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

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Abstract

This study aims to evaluate some technological and functional potential of lactic acid bacteria (LAB) isolated from fermented stinky bean (*Sataw-Dong*). After screening, 114 LAB isolated from spontaneously fermented stinky bean for their inhibition towards two food-borne pathogens (*Staphylococcus aureus* DMST 8840 and *Escherichia coli* DMST 4212), 5 isolates namely KJ03, KJ15, KJ17, KJ22 and KJ23 exhibited antagonistic activity. These 5 strains gave titratable acidity as lactic acid in the range of 1.47-1.55% and strains KJ03 and KJ23 exhibited great NaCl tolerance over 7% (w/v). Using 16S rRNA gene sequence analysis, KJ03 and KJ23 were identified as *Lactobacillus plantarum* and *Lactobacillus fermentum*, respectively. They were further investigated for their functional properties *in vitro*. Both *L. plantarum* KJ03 and *L. fermentum* KJ23 survived best in the simulated gastrointestinal tract with less than one log cell decrease over 8 h (>8 log CFU/ml). *L. plantarum* KJ03 performed the best in cholesterol removal (53%). *L. fermentum* KJ23 gave the highest cell surface hydrophobicity (39.5%). None of the two strains showed any haemolysis activity. They also hydrolyzed glycodeoxycholic and taurodeoxycholic acids. Regarding antibiotic susceptibility, it was found that *L. fermentum* KJ23 was not sensitive to tetracycline. For all obtained results, *L. plantarum* KJ03 was found to possess desirable *in vitro* functional properties. This strain is a good candidate for further investigation in *Sataw-Dong* fermentation process to assess its technological performance as a potential probiotic starter.

Keywords: lactic acid bacteria, *Lactobacillus fermentum*, *Lactobacillus plantarum*, probiotic, *Sataw-Dong*

1. *Introduction*

Fermented foods make an important contribution to human diet in many countries, especially at household level, because fermentation is an inexpensive way that potentially preserves food, improves its nutritional value and enhances its aroma and taste (Aloys and Angeline 2009). Besides, indigenous fermented foods are strongly linked to culture and tradition of their origin (Guo et al. 2015).

Foods containing probiotics are a group of health-promoting, functional foods, with a large commercial interest and big growing in market share (Vuyst et al. 2008). Most of probiotic bacteria belong to lactic acid bacteria (LAB) groups, especially the species of the genus *Lactobacillus* representing one of the fundamental microbial groups. These LAB have been introduced in several of fermented food products. Many studies have reported that dairy products are the most commonly used as food vehicles for the delivery of probiotic (Rubio et al. 2014). Recently, there is a growing interest in developing non-dairy probiotics and it has been 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 new opportunity for non-dairy probiotic products development has interested from the high number 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-Navarro 2010). Thus, non-dairy products made from fruits, vegetables and cereals 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 the 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) as well as hypoglycemic (Jamaluddin and Mohamed, 1993). Stink beans are preserved in brine with lactic acid (LA) fermentation for several days, called fermented stink bean or *Sataw-Dong* (in Thai language) and consumed as raw pickle. The similar products of LA fermentation, although so far cucumber, cabbage and olives are the only vegetables that are fermented in large volumes for human consumption (Montet et al. 2006).

However, the common problems in natural fermentation are the uncontrollable fermentation process that result in variations of product stability and quality attributes (Parkouda et al. 2010). It was reported that the problems can be overcome through starter culture by controlling the microflora, ripening time acceleration, inhibition 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 et al. 1999).

Therefore, the purposes of this paper were: (i) to characterize LAB isolated from traditionally fermented stinky bean; (ii) to select the most suitable strains according to their technological characteristics including and antagonistic activity towards foodborne pathogens and (iii) to investigate some functional properties the isolates may have.

2. Materials and Methods

2.1 Sataw-Dong preparation

Sataw-Dong was prepared according to the traditional recipe. Whole pods were blanched and added of a brine prepared by boiling clean water containing; salt 3-4%, sugars 5%, and Malabar tamarind (*Garcinia Cambogia*.) 0.5%. The beans with the brine were transferred to 12 glass jars (100 g), which were covered with lids and spontaneously fermented 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 for LAB isolation.

