p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Strengthening for Research and Development No: 30/E/KPT/2018

Tropical Animal Science Journal, March 2022, 45(1):112-120
DOI: https://doi.org/10.5398/tasj.2022.45.1.112
Available online at http://journal.ipb.ac.id/index.php/tasj

The Probiotic Properties of *Lactobacilli* in Organic Pigs

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(Received 15-06-2021; Revised 25-07-2021; Accepted 16-08-2021)

ABSTRACT

Indigenous *Lactobacilli* are suitable probiotics because they adapt well in the hosts and ecological niches. Here we test local *Lactobacillus* for future application in the pigs as the farm-autogenous strains. The objectives of this study were to evaluate the probiotic properties of *Lactobacillus* isolated from the feces of antibiotic-free organic pigs. The properties include bile salt and pepsin tolerance, survival in storage (37 & 4 °C) and probiotic-packaging (50 °C) temperatures, antibiogram, and antagonistic activity against *Salmonella typhimurium* ATCC 13311 and *Escherichia coli* ATCC 25922. Eighteen isolates with three different species were tested in this study as follows: *L. reuteri* (seven strains), *L. mucosae* (ten strains), and *L. plantarum* (one strain). Four isolates—*L. reuteri*-OP1, *L. mucosae*-OP2, *L. mucosae*-OP3, and *L. reuteri*-OP17—had good *in vitro* probiotic characteristics. Eleven isolates completely inhibited both *E. coli* and *S. typhimurium*. The other isolates are perfectly disabled, either *E. coli* or *S. typhimurium*. Despite that, they caused a reduction in the numbers of each pathogen. All *Lactobacilli* tested were susceptible to amoxicillin-clavulanate, ampicillin, and imipenem. Most isolates were sensitive to clindamycin (72%), gentamicin (56%), and tetracycline (50%). Half of the proportions were somewhat sensitive/resistant to cefotaxime (39/44%), tetracycline (50/39%), and streptomycin (39/56%). One hundred percent of *Lactobacilli* were resistant to norfloxacin, sulfamethoxazole-trimethoprim, and vancomycin, while 94% were resistant to enrofloxacin. Most of the local *Lactobacilli* passed *in vitro* tests, but the efficacy of probiotics in pigs awaits further *in vivo* investigation. Therefore, the potential probiotic strains derived from this study could be selected for further evaluation of their probiotic roles in economic pigs.

Keywords: antibiotic-free pig; antibiotic resistant; Escherichia coli; farm-autogenous strain; indigenous Lactobacilli; Salmonella typhimurium

INTRODUCTION

Lactobacillus is a probiotic bacterium commonly used to alleviate diarrhea and improve nutrient efficiency, promote growth, and enhance the carcass quality of pigs (Hou *et al.*, 2015; Sayan *et al.*, 2018). Another benefit is that *Lactobacillus* can incapacitate enteric pathogens (Fijan, 2018). A few studies have concerned swine *Lactobacilli* and the rising trend toward probiotic usage in the pig production industry. The application of *Lactobacillus* probiotics in livestock production is an alternative to medicine utilization for disease prevention and growth promotion (Hou *et al.*, 2015; Śliżewska *et al.*, 2021). Since livestock is the primary protein source for humans, *Lactobacillus* probiotics are attractive options concerning the risk minimization of antimicrobial-resistant bacteria, drug residues, and pathogen-free foods. However, not all probiotic products are equally effective (Sniffen *et al.*, 2018). The issue is whether non-host-specific probiotics exert maximum beneficial effects; for example, a non-swine origin of probiotic strains may pose difficulties in intestinal colonization and competition with normal flora (Fijan, 2014).

Most commercial *Lactobacillus* probiotic seed strains are obtained from humans, foods, and plants. Furthermore, the species-specific characteristics of *Lactobacilli,* particularly for local pigs, are still not well understood, especially in Thailand. Orally administered probiotics should be able to survive the harsh gastrointestinal environment, which includes such components as gastric acid and bile salt; compete with other intestinal residential floras; settle well in the gastrointestinal tract; maintain sufficient numbers; confer positive health effects; and inactivate pathogens (Fenster *et al.*, 2019). In addition to viability, a safety precaution to be considered when using *Lactobacillus* is that probiotic seeds must not be antibiotic-resistant (Hou *et al.*, 2015). In the present study, indigenous *Lactobacillus* strains are promising for attaining probiotic effects because they should adapt well in pigs, fit ecological niches, and reduce the risk of exotic bacteria importation. Therefore, studying the probiotic characteristics of *Lactobacilli*

isolated from organic pigs is the right foundation for sustainable swine production in northeastern Thailand. This study aimed to evaluate the *in vitro* probiotic properties of *Lactobacillus* spp. isolated from the feces of antibiotic-free organic pigs.

