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Characterization and Antibacterial Activity Against *Helicobacter pylori* of Lactic Acid Bacteria Isolated from Thai Fermented Rice Noodle

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Abstract
A total of 32 lactic acid bacteria (LAB) were isolated from Khanom-jeen, a Thai traditional fermented rice noodle. They belonged to the genus *Leuconostoc* (*Ln*), *Lactobacillus* (*Lb*), *Enterococcus* (*E*), *Lactococcus* (*Lc*), and *Weissella* (*W*), based on their phenotypic characteristics and 16S rRNA gene sequence analyses. The strains were identified as *Ln. pseudomesenteroides* (group 1, two strains), *Ln. citreum* (group 2, three strains), *Ln. lactis* (group 3, three strains), *Lb. paracasei* subsp. *tolerans* (group 4, two strains), *E. faecium* (group 5, three strains), *Lc. lactis* subsp. *lactis* (group 6, one strain), *W. confusa* (group 7, six strains), *Lb. fermentum* (group 8, seven strains), and *Lb. plantarum* subsp. *plantarum* and *Lb. pentosus* (group 9, five strains). Fifteen strains exhibited the inhibitory activity against *Helicobacter pylori* clinical isolates by spot-on-lawn method. *Lb. fermentum* P43-01 resisted to bile acids showed the broad spectrum of antimicrobial activity against *H. pylori* strains MS83 and BK364. These antagonistic effects were associated with proteinaceous compounds which are sensitive to α-chymotrypsin and pepsin. Results indicated that production of bacteriocin-like substances of selected strain might be the significant mechanism that exerted the inhibition on *H. pylori*. A potential strain could be used as probiotics in alternative or adjunctive therapy for a patient suffering from *H. pylori* infection.

Keywords Antimicrobial activity · Fermented rice noodle · *Helicobacter pylori* · Lactic acid bacteria · Bacteriocins

Introduction

Helicobacter pylori strain has been first successfully cultivated by Warren and Marshall in 1983 [1]. This bacterium is now recognized as a major cause of chronic gastritis and peptic ulcer and is a risk factor for gastric carcinoma [2]. *H. pylori* strains were found in the human stomach of about half of the world populations. The prevalence of infection of developing countries was higher (> 80%) than the developed ones (< 40%) [3]. Currently, the standard triple and sequential therapies have been widely used for *H. pylori* eradication. These therapies required proton pump inhibitor (PPI) plus antibiotics including amoxicillin, metronidazole, tinidazole, and clarithromycin [4]. However, antibiotic-based therapy had the significant effect on the composition of intestinal microbiota, would not be cost-effective, causes side effects, and in particular, encouraging widespread antibiotic resistance [5, 6]. Hence, the alternative or adjunctive therapy has been proposed.

Probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit to the host" [7]. The use of probiotics to improve intestinal health of humans has been purposed for many years [8]. Lactic acid bacteria (LAB; *Lactobacillus*, *Enterococcus*, etc.) and *Bifidobacterium* are known to be commonly used as probiotics [9]. The studies involving the antimicrobial activity against *H. pylori* in vitro and in vivo of probiotic LAB such as *Lb. acidophilus* LB [10], *Lactobacillus johnsonii* La1 [11], *Lb. casei* Shirota [12], *Lb. rhamnosus* GG [13], and *Lb. gasseri* OLL2716 have been reported [14].

LAB could inhibit the growth of *H. pylori* by production of organic acids, competitive adhesion, and reduction of cytokines [15, 16]. Moreover, antimicrobial peptide or bacteriocin secretion is one of the most interesting mechanisms which

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suppress the growth of pathogenic bacteria because it acts specifically to target bacteria only at nonamolar level [17]. Several investigations found that antimicrobial peptides produced by LAB exhibited antimicrobial activity against *H. pylori*; however, there are rarely [18–22]. Furthermore, there are only few reports on screening of LAB isolated from Thai traditional food products that have the ability to combat *H. pylori* clinical isolates and no one has studied on bacteriocin. Thus, the objective of this study was to isolate and identify LAB presented in Khanom-jeen, a Thai traditional fermented rice noodle, and to screen their ability to inhibit the growth of *H. pylori* in vitro.

Materials and Methods

Sample Collection, Isolation, Indicator Strains, and Cultivation

Thai fermented rice noodle, also called Khanom-jeen, was produced by a traditional lactic acid fermentation of rice flour [23]. Sixteen samples were collected from traditional Khanom-jeen manufacturer in Songkhla and Nakhonratchasima provinces, Thailand (Table 1). LAB were isolated by pour plate technique using MRS [24] agar containing 0.3% $CaCO_3$ and incubated at 30 °C for 2–3 days. The number of viable LAB found in samples was determined by counting with the colony-forming unit. Colonies surrounded by a clear zone were selected for purification. Pure cultures were maintained in MRS broth with 20% glycerol at − 80 °C and lyophilized.

H. pylori strains 2649, MS83, and BK364 isolated from a patient suffering from gastritis, peptic ulcer diseases, and gastric cancer, respectively were used as the indicator strains. These three clinical *H. pylori* strains were obtained from the Faculty of Medicine, Thammasat University, Pathum Thani, Thailand. *H. pylori* strains were cultivated on Columbia blood agar supplemented with 7% sheep blood and incubated at 37 °C for 3 days under microaerobic conditions using a gas-generating kit (Anaero Pack-MicroAero, Mitsubishi Gas Chemical, Japan). They were subcultured twice on the same medium before experimental use.

