Original Article

Screening and Characterization of Lactic Acid Bacteria from Animal Faeces for Probiotic Properties

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Abstract

Twenty-two bacterial isolates from wild elephant faeces and 4 isolates from buffalo faeces in Thailand were investigated for probiotic properties and their lactic acid production. On the basis of their phenotypic characteristic clustering and 16S rRNA gene sequence analysis, the isolates were divided into seven groups. Group I (10 isolates) was identified as *Enterococcus hirae,* group II (4 isolates) as *E. avium*, group III (3 isolates) as *Pediococcus pentosaceus* , IV (3 isoaltes) as *P. acidilactici*, group V (3 isolates) as *Lactobacillus pentosus,* group VI (1 isolate) as *Lactococcus garvieae* and group VII (2 isolates) as *Weissella paramesenteroides.* All of the bacterial isolates were examined for their potentially probiotic properties in acid and bile tolerance as well as in inhibition of cancer cells proliferation by MTT assay. Although the isolates showed no significant effects on human monocytic U937 cells and colon carcinoma Caco-2 cells proliferation, *P. acidilactici* EL8-2 exhibited the ability to tolerate in the acidic conditions and bile salts. Therefore, *P. acidilactici* EL8-2 may be regarded as probiotic strains. For lactic acid fermentation with glucose, it was found that *E. hirae* BF14-1 produced 100% optically pure L(+)-lactic acid while *W. paramesenteroides* BF13-3 produced 99.79% D (-)-lactic acid.

Keywords: animal faeces, *Enterococcus*, lactic acid bacteria, *Lactobacillus, Lactococcus, Pediococcus*, probiotic, *Weissella*

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บทคัดย่อ

การคัดกรองและอธิบายคุณสมบัติโพรไบโอติกของแบคทีเรียกรดแลกติกจากมูลสัตว์

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ศึกษาคุณสมบัติโพรไบโอติกและการผลิตกรดแลกติกของแบคทีเรียจำนวน 22 สายพันธุ์ที่แยกได้จากมูลช้างป่าและ 4 สายพันธุ์ที่แยกได้จากมูลกระบือในประเทศไทย การจัดกลุ่มโดยอาศัยลักษณะทางฟีโนไทป์และผลการวิเคราะห์ลำดับเบสบนยีน 16S rRNA สามารถจัดแบ่งกลุ่มแบคทีเรียได้เป็น 7 กลุ่ม คือ กลุ่มที่ 1 (10 สายพันธุ์) พิสูจน์เอกลักษณ์ได้เป็น *Enterococcus hirae* กลุ่มที่ 2 (4 สายพันธุ์) เป็น *E. avium* กลุ่มที่ 3 (3 สายพันธุ์) เป็น *Pediococcus pentosaceus* กลุ่มที่ 4 (3 สายพันธุ์) เป็น *P. acidilactici* กลุ่มที่ 5 (3 สายพันธุ์) เป็น *Lactobacillus pentosus* กลุ่มที่ 6 (1 สายพันธุ์) เป็น *Lactococcus garvieae* และกลุ่มที่ 7 (2 สายพันธุ์) เป็น *Weissella paramesenteroides* ศึกษาคุณสมบัติโพรไบโอติกในการทนต่อกรดและน้ำดีของแบคทีเรียทุกสายพันธุ์ เช่นเดียวกับการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง โดยการวิเคราะห์ MTT ถึงแม้ว่าทุกสายพันธุ์จะไม่มีผลยับยั้งต่อเซลล์มะเร็งเม็ดเลือดขาว U937 และเซลล์มะเร็งลำไส้ Caco-2 อย่างมีนัยสำคัญ พบว่า *P. acidilactici* EL8-2 แสดงความสามารถในการอยู่รอดในสภาพแวดล้อมที่เป็นกรดและเกลือน้ำดี และอาจนำมาพิจารณาใช้เป็นสายพันธุ์โพรไบโอติก จากกระบวนการหมักกรดแลกติกด้วยน้ำตาลกลูโคส พบว่า *E. hirae* BF14-1 ผลิตกรดแลกติกที่ให้ค่าความบริสุทธิ์เชิงแสงของกรดแลกติกชนิดแอลร้อยละ 100 ในขณะที่ *W. paramesenteroides* BF13-3 ให้ค่าความบริสุทธิ์เชิงแสงของกรดแลกติกชนิดดีร้อยละ 99.79

คำสำคัญ: มูลสัตว์ *Enterococcus* แบคทีเรียกรดแลกติก *Lactobacillus* *Lactococcus Pediococcus* โปรไบโอติก *Weissella*

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Introduction

Lactic acid bacteria (LAB) are Gram-positive bacteria that consist of several genera, *Lactobacillus (Lb.), Lactococcus (Lc.), Enterococcus, Carnobacterium, Leuconostoc, Oenococcus, Pediococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Von Wright and Axelsson, 2011). They were recognized by their ability to produce lactic acid and are generally found in the environment such as fermented foods, soil, human and animal digestive systems (Parente et al., 2001; Heilig et al., 2002; Callon et al., 2004).

