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Encapsulating Viability of Multi-strain Lactobacilli as Potential Probiotic in Pigs

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Abstract

Important aspects of the selection of probiotics to be used for mixing in animal feed include host species specificity and probiotic cell survival during production and storage of their products. The research was to screen and investigate some probiotic properties of lactic acid bacteria (LAB) isolated from pig fecal samples. One hundred and thirty-eight representative LAB isolates, which were isolated from 51 pig fecal samples, were tested for acid and bile tolerance, antimicrobial susceptibility, antibacterial activity, potential adhesion to the cell surface, and survival rates when stored in varied microencapsulation forms: freeze-dried, spray-dried, and micro-beads. The antibacterial activity results of the ten LAB isolates, which were acid- (pH 2, 3 h) and bile- (50% (*v*/*v*) fresh pig bile, 8 h) tolerant and suitable for resisting the five antibiotics commonly used for treating pig infections with pathogenic indicator strains, showed that three isolates (L21, L80, L103) had strong inhibition to *Escherichia coli*, *Salmonella* group B, and *Salmonella* group D using co-culturing and agar spot assays. The three isolates had high hydrophobicity (65–73%) and did not show antagonistic growth against each other. All three selected isolates had greater than 80% survival in freeze-dried and micro-bead forms at 25–30 °C after 2 days of storage (80.4–86.75%, 7.31–7.89 log CFU/ml). Sequence analysis of the 16S rRNA genes demonstrated that the three isolates belong to *Lactobacillus plantarum* (strain L21 and strain L80) and *L. paraplantarum* (strain L103). The single and multiple strains of these bacteria may have potential use as probiotics in pig diets.

Keywords Encapsulation · Multi-strain · Probiotic · Pigs

Introduction

Probiotics are recently defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. The European Commission has banned the use of antibiotic growth promoters (AGP) in animal feed since January 2006. This concern resulted in choosing an alternative for animal farming, such as "probiotic" application. Pig-raising management may provide biodiversity of microorganisms in pig gastrointestinal tracts (GITs). The farming under conventional raising may have a different biodiversity from that under free-range raising. Many factors cause social and environmental stresses during the weaning period of pigs, including piglet separation from their mother, handling, transport, unfamiliar physical environments, and mixed litters which lead to performance decreases and mortality increases [2]. In this period, probiotics were applied to control diarrhea and maintain an acidic environment in the GITs, which produced lactic acid and other antibacterial substances. One important aspect of probiotic selection is host species specificity for good functionality. Therefore, probiotics used in animal species have usually been isolated from their bodies [3].

Considering that the selected microorganism is not pathogenic, to be used as probiotic, it should able to survive in the gastrointestinal environment and has the ability to withstand low pH and high concentrations of bile acids. For safety assessment, probiotics should not have any transferable antibiotic resistance genes that transfer to other bacteria [4]. In addition, the chosen strain should be tolerant during manufacturing, transportation, and application processes in terms of maintaining its viability and desirable characteristics and storage stability [5]. The combination of different probiotic strains has been reported that was more effective than single-strain probiotics [6]. Microencapsulation techniques (MEs) are one of the most efficient methods used for keeping viable cells of

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probiotics to store longer and maintain functionality when applied on the host [7]. Pringsulaka et al. [8] reported that multi-strain probiotics maintained at 10^7 colony-forming units per gram (CFU/g) in broiler chicken diets for 3 days of storage at 30 °C. However, information about the survival of these multi-strain probiotics during microencapsulation processes and storage stability tests, before uses in animals, are scarce.

Lactic acid bacteria (LAB) are widely used as probiotics in humans and animals. They are a genetically diverse group of bacteria, including species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. LAB are Gram-positive and usually catalase-negative bacteria, which grow under microaerophilic to strictly anaerobic conditions, and are non-spore forming. The purpose of this research was to isolate, characterize, and evaluate LAB from conventionally and free-range raised pig feces. The tests of probiotic properties include acid and bile tolerance, antimicrobial susceptibility, antibacterial activity, cell surface hydrophobicity, coexistence tests, and genotypic identification. The storage stability was tested in various forms of microencapsulation techniques.

Materials and Methods

Sample Collection and Isolation of LAB

All 51 fecal samples, from healthy pigs that had no history of gastrointestinal disease and had not taken antibiotics for at least 2 weeks before sampling, were collected from 11 free-range raised pigs (FRP) and 40 conventionally raised pigs (CRP) in Surin, Thailand.

Tenfold serial dilutions from homogenate fecal samples in 0.85% NaCl were obtained and plated in De Man, Rogosa, and Sharpe (MRS) agar [9], with modification by adding 0.3% (*w*/*v*) $CaCO_3$ using the pour plate method. Incubation was carried out aerobically with 5% carbon dioxide (CO_2) for 2 days at 37 °C. Bacterial colonies that showed a clear zone in the surrounding acid on the plate were picked, purified by streaking on MRS agar, and incubated aerobically with 5% CO_2 for 2 days at 37 °C. The pure colonies were stored in a stock medium (10% skimmed milk and 20% glycerol) at − 70 °C for further testing.