Identification of Strains

Phenotypic Characterization

Phenotypic characteristics of LAB strains were performed as described previously [25]. Cell morphology and colony appearance were determined after cultivating the strains on MRS agar plate at 30 °C for 48 h. Catalase and nitrate reduction, gas production, slime formation, hydrolysis of arginine and starch, acid production from carbohydrates, growth at temperature of 45 °C and at various pH values (3.5, 9.0, and 9.6), and NaCl tolerance (6% NaCl) were investigated. *Meso*-diaminopimelic acid (DAP) was determined as reported previously [26]. Lactic acid isomers were analyzed by the enzymatic method using D- and L-lactate dehydrogenases (Boehringer, Germany) [27].

Table 1 Source, LAB count, strain number and number of strains isolated from fermented rice noodle

Province	Sample	LAB count (cfu/g)	Strain no.	Number of strain
Songkhla	HB3	1.5×10^7	HB3-3, HB3-4	2
	SB1	4.0×10^7	SB1-2	1
	SC2	3.5×10^6	SC2-1	1
	SC3	3.6×10^7	SC3-4	1
Nakhonratchasima	P1	1.5×10^9	P1-1, P1-2A, P1-3	3
	P2	1.2×10^{10}	P2-2A, P2-4	2
	P3	3.5×10^9	P3-1	1
	P4	4.8×10^{11}	P4-1, P4-3, P4-6, P4-6A	4
	N11	2.4×10^{12}	N11-5, N11-7	2
	P22	2.0×10^{13}	P22-7, P22-7A, P22-9	3
	P23	4.0×10^{14}	P23-10, P23-13	2
	P32	1.8×10^{13}	P32-9	1
	N21	4.0×10^{13}	N21-9	1
	P41	6.0×10^4	P41-3	1
	P42	1.2×10^4	P42-1, P42-1A, P42-4, P42-4A	4
	P43	1.2×10^4	P43-1, P43-1A, P43-01	3
Total				32

Statistical Analysis

The LAB strains were grouped based on their phenotypic characteristics by cluster analysis. Hierarchical cluster analysis was performed by using SPSS for Windows version 16.0.

Genotypic Characterization

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the primers 20F (5′-AGTT TGATCCTGGCTC-3′) and 1530R (5′-AAGGAGGTGAT CCAGCC-3′). Amplification reaction was carried out in 50 μl volume, using *Taq* DNA polymerase and buffer system (Vivantis Technologies, Malaysia). The final PCR mixture consisted of 10× ViBuffer A (without $MgCl_2$; 50 mM KCl, 50 mM Tris-HCl (pH 9.1 at 20 °C) and 0.1% Triton™ X-100), 5 mM $MgCl_2$, 0.2 μM concentration of each deoxynucleoside triphosphate, 0.2 μM concentration of each primer, one unit of *Taq* DNA polymerase, and 50 ng of template DNA. Amplification was carried out in a thermocycler (C1000 Touch™, Bio-Rad Laboratories Inc., Berkeley, California) with the following cycling program: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 2 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 5 min [28]. Three microliters of the PCR product was electrophoresed for 40 min at 100 V on a 1.5% (*w*/*v*) agarose gel in 1× TAE buffer (40 mM Tris base (pH 7.6), 20 mM acetic acid, 1 mM EDTA, pH 8.0). A 10-kb DNA molecular weight marker (Vivantis Technologies, Malaysia) was included as standard for the calculation of the fragments. After staining with ethidium bromide (0.5 μg ml^{-1}) for 5 min, the fingerprint patterns were visualized under ultraviolet light followed by digital capture using the Gel Documentation system (Bio-Rad Laboratories Inc., Berkeley, California). PCR products were sequenced (Macrogen, Seoul, Korea) by using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [29]. The 16S rRNA gene sequences were multiple-aligned with the selected sequences obtained from the EzTaxon-e database [30] by using the CLUSTAL_X program [31]. Gaps and ambiguous bases were eliminated before reconstructing the phylogenetic tree by the neighbor-joining method [32] in the MEGA 6 program [33]. Confidence values of the branches of phylogenetic tree were evaluated by using the bootstrap resampling method with 1000 replications [34].

Screening of Anti-*H. pylori* Activity

Cells of LAB strain cultivated in MRS broth at 30 °C for 18 h were removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatant was collected and then filtered through a 0.2-μm pore-size filter. The antimicrobial activity was tested by the spot-on-lawn method as previously described with minor modification [35]. This assay was performed by spotting 10 μl of cell-free supernatant (CFS) onto a double layer including 5 ml of brain heart infusion (BHI) agar (0.7% agar) containing 10^7 cfu/ml of *H. pylori* culture which overlaid on the BHI agar medium (1.5% agar). After incubation under microaerobic conditions at 37 °C for 72 h, the inhibition zone (mm) on *H. pylori* lawn was measured.

