Original Article

Identification and antimicrobial susceptibility of lactic acid bacteria from fecal samples of indigenous and commercial pigs

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Abstract

Lactic acid bacteria (LAB) are currently applied as probiotics due to their benefit to pig performance. This study aimed to identify candidate LAB from pig feces and determine their antimicrobial susceptibility following an international standard recommendation. A total of 204 LAB isolates from 60 fecal samples of 30 antibiotic-free healthy fattening indigenous pigs and 30 antibiotic-free healthy fattening commercial pigs were initially screened for viability in acidic and bile conditions. Thirty-four of the isolates as acid- and bile-tolerant LAB were selected for identification and determination of antimicrobial susceptibility. They were characterized and identified by a set of 26 phenotypic tests, whole-cell protein patterns (SDS-PAGE analysis), and 16S rDNA sequencing analysis. They were identified as *Enterococcus faecium* (11 isolates), *E. hirae* (nine isolates), *Lactobacillus agilis* (three isolates), *L. plantarum* (four isolates), *Pediococcus acidilactici* (one isolate) and *P. pentosaceus* (six isolates). The identification by 16S rDNA sequence analysis was strongly consistent with the whole-cell protein profiles, but not with the biochemical profiles. LAB presenting multidrug resistance could be found in antibiotic-free pigs. Determination of minimum inhibitory concentration (MIC) values showed that among the 34 LAB isolates, only four (*P. pentosaceus* 77F, and *L. plantarum* 22F, 25F, 31F) from commercial pigs and one (*P. acidilactici* 72N) from an indigenous pig were susceptible to all eight antibiotics including ampicillin, chloramphenicol, gentamicin, kanamycin, erythromycin, tetracycline, streptomycin and vancomycin according to EFSA criteria. In conclusion, five LAB strains derived from healthy pigs displayed potential as porcine probiotics and will be screened in further clinical studies.

Keywords: antimicrobial susceptibility, identification, lactic acid bacteria, pig feces

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Introduction

Lactic acid bacteria (LAB) are beneficial commensals in the gut with a long history of safe use as probiotics for animals and humans. They are Gram-positive, catalase-negative, non-spore forming bacteria which anaerobically produce lactic acid as the major end product from carbohydrate (Parente et al., 2001). The major LAB generally used as probiotics in livestock are *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp., and *Enterococcus faecium*. The increased attention to LAB in pig production is due to their potential benefits in antibiotic replacement, maintaining and promoting animal health status at farm level (Téllez et al., 2015).

In general, the isolation of LAB from a healthy host and subsequent use in the same host species is an ideal procedure, due to their specific adaptation to the GI environment, competitive adaptation to endemic pathogens, eco-friendly status and long-term maintainable within the GI tract (Téllez et al., 2015). Moreover, LAB used as probiotic strains function better in an environment similar to their original hosts (Saarela et al., 2000). Thus, the isolation of potential LAB from local healthy pigs in antibiotic-free farms will provide a higher opportunity to select safer LAB as a putative probiotic that shows a lack of transferable antimicrobial resistance gene, especially *tet* gene family and *cat* gene (EFSA, 2012; Gueimonde et al., 2013).

The classification and identification of LAB species are the fundamental safety aspect of probiotics (Saarela et al., 2000). To identify and classify LAB, physiological characteristics such as morphology and carbohydrate fermentation patterns can be used for bacterial screening, but unreproducible outcomes may occur among intraspecies biodiversity (AxelssonandAhrné, 2000). To date, the genome base analysis using 16S rRNA gene is considered as a gold standard method which is comparable to global database (AxelssonandAhrné, 2000; Vandamme et al., 1996). However, the data set of 16S rRNA gene sequences shares the highest similarity with those of the related species, i.e. *L. amylovorus* and *L. sobrius*, both affiliated to *L. acidophilus* group (Klein et al., 1998). On the other hand, the use of whole-cell protein pattern is also a presumptive identification tool for LAB, once reference species are available to be compared between strains (Vandamme et al., 1996). The protein patterns can be incorporated in the identification process to reflect the dominance of LAB species from all GIT sources (Klose et al., 2010).

Although a considerable amount of research has been done on commercially utilized LAB isolates in the pig rearing industry (Kenny et al., 2011), there is still a lack of knowledge of the comparative identity of wild LAB isolates from pig fecal sources. This understanding will be helpful for further study of the development of appropriate probiotics suitable for pig productions. LAB cannot be termed probiotics until they have been isolated, identified, proved to survive acidic and bile within the GI tract and safe to use (Hill et al., 2014). Therefore, this study attempted to screen for the acid- and bile-tolerant LAB from feces of antibiotic-free healthy fattening indigenous and commercial pigs in Thailand, and identify them using a set of 26 phenotypic tests, whole-cell protein pattern analysis and 16S rRNA gene analysis. The antimicrobial susceptibility and resistance genes of all selected strains were determined following international standard guidelines.