Acid and Bile Tolerance Test

Testing of the bile tolerance was similar to that described by Park et al. [10]. Briefly, LAB isolates were grown overnight, subcultured in 50% (*v*/*v*) fresh pig bile (collected from the gallbladder of slaughtered pigs) broth at pH 8 (1:1 (*v*/*v*) ratio of MRS broth to filtered fresh pig bile), and incubated aerobically in 5% CO_2 at 37 °C for 8 h. A tenfold dilution from homogenate samples was plated in MRS agar, incubated aerobically in 5% CO_2 at 37 °C for 2 days and enumerated in terms of their growth (30–300 colonies). The acid tolerance of LAB was tested as described by Park et al. [10]. Briefly, the cultures were grown in MRS broth at 37 °C overnight, then subcultured into 10 ml of fresh MRS broth adjusted to pH 2 using 1 M HCL and incubated aerobically in 5% CO_2 for 3 h at 37 °C. Tenfold serial dilutions of the homogenate samples were plated on MRS agar, incubated aerobically in 5% CO_2 for 2 days at 37 °C, and enumerated in terms of their growth (30–300 colonies). Bacterial concentrations were expressed as colony-forming units per gram of sample (CFU/g).

Antimicrobial Susceptibility Test

A modified version of the Kirby-Bauer disc diffusion method was used, in which Mueller-Hinton medium was replaced by LAB susceptibility test medium (LSM) (Fig. 1) [11, 12]. LAB were spread on the LSM agar at 10^8 CFU/ml and then placed 6-mm diameter discs of five chosen antibiotics (Oxoid, Hampshire, England) commonly used in treating human or pig infections and different antibacterial activities including inhibitors of cell wall synthesis: amoxicillin-clavulanic acid (AMC 30 μg), Gram-positive spectrum: vancomycin (VA 30 μg), broad spectrum: tetracycline (OT 30 μg), inhibitors of folate synthesis: sulfamethoxazole-trimethoprim (SXT

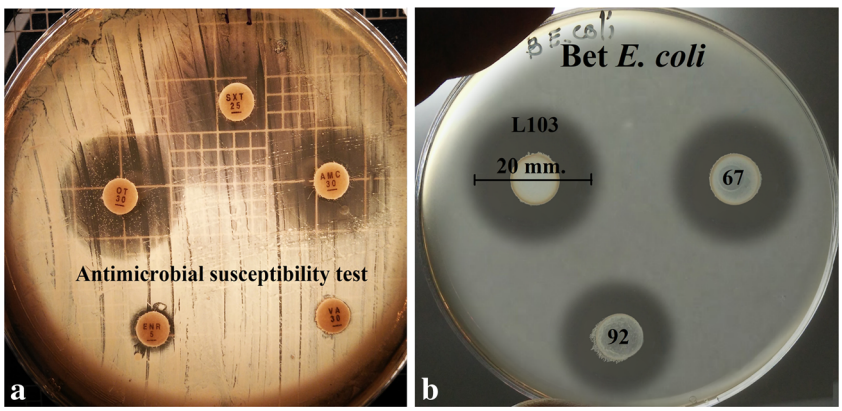

Fig. 1 Antimicrobial susceptibility of the selected LAB isolates using disc diffusion method on LSM agar (**a**) and antibacterial activity of the strain L103 against *E. coli* using agar spot assay (**b**) (20 mm of the inhibition diameter zone indicated with the arrow)

25 μg), and inhibitors of DNA replication: enrofloxacin (ENR 5 μg) and incubated at 37 °C for 24 h. The diameter of the antibiotic inhibition zone was measured using a ruler. All antibiotics were tested in duplicate. The inhibition zone diameters in millimeters were measured, including the diameter of the discs. The results regarding the inhibition zone diameters were resistant (*R*), intermediate (*I*), and sensitive (*S*) using cutoff values following AMC—$R \leq 18$, *I* 19–20, $S \geq 21$; VA—$R \leq 14$, *I* 15–16, $S \geq 17$; OT—$R \leq 14$, *I* 15–18, $S \geq 19$; SXT—$R \leq 10$, *I* 11–15, $S \geq 16$; and ENR—$R \leq 13$, *I* 14–18, $S \geq 19$) [13].

Antibacterial Activity Test

The antibacterial activity of selected LAB isolates against five pathogenic indicator strains (β *Escherichia coli*, *Salmonella* group B, *Salmonella* group D, *Enterococcus faecalis* ATCC29218, and *Streptococcus suis*, obtained from Northeastern Veterinary Research and Development Center, lower zone (Surin), Thailand) was tested using an agar spot assay and co-culture techniques. For the agar spot assay, a 5-μl sample of an 18-h MRS broth culture was spotted onto the surface of the MRS agar, four spots each, and was incubated in 5% CO_2 at 37 °C for 24 h. Then, the MRS agar plate was overlaid with 7 ml of soft agar (0.7%) inoculated with these pathogenic indicator strains at a final concentration of 10^7–10^9 CFU/ml. Plates were then incubated in 5% CO_2 at 37 °C for 24 h. The antagonistic activity was investigated by the appearance of a growth inhibition zone around the spots [14].

