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Identification and characterization of lactic acid bacteria from forest musk deer feces

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The aims of this study were to identify and characterize lactic acid bacteria (LAB) isolated from healthy forest musk deer (FMD) feces which have good potential to develop orally delivered probiotics applied in FMD. Eight LAB isolates were obtained from feces samples and were identified as *Lactobacillus acidipiscis*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus durans* and *Leuconostoc fallax* using morphology, physiology, biochemical and 16s rDNA PCR methods. Five strains were selected after safety and function tests which showed no hemolytic activity, no harmful indole and hydrogen sulphide produced, safety to KM mouse, growth inhibition to *Escherichia coli* (ATCC25922), *Salmonella pullorum* (CVCC527) and *Staphylococcus aureus* (ATCC29213), and no acquired resistance genes detected. The growth of five strains could not be inhibited by 0.9% pig bile. Two *Lactobacillus* strains could survive at as low as pH 2.0. One *L. acidipiscis* strain and one *E. durans* strain could survive at as high as 70°C. Five strains identified as *L. acidipiscis*, *L. plantarum*, *Enterococcus faecium* and *L. fallax* could be well suitable for developing an orally delivered probiotic. To our knowledge, this is the first time to isolate and identify LAB from forest musk deer feces and the characterized strains have good potential for developing an orally delivered probiotic to improve health condition of forest musk deer.

Key words: Lactic acid bacteria (LAB), probiotics, *Lactobacillus*, *Enterococcus*, *Leuconostoc*.

INTRODUCTION

Forest musk deer (FMD, *Moschus berezovskii*) is on the World Conservation Union IUCN Red List of Threatened International Trade in Endangered Species of Wild Fauna

Species and in the Appendices II of the Convention on and Flora (CITES; Zhang, 1998). In addition, FMD is also considered as a Category I key species under the Wild Animal Protection Law in China. Wild FMD is endangered because of over poaching. For sustainable use of musk resource, musk deer breeding farms have been established since 1958 in China. However, some diseases hinder the development of musk deer breeding farms in the past decades.

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Abbreviations: LAB, Lactic acid bacteria; FMD, forest musk deer; GRAS, general regard as safe; MRS, Man-Ragosa-Sharpe agar; PCR, polymerase chain reaction; BLAST, basic local alignment search tool; NCBI, national center for biotechnology information; KM mouse, Kun-Ming mouse; MH, Mueller-Hinton agar; CLSI, clinical and laboratory standards institute; RFLP, restriction fragment length polymorphism; RAPD, randomly amplified polymorphic DNA; M.D.L, musk deer *lactobacillus*; M.D.E, musk deer *enterococcus*.

Nowadays, enteritis disease in FMD causes a high mortality among young forest musk deer. Frequently, antibiotics were used to treat this disease. However, overuse of antibiotics could cause antibiotic resistance to the gut microbiome of FMD (Alfonso, 2005; Levy, 1992). So, we expect to develop a host-specific probiotic via isolating lactic acid bacteria (LAB) from healthy forest musk deer intestine for treatment of this disease.

LAB was widely used in food industries and also for animal and human health improvements because of their lactic acid production and antibacterial activity (Ulrich and Jéssica, 2010). Recently, LAB have been receiving more and more attention as probiotic strains. LAB could significantly improve microflora ecosystem balance of animal gastrointestinal tract (Soomro et al., 2002). Moreover, due to the production of inhibitory compounds like organic acids, hydrogen peroxide, bacteriocin, reuterin, and competition for epithelium adhesion, LAB seems to be a better choice for inhibiting pathogenic bacteria (Sven and Walter, 1990). Gu et al. reported that LAB could inhibit growth of several pathogenic bacteria, such as *Escherichia coli* O13, *Helicobacter pylori*, *Shigella flexneri* and *Salmonella typhimurium* (Gu et al., 2008).

LAB was widely distributed in environment and mammal intestine. According to previous studies, many LAB were isolated from fermented products, such as acidophilus milk (Sun et al., 2010; Chen et al., 2010), fermented vegetables (Rodolphe et al., 2002), fermented beans (Chen et al., 2006) and fermented pork sausage (Pornpan et al., 2010). Hidetoshi et al. reported that some LAB was isolated from intestines of healthy thoroughbreds (Hidetoshi et al., 2007). However, identifying LAB to species level was difficult, since there were no golden standards of biochemical characteristics of LAB especially in genera *Enterococcus*, *Leuconostoc* and *Weissella*, and only a few data about biochemical characteristics of these species were available (Devrlese et al., 1993; Villani et al., 1997; Albert and Anicet, 1999; Myung et al., 2003). Molecular identification methods such as 16s rDNA sequence analysis, genotyping analysis and molecular marker amplification were also commonly used to identify LAB (Jonathan, 1995; Rosalinda et al., 2006; Chen, 2010). Unfortunately, molecular identify methods do not always give unequivocal results for species identification although it is usually time-saving (Smita et al., 2009). Thus, in this study, both traditional and molecular identification methods were carried out to identify the isolates.