MATERIALS AND METHODS

Standard Bacteria

Standard bacteria included in the study were *Salmonella typhimurium* ATCC 13311 (*S. typhimurium*) and *Escherichia coli* ATCC 25922 (*E. coli*) for antagonistic assay and quality control of the susceptibility test, *Enterococcus faecalis* ATCC 7212 (*E. faecalis*) for quality control of susceptibility test and bile salt quality control, and *Staphylococcus aureus* ATCC 25923 (*S. aureus*) for quality control of the susceptibility test. All standard strains were obtained from the American Type Culture Collection (ATCC).

Lactobacillus Strains

Lactobacilli were selected from our laboratory collection, with isolates derived from 18 different healthy organic pigs raised under a strict "permaculture farm practice" in Khon Kaen Province, Thailand. Since its establishment 10 years ago, the farm has avoided using any medicines, chemical substances, or vaccines. The farm produces and formulates its feed. Eighteen isolates of the three following species of *Lactobacillus* have been used in this study: *L. mucosae* (n= 10), *L. reuteri* (n= 7), and *L. plantarum* (n= 1). Names and details of each strain are shown in Table 1. The 18 *Lactobacillus* cultures were stored at −20 °C in De Man–Rogosa–Sharpe broth (MRS, Hi-Media Pvt. Ltd., India) supplemented with horse serum (20% v/v) and L-cysteine (0.05% w/v; No. 30089 BioUltra, Sigma-Aldrich (Thailand) Co. Ltd.).

Preparation of Stock Bacteria

The frozen stored *Lactobacillus* isolates were cultivated on MRSc agar (MRS broth supplemented with 0.05% w/v of L-cysteine, and 1.5 % w/v of bacteriological agar (Hi-Media Pvt. Ltd., India), pH 6.5, and incubated under an anaerobic atmosphere (17% CO2, 80% N2, and 3% H2) for 48 h at 37 °C. Fresh and active-grown *Lactobacillus* colonies were transferred into MRSc broth, pH 6.5. Following this, the tubes were incubated at 37 °C for 24 h. Before the assays, this *Lactobacillus* stock was diluted in phosphate-buffered saline supplemented with 0.05% L-cysteine (PBSc) to prepare the desired concentrations (MacFarland No. 1 to 5) for specific assays. Frozen stored *S. typhimurium*, *E. coli*, and *E. faecalis* were cultured on nutrient agar (Oxoid, United Kingdom) incubated at 37 °C overnight. Frozen stored *S. aureus* was cultured on 5% bovine blood agar (Columbia agar, Oxoid) incubated at 37°C overnight. Fresh stock of these reference bacteria was prepared by transferring colonies from nutrient agar grown overnight into brain heart infusion broth (BHI, Hi-Media Pvt. Ltd.) and incubated at 37 °C for 18–24 h. Before use, the stocks were diluted with PBSc to equal MacFarland No. 1 to 5 as specific concentrations for the assays.

Pepsin Tolerance

One hundred microliters of *Lactobacillus* bacterial solution (1.0 MacFarland concentration) was transferred into 1 mL of 0.3% pepsin (PanReac-AppliChem, GmbH, Germany) in PBSc, adjusted to pH 3, and 1 mL PBSc (control) tubes, incubated at 37 °C for 2 h (Arboleya *et*

Table 1. *Lactobacillus* spp. isolates used in the study

No.	Isolate	*Lactobacillus* spp.	Source (pig)	NCBI accession numbers
1	OP1	*Lactobacillus reuteri*	Sow	MZ382874
2	OP2	*Lactobacillus mucosae*	Piglet	MZ382875
3	OP3	*Lactobacillus mucosae*	Sow	MZ382876
4	OP4	*Lactobacillus mucosae*	Finishing	MZ382877
5	OP5	*Lactobacillus mucosae*	Finishing	MZ382878
6	OP6	*Lactobacillus mucosae*	Finishing	MZ382879
7	OP7	*Lactobacillus plantarum*	Finishing	MZ382880
8	OP8	*Lactobacillus mucosae*	Finishing	MZ382881
9	OP9	*Lactobacillus reuteri*	Finishing	MZ382882
10	OP10	*Lactobacillus reuteri*	Finishing	MZ382883
11	OP11	*Lactobacillus mucosae*	Piglet	MZ382884
12	OP12	*Lactobacillus mucosae*	Piglet	MZ382885
13	OP13	*Lactobacillus mucosae*	Finishing	MZ382886
14	OP14	*Lactobacillus reuteri*	Sow	MZ382887
15	OP15	*Lactobacillus reuteri*	Sow	MZ382888
16	OP16	*Lactobacillus mucosae*	Growing	MZ382889
17	OP17	*Lactobacillus reuteri*	Growing	MZ382890
18	OP18	*Lactobacillus reuteri*	Growing	MZ382891

Note: All strains were identified by genomic sequencing of 16S rRNA using universal primers; 27F: 5′ – AGAGTTTGATCCTGGCTCAG – 3′ and 1492R: 5′ –GGTTACCTTGTTAGGACTT– 3′ and compared to the sequence database in GenBank (http://www.ncbi.nim.nih.gov) by BLAST program. OP= organic pigs.

al., 2011). Surviving *Lactobacilli* from the tubes were checked by inoculating the loop of the bacterial solution on MRSc agar plates and incubated anaerobically at 37 °C for 24–48 h, observed for *Lactobacillus* characteristic colonies. Growth ability was reported by grading the colonies with one to three plus signs compared with control. An invisible colony on agar plates was read as no growth.