Characterization of Antimicrobial Compound

LAB strain which showed a highest anti-*H. pylori* activity was selected to determine the nature of compounds responsible for antimicrobial activity. To exclude the effect of organic acids, CFS collected from the selected strain was neutralized to pH 6.5 by using 3 M Tris base. In addition, CFS was treated with different enzymes including pepsin, trypsin, α-chymotrypsin, proteinase K, and catalase at final concentration of 1 mg/ml. After incubation for 3 h at optimal pH and temperature for each specific enzyme, the antimicrobial activity of enzyme-treated samples was tested by critical dilution spot-on-lawn assay. The antimicrobial activity was expressed as arbitrary activity units (AU) per milliliters which were defined as the reciprocal of the highest dilution causing a clear zone of inhibition in the indicator lawn according to the following formula [35];

$$\textit{The antibacterial activity}\ (\mathrm{AU/ml}) = 2^N \times \ 100$$

where, AU is the arbitrary unit and N is the highest two-fold serial dilution showing an inhibition zone of the bacterial indicator strain.

Probiotic Properties Assays

Acid Tolerance

Acid tolerance was determined as a minor modification of the previous report [36]. Single colony of LAB strain was anaerobically cultivated in MRS broth at 37 °C for 18 h. Cells (4.0×10^9 cfu/ml) were centrifuged (8000×*g* for 5 min at 4 °C), washed twice with sterile normal saline (0.85% NaCl), and re-suspended in 10 ml of MRS broth. Cell suspension (1%) was inoculated into 10 ml of MRS with adjusting pH to 2.0 and 3.0 using 6 N hydrochloric acid (HCl). The cultures were then anaerobically incubated at 37 °C. At 0, 1.5, and 3 h of incubation, viable cell counts were determined on a MRS agar plate using the spread plate technique.

Bile Tolerance

Bile tolerance was evaluated with some modifications [37]. One hundred microliters of cell suspension was inoculated

into 10 ml of MRS broth supplemented with 0.3 and 0.5% of oxgall (Sigma-Aldrich, MD, USA). The cultures were then anaerobically incubated at 37 °C. After incubation at 0, 3, and 6 h, viable cell counts were examined on a MRS agar plate using the spread plate technique.

Results

The total number of LAB found in samples collected from Songkhla and Nakhonratchasima was ranged from 3.5×10^6 to 4.0×10^7 cfu/g and 1.2×10^4 to 4.0×10^{14} cfu/g, respectively. Thirty-two strains of LAB were isolated from 16 samples of the fermented rice noodle as shown in Table 1.

Identification of Strains

From the total 32 LAB strains, they were 14 rods, 6 coccobacilli, and 12 cocci and they appeared singly, in pair or in chain. All LAB strains were Gram-positive, catalase-negative, non-motile, non-spore forming bacteria. They were divided into nine groups based on their phenotypic characteristics as shown in Fig. 1. Based on the 16S rRNA gene sequence, LAB strains belonged to the genus *Lactobacillus*, *Weissella*, *Enterococcus*, *Lactococcus*, and *Leuconostoc* (Fig. 2).

Group 1 included two coccal strains (P4-6 and P4-6A). All produced gas from glucose. They grew at pH 9.0 and 9.6 and in 6% NaCl but did not grow at 45 °C and pH 3.5. The strains did not contain *meso*-DAP in the cell wall. They produced D-lactic acid. All strains could ferment tested carbohydrates but did not ferment glycerol, lactose, and raffinose (Table 2). The strains P4-6 and P4-6A showed 99.64% 16S rRNA gene sequence similarity to *Ln. pseudomesenteroides* JCM 9696^T (Fig. 2).

Group 2 contained three coccal strains (P42-1, P42-1A, and P41-3). These strains produced gas from glucose. All strains grew in 6% NaCl but did not grow at 45 °C. Variable pH growth was found from 3.5 to 9.6. They did not have *meso*-DAP in the cell wall. Tested strains produced D-lactic acid. All strains produced acid from tested carbohydrates except for glycerol, maltose, D-mannitol, ribose, and xylose (Table 2). The strains P42-1, P41-3, and P42-1A were closely related to *Ln. citreum* ATCC 49370^T with 99.78, 99.86, and 99.85% 16S rRNA gene sequence similarity, respectively (Fig. 2).

Group 3 consisted of three coccal strains (P3-1, P4-1, and P32-9). All strains produced gas from glucose. They grew at pH 3.5 and 9.0 and in 6% NaCl. Variable growth was observed at 45 °C and pH 9.6. These strains did not have *meso*-DAP in the cell wall. They produced only D-lactic acid. All strains fermented D-amygdalin, L-arabinose, cellobiose, D-galactose, gluconate, maltose, D-mannose, melibiose, raffinose, salicin, sucrose, trehalose, and xylose (Table 2).