LAB have a long history of safe use as functional foods and acquired the "Generally Regarded As Safe" (GRAS) status (Donohue and Salminen, 1996; Marteau and Salminen, 1997). Nevertheless, potential health risk due to antibiotic resistance genes transferred from LAB reservoir strains to pathogenic bacteria, and opportunity infection to host had been addressed (Donohue and Salminen, 1996; Norio and Shoji, 2001; Sung et al., 2005). Therefore, safety assessment of LAB is very important especially those marked as foodstuffs and drugs. A finding demonstrated that many species of LAB have intrinsic antibiotic resistant genes which could be horizontally transferred to patho-genic bacteria (Shalini and Rameshwar, 2005). So, it is significant to determine antimicrobial susceptibility of the functional foods which were developed by LAB. In this study, all candidate strains

were screened for their antimicrobial susceptibility.

The aims of this study were to isolate and identify LAB which has good potential to develop oral delivered probiotics applied in deer. To our knowledge, this is the first time to isolate and identify LAB from healthy forest musk deer feces.

MATERIALS AND METHODS

Sample collection and cultivation

Feces samples of healthy forest musk deer were collected from Sichuan Institute of Musk Deer Breeding in Dujiangyan, China. Samples were serially diluted in sterile physiological saline solution (0.85% NaCl) and spread on LAB selective media, Man-Ragosa-Sharpe agar (MRS, Hangzhou Microbial Reagent CO., LTD, China), which contains 1% CaCO₃ to isolate LAB according to Chen reported (Chen et al., 2005). The plates were anaerobically incubated at 37°C for 3 days. Typical LAB colonies were picked out and anaerobically subcultured on MRS agar at 37°C for another 24 h. LAB colonies were identified by using Gram-staining, catalase activity, colony morphology and bacterial morphology. Only Gram-positive and catalase-negative strains were selected for further studies. All selected strains were stored at -70°C in MRS broth with 10% glycerol.

Traditional identification and genetic analysis

Identification and phenotypical characterization

Bacterial morphology and Gram-stain reaction of isolates were identified by a microscope. Catalase reaction was determined by transferring fresh colonies from agar to a slide glass and adding 3% hydrogen peroxide reagent. Physiological and biochemical characterization of isolates were performed by Bacteria Trace Quantities Reaction Kits (Hangzhou Microbial Reagent Co., Ltd, China) according to manufacturer's instructions. All the used physiological and biochemical characterization test kits are shown in Table 1.

16s rDNA analysis and phylogenetic identification

LAB strains were selected to perform colony polymerase chain reaction (PCR) as described by Sheu et al. (2000), Colony PCR was carried out using a TIANGEN 2xTaq PCR MasterMix Kit (TIANGEN Biotech, Beijing, China) according to manufacturer's instructions by following universal primers: 5' AGA GTT TGA TCC TGG CTC AG 3' (27F) and 5' TAC GGY TAC CTT GTT ACG ACT T 3' (1492R). The PCR mixture contained 25 µl TIANGEN 2xTaq PCR MasterMix, 1 µl of 20 mmol/L 27F primer, 1 µl of 20 mmol/L 1492R primer, a small quantity of bacterial cells. PCR reaction was performed at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, and 72°C for 1 0 min, 4°C for completion. The expected size of PCR product was 1500 bp. Reaction products were electrophoresed in 1% agarose gels and stained with Gold-View (0.005% v/v). Hereafter, complete sequencing was performed by Sangon Biotech (Shanghai, China). The closest known relatives of the isolates were determined by performing Basic Local Alignment Search Tool (BLAST) program at National Center of Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple sequence alignments were performed and drawing of phylogenetic tree was

Table 1. Physiological and biochemical characteristics of eight LAB isolates.

Tests	Isolates							
	M.D. L1	M.D. L2	MRS 4-5	YAN 2-2	YAN 1-4	M.D.E.S8	MRS 2-2	MRS 2-5
Gram-staining	+	+	+	+	+	+	+	+
Cell morphology	R	R	C	C	C	C	C	C
Catalase reaction	-	-	-	-	-	-	-	-
MR	NT	-	-	-	-	+	+	+
VP	-	NT	NT	NT	NT	NT	-	-
Indole	-	-	NT	NT	NT	-	-	-
H ₂ S	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-
Rhamnose	+	-	-	(d)-	(d)-	(d)+	-	-
D-Ribose	+	(d)+	+	+	+	+	W	W
Mannose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	(d)-	(d)-
Sucrose	+	+	+	(d)-	(d)-	(d)-	+	+
Maltose	+	+	+	-	-	+	(d)-	(d)-
Cellobiose	+	+	+	+	+	+	-	-
Lactose	+	+	+	+	+	+	-	-
Starch	-	-	-	-	-	-	-	-
Sorbitol	+	-	-	-	-	-	-	-
Aesculin	+	+	+	+	+	+	-	-
Amygdalin	NT	NT	+	+	+	+	-	-
Raffinose	-	-	-	+	+	-	-	-
Arabinose	-	W	-	(d)-	(d)-	(d)-	-	-
Sorbose	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	(d)-	-	-
Gelatin	-	-	NT	NT	NT	NT	-	-

Note: +: Positive; -: Negative; R: Rod; C: Cocci; NT: Not tested; W: weak reaction; d: different from reference studies.

carried out by Neighbor-Joining method (Naruya and Masatoshi, 1987). The robustness of individual branches was estimated by using bootstrapping with 1000 replications (Felsenstein, 1985) and the phylogenetic tree was conformed by maximum-parsimony method (Kluge and Farris, 1969) and maximum-likelihood method (Cavalli-Sforza and Edwards, 1967). Phylogenetic and molecular evolutionary analyses of the sequences were conducted using MEGA version 4 (Tamura et al., 2007).