Bile Salt Tolerance

The endurance test to 2% bile salt was similar to the earlier method (Awasti *et al*., 2016) with a slight change. Fifty microliters (1.0 MacFarland concentration) each of *Lactobacillus* and *E. faecalis* (Standard bile resistance control) suspension was inoculated into 2 mL MRSc broth supplemented with 150 µL of 29% stock solution of bile salt (Hi-Media Pvt. Ltd.) to obtain a 2% final concentration of bile salt in the MRSc broth. At the same time, control tubes were prepared by transferring 50 µL of the same stock *Lactobacillus* and *E. faecalis* into 2 mL MRSc broth without bile salt. After 3 h of incubation at 37 °C, a loop of the bacterial solution was plated on MRSc or nutrient agars (*E. faecalis*) and incubated anaerobically at 37 °C for 24–48 h. Then, the plates were observed for *Lactobacillus* or *E. faecalis* characteristic colonies. Qualitative survived *Lactobacillus* was graded the colonies compared with control as an aforesaid method.

Temperature Tolerance

Temperature and duration sets were for survival ability of *Lactobacillus* storage and shelf life (37 °C, 4 °C) and after manufacturing or packaging of probiotic seeds (50 °C). One hundred microliters of overnight grown *Lactobacillus* (1.0 MacFarland concentration) were inoculated into a 4 mL MRSc broth tube and incubated anaerobically at 37 °C for 5 and 7 days; 50 °C for 1, 6, and 24 h; and 4 °C for 1, 6, 10, and 14 days. For each specific time point, bacterial enumeration proceeded by plate count agar. Serial tenfold dilutions of the bacterial solutions were prepared and spread (100 µL) on MRSc agar plates. After anaerobic incubation at 37 °C for 24-48 h, *Lactobacillus* colonies were counted and calculated following the dilutions and expressed as colony-forming units per milliliter (CFU/mL). Except for the solution treated at 50 °C for 24 h, these were evaluated as alive or inactivated.

Aerotolerance

One hundred microliters of overnight grown *Lactobacillus* suspension (1.0 MacFarland concentration) were transferred into 4 mL MRSc broth, and one mixture in a loose-cap tube and another one in a tightened screw-cap tube (control) were prepared. The loose-cap tubes were on a rack intact, and the tightened screw-cap tubes were in an anaerobic chamber for aerobic and anaerobic incubation, respectively. After incubation at 37 °C overnight, one solution loop was streak on MRSc agar, incubated anaerobically at 37 °C for 24–48 h. Growth ability was reported by grading the colonies with one to three plus signs compared with control as the aforesaid method.

Antimicrobial Susceptibility Test

The *in vitro* antibiotic susceptibility test was employed with the disk diffusion method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). Twelve antibiotics (Oxoid) were included as follows: gentamicin 10 µg, streptomycin 10 µg, cefotaxime 30 µg, norfloxacin 10 µg, amoxicillin clavulanate 30 µg, clindamycin 2 µg, ampicillin 10 µg, sulfamethoxazole–trimethoprim 25 µg, enrofloxacin 5 µg, tetracycline 30 µg, imipenem 10 µg, and vancomycin 30 µg. The standard quality control bacteria were also included. Susceptibility interpretation followed the standard breakpoints of *E. faecalis* and *S. aureus* recommended by the CLSI-M100-S28 guideline (CLSI, 2018).

Antagonistic Test

The antagonist effect of *Lactobacillus* on the growth of *S. typhimurium* ATCC 13311 and *E. coli* ATCC 25922 was tested by co-culture in MRSc broth (Ahn e*t al.,* 2002). Single cultures of *Lactobacillus, S. typhimurium*, or *E. coli* were prepared along the co-culture inoculums. In brief, 50 µL (1.0 MacFarland concentration) of each bacterial solution was inoculated into 2 mL MRSc broth tubes. The co-culture tubes composed of 50 µL of either *S. typhimurium* or *E. coli* and 50 µL of each *Lactobacillus* strain were transferred into a 2 mL MRSc broth tube. The preparation obtained was three different sets of bacteria—namely, *Lactobacillus* only (Lacto), *S. typhimurium* only (Sal), and *Lactobacillus* plus *S. typhimurium* (L+S). Similar processes were done for *E. coli*, with sets of *Lactobacillus* only (Lacto), *E. coli* only (Ecoli), and *Lactobacillus* plus *E. coli* (L+E). The single or combination inoculums were incubated anaerobically at 37 °C for 24 h. Live bacteria from the co-culture broth were then enumerated using MRSc agar (adjusted to pH 5) selectively for *Lactobacillus* and MacConkey agar (Oxoid) for *E. coli* and *S. typhimurium*.