LAB are known as "probiotics" that exhibit potential health and nutritional benefits. Their ability to provide relief for lactose intolerance and reduce diarrhea, remodel microbial communities, suppress pathogens, stimulate immune system and suppress cancer is clearly established (Von der Weid et al., 2001; Lin et al., 2008). The most commonly used probiotics of lactic acid bacteria are *Lactobacillus, Bifidobacterium, Pediococcus, Lactococcus* and *Streptococcus*. They are important components of the gastrointestinal flora (Isolauri et al., 2004). Probiotic bacteria which are selected for commercial use in foods and in therapeutics should retain their characteristics such as growth and survival after consumption or during transit through the stomach and small intestine (Tuomola et al., 2001). Numerous probiotics have been investigated for their benefit in prophylaxis and therapeutic gastrointestinal diseases. The effectiveness of probiotics is based on the knowledge that normal flora can protect humans against infection and disturbance of this flora can increase susceptibility to diseases. Examples of such diseases include gastrointestinal tract infections caused by rotavirus, enterotoxigenic *E. coli*, antibiotic-associated diarrhea including *Clostridium difficile*-associated disease, *Helicobacter pylori* gastroenteritis, irritable bowel syndrome, inflammatory bowel disease and colorectal cancer (Rolfe, 2000).

Colorectal cancer is the third prevalence of cancer in men and women. Mortality and incidence of colorectal cancer are also the third subordinate of prostate and lung cancer in men, breast and lung cancer in women. Diet makes an important contribution to colorectal cancer risk. Evidence also supports that the colonic microflora are involved in the etiology of colorectal cancer (Fotiadis et al., 2008). Studies on the effect of LAB consumption on cancer indicated that LAB might reduce the risk, incidence and number of colorectal cancer. These effects may involved several mechanisms such as alteration of the physicochemical conditions in the colon, quantitative or qualitative alterations in the intestinal microflora, production of anti-tumorigenic or antimutagenic compounds, alteration of the metabolic activities of

the intestinal microflora, binding and degradation of potential carcinogens and enhancement of the host's immune response (Rafter, 2003; Commane et al. 2005). Therefore, the screening of probiotic and their applications from various sources for human and animal health such as colorectal cancer are interesting nowadays.

Recently, there has been an increased interest in lactic acid production for industrial uses such as food, pharmaceuticals, textile and chemical feed stock (Yun et al., 2003) including the production of polylactic acid, a polymer which is substituted for synthetic plastics derived from petroleum. The physical properties of polylactic acid depend on the isomeric composition of lactic acid. Therefore, the production of optically pure lactic acid is essential for the polymer synthesis (Nampoothiri et al., 2010).

Elephant and buffalo are herbivorous animals that consume plant materials. Their common diets are leaves, grasses, vegetables and fruits (Hackmann and Spain, 2010). Most microbial species present in rumen or stomach in order to digest plants are obligate or facultative anaerobes bacteria (Kamra, 2005). Many LAB are isolated from plant sources, fermented food (Kim et al., 2002; Tamang et al., 2005; Chiu et al., 2008), and human GI tracts, regarding the identification and characterization of the predominant strains in order to be used as probiotics (Guo et al., 2010; Mohankumar et al., 2011; Yuliana et al., 2011; Noguchi et al., 2012). In Thailand, there have been no reports on the isolation of LAB from elephant and buffalo faeces of which probiotic properties and acid production are interesting. The aim of this study was to isolate and identify LAB present in faeces of elephant and buffalo as well as to screen probiotic properties and identify optical purity D (-) or L (+)-lactic acid.

Materials and Methods

Faeces samples and isolation of lactic acid bacteria: One gram of animal faeces from ten wild elephant and two buffalo samples collected in Petchaburi and Mahasarakham provinces, Thailand (Table 1) was enriched in 5 ml MRS broth and incubated at room temperature for 48-72 hours (Tanasupawat et al., 2002). A loopful of the enrichment broth was streaked on MRS agar containing 0.3% calcium carbonate. Acid-producing bacterial isolates were selected by presence of a clear zone around the colonies after incubated at room temperature for 48-72 hours. Pure cultures were maintained in MRS broth, in freezer, and were lyophilized.

Identification of LAB isolates

Phenotypic characterization: Phenotypic characteristics of isolates were performed according to their cultural, morphological (i.e. shape, form and cell arrangement), physiological and biochemical characteristics. Gram reaction, oxygen requirement, gas formation and catalase production were determined. Nitrate reduction, arginine hydrolysis, growth at 50^0C, at various pH value (3.5, 4.0, 8.5 and 9.0) and various NaCl concentrations (3, 6 g/100 ml) were tested as described by Tanasupawat et al. (2002). Ability to ferment in different types of sugar was performed using a conventional method. *Meso*-diaminopimelic acid (DAP) was determined as reported by Tanasupawat et al. (2002). Isomer of lactic acid was preliminarily determined by enzymatically following the method of Tanasupawat et al. (2007), which used D- and L-lactate dehydrogenases (Boehringer, Germany). The isolates were grouped based on their relationships among the phenotypic characteristics by cluster analysis. Hierarchical cluster analysis was conducted by using SPSS for Windows version 13.0.

Genotypic characterization: The 16S rRNA gene of isolates was PCR amplified using primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and the amplified 16S rRNA gene sequence was analyzed by Macrogen®, Korea. Sequence alignment was determined by BLAST software from GenBank. Multiple alignments of sequences were performed by CLUSTAL X program. Gaps and ambiguous bases were eliminated prior to construction of a phylogenetic tree. A phylogenetic tree was constructed by the neighbor-joining method with MEGA 4 program (Tamura et al., 2007). Bootstrap analysis was performed to determine confidence values of individual branches in the phylogenetic tree with 1000 replications.

