JOURNAL OF CULTURE COLLECTIONS
Volume 5, 2006-2007, pp. 46-57

CHARACTERIZATION OF LACTIC ACID BACTERIA FROM TRADITIONAL THAI FERMENTED SAUSAGES

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Summary

Lactic acid bacteria from traditional Thai fermented sausages were characterized. The fermented sausages were mainly produced from minced pork/beef or pork/beef liver. Sixty five strains were isolated from 12 samples collected from the central and northeastern parts of Thailand. The strains were identified by conventional morphological, cultural, physiological and biochemical tests as well as by 16S rDNA sequence analysis. The isolates were identified as Weissella cibaria/kimchii (5), W. confusa (3), Pediococcus pentosaceus (20), P. acidilactici (2), Lactobacillus fermentum (3), L. brevis (4), L. farciminis (4), L. plantarum (23), and L. sakei (1). Some of these species have not been previously isolated from Thai fermented sausages. The inhibition tests against Bacillus cereus and Staphylococcus aureus showed that 5 isolates could inhibit the growth of B. cereus and 2 of them could also inhibit S. aureus. The isolates were identified as W. confusa (1), P. acidilactici (1), L. plantarum (3). Three strains identified as L. plantarum and one as Weissella spp. could produce diacetyl.

Key words: *fermented sausage, lactic acid bacteria, Weissella, Lactobacillus, Pediococcus, 16S DNA, diacetyl, bacteriocin.*

Introduction

"Sai-krork-prieo" is a traditional Thai fermented sausage, consumed all around the country and produced from minced pork, garlic, pepper, spices, salt and sugar. "Mum" is a product similar to "Sai-krork-prieo", but can be made from beef as well as pork, and is popular only in the Northeastern of Thailand.

Lactic acid bacteria (LAB) play an important role in the ripening process of raw fermented sausages. However, the production of traditional fermented sausages in Thailand has utilized the naturally occurring LAB, resulting in various and inconstant products. Natural fermentations could vary from the simple to the complex ones. In general, each fermentation takes place under conditions that the producers have found to be favorable for the appropriate growth and action of microorganisms. Alternative to the natural fermentation, the use of well-studied starter cultures would result in more constant and safe food products.

The aim of this study was the isolation and identification of LAB from traditional fermented sausages in Thailand. In addition, their capability of producing metabolic compounds with antimicrobial property, i.e., diacetyl and bacteriocins, is reported. The strains found in this study have a potential use in the establishing of the so-called "functional foods" [12, 17].

Materials and methods

Sample collection, bacterial cell counts, and isolation method. Twelve fermented sausage samples of various brands were collected from factories and local markets. The pH of the samples was represented by the pH of the suspension of a 5-gram portion in 10 ml deionized water. The cell numbers were counted by a plating method on De Man, Rogasa and Sharpe agar (MRS agar) [3] at 30 ^{o}C after a 3- to 5-day incubation. Pure cultures were obtained by streaking cultured cells on MRS agar plates with 0.2 % $CaCO_3$.

Morphological and cultural characteristics. Cell form, cell size, cell arrangement, and colony appearance were examined on cells grown on a half strength MRS (MRSH) agar incubated for 3 days. Hucker-Conn modification [8] was used for gram stain. Spore formation was examined in gram-stained specimens. Motility was detected by the appearance of stab cultures in soft agar [30].

Biochemical and physiological characteristics. Catalase, nitrate reduction, hydrolysis of esculin, arginine, slime formation and reactions in litmus milk were investigated as described by Tanasupawat et al. [20, 23]. Catalase activity was detected on cells grown on MRSH agar. Nitrate reduction was tested after incubation of cells for 7 days in a medium composed of 1.0 g KNO_3, 3.0 g yeast extract, 5.0 g peptone, 0.2 g beef extract, 5.0 g NaCl, 0.25 g Tween 80, 1.0 g agar, and 1000 ml deionized water, adjusted to pH 6.8. Growth at different temperature (30, 45, and 50 ^{o}C), at different starting pH (3.5, 4.0, 4.5, 8.0, 8.5 and 9.6), and at different concentrations of NaCl (4, 6, 8, and 10 %) were tested by using MRSH broth. The production of gas from D-glucose was examined by using MRS broth with a Durham tube. Acid production from carbohydrates was determined as described in Tanasupawat et al. [23] by the use of a basal medium of GYPB broth with the omission of glucose. The GYPB medium contained 10.0 g glucose, 5.0 g yeast extract, 5.0 g peptone, 2.0 g beef extract, 2.0 g sodium acetate, 0.25 g Tween 80, 200 mg $MgSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot 4H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5.0 g NaCl, and 1000 ml deionized water, adjusted to pH 6.8. The acid produced in 3 ml broth was titrated with 0.1 N NaOH.

Peptidoglycan type of the cell wall. Meso-diaminopimelic acid (DAP) in the cell wall was detected by hydrolysis of 3 mg dried cells grown in GYPB broth. Hydrolysis was performed with 1 ml 6 N HCl at 100 ^{o}C for 18 h, and the hydrolysate was applied on cellulose TLC plate (Merck no. 5577). The TLC plate was developed with the solvent system of methanol-pyridine-12 N HCl-water (80: 10: 1.5: 17.5) (v/v) [11]. Spots were visualized by spraying with 0.5 % ninhydrin solution in n-butanol followed by heating at 100 ^{o}C for a few minutes.