RESULTS

Tolerance to Bile Salt, Pepsin, 50 °C for 24 h, and the Presence of Oxygen

A qualitative assessment of *Lactobacillus* was determined for the tolerance to bile salt, pepsin, 50 °C for 24 h, and the presence of oxygen. Ten strains isolated from organic pigs or OP (OP1, OP2, OP3, OP7, OP9, OP10, OP11, OP15, OP17, and OP18) were qualitatively alive after 3 h of exposure to 2% bile salt; OP4, OP5, OP6, OP12, and OP16 did not survive after bile salt exposure. The activity of OP8, OP13, and OP14 diminished noticeably when incubated with 2% bile salt for 3 h (Table 2). All the strains endured 0.3% pepsin for 2 h, and no obvious reduction in the activity was observed (Table 2). Eight strains—OP1, OP2, OP3, OP8, OP9, OP10, OP11, and OP15—stayed alive after being incubated at 50 °C for 24 h. However, seven strains—OP5, OP7, OP12,

OP13, OP16, OP17, and OP18—were completely inactivated, and three strains—OP4, OP6, and OP14—were barely alive (Table 2).

Temperature Tolerance

From the results of *Lactobacillus* enumeration, there was no apparent difference between day zero (D0, the starting point) and incubation at 50 °C for 1 h (range: −0.43 to 0.44 $\log_{10}$ CFU/mL). In comparison, viable bacteria for all the isolates decreased (from D0) from 1.2 to 4.93 $\log_{10}$ CFU/mL after incubation at 50 °C for 6 h (Table 3). Five isolates—OP1, OP9, OP14, OP15, and OP18—remained stable after incubation at 50 °C for 6 h (decreased less than 2 $\log_{10}$ CFU/mL). However, OP7 and OP16 were the most affected by high temperatures (decreased more than 4 $\log_{10}$ CFU/mL; Table. 3). There were two isolates, OP1 and OP2, that performed well after being placed at 37 °C for 5 days (decreased 1.45 and 2.38 $\log_{10}$ CFU/mL, respectively). With this condition, OP6, OP7, OP16, and OP17 decreased more than 4 $\log_{10}$ CFU/mL; OP14 decreased 2.74 $\log_{10}$ CFU/mL, and the other isolates decreased more than 3 $\log_{10}$ CFU/mL (Table 3). After 7 days at 37 °C, OP2, OP6, OP16, and

Table 2. Qualitative assessment of *Lactobacillus* spp. isolates regarding tolerance to bile salt, pepsin, 50 °C for 24 h, and the presence of oxygen

Isolate	2% bile salt	0.3% pepsin	PBSc (control)	50 °C 24 h	Aerotolerance
OP1	+++	+++	+++	+++	+++
OP2	+++	+++	+++	+++	+++
OP3	+++	+++	+++	+++	+++
OP4	-	+++	+++	+	+++
OP5	-	+++	+++	-	+++
OP6	-	+++	+++	+	+++
OP7	+++	+++	+++	-	+++
OP8	+	+++	+++	+++	+++
OP9	+++	+++	+++	+++	+++
OP10	+++	+++	+++	+++	+++
OP11	+++	+++	+++	+++	+++
OP12	-	+++	+++	-	+++
OP13	+	+++	+++	-	+++
OP14	+	+++	+++	+	+++
OP15	+++	+++	+++	+++	+++
OP16	-	+++	+++	-	+++
OP17	+++	+++	+++	-	+++
OP18	+++	+++	+++	-	+++

Note: +++ = grow well; + = poor growth; - = no growth; OP= organic pigs; PBSc= phosphate-buffered saline supplemented with 0.05% L-cysteine.

Table 3. Enumeration of *Lactobacillus* spp. isolates after incubation to various temperatures ($\log_{10}$ CFU/mL)

Isolate	D0	50 °C		4 °C				37 °C	
		1h	6h	D1	D6	D10	D14	D5	D7
OP1	8.22	8.32	7.00	8.48	8.61	7.98	9.38	6.77	5.65
OP2	9.51	9.36	6.60	10.04	9.54	9.27	9.26	7.13	3.95
OP3	9.16	8.89	7.26	8.96	8.54	8.55	9.26	6.07	5.88
OP4	9.51	9.30	6.08	9.44	9.51	9.20	9.19	5.74	4.90
OP5	9.52	9.36	6.91	9.43	9.12	9.45	8.99	5.83	5.26
OP6	10.08	9.86	7.61	10.30	9.36	9.93	9.56	4.40	3.53
OP7	10.43	10.31	5.72	10.08	10.33	10.5	10.49	4.88	NG
OP8	10.34	9.94	7.05	9.53	9.70	9.55	9.25	6.53	5.99
OP9	10.04	10.47	8.72	9.76	9.73	9.55	9.60	7.01	5.24
OP10	9.72	9.75	7.03	9.42	9.88	9.77	9.43	6.42	5.08
OP11	9.23	9.10	6.97	9.12	9.38	9.53	8.66	5.89	5.07
OP12	10.04	10.38	7.63	9.54	9.48	9.13	8.39	6.40	5.63
OP13	9.44	9.62	7.11	9.35	9.49	9.17	8.88	6.13	5.51
OP14	9.16	10.19	7.96	9.05	9.12	9.08	8.72	6.42	5.98
OP15	10.02	9.67	8.70	9.46	9.31	9.29	8.65	6.71	6.99
OP16	10.39	9.95	5.46	9.81	9.45	9.32	8.86	5.66	1.60
OP17	9.67	9.68	6.64	9.51	9.57	8.86	8.87	3.74	3.78
OP18	9.42	9.52	7.43	9.94	7.79	7.00	7.95	5.62	5.35

