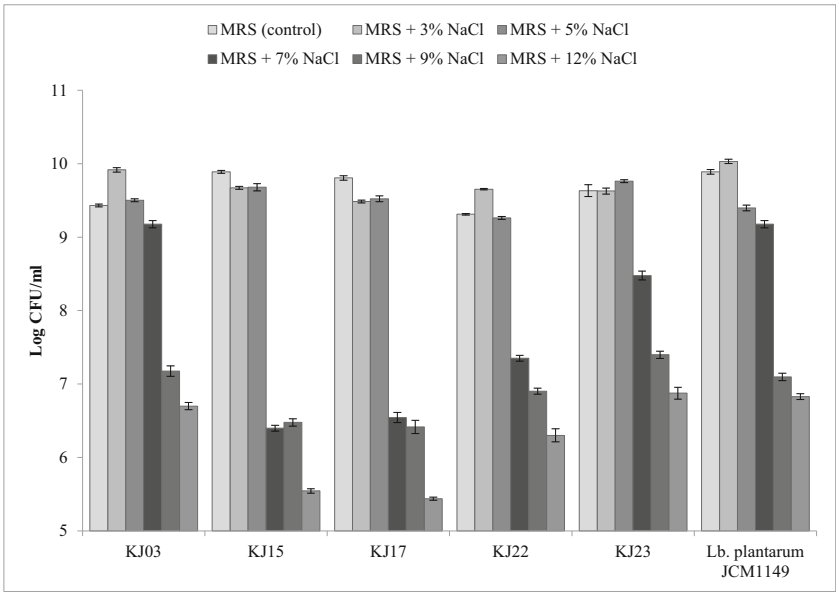

Fig. 1 Viability of lactic acid bacteria isolates KJ03, KJ15, KJ17, KJ22, and KJ23 after cultivation in MRS broth supplemented with various concentrations of NaCl (0–12 %) at 37 °C for up to 24 h. *Lb. Lactobacillus*

lysozyme has been attributed to the peptidoglycan structure in the cell wall, the physiological state of the cell, and lysozyme structure in the medium (Cunningham et al. 1991). This result confirmed the high resistance of the two *Lactobacillus* strains to 100 mg/l of lysozyme under conditions stimulating the in vivo dilution by saliva (Zago et al. 2011).

Survival of LAB under simulated gastric and intestinal juices The behavior of *L. plantarum* KJ03 and *L. fermentum* KJ23 under simulated GIT conditions enables strains to be selected which are likely to survive these conditions, a necessary prerequisite for probiotic cultures. Stresses to microorganisms begin in the mouth, with lysozyme-containing saliva, and continue in the stomach (pH 1.5–3.0) and in the upper intestine which contains bile (Zago et al. 2011). *Lactobacillus plantarum* KJ03 and *L. fermentum* KJ23 survived more than 9 log CFU/ml (>95 %) after their cell suspensions were exposed to the simulated gastric juice. The surviving cells were further tested for bile salt tolerance. *Lactobacillus plantarum* KJ03 exhibited better survival, at >9 log CFU/ml (92.55 %), than *L. fermentum* KJ23 (81.32 %) (Table 5).

The ability to survive the acidic and bile challenges in the GIT is advantageous for probiotics. Acid tolerance of *Lactobacillus* strains was also reported in previous studies. Srinu et al. (2013) revealed that all of the *L. plantarum* and *L. casei* strains they tested showed good survival in the acidic pH range (1.5–3.5) tested. Jamaly et al. (2011) also reported that *L. plantarum* was able to tolerate 3 h of acid exposure (pH. 2.0 and 3.0). Good probiotic bacteria should survive well in a pancreatin solution at pH 8.0 in the presence of bile salts

Table 4 Physiological properties of the five selected lactic acid bacterial strains isolated from *Sataw-Dong*

Property	*Lactobacillus plantarum* KJ03	*Lactobacillus fementum* KJ23	*Lactobacillus plantarum* JCM1149[a]
Shape	Rod	Short rod	Rod
Gram stain	Positive	Positive	Positive
Catalase test[b]	–	–	–
Growth at 15/45 °C[c]	+/+	+/+	+/+
Growth at pH[c]			
2.0	+	+	+
3.0	++	+	++
4.0	+++	+++	+++
5.0	+++	+++	+++

[a] *Lactobacillus plantarum* JCM1149 was used as a positive control for the two probiotic strains isolated in this study (KJ03 and KJ23)