Determination of probiotic properties

Acid and bile tolerance: Acid and bile tolerance were determined according to the modified method of Hyronimus et al. (2000). The isolates were cultivated in MRS medium at 37^0C for 24 hours. Each strain (10^6 -10^7 CFU/ml concentrated culture) was inoculated into MRS broth at various pH values (pH 2.0 and 3.0) with hydrochloric acid as well as broth with various concentrations of bile salt or oxgall (0.1%, 0.3%, 0.5% and 1.0%). The samples were incubated at 37^0C for 3 hours and were diluted into 10-fold serial dilution in phosphate buffer (0.1 M, pH 6.2). Then, 0.1 ml inoculums were transferred onto MRS agar by spreading plate method and incubated at 37^0C for 24-48 hours. Total viable counts were determined before incubation and after 3 hours incubation and expressed as the $\log_{10}$.

Cytotoxicity assay: Human colon adenocarcinoma cell lines (Caco-2 cells) and human monocytic leukemic cell lines (U937 cells) were kindly provided by Faculty of Medicine, Srinakharinwirot University, Bangkok. Caco-2 cells and U937 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM medium) and Roswell Park Memorial Institute medium (RPMI 1640 medium), respectively, supplemented with 10% fetal bovine serum. Cell lines were incubated at 37^0C in 5% CO_2 with a humidified incubator.

Effects of lactic acid from supernatant LAB on the cytotoxic effects was determined by 3-(4, 5-dimethythiazol-2-yl) - 2,5-diphenyl tetrazolium bromide (MTT assay) as described by Iyer et al. (2008) with slight modification. Briefly, Caco-2 cells and

U937 cells were incubated with lactic acid (10% v/v) (testing group), MRS broth (control group), cell lines alone (standard group) in triplicate in 96-well plates at 37^{0}C in 5% CO_2 incubator for 24 hours. Then, MTT solution was added to each well and incubated at 37^{0}C in 5% CO_2 for 3 hours. DMSO was added to dissolve formazan crystal formed. Optical density was measured at 595 nm with microplate reader (Biotek®, Instrument, Inc, USA). According to cytotoxicity test, statistical analysis was determined by one way ANOVA at significant value $p < 0.05$ and the values were shown as mean OD±SE.

Determination of total and optical purity lactic acid

Total lactic acid and optical purity lactic acid were determined as described by Prasirtsak et al. (2013). The isolates grew on GYP slant at 37^{0}C for 48 hours were transferred to GYP preculture medium containing glucose 10 g/l and incubated at 37^{0}C for 48 hours. The preculture broth (1 ml) was inoculated into the fermentation medium with glucose 120 g/l and incubated at 37°C for 72 hours. After fermentation, the supernatant was collected for analysis of total and optical purity lactic acid and were also examined for remaining glucose using high-performance liquid chromatography.

Results

Identification of the isolates

Twenty-six isolates were Gram-positive, catalase-negative, non-spore forming, non motility, facultative anaerobic and did not produce gas from glucose except the isolates in group VII. They did not reduce nitrate and did not grow at 50^{0}C except the isolates in group IV (Table 2). All isolates were clustered and divided into seven groups based on the cell form, cell arrangements, motility, Gram reaction, catalase production, spore formation, gas production, hydrolysis of arginine, growth at high temperatures, different pH and different NaCl concentrations, type of peptidoglycan cell wall as well as acid production from carbohydrates as shown in Fig 1. They belonged to the genera *Enterococcus*, *Pediococcus, Lactobacillus, Lactococcus* and *Weissella* based on 16S rRNA gene sequence and phylogenetic analysis (Fig 2).

Group I consisted of ten isolates, which were EL1-3, EL2-3, EL4-1, EL4-2, EL4-3, EL7-1, EL7-2, EL10-1, BF13-2 and BF14-1. They were cocci in chains. They could hydrolyse arginine and grew at pH 8.5, 9.0 and in 3% NaCl but did not grow at pH 4.0. They did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. Variable growth was found in 6% NaCl and at pH 3.5. They fermented cellobiose, D-fructose, D-galactose, D-mannose, ribose and trehalose but did not ferment L-arabinose, gluconate, D-mannitol, methyl-α-D-glucoside and sorbitol. Variable fermentation was found in raffinose, lactose, maltose, melibiose, rhamnose, salicin, sucrose, and D-xylose (Table 3). The 16S rRNA gene sequence of representative strain in this group (EL4-1, EL4-3 and BF14-1) showed 100%, 100% and 99.93% similarity (1,404, 1,380 and 1,408 bps), respectively, to *E. hirae* ATCC 9790^T as shown in Fig 2.