A co-culture technique was tested according to Lee et al. [15]. Briefly, 1 ml of overnight culture of selected LAB cultures (10^8 CFU/ml) was mixed with 1 ml of overnight culture for each strain of three pathogenic indicator strains (β *E. coli*, *Salmonella* group B, and *Salmonella* group D) (10^8 CFU/ml) and completed with 1 ml of Mueller-Hinton broth, incubated at 37 °C for 48 h. Then, selected LAB isolates, β *E. coli* and *Salmonella*, were plated on MRS, MacConkey, and brilliant green agars, respectively. These isolates were diluted using tenfold serial dilutions and incubated in appropriate conditions for counting viable cells, including in 5% CO_2 at 37 °C for 48 h for LAB isolates and at 37 °C for 24 h for β *E. coli* and *Salmonella*.

Cell Surface Hydrophobicity

The hydrophobicity was measured as the ability of selected LAB isolates to adhere to hydrocarbons as described by García-Hernández et al. [16]. Briefly, bacterial cultures in exponential growth phase were harvested and pelleted by centrifugation at 4000*g* for 15 min, washed twice with PBS (pH 7.0), and re-suspended in the same buffer solution. The suspension was adjusted with PBS (pH 7.0) to approximately 10^8 CFU/ml (OD 640 nm = 1.0 (*A*)). We added 3.5 ml of this bacterial suspension to 0.5 ml of hexadecane, mixed using a vortex mixer for 2 min, and allowed the mixture to stand until phase separation (10–15 min). The lower aqueous phase was carefully removed, and its optical density was determined at 640 nm (*B*). The hydrophobicity index (HPBI) percentage was calculated as follows: HPBI = [(*A* − B) *A*] × 100]. Isolates with an HPBI greater than 70%, between 50 and 70%, and lower than 50% were classified as having high, moderate, and low hydrophobicity, respectively [8].

Coexistence Test

Coexistence among the selected LAB strains was tested using the cross-streak method. Briefly, the selected LAB strains were streaked perpendicularly and across from each other on MRS agar plates. The plates were incubated aerobically in 5% CO_2 for 24–48 h at 37 °C to observe the strains' antagonism against each other [8].

Genotypic Identification

Bacterial strains that have all the properties tested above were identified at the species level by sequencing the 16S rRNA gene. Selected strain cultures grown overnight to log phase were centrifuged at 6000*g* for 2 min. The supernatant was decanted completely. Genomic DNA was extracted and purified using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified with the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [17]. Each reaction mixture (50 μl) contained 41.5 μl of Milli-Q water, 5 μl of 10× reaction buffer (1 μl of 10× reaction buffer plus 9 μl of other reagents), μl of 0.2 mM deoxynucleoside triphosphate (dNTP), 1 μl of 2.0 μmoles each of the two primers, one selected colony of each sample (15–20 ng of template DNA), and 0.5 μl of 2.5 units of *Taq* DNA polymerase. The following amplification program was used: 95 °C for 5 min, 30 cycles consisting of 95 °C for 90 s, 55 °C for 90 s, 72 °C for 1.5 min, and 10 min at 72 °C. The purified PCR products (one product for one isolate) were sent to Solgent Co. Ltd. (Daejeon, Korea) for sequencing. The related taxa based on the 16S rRNA gene sequences were obtained from the GenBank database. Phylogenetic trees were reconstructed using neighbor-joining and maximum likelihood methods in the MEGA7 program [18], with bootstrap values based on 1000 replications.

Storage Stability Test in Freeze-Drying, Spray-Drying, and Micro-bead Forms

Culture Preparation

Three selected probiotics (*Lactobacillus paraplantarum*, *L. plantarum* L21, *L. plantarum* L80) were cultured in MRS

broth at 37 °C for 15 h (10^9 CFU/ml). A stability test was measured in three cultured forms, including single strains (SS) and multiple strains (MS, the three selected probiotics combined) of the selected probiotics. Microencapsulation techniques (MEs) were tested in three preparations as described below:

Cultures in Freeze-Drying Form

Freeze-dried cells were prepared according to Sánchez et al. [19] with minimal modification. Briefly, the SS and MS of the selected three probiotics were cultured in MRS broth at 37 °C for 15 h (10^9 CFU/ml). The cultures were centrifuged at 10000×*g* for 15 min for harvesting cell pellets. The cell pellets were re-suspended with 1 ml of phosphate buffered saline (PBS, pH 7.2), centrifuged at 10000×*g* for 15 min to pick the cells, and then mixed with 1 ml of ultra-high temperature (UHT) milk. A 3-ml glass bottle containing 1 ml of each prepared probiotic was frozen at − 80 °C for 8 h and then freeze-dried using a freeze dryer (CHRIST, GAMMA 2-16 LSC, Germany) for 34 h. The freeze-dried cells were stored for different times at 4 °C until testing.

Cultures in Spray-Drying Form

The three chosen probiotics were prepared as described in the culture preparation process. Carrier materials (gum arabic 30% and gelatin 15%) were mixed with deionized water and autoclaved at 121 °C for 15 min (water phase). The capsules of selected probiotics prepared in filtered coconut oil (45%) were then homogenized at the lowest speed, 3000 rpm, for 10–15 min. The oil phase was mixed with the water phase using a homogenizer, and all processes were done under sterile conditions. The homogenized mixtures were dried using a spray dryer (mini spray dryer BUCHI, B-290, Germany) at a 110 °C inlet temperature, a 75 °C outlet temperature, and 4% moisture with a feed rate at 100 rpm. The product was stored at 4 °C until testing [20].