Isomers of lactic acid. The strains tested were cultivated in GYPB broth for 3 to 5 days. Lactic acid was analyzed enzymatically according to Okada et al. [15] using D- and L-lactate dehydrogenase (Boehringer, Germany).

Screening for diacetyl formation. Diacetyl formation was screened by the method modified from Mattessich and Cooper [13] and Phalip et al. [16], which yielded qualitative results. Cells were grown in MMRS broth at 35 ^{o}C for 3-5 days. Hundred ml of the MMRS broth contained 0.18 g glucose, 0.5 g yeast extract, 1.0 g peptone, 1.0 g beef extract, 0.5 g sodium acetate, 1.29 g trisodium citrate x $2H_2O$, 0.15 g Tween 80, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.005 g $MnSO_4 \cdot H_2O$, and 0.2 g K_2HPO_4. One ml of each test solution (0.5 % creatine solution and 7.5 % α-naphthol in 2.5 N NaOH) was added into each 3 ml of culture medium. The degree of change in the culture medium color was recorded.

Screening for bacteriocin production. The screening method was modified from Yin et al. [31]. Each LAB strain was grown on Trypticase soy agar (without glucose) with a 2.0 % yeast extract supplement (TSAYE) slant at 37 ^{o}C for 2 days. The cultured LAB cells were streaked on TSAYE plate. Working pathogens (*Bacillus cereus* TISTR 037 and *Staphylococcus aureus* TISTR 029 from Thailand Institute of Scientific and Technological Research) were cultured in nutrient agar (NA) slant at 37 ^{o}C for 2 days. The cultured pathogen cells were transferred to 0.85 % saline solution to obtain a cell density of 10^5-10^6 colony-forming units (CFU) per ml. One ml of the suspended pathogen cells was mixed with 15 ml of melted Trypticase soy agar (TSA) at 50 ^{o}C. The melted TSA was then overlaid on the TSAYE plate with growing LAB. The plate with the overlay was incubated anaerobically at 37 ^{o}C for 2 days. The control TSAYE plate with a pathogen overlay but without cultured LAB was incubated in parallel for comparison. Inhibition zones revealed by colony appearance were recorded.

Sequencing and comparison of 16S rRNA gene. Template DNA for 16S rRNA gene amplification was prepared by the method modified from Nilsson et al. [14]. Two loops from an overnight MRS plate culture were transferred into 100-500 µl of TE buffer (10 mM Tris-Cl, pH 7.5, and 1 mM EDTA). Samples were boiled for 10-15 min. Then, the debris was pelleted by centrifugation at 12000 rpm for 10 min, and 50-

200 µl of the supernatant was collected. The 16S rRNA gene was amplified using the PCR method with a 1U *Taq* DNA polymerase (Bio-Lab Ltd., Auckland, New Zealand) and the primers UFUL (GCCTAACACATGCAAGTCGA) and URUL (CGTATTACCGCGGCTGCTGG) (Great American Gene Co., California, USA). These primers target two highly conserved regions known to be variable among bacterial species [4, 29]. The 16S rRNA gene was sequenced by using a BigDye v. 3.1 cycle sequencing kit (Applied Biosystems, California, USA), according to the manufacturer's protocol, with UFUL as primer. The 16S rRNA gene sequences determined (ca. 400-500 bases) were aligned along with the sequences of type strains obtained from the GenBank by using the program CLUSTAL X (version 1.82) [28]. Distance matrices for aligned sequences were calculated by the two-parameter method of Kimura [10]. A phylogenic tree was constructed by the neighbor-joining method [18] with the program PHYLIP (version 3.64) available at http://evolution.genetics.washington.edu/phylip.html. Confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein [5] based on 1,000 samplings.

Reference strains. *Weissella confusa* ATCC 10881^T, *W. cibaria/kimchii* LMG 17699^T, *Pediococcus pentosaceus* ATCC 33316^T, *P. acidilactici* DSM 20284^T, *Lactobacillus fermentum* ATCC 14931^T, *L. brevis* ATCC 14869^T, *L. farciminis* ATCC 29644^T, *L. plantarum* ATCC 14917^T and *L. sakei* ATCC 15521^T were used as reference strains.

Results

Bacterial cell counts and sausage characteristics

Twelve samples from Thai fermented sausages collected from the central and northeastern parts of Thailand were studied. The tested samples contained 1.4 x 10^{11}-5.5 x 10^{13} bacterial cells/g, and showed a pH between 4.2 and 5.0 (Table 1).

Table 1. Bacterial cell counts and sausage characteristics.