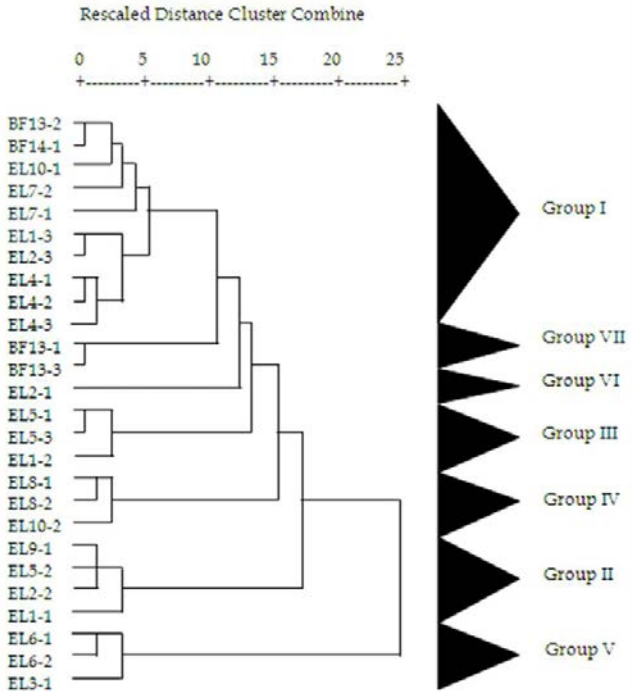

Figure 1 Dendrogram using average linkage (between groups) showing the hierarchical cluster of the lactic acid bacteria isolates based on their phenotypic characteristics

Table 1 Isolation and Identification of lactic acid bacteria from animal faeces

Isolation source	Province	Group	Isolate no.	Identification
Elephant faeces	Petchaburi	I	EL1-3, EL2-3, EL4-1, EL4-2, EL4-3, EL7-1, EL7-2 and EL10-1	*E. hirae*
Elephant faeces	Petchaburi	II	EL1-1, EL2-2, EL5-2 and EL9-1	*E. avium*
Elephant faeces	Petchaburi	III	EL1-2, EL5-1and EL5-3	*P. pentosaceus*
Elephant faeces	Petchaburi	IV	EL8-1, EL8-2 and EL10-2	*P. acidilactici*
Elephant faeces	Petchaburi	V	EL3-1, EL6-1and EL6-2	*Lb. pentosus*
Elephant faeces	Petchaburi	VI	EL2-1	*Lc. garvieae*
Buffalo faeces	Mahasarakham	VII	BF13-1 and BF13-3	*W. paramesenteroides*
Buffalo faeces	Mahasarakham	I	BF13-2 and BF14-1	*E. hirae*

Table 2 Phenotypic characteristics of LAB isolates from animal faeces

Characteristics		Group I (10)[a]	Group II (4)	Group III (3)	Group IV (3)	Group V (3)	Group VI (1)	Group VII (2)
Cell form		Cocci in chains		Tetrads		Rods	Cocci in chains	
Gas from Glucose		-	-	-	-	-	-	+
Nitrate reduction		-	-	-	-	-	-	-
Arginine hydrolysis		+	-	-	+	-	+	-
Growth at 50^{0}C		-	-	-	+	-	-	-
Growth at	pH 3.5	- (+3)	+(-2)	+	-	+	-	-
	pH 4.0	-	+(-2)	+	+	+	+	-
	pH 8.5	+	+	+	+	+	+	+
	pH 9.0	+	+	+	-	+(-1)	+	-
Growth in	6% NaCl	+ (-1)	+	+	+	+	+	+
Meso-Diaminopimelic acid		-	-	-	-	+	-	-
Lactic acid isomer		L	L	DL	DL	DL	L	D

+ : positive, - : negative reaction, [a] : Number of isolates
Numbers in parentheses indicate the number of isolates showing the reaction

Group II consisted of four isolates, which were EL1-1, EL2-2, EL5-2 and EL9-1. They were cocci in chains. They grew at pH 8.5, 9.0 and in 3%, 6% NaCl. They did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. Variable growth was found at pH 3.5 and 4.0. They fermented raffinose, L-arabinose (weakly), cellobiose, D-fructose, D-galactose, gluconate (weakly), maltose, D-mannitol, D-mannose, melibiose, methyl-α-D-glucoside, rhamnose, ribose and sorbitol but did not ferment lactose, trehalose and D-xylose. Variable fermentation was found in salicin and sucrose (Table 3). The 16S rRNA gene sequence of representative strain in this group (EL1-1 and EL2-2) showed 99.86% and 99.78% similarity (1,393 and 1,384 bps), respectively, to *E. avium* NCDO 2369^T as shown in Fig 2.

Group III consisted of three isolates, which were EL1-2, EL5-1, EL5-3. They were tetrad forming cocci and did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. Hydrolysis of arginine was negative. They grew at pH 3.5 to pH 9.0 and in 3%, 6% NaCl but did not grow at 50^{0}C. They fermented L-arabinose, cellobiose, D-fructose, D-galactose, maltose, D-mannose, melibiose, sucrose, salicin, trehalose and D-xylose but did not ferment raffinose, gluconate, methyl-α-D-glucoside, rhamnose or sorbitol. Variable fermentation was found in lactose, D-mannitol and ribose (Table 3). The 16S rRNA gene sequence of representative strain in this group (EL1-2) showed 99.51% (1,434 bps) similarity to *P. pentosaceus* DSM 20336^T as shown in Fig 2.

Group IV consisted of three isolates, which were EL8-1, EL8-2 and EL10-2. They were tetrad forming cocci and did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan. They hydrolysed arginine and grew at 50^{0}C, at pH 4.0, 8.5 and in 3%, 6% NaCl but did not grow at pH 3.5 and 9.0. They fermented L-arabinose, cellobiose, D-fructose, D-galactose, D-mannose, ribose, salicin, trehalose and D-xylose but did not ferment raffinose, gluconate, lactose, melibiose, methyl-α-D-glucoside, sorbitol and sucrose. Variable fermentation was found in maltose, D-mannitol and rhamnose (Table 3). The 16S rRNA gene sequence of representative strain in this group (EL8-2) showed 99.5% (1,411 bps) similarity to *P. acidilactici* DSM 20284^T as shown in Fig 2.