Note: NG= no growth; D0= Day0; D1= Day1; D5= Day 5; D6= Day6; D7= Day7; D10= Day10; D14= Day 14; OP= organic pigs.

OP17 decreased more than 5 $\log_{10}$ CFU/mL and OP7 was completely lost all activity (Table 3). For the refrigerated storage temperature of 4 °C, the numbers for live bacteria fluctuated between 1.16 and 2.42 $\log_{10}$ CFU/mL for all the isolates as counted on D1, D6, D10, and D14 (Table 3).

Antagonistic Activity

Whether a single *Lactobacillus* culture was used or a combination with either *S. typhimurium* ATCC 13311 or *E. coli* ATCC 25922, the numbers of *Lactobacillus* were almost the same (Figure 1 and 2). In contrast, the numbers of both pathogens distinctly reduced in the co-culture tubes until they were unculturable. Eleven strains of all tested *Lactobacilli*—OP1–4, OP7, OP10, OP12, OP14, and OP16–18—completely inhibited *S. typhimurium* and *E. coli* (Figure 1 and 2). The OP9 inactivated *S. typhimurium* completely but only caused a reduction in the amount of *E. coli* (31.2% reduction). Four strains—OP5, OP6, OP8, and OP15—perfectly antagonized *E. coli* growth but only diminished numbers of *S. typhimurium*, with 71%, 44%, 27%, and 45.6% reduction rates, respectively. Two isolates, OP11 and OP13, reduced the number of *S. typhimurium* by 88.8% and 34.9% and reduced the number of *E. coli* by 27.8% and 48.2%, respectively.

Antimicrobial Susceptibility

All *Lactobacilli* were completely susceptible to amoxicillin-clavulanate, ampicillin, and imipenem. Varying susceptibility (range: 39%–56%) was found among the 18 *Lactobacillus* isolates to the following drugs: gentamicin, streptomycin, cefotaxime, and tetracycline (Figure 3). Most isolates were sensitive to clindamycin (72%), gentamicin (56%), and tetracycline (50%). A small proportion of isolates were sensitive to cefotaxime (39%) and streptomycin (39%). Inhibition zones that fell into intermediate susceptibility were found in cefotaxime (17%), clindamycin (11%), tetra-

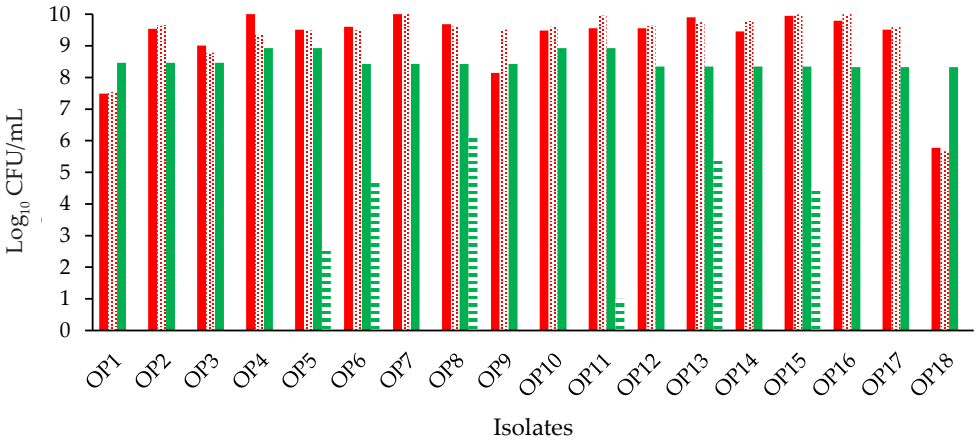

Figure 1. Bacterial numbers determined by the viable plate count agar method after single culture (Lacto) or co-culture (L+S) between *Lactobacillus* spp. isolates and *Salmonella typhimurium* ATCC 13311.
Note: 0 = no growth, Lacto ( )= *Lactobacillus*, Sal ( )= *Salmonella*, Lacto (L+S) ( )= *Lactobacillus* in the co-cultured tube, Sal (L+S) ( )= *Salmonella* in the co-cultured tube; OP= organic pigs.