Sample No.	Sausage names and province where collected	Days of fermentation	pH	Bacterial counts (cells/g)	Isolate No. (65 isolates)
CP1	Sai-krork-prieo Bangkok	4	4.2	3.7x10^{11}	CP1-5, CP1-8, CP1-13, CP1-14, CP1-15, CP1-17, CP1-18, CP1-19, CP1-20
CP2	Sai-krork-prieo Pathumthani	5	4.2	2.5x10^{12}	CP2-3A, CP2-3B, CP2-10, CP2-11, CP2-16
CP3	Sai-krork-prieo Pathumthani	4	4.9	1.4x10^{11}	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP3-16
CP4	Sai-krork-prieo Pathumthani	4	4.8	1.9x10^{12}	CP4-5, CP4-6, CP4-11, CP4-12, CP4-16, CP4-17, CP4-18
CP5	Sai-krork-prieo Bangkok	2	4.3	4.0x10^{13}	CP5-2, CP5-6, CP5-9
CP6	Mum (beef) Chaiyaphoom	3	4.5	3.0x10^{13}	CP6-2, CP6-3, CP6-8, CP6-10,
CP7	Mum (beef) Chaiyaphoom	3	4.4	5.5x10^{13}	CP7-2, CP7-3, CP7-4, CP7-5, CP7-7, CP7-8, CP7-9, CP7-10, CP7-12, CP7-13
CP8	Mum (beef) Konkean	4	4.6	1.5x10^{12}	CP8-1, CP8-4
CP10	Mum (pork) Chaiyaphoom	4	5.0	8.0x10^{11}	CP10-2, CP10-3
CP11	Sai-krork-prieo Chaiyaphoom	4	4.6	1.0x10^{13}	CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15
CP12	Mum (pork) Chaiyaphoom	4	5.0	2.0x10^{13}	CP12-4
CP13	Sai-krork-prieo Chaiyaphoom	4	4.8	8.5x10^{12}	CP13-1, CP13-2, CP13-3, CP13-4, CP13-7, CP13-11

Morphological and cultural characteristics

All isolates were Gram-positive, non-motile, and non-sporing. The cells of 30 sphere-shaped strains measured 0.8 to 1.0 µm in size and appeared in pairs or in tetrads (Table 2). Their colonies on MRS agar plates were circular, low convex with entire margin, and non-pigmented. The cells of 35 rod-shaped strains measured 0.8-1.0 x 1.5-5.0 µm in cell size, and appeared singly, in pairs, or in chains (Table 3). Their colonies on MRS agar plates were circular, low convex with entire margin, and non-pigmented.

Physiological and biochemical characteristics

Isolates were divided into two major groups, Groups A and B by cell shape. Group A consisted of 30 sphere-shaped isolates, which were further divided into 2 subgroups according to gas production from glucose. Subgroup A1 produced gas from glucose (8 strains), while sub-

Table 2. General characteristics of group A strains.

Characteristics	A1	[1] *W. confusa* ATCC 10881^T	[2] *W. kimchii* JCM 12495^T	A21	[3] *P. pentosaceus* ATCC 33316^T	A22	[3] *P. acidilactici* DSM 20284^T
Number of strains	8	-	-	20	-	2	-
Cell form	Cocci						
Cell arrangement	In pairs or tetrads						
Gas from glucose	+	+	+	-	-	-	-
Growth at							
45 °C	-	-	-	+	-	+	+
50 °C	-	-	-	-	-	+	+
Isomers of lactic acid	DL	DL	D	DL	DL	DL	DL
Arginine hydrolysis	+	+	+	+	-	+	+
Esculin hydrolysis	+	+	+	+	+	+	+
Nitrate Reduction	-	ND	ND	-	-	-	-
Reaction in litmus milk							
Acidification	- (+2)	ND	ND	-	-	-	-
Coagulation	- (+2)	ND	ND	-	-	-	-
Reduction	-	ND	ND	-	-	-	-
Growth at pH							
3.5	- (+1)	-	-	+(-2)	ND	+(-1)	ND
4.0	+	+	W	+	+	+	+
4.5	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+
8.5	- (+3)	-	-	+(-2)	-	+	-
9.6	-	-	-	-	-	-	-
Salt tolerance							
4 % NaCl	+	+	+	+	+	+	+
6 % NaCl	+	+	+	+(-1)	+	+	-
8 % NaCl	+	-	-	+(-2)	-	+	-
10 % NaCl	-	-	-	-	-	-	-
Slime from sucrose	+	+	+	-	-	-	-
Peptidoglycan type: DAP	-	-	-	-	-	-	-

Legend: positive (+), weakly positive (W), negative reaction (-), not determined (ND); numbers in parenthesis indicate the number of strains showing a positive or negative reaction; diaminopimelic acid (DAP); American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Japan Collection of Microorganisms (JCM).
[1] Data from [1, 2, 7, 9, 19, 24, 26]; [2] Data from [1]; [3] Data from [25].

group A2 did not (22 strains). All strains in subgroup A1 did not grow at 45 °C. Subgroup A2 was further divided into 2 subgroups according to their growth at different temperatures. Subgroup A21 grew at 45 °C (20 strains); and subgroup A22 grew at 50 °C (2 strains).