Group V consisted of three isolates, which were EL3-1, EL6-1 and EL6-2. They were rod-shaped and contained *meso*-diaminopimelic acid in cell wall peptidoglycan. Hydrolysis of arginine was negative. They grew at pH 3.5 to pH 8.5, in 3% and 6% NaCl. Variable growth was found at pH 9. They almost fermented all carbohydrates including raffinose, L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, melibiose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and D-xylose. Variable fermentation was found in gluconate and methyl-α-D-glucoside (Table 3). The 16S rRNA gene sequence of representative strain in this group (EL3-1 and EL6-2) showed 99.93% and 100% similarity (1,434 and 1,399 bps), respectively, to *Lb. pentosus* JCM 1558^T as shown in Fig 2.

Group VI consisted of one isolate, EL2-1, and was cocci in chains. This isolate could hydrolyse arginine. It did not contain *meso*-diaminopimelic acid in cell wall peptidoglycan and did not grow at pH 3.5 but grew at pH 4.0 to pH 9.0, in 3% and 6% NaCl. The isolate fermented cellobiose, D-fructose, D-galactose, D-mannose, ribose, salicin and trehalose but did not ferment raffinose, L-arabinose, gluconate, lactose, maltose, D-mannitol, melibiose, methyl-α-D-glucoside, rhamnose, sorbitol, sucrose, or D-xylose (Table 3). The 16S rRNA gene sequence of strain EL2-1 showed 100% (1,385 bps) similarity to *Lc. garvieae* ATCC 49156^T as in Fig 2.

Group VII consisted of two isolates, which were BF13-1 and BF13-3. They were cocci in chains. The isolates produced gas from glucose. Hydrolysis of arginine was negative. They did not grow at pH 3.5, 4.0 and 9.0 but grew at pH 8.5, in 3% and 6% NaCl. They did not contain *meso*-Diaminopimelic acid. They fermented raffinose, L-arabinose, cellobiose, D-fructose, D-galactose, gluconate, lactose, maltose, D-mannose, melibiose, methyl-α-D-glucoside, sucrose and trehalose but did not ferment D-mannitol, rhamnose, ribose, salicin, sorbitol or D-xylose (Table 3). The 16S rRNA gene sequence of representative strain in this group (BF13-1) showed 99.93% (1,384 bps) similarity to *W. paramesenteroides* ATCC 33313^T as in Fig 2.