2.2 Isolation of LAB with antagonistic activity towards foodborne pathogens

Samples (25 g) were added into 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. Appropriate dilutions were spread onto MRS agar (LAB, UK) supplemented with bromocresol purple (0.02%, w/v) and NaCl (3%, w/v), and incubated at 37°C for 24 h for isolation of presumptive LAB. The overnight plates of LAB were individually overlaid with Tryptone Soy Agar (TSA; Himedia, India) (0.75%, w/v agar) seeded with *Staphylococcus aureus* DMST 8840 and *Escherichia coli* DMST 4212 (ca. 10^6 CFU/mL). The plates were 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 inhibition zone were individually picked and streaked on MRS agar 2-3 times to purify the isolates. All Gram-positive and catalase-negative isolates were defined as LAB (Hwanhlem *et al.*, 2011) and they maintained in MRS broth containing glycerol (60%) at -20°C. For routine analysis, strains were subculture twice in MRS broth at 37°C for 24 h.

2.3 Confirmation of the antagonistic activity

Antagonistic activity against bacterial indicators was investigated by agar spot test and agar well diffusion assay as described by Jones et al. (2008) and Schillinger and Lüche (1989), respectively. The agar spot test experiment was conducted by individual spotting an overnight LAB culture (5 µl) onto the surface of a MRS

agar plate and incubating at 37°C for 24 h. These plates were overlaid with 10 ml of TSA (0.75% w/v, agar) seeded with 100 µl of each tested indicator strains. After 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 (8,000×*g*, 10 min, 4°C) from overnight cultures. Two samples of supernatant taken from each strain were used in trials as follows: (i) pH was adjusted to 6.5 and heated at 90 °C for 10 min, and (ii) the supernatant without adjustment was used as control. Twenty ml of TSB (1.0% w/v, agar) seeded with each bacterial indicators were poured onto sterile plates. Wells (7 mm diameter) were punched in the agar layer and cell-free supernatants (50 µl) were placed into each well, and incubated at 37°C for 24 h. The inhibition activity was examined by the diameters of inhibition zones.

2.4 Determination of pH value and total titratable acidity (TTA)

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

2.5 Determination of NaCl tolerance of LAB

The NaCl concentration in MRS broth was adjusted to 0, 3, 5, 7, 9 and 12% (w/v). One percent (v/v) of active LAB was inoculated into each MRS broth containing NaCl and cultivated at 37°C for 24 h. The survival of LAB was enumerated by drop plate method on MRS agar and reported as CFU/mL.

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

DNA of selected LAB was extracted using an extraction kit (QIAgen, Germany) according to the manufacturer's protocol and stored at -20°C. The 16S rRNA gene amplification was performed in a Thermocycler (Techne, UK) with the universal primers: 27F (5'-AGAGTTTGATCCTGG CTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') according to protocol described by Cai et al. (1999). The PCR products were purified using a PCR purification kit (QIAgen, Germany) and sequenced (Ward Medic Ltd., 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 number LC016617 and LC037354).

2.7 Determination of functional properties in vitro

2.7.1 Resistance to lysozyme

Evaluation of lysozyme resistance was performed to assess the *in vitro* ability of the strains to survive transit through the oral cavity, as described by Turchi et al. (2013) with slightly modification. LAB grown overnight were harvested by centrifugation (8,000×*g*, 10 min) and resuspended in 2 ml of phosphate buffer saline (PBS) in the presence of lysozyme (100 mg/L) (Sigma-Aldrich, Germany). Bacterial suspensions without lysozyme were used as control. Samples were incubated at 37 ºC and viable cell counts after 10 and 20 min were enumerated on MRS agar by drop plate method. Survival rates were calculated as percentage of the growth.

2.7.2 Survival of LAB under simulated gastric and intestinal juices

The cells were collected by centrifugation (8,000×*g*, 10 min) and washed twice with PBS before being resuspended in PBS solution at pH 2.5 containing pepsin (3 mg/mL, Sigma, Germany) 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 in PBS solution at pH 8.0 containing pancreatin (1 mg/mL) and ox bile salts (3 mg/mL, Sigma) 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.