Materials and Methods

Sample collection and LAB isolation: A total of 60 fecal samples were collected directly from the rectum of indigenous pigs in Nan province and commercial pigs in Chai-nart province. All samples were collected from eight-month-old healthy, antibiotic-free pigs weighing around 120 to 130 kg with no evidence of clinical signs of enteric diseases and having perfect body condition score (ribs, hips and backbone could not be observed). LAB were isolated by dilution and plating. An amount of 10 g of the sample was 10-fold serially diluted and inoculated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) supplemented with 0.3% (w/v) calcium carbonate. Three to five bacterial colonies presenting clear zone were selected, and re-streaked on MRS agar to ensure purity. The bacterial isolates were subjected to Gram's stain and catalase test to be identified as presumptive LAB. Only isolates which were Gram-positive and catalase-negative rods and cocci were selected for further studies (Maragkoudakis et al., 2006). Approval for use of the experimental animals was obtained from the ethical committee of Faculty of Veterinary Science, Chulalongkorn University (No. 1531011).

Acid and bile tolerance of LAB: A total of 204 presumptive LAB isolates comprising 90 isolates from the indigenous pigs and 114 isolates from the commercial pigs were examined in MRS broth for the ability to tolerate acidic and bile conditions in order to select resistant isolates for further studies. Overnight cultures (24 h) of the isolates were harvested (10,000 x g, 10 min) and re-suspended in MRS broth adjusted to pH 2.0 with 1 N Hydrochloric acid (HCl) at a bacterial concentration of 1 x 10^8 CFU/mL. The same procedure was conducted to test bile tolerance. Overnight cultures of each isolate was inoculated in MRS broth supplemented with 0.3% (w/v) Oxgall powder (Sigma-Aldrich, Louise, USA) at pH 6.5. Normal MRS broth was used as the control. Acid and bile resistance was assessed after incubation at 37°C for 12 h using viable bacterial counts and enumerated after plating serial dilutions on MRS agar (Oxiod, Basingstoke, UK). Strains with $\geq 10^4$ CFU/mL were chosen as acid- and bile-tolerant LAB for further examinations. Pure isolates were stored in MRS broth (Oxoid, Basingstoke, UK) supplemented with 20% (w/v) glycerol at -80°C (Federici et al., 2014).

Phenotypic and biochemical characterization: Based on LAB morphologies and ability to tolerate acidic and bile conditions, 34 isolates were selected for this study. A set of 26 tests including cell morphology, CO_2 production from glucose, ability to grow at 45°C and 50°C, and acid production from 21 types of carbohydrates (amygdalin, L-arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose,

rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-Xylose) were used to classify and characterize the isolates (Ricciardi et al., 2005; Tanasupawatand and Komagata, 1995).

Whole-cell protein profiling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): The overnight culture of 34 selected acid- and bile-tolerant isolates in MRS broth was harvested and washed twice with 0.85% (w/v) sterile saline solution. The washed packed cells were extracted by the freeze-thaw method with stirring glass beads. Supernatant was collected, boiled at 100ºC with denaturing buffer for 10 min and clarified by centrifugation at 9,000 x g for 10 min. The supernatant was collected for whole-cell protein pattern determination (Ghazi et al., 2009). A total of 10 µL supernatant was applied per track and resolved by discontinuous 1D-SDS-PAGE through a 5% (w/v) stacking gel and a 12% (w/v) separating gel at a constant of 10 mA (ATTO, Tokyo, Japan). The separating gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich, Louise, USA) (Descheemaeker et al., 1994). Molecular weight of the stained protein bands was calculated by GeneTools software (Syngene, Cambridge, UK). The protein pattern of each isolate was analyzed on a similarity matrix before being clustered by the unweighted pair group method with arithmetic mean (UPGMA) for dendrogram illustration using the GeneDirectory software (Syngene, Cambridge, UK). The protein patterns of *Enterococcus faecium* ATCC 19434, *E. hirae* ATCC 9790, *Pediococcus acidilactici* DSM 20284, *P. pentosaceus* ATCC 25745, *Lactobacillus agilis* DSM 20509 and *L. plantarum* JCM 1149 were used as reference strains (Ricciardi et al., 2005).