Group B consisted of 35 rod-shaped isolates, which were further divided into 2 subgroups according to gas production from glucose. Subgroup B1 produced gas from glucose (7 strains), while subgroup B2 did not (28 strains). Subgroup B1 was further divided into 2 subgroups according to their growth at different temperatures. Subgroup B11 grew at 45 °C (3 strains); and subgroup B12 did not grow at 45 °C (4 strains). All strains in subgroup B2 did not grow at 45 °C. Subgroup B2 was further divided into 2 groups according to isomers of lactic acid. Subgroup B21 produced L-lactic acid from glucose (4 strains), while subgroup B22 produced DL-lactic acid (23 strains). All strains in subgroup B22 did not produce NH_3 from arginine. One strain (CP8-1) was separated from subgroup B22 and located in subgroup B23 because it hydrolyzed arginine. The distribution of isolated strains among groups and their other general characteristics are shown in Tables 2 and 3. Tables 4 and 5 present the characteristics regarding the acid production from carbohydrates by group A and B strains, respectively.

Table 3. General characteristics of group B strains.

Characteristics	B11	[1] *L. fermentum* ATCC 14931^T	B12	[2] *L. brevis* ATCC 14869^T	B21	[2] *L. farciminis* ATCC 29644^T	B22	[3] *L. plantarum* ATCC 14917^T	B23	[4] *L. sakei* ATCC 15521^T
Number of strains	3	-	4	-	4	-	23	-	1	-
Cell forms	Rods									
Cell arrangement	Singly, in pairs or in chains									
Gas from glucose	+	+	+	+	-	-	-	-	-	+
Growth at										
45 °C	+	+	-	-	-	-	-	-	-	-
50 °C	-	ND	-	-	-	-	-	-	-	-
Isomers of lactic acid	DL	DL+ L(+)	DL	DL	L	L	DL	DL	DL	DL+ D(-)
Arginine hydrolysis	+	-	+(-1)	-	+	+	-	-	+	+
Esculin hydrolysis	-(+1)	-	+	-	+	+	+	+	-	+
Nitrate Reduction	-(+1)	ND	-	ND	-	ND	-	ND	-	ND
Reaction in litmus milk										
Acidification	-(+1)	-	-	-	+	ND	+(-5)	-	-	ND
Coagulation	-	-	-	-	-	ND	-(+2)	-	-	ND
Reduction	-	-	-	-	-	ND	-(+1)	-	-	ND
Growth at pH										
3.5	+	-	+(-1)	-	+(-2)	ND	+(-2)	+	-	ND
4.0	+	+	+	+	+	+	+	+	-	+
4.5	+	+	+	+	+	+	+	+	+	+
8.0	+	+	-(+1)	+	+	+	+	+	+	+
8.5	-(+1)	+	-	-	+(-1)	+	-(+9)	+	+	+
9.6	-	-	-	-	-	-	-	-	-	-
Salt tolerance										
4 % NaCl	+	+	+	+	+	+	+	+	+	+
6 % NaCl	+	-	-	-	+	+	+(-3)	+	+	-
8 % NaCl	-(+1)	-	-	-	+	+	-(+11)	-	-	-
10 % NaCl	-	-	-	-	+	+	-	-	-	-
Slime from sucrose	-	-	-	-	-	-	-	-	-	-
Peptidoglycan type: DAP	-	-	-	-	-	-	+	+	-	-

Legend: positive (+), weakly positive (W), negative reaction (-), not determined (ND); numbers in parenthesis indicate the number of strains showing a positive or negative reaction; diaminopimelic acid (DAP); American Type Culture Collection (ATCC).
[1] Data from [24]; [2] Data from [23, 27]; [3] Data from [20, 24, 26]; [4] Data from [24].

Table 4. Acid production from carbohydrates by group A strains.

Characteristics	A1	[1] *W. confusa* ATCC 10881^T	[2] *W. kimchii* JCM 12495^T	A21	[3] *P. pentosaceus* ATCC 33316^T	A22	[3] *P. acidilactici* DSM 20284^T
Number of strains	8	-	-	20	-	2	-
D-Amygdalin	+(-1)	+	+	+	ND	+	ND
L-Arabinose	+	-	-	+(-1)	+	+(-1)	+
D-Cellebiose	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+
D-Galactose	+(-3)	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+
Gluconate	-(+3)	+	+	-	ND	-	ND
Glycerol	-	ND	-	-	-	-	-
Inulin	-	ND	-	-(+4)	-	-	-
Lactose	-(+1)	-	-	-(+7)	-	-	-
Maltose	+	+	+	-(+2)	+	-	-
D-Mannitol	-(+1)	-	-	-	-	-	-
D-Mannose	+	+	+	+	+	+	+
D-Melibiose	-	-	-	-(+1)	+	-	-
D-Melezitose	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	ND	-	-	-	-	-
Raffinose	-	-	-	-	W	-	-
L-Rhamnose	-(+1)	-	-	+(-3)	-	+(-1)	-
D-Ribose	-(+1)	+	-	+	+	+	+
Salicin	+	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-	-
Sucrose	+	+	+	+(-5)	+	+	-
D-Trehalose	-	-	-	-(+8)	+	+	+
D-Xylose	+	+	+	+(-1)	W	+	+

Legend: positive (+), weakly positive (W), negative reaction (-), not determined (ND); numbers in parenthesis indicate the number of strains showing a positive or negative reaction.
[1] Data from [1, 2, 7, 9, 19, 24, 26]; [2] Data from [1]; [3] Data from [25].

Peptidoglycan type of cell walls and isomers of lactic acid

Only subgroup B22 strains contained DAP in the whole cell hydrolysate (Table 3), while the others (subgroup A1, A22, A23, B11, B12, B21, B23 strains) had no DAP.