Safety assessment

Hemolysis, pathogenicity and harmful metabolites production were evaluated to determine the safety of LAB strains. Hemolysis assay was performed by culturing strains on agar containing 5% defibrinated sheep red blood cells (Deog et al., 2009). Pathogenicity assessment was performed as follows: Tested strains were cultured in MRS broth at 37°C for 18 h, and then harvested by centrifugation at 10000 g for 10 min at 4°C, followed by washed three times and then diluted to 10⁹ cells per milliliter by physiological saline (0.85% NaCl). 0.5 ml inoculum was injected in abdominal cavity of white mouse (KM mouse, each weighting 20 to 25 g, from Animal Experimental Center of Sichuan University) to investigate health conditions of the tested mice in 7 days. The

animal use protocols were approved by Sichuan Agricultural University. Each strain dilution was injected into three white mice. Physiological saline was injected as negative control. The production of indole and hydrogen sulphide was also assayed using Bacteria Trace Quantities Reaction Kits.

Functional characteristics

Antibacterial activity assay

Well diffusion method was used to assay antibacterial activity of tested strains as described by Kekessy with some brief modifications (Kekessy and Piguet, 1970; Tagg and Mcgiven, 1971; Takumi et al., 1999). Wells in Mueller-Hinton (MH) agar plate were punched out with a cork borer (diameter is 6 mm). Each bottom of wells was sealed with 50 µl melted MH medium. In the next step, wells were filled with 200 µl supernatant of 24 h tested strain cultures, and the plate were left for 2 to 3 h to allow diffusion of culture supernatant into the agar. The agar was detached from the edges of the plate with a sterile spatula, thus could expose bottom surface of the agar to inoculate indicator strains. *Escherichia coli* (ATCC25922), *Salmonella pullorum* (CVCC527), *Salmonella enteritidis* (CVCC3377) and *Staphylococcus aureus* (ATCC29213)

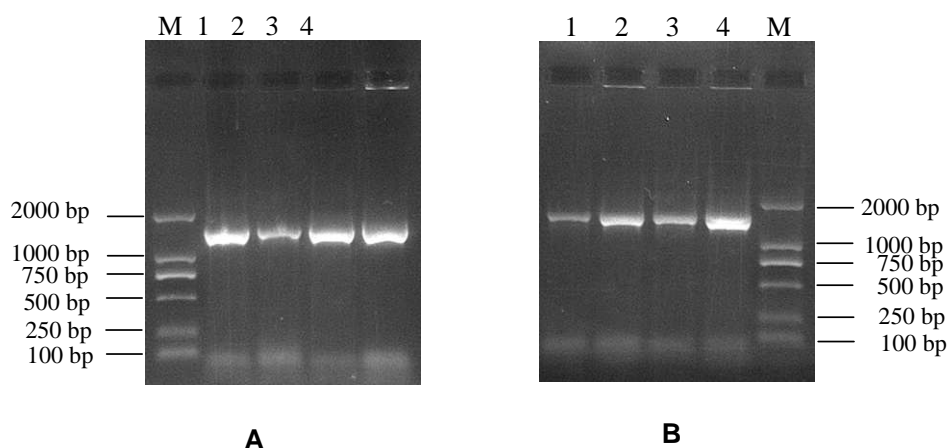


Figure 1. Electrophoretic analysis of colony PCR products. (A) lane M: D2000 DNA marker (TINAGEN); 1: M.D.L1; 2: M.D.L2; 3: M.D.E.S8; 4: M.D.L.MRS2-2; (B) lanes M: D2000 DNA marker; 1: M.D.L.MRS2-5; 2: M.D.E.MRS4-5; 3: M.D.E.YAN2-2; 4: M.D.E.YAN1-4.

were used as indicator strains. Lactic acid was used as positive control and sterilized normal MRS broth was used as negative control, each pate was repeated in triplicate. The plates were incubated at 37°C for 24 h to measure the diameter of each antibacterial zone.