16S rDNA sequence analysis: A pure colony of each selected isolate was grown for 24 h in MRS broth and prepared for DNA extraction. The cells were harvested and washed twice with 0.85% physiological saline and centrifuged at 8,000 x g for 2 min. The bacterial DNA was extracted using a Nucleospin® tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The 16S rDNA of selected isolates were amplified by PCR using the universal 16S ribosomal gene primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), as reported previously (Angmo et al., 2016). The reference strains used for the whole-cell protein pattern analysis were also used as control strains for the PCR amplification, while DNase free water was used as a negative control. The PCR products were purified using a QIAquick purification kit (Qiagen, Tokyo, Japan) prior to submission for commercial sequencing (WardMedic, Bangkok, Thailand). The obtained 16S rDNA sequences were compared with the sequences of type strains available in the GenBank database on the National Center for Biotechnology Information (NCBI) for species identification and nucleotide identity using the online BLASTn algorithm. A phylogenetic tree was constructed from the aligned 1,400-1,500 bp sequences (after removal of indels) using the neighbor-joining (NJ) distance method with bootstrap resampling of 1000 replicates in the MEGA6 software program (Tamura et al., 2007). The nucleotide sequences of all the analyzed isolates were deposited in the DDBJ gene databank (Shizuoka, Japan), with the accession numbers presented in Table 1.

Antimicrobial susceptibility: The antimicrobial susceptibility of the 34 selected acid- and bile-tolerant LAB was evaluated by the disc diffusion method modified from Clinical and Laboratory Standards Institute (CLSI, 2012) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013). The susceptibility testing of all isolates was performed on MRS agar except for enterococci which was performed on Mueller-Hinton agar. Antibiotic disks (Oxoid, Basingstoke, UK), consisting of amoxicillin (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), vancomycin (30 µg), colistin sulfate (10 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), erythromycin (15 µg) and clindamycin (2 µg), were used for the susceptibility determination. Inhibition zone diameters were interpreted according to Charteris et al. (1998) and Temmerman et al. (2003). Out of the 34 acid- and bile-tolerant LAB, seven isolates that were susceptible to most of the tested antibiotics were further evaluated for the respective minimum inhibitory concentration (MIC) to nine antibiotics (Sigma-Aldrich, Louise, USA) including ampicillin (0.0625-16 µg/mL), chloramphenicol (0.5-128 µg/mL), erythromycin (0.125-32 µg/mL), gentamicin (0.125-32 µg/mL), kanamycin (0.5-1024 µg/mL), streptomycin (0.5-256 µg/mL), tetracycline (0.125-64 µg/mL), vancomycin (0.125-32 µg/mL) and tylosine (0.0625-16 µg/mL). The tests were performed using broth microdilution according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2012; Jorgensen et al., 2007). Breakpoints for the studied MICs followed the microbiological cut-off values proposed by the EFSA FEEDAP Panel (EFSA, 2012). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as standard controls.

Confirmation of antimicrobial resistance genes: The existence of thirteen antimicrobial resistance genes in the seven selected acid- and bile-tolerant LAB isolates was confirmed by selective PCR amplification using the gene-specific primers (*erm(A)*, *erm(B)*, *erm(C)*, *aac(6')aph(2")*, *aac(3")II*, *aac(3")IV*, *ant(2")-I*, *aph(3")-I*, *aph(3")-III*, *strA*, *strB*, *aadA* and *aadE*). All PCR amplifications were performed using a thermal cycling profile of 94ºC for 3 min, followed by 35 cycles of 94ºC for 1 min, 48-68ºC for 1 min and 72ºC for 1 min, followed by a final 72ºC for 10 min. The PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Ouoba et al., 2008).

Statistical analysis: The phenotypic tests were coded as 0 = negative, or 1 = positive for the ability to produce acid from the 21 types of carbohydrates. For cell morphology, two variables were applied: C/R (0 = cocci, 1 = rods) and C/ST (0 = chains, 1 = single cells or tetrads). A similarity matrix was built using Jaccard coefficient (S_j). Hierarchical clustering of the phenotypic tests was performed using Unweighted Pair-Group Average Linkage Analysis (Ricciardi et al.,

2005; Tanasupawat and Komagata, 1995). Statistical analyses were performed using Systat 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

Table 1 Source, isolate number and identification results of 34 selected acid- and bile-tolerant lactic acid bacteria from pig feces in Thailand