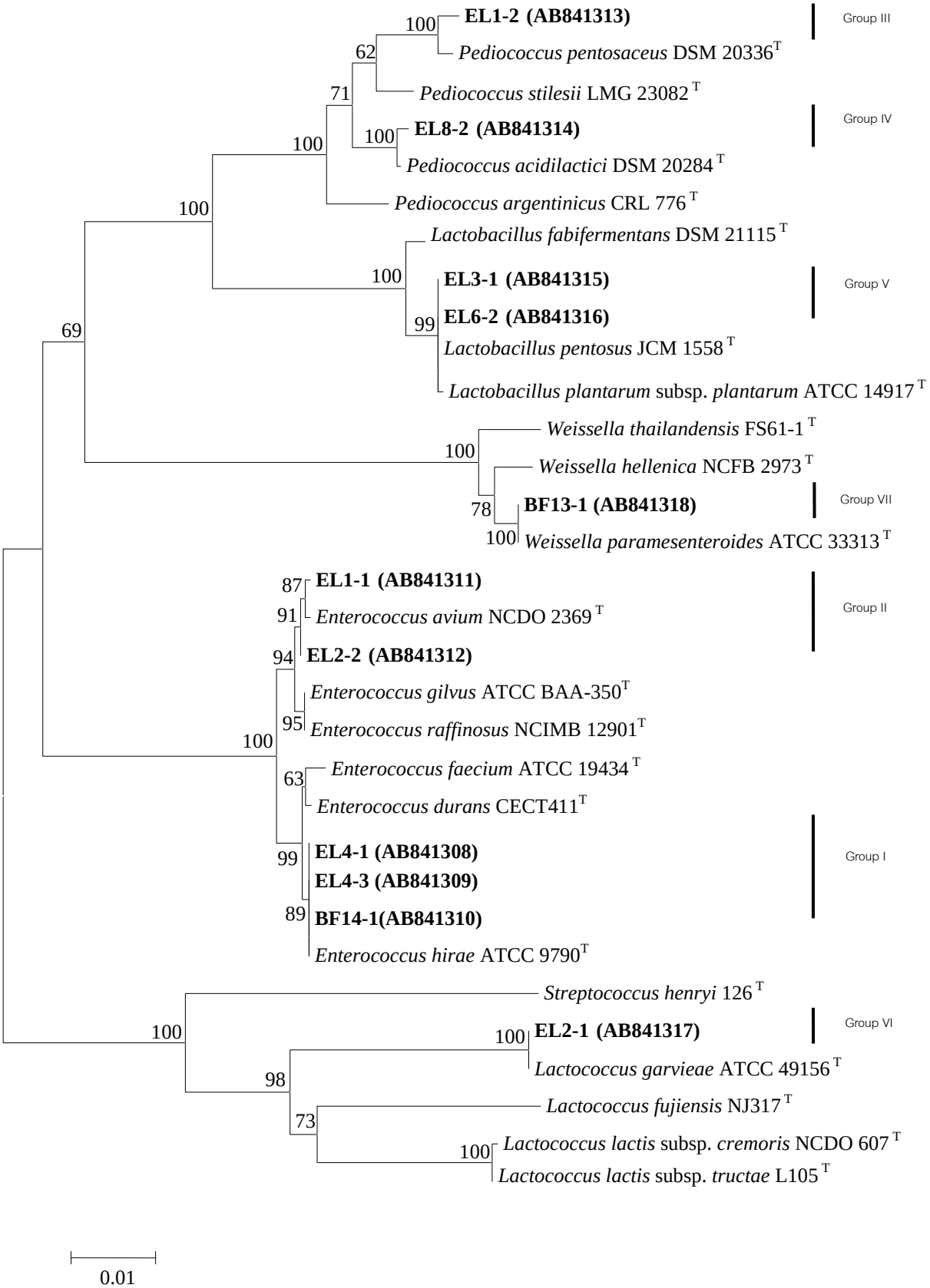

Figure 2 Phylogenetic relationships of isolates in group I to group VII based on 16s rRNA gene sequences. Phylogenetic tree was constructed by neighbor-joining method. Bar 0.005 substitutions per nucleotide position. Bootstrap values are expressed as percentages of 1000 replications.

Probiotic properties

The selected isolates of each group were observed for their tolerance to low pH (2.0 and 3.0) and bile salt at varied concentrations (0.1, 0.3, 0.5 and 1.0%) as shown in Table 4. As a result, all isolates were able to grow in the bile salt. They showed that the survival increased about 0.3-2 log values after 3 hours of incubation. Contrastly, in acidic condition, none of the isolates grew in the presence of pH 2.0 with the exception of isolates EL4-1 and EL8-2. Survival at pH 3.0 of the isolates was stable or slightly decreased about 0.5-0.7 log values after 3 hours of incubation. Only EL2-1 isolate could not survive at pH 2.0 and 3.0.

In order to determine the effects of LAB on the cytotoxic effects, we incubated Caco-2 cells and U937 cells with or without supernatants of LAB. At 24 hours post incubation, cytotoxicity was measured by MTT assays. In Caco-2 cells, it was shown that the OD values of LAB in groups I, II, III, IV, V, VI and VII were 1.234±0.018, 1.306±0.027, 1.293±0.007, 1.201±0.016, 1.313 ±0.057, 1.314±0.002, 1.236±0.010, respectively, and were not significantly different compared with control Caco-2 cells and standard that were 1.375±0.019 and 1.341±0.007, respectively, in OD value (Fig 3). In U937 cells, the cytotoxic effects observed from OD value were found insignificantly different as well as on Caco-2 cells. The OD values of control, standard, LAB in groups I, II, III, IV, V, VI, VII were 1.010±0.010, 1.007±0.028, 0.907±0.026, 0.852±0.029, 0.963±0.009, 0.812±0.037, 0.796±0.043, 0.940±0.001, 0.976±0.043, respectively (Fig 4). Thus, the supernatant of LAB showed no significant effects on human monocytic U937 cells and colon carcinoma Caco-2 cells proliferation.

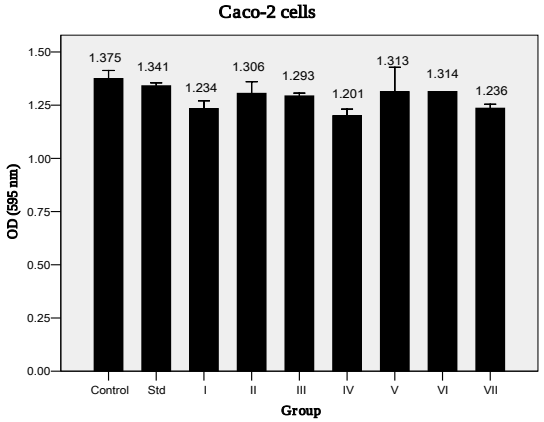

Figure 3 Effect of LAB on cytotoxicity assay of Caco-2 cells. Reduction in MTT values in LAB group did not significantly differ from the controls. Data shown above the bars are mean OD of each group. Error bars represent standard errors.

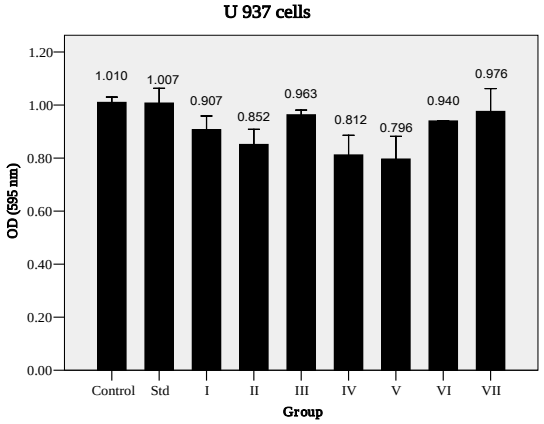

Figure 4 Effect of LAB on cytotoxicity assay of U937 cells. Reduction in MTT values in LAB groups did not significantly differ from the controls. Data shown above the bars are mean OD of each group. Error bars represent standard errors.