Subgroup A1, A21, A22, B11, B12, B22, and B23 strains produced DL-lactic acid, while subgroup B21 strains produced L-lactic acid from D-glucose.

Diacetyl production and bacteriocins production

Three strains from subgroup B22 (CP2-3A, CP2-11 and CP3-16) and one from A1 (CP3-11) produced diacetyl, while the other isolates did not.

The inhibition tests against *B. cereus* and *S. aureus* showed that five isolates could inhibit the growth of *B. cereus* (CP1-15, CP2-11, CP3-1, CP7-3, CP10-3) and two of them could also inhibit *S. aureus* (CP1-15, CP7-3).

These isolates were from subgroups A1 (CP3-1), A22 (CP7-3), and B22 (CP1-15, CP2-11, CP10-3).

Table 5. Acid production from carbohydrates by group B strains.

	B11	[1] *L. fermentum* ATCC 14931^{T}	B12	[1] *L. brevis* ATCC 14869^{T}	B21	[2] *L. farciminis* ATCC 29644^{T}	B22	[3] *L. plantarum* ATCC 14917^{T}	B23	[4] *L. sakei* ATCC 15521^{T}
Number of strains	3	-	4	-	4	-	23	-	1	-
D-Amygdalin	-(+1)	-	-	-	+(-2)	+	+	ND	-	+
L-Arabinose	+(-1)	-	+	+	-	-	+(-2)	+	+	+
D-Cellebiose	-(+1)	-	-	-	+	+	+	+	W	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	-	-(+2)	-	+	+	+	+	+	ND
D-Glucose	+	+	+	+	+	+	+	+	+	+
Gluconate	-	-	-	-	-	-	+(-11)	+	W	+
Glycerol	-	-	-	-	-	-	-	-	W	ND
Inulin	-(+1)	ND	-	ND	-(+1)	ND	+(-11)	ND	-	ND
Lactose	+(-1)	+	-	-	+	+	+(-1)	+	+	ND
Maltose	+	-	+	-	+(-2)	+	+	+	-	ND
D-Mannitol	-(+1)	-	-	-	-	-	+	+	-	-
D-Mannose	+(-1)	-	-	-	+	+	+	+	+	ND
D-Melibiose	+	-	-	-	-	-	+(-1)	+	+	+
D-Melezitose	-(+1)	-	-	-	-	ND	+(-2)	+	-	-
α-Methyl-D-glucoside	-	-	+	-	-	-	-(+7)	ND	-	ND
Raffinose	+	-	-	-	-	-	+(-1)	+	-	-
L-Rhamnose	-(+1)	-	-	-	-	-	-(+11)	-	-	ND
D-Ribose	+	+	+	-	-	-	+	+	+	+
Salicin	-(+1)	ND	-	ND	+	+	+	+	W	
D-Sorbitol	-(+1)	-	-	-	-	-	+(-4)	+	-	-
Sucrose	+	+	-	-	+	+	+	+	+	+
D-Trehalose	+	-	-	-	+	+	+	+	+	ND
D-Xylose	+	-	+	+	-	-	-(+2)	-	W	-

Legend: positive (+), weakly positive (W), negative reaction (-), not determined (ND); numbers in parenthesis indicate the number of strains showing a positive or negative reaction.
[1] Data from [24]; [2] Data from [23, 27]; [3] Data from [20, 24, 26]; [4] Data from [24].

Discussion

The isolates were assigned to the genera *Lactobacillus* (35), *Pediococcus* (22), and *Weissella* (8) on the basis of morphological, cultural, biochemical, and physiological characteristics, fermentation type, isomer of lactic acid and 16S rRNA gene sequences. They were grouped at species level on the basis of key phenotypic characteristics and 16S rRNA gene sequences.

Group A1 contained 8 isolates (CP1-8, CP3-1, CP3-8, CP3-9, CP3-10, CP3-11, CP4-6, and CP4-11). The strains in this group were heterofermentative sphere-shaped, produced slime on sucrose, did not grow at 45 °C, had no DAP in peptidoglycan, produced DL-lactic acid, and were identified as belonging to genus *Weissella*. Similarly to the type strain *W. confusa* ATCC 10881^{T}, the isolates produced NH_3 from arginine and hydrolyzed esculin, produced acids from D-cellobiose, D-fructose, D-glucose, maltose, D-mannose, salicin, sucrose and xylose, but not from D-melezitose, D-melibiose, raffinose, D-sorbitol, and D-trehalose [2, 7, 9, 19, 24, 26]. Based on 16S rRNA gene sequences of selected isolates, CP3-8 represented 99 % similarity to *W. confusa* ATCC 10881^{T} and CP1-8, CP3-9, CP4-6, and CP4-11 represented 98 %, 98 %, 99 %, and 99 % similarities respectively to *W. kimchii* JCM 12495^{T}.

Group A21 contained 20 strains (CP1-5, CP1-18, CP7-4, CP7-8, CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15, CP12-4, CP13-2, CP13-3, CP13-4, CP13-7, and CP13-11). The strains in this group were homofermentative sphere-shaped, grew at 45 °C but not at 50 °C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production, reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose and D-ribose, but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics [25], they were identified as *P. pentosaceus*. CP1-18, CP7-4, and CP11-11 were selected for 16S rRNA gene sequence analysis, and represented 99 %, 98 %, and 98 % similarities respectively to *P. pentosaceus* ATCC 33316^T.