2.7.3 Effect of micro-aerobic and anaerobic conditions on growth of LAB

One percent (v/v) of LAB strains was inoculated to MRS broth (micro-aerobic condition) and MRS broth supplementing 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.

2.7.4 Cell-surface hydrophobicity assay

Cell-surface hydrophobicity was determined by a microbial adhesion to hydrocarbon (MATH) 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. 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).

2.7.5 Blood haemolytic activity

Fresh overnight LAB cultures were streaked in triplicate on TSA plates supplemented with 5% (v/v) of human blood (obtained from Songklanagarind Hospital) and incubated at 37°C for 48 h. Haemolytic activities of the bacterial culture were examined for signs of *β*-haemolysis (clear zones around colonies), *α*-haemolysis (green zones around colonies) or *γ*-haemolysis (no clear zones around colonies) (Hargrove and Alford 1978).

2.7.6 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, Germany) as follows: glycocholic acid (GC), taurocholic acid (TC), 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 as a positive result. MRS agar plate without supplementation was used as control.

2.7.7 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 (Himedia, India). Water-soluble cholesterol (polyoxyethylene cholesteryl sebacate; Sigma, Germany) 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 the residual of cholesterol concentration in the broth was determined using modified colorimetric method. One mL of the broth was added with 1 mL of KOH (33%, w/v) and 2 mL of absolute ethanol, mixed for 1 min, thereafter heated at 70°C for 30 min. After cooling, 5 mL of hexane were added into the tube and vortexed for 1 min. The hexane layer (4 mL) was transferred into a clean glass tube and evaporated. The residual was dissolved in 2 mL of *O*-phthalaldehyde reagent. After mixing, 1 mL of concentrated sulfuric acid was added and the mixture was vortexed for 1 min. Absorbance was read at 540 nm. All experiments were performed in duplicate.

2.7.8 Determination of antibiotic susceptibility

The LAB strains were tested for antibiotics resistance 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 concentration 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. One hundred µL of bacterial suspension was mixed to antibiotic solution (100 µL) 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 EFSA (EFSA, 2012). Accordingly, strains showing MICs higher than the respective cutoff values were considered as resistant.

2.7.9 Detection of the presence of virulence genes

For evaluation of the safety selected LAB for their application in fermentation processes, the detection of genes encoding potential virulence genes was carried out by PCR using genomic DNA obtained as described previously and the specific primers described in Table 1 (Vankerckhoven et al. 2004). The positive strains were *Enterococcus feacalis* Van B for *ace, asa*1, *Cyl*A/B, *efaAfs* and *gelE* genes and *Enterococcus feacalis* 13-5 for *esp* gene. 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).

2.8 Statistical analysis

Statistical analysis was conducted using one-way ANOVA with post hoc Duncan's test ($p < 0.05$ was considered significant). All data are expressed as mean ± S.D.

3. Results and Discussion

3.1 Isolation of LAB with primary antagonistic activity

LAB originally isolated from vegetable are probably the most suitable candidates for improving the microbiological safety of fermented vegetable products, because they are well adapted to the condition of these kinds matrices and should therefore be more competitive than LAB obtained from other sources (Ponce et al. 2008). The colonies which showed clear zone against *S. aureus* DMST 8840 and *E. coli* DMST 4212 were 114. All of those isolates were identified as LAB using the criteria of Gram-positive and catalase negative (data not shown).

3.2 Screening for antagonistic activity

Antagonistic activity of LAB strains may contribute to the quality improvement of fermented foods, which is achieved through the control of spoilage and pathogenic bacteria, thus extending shelf-life and improving

sensory quality (Beganović et al. 2014). All 114 LAB strains were tested for antagonistic activity toward *S. aureus* DMST4480 and *E. coli* DMST4212 followed by the safety criteria of Thai Community Product Standard (317/2004). However, only 5 strains (KJ03, KJ15, KJ17, KJ22 and KJ23) showed excellent inhibition diameter zone larger than 15 mm (Table 2), which strain KJ03 gave the highest inhibition zone.