Cultures in Micro-bead Encapsulation

The prepared cultures as described in the culture preparation process were formed into alginate capsules according to Ross et al. [21]. Briefly, the cultures (10^9 CFU/ml) in 20% skimmed milk were mixed with 1.8% sodium alginate sterile solution. Capsules were prepared aseptically by dropping the alginate mixture in 500 ml of 0.1 M calcium chloride sterile solution under gentle stirring for hardening. The micro-beads were rinsed twice with distilled water and kept in a vacuum plastic sterilized bag. The capsules were stored at 4 °C until testing. Twenty four capsules or two teaspoons were dissolved in PBS (pH 7.2) for the evaluation of the number of released viable probiotics.

For stability testing, all culture preparation was stored at 4 and 25–30 °C for 96 h and stored longer at 4 °C for 4 weeks. The viable cells were measured every 24 h and every week for 4 weeks. The stored probiotic products were serially tenfold diluted and plated in triplicate onto MRS agar. The plates were incubated in 5% CO_2 at 37 °C for 48 h. The number of CFUs was calculated as the log CFU/ml according to the equation log (CFU/ml) = log [(number of colonies × dilution factor) 0.1]. The survival rate of LAB during the storage was calculated by applying survival rate = (N_{A1} N_{A2}) × 100 where N_{A1} (log CFU/ml) is the number of initial viable cells after encapsulation (time 0) and N_{A2} (log CFU/ml) is the number of viable cells within encapsulation forms after storage.

Statistical Analysis

An analysis of variance (ANOVA) was performed by comparing the mean difference of each variable with the least significant difference (LSD) test at a 95% significance level using the Program R (version 3.2.3).

Results

Five different morphologic colonies which grew on MRS agar with 0.4% (*w*/*v*) $CaCO_3$ added and showed acid production, which reacts with the mixed $CaCO_3$ medium, resulting in a clear zone surrounding from each sample was randomly picked for further testing. Of 255 isolates, 138 were catalase-negative and Gram-positive properties which confirmed to be LAB. These isolates were further tested for acid (pH 2.5, 3 h) and bile (50% (*v*/*v*) fresh pig bile, 8 h) tolerance.

All 92 acid- and bile-tolerant LAB isolates (19 isolates from FRP and 73 isolates from CRP) were tested for antimicrobial susceptibility to 5 antibiotics. The results showed that almost all isolates from FRP were susceptible to SXT (80%) and AMC (58%) but were resistant to VA, ENR, and OT at 100, 84, and 58%, respectively. The isolates from CRP were resistant to SXT, VA, ENR, and OT at 70, 85, 71, and 85%, respectively, but showed susceptibility to AMC at 59%, as shown in Table 1. LAB antibiotic resistance may present two characteristics including natural or intrinsic resistance, being non-transmissible and acquired resistance, usually caused from bacterial mutation or may carry mobile genetic elements (plasmids and transposons) and potentially transmissible to other bacteria [22]. *Lactobacillus* species were intrinsically resistant to vancomycin, streptomycin, ciprofloxacin, and sulphamethoxazole-trimethoprim [23]. Therefore, we selected ten isolates which were intrinsic but not acquired resistance to these five antibiotics for further testing.

The antibacterial activity against five pathogenic bacteria for the ten isolates resistant to acid and bile and suitable for resisting to the antibiotics is shown in Table 2. Viable cell

Table 1 Antimicrobial susceptibility of 92 LAB isolates isolated from free-range raised (FRP) and conventionally raised pigs (CRP) in Surin Province, Thailand

Sources	Number of LAB isolates isolated from FRP and CRP for resisting to five antibiotics (%)														
	Antibiotics														
	SXT (25 μg)			AMC (30 μg)			VA (30 μg)			ENR (5 μg)			OT (30 μg)		
	R	*I*	*S*	*R*	*I*	*S*	*R*	*I*	*S*	*R*	*I*	*S*	*R*	*I*	*S*
CRP	51 (70)	0 (0)	22 (30)	24 (33)	6 (8)	43 (59)	62 (85)	2 (3)	9 (12)	52 (71)	11 (15)	10 (14)	62 (85)	7 (10)	4 (5)
FRP	3 (16)	0 (0)	16 (84)	3 (16)	5 (26)	11 (58)	19 (100)	0 (0)	0 (0)	16 (84)	3 (16)	0 (0)	11 (58)	1 (5)	7 (37)

Antimicrobial susceptibility interpreted using cutoff values of inhibition zone diameters in millimeters following AMC—$R \leq 18$, I 19–20, $S \geq 21$; VA—$R \leq 14$, I 15–16, $S \geq 17$; OT—$R \leq 14$, I 15–18, $S \geq 19$; SXT—$R \leq 10$, I 11–15, $S \geq 16$; and ENR—$R \leq 13$, I 14–18, $S \geq 19$) [13]

R resistant, *I* intermediate, *S* susceptible, *SXT* sulphamethoxazone/trimethoprim, *AMC* amoxycillin/clavulanic acid, *VA* vancomycin, *ENR* enrofloxacin, *OT* oxytetracycline

counts between the LAB isolates and pathogens using co-culturing techniques are also shown in Table 2.