Table 3 Acid production from carbohydrates of LAB isolates from animal faeces

Carbohydrate	Isolate in Group						
	I	II	III	IV	V	VI	VII
L-Arabinose	-	+w	+	+	+	-	+
Cellobiose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+
Gluconate	-	+w	-	-	-(w1)	-	+
Lactose	+(-2)	-(+2)	-(+1)	-	+	-	+
Maltose	+(-3)	+	+	-(+1)	+	-	+
D-Mannitol	-	+	-(+1)	-(+1)	+	-	-
D-Mannose	+	+	+	+	+	+	+
Melibiose	+(-2)	+	+	-	+	-	+
Methyl-α-D-glucoside	-	+	-	-	+(-1)	-	+
Raffinose	-(+1)	+	-	-	+	-	+
Rhamnose	-(+2)	+	-	+(-1)	+	-	-
Ribose	+	+	+(-1)	+	+	+	-
Salicin	+(-1)	-(+1)	+	+	+	+	-
Sorbitol	-	+	-	-	+	-	-
Sucrose	+(-4)	-(+1)	+	-	+	-	+
Trehalose	+	-	+	+	+	+	+
D-Xylose	-(+2)	-	+	+	+	-	-

+ : positive, - : negative reaction, [a] : Number of isolates.
Numbers in parentheses indicate the number of isolates showing the reaction

Total and optical purity of lactic acid production

Results of lactic acid production and stereoisomer by using enzymatic reaction and HPLC analysis are shown in Table 5. After glucose fermentation, it was found that groups I to VII had an average between 21.68-112.15 g/l in total lactic acid production, 0.25-80.26 g/l in remaining glucose, 25.49-93.65% in yield of lactic acid and 0.30-1.56 value in lactic acid productivity. High lactic acid production, yield and productivity were found in EL6-2 (112.15/93.65/1.56; lactic acid/ %yield/ productivity). EL6-2 was DL-lactic acid producer. Lactic acid bacteria in groups I, II and VI were optically pure L(+)- lactic acid producers; they could produce an average L(+)-isomer of 92.21%, 98.90% and 98.87%, respectively. Especially, EL4-3 and BF14-1, identified as *E. hirae* produce the highest 100% of optically pure L (+)- lactic acid. Group VII was optically pure D(-)- lactic acid producers, producing an average D(-)-isomer of 99.16%. The BF13-3 isolate, identified as *W. paramesenteroides*, produced the highest amount of optically pure D (-)- lactic acid, 99.79%. In contrast, groups III, IV and V produced racemic DL-lactic acid.

Table 4 Acid and bile tolerance of isolates

Isolate no.	Viable counts (log_{10} CFU/ml)						
	0 hour	3 hours					
		pH 2.0	pH 3.0	0.1% oxgall	0.3% oxgall	0.5% oxgall	1.0% oxgall
EL4-1	6.40	5.05	6.41	8.1	7.87	7.83	7.74
EL2-2	6.38	0.00	5.62	7.33	7.30	7.20	7.07
EL1-2	6.89	0.00	6.90	8.05	7.88	7.78	7.50
EL8-2	7.02	3.90	7.01	7.99	7.98	7.90	7.87
EL3-1	6.31	0.00	6.35	6.76	6.75	6.76	6.61
EL2-1	7.03	0.00	0.00	8.32	8.14	8.02	7.87
BF13-1	6.74	0.00	6.20	7.60	7.51	7.38	7.53

Table 5 Lactic acid and remaining glucose of isolates

Isolate no.	Group	Lactic acid (g/l)			Remaining glucose (g/l)	Isomer (% Optical purity)
		Total lactic acid (g/l)	% Yield	Productivity (g/l.h)		
EL1-3	I	33.87	74.70	0.47	74.66	L (98.11)
EL2-3	I	37.95	71.77	0.53	67.12	L (38.87)
EL4-1	I	41.01	88.44	0.57	73.63	L (99.63)
EL4-2	I	32.49	62.72	0.45	68.20	L (99.75)
EL4-3	I	42.37	69.13	0.59	58.71	L (100.0)
EL7-1	I	38.18	70.31	0.53	65.70	L (98.66)
EL7-2	I	32.39	70.71	0.45	74.19	L (98.02)
EL10-1	I	68.49	85.08	0.95	39.50	L (89.27)
BF13-2	I	30.38	44.47	0.42	51.68	L (99.78)
BF14-1	I	38.54	73.38	0.54	67.48	L (100.0)
EL1-1	II	38.79	65.13	0.54	60.45	L (98.90)
EL2-2	II	38.26	54.80	0.53	50.18	L (nd)
EL5-2	II	21.68	25.49	0.30	34.96	L (nd)
EL9-1	II	25.41	33.65	0.35	44.48	L (nd)
EL1-2	III	31.39	46.73	0.45	52.83	DL (nd)
EL5-1	III	30.45	49.68	0.42	58.71	DL (nd)
EL5-3	III	31.75	60.85	0.44	67.82	DL (nd)
EL8-1	IV	53.94	88.02	0.75	58.72	DL (nd)
EL8-2	IV	50.32	84.50	0.70	60.45	DL (nd)
EL10-2	IV	42.84	65.24	0.60	54.33	DL (nd)
EL3-1	V	54.95	55.34	0.76	20.71	DL (nd)
EL6-1	V	89.14	86.58	1.24	17.04	DL (nd)
EL6-2	V	112.15	93.65	1.56	0.25	DL (nd)
EL2-1	VI	35.27	88.75	0.49	80.26	L (98.87)
BF13-1	VII	22.30	25.52	0.31	32.64	D (98.53)
BF13-3	VII	28.51	43.78	0.40	54.89	D (99.79)