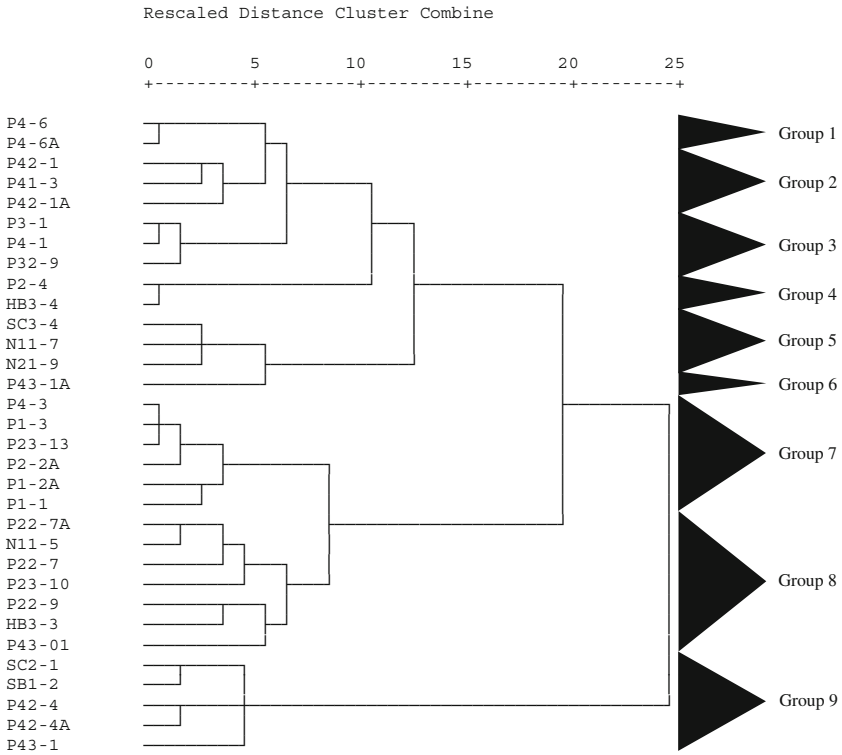

Fig. 1 Dendrogram using average linkage (between groups) showing the hierarchical cluster of the LAB strains based on their phenotypic characteristics

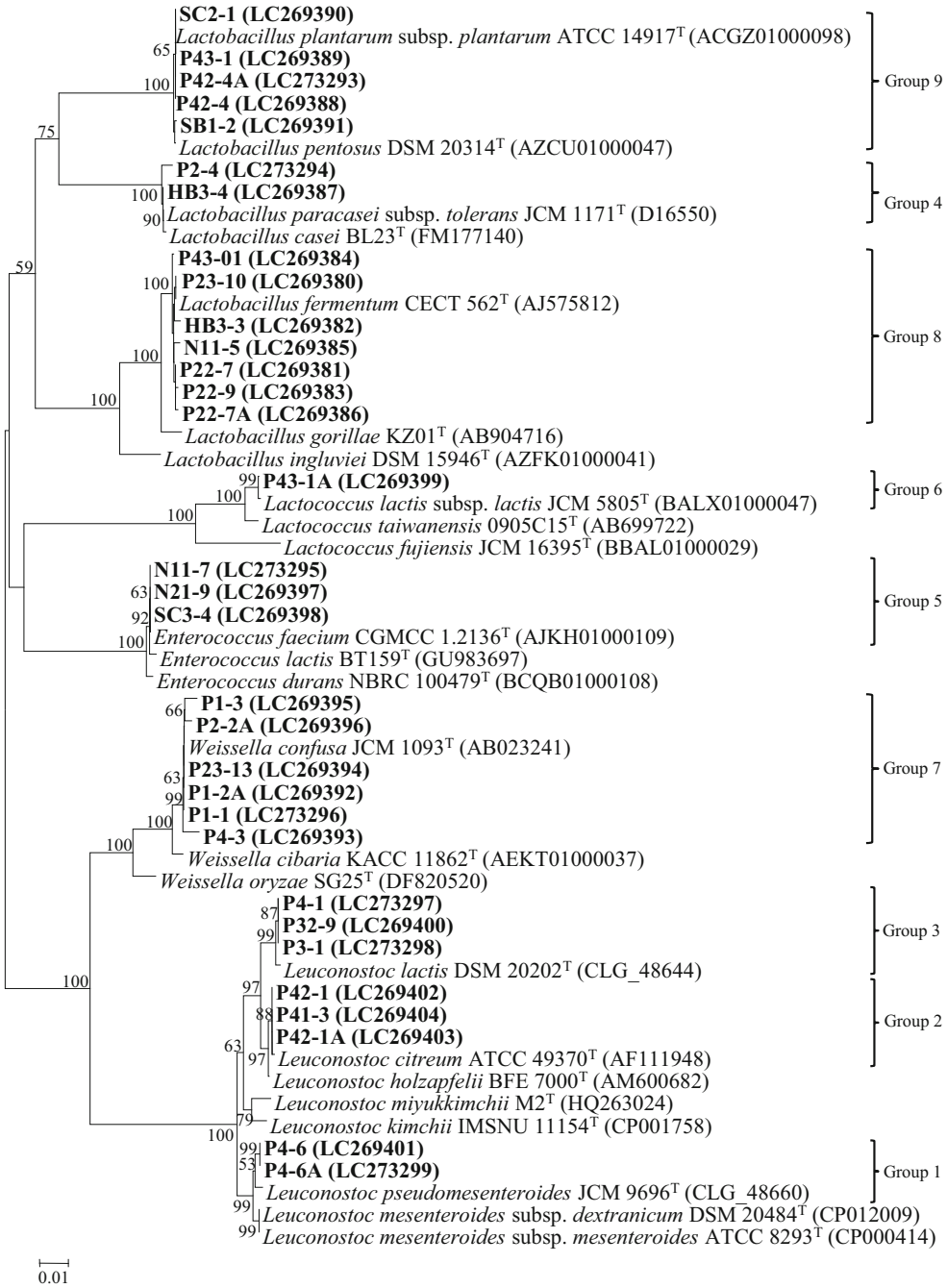

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequences showing the relationship among the representative strains in group 1 to group 9 and related species. Bootstrap percentages > 50%, based on 1000 replications, are given at nodes. Bar 0.01 substitutions per nucleotide position