Those 5 LAB strains were subsequently analyzed for their production of antibacterial compounds by agar well diffusion method. Supernatants obtained from the 5 strains did not exhibit the inhibition zones after pH adjustment to 6.5 (data not shown). The antimicrobial activity from the 5 LAB strains may come from organic acids. In fact, the drop in pH arising from the production of lactic acid can be enough to inhibit certain bacteria. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence having 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. 2008b), and fermented Himalayan vegetables (Dewan and Tamang 2007).

3.3 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 towards food spoilage and poisoning bacteria (Hwanhlem et al. 2011). LAB ferment sugars via different pathways resulting in homo-, or mixed acid fermentation. Homofermentation gives lactic acid as the sole end product of glucose metabolism when the Embden-Meyerhof-Parnas pathway is used (Hofvendahl and Hahn–Hägerdal 2000).

These 5 LAB isolates exhibiting excellent antagonistic activity were chosen for lactic acid production and pH reduction measurement. Strain KJ23 was the highest acid production of 1.55% (equivalent to % lactic acid), while strain KJ15 gave the lowest acid production of 1.47% after 24 h of incubation. The other strains showed no significant lactic acid production except strain KJ15. The pH values were correlated with these results in the range of 3.86-4.00 (Table 3).

3.4 Effect of NaCl on growth of isolates

Figure 1 reports the cell viability after incubation in MRS broth supplemented with NaCl. A concentration of salt of 3 to 5% did not significantly affect growth of LAB, as they grew over 9 log CFU/mL. In addition, some isolates (KJ03, KJ22 and KJ23) appeared slightly stimulated when 3% NaCl was added. As predicted, the increase of NaCl concentration showed a negative influence on the growth of all isolates with a

subsequent decrease of cell viability (Fig. 1). In particular, LAB viability was strongly affected by 7% NaCl, meanwhile isolates KJ03 and KJ23 tolerated the highest ones (>8 log CFU/mL). Strain KJ03 and KJ23 were selected for further investigations. At 9-12% of NaCl, LAB were not able to grow throughout 24 h. However, cell counts remained constant (ca. 5 to 6 log CFU/mL) for the entire running 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 Vuyst 1999). Consequently, a salt tolerance of at least 7% was considered for selection as potential starter cultures in *Sataw-Dong* fermentation because the presence of 5-7% NaCl is normally found in many recipes of *Sataw-Dong* production.

3.5 Identification of LAB isolates

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

3.6 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 human gastrointestinal tract (GIT). The *L. plantarum* subsp. *plantarum* JCM1149, originally isolated from pickled cabbage, was commercial type strain with probiotic properties (Zago et al. 2011; Nazzaro et al. 2012) and also used in this study to compare properties among our isolates. The results corresponding to the functional properties are shown in Table 5.

3.6.1 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%. Among these isolates revealed a high lysozyme resistance, after both 10 and 20 min incubation, with a survival rate >98%

(Table 5). Lysozyme presented in human saliva is the first step to be passed. Resistance to lysozyme has been attributed to the peptidoglycan structure in the cell wall, the physiological state of the cell and lysozyme structure in the medium (F. E. Cunningham, 1991). This result confirmed the high resistance of *Lactobacillus* strains to 100 mg/L of lysozyme under condition stimulating the *in vivo* dilution by saliva (Zago et al. 2011).

3.6.2 Survival of LAB under simulated gastric and intestinal juices

The strains behavior under conditions simulated the GIT allows a selection of strains likely to survive such conditions, and which can be further investigated for their potential as probiotic cultures. Stresses to microorganisms begin in the mouth, with lysozyme-containing saliva, and continue in the stomach (pH 1.5-3.0) and the upper intestine, which contains bile (Zago et al. 2011). *L. plantarum* KJ03 and *L. fermentum* KJ23 survived more than 9 log CFU/mL (>95%) after exposing the cell suspensions to the simulated gastric juice. The surviving cells were further tested for bile salt tolerance. *L. plantarum* KJ03 exhibited the greater survival over 9 log CFU/mL (92.55%) than *L. fermentum* KJ23 (81.32%) (Table 5).