nd : not detected

Discussion

In this study, we focused on the isolation and identification of lactic acid bacteria presented in elephant and buffalo faeces in Thailand. Based on the phenotypic characteristic results as described above, some characteristics such as cell form, arginine hydrolysis, cell wall type, isomer of lactic acid, growth at 50^0C, at pH 3.5, 4.0 and 9.0 and acid production from carbohydrates (Tables 2, 3 and 4) are useful for the differentiation of species in each group. The cocci in chains were identified as the genus *Enterococcus, Lactococcus* and *Weissella* whereas the tetrad forming cocci were identified as the genus *Pediococcus.* Rod-shaped isolates were identified as the genus *Lactobacillus* based on their 16S rRNA gene sequence and phylogenetic analysis (Fig 2) (Von Wright and Axelsson, 2011). Ten isolates in group I were identified as *E. hirae* (Devriese et al., 2006) while 4 isolates in group II were identified as *E. avium* (Devriese et al., 2006). Three isolates in group III could not grow at 50^0C and were identified as *P. pentosaceus*

while 3 isolates in group IV did grow at 50^{0}C and were identified as *P. acidilactici* (Holzapfel et al., 2006). Three rod-shaped isolates in group V containing *meso*-diaminopimelic acid in cell wall peptidoglycan were identified as *Lb. pentosus* (Tanasupawat et al., 2002). One coccal isolate in group VI was identified as *Lc. garvieae* (Fihman et al., 2006). Two coccal isolates in group VII producing gas from glucose were identified as *W. paramesenteroides* (Schillinger et al., 2008).

According to probiotic properties, the result demonstrated that all of the selected isolates were able to tolerate in bile salt while some isolates could tolerate in acid environment. It was found that only EL4-1 and EL8-2 had the strongest capacity for surviving acidic conditions and bile salts. However, the safety of strain is an important factor in probiotic properties. Thus, only *P. acidilactici* EL8-2 was possibly considered as a probiotic strain, which in agreement with a previous report by Guerra (2006). In cytotoxicity assay, it was found that all isolates showed no significant effects on human monocytic U937 cells and colon carcinoma Caco-2 cells proliferation. Therefore, this study might benefit for other research such as antimicrobial properties.

In lactic acid production, it was found that high lactic acid production, yield and productivity were found in EL6-2 (112.15/93.65/1.56; lactic acid/ %yield/ productivity). EL6-2 was DL-lactic acid producer. Development of the physical properties of polylactic acid depends on the isomeric compositions. The production of optically pure lactic acid is essential for the polymer synthesis (Wee et al., 2006). In our study, it was found that *E. hirae* EL4-3 and BF14-1 produced the highest amount of optically pure L (+)-lactic acid 100%. *W. paramesenteroides* BF13-3 produced the highest amount of optically pure D (-)-lactic acid 99.79%. However, the total lactic acid and productivity of these isolates were quite low. Improvement should be considered further to enhanced lactic acid concentration with higher productivity.

The isolation of LAB from poultry feces such as *E. faecalis* strain S37 exhibited an antilisterial activity and a slight anti-*Campylobacter* activity and *Lb. reuteri* strain S42 exhibited only anti-*Campylobacter* activity (Nazef et al., 2008); *Lb. salivarius, Lb. reuteri, Lb. johnsonii, P. acidilactici,* and *Lb. paralimentarius* strains from chicken carcasses (Sakaridisa et al., 2012); *Lb. ruminis* strains in the caecum and rectum of the pig (Al Jassim, 2003); *Lb. fermentum, Lb. salivarius, Lb. plantarum,* and *Lb. reuteri* from pig feces (Yun et al., 2009) including LAB in the foregut of the feral camel (Ghali et al., 2011) were studied.

In comparison to this study, we found *E. hirae*, *E. avium* and *Lc. garvieae* strains distributed in elephant faeces and only *E. hirae* and *W. paramesenteroides* strains were in buffalo faeces. Some strains of *E. hirae*, *E. avium* and *Lc. garvieae* were opportunistic and nosocomial pathogens that infected human (Watanabe et al., 2011). On the other hand, *P. pentosaceus, P. acidilactici* and *Lb. pentosus* strains were found only in elephant faeces (Table 1), however, the strains in these species were previously reported as probiotics (Yateem et al., 2008; Castex et al., 2008). The majority of LAB belonged to the genera *Enterococcus*, 38.46% were *E. hirae*. In lactic acid production, *E. hirae* and *W. paramesenteroides* were the potential L(+)-lactic acid and D(-)-lactic acid producing bacteria, respectively. Although the probiotic properties of isolates in inhibition of cancer cells proliferation showed no significant effects, *P. acidilactici*, EL8-2, may be regarded as a probiotic strain due to the safety and ability to tolerate low pH and bile salts.

This is the first report on the identification of LAB diversity in elephant and buffalo faeces as well as on the investigation of probiotic properties and pure stereoisomer lactic acid. Further studies will be conducted on the use of the isolate as a probiotic strain in other applications or in animal models.

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