Antimicrobial susceptibility test

Antimicrobial susceptibility tests were performed according to the standard disk diffusion method recommended by Clinical and Laboratory standards Institute (CLSI, 2009). Briefly, candidate strains were cultured overnight in MRS broth and diluted by physiological saline to 10^8 cells per milliliter. The diluted strain solutions were spread onto MRS agar. Antibiotic discs were put onto MRS agar spread with strain solutions. The diameter of each antibacterial zone was measured after 24 h incubation at 37°C. Test of *E. coli* (ATCC25922) was carried out as quality control for selected antibiotics. 37 antimicrobial agents were selected to test antimicrobial susceptibility of *Lactobacillus* and *Leuconotoc* (Charteris et al., 1998), including inhibitors of cell wall synthesis, protein synthesis, nucleic acid synthesis, and cytoplasmic membrane function plus some other antimicrobials. Nine antibiotics were selected for *Enterococcus* according to CLSI (CLSI, 2009). All antimicrobial agents were listed in Table 3, and all were from Hangzhou Microbial Reagent CO., LTD (Hangzhou, China).

Tolerance to pH and bile acid and acid production

Tests were carried out in 10 ml tubes with 3 ml MRS broth supplemented by acid (pH 2.0, pH 3.0, pH 4.0 adjusted with 1.0 M HCl), bile acid (0.3, 0.6, 0.9% pig bile), or normal MRS broth only. All tubes were inoculated by 1% diluted cultures ($OD_{620} = 0.006$) and incubated at 37°C for 24 h and optical density of each tube at 620 nm was measured. Treated cells were cultured on MRS agar after 4 h of incubation under different conditions. Colony formations on MRS agar were counted after 24 h incubation at 37°C to indicate survival. Comparison of the pH values of MRS broth before and after the incubation of the strains was performed by a pH meter (FA2004, Shanghai Shunyuheping Science Instrument LTD) to assay acid production of the candidate strains.

Heat tolerance

LAB candidate strains were cultured for 18 h in eppendorf tubes and then treated at 50, 55, 60, 65, 70, 75 or 80°C for 10 min, respectively. 50 μ l (10^9 CFU/ml) of heated cultures were spread onto MRS agar and incubated for 24 h. Colony formation units were counted. Survival rate was expressed as the survival colonies after heating treatment divided by the total number of viable cells.

RESULTS

Traditional identification

Eight LAB strains were obtained after phenotype characterization of the selected isolates. The carbohydrate fermentation results of M.D.L1 and M.D.L2 strains agreed with the descriptions of *L. plantarum* and *L. acidipiscis* in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Strain MRS4-5 was identified as *Enterococcus durans* and strains M.D.E.YAN2-2 and M.D.E.YAN1-4 were identified as *Enterococcus mundtii* according to previous study (Albert and Anicet, 1999). Strains M.D.L.MRS2-2 and M.D.L.MRS2-5 were similar to *Leuconotoc fallax* described by Villani et al. (1997). Strain M.D.E.S8 could not be accurately identified because the fermentation results of rhamnose, sucrose, arabinose and trehalose were different from previous study (Albert and Anicet, 1999). All the traditional identification results were shown in Table 1.

Genetic analysis

Electrophoresis indicated that the size of PCR products of 16s rDNA were about 1500 bp as shown in Figure 1. PCR products of eight strains were sequenced and their