All ten LAB isolates showed inhibition to β *E. coli* in both co-culturing and agar spot assays. Antibacterial activities of the three selected LAB isolates can reduce and inhibit the growth of β *E. coli*, *Salmonella* group B, and *Salmonella* group D by 1.92 to 3.72 log cycles and showed diameter zones from 16 to 34 mm using co-culturing techniques and agar spot assay, respectively. The three selected LAB isolates were L21, L80, and L103.

Microbial adhesion to the solvents of three selected isolates (L21, L80, L103) was expressed as % HPBI. In this study, high hydrophobicity (73%) was observed for the L21 and L80 isolates. The L103 isolates showed moderate hydrophobicity (65%).

In this study, the three selected isolates did not show antagonism against each other on MRS agar after incubation for 48 h. Therefore, the three strains, *L. plantarum* (L21 and L80), and *L. paraplantarum* (L103), were combined as multi-strain probiotics for further application.

All three selected isolates had greater than 80% survival in all the microencapsulation forms except for spray drying when stored at 4 °C for 28 days (87.77–94.96%, 7.99–8.00 log CFU/ml). The reduction rate of the isolates' viable cells was 0.4–0.57 log cycles (Table 3). The viable cells of the three isolates in storage at 4 °C for 28 days in freeze-drying (FD) and micro-bead (MB) forms had a higher reduction than those in storage for 4 days (0.03–0.07 log cycles) but their viable cells were still acceptable in quantity, as shown in Table 3. All three selected isolates had greater than 80%

Table 2 Anti-pathogen of ten LAB isolates isolated from free-range raised (FRP) and conventionally raised pigs (CRP) in Surin Province, Thailand

Sources	LAB isolates	Co-culture between LAB isolates and bacterial pathogen at 48-h incubation (log CFU/ml)				Inhibition diameter zone using spot agar assay (mm)				
		β *E. coli*	*Salmonella* group B	*Salmonella* group D	LAB	β *E. coli*	*Salmonella* group B	*Salmonella* group D	*E. faecalis* ATCC29218	*S. suis*
FRP	L67	4.30	6.98	5.65	6.26	20	24	17	10	0
	L80	4.30	6.08	5.48	5.38	34	16	18	10	0
	L92	4.30	6.11	5.78	5.95	22	19	22	0	0
CRP	L21	4.30	6.00	5.48	6.60	30	17	24	0	10
	L103	4.30	5.26	5.95	5.26	20	18	20	12	0
	L57	4.30	5.65	5.40	6.56	14	22	18	11	0
	L58	4.30	6.16	5.70	5.89	14	18	30	11	0
	L20	4.30	6.06	6.56	6.18	24	24	20	0	0
	L38	4.30	5.60	5.85	6.27	30	18	18	0	0
	L73	4.30	5.89	5.78	6.60	25	20	18	0	0

Initial viable cells at 10^8 CFU/ml in both LAB isolates and bacterial pathogen

The degree of growth inhibition interpreted using cutoff values of inhibition zone diameters (mm) following no inhibition 0, low inhibition 5–10 mm, medium inhibition 11–17 mm, high inhibition > 17 mm

Table 3 Cell survival (log CFU/ml) of selected LAB isolates in various forms stored at 4 °C