Strains P3-1, P4-1, and P32-9 were closely related to *Ln. lactis* DSM 20202^T with 99.78% 16S rRNA gene sequence similarity (Fig. 2).

Group 4 included two rod-shaped strains (P2-4 and HB3-4). They did not produce gas from glucose. These strains grew at pH 3.5 and 9.0, 45 °C, and in 6% NaCl but did not grow at pH 9.6. They did not contain *meso*-DAP in the cell wall. They produced only L-lactic acid. All strains could produce acid from tested carbohydrate except for glycerol and rhamnose (Table 2). Strains P2-4and HB3-4 were closely related to *Lb. paracasei* subsp. *tolerans* JCM 1171^T with 99.57 and 100% similarity based on their 16S rRNA gene sequence (Fig. 2).

Group 5 contained three coccal strains (N21-9, SC3-4, N11-7). All strains could hydrolyze arginine. They grew at 45 °C, pH 9.0, and in 6%NaCl but did not grow at pH 3.5. These strains did not contain *meso*-DAP in the cell wall and tested strains produced L-lactic acid. All strains produced acid from tested carbohydrates but did not produce acid from gluconate, glycerol, α-methyl-D-glucoside, and xylose. Variable fermentation was observed from D-mannitol, rhamnose,

Table 2 Characteristics of LAB strains isolated from fermented rice noodle

Characteristics	Group 1 (2)[a]	Group 2 (3)	Group 3 (3)	Group 4 (2)	Group 5 (3)	Group 6 (1)	Group 7 (6)	Group 8 (7)	Group 9 (5)
Cell form	Cocci	Cocci	Cocci	Rods	Cocci	Cocci	Coccobacilli	Rods	Rods
Gas from glucose	+	+	+	–	–	–	+	+	–
Arginine hydrolysis	–	–	–	–	+	+	–	+ (−3)	–
Growth at 45 °C	–	–	+ (−1)	+	+	–	− (+2)	+ (−1)	− (+1)
Growth at pH 3.5	–	+ (−1)	+	+	–	–	–	+	+
pH 9.0	+	+ (−1)	+	+	+	+	+ (−1)	–	− (+2)
pH 9.6	+	+ (−1)	− (+1)	–	− (+1)	+	+ (−1)	–	− (+1)
Growth in 6% NaCl	+	+	+	+	+	+	+ (−1)	+ (−1)	+
D-Amygdalin	+	+	+	+	+	+	–	–	+
L-Arabinose	+	+ (−1)	+	+ (−1)	+	+	–	+ (−3)	− (+1)
Cellobiose	+	+	+	+	+	+	–	–	+
D-Galactose	+	+	+	+	+	+	+	+ (−1)	+ (−2)
Gluconate	+	+	+	+	–	–	+ (−1)	+ (−2)	+
Glycerol	–	–	–	–	–	–	–	− (+2)	− (+1)
Lactose	–	+	− (+1)	+	+	+	–	+ (−3)	+ (−2)
Maltose	+	–	+	+	+	+	+ (−1)	+ (−3)	+
D-Mannitol	+	–	–	+	+ (−1)	+	–	− (+3)	+
D-Mannose	+	+	+	+	+	+	+ (−1)	+ (−3)	+
Melibiose	+	+	+	+	+	–	–	+ (−2)	+ (−1)
α-Methyl-D-glucoside	+	+	–	+	–	+	–	− (+3)	–
Raffinose	–	+	+	+	+	–	–	+ (−2)	− (+2)
Rhamnose	+	+	–	–	− (+1)	–	–	–	–
Ribose	+	–	–	+	+ (−1)	–	+ (−3)	− (+1)	+
Salicin	+	+	+	+	+	+	− (+1)	–	+
Sorbitol	+	+	–	+	+	–	–	–	− (+2)
Sucrose	+	+ (−1)	+	+	+ (−1)	+	− (+2)	+ (−2)	+
Trehalose	+ (−1)	+ (−1)	+	+	+	+	–	− (+3)	+
Xylose	+	–	+	+	–	–	+ (−1)	− (+2)	− (+1)
meso-Diaminopimelic acid	–	–	–	–	–	–	–	–	+
Isomer of lactic acid	D	D	D	L	L	L	DL	DL	DL

+, positive; −, negative reactions

[a] Number of strains. Numbers in parentheses indicate the number of strains showing the reaction

ribose, and sucrose (Table 2). The strains (N21-9, SC3-4, and N11-7) were closely related to *E. faecium* CGMCC 1.2136^T with 100, 99.93, and 99.93% 16S rRNA gene sequence similarity (Fig. 2).

