การคัดเลือกสายพันธุ์แบคทีเรียกรดแลคติกจากสุกรเพื่อต่อต้านแบคทีเรียก่อโรคและไวรัส porcine epidemic diarrhea (PED)

นางสาววันดี ศิริโชคชัชวาล

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SELECTION OF LACTIC ACID BACTERIA ISOLATED FROM SWINE AGAINST PATHOGENIC BACTERIA AND PORCINE EPIDEMIC DIARRHEA (PED) VIRUS

Miss Wandee Sirichokchatchawan

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By	Miss Wandee Sirichokchatchawan
Field of Study	Veterinary Pathobiology
Thesis Advisor	Associate Professor Dr. Nuvee Prapasarakul, D.V.M., Ph.D.
Thesis Co-Advisor	Professor Dr. Somboon Tanasupawat, B.Sc, Ph.D.
	Assistant Professor Dr. Dachrit Nilubol, D.V.M., M.Sc., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Veterinary Science
(Professor Dr. Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

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.....Chairman
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.....Thesis Advisor
(Associate Professor Dr. Nuvee Prapasarakul, D.V.M., Ph.D.)

.....Thesis Co-Advisor
(Professor Dr. Somboon Tanasupawat, B.Sc, Ph.D.)

.....Thesis Co-Advisor
(Assistant Professor Dr. Dachrit Nilubol, D.V.M., M.Sc., Ph.D.)

.....Examiner
(Associate Professor Dr. Nopadon Pirarat, D.V.M., Ph.D.)

.....Examiner
(Assistant Professor Dr. Channarong Rodkhum, D.V.M., Ph.D.)

.....External Examiner
(Associate Professor Dr. Kampon Kaeoket, D.V.M., M.Sc., Ph.D.)

วันดี ศิริโชคชัชวาล : การคัดเลือกสายพันธุ์แบคทีเรียกรดแลคติกจากสุกรเพื่อต่อต้านแบคทีเรียก่อโรคและไวรัส porcine epidemic diarrhea (PED) (SELECTION OF LACTIC ACID BACTERIA ISOLATED FROM SWINE AGAINST PATHOGENIC BACTERIA AND PORCINE EPIDEMIC DIARRHEA (PED) VIRUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. สมบูรณ์ ธนาศุภวัฒน์, ผศ. น.สพ. ดร. เดชฤทธิ์ นิลอุบล, 130 หน้า.