Sample origins	[a] Strain and accession numbers	[b] 16 rDNA sequencing	[c] Phenotypic cluster	[d] Whole-cell protein cluster	[e] Species identification (≥ 99% similarity to type strain)
Indigenous pig	73N (LC035112)	II	1	A	*E. hirae*
Indigenous pig	69N (LC035131)	II	2	A	*E. hirae*
Indigenous pig	61N (LC035130)	II	1	A	*E. hirae*
Indigenous pig	71N (LC035122)	II	2	A	*E. hirae*
Indigenous pig	77N (LC035118)	II	2	A	*E. hirae*
Commercial pig	69F (LC035114)	II	2	A	*E. hirae*
Commercial pig	85F (LC035113)	II	2	A	*E. hirae*
Commercial pig	84F (LC035117)	II	1	A	*E. hirae*
Commercial pig	68F (LC035115)	II	1	A	*E. hirae*
Indigenous pig	38N (LC035121)	I	4	F	*E. faecium*
Indigenous pig	29N (LC035124)	I	4	F	*E. faecium*
Indigenous pig	54N (LC035120)	I	4	F	*E. faecium*
Indigenous pig	40N (LC035104)	I	4	F	*E. faecium*
Indigenous pig	79N (LC035103)	I	4	F	*E. faecium*
Indigenous pig	51N (LC035110)	I	3	F	*E. faecium*
Indigenous pig	49N (LC035125)	I	3	F	*E. faecium*
Indigenous pig	39N (LC035119)	I	3	F	*E. faecium*
Indigenous pig	80N (LC035132)	I	1	F	*E. faecium*
Commercial pig	67F (LC035123)	I	4	F	*E. faecium*
Commercial pig	28F (LC035109)	I	3	F	*E. faecium*
Commercial pig	101F (LC035133)	VI	6	C	*P. pentosaceus*
Commercial pig	40F (LC035129)	VI	7	C	*P. pentosaceus*
Commercial pig	39F (LC035128)	VI	8	C	*P. pentosaceus*
Commercial pig	90F (LC035134)	VI	6	C	*P. pentosaceus*
Commercial pig	76F (LC035126)	VI	6	C	*P. pentosaceus*
Commercial pig	77F (LC035102)	VI	6	C	*P. pentosaceus*
Indigenous pig	72N (LC035107)	V	9	B	*P. acidilactici*
Commercial pig	31F (LC035106)	IV	5	E	*L. plantarum*
Commercial pig	25F (LC035105)	IV	5	E	*L. plantarum*
Commercial pig	22F (LC035101)	IV	5	E	*L. plantarum*
Commercial pig	44F (LC035111)	IV	5	E	*L. plantarum*
Commercial pig	56F (LC035108)	III	11	D	*L. agilis*
Commercial pig	74F (LC035116)	III	10	D	*L. agilis*
Commercial pig	75F (LC035127)	III	10	D	*L. agilis*

[a] Accession number: sequences determined in this study were deposited in the DDBJ gene databank in Japan.
[b] Group: isolates were grouped and identified by 16S rRNA gene.
[c] Cluster: isolates were clustered and analyzed by hierarchical clustering of a set of 26 phenotypic tests.
[d] Cluster: isolates were clustered and analyzed by SDS-PAGE according to whole-cell protein profiles.
[e] Type strains: *E. hirae* ATCC 9790^T, *E. faecium* ATCC 19434^T, *P. pentosaceus* DSM 20336^T, *P. acidilactici* DSM 20284^T, *L. plantarum* JCM 1149^T, and *L. agilis* JCM 1187^T

Results

Isolation and selection of LAB for further identification: From the 60 fecal samples (one per pig), 204 presumptive LAB isolates (90 from the indigenous pigs and 114 from the commercial pigs) were initially isolated. They were Gram-positive, non-motile and catalase-negative bacteria of a rod or cocci shape. Only 34 isolates, 15 from the indigenous pigs and 19 from the commercial pigs, showed resistance to acidic (pH 2) and bile environments for 12 h at $\geq 1 \times 10^4$ CFU/mL yield when re-enumerated on MRS agar. Thereafter, these 34 acid- and bile-tolerant LAB strains were identified by phenotypic and genotypic characteristics (Table 1), and their antimicrobial susceptibility was determined.