Group 6 contained one coccal strain (P43-1A). It could hydrolyze arginine. The strain grew at pH 9.0 and 9.6 and in 6% NaCl but did not grow at 45 °C and pH 3.5. It did not have *meso*-DAP in the cell wall. This strain produced L-lactic acid. It produced acid from D-amygdalin, L-arabinose, cellobiose, D-galactose, lactose, maltose, D-mannitol, D-mannose, α-methyl-D-glucoside, salicin, sucrose, and trehalose (Table 2). This strain was closely related to *Lc. lactis* subsp. *lactis* JCM 5805^T with 99.93% 16S rRNA gene sequence similarity (Fig. 2).

Group 7 consisted of six coccobacillary strains (P1-1, P1-2A, P23-13, P1-3, P2-2A, and P4-3). All strains produced gas from glucose. They did not grow at pH 3.5. Variable growth was observed at 45 °C, pH 9.0 and 9.6, and in 6% NaCl. Tested strains did not have *meso*-DAP in cell wall and produced DL-lactic acid. All strains produced acid from D-galactose. Variable acid production was found in gluconate, maltose, D-mannose, ribose, salicin, sucrose, and xylose (Table 2). The strains P1-1, P1-2A, P4-3, P23-13, P1-3, and P2-2A were closely related to *W. confusa* JCM 1093^T with similarity 99.78, 100, 100, 100, 99.64, and 99.64% based on their 16S rRNA gene sequences (Fig. 2).

Group 8 included seven rod-shaped strains (P23-10, P22-7, HB3-3, P22-9, P43-01, N11-5, and P22-7A). All strains

produced gas from glucose. Some strains could hydrolyze arginine. They grew at pH 3.5 but did not grow at pH 9.0 and 9.6. Variable growth was observed at 45 °C and in 6% NaCl. These strains did not contain *meso*-DAP in cell wall. They produced DL-lactic acid. All strains did not produce acid from D-amygdalin, cellobiose, rhamnose, salicin, and sorbitol. Variable acid production was found in L-arabinose, D-galactose, gluconate, glycerol, lactose, maltose, D-mannitol, D-mannose, melibiose, α-methyl-D-glucoside, raffinose, ribose, sucrose, trehalose, and xylose (Table 2). Based on 16S rRNA gene sequences, these strains (P23-10, P22-7, HB3-3, P22-9, P43-01, N11-5, and P22-7A) were closely related to *Lb. fermentum* CECT 562^T with 100, 99.93, 99.78, 99.57, 99.86, 99.64, and 99.64% similarity, respectively (Fig. 2).

Group 9 comprised five rod-shaped strains (SB1-2, P42-4, P42-4A, P43-1, and SC2-1). They did not produce gas from glucose. All strains grew at pH 3.5 and in 6% NaCl. Variable growth was found at 45 °C and at pH 9.0 and 9.6. These strains contained *meso*-DAP in the cell wall and produced DL-lactic acid. All strains produced acid from tested carbohydrates but did not produce acid from α-methyl-D-glucoside and rhamnose. Variable acid production was found in L-arabinose, D-galactose, glycerol, lactose, melibiose, raffinose, sorbitol, and xylose (Table 2). Strain SB1-2 was closely related to *Lb. pentosus* DSM 20314^T with 99.93% similarity of 16S rRNA gene sequence. Strains P42-4, P42-4A, P43-1, and SC2-1 were closely related to *Lb. plantarum* subsp. *plantarum* ATCC 14917^T with 100% similarity of 16S rRNA gene sequence (Fig. 2).

Antimicrobial Activity Against *H. pylori* Strains

Thirty-two strains of LAB isolated from the fermented rice noodle were tested for their anti-*H. pylori* activity by the spot-on-lawn method. Fifteen LAB strains exhibited different levels of *H. pylori* inhibition as shown in Table 3. Eleven strains of LAB (P42-1, P42-1A, P32-9, N21-9, P4-3, P23-13, HB3-3, P22-7, P22-7A, P22-9, and P43-01) could suppress the growth of *H. pylori* BK364, which were in the range of 6.0 to 11.5 mm. Four strains (HB3-4, P43-1A, P43-01, and SB1-2) displayed the inhibition against *H. pylori* MS83, which were in the range of 5.8 to 9.0 mm. Only one strain (SC3-4) showed the suppression against *H. pylori* 2649 (5.8 mm). *H. pylori* strain BK364 was the most sensitive whereas strain 2649 was less sensitive to CFSs collected from LAB. CFS from strain P43-01 showed the highest activity which was able to inhibit the growth of two clinical strains of *H. pylori* (strains MS83 and BK364) whereas CFSs from other strains inhibited only one strain of *H. pylori*. Strain P43-01 was selected and identified. Based on 16S rRNA gene sequence similarity, the selected strain was identified as *Lb. fermentum* P43-01 as describe previously.