Isolates	Storage forms	Initial cells	Mean ± SD of viable cell counts (log CFU/ml) and cell survival (%)				
			Times (day)				
			4	7	14	21	28
L103	FD	9.09 ± 0.01	8.32 ± 0.02 (91.64)	8.33 ± 0.01 (91.64)	8.31 ± 0.01 (91.45)	8.29 ± 0.00 (91.20)	8.26 ± 0.00 (90.97)
	SD	9.09 ± 0.01	4.48 ± 0.00^{a} (49.28)	4.48 ± 0.00^{a} (49.26)	4.43 ± 0.02^{a} (48.75)	4.40 ± 0.01^{a} (48.45)	4.36 ± 0.01^{a} (47.91)
	MB	9.09 ± 0.01	8.35 ± 0.01^{b} (91.89)	8.33 ± 0.01 (98.89)	8.26 ± 0.01^{b} (98.08)	8.15 ± 0.01^{b} (96.80)	8.00 ± 0.00^{b} (94.96)
L80	FD	9.10 ± 0.00	8.38 ± 0.00^{a} (92.13)	8.37 ± 0.00 (92.02)	8.34 ± 0.00 (91.73)	8.29 ± 0.01 (91.14)	8.25 ± 0.01 (90.71)
	SD	9.07 ± 0.02^{a}	4.31 ± 0.01 (47.52)	4.30 ± 0.00^{a} (47.48)	4.27 ± 0.01^{a} (47.06)	4.19 ± 0.01^{a} (46.22)	4.15 ± 0.03^{a} (45.72)
	MB	9.08 ± 0.00^{a}	8.49 ± 0.00^{b} (93.53)	8.48 ± 0.00^{b} (93.38)	8.34 ± 0.00 (91.88)	8.24 ± 0.01^{b} (90.72)	7.99 ± 0.00^{b} (87.96)
L21	FD	9.10 ± 0.01	8.40 ± 0.00^{a} (92.36)	8.39 ± 0.00 (92.17)	8.37 ± 0.00 (91.98)	8.34 ± 0.00 (91.70)	8.30 ± 0.00 (91.27)
	SD	9.09 ± 0.00	4.61 ± 0.00 (50.7)	4.61 ± 0.00^{a} (50.67)	4.54 ± 0.01^{a} (49.92)	4.50 ± 0.01^{a} (49.44)	4.43 ± 0.02^{a} (48.72)
	MB	9.09 ± 0.00	8.49 ± 0.00^{b} (93.4)	8.48 ± 0.00^{b} (93.27)	8.35 ± 0.00^{b} (91.82)	8.27 ± 0.00^{b} (90.93)	8.00 ± 0.00^{b} (87.93)
MS	FD	9.10 ± 0.01	8.51 ± 0.00 (93.52)	8.43 ± 0.00 (92.64)	8.39 ± 0.00 (92.16)	8.37 ± 0.00 (91.93)	8.34 ± 0.01 (91.58)
	SD	9.11 ± 0.01	4.64 ± 0.00^{a} (50.91)	4.63 ± 0.00^{a} (50.89)	4.57 ± 0.01^{a} (50.15)	4.55 ± 0.00^{a} (49.91)	4.45 ± 0.02^{a} (48.82)
	MB	9.11 ± 0.01	8.54 ± 0.00^{b} (93.81)	8.53 ± 0.00^{b} (93.71)	8.37 ± 0.01^{b} (91.89)	8.26 ± 0.01^{b} (90.70)	7.99 ± 0.00^{b} (87.77)

The means with different superscript letters within the same column in each isolate group are significantly different ($p < 0.05$). Data are expressed as the means ± SD in triplicate. MS (multiple strains) consisted of the three *Lactobacillus* strains (L103, L80, L21)

L103 *Lactobacillus paraplantarum*, L80 *Lactobacillus plantarum* L80, L21 *Lactobacillus plantarum* L21

Storage forms: *FD* freeze-dried, *SD* spray-dried, *MB* micro-beads

survival in the FD and MB forms, when stored at 25–30 °C for 48 h (80.4–86.75%, 7.31–7.89 log CFU/ml). The reduction rate of the isolates' viable cells was 1.08–1.14 log cycles, as shown in Table 4. Isolate L21 in the micro-beads form had the best shelf life at 81% survival (7.37 log CFU/ml) after 72 h of storage at 25–30 °C.

Table 4 Cell survival (log CFU/ml) of selected LAB isolates in various forms stored at 25–30 °C

Isolates	Storage forms	Initial cells	Mean ± SD				
			Viable cell counts (log CFU/ml) and cell survival (%)				
			Times (h)				
			0	24	48	72	96
L103	FD	9.09 ± 0.01	8.39 ± 0.01 (92.33)	8.39 ± 0.05 (92.4)	7.34 ± 0.04 (80.75)	7.27 ± 0.01 (79.9)	6.51 ± 0.35 (70.58)
	SD	9.09 ± 0.01	4.75 ± 0.00^{a} (52.29)	4.65 ± 0.01^{a} (51.2)	4.39 ± 0.02^{a} (48.28)	3.99 ± 0.01^{a} (43.84)	3.33 ± 0.01^{a} (36.62)
	MB	9.09 ± 0.01	8.40 ± 0.00^{b} (92.46)	8.49 ± 0.02^{b} (93.4)	7.45 ± 0.01^{b} (82.03)	6.85 ± 0.04^{b} (75.37)	5.97 ± 0.02^{b} (65.69)
L80	FD	9.10 ± 0.00	8.39 ± 0.01 (92.24)	8.40 ± 0.02^{a} (92.29)	7.31 ± 0.02^{a} (80.4)	6.98 ± 0.01^{a} (76.76)	6.42 ± 0.03 (70.65)
	SD	9.07 ± 0.02^{a}	4.65 ± 0.00^{a} (51.27)	4.51 ± 0.00 (49.72)	4.33 ± 0.01 (47.75)	3.98 ± 0.01 (43.87)	3.26 ± 0.03^{a} (35.93)
	MB	9.08 ± 0.00^{a}	8.52 ± 0.00^{b} (93.8)	8.56 ± 0.01^{b} (94.22)	7.50 ± 0.03^{b} (82.62)	6.95 ± 0.03^{b} (76.58)	5.93 ± 0.04^{b} (65.28)
L21	FD	9.10 ± 0.01	8.43 ± 0.01 (92.69)	8.51 ± 0.04^{a} (93.52)	7.48 ± 0.01^{a} (82.15)	7.22 ± 0.06 (79.32)	6.92 ± 0.08 (76.11)
	SD	9.09 ± 0.00	4.82 ± 0.00^{a} (53.03)	4.61 ± 0.00 (50.69)	4.45 ± 0.01 (48.93)	3.98 ± 0.01^{a} (43.8)	3.33 ± 0.01^{a} (36.68)
	MB	9.09 ± 0.00	8.51 ± 0.00^{b} (93.59)	8.52 ± 0.02^{a} (93.64)	7.89 ± 0.00^{b} (86.75)	7.37 ± 0.01^{b} (81)	6.75 ± 0.00^{b} (74.17)
MS	FD	9.10 ± 0.01	8.55 ± 0.00 (93.91)	8.55 ± 0.03^{a} (93.92)	7.47 ± 0.01^{a} (82.08)	7.11 ± 0.20^{a} (78.1)	6.70 ± 0.11 (73.56)
	SD	9.11 ± 0.01	4.83 ± 0.00^{a} (53.04)	4.81 ± 0.00 (52.86)	4.49 ± 0.01 (49.29)	4.11 ± 0.03 (45.1)	3.41 ± 0.01^{a} (37.4)
	MB	9.11 ± 0.01	8.56 ± 0.00^{b} (94.02)	8.58 ± 0.01^{b} (94.19)	7.48 ± 0.00^{a} (82.16)	6.95 ± 0.03^{a} (76.38)	6.36 ± 0.01^{b} (69.88)