เชื้อแบคทีเรียกรดแลคติก (lactic acid bacteria; LAB) เป็นแบคทีเรียประจำถิ่นที่พบได้ในระบบทางเดินอาหารของสุกร และนิยมนำมาใช้เป็นโปรไบโอติค (probiotics) สำหรับให้ประโยชน์ต่อสุขภาพของสุกร การค้นหาสายพันธุ์ของแบคทีเรียกรดแลคติกที่เหมาะสมในกระบวนการผลิตสุกรในประเทศไทยเป็นการช่วยเพิ่มมูลค่าการผลิตและทดแทนการใช้ยาปฏิชีวนะในอนาคต การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อ 1) คัดเลือกแบคทีเรียกรดแลคติกที่มีความสามารถในการทดกรด (pH2) และ น้ำดี (0.3% bile) จากอุจจาระสุกรพื้นเมืองและสุกรฟาร์มสุขภาพดีปลอดยาปฏิชีวนะในประเทศไทย จำแนกชนิดของสายพันธุ์ที่คัดเลือกด้วยความสามารถในการใช้น้ำตาล 21 ชนิด รูปแบบโปรตีน และการหาลำดับพันธุกรรมที่ยีนส์ 16S rRNA และตรวจสอบความไวรับต่อยาต้านจุลชีพต่อเชื้อแบคทีเรียกรดแลคติกตามข้อแนะนำของ European Food Safety Authority (EFSA) 2) เพื่อหาความสามารถในการต้านเชื้อไวรัส porcine epidemic diarrhea ของเชื้อแบคทีเรียกรดแลคติกที่ผ่านการคัดเลือกในวัตถุประสงค์ที่หนึ่ง 3) เพื่อทำการทดสอบความสามารถเป็นโปรไบโอติคของเชื้อแบคทีเรียกรดแลคติกที่คัดเลือก และทดสอบความสามารถการต่อต้านเชื้อแบคทีเรียก่อโรคที่สำคัญในลำไส้สุกร ได้แก่ เชื้อแบคทีเรียก่อโรค Enterohemorrhagic *Escherichia coli* (EHEC) Enterotoxigenic *Escherichia coli* (ETEC) *Salmonella Typhimurium* และ *Salmonella Choleraesuis* ผู้วิจัยสามารถแยกและคัดเลือกเบื้องต้นได้เชื้อแบคทีเรียกรดแลคติกที่มีความสามารถความทนกรดและน้ำดี 34 สายพันธุ์ จากสุกรฟาร์ม 19 สายพันธุ์ และสุกรพื้นเมือง 15 สายพันธุ์ จึงนำไปทำการจำแนกชนิดของสายพันธุ์ พบว่าเป็นสายพันธุ์ *Enterococcus faecium* 11 เชื้อ *Enterococcus hirae* 9 เชื้อ สายพันธุ์ *Lactobacillus plantarum* 4 เชื้อ *Lactobacillus agilis* 3 เชื้อ สายพันธุ์ *Pediococcus pentosaceus* 6 เชื้อ และ *Pediococcus acidilactici* 1 เชื้อ หลังทำการทดสอบความไวรับต่อยาต้านจุลชีพด้วยวิธี disk diffusion พบว่ามี 7 สายพันธุ์ที่คุณสมบัติของกลุ่มตัวอย่างที่เลือกเข้าศึกษาโดยมีความไวรับต่อยาในกลุ่ม broad spectrum และยาปฏิชีวนะต้านแบคทีเรียแกรมบวกที่ทดสอบ จึงได้นำสายพันธุ์เหล่านี้มาทำการยืนยันทดสอบความไวรับต่อยาต้านจุลชีพด้วยวิธี Minimum Inhibitory Concntration ตามข้อแนะนำของ EFSA และ 7 สายพันธุ์นี้ไปทำการทดสอบความสามารถในการต้านเชื้อไวรัส porcine epidemic diarrhea (PED) พบว่าสายพันธุ์ *L. plantarum* (22F 25F 31F) *P. acidilactici* (72N) และ *P. pentosaceus* (77F) ผ่านตามข้อเกณฑ์กำหนดค่าความไวรับต่อยาต้านจุลชีพของ EFSA สำหรับผลการต่อต้านเชื้อไวรัส PED พบว่าทั้ง cell-free supernatant (CFS) และเชื้อเป็นของ *L. plantarum* (25F) มีความสามารถในการต่อต้านเชื้อไวรัส PED ได้ดีที่สุดโดยสามารถลดการเกิด cytopathic effect (CPE) เหลือเท่ากับ <50% of high power field area หลังจากนั้นได้นำ 5 สายพันธุ์นี้มาทำการทดสอบความสามารถในการเป็นโปรไบโอติกที่ดีและความสามารถในการต่อต้านเชื้อก่อโรคในลำไส้ที่สำคัญในสุกรได้แก่เชื้อแบคทีเรียก่อโรค EHEC, ETEC, *Salmonella* Typhimurium, *Salmonella Choleraesuis,* และ *Streptococcus suis* type II พบว่าเชื้อ *L. plantarum* 22F แสดงความสามารถเป็นโปรไบโอติกที่ดีในการทดสอบส่วนใหญ่ เมื่อนำทุกการทดลองในการศึกษาครั้งนี้มารวมกันพบว่าเชื้อ *L. plantarum* 22F และ 25F เหมาะที่จะนำมาพัฒนาเป็นโปรไบโอติกสำหรับอุตสาหกรรมสุกรต่อไปเนื่องจาก *L. plantarum* 22F มีคุณสมบัติของโปรไบโอติกที่ดีในการทดสอบส่วนใหญ่ ในขณะที่เชื้อเป็นของ *L. plantarum* 25F มีความสามารถในการต่อต้านเชื้อจุลชีพก่อโรคและไวรัส PED ได้ดี

ภาควิชา พยาธิวิทยา
สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์
ปีการศึกษา 2559

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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WANDEE SIRICHOKCHATCHAWAN: SELECTION OF LACTIC ACID BACTERIA ISOLATED FROM SWINE AGAINST PATHOGENIC BACTERIA AND PORCINE EPIDEMIC DIARRHEA (PED) VIRUS. ADVISOR: ASSOC. PROF. DR. NUVEE PRAPASARAKUL, D.V.M., Ph.D., CO-ADVISOR: PROF. DR. SOMBOON TANASUPAWAT, B.Sc, Ph.D., ASST. PROF. DR. DACHRIT NILUBOL, D.V.M., M.Sc., Ph.D., 130 pp.