Characterization of Anti-*H. pylori* Substances

Effect of neutralization, catalase, and protease treatment on inhibitory activity of CFS obtained from *Lb. fermentum* P43-01 was presented as Table 4. The results showed that the anti-*H. pylori* activity of untreated CFS against *H. pylori*

Table 3 Anti-*H. pylori* activity of CFSs collected from LAB

Group	Identification	Strain no.	Diameter of inhibition zone (mm) against *H. pylori* strains		
			2649	MS83	BK364
2	*Ln. citreum*	P42-1	–	–	7.0 ± 1.8[a]
		P42-1A	–	–	8.3 ± 1.3
3	*Ln. lactis*	P32-9	–	–	6.3 ± 1.0
4	*Lb. paracesei* subsp. *tolerans*	HB3-4	–	8.5 ± 0.6	–
5	*E. faecium*	N21-9	–	–	6.0 ± 1.2
		SC3-4	5.8 ± 1.0	–	–
6	*Lc. lactis* subsp. *lactis*	P43-1A	–	7.0 ± 2.4	–
7	*W. confusa*	P4-3	–	–	6.5 ± 0.7
		P23-13	–	–	6.3 ± 1.0
8	*Lb. fermentum*	HB3-3	–	–	6.5 ± 0.6
		P22-7	–	–	11.0 ± 0.6
		P22-7A	–	–	9.3 ± 1.0
		P22-9	–	–	8.0 ± 0.0
		P43-01	–	9.0 ± 1.2	11.5 ± 1.2
9	*Lb. pentosus*	SB1-2	–	5.8 ± 1.0	–

–, no inhibition

[a] Results indicate mean ± SD of two independent experiments

Table 4 Inhibitory activity against *H. pylori* of CFS collected from *Lb. fermentum* P43-01 after neutralization and enzyme treatments

Indicator strain	Anti-*H. pylori* activity (AU/ml)						
	Untreated	Neutralized	Catalase	Pepsin	Trypsin	α-Chymotrypsin	Proteinase K
H. pylori MS83	200	200	200	100	200	–	200
H. pylori BK364	200	200	200	100	200	–	200

–, no inhibition

MS83 and BK364 was 200 AU/ml, and after CFS neutralization, their antimicrobial activity did not change. In addition, the inhibitory activity of CFS against two clinical *H. pylori* strains did not alter when treated with catalase. However, CFS was inactivated after incubation with α-chymotrypsin and reduced from 200 to 100 AU/ml when treated with pepsin. Trypsin and proteinase K were not effective on the anti-*H. pylori* activity of CFS. These results indicated that the inhibitory substance might be the proteinaceous compounds or bacteriocins.

Probiotic Properties of Selected Strain

Survival of *Lb. fermentum* P43-01 after exposures to low pH (2.0 and 3.0) and bile salts (0.3 and 0.5%) was shown in Table 5. Decreases in viable cells were observed through the incubation period at pH 2.0. The survival of a selected strain was decreased about 2 log cycles after incubation at pH 2.0 for 3 h. At pH 3.0, the viability of a selected strain was constant at 7.6 $\log_{10}$ cfu/ml during 3 h of incubation. A strain was able to grow in the presence of 0.3 and 0.5% of oxgall. There were increases in viable cell count around 1.2 and 1 log cycles after exposures to 0.3 and 0.5% of oxgall for 6 h.

Discussion

This study focused on isolation and identification of lactic acid bacteria presented in fermented rice noodle produced in Thailand. The number of LAB presented in fermented rice noodle samples obtained from two producers in Thailand ranged from 10^4 to 10^{14} cfu/g (Table 1). Samples collected from Songkhla had lower LAB counts than that obtained from Nakhonratchasima. LAB that involved in Khanom-jeen process were *Lb. plantarum*, *Lb. fermentum*, and *Pediococcus acidilactici* [23]. In this study, the differentiation of LAB in each group was carried out based on their phenotypic characteristics such as cell form; gas production; arginine hydrolysis; growth at 45 °C, at pH 3.5, 9.0, and 9.6, and in 6% NaCl; cell wall type; lactic acid isomer; and acid production from carbohydrates (Table 2). The coccal strains belonged to the genera *Leuconostoc*, *Enterococcus*, *Lactococcus*, and *Weissella* whereas rod-shaped strains belonged to the genus *Lactobacillus* based on their 16S rRNA gene sequence and phylogenetic analysis (Fig. 2) [38]. Two heterofermentative cocci that produced D-lactic acid in group 1 were identified as *Ln. pseudomesenteroides* while three heterofermentative cocci in group 2 were identified as *Ln. citreum* and three heterofermentative cocci in group 3 were identified as *Ln. lactis* [39]. Two heterofermentative rods in group 4 which did not have *meso*-DAP in the cell wall were identified as *Lb. paracasei* subsp. *tolerans* [40]. Three homofermentative cocci in group 5 were identified as *E. faecium* while one homofermentative coccus in group 6 was *Lc. lactis* subsp. *lactis* [41, 42]. Six heterofermentative coccobacilli in group 7 were identified as *W. confusa* [43]. Seven heterofermentative rods in group 8 were identified as *Lb. fermentum* while five homofermentative rods in group 9 that contained *meso*-DAP were identified as *Lb. plantarum* subsp. *plantarum* and *Lb. pentosus* [40].