The means with different superscript letters within the same column in each isolate group are significantly different ($p < 0.05$). Data are expressed as the means ± SD in triplicate. MS (multiple strains) consisted of the three *Lactobacillus* strains (L103, L80, L21)

L103 *Lactobacillus paraplantarum*, L80 *Lactobacillus plantarum* L80, L21 *Lactobacillus plantarum* L21

Storage forms: *FD* freeze-dried, *SD* spray-dried, *MB* micro-beads

According to the analysis of 16S rRNA genes sequence, all three selected *Lactobacillus* strains (L21, L80, and L103) were 98–99% nucleotide sequence identities to *L. plantarum* (1455/1485 sequence length compared to the accession no. NR042254), *L. plantarum* (1444/1447 sequence length compared to the accession no. NR104573), and *L. paraplantarum* (1445/1450 sequence length compared to the accession no. NR025447), respectively (Fig. 2).

Discussion

Ninety two of the 138 representative LAB isolates, of which 73 and 19 fecal isolates were isolated from CRP and FRP systems, respectively, can tolerate acid (pH 2.5, 3 h) and fresh pig bile (50% (*v*/*v*), 8 h). These isolates demonstrated that they have an important basic property for the selection of probiotics for further testing, similar to the previous study [24].

The two kinds of pig-raising systems, which have different husbandry management, such as feed composition, out-pen activities, and overuse or misuse of antibiotics, may affect the antimicrobial susceptibility of the pigs' intestinal microflora, such as LAB. In this study, LAB isolates from both CRP and FRP were resistant to the antibiotics, which showed Gram-positive bacteria spectrum such as vancomycin (85 and 100%, respectively). The resistance to some antibiotics, such as vancomycin, sulfamethoxazone/trimethoprim, and the aminoglycosides group, of the genera *Lactobacillus* is usually natural and does not involve any transferable antibiotic-resistant genes that could possibly be transferred to other bacteria [25, 26]. For the genera *Lactobacillus* or other LAB, we should be conscious of the resistance to other antibiotics that are commonly used for treating diseases in both humans and animals because it may lead to acquisition of transferable antibiotic-resistant genes [26]. In the present study, ten LAB isolates had suitable resistance to the five antibiotics and were selected for further study.

A major antibacterial substance of the LAB isolates is lactic acid. Other antibacterial substances, such as bacteriocins and hydrogen peroxide, produced from LAB can make them more strongly inhibitory to the more pathogenic bacteria. In this study, the three best LAB isolates (L21, L103, L80) could reduce the viable cells of Gram-negative bacteria, including β *E. coli*, *Salmonella* group B, and *Salmonella* group D at 3.70 and 1.02–2.74 log cycles, when they were co-cultured. This result is similar to the study of Aguilar et al. [27], who reported that LAB isolates can reduce the viable cells of *E. coli* at 4 log cycles using co-culture techniques.

The three selected isolates also inhibited β *E. coli*, *Salmonella* group B, and *Salmonella* group D, which showed clear zones of inhibition in the range of 20–34, 16–18, and 18–24 diameters (mm), respectively, using an agar spot assay. The anti-β *E. coli* result closely resembled the results from the study by Chapman et al. [6], who reported that LAB isolates inhibited β *E. coli*, showing clear zones of inhibition in the range of 14–34 diameters (mm) using the agar spot assay. The major lactic acid produced from these LAB isolates caused growth inhibition of these pathogens, similar to the study by Sornplang et al. [28], who reported that the supplementation of *Lactobacillus* isolates could reduce the amount of *Salmonella enteritidis* in chicks.