[b] +, Positive reaction; −, negative reaction;

[c] +, ++, +++: Low, moderate, and high turbidity of growth, respectively

Table 5 In vitro probiotic properties of the selected isolates of lactic acid bacteria

Properties	*Lactobacillus plantarum* KJ03	*Lactobacillus fementum* KJ23	*Lactobacillus plantarum* JCM1149
Survival (log CFU/ml)			
Control (0 h)	9.584 ± 0.193 [a]	9.498 ± 0.147 [a]	9.315 ± 0.229 [a]
Simulated lysozyme, 10 min	9.550 ± 0.146 [a]	9.475 ± 0.285 [a]	9.280 ± 0.167 [a]
Simulated lysozyme, 20 min	9.445 ± 0.140 [a]	9.392 ± 0.121 [a]	9.225 ± 0.181 [a]
Simulated gastric juice (pH 2.5), 2 h	9.211 ± 0.061 [a]	9.011 ± 0.008 [b]	9.004 ± 0.083 [b]
Simulated intestinal juice (pH 8.0), 3 h	9.020 ± 0.010 [a]	8.542 ± 0.022 [b]	8.788 ± 0.103 [b]
Simulated intestinal juice (pH 8.0), 6 h	8.735 ± 0.015 [a]	7.621 ± 0.035 [c]	8.312 ± 0.045 [b]
Viable cell count (log CFU/ml)			
Micro-aerobic condition	9.541 ± 0.112 [a,A]	9.522 ± 0.075 [a,A]	9.122 ± 0.063 [b,A]
Anaerobic condition	9.531 ± 0.065 [a,A]	9.538 ± 0.082 [a,A]	9.250 ± 0.008[a,A]
Cell surface hydrophobicity (%)	34.82 ± 1.57 [b]	39.53 ± 1.34 [a]	19.90 ± 1.33 [c]
Bile salt hydrolase activity[a]			
Glycocholic acid	–	–	–
Taurocholic acid	–	–	–
Glycodeoxycholic acid	+	+	+
Taurodeoxycholic acid	–	–	–
Blood hemolysis	γ	γ	γ
Cholesterol removal (%)	53.0 ± 0.6	49.2 ± 1.4	38.0 ± 1.1

Where applicable, data are presented as the mean ± SD. Means followed by different superscript lowercase letters in the same row are significantly different at $p \leq 0.05$. Means followed by different superscript uppercase letters in the same row are significantly different at $p \leq 0.05$

[a] +, precipitated bile salt acid around colonies

(0.3 %, w/v), simulating the near neutral small intestine environment. Many studies have reported that the majority of the strains survived well under such conditions, suggesting a potential recuperation of the initial levels during the passage of the small intestine (Maragkoudakis et al. 2006). Zielińska et al. (2015) reported that *L. plantarum* strains K1 and O23 isolated from pickled vegetables showed good resistance under the bile salt stress condition.

Effect of micro-aerobic and anaerobic conditions on LAB isolates growth There was no significant difference in the growth of *L. plantarum* KJ03 or *L. fermentum* KJ23 when tested under micro-aerobic and anaerobic conditions (Table 5). Moreover, both strains grew >log 9 CFU/ml in all conditions after a 24-h incubation. These results are in accordance with those reported by Smetankova et al. (2012) who observed that wild strains of *L. plantarum* produced lactic acid equally well under aerobic and anaerobic conditions. *L. fermentum* was able to grow and show probiotic properties in the absence of oxygen (Lingani et al. 2008). Probiotic bacteria generally grow and colonize the small intestine, which is a strictly anaerobic environment, and have been reported to survive under these conditions (Talwalkar and Kailasapathy 2003). Oxygen toxicity is a major problem in the survival of probiotics. Screening probiotics for oxygen tolerance before their use in commercial products could ensure high cell survival during storage (Talwalkar et al. 2001).