Identification of acid- and bile-tolerant LAB*:* The 34 selected LAB were characterized using a set of 26 phenotypic tests (Table S1). They were statistically grouped into eleven clusters at 80% similarity level. Most strains were identified as *E. faecium* (cluster 4, 6 isolates), *E. hirae* (cluster 1, 5 isolates) and *L. plantarum* (cluster 5, 4 isolates), whereas 19 isolated strains could not be classified into a species (Fig. S1). To confirm the identification of the selected LAB obtained from phenotypic tests, a near full-length 16S rDNA sequence was obtained for all 34 acid- and bile-tolerant LAB and depicted as a phylogenetic relationship as inferred by the neighbor-joining analysis (Fig. 1). The isolates were placed into six clusters (designated as I to VI) and were identified as genus *Enterococcus* (11 isolates as *E. faecium* and 9 isolates as *E. hirae*), 7 isolates were assigned to the genus *Lactobacillus* (4 isolates as *L. plantarum* and 3 isolates as *L. agilis*) and 7 isolates to genus *Pediococcus* (6 isolates as *P. pentosaceus* and 1 isolate as *P. acidilactici*) (Table 1). From the mirror image, isolate number 80N, which was identified as *E. hirae* by the phenotypic dendrogram, was identified as *E. faecium* by the 16S rDNA sequencing and also later identified as *E. faecium* by the whole-cell protein pattern analysis (Fig. S1).

The analysis of whole-cell protein patterns classified the isolates on provisional species level into six clusters (A to F) (Table 1 and Fig. S2) of *E. faecium* ATCC 19434 (82% similarity), *E. hirae* ATCC 9790 (82% similarity), *P. acidilactici* DSM 20284 (91% similarity), *P. pentosaceus* ATCC 25745 (96% similarity), *L. agilis* DSM 20509 (82% similarity) and *L. plantarum* JCM 1149 (80% similarity), and was confirmed by the mirror image with the phylogenetic relationships as shown in Fig. S2. The distinctions on the acid production from carbohydrates of *E. faecium*, *L. agilis*, *L. plantarum*, *P. acidilactici* and *P. pentosaceus* isolates in this study were the ability to produce acid from mannitol, mannose, trehalose and D-Xylose as shown in Table 2.

Determination of antimicrobial susceptibility and resistance gene detection: The antimicrobial susceptibility by disc diffusion of the 34 selected LAB isolates is summarized in Table S2. All isolates were susceptible to amoxicillin but resistant to colistin sulfate, gentamicin, kanamycin and streptomycin. Moreover, 94.1%, 79.4%, 58.9%, and 52.9% of the isolates were susceptible to ampicillin, chloramphenicol, tetracycline, and vancomycin, respectively. Furthermore, over 75% of the tested strains showed resistance to erythromycin and clindamycin, especially among *E. hirae*, *E. faecium* and *L. agilis*. From the MIC values (Table 3), the final five selected LAB strains (*P. pentosaceus* 77F, *P. acidilactici* 72N, *L. plantarum* 22F, 25F, and 31F) were susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, vancomycin and tylosine; while the two strains of *E. faecium* (79N and 40N) were only susceptible to tetracycline and vancomycin. With respect to the antimicrobial resistance gene profile, none of the genes studied were detected in all three *L. plantarum* strains (22F, 25F, and 31F), whereas genes associated with resistance to four antibiotics were detected in *E. faecium* (*erm(B)*, *aac(6')aph(2)'*, *aph(3)'-III* and *aadE*) and to three and two antibiotics in *P. acidilactici* (*erm(B)*, *aac(6')aph(2)'* and *aph(3)'-III*) and *P. pentosaceus* (*erm(B)* and *aph(3)'-III*), respectively (Table S3).

Table 2 Consensual agreement between phenotypic and genotypic characterizations for identification purpose of six lactic acid bacteria species

LAB species	16S rRNA clusters	Protein clusters	Cell morphology	Profiles of acid production from carbohydrates				
				Fructose	Mannitol	Mannose	Trehalose	D-Xylose
E. faecium	I	F	Cocci	+	+	+	+	-
E. hirae a)	II	A	Cocci	+	-	+	+	-
L. agilis	III	D	Rods	+	-	-	-	-
L. plantarum	IV	E	Rods	+	+	+	+	-
P. acidilactici	V	B	Tetrads	+	-	+	+	+
P. pentosaceus a)	VI	C	Tetrads	+	-	+	+	-

a) By phenotypic characteristics, the profile of *E. hirae* is identical to that of *P. Pentosaceus*.