The ability of probiotics to adhere to the gut epithelial cells and their colonization can be measured indirectly by the cell surface hydrophobicity test, which measures the ability to

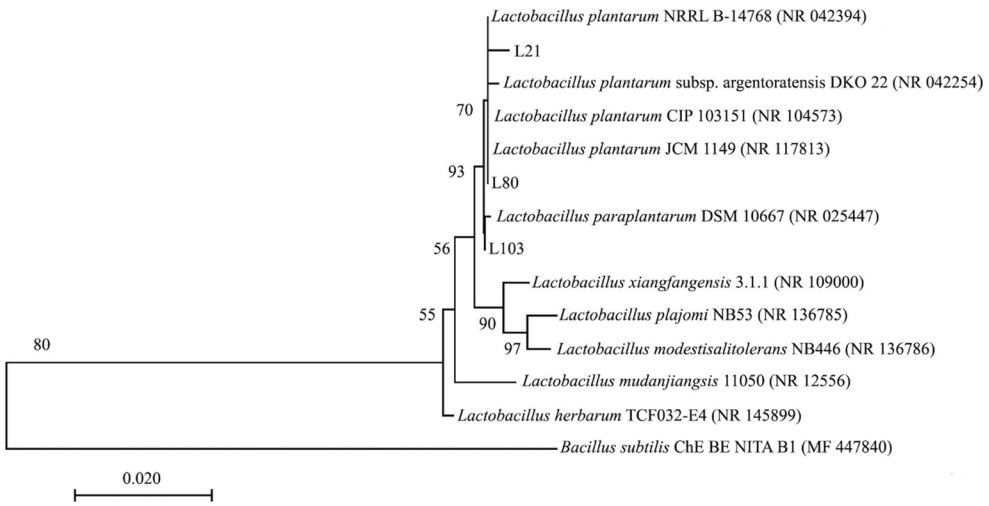

Fig. 2 Phylogenetic trees based on the neighbor-joining method of partial 16S rRNA sequences of L21, L80, and L103 with the sum of branch length at 0.02594222. The evolutionary distances were computed using the maximum likelihood method in MEGA7 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test with 1000 replicates is represented on the branches (only values above 50% are reported). Eleven nucleotide sequences with their accession numbers obtained from NCBI database are compared to the three isolates. *Bacillus subtilis* was used as an out-group organism

adhere to hydrocarbons. In this study, the hydrophobicities of the three LAB isolates (65–73%) classified them as having good hydrophobicity, similar to the study by Han et al. [29], who reported that the good adhesion rate of lactobacilli to Caco-2 cell line is related to the hydrophobicity values greater than 70%.

The survival of all the selected lactobacilli when stored at 4 °C for 4 days in both the single- and multi-strain forms using the FD and MB encapsulation techniques was higher than 80% viability (viable cell count in the range of 8.32–8.54 log CFU/ml). The formulation including the three mixed strains had the highest viability (8.54 log CFU/ml) when encapsulated in the MB form. This result demonstrated that these strains can combine to keep themselves viable for more time, resulting in higher efficacy. All isolates in the spray-drying form had a percentage survival in the range of 47.52–50.91 (4.31–4.64 log CFU/ml). This survival was lower than that in the study by Behboudi-Jobbehdar et al. [30], which may be due to the proper selection of the encapsulated substance before spray drying. All isolates stored in the FD form, except for L103, had higher survivability than those stored in the MB form during longer storage at 4 °C for 28 days. This result may be due to the selection of cryoprotectants with proper proportions and ingredients during freeze drying, such as 24.06% milk powder, 6.22% sucrose, and 5.63% trehalose [31]. The best number of cells surviving preparation in the MB form (L103) in this study was 8 log CFU/ml when stored at 4 °C for 28 days. This result seems similar to the study by Ozer et al. [32], who reported that *Lactobacillus acidophilus* encapsulated in 2% alginate gel and formed into capsules by the extrusion technique provided counts above 6 log CFU/ml when stored at 4 °C for 90 days. The viability of all the MB-encapsulated lactobacilli in this study was reduced in the range of 0.14–0.19 log CFU/ml after 14 days of storage at 4 °C, as shown in Table 3. The viable cells of this study were slightly lower than the study by Shi et al. [33], who reported that the viability of the encapsulated *Enterococcus faecalis* HZNU P2 was reduced by 0.06 log CFU/ml. This result may be due to many factors affecting the storage stability of probiotics including bacterial strains, storage times and temperatures, and suitable encapsulated substances [34]. The storage stability of the selected lactobacilli when stored at 25–30 °C for 96 h in both single- and multi-strain forms showed that *Lactobacillus* L21 had the most viable cells at 7.37 log CFU/ml (81% survival) after 72 h of storage in the MB form, as shown in Table 4.

The qualified *Lactobacillus* strain L21, L80, and L103 were identified as *L. plantarum* (accession no. NR042254), *L. plantarum* (accession no. NR104573), and *L. paraplantarum* (accession no. NR025447), respectively, using nucleotide sequencing analysis of the 16S rRNA genes compared to GenBank with 98–99% similarity (Fig. 2). Some species from this study, such as *L. plantarum* and *L. paraplantarum*, were commonly isolated from pig feces and were suitable for mixing in pig feed and potential probiotic use, similar to the study of Pringsulaka et al. [8].

Conclusion

Our study showed that the three selected strains, including *L. plantarum* (strain L21 and strain L80), and *L. paraplantarum* (strain L103), could be used as multi-strain probiotics. These strains showed the best probiotic properties in vitro. All three strains were prepared in freeze-dried and micro-bead encapsulation forms to prolong their viability. Feeding trials should be performed to evaluate their effects on pig performance and health.

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Compliance with Ethical Standards

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

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