Lactic acid bacteria (LAB) are commensal microbes in pig gastrointestinal tract and have been applied as probiotics due to their ability to benefit and improve pig health. The finding of suitable LAB strains to use as probiotics as substitution of antibiotic growth promoter in pig production in Thailand would lead to the increase of product quality and value. The objective of this research was to 1) isolate and pre-select acid- and bile- tolerant LAB from healthy antibiotic-free indigenous and commercial pigs in Thailand, characterize and identify the pre-selected acid and bile tolerant LAB by phenotypic characteristics (21 sugars), whole cell protein profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and complete 16S ribosomal RNA gene analysis, and investigate antimicrobial susceptibility following European Food Safety Authority (EFSA) recommendations; 2) study and observe their antiviral activity against porcine epidemic diarrhea virus (PEDV); 3) evaluate the probiotic properties and antibacterial activity against porcine enteric pathogenic bacteria included Enterohemorrhagic *Escherichia coli* (EHEC) Enterotoxigenic *Escherichia coli* (ETEC) *Salmonella Typhimurium* and *Salmonella Choleraesuis*. We were able to pre-select 34 acid and bile tolerant LAB strains (15 isolates from indigenous pigs and 19 isolates from commercial pigs) which were identified as *Enterococcus faecium* (11 isolates), *Enterococcus hirae* (9 isolates), *Lactobacillus plantarum* (4 isolates), *Lactobacillus agilis* (3 isolates), *Pediococcus pentosaceus* (6 isolates) and *Pediococcus acidilactici* (1 isolate). After, the results of their antimicrobial susceptibility profiles by disk diffusion showed seven isolates with susceptible to the broad spectrum antibiotics and antibiotics against Gram-positive bacteria. These seven isolates were selected for further confirmation of their antimicrobial susceptibility profiles by MICs following European Food Safety Authority (EFSA) microbiological cut-off values, and investigated for antiviral activity against PEDV. We found that five isolates included *L. plantarum* (22F, 25F, 31F), *P. acidilactici* (72N) and *P. pentosaceus* (77F) were acceptable as probiotic candidates followed the antimicrobial susceptibility recommendation by EFSA, and among the five isolates, both cell-free supernatant and live cells of *L. plantarum* (25F) possessed the best antiviral activity against PEDV by reducing cytopathic effects (CPE) from PEDV to <50% of high power field area. In addition, these five isolates were selected for *in vitro* evaluation of probiotic properties and antibacterial activity against EHEC, ETEC, *Salmonella Typhimurium*, *Salmonella Choleraesuis* and *Streptococcus suis* type II. The resuls revealed that *L. plantarum* 22F possessed better functional probiotic properties for most *in vitro* evaluations. When taken together all the experiments in this research, we could summarize that *L. plantarum* 22F and 25F were the most suitable for development as probiotic candidates for pig industry since *L. plantarum* 22F exhibited most of functional probiotic properties and live cells of *L. plantarum* 25F showed the best antibacterial and antiviral activity against pathogenic bacteria and PEDV

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Field of Study: Veterinary Pathobiology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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CONTENTS

Page

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
℃	degree celcius
AM	amoxycillin
ANOVA	Analysis of variance
AP	ampicillin
ATCC	American type culture collection
bp	base pair
BSH	bile salt hydrolase
C	chloramphenicol
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
CFU/mL	colony-forming units per milliliter
CFS	Cell-free supernatants
CLSI	Clinical and Laboratory Standards Institute
CPE	cytopathic effect
CM	clindamycin
CS	colistin sulfate
Da	Dalton
DCs	Dendritic cells
DNA	Deoxyriboneucleic acid
E. facium	*Enterococcus facium*
E. hirae	*Enterococcus hirae*
E. coli	*Escherichia coli*

EFSA	European Food Safety Authority
EHEC	enterohemorrhagic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EVD	epidemic viral diarrhea
ER	erythromycin
FAO	Food and Agriculture Organization of the United Nations
FBS	fetal bovine serum
g	grams
G	gentamicin
GIT	gastrointestinal tract
H	hours
HIV	human immunodeficiency virus
HCl	Hydrochloric acid
IECs	Intestinal epithelial cells
K	kanamycin
kg	kilogram
KCl	potassium chloride
KNO_3	potassium nitrate
L. agilis	*Lactobacillus agilis*
L. plantarum	*Lactobacillus plantarum*
LAB	Lactic acid bacteria
M	molar
MATH	microbial adhesion to hydrocarbon

mg	milligram
mA	milliampere
MEM	Modified Eagle's Medium
MIC	Minimal inhibitory concentration
min	minutes
mL	milliliter
MRS	de Man, Rogosa and Sharpe
nm	nanometer
NaCl	sodium chloride
NaOH	sodium hydroxide
NJ	neighbor-joining
NP	nucleoprotein
n.r.	not required
OD	optical density
P. acidilactici	*Pediococcus acidilactici*
P. pentosaceus	*Pediococcus pentosaceus*
PBS	phosphate buffer solution
PCA	Principal component analysis
PCR	polymerase chain reaction
PEDV	porcine epidemic diarrhea virus
PRR	pattern recognition receptor
R	resistant
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid

RPM	round per minute
RV	rotavirus
s	second
S	susceptible
S. Choleraesuis	*Salmonella Choleraesuis*
S. Typhimurium	*Salmonella Typhimurium*
S. aureus	*Staphylococcus aureus*
S. suis	*Streptococcus suis*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SD	standard deviation
SES	sterile electrolyte solution
ST	streptomycin
TC	tetracycline
TCID	tissue culture infective dose
TDCA	taurodeoxycholic acid
TGEV	transmissible gastroenteritis virus
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSA	tryptic soy agar
TSB	tryptic soy broth
UPGMA	unweighted pair group method with arithmetic mean
v/v	volume by volume
VA	vancomycin

w/v	weight by volume
WHO	World Health Organization
wk	week
µg	microgram
µg /mL	microgram per milliliter
µl	microliter
µm	micrometer

CHAPTER I

INTRODUCTION

1.1 Importance and rationale

The forbidden use of antibiotics as growth promoters due to the concern of spreading of antimicrobial resistance and the transference of resistance genes from meat products to consumers have contributed to the searching for safer alternatives use of antibiotics that could prevent enteric diseases and improve animal health and production (Casewell et al., 2003). Commensal gut microbiota receives high interest as an alternative use of antibiotics growth promoters due to their history of safe use and beneficial effects towards the host. These beneficial bacteria are named "probiotics" and are officially defined by FAO/WHO in the year 2002 as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The characteristics that are important for probiotics include non-toxic, non-pathogenic, able to identify the accurate taxonomy, of the normal inhabitant of the targeted species, able to survive, colonize, and active at the targeted organ, resist to gastric juice and bile, able to compete with pathogenic organisms, produce certain antimicrobial substances, modulate immune responses, able to confer at least one scientifically supported health promoting properties, and genetically stable throughout the production and delivery processes. The common probiotics use in livestock are from a species of lactic acid bacteria (LAB) such as *Lactobacillus, Enterococcus, Bacillus* and *Bifidobacterium* species (Maria Carmen Collado et al., 2012; Iournals, 2011; Tuomola et al., 2001).

Enteric diseases and post-weaning anorexia are common concerned in pig-producing countries. Neonatal and weaning is the most critical and susceptible periods for the enteric diseases due to various stresses ranging from changing in environments and diet to the end of lactation immunity that result in an imbalance of intestinal microbiota and allow enteric pathogens to inhabit the host gastrointestinal tract (GIT) (Lallès et al., 2007). Pathogenic *Escherichia coli* (*E. coli*), *Salmonella* spp., and porcine epidemic diarrhea virus (PEDV) are the major causative agents of enteric diseases in piglets. These pathogens cause significant economic losses due to high mortality and morbidity, and decrease of growth rate in pigs of all ages. For examples, colibacillosis

and salmonellosis contribute to approximately 10 to 15 percent mortality rate in pigs (X. Guo et al., 2006; Kyriakis et al., 1999; Seo et al., 2010) and the widespread of PEDV in Thailand results in 95 to 100 percent death in infected farms (Pan et al., 2012; SongandPark, 2012). The probiotic supplementation using LAB would encourage the formation of beneficial gut microflora which benefit the health of neonatal piglets by improving the intestine development, strengthen the intestine barrier function by increased butyrate concentration which lead to the defence and reduction of potential enteric pathogens, modulation of immune system (reduced expression of inflammatory cytokine, and enhanced T-cell differentiation and cytokine expression). In weaning piglets, supplementation of LAB can improve feed intake, weight gain, and feed conversion, and lessened weaning stress by increasing the protein levels associated with energy and lipid metabolism, and protein synthesis. In addition, LAB colonized gut epithelial cells created protection barrier against enteric pathogens, and modulated immune response facilitating cellular proliferation and reduction of cell apoptosis (F. Yang et al., 2015).