Table 3 Confirmation of antimicrobial susceptibility of seven selected acid- and bile-tolerant lactic acid bacterial strains by minimum inhibitory concentration (MIC) values

Antimicrobials	MIC (μg/mL)						
	Enterococcus		*Lactobacillus*			*Pediococcus*	
	E. faecium	*E. faecium*	*L. plantarum*	*L. plantarum*	*L. plantarum*	*P. acidilactici*	*P. pentosaceus*
	79N	**40N**	**22F**	**25F**	**31F**	**72N**	**77F**
Ampicillin	4 (R)	4 (R)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)
Chloramphenicol	64 (R)	64 (R)	8 (S)	4 (S)	8 (S)	4 (S)	2 (S)
Erythromycin	> 32 (R)	> 32 (R)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)
Gentamicin	> 32 (R)	> 32 (R)	0.125 (S)	0.125 (S)	0.25 (S)	0.125 (S)	0.125 (S)
Kanamycin	> 1024 (R)	> 1024 (R)	8 (S)	4 (S)	16 (S)	8 (S)	4 (S)
Streptomycin	> 256 (R)	> 256 (R)	n.r.	n.r.	n.r.	4 (S)	2 (S)
Tetracycline	0.25 (S)	0.25 (S)	16 (S)	16 (S)	16 (S)	4 (S)	4 (S)
Vancomycin	0.25 (S)	0.25 (S)	n.r.	n.r.	n.r.	n.r.	n.r.
Tylosine	> 16 (R)	> 16 (R)	n.r.	n.r.	n.r.	n.r.	n.r.

n.r., not required by EFSA; (R), resistant; (S), susceptible according to EFSA microbiological cut-off values (EFSA, 2012)

Discussion

The study scoped on the group of LAB that showed high viability in low pH and bile salt conditions since it reflects the potential for long-term survival within the pig GI tract. Regarding the criteria of sample collection, pig farms that did not administer antibiotics might raise a chance to obtain non-antimicrobial-resistant isolate (Gueimonde et al., 2013). Given that differences in pig breeds might also enhance the possibility of obtaining a greater variety, including potential novel isolates among LAB species (Seo et al., 2010). The LAB derived from indigenous pigs were viewed as a potential source of local LAB strains due to their natural adaptation within the GI tract and environment that differed from commercial pigs (Saarela et al., 2000). However, one strain was finally obtained from an indigenous pig and the commercial pigs were likely to possess a more diverse LAB species.

The 34 acid- and bile-tolerant LAB isolates were successfully identified by the 16S rDNA sequence and protein pattern analysis to the species level, or strictly to the operational taxonomic unit level, with congruency between these two methods, although the phylogenetic analysis provided greater resolution on their potential sub-species (strain) relationships. Meanwhile, the biochemical profiles using the ability to produce acid from 21 sugars, which has been proposed as an alternative tool, gave incongruent results to the other two methods. In detail, it could not distinguish *E. faecium* from *E. hirae* in this study, as well as in a previous study (Devriese et al., 1995). Nevertheless, there was a pronounced agreement in the detection of six LAB species between the genotype and phenotype. Use of the cell morphology coupled with acid production ability of five sugar types (fructose, mannitol, mannose, trehalose and D-Xylose) might give quick and reasonable initial classification to the genus and potential species level in the early stages of LAB selection (Parente et al., 2001; Ricciardi et al., 2005), except between *E. hirae* and *P. pentosaceus*, with confirmation by subsequent molecular phylogenetic studies of the final samples. Although the 16S rDNA sequencing analysis is generally recommended for LAB identification, it is costly, inconvenient and time-consuming in case of a routine laboratory service (Moraes et al., 2013), whereas the whole-cell protein analysis is less expensive and requires less time in the case of available databases and/or of the reference strains (Leisner et al., 1999).