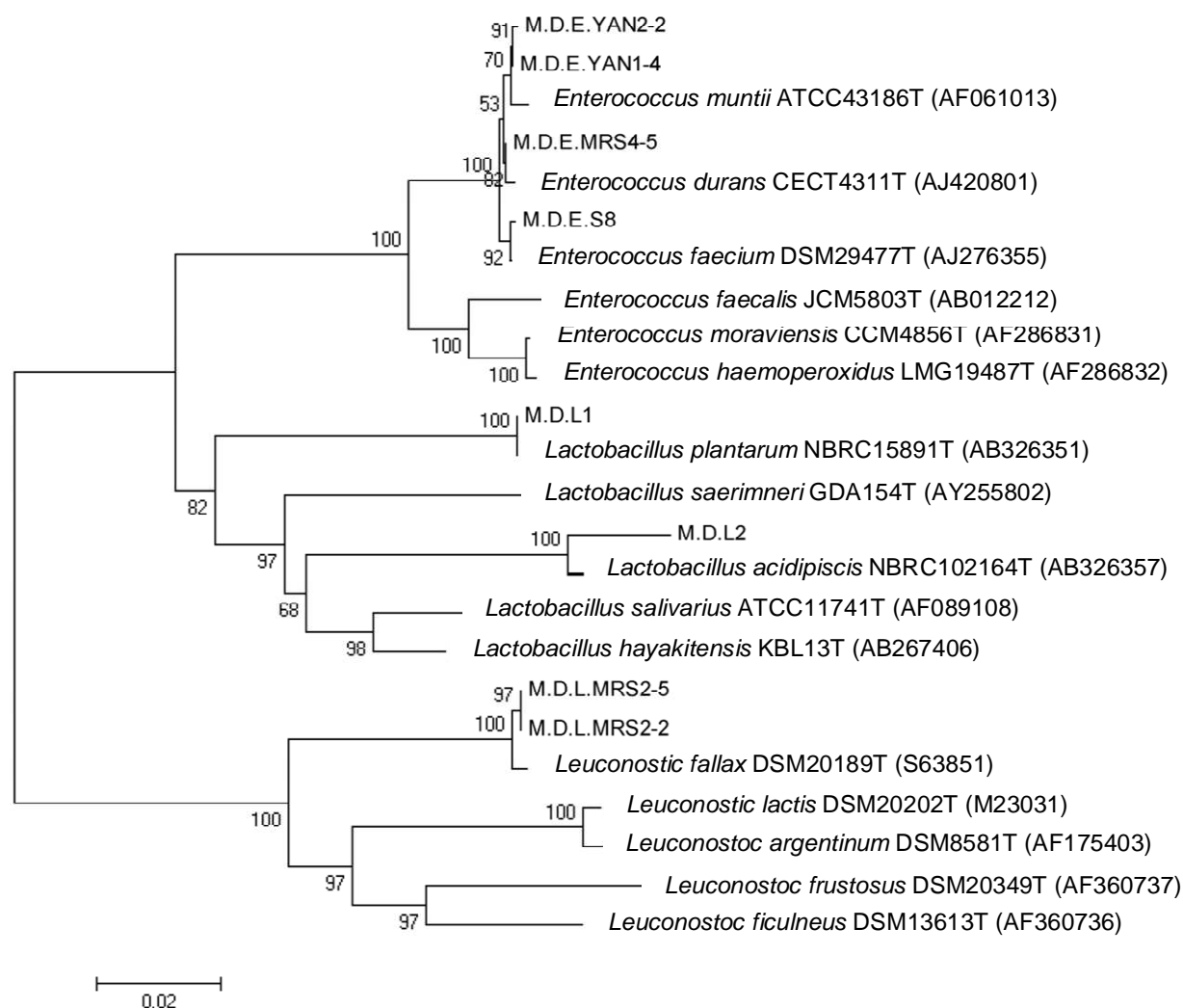


Figure 2. Phylogenetic tree of eight LAB isolates. The phylogenetic tree was constructed by Kimura 2-parameter model and Neighbor-Joining method. The robustness of individual branches was estimated by using bootstrapping with 1000 replications and the phylogenetic tree was conformed by the maximum-parsimony method and maximum-likelihood method. The GenBank accession number of each typical strain is given in parentheses.

16s rDNA sequences were analyzed by BLAST program on NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree was constructed using the sequences of candidate strains and the sequences of closely related typical strains for species identification (Figure 2).

M.D.L.1 was identified as *L. plantarum* with 100% identity to *L. plantarum* NBRC15891. M.D.L.2 shared 98.1% identity with *L. acidipiscis* NBRC102164. M.D.E.MRS4-5 was 99.8% identical to *E. durans* CECT411. M.D.E.YAN2-2 and M.D.E.YAN1-4 were 99.6 and 99.7% identical to *E. mundtii* ATCC43186, respectively. M.D.E.S8 was 99.9% identical to *E. faecium* DSM29477. M.D.L.MRS2-2 and M.D.L.MRS2-5 was 99.6% identical to *L. fallax* DSM20189 (Figure 2). 16s rDNA sequences of eight stains were submitted to

GenBank database and accession numbers were given as follows: M.D.L1 (HM753266), M.D.L2 (HM753265), M.D.E.MRS4-5 (JF690867), M.D.E.YAN2-2 (JF690893), M.D.E.YAN1-4 (JF690892), M.D.E.S8 (JF690891), M.D.L.MRS2-2 (JF690863) and M.D.L.MRS2-5 (JF690864).

Safety assessment

All tested LAB strains showed no hemolysis activity on sheep blood agar. Indole and hydrogen sulphide production were not detected. No harmful clinical symptoms were observed from 27 tested mice after 24 h and 48 h of injection 0.5 ml LAB strains. None of the tested

Table 2. Safety assessment and functional characteristics of eight LAB isolates.

Characteristics	<i>Lactobacillus</i>		<i>Enterococcus</i>				<i>Leuconostoc</i>		
	M.D.L1	M.D.L2	MRS4-5	YAN2-2	YAN1-4	M.D.E.S8	MRS2-2	MRS2-5	
Safety	Hemolysis	-	-	-	-	-	-	-	
	Indole test	-	-	-	-	NT	-	-	
	H ₂ S product	-	-	-	-	NT	-	-	
	Pathogenicity test	-	-	-	-	-	-	-	
Function	pH tolerance*								
	pH 2.0	0.120	0.129	0.012	-0.016	0.000	0.039	0.006	0.003
	pH 3.0	0.165	0.186	0.006	0.006	0.000	0.027	0.000	0.007
	pH 4.0	1.344	1.389	0.021	0.016	0.023	0.030	0.026	0.033
	Bile tolerance								
	0.3%	1.354	1.356	1.124	1.125	1.023	1.632	1.898	1.624
	0.6%	1.363	1.329	1.109	1.204	1.105	1.667	1.728	1.536
	0.9%	1.105	1.222	1.003	1.036	1.006	1.569	1.862	1.236
	Antibacterial activity [†]								
ATCC25922	11 mm	11 mm	6 mm	6 mm	6 mm	6 mm	6 mm	6 mm	
ATCC29213	13 mm	13 mm	6 mm	6 mm	6 mm	6 mm	6 mm	6 mm	
CVCC527	11 mm	9 mm	6 mm	6 mm	6 mm	6 mm	11 mm	6 mm	
CVCC3377	11 mm	12 mm	6 mm	6 mm	6 mm	6 mm	6 mm	6 mm	
Lactic acid	ATCC25922 = 35 mm		ATCC29213 = 35 mm		CVCC527 = 32 mm		CVCC3377 = 32 mm		
MRS broth	ATCC25922 = 0 mm		ATCC29213 = 0 mm		CVCC527 = 0 mm		CVCC3377 = 0 mm		
Acid product (pH)	2.88	2.91	4.33	4.42	4.48	3.46	4.28	4.32	
Heat tolerance	55°C	70°C	70°C	NT	NT	65°C	NT	55°C	
Growth at 15°C	-	-	-	-	-	-	-	-	