Group A22 contained 2 strains (CP6-2 and CP7-3). The strains in this group were homofermentative sphere-shaped, and grew at 50 °C. They were negative for catalase activity, nitrate reduction, slime formation from sucrose, acid production and reduction in litmus milk, and had no DAP in peptidoglycan. They all hydrolyzed esculin, and produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose, and D-ribose but failed to produce acid from α-methylglucoside, D-melezitose, glycerol, D-mannitol, and D-sorbitol. On the basis of these key characteristics [25], they were identified as *P. acidilactici*. Based on 16S rRNA gene sequence analysis, CP6-2 and CP7-3 represented 98 % similarities to *P. acidilactici* DSM 20284^T.

Group B11 contained 3 strains (CP7-2, CP7-9, and CP13-1). The strains in this group were heterofermentative rod-shaped, grew at 45 °C, produced DL-lactic acid and had no DAP in peptidoglycan. Similarly to the type strain *L. fermentum* ATCC 14931^T, these strains produced acid from D-glucose, D-fructose, D-ribose and sucrose, but failed to produce acid from gluconate, glycerol, and α-methyl-D-glucoside. Based on 16S rRNA gene sequence analysis, CP7-2, CP7-9 and CP13-1 represented 100 %, 100 % and 99 % similarities respectively to *L. fermentum* ATCC 14931^T.

Group B12 contained 4 strains (CP1-13, CP1-14, CP1-19, and CP6-8). The strains in this group were heterofermentative rod-shaped, and did not grow at 45 °C, produced DL-lactic acid and had no DAP in peptidoglycan. All strains had the following characteristics in common with *L. brevis* ATCC 14869^T and 3 other strains identified as *L. brevis* by Tanasupawat et al. [24] on the basis of DNA relatedness: negative for acid production and reduction in litmus milk; negative for growth at pH 8.5 and at 6 % NaCl; positive for acid production from L-arabinose, fructose, D-glucose and D-xylose; negative for acid production from D-amygdalin, D-cellobiose, gluconate, glycerol, lactose, D-mannose, D-mannitol, D-melezitose, D-melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, and D-trehalose. The two strains selected for 16S rRNA gene sequence analysis, CP1-19 and CP6-8, represented 98 % similarities with *L. brevis* ATCC 14869^T.

Group B21 contained 4 strains (CP2-3B, CP5-2, CP5-6, and CP5-9). The strains in this group were homofermentative rod-shaped, did not grow at 45 °C, had no DAP in peptidoglycan, and produced L-lactic acid from glucose. All strains had the following key characteristics of *L. farciminis* [6]: positive for NH_3 production from arginine; positive for acid production from D-cellobiose, D-galactose, D-glucose, lactose, D-mannose, salicin, sucrose and D-trehalose; negative for acid production from D-mannitol, D-melibiose, and raffinose; positive for growth at 10 % NaCl. The two strains selected for 16S rRNA gene sequence analysis, CP5-2 and CP5-9, represented 98 % and 99 % similarities respectively to *L. farciminis* ATCC 29644^T.

Group B22 contained 23 strains (CP1-15, CP1-17, CP1-20, CP2-3A, CP2-10, CP2-11, CP2-16, CP3-16, CP4-5, CP4-12, CP4-16, CP4-17, CP4-18, CP6-3, CP6-10, CP7-5, CP7-7, CP7-10, CP7-12, CP7-13, CP8-4, CP10-2, and CP10-3). The strains in this group were homofermentative rod-shaped, did not grow at 45 °C, had DAP in peptidoglycan, and produced DL-lactic acid from glucose. All isolates hydrolyzed esculin, but not arginine. They all produced acid from D-cellobiose, D-fructose, D-galactose, D-glucose, D-maltose, D-mannitol, D-mannose, D-ribose, salicin, sucrose and D-trehalose, but not from glycerol. These are the common characteristics with *L. plantarum* ATCC 14917^T and the other 16 strains identified as *L. plantarum* by Tanasupawat et al. [20] on the basis of DNA relatedness. The eight strains selected for 16S rRNA gene sequence analysis, CP1-15, CP1-20, CP2-3A, CP3-16, CP6-10, CP7-7, CP8-4, and CP10-3, represented 98 % similarities to *L. plantarum* ATCC 14917^T.

Group B223 contained one strain, CP8-1. This strain was homofermentative rod-shaped, did not grow at 45 °C, had no DAP in peptidoglycan and produced DL-lactic acid from glu-

cose. This strain was removed from group B22 because it hydrolyzed arginine. Similarly to *L. sakei* ATCC 15521^{T}, CP8-1 produced acid from L-arabinose, D-cellebiose, D-fructose, D-glucose, gluconate, D-melibiose, D-ribose and sucrose, but failed to produce acid from D-mannitol, D-melezitose, raffinose and D-sorbitol. The 16S rDNA sequence analysis of this strain showed 99 % similarity to *L. sakei* ATCC 15521^{T}. Fig. 1 shows a phylogenetic tree based on 16S DNA sequences of type strains and of representative strains of each group.