Cell surface of hydrophobicity of LAB isolates The analysis of the adhesion ability of the food bacteria with probiotic potential has been conducted in many species and strains. In fact, it is commonly accepted that adhesion properties and mechanisms in *Lactobacillus* are strain- and matrix-dependent (Federici et al. 2014). In this study, *L. fermentum* KJ23 gave the highest affinities value of 39.5 %, followed by 34.8 % and 19.9 % in *L. plantarum* KJ03 and *L. plantarum* JCM 1149, respectively, toward *n*-hexadecane (Table 5). Nevertheless, both strains showed moderate affinity to *n*-hexadecane, which were in range of 30–40 % cell-surface hydrophobicity. The determination of microbial adhesion to hexadecane as a method to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson 2000). Adhesion is believed to be a requirement for the realization of probiotic effect, as it is required for colonization of the GIT; it is also an important prerequisite for competitive exclusion of enteropathogens and immunomodulation of the host (Begonović et al. 2014). Our results indicate that the LAB strains isolated from our food source have the potential to adhere to and colonize the gut epithelial cells of the human intestine.

Blood hemolytic activity and bile salt hydrolase All strains displayed no haemolysis (γ -hemolysis) when tested with human blood agar (Table 5). Absence of hemolytic activity is considered to be a safety prerequisite for the selection of a probiotic strain (Ruiz-Moyano et al. 2009). Results from recent studies strongly suggest that BSH activity is a relevant property of a candidate probiotic. BSH activity is associated with the protection of bacteria from toxicity through the detoxification of bile salts, thereby increasing the intestinal survival and persistence of the BSH-producing strains (Guo et al. 2010). Du Toit et al. (1998) reported a beneficial effect of BSH-positive *Lactobacillus* strains in vivo. In our study, *L. plantarum* KJ03 and *L. fermentum* KJ23 both showed similar BSH activity in culture, as they both hydrolyzed GDC and TCD. In populations with a high incidence of colorectal cancer, fecal concentrations of bile acids are increased, suggesting that increased exposure of the colonic lumen to high levels of bile acids plays a role in the natural course of development of colon cancer. Bayerdörffer et al. (1995) also reported a positive association between deoxycholic acid (DOC) in the serum and colorectal adenomas, the precursors of colorectal cancer, further indicating a pathogenic role of DOC in colonic carcinogenesis. DOC also appears to be the most significant bile acid with respect to human colorectal cancer (Hill 1990).

Cholesterol-lowering property A high level of serum cholesterol in humans is generally considered to be a risk factor for coronary heart disease (Klaver and van der Meer 1993). Among the isolates tested for the removal of cholesterol, *L. plantarum* KJ03 showed the highest cholesterol reduction (52.27 %), followed by *L. fermentum* KJ23 (49.33 %); the lowest value was found for *L. plantarum* JCM 1149 (36.73 %). Studies have indicated that many *Lactobacillus* spp. have cholesterol-reducing effects in vitro or in vivo (Kumar et al. 2012). It was hypothesized that deconjugated bile salts may contribute to lower cholesterol levels as free bile salts may be more readily excreted from the GIT than conjugated bile salts (Fukushima et al. 1999). However, the exact mechanism of serum cholesterol reduction by probiotic bacteria is not completely understood (Choi and Chang 2015). Klaver and van der Meer (1993) suggested that in vitro cholesterol reduction by some *Lactobacillus* spp. results from their coprecipitation with deconjugated bile salts. Interestingly, Mann and Spoerry (1974) studied cholesteremia in a tribe of Maasai and found that serum cholesterol levels of Maasai men decreased after consumption of large amounts of milk fermented with a wild *Lactobacillus* strain.

Determination of antibiotic susceptibility The selected strains were tested for their susceptibility to nine antibiotics by the broth microdilution assay. *Lactobacillus plantarum* KJ03 was found to be susceptible to six antibiotics

Table 6 Minimum inhibitory concentrations for selected strains of lactic acid bacteria

Strain	Minimum inhibitory concentration[a] (μg mL^{-1})							
	Ampicillin	Vancomycin	Ciprofloxacin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
Lactobacillus plantarum KJ03	2	>64	16	16	8	<0.5	<0.125	32	8
Break point for *Lactobacillus plantarum*[b]	2	Not required	–	64	Not required	1	2	32	8
Lactobacillus fermentum KJ23	1	>64	32	16	8	<0.5	<1.25	32	4
Breakpoint for *Lactobacillus fermentum*[b]	2	Not required	–	32	64	1	1	8	4

[a] Strains with a minimum inhibitory concentration lower than or equal to the breakpoints are considered here to be susceptible

[b] Breakpoints are according to the guidelines of the European Food Safety Authority (2012)