Note: *: Optical density of each tube at 620 nm; [†]: diameter of each antibacterial zone; +: Positive; -: Negative; NT: Not tested.

mice was dead after 7 days of injection. The eight LAB strains were no harmful characteristics *in vitro* and *in vivo*.

Functional characteristics

Antibacterial activity assay

Results revealed that M.D.L1 and M.D.L2 could

significantly inhibit growth of indicator strains and the other strains showed no antibacterial activities as shown in Table 2. However, M.D.L.MRS2-2 strain could specifically inhibit growth of *S. pullorum* (CVCC527) with an 11 mm antibacterial zone but could not inhibit growth of other indicator strains. The antibacterial zone of positive control (lactic acid) was more than 32 mm, and no antibacterial zone was detected in the negative

control (normal MRS broth).

Antimicrobial susceptibility test

There are neither golden standard for susceptibility testing of *Lactobacillus* and *Leuconotoc* nor approved guidelines for interpreting test results for now. In our study, Charteris's results were used as

susceptibility tests standard of *Lactobacillus* and *Leuconotoc* to interpret our results (Table 3). Results showed that *Lactobacillus* M.D.L1 and M.D.L2 were resistant to all tested sulfafurazoles agents and aminoglycosides agents except for *streptomycin*, M.D.L1 and M.D.L2 were also resistant to some antimicrobials: aztreonam, vancomycin, metronidazole, and polymyxin B, and susceptible to some other antimicrobials: tetracycline, chloramphenicol, erythromycin, clindamycin, nitrofurantoin, imipenem and meropenem. Furthermore, M.D.L1 and M.D.L2 were susceptible to all of the tested cephalosporins except that with intermediate zone for cefixime. M.D.L2 was found resistant to ceftiofur, but M.D.L1 was with an intermediate zone for this antimicrobial. Moreover, M.D.L1 and M.D.L2 were both with intermediate zones to *penicillin* but susceptible to other tested antimicrobials among penicillins.

L. fallax M.D.L.MRS2-2 and M.D.L.MRS2-5 were susceptible to ampicillin but resistant to all the other penicillins and all the selected cephalosporins, except that M.D.L.MRS2-2 was with intermediate zone for cephadrine and cefixime. For aminoglycosides agents and sulfafurazoles agents, M.D.L.MRS2-2 was susceptible to gentamicin and netilmicin, however, M.D.L.MRS2-5 strain was susceptible to amikacin, netilmicin, and ciprofloxacin. Furthermore, M.D.L.MRS2-2 was resistant to some antimicrobials, such as aztreonam, vancomycin, polymyxin B, nitrofurantoin. And M.D.L.MRS2-5 was resistant to aztreonam, tetracycline, erythromycin, metronidazole and polymyxin B. M.D.L.MRS2-2 was with intermediate zone for cephadrine and cefixime. M.D.L.MRS2-5 was with intermediate zone for vancomycin.

Antimicrobial susceptibility results of *Enterococcus* M.D.E.S8, M.D.E.MRS4-5, M.D.E.YAN1-4 and M.D.E.YAN2-2 were interpreted according to CLSI guidelines as described earlier. Susceptibility results of *Enterococcus* varied. For instance, M.D.E.S8 strain was resistant to penicillin, erythromycin, norfloxacin and ciprofloxacin. However, M.D.E.MRS4-5 and M.D.E.YAN2-2 strains were susceptible to them and M.D.E.YAN1-4 was intermediate to erythromycin and ciprofloxacin. In contrast, M.D.E.S8, M.D.E.MRS4-5, M.D.E.YAN1-4 and M.D.E.YAN2-2 were susceptible to ampicillin, vancomycin, chloramphenicol and nitrofurantoin.

Tolerance to pH and bile acid and acid production

All strains survived at pH 4.0, and *Lactobacillus* strains (M.D.L1 and M.D.L2) could survived as low as pH 2.0 (Table 2), although with a suppressed growth compared to the positive control (normal MRS broth). Growth of tested strains could not be inhibited by 0.9% pig bile ($OD_{620} > 1.000$). The culture pH values of normal MRS broth were at the range of 2.88 to 4.48 as shown in Table 2.