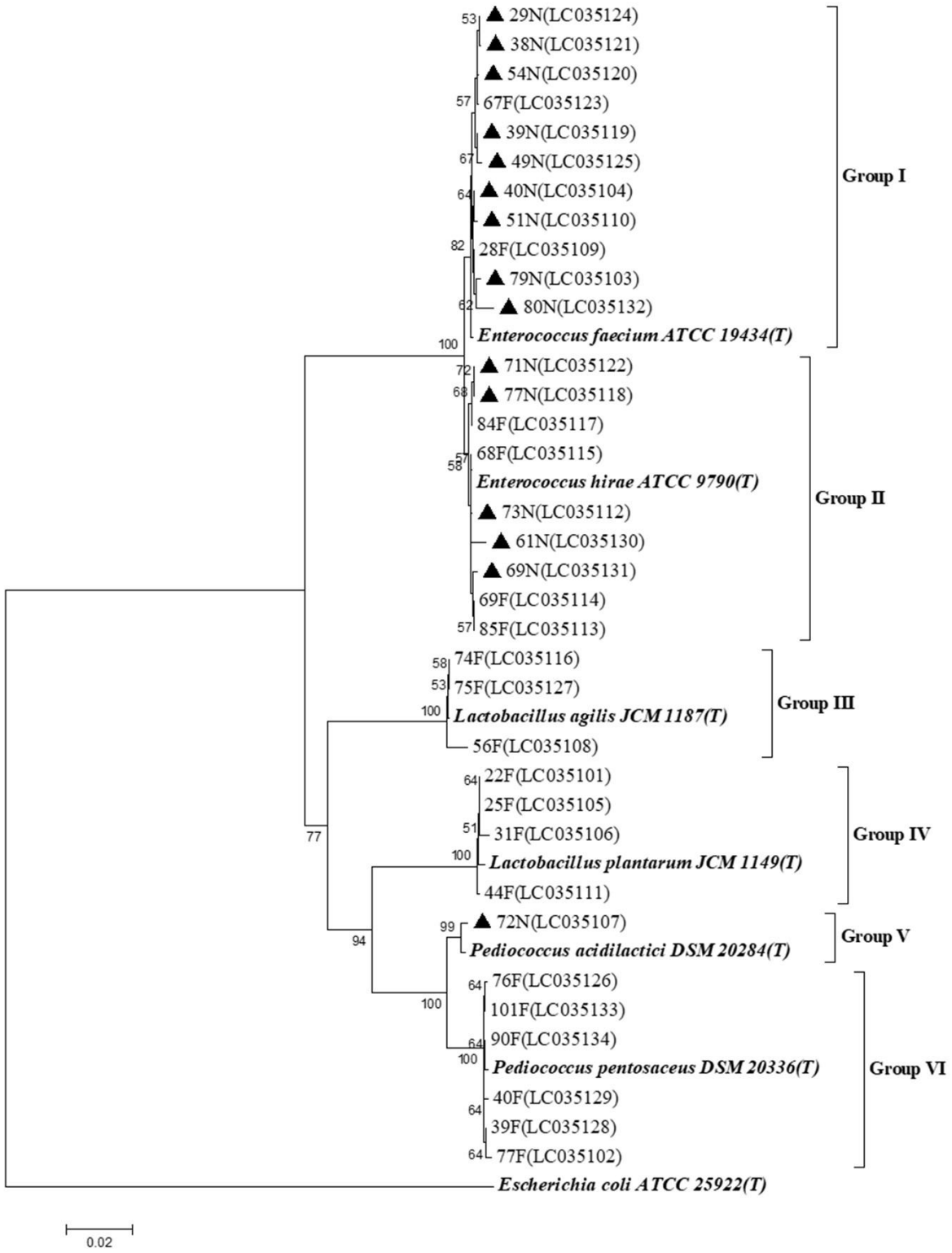

Figure 1 Phylogenetic relationship of 34 selected LAB isolates and reference strains based on 16S rRNA gene sequences. Accession numbers are given in parentheses. Bootstrap replicate values (1000 replicates) of > 50% are shown above the node. *Escherichia coli* is used as the outgroup. Scale represents 0.02 substitutions per site. ▲ represents isolates from indigenous pig feces. Isolates without a symbol are from commercial pig feces. Reference strains are shown in bold italic with (T).

The antimicrobial resistance phenotype and genotype are also the essential selection criteria for screening candidate probiotics (EFSA, 2012). In the present study, isolates that showed resistance toward clindamycin, erythromycin, chloramphenicol and gentamicin were excluded, as they pose a high risk of harboring transferable resistance genes (Muñoz-Atienza et al., 2013). The confirmation of antimicrobial susceptibility on these thirty-four acid- and bile-tolerant LAB revealed that only five isolates (three *L.*

plantarum, *P. pentosaceus* and *P. acidilactici*) were found to be secured from being a possible source of antimicrobial resistance gene transmission, and were acceptable as potential candidates for further studies on probiotics following the European Food Safety Authority (EFSA) recommendations (EFSA, 2012).

In conclusion, in screening for potential probiotic strains from pig feces, 204 LAB isolates were obtained from 60 fecal samples of antibiotic-free, healthy, Thai fattening indigenous and commercial pigs. Of these, 34 isolates showed good resistance to gastric acidity and bile salts, and were selected for species identification and determination of antimicrobial susceptibility. Although the sequencing of 16S rRNA gene is still the gold standard in bacterial species identification (strictly speaking as molecular operational taxonomic units), the analysis of whole-cell protein patterns, but not biochemical profiles, could potentially be used for initial LAB species-specific screening. The final 5 LAB, three *L. plantarum* (22F, 25F and 31F), *P. pentosaceus* 77F from commercial pigs, and *P. acidilactici* 72N from indigenous pigs, which showed the acceptable profiles, *in vitro*, as presumptive probiotics still needs to be proven for their antimicrobial activity and clinical efficacy in further study.