Most lactic acid bacteria isolated in this study were assigned to *P. pentosaceus* (31 %) and *L. plantarum* (35 %). *L. pentosus* and *L. plantarum* that contain *meso*-diaminopimelic acid in the cell wall were previously reported to be the predominant rod-shaped LAB, and *P. pentosaceus* strains were the major coccal bacteria in other fermented Thai foods, e.g. fish cake, sweetened rice, fermented rice noodle and pickle [21]. Table 6 summarizes the identification of isolates and their distribution in Thai traditional fermented sausages. It is noteworthy that *L. plantarum* was distributed in at least 8 (of total 12) samples and *P. pentosaceus* in 5 (of total 12) samples used in this study. On the other hand, *Weissella* sp., *P. acidilactici*, *L. fermentum*, *L. brevis*, *L. farciminis* and *L. sakei* were isolated from much fewer samples.

Table 6. Identification of isolates and their distribution in Thai traditional fermented sausages.

Sausage names and province where collected	Days of fermentation	Isolate No.	Group or subgroup	Identification
Sai-krork-prieo Bangkok	4	CP1-8	A1	*W. cibaria/kimchii*
		CP1-5, CP1-18	A21	*P. pentosaceus*
		CP1-13, CP1-14, CP1-19	B12	*L. brevis*
		CP1-15, CP1-17, CP1-20	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	5	CP2-3B	B21	*L. farciminis*
		CP2-3A, CP2-10, CP2-11, CP2-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP3-1, CP3-8, CP3-9, CP3-10, CP3-11	A1	*W. confusa*
		CP3-16	B22	*L. plantarum*
Sai-krork-prieo Pathumthani	4	CP4-6, CP4-11	A1	*W. cibaria/kimchii*
		CP4-5, CP4-12, CP4-16, CP4-17, CP4-18	B22	*L. plantarum*
Sai-krork-prieo Bangkok	2	CP5-2, CP5-6, CP5-9	B21	*L. farciminis*
Mum (beef) Chaiyaphoom	3	CP6-3, CP6-10	B22	*L. plantarum*
		CP6-2	A22	*P. acidilactici*
		CP6-8	B12	*L. brevis*
Mum (beef) Chaiyaphoom	3	CP7-4, CP7-8	A21	*P. pentosaceus*
		CP7-3	A22	*P. acidilactici*
		CP7-2, CP7-9	B11	*L. fermentum*
		CP7-5, CP7-7, CP7-10, CP7-12, CP7-13	B22	*L. plantarum*
Mum (beef) Konkean	4	CP8-1	B23	*L. sakei*
		CP8-4	B22	*L. plantarum*
Mum (pork) Chaiyaphoom	4	CP10-2, CP10-3	B22	*L. plantarum*
Sai-krork-prieo Chaiyaphoom	4	CP11-2, CP11-3, CP11-5, CP11-7, CP11-8, CP11-10, CP11-11, CP11-13, CP11-14, CP11-15	A21	*P. pentosaceus*
Mum (pork) Chaiyaphoom	4	CP12-4	A21	*P. pentosaceus*
Sai-krork-prieo Chaiyaphoom	4	CP13-1	B11	*L. fermentum*
		CP13-2, CP13-3, CP13-4, CP13-7, CP13-11	A21	*P. pentosaceus*