H. pylori infection remains a worldwide spread disease with a definite morbidity and mortality. Antibiotic-based therapy has been applied to treat patients who were infected with *H. pylori*. Unfortunately, no current therapy is able to achieve a 100% success rate due to the antibiotic resistance of

Table 5 Acid and bile tolerances of *Lb. fermentum* P43-01

Incubation time (h)	Viable counts ($\log_{10}$ cfu/ml)[a]		Incubation time (h)	Viable counts ($\log_{10}$ cfu/ml)	
	pH 2.0	pH 3.0		0.3% oxgall	0.5% oxgall
0	7.5 ± 0.2	7.6 ± 0.0	0	7.6 ± 0.1	7.7 ± 0.0
1.5	6.2 ± 0.0	7.6 ± 0.0	3	8.0 ± 0.1	7.8 ± 0.1
3	5.7 ± 0.1	7.6 ± 0.0	6	9.1 ± 0.1	8.7 ± 0.1

[a] Results indicate mean ± SD of two independent experiments

H. pylori [4, 44]. Thus, the need for alternative or adjunctive therapy such as the use of probiotics for the *H. pylori* eradication has claimed by many researches [2, 45].

This study reported the anti-*H. pylori* activity of LAB strains isolated from Khanom-jeen. Fifteen LAB strains exhibited different spectrum of antimicrobial activity against clinical strains of *H. pylori*. The susceptibility of clinical *H. pylori* isolates to CFSs collected from LAB seemed to be strain-dependent sensitivity. *H. pylori* 2649 isolated from patient with gastritis was more tolerant than strains MS83 and BK364. It was well known that antimicrobial metabolites produced by LAB included organic acids (mainly lactic and acetic acid), diacetyl, hydrogen peroxide, and bacteriocin [46]. The broad antimicrobial spectrum that showed inhibition on *H. pylori* strains MS83 and BK364 was found in CFS produced by *Lb. fermentum* P43-01. Although, CFS collected from selected strain was neutralized by adjusting pH to 6.5, but the anti-*H. pylori* activity was still stable. Moreover, catalase treatment did not change its inhibitory activity. The results indicated that antimicrobial activity did not form organic acids and H_2O_2. However, the inhibitory activity of CFS from selected strains was destroyed by α-chymotrypsin and reduced by pepsin. Antimicrobial metabolites secreted by *Lb. fermentum* P43-01 which suppressed the growth of clinical isolates of *H. pylori* might be bacteriocins.

Anti-*H. pylori* activity from LAB has been reported in many investigations. In addition to lactic acid and low pH, bacteriocin-like substances produced by LAB seem to be an important factor which exerts the inhibition of *H. pylori* growth. *E. faecium* TM39 produced bacteriocin-like substance with antagonistic activity against *H. pylori*, in addition to lactic acid and pH [18]. Bacteriocin secreted by *W. confusa* PL9001 was the major substance which suppressed the growth of *H. pylori* [19]. Six strains of *Bifidobacterium* from human feces showed inhibitory activity against clinical *H. pylori* isolates by producing heat-stable proteinaceous compound [20]. Bulgaricin BB18 secreted by *Lb. bulgaricus* BB18 showed the inhibition against *H. pylori* [21]. *Lb. brevis* BK11 and *E. faecalis* BK61 produced bacteriocin which displayed strong inhibition on the growth of *H. pylori* [47]. Among bacteriocins tested (nisin A; lacticins A164, BH5, JW3, and NK24; pediocin PO2; and leucocin K), lacticins A164 and BH5 produced by *Lc. lactis* A164 and *Lc. lactis* BH5 showed the strongest inhibition on the growth of *H. pylori* [48]. The effects of antimicrobial peptides on the growth of *H. pylori* have been less infrequently reported when compared with those of other pathogenic bacteria such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium difficile*, *Escherichia coli*, and *Salmonella* spp. More researches that involved antimicrobial peptides with anti-*H. pylori* activity which produced by LAB were thus needed. However, this is the first report regarding the antimicrobial peptides produced by *Lb. fermentum* P43-01 which showed the inhibition against *H. pylori*. To evaluate their possible use in the treatment of *H. pylori* infection, bacteriocin purification and their characterization should be performed in further studies.

Prior to passing through the intestinal tract, probiotics must survive under severe conditions in the stomach with very low pH. Moreover, they have to resist bile acids when they reach the intestine [49]. According to acid and bile tolerance, the viable cells of selected strain were substantially declined during incubation at pH 2.0, but the survived cells was remained at about 5.7 log cycles. A strain showed the resistance when it was exposed to pH 3.0. In addition, it could grow in the medium supplemented with 0.3 and 0.5% of bile salts. In agreement with the study of [50], most *Lb. fermentum* isolates exhibited high tolerance to low pH as well as high bile concentration with minimum cell count loss. These results revealed that *Lb. fermentum* P43-01 displayed an ability to tolerate an acidic conditions and bile salts, thus, it could be a potential probiotic candidate.

Conclusions

This study provides the distribution of *Lactobacillus*, *Weissella*, *Enterococcus*, *Lactococcus*, and *Leuconostoc* presented in Khanom-jeen, a Thai fermented rice noodle. Fifteen strains exhibited the antimicrobial activity against clinical *H. pylori* isolates. *Lb. fermentum* P43-01 is a candidate strain to use as probiotics for alternative or adjunctive therapy in patient infected with *H. pylori*. This strain could resist bile acids and produce antimicrobial peptides against *H. pylori* in vitro.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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