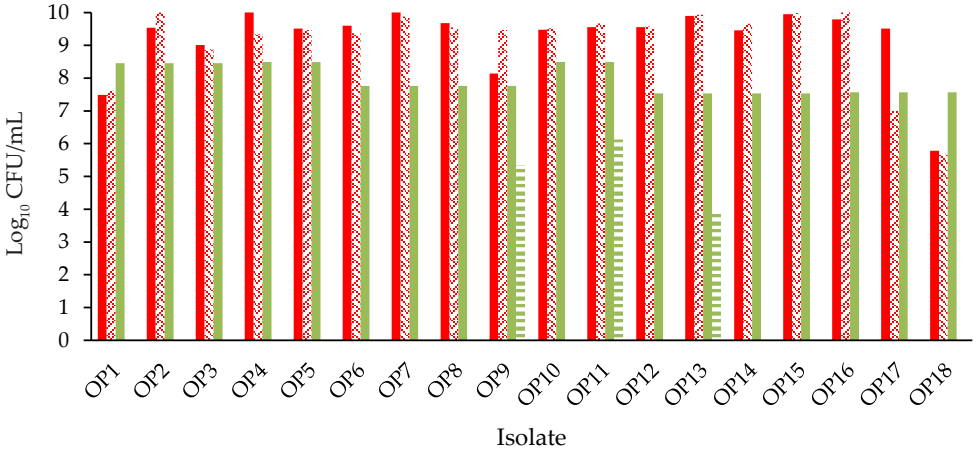

Figure 2. Bacterial number determined by the viable plate count agar method after single culture (Lacto) or co-culture (L+E) between *Lactobacillus* spp. isolates and *Eschericia coli* ATCC 25922.
Note: 0 = no growth, Lacto ( )= *Lactobacillus*, Ecoli ( )= *Eschericia coli*, Lacto (L+E) ( )= *Lactobacillus* in the co-cultured tube, Ecoli (L+E) ( )= *Eschericia coli* in the co-cultured tube; OP= organic pigs.

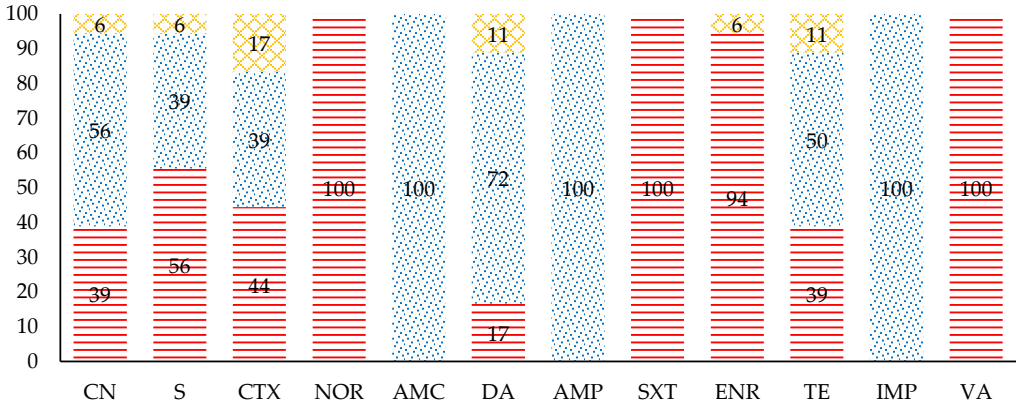

Figure 3. Antibiogram results of 18 *Lactobacillus* spp. isolates against 12 drugs in the study, = % Resistance, = % Susceptible, =% Intermediate.
Note: CN= Gentamicin (10 µg), S= Streptomycin (10 µg), CTX= Cefotaxime (30 µg), NOR= Norfloxacin (10 µg), AMC= Amoxicillin-clavulanate (30 µg), DA= Clindamycin (2 µg), AMP= Ampicillin (10 µg), SXT= Sulfamethoxazole -trimethoprim (25 µg), ENR= Enrofloxacin (5 µg), TE= Tetracycline (30 µg), IPM= Imipenem (10 µg), VA= Vancomycin (30 µg).

cycline (11%), gentamicin (6%), and enrofloxacin (6%; Figure 3). All strains showed 100% resistance against norfloxacin, sulfamethoxazole-trimethoprim, vancomycin resistance, and 94% against enrofloxacin. None of the *Lactobacilli* tested was susceptible to enrofloxacin, but some strains had intermediate susceptibility to this drug.

Variable resistance was detected for streptomycin (56%), cefotaxime (44%), gentamicin (39%), tetracycline (39%), and clindamycin (17%). The result indicated drug resistance if equal to or more than half of the isolates were resistant. Hence, streptomycin (56%) was associated with moderate resistance. Half the proportions of *Lactobacilli* were somewhat sensitive/resistant to cefotaxime (39/44%), streptomycin (39/56%), and tetracycline (50/39%). Except for OP7, OP9, and OP18, the other *Lactobacillu*s isolates were sensitive to clindamycin (Table 3). More than half of the isolates exhibited streptomycin resistance, but OP1, OP2, OP3, OP13, OP14, OP17, and OP18 were sensitive to this drug.