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บทคัดย่อ

การจำแนกเชื้อและหาค่าความไวรับต่อยาปฏิชีวนะของแบคทีเรียกรดแลกติก จากมูลสุกรพื้นเมืองและสุกรฟาร์ม

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แบคทีเรียกรดแลกติกถูกประยุกต์ใช้เป็นโปรไบโอติกเพื่อเพิ่มผลผลิตในการเลี้ยงสุกร การศึกษานี้มีวัตถุประสงค์เพื่อจำแนกชนิดแบคทีเรียกรดแลกติกจากมูลสุกรและหาค่าความไวรับต่อยาปฏิชีวนะตามข้อกำหนดมาตรฐานสากล แบคทีเรียกรดแลกติกจำนวน 204 สายพันธุ์ถูกคัดแยกจากมูลสุกรที่มีสุขภาพดีและเลี้ยงโดยปลอดยาปฏิชีวนะจำนวน 60 ตัวอย่าง โดย 30 ตัวอย่างมาจากสุกรพื้นเมืองและ 30 ตัวอย่างมาจากสุกรฟาร์ม แบคทีเรียกรดแลกติกทั้งหมดถูกทดสอบความทนต่อกรดและน้ำดี พบว่ามีเชื้อแบคทีเรียกรดแลกติกจำนวน 34 สายพันธุ์ที่สามารถทนกรดและน้ำดีได้ดี จึงนำเชื้อทั้งหมดนี้มาทดสอบความไวรับต่อยาปฏิชีวนะ และทำการจำแนกสายพันธุ์ด้วยคุณสมบัติทางชีวเคมียี่สิบหกชนิด รูปแบบการแยกวินิจฉัยด้วยโปรตีน และการวิเคราะห์ลำดับเบสบนยีน 16S rRNA พบว่าเป็นสายพันธุ์เชื้อ *Enterococcus faecium* จำนวนสิบเอ็ดเชื้อ เชื้อ *E. hirae* จำนวนเก้าเชื้อ เชื้อ *Lactobacillus agilis* จำนวนสามเชื้อ เชื้อ *L. plantarum* จำนวนสี่เชื้อ เชื้อ *Pediococcus pentosaceus* จำนวนหกเชื้อ และ เชื้อ *Pediococcus acidilactici* จำนวนหนึ่งเชื้อ และการจำแนกเชื้อด้วยการวิเคราะห์ลำดับเบสบนยีน 16S rRNA และรูปแบบโปรตีนมีความสอดคล้องกันในการจำแนกเชื้อแบคทีเรียกรดแลกติก แต่ไม่สอดคล้องกับการจำแนกเชื้อด้วยวิธีทางชีวเคมี การทดสอบความไวรับต่อยาปฏิชีวนะพบว่า เชื้อแบคทีเรียกรดแลกติกจากสุกรปลอดยาปฏิชีวนะสามารถดื้อยาได้หลายชนิด และพบว่า มีเพียงสี่สายพันธุ์จากสุกรฟาร์ม คือ สายพันธุ์ *L. plantarum* (22F 25F และ 31F) และสายพันธุ์ *P. pentosaceus* (77F) และหนึ่งสายพันธุ์จากสุกรพื้นเมือง คือ สายพันธุ์ *P. acidilactici* (72N) ที่มีระดับความไวรับต่อยาปฏิชีวนะแปดชนิด ได้แก่ แอมพิซิลลิน คลอแรมเฟนิคอล เจนตามัยซิน กานามัยซิน อิริโทรมัยซิน เตตราไซคลีน สเตรปโตมัยซิน และแวนโคมัยซิน ผ่านตามข้อกำหนดขององค์การความปลอดภัยของอาหารแห่งสหภาพยุโรป จากการศึกษาสรุปได้ว่า มีเชื้อแบคทีเรียกรดแลกติกห้าสายพันธุ์จากสุกรสุขภาพดีที่เหมาะสมกับการนำมาศึกษาต่อเพื่อใช้เป็นโปรไบโอติกสำหรับสุกรต่อไป

คำสำคัญ: ความไวรับต่อยาปฏิชีวนะ การจำแนกเชื้อ แบคทีเรียกรดแลกติก มูลสุกร

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