The ability to survive the acidic and bile challenges in GIT is advantageous for probiotic. Acid tolerance of *Lactobacillus* strains was also reported. Srinu et al. (2013) revealed that all the selected LAB strains, *L. plantarum* and *L. casei*, had good survival abilities in the tested acidic pH range (1.5-3.5). Also Jamaly et al. (2011) reported that *L. plantarum* was able to tolerate 3 h of acid exposure (pH. 2.0 and 3.0). In contrast to gastric juice, good probiotic bacteria should survive well in a pancreatin solution at pH 8.0 in the presence of bile salts (0.3%, w/v), simulating the near neutral small intestine environment. Many studies so far 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). It was reported that *L. plantarum* K1 and O23 isolated from pickled vegetables showed good resistance under bile salt stress condition (Zielińska et al. 2015).

3.6.3 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 tested under micro-aerobic and anaerobic conditions (Table 5). Moreover, both strains grew over log 9 CFU/mL in all conditions after 24 h incubation. These results were in accordance with Smetankova et al. (2012) who reported that wild strains of *L. plantarum* produced lactic acid no significantly 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 at the small intestine which has strictly anaerobic condition and

have been reported to survive under these conditions (Talwalkar and Kailasapathy 2003). Moreover, oxygen toxicity is a major problem in the survival of probiotics. Screening probiotic for oxygen tolerance before their incorporation could ensure high cell survival during storage (Talwalkar et al. 2001).

3.6.4 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 way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson 2000). Because adhesion is believed to be a requirement for the realization of probiotic effect, as it is required for colonization of the GIT and an important prerequisite for competitive exclusion of enteropathogens and immunomodulation of the host (Beganović et al. 2014). This indicated that LAB from the food source have the potential to adhere to and colonize the gut epithelial cells of human intestine.

3.6.5 Blood haemolytic activity and bile salt hydrolase (BSH)

All strains displayed no haemolysis (γ-haemolysis) when tested with human blood agar (Table 5). Absence of haemolytic activity considered a safety prerequisite for the selection of a probiotic strain (Ruiz-Moyano et al. 2009). Recent research data strongly suggested that the relevant properties of a candidate probiotic is a BSH function, which involves the protection of bacteria from toxicity by detoxification of bile salts, increasing the intestinal survival and persistence of producing strains (Guo et al. 2010). Beneficial effect of BSH-positive *Lactobacillus* strains *in vivo* was also observed in some reports (Du Toit et al. 1998). The result demonstrated that *L. plantarum* KJ03 and *L. fermentum* KJ23 gave the same result on BSH activity on plates, as they both hydrolyzed GDC and TCD. Besides, in populations with 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. The DOC also appears to be the most significant bile acid with respect to human colorectal cancer (Hill 1990).

3.6.6 Cholesterol-lowering property

A high level of serum cholesterol in humans is generally considered to be risk factor for coronary heart disease (Klaver and van der Meer 1993). Among the isolates tested for removal of cholesterol, *L. plantarum* KJ03 showed the highest cholesterol reduction (52.27%), followed by *L. fermentum* KJ23 (49.33%) and the lowest value was found by *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 clear (Choi and Chang 2015). Klaver and van der Meer (1993) suggested that *in vitro* cholesterol reduction by some *Lactobacillus* spp. resulted from their coprecipitation with deconjugated bile salts. Interestingly, Mann and Spoerry (1974) studied the cholesteremia in a tribe of Maasai revealed that serum cholesterol levels of Maasai men decreased after consumption of large amounts of milk fermented with a wild *Lactobacillus* strain.