DISCUSSION

Lactobacilli can be found along the length of the gastrointestinal tract and are abundant in the large intestine (Wylensek *et al.*, 2020), which is the targeted residency of exogenous probiotic administration. Furthermore, *Lactobacillus* probiotics utilized in pigs are mostly administered in feed additives (Alayande *et al.*, 2020). Our results indicated that all *Lactobacillus* isolates tolerated gastrointestinal conditions. Survival of *Lactobacillus* may be affected by the duration of pepsin and bile salt exposure as well. The duration of 2–3 h conducted in our study was compatible with the gastric transit time of the pig (Henze *et al.*, 2021). It may be that certain amounts of the bacteria had lost activity, but our result identified them simply as qualitatively alive. The pepsin used in our study was derived from swine, which affirms that pigs' *Lactobacilli* can resist the gastric environment. For the bile salt test, our result corroborates another study finding that *Lactobacillus* of pig origin remained alive after exposure to 2% bile salt (Betancur *et al.*, 2020). Bile salt is present in the small and large intestines; this is why it is assumed that *Lactobacilli* obtained from pig feces can endure the bile salt test (Ruiz *et al.*, 2013). Ten isolates evaluated in this study are promising for oral probiotic preparations. Although the study did not evaluate the exact numbers of *Lactobacilli* remaining after bile salt and pepsin exposure, live bacteria can proliferate in the intestine once residing there. Although the intestinal adherence ability of *Lactobacilli* was not investigated in this study, all strains were procured from the rectums of healthy pigs, which ensured their natural habitat.

For manufacturing criteria, probiotic starter culture must maintain viability in sufficient numbers after undergoing probiotic production steps and storage temperatures. Oxygen exposure during handling is another issue involved in anaerobic bacteria manipulation. The species of the genus *Lactobacillus* have been classified as oxygen-tolerant anaerobes (Zotta *et al.*, 2017). The results of our study indicated that all isolates could grow in the microaerophilic atmosphere. An additional property of probiotics is that they must tolerate high temperatures. All *Lactobacillus* strains tested could resist 50 °C for 6 h, although they were slightly reduced in number. Generally, *Lactobacilli* were not able to grow under high-temperature conditions (Praepanitchai *et al.*, 2019). As a result, most isolates diminished in terms of their viable numbers when incubated at 50 °C for 24 h or 37 °C for 7 days, although some isolates still had growth activity. The high-temperature resistance of the pig's *Lactobacilli* could be explained by the pigs' high body temperature. The normal rectal temperature of pigs is 38.5 °C to 39.5 °C (Zhang *et al.*, 2019), and the temperature in the abdominal cavity (where the intestine is located) can be much higher than this. For the storage temperature, there was no dispute that most of our *Lactobacilli* could maintain good activity at 4 °C for up to 14 days, and this result agreed well with another study (Watkins *et*

al., 2018). Nevertheless, keeping them at 4 °C for longer than 14 days was not attempted in our study.

Beyond good attributes concerning the handling, safety issues of probiotic seeds, such as drug resistance, must be cautiously considered (Anisimova & Yarullina, 2020). The present study established the antimicrobial susceptibility standard of *Lactobacillus* from antibiotic-free pigs raised on a 10-year-old farm that had never used any chemicals or medicines. As a matter of fact, the true origin of *Lactobacillus* strains was unknown. Although procured from pigs on the same farm, various reactions of antimicrobial susceptibility were found among 18 *Lactobacillus* isolates. The previous study analyzed six standard reference *Lactobacillus* strains that yield variable activity against antimicrobials, similar to our results (Sharma *et al.,* 2017). The variable antibiogram patterns could be due to the species and origin of *Lactobacillus*. According to a study that investigated *L. salivarius* and *L. mucosae* derived from the same wild boar, these *Lactobacilli* exhibited different drug susceptibility patterns (Fukuda *et al.,* 2020). In our study, all *Lactobacillus* isolates were completely susceptible to ampicillin, amoxicillin-clavulanate, and imipenem, as expected for Gram-positive bacteria. Most strains were sensitive to clindamycin. Moderate sensitivity of a few *Lactobacilli* toward clindamycin was also indicated in a previous study (Schmitt *et al.,* 2018). Our isolates exhibited variable sensitivity/resistance to gentamicin and streptomycin, while another study reported that their isolates were highly sensitive to these two antibiotics (Georgieva *et al.,* 2015). Resistance phenotype to aminoglycosides of *Lactobacillus* has been addressed as intrinsic (Hummel *et al.,* 2007). However, another study demonstrated aminoglycosides' resistance genes in *Lactobacilli* procured from chickens (Dec *et al.,* 2017).