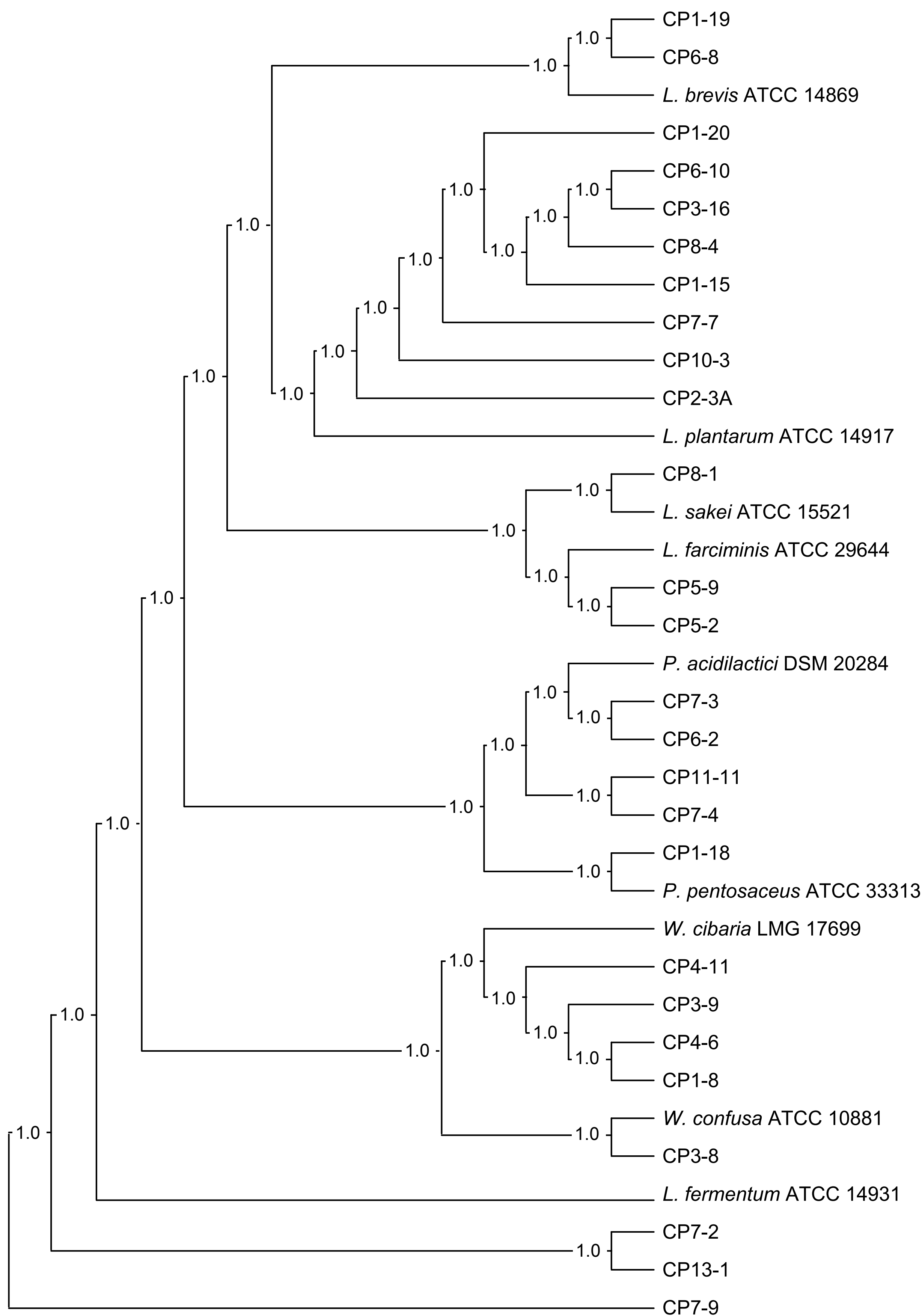

Fig. 1. Phylogenetic relationships based on 16S rDNA sequences of type strains and representative strains of each group. The phylogenetic tree was constructed by the neighbor-joining method. The numbers on the branches indicate how many times the partition of the species into the two sets, separated by that branch, occurred among the trees; out of 1.00 trees (bootstrap values derived from 1,000 replications).

Previous studies reported the isolation of *L. pentosus*, *L. plantarum*, *L. fermentum*, *P. pentosaceus*, and *Enterococcus hirae* from "Sai-krog-prieo", and of *L. plantarum* and *Enterococcus* sp. from "mum" [21, 22]. This study first reports the isolation of *W. cibaria/kimchii*, *W. confusa*, *L. brevis*, and *L. farciminis*, from "Sai-krog-prieo", and *P. pentosaceus*, *P. acidilactici*, *L. fermentum*, *L. brevis*, and *L. sakei* from "mum".

Certain strains from this study were found to produce diacetyl and bacteriocins. *B. cereus* and *S. aureus* were selected as test pathogens for preliminary bacteriocin screening, while further studies on activity spectrum and bacteriocin characterization, i.e., minimum inhibitory concentration, thermostability, synergism of each purified bacteriocin, are being pursued at our laboratory.

Acknowledgements. We gratefully acknowledge the support of Thailand Research Fund and NRCT-JSPS for the research grants. We thank the Faculty of Pharmaceutical Sciences, Chulalongkorn University and Mahidol University-Osaka University Collaborative Research Center for providing research facilities. Special thanks to Mr. Amnat Pakdeeto and Mr. Veerapon Chavasirikunton for their technical assistances and to Prof. Watanalai Panbangred and Assoc. Prof. Nachol Chaiyaratana for their discussions on 16S DNA sequence analysis and the phylogenetic tree construction.

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ХАРАКТЕРИЗИРАНЕ НА МЛЕЧНОКИСЕЛИ БАКТЕРИИ ОТ ТРАДИЦИОННИ ФЕРМЕНТИРАЛИ НАДЕНИЦИ THAI

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Резюме

Характеризирани са млечнокисели бактерии, изолирани от традиционни ферментирали наденици Thai. Надениците са произведени предимно от смляно свинско и говеждо месо или черен дроб. Изолирани са шестдесет и пет щама от 12 проби, събрани от централните и североизточни части на Тайланд. Щамовете са идентифицирани чрез стандартни морфологични, културални, физиологични и биохимични тестове, както и чрез 16S рДНК секвенционен анализ. Изолатите са определени като Weissella cibaria/kimchii (5), W. confusa (3), Pediococcus pentosaceus (20), P. acidilactici (2), Lactobacillus fermentum (3), L. brevis (4), L. farciminis (4), L. plantarum (23), и L. sakei (1). Някои от тези видове не са били изолирани досега от ферментирали наденици Thai. Инхибиторният тест срещу Bacillus cereus и Staphylococcus aureus показва, че 5 изолата могат да подтискат растежа на B. cereus, а 2 от тях също инхибират и S. aureus. Изолатите са идентифицирани като W. confusa (1), P. acidilactici (1) и L. plantarum (3). Три щама, определени като L. plantarum и един като Weissella spp. могат да продуцират диацетил.