3.6.7 Determination of antibiotic susceptibility

The selected strains were tested for their susceptibility to 9 antibiotics by broth microdilution assay. *L. plantarum* KJ03 was susceptible to 6 antibiotics (erythromycin, chloramphenicol, ampicillin, kanamycin, tetracycline and clindamycin) but not to streptomycin and vancomycin. For *L. fermentum* KJ23 was susceptible to 6 antibiotics (erythromycin, chloramphenicol, ampicillin, kanamycin, streptomycin and clindamycin) but not to tetracycline according to the breakpoint levels suggested by EFSA (2012). All strains were resistant to ciprofloxacin in accordance with Scientific Committee for Animal Nutrition (SCAN) (SCAN, 2002). However, they were susceptible to the breakpoints values proposed by Danielsen and Wind (2003) who reported the relevant microbiological breakpoints for a wider range of *Lactobacillus* species (Table 6). Strain KJ03 and KJ23 showed resistance to vancomycin at the highest amount, 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 toward 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 exchange of genetic material, mutations and the incorporation of new genes (Ammor et al. 2007). Moreover, a drawback of antibiotic resistance is that 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 Gürakan 2003). This may result in highly antibiotic resistance of pathogenic bacteria. Owing to tetracycline resistance of *L. fermentum* KJ23, therefore *L. plantarum* KJ03 was selected as a potential LAB for further starter culture application.

3.6.8 Detection of virulence genes

Depending on their type and combination of virulence factors, they become crucial for the strain pathogenicity. The presence of virulence genes in both isolate strains were investigated by PCR technique. 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 *Ent. feacalis* 13-5 and *Ent. feacalis* VanB as positive control (Table 7). The positive control also exhibited negative result for cell wall adhesion (*Cyl*) due to the low levels or down regulation of gene expression or an inactive gene product.

It is well known that virulence of microorganism is regulated with virulence coding genes present on the genome in special regions. Several putative virulence factors have been described and caused 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 procedure is important due to the risk of genetic transfer, because these genes are usually located in conjugative plasmids (Eaton and Gasson 2001).

4. Conclusion

Our research effort demonstrated that *L. plantarum* KJ03 isolated from fermented stinky bean was found to possess desirable *in vitro* functional properties similar or superior to the reference probiotic strain, *L. plantarum* subsp. *plantarum* JCM 1149. This strain 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 was considered 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 stinky 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 originate from less developed regions.

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Figure captions

Fig. 1 Viability of LAB strains after cultivation in MRS broth supplemented with various NaCl concentration (0-12%) incubated at 37 °C for 24 h.