Small numbers of our isolates were resistant to tetracycline, but more than half of these *Lactobacilli* were susceptible to this drug. It can be concluded that our isolates were tetracycline susceptible. Although 100% tetracycline resistance was reported by other researchers (Anisimova & Yarullina, 2018), *Lactobacilli* were found to be completely susceptible to tetracycline in a different study (Jomehzadeh *et al.,* 2020). Extensive exploration of tetracycline resistance using 128 strains of the *L. buchneri* group, the research team concluded that the tetracycline resistance was intrinsic (Feichtinger *et al.,* 2016). They found that 96.9% of the strains could be categorized as tetracycline-resistant, but none of the *Lactobacilli* carried tetracycline resistance genes (Feichtinger *et al.,* 2016). Less than half the 18 isolates fell into either sensitivity or resistance against cefotaxime (with almost equal proportions). It can be inferred that our *Lactobacilli* were somewhat sensitive to this drug, which is similar to the findings of a previous study in that *Lactobacillus* strains were completely sensitive to β-lactam antibiotics but moderately sensitive to cefotaxime (Sharma *et al.,* 2017).

All *Lactobacilli* in our study were completely resistant to norfloxacin, sulfamethoxazole-trimethoprim, and vancomycin, and 94% of the isolates were resistant to enrofloxacin. None of the *Lactobacilli* tested were susceptible to enrofloxacin; this was considered to indicate enrofloxacin resistance. High resistance toward glycopeptides (vancomycin, teicoplanin) and quinolones (ciprofloxacin, ofloxacin) have been demonstrated in reference *Lactobacillus* commercial strains (Sharma *et al.,* 2017). It has been indicated that *Lactobacilli* possess intrinsic resistance to these two antibiotic classes (Casado Muñoz *et al.,* 2014). Natural resistance to vancomycin is due to *Lactobacillus* having peptidoglycan precursors terminating in D-alanyl-D-lactate. Vancomycin binds relatively poorly to this peptidoglycan ending, whereas it binds with high affinity to peptidoglycan ending in D-alanyl-D-alanine, causing natural vancomycin resistance of *Lactobacillus* (Zhang *et al.,* 2018). Insensitivity to enrofloxacin was also detected in *Lactobacillus* isolated from weaned pigs (Zou *et al.,* 2017). Nevertheless, no mutations in the quinolone resistance determining regions of the genes encoding *GyrA* and *ParC* of *Lactobacilli* were found, indicating an intrinsic resistance (Casado Muñoz *et al.,* 2014). Intrinsic resistance is usually nontransferable, and hence, poses no risk for using *Lactobacillus* as probiotics. Further screenings and in-depth characterization of the resistant determinants are required to use *Lactobacillus* starter culture safely.

Some beneficial bacteria, including *Lactobacillus,* can produce substances to prevent or inhibit harmful bacteria, and their metabolites contribute to nutritional efficiency and immunological modification of the hosts (Novik & Savich, 2020). In modern intensive farming systems, high stocking rates predispose pigs to many infectious diseases (Lee *et al.,* 2016). The most costly disease is pre-and post-weaning diarrhea (Breda *et al.,* 2017). The common etiology of this problem relates to *Salmonella* spp. and *E. coli* infections (Komatsu *et al.,* 2019). Manipulations of intestinal microbial ecosystems have been attempted to prevent diarrhea, improve health status, and promote growth performance in pigs (Fouhse *et al.,* 2016; Novik & Savich, 2020). In the present study, autogenous pig *Lactobacilli* showed good ability to inhibit *E. coli* and *S. typhimurium*. The numbers of pathogens were markedly reduced until they were not culturable. Eleven isolates of *Lactobacilli* completely inactivated both *S. typhimurium* and *E. coli*. Notably, some isolates could kill either *S. typhimurium* or *E. coli* but not both pathogens. Nevertheless, they could substantially reduce the numbers of culturable *S. typhimurium* and *E. coli*. From this *in vitro* result, *Lactobacilli* are promising probiotics to be used for fighting enteric pathogens in swine. It appears that most of our *Lactobacilli* can combat *E. coli* better than they can combat *S. typhimurium*. However, this result is preliminary *in vitro* experiment and cannot elucidate the *Lactobacilli*'s mechanisms. Theoretically, *Lactobacillus* can produce acids, antimicrobials, and/or other metabolic substances to combat pathogens (Fijan, 2018). In addition, probiotics are acknowledged for being good at completing with pathogens on mucosal adhesion in the intestinal tract (Singh *et al.,* 2017). Further investigation on this attribute is required.

CONCLUSION

Four isolates among 18 Lactobacilli—OP1, OP2, OP3, and OP17—had good *in vitro* probiotic characteristics. These local *Lactobacillus* strains are promising commercial probiotics for pigs and are worth further *in vivo* testing.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations related to the materials discussed in the manuscript.

ACKNOWLEDGEMENT

This work was funded by the Research grant for graduate students of the Faculty of Veterinary Medicine, Khon Kaen University (Project No. 625180005-7/2020).

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