Heat tolerance

Most tested strains tolerated 10 min incubation at 60°C, except that M.D.L.MRS2-5 could only survive under 50°C. M.D.L2 and M.D.E.MRS4-5 tolerated up to 70°C (Table 2) and with a 10% survival rate after incubating 10 min at 55°C (data not shown).

DISCUSSION

Several molecular methods have been developed to identify LAB. These include genotyping, ribotyping (Schleifer et al., 1995), pulsed-field gel electrophoresis (Schwartz and Cantor, 1984), 16s rDNA sequencing, restriction fragment length polymorphism (RFLP) (Stiles and Holzappel, 1997) and randomly amplified polymorphic DNA (RAPD) (Aymerich et al., 2006). In our study, both conventional method and 16s rDNA sequence analysis method were used. M.D.E.S8 was difficult to be identified based on the traditional identification results alone (Table 1). A few biochemical characteristics of M.D.E.S8 were variable compared to the typical strain, however, 16s rDNA sequencing result revealed that it was 99.9% identical to *E. faecium*; it might due to host-specificity which attribute to the variable phenotype. Therefore, we identified M.D.E.S8 as *E. faecium* by both of the traditional and molecular results.

The tolerance against pH was genera dependent. This might be due to acid production differences. Heat tolerance of each species was very different, which was similar with previous reports (Deog et al., 2009). However, there are few differences of the tested strains in bile tolerance activity; all tested strains grew well in MRS broth with 0.9% pig bile. Tolerance to bile is a good characterization for probiotics which benefits the colonization in host gastrointestinal tract.

Antibacterial activity of the tested strains was related to their fermented pH value. The lower its fermented pH value is, the stronger antibacterial activity was detected (Table 2).

In recent years, increasing attention has been paid to antimicrobial susceptibility of the starter cultures and food additives of LAB. In this study, we intend to exploit new LAB as candidate probiotics which should be able to survive in the gut even during antibiotic treatment (Gupta et al., 1995). Therefore, it is important to test their susceptibility toward commonly used antimicrobial agents. Moreover, as a probiotic, LAB should not contain transferable resistance genes for safety use. Usually, resistance genes in LAB were distinguished into two kinds, intrinsic and acquired. Intrinsic resistance is not horizontally transferable, and poses no risks on non-pathogenic bacteria. In contrast, acquired resistance is present in some strains usually susceptible to antibiotic, and might be horizontally spread among bacteria. Thus, antimicrobial susceptibility test was performed to demonstrate the absence of acquired resistance genes and the

Table 3. The antimicrobial susceptibility results and interpretations.

Groups	Antimicrobial		Antibacterial zone diameters (mm)							
	Name	Disc conc. (μg)	M.D.L1	M.D.L2	MRS4-5	YAN2-2	YAN1-4	M.D.E.S8	MRS2-2	MRS2-5
Group 1-Inhibitors of cell wall synthesis										
Penicillins	Penicillin	10U	26.0/I	24.5/I	24.3/S	27.0/S	9.0/R	6.0/R	18.0/R	11.0/R
	Ampicillin	10	35.0/S	35.0/S	34.0/S	33.0/S	18.0/S	18.7/S	20.4/S	17.0/S
	Amoxicillin	10	32.0/S	33.5/S					17.0/R	18.0/R
	Piperacillin	100	34.5/S	36.0/S					16.8/R	12.0/R
Cephalosporins	Cephalothin	30	23.0/S	30.0/S					23.0/S	30.0/S
	Cephradine	30	25.5/S	20.0/S					16.0/I	25.0/S
	Cefazolin	30	29.0/S	28.0/S					32.3/S	35.0/S
	Cefuroxime	30	30.0/S	32.0/S					27.8/S	30.0/S
	Cefaclor	30	30.0/S	29.0/S					21.0/S	25.0/S
	Cefoxitin	30	15.0/I	9.5/R					28.0/S	32.0/S
	Cefixime	5	15.5/I	17.0/I					15.7/I	18.0/S
	Ceftizoxime	30	33.0/S	27.5/S					29.0/S	30.0/S
	Cefotaxime	30	32.0/S	34.0/S					24.5/S	27.0/S
	Ceftazidime	30	27.0/S	25.0/S					19.0/S	20.0/S
	Cefoperazone	75	24.0/S	24.0/S					30.0/S	24.0/S
	Ceftriaxone	30	25.0/S	24.0/S					23.5/S	30.5/S
Monobactams	Aztreonam	30	6.0/R	6.0/R					6.0/R	9.0/R
Glycopeptides	Vancomycin	30	6.0/R	6.0/R	22.0/S	19.0/S	19.2/S	19.0/S	6.0/R	16.0/I
Group 2-Inhibitors of protein synthesis										
Aminoglycosides	Amikacin	30	6.0/R	6.0/R					6.0/R	19.0/S
	Gentamicin	10	6.0/R	6.0/R					18.0/S	6.0/R
	Kanamycin	30	6.0/R	6.0/R					10.5/R	6.0/R
	Netilmicin	30	12.0/R	11.5/R					20.0/S	19.0/S
	Streptomycin	10	13.3/I	12.5/I					6.0/R	9.0/R
Tetracyclines	Tetracycline	30	20.5/S	21.0/S	15.0/I	32.0/S	28.0/S	31.6/S	31.0/S	14.5/R
Chloramphenicols	Chloramphenicol	30	30.0/S	29.0/S	25.5/S	30.0/S	22.0/S	25.0/S	28.0/S	25.0/S
Macrolides	Erythromycin	15	26.0/S	26.0/S	27.5/S	22.0/S	21.0/I	10.0/R	26.0/S	6.0/R
Licosamides	Clindamycin	2	11.5/I	13.5/S					17.6/S	21.0/S