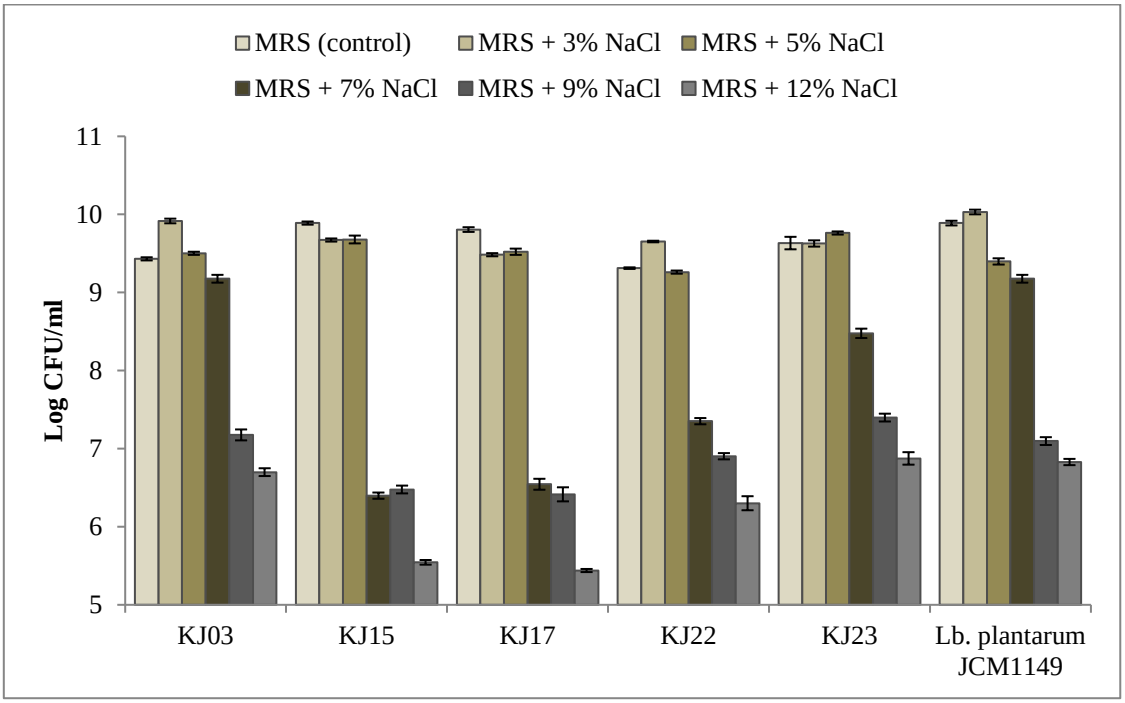

Figure 1

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Table 1 PCR primers and products for detection of virulence determinants

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

Table 2 Inhibition zone diameter of LAB isolated from *Sataw-Dong* on agar when *S. aureus* DMST8840 and *E. coli* DMST4212 were used as indicators.

Isolated no.	Inhibition zone (mm)	
	S. aureus DMST8840	*E. 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

[a]Mean ± SD from triplicated determinations

Table 3 pH values and titratable acidity of cell-free supernatant of LAB isolated from *Sataw-Dong*

Isolated	pH				Titratable acidity (%)			
no.	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]

* Mean ± SD from triplicated determinations.

Mean values with different lowercase letter in the same column indicate significant difference ($P \leq 0.05$).

Table 4 Physiological properties of selected LAB isolated from *Sataw-Dong*

Property	*L. plantarum* KJ03	*L. fementum* KJ23	*L. plantarum* JCM1149**
Shape	rod	Short rod	rod
Gram stain	positive	positive	positive
Catalase test	-	-	-
Growth at 15/45 °C	+/+	+/+	+/+
Growth at pH			
2.0	+*	+	+
3.0	++	+	++
4.0	+++	+++	+++
5.0	+++	+++	+++

+, positive reaction; -, negative reaction

*+, ++ and +++ represent low, moderate and high turbidity of growth, respectively.

** *L. plantarum* JCM1149 was used as a positive control of probiotic strain.

Table 5 In vitro probiotic properties of selected LAB isolates

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

Mean values with different lowercase letters in the same row indicate significant differences ($P \leq 0.05$). Mean values with different uppercase letters in the same column indicate significant differences ($P \leq 0.05$).

*+, precipitated bile salt acid around colonies

Table 6 Minimum inhibitory concentrations (MICs) for selected LAB strains

Strain	MIC** (μg mL^{-1})								
	AMP	VAN	CIP	KAN	STR	ERY	CLI	TET	CHL
Lb. plantarum KJ03	2	>64	16	16	8	<0.5	<0.125	32	8
Break point for *L. plantarum**	2	n.r.	-	64	n.r.	1	2	32	8
Lb. *fermentum* KJ23	1	>64	32	16	8	<0.5	<1.25	32	4
Break point for *L. fermentum**	2	n.r.	-	32	64	1	1	8	4

*Breakpoints are according to the guidelines of EFSA (EFSA, 2012)

**Strains with MICs lower than or equal to the breakpoints are considered susceptible. AMP: ampicillin; VAN: vancomycin; CIP: ciprofloxacin; KAN: kanamycin; STR: streptomycin; ERY: erythromycin; CLI: clindamycin; TET: tetracycline; CHL: chloramphenicol.

***n.r. Not required

Table 7 The evaluation of virulence genes in LAB strains

Isolates	Virulence genes*						
	esp	*ace*	*asa*1	*gelE*	*efaAfs*	*Cyl*A	*Cyl*B
L. plantarum KJ03	-**	-	-	-	-	-	-
L. fermentum KJ23	-	-	-	-	-	-	-
Ent. feacalis VanB	-	+	+	+	+	-	-
Ent. feacalis 13-5	+	ND***	ND	ND	ND	ND	ND

**esp* (enterococcal surface protein), *ace* (adhesion of collagen), *asa1* (aggregation substance), *gel*E (gelatinase), *efaAfs* (adhesion to cell wall) and *Cyl*A/B (cytolysin)

** +, Positive; –, Negative

***ND, not detected