(erythromycin, chloramphenicol, ampicillin, kanamycin, tetracycline, clindamycin) but not to streptomycin and vancomycin. *Lactobacillus fermentum* KJ23 was also susceptible to six antibiotics (erythromycin, chloramphenicol, ampicillin, kanamycin, streptomycin, clindamycin) but not to tetracycline according to the breakpoint levels suggested by European Food Safety Authority (2012). All strains were resistant to ciprofloxacin in accordance with the Scientific Committee for Animal Nutrition (2002). However, they were susceptible to the breakpoints values proposed by Danielsen and Wind (2003) who reported relevant microbiological breakpoints for a wider range of *Lactobacillus* species (Table 6). Strains KJ03 and KJ23 showed resistance to vancomycin at the highest amount tested (64 μg). Since vancomycin is an antibiotic belonging to glycopeptide antibiotics, it inhibits the synthesis of peptidoglycan which is an important structural component of the bacterial cell wall. Therefore, Gram-positive bacteria, including LAB, are especially vulnerable to vancomycin treatment (Reynolds 1989). Moreover, resistance to vancomycin has been reported as a natural or an intrinsic property of many LAB (Gotcheva et al. 2002).

The *Lactobacillus* spp. exhibited resistance against inhibition of nucleic acid synthesis (ciprofloxacin), resulting in its natural resistance, which may be inherent to a bacterial genus or species, but may also be acquired through an exchange of genetic material, mutations and the incorporation of new genes (Ammor et al. 2007). Moreover, a drawback to antibiotic resistance is that a transfer of antibiotic resistance genes is possible because antibiotic resistance genes are generally carried on plasmids, which can be transferred to other bacteria by means of conjugation (Cebeci and Gurakan 2003). This may result in pathogenic bacteria possessing a high level of antibiotic resistance. Owing to tetracycline resistance of *L. fermentum* KJ23, we selected *L. plantarum* KJ03 as a potential LAB for further starter culture applications.

Detection of virulence genes Virulence factors can be crucial for strain pathogenicity depending on their type. The presence of virulence genes in both LAB strains KJ03 and KJ23 was investigated using PCR technology. The genes for adhesion collagen protein (*ace*), aggregation substances (*agg* and *asa*), enterococcal surface protein (*esp*) and gelatinase (*gelE*) were absent, whereas the expected PCR products were observed for *Enterococcus faecalis* 13–5 and *E. faecalis* VanB as positive control (Table 7). The positive control also exhibited negative results for cell wall-adhesion (*Cyl*) due to the low levels or downregulation of gene expression or an inactive gene product.

It is well known that virulence of microorganisms is regulated by virulence coding genes present on the genome in special regions. Several putative virulence factors have been described which cause serious disease in humans, such as aggregation substance protein, gelatinase, cytolysin, enterococcal surface proteins, accessory colonization factors and endocarditis antigens (Moraes et al. 2012). The verification of virulence factors by molecular procedures is important due to the risk of genetic transfer because these genes are usually located in conjugative plasmids (Eaton and Gasson 2001).

Conclusion

The results of our study demonstrate that *L. plantarum* KJ03 isolated from fermented stinky bean possesses desirable in vitro functional properties that are similar or superior to those of the reference probiotic strain, *L. plantarum* subsp. *plantarum* JCM 1149. Strain KJ03 was able to survive and establish in an environment similar to the human GIT, inhibit potential pathogenic bacteria under the safety of Thai Community Product Standard (317/2004), and considered to be safe to be used with regard to their antibiotic resistance pattern. Therefore, *L. plantarum* KJ03 can be considered as a starter culture to improve the quality of *Sataw-Dong*. A probiotic potential is expected to greatly enhance the already important nutritional value of stink bean, which is regarded as a source of organic acids, vitamins, and minerals. Development of a functional product may indeed convey a favorable impact in rural economy, especially knowing that such product originates from less well-developed regions.

Table 7 The evaluation of virulence genes in strains of lactic acid bacteria

Isolates	Virulence genes[a]						
	esp	*ace*	*asa*1	*gelE*	*efaAfs*	*Cyl*A	*Cyl*B
Lactobacillus plantarum KJ03	−	−	−	−	−	−	−
Lactobacillus fermentum KJ23	−	−	−	−	−	−	−
Enterococcus faecalis VanB	−	+	+	+	+	−	−
Enterococcus faecalis 13-5	+	ND	ND	ND	ND	ND	ND

The results are presented as: +, Positive; −, negative; ND, not detected

[a] See Table 1 for definition of genes

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