Table 3. Contd.

Group 3-Inhibitors of nucleic acid synthesis											
Sulfafurazoles	Sulfafurazole	300		6.0/R	6.0/R					6.0/R	6.0/R
	Trimethoprim	5		6.0/R	6.0/R					6.0/R	6.0/R
	Norfloxacin	10		6.0/R	6.0/R	19.0/S	22.0/S	18.0/S	10.3/R	6.0/R	17.0/R
	Ciprofloxacin	5		6.0/R	6.0/R	21.5/S	22.0/S	16.8/I	13.0/R	12.0/R	21.0/S
	Nalidixic acid	30		6.0/R	6.0/R					6.0/R	6.0/R
Metronidazoles	Metronidazole	5		6.0/R	6.0/R					6.0/R	6.0/R
Group 4-Inhibitors of cytoplasmic membrane function											
Polmycins	Polymycin B	300		6.0/R	6.0/R					6.0/R	6.0/R
Group 5 - Other antimicrobials											
	Nitrofurantoin	300		32.5/S	31.5/S	24.0/S	24.0/S	19.5/S	19.5/S	6.0/R	24.0/S
	Imipenem	10		41.5/S	43.0/S					18.0/S	33.0/S
	Meropenem	10		42.0/S	42.0/S					22.0/S	33.0/S

Susceptibility expressed as R (resistant), I (intermediate), S (susceptible).

presence of intrinsic resistance genes which make the isolates suitable and safe for probiotics.

In our study, *Lactobacillus* has a high resistance to inhibitors of cell wall synthesis group: aztreonam, vancomycin; inhibitors of protein synthesis group: amikacin, gentamicin, kanamycin, netilmicin; inhibitors of nucleic acid synthesis group: sulfafurazole, trimethoprim, norfloxacin, ciprofloxacin, nalidixic acid, metronidazole and inhibitors of cytoplasmic membrane function group: polymycin B. We considered that high resistance as intrinsic resistance according to earlier studies (Simpson et al., 1988; Charteris et al., 1998; Francesca and Lucia, 2011).

The antimicrobial susceptibility results of *L. fallax* were impressive. It was dissimilar with the results reported by Swenson et al. (1990). In our study, we found M.D.L.MRS2-5 was resistant to

gentamicin, streptomycin, and erythromycin, susceptible to ampicillin, clindamycin and ciprofloxacin, and intermediate to vancomycin. In contrast, Swenson et al. (1990) reported that all tested *Leuconostoc* spp. were very susceptible to gentamicin and erythromycin, resistant to ampicillin, clindamycin and ciprofloxacin, and highly resistant to vancomycin (MIC $\geq 256 \mu\text{g ml}^{-1}$) with no antibacterial zone in the disk diffusion testing. We considered that discrepancy is due to the difference of the testing media. Mueller-Hinton agar with 5% sheep blood was used in Swenson's research and plates were incubated in 5% CO₂, while the antimicrobial agents listed earlier are known to be affected by either media or pH differences caused by CO₂ incubation (Grayson et al., 1989).

The antimicrobial susceptibility to *Enterococcus* was species-dependent. There is a big difference

among M.D.E.S8, M.D.E.MRS4-5, M.D.E.YAN1-4 and M.D.E.YAN2-2 as shown in Table 3. *Enterococcus*, particularly *E. faecium*, was frequently isolated from clinical specimen and always had high resistance to vancomycin which was called vancomycin-resistant enterococci (Steven et al., 1992; Schouten et al., 1999; Glenn et al., 1995). However, in our study, it was noticeable that three of the tested *Enterococcus* strains were susceptible to vancomycin.

Based on safety and function test results, *L. acidiphiscis* (M.D.L2), *L. plantarum* (M.D.L1), *E. faecium* (M.D.E.S8), *L. fallax* (M.D.L.MRS2-2 and M.D.L.MRS2-5) were considered as potential LAB to develop oral delivered probiotics. Though *E. faecium* (M.D.E.S8) strain showed no antibacterial activity to indicator strains, it might have synergistic activity and promote immune system as a time famous probiotics when colonizing together

with the other LAB in host intestine (Jalil et al., 2003; Scharek et al., 2005). Further tests should be performed to study effect of five LAB isolated from forest musk deer on health.

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