According to characteristics of probiotics, many studies have been conducted to observe their effects on growth performances and antagonistic effects towards enteric pathogens in pigs. Feeding experiments using *Enterococcus faecium*, *Bacillus cereus* var. toyoi, or *Bacillus cereus* CenBiot as a probiotic supplementation showed significant decrease of diarrhea comparing with the control group without probiotics (Lin et al., 2006; Sathyabama et al., 2012). Studies using *Lactobacillus* species showed a broad antimicrobial activity and growth inhibition against many pathogenic bacteria including *Salmonella, E. coli, Listeria,* and *Campylobacter* (Hudault et al., 1997; Jacobsen et al., 1999; Servin, 2004). *Lactobacillus* spp. isolated from pigs showed antiviral activity against coronavirus, a common cause of enteric diseases in pigs with mortality rate close to 100 percent (Seo et al., 2010). Moreover, researchers suggested that probiotics given as a feed supplement in piglets maintain intact epithelial cell lines which might inhibit the infection by enteric virus (Kyriakis et al., 1999; Le Bon et al., 2010).

Researches on rearing environments in piglets reported that outdoor rearing contributed to better growth and better adaptation to weaning when compared with indoor reared piglets. This may be the result of better development of GIT due to the more variety of outdoor/ natural stimuli (Loncaric et al., 2009). Exposure to more extreme environments and forage for variety of foods possibly increase wider range of pig GIT's microbiota; obtaining from plant and soil materials such as beneficial bacteria since differences in diets result in different populations of gut microbiota.

Environments and geographical locations may also contribute to different bacterial group in GIT (Sathyabama et al., 2012; Yatsunenko et al., 2012).

In the last decades, the increasing number of commercial probiotic products available on the market in Thailand. Most are imported products which when evaluated found to be not raise up to their statement; either contain less to no viable probiotics, or they do not contain probiotic species as claimed on the label. Moreover, those products when use in Thailand might not provide the expected results as claimed. Because both internal and external factors such as host age, geographical areas, rearing systems, health and genetics are the reasons to differences in species of commensal LAB (Maria Carmen Collado et al., 2012).

The concept of species specific for probiotics used has been introduced to us and as mentioned above that piglets raising in natural environment will have better chance to acquire a better complexity of beneficial bacterial community in their gut. Therefore, our study aims to isolate and select LAB that could represent as the potential probiotic candidates use in pig supplementation from feces of a healthy indigenous and commercial pigs in Thailand, and to study the antibacterial and antiviral activities against pathogenic *E. coli, Salmonella*, and PEDV; with the notion that these beneficial bacteria should be a better and more suitable probiotics candidate to use for Thai pig's farm.

1.2 Literature review

History and concept of probiotics

The concept of probiotics was first introduced in 1908 by Elie Metchnikoff, the deputy director of the Pasteur Institute laboratory in Paris. He had observed and suggested that the consumption of LAB in fermented dairy products regularly contributed to healthy and longevity of Bulgarian peasant individuals (AnukamandReid, 2007). 'Probiotic' is from the Greek word and mean 'for life'. The term probiotics was first used by Lilly and Stillwell in 1965 to describe the growth promoting substances from ciliate protozoan, and was redefined by Parker in 1974 to cover both living organisms and substances that contribute to the balance of intestinal microbiota. However, in 1989, Fuller had narrowed the term to only lived microorganisms as feed supplements that benefit the host by improving the intestinal microbial balance (Roy Fuller, 1992). Only in the past decades that the concept of probiotics has globally accepted by scientists and consumers. In 2001, the joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) had worked together and came up with the most widely accepted definition of probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (PineiroandStanton, 2007).

Many genera of microorganisms are being applied as probiotics; however, the most commonly used bacteria are member of lactic acid bacteria (LAB). They have been well-known as an active portion, and most important strains of probiotics. Of the members in LAB, the genera *Lactobacillus, Lactococcus, Pediococcus,* and *Bifidobacterium* are given as Generally Recognized as Safe (GRAS) status while members in the genera *Streptococcus* and *Enterococcus* regarding opportunistic pathogens (AguirreandCollins, 1993; Gasser, 1994). LAB are Gram-positive bacteria those lack cytochromes and favor anaerobic condition, but able to grow in aerobic condition. Most of the LAB members have low G+C content as less than 55% mol% DNA, which belong to the *Clostridium branch* (Vandamme et al., 1996). As the name implies, LAB produce lactic acid as a main product. They are usually divided into two groups on the basis of their carbohydrate fermentations. Homofermentative LAB produce only lactic acid as their by-product from carbon sources including species of *Enterococcus, Pediococcus, Lactococcus, Streptococcus*, and some members of *Lactobacillus.* Whereas, heterofermentors produce lactate, carbon dioxide, acetate or ethanol as their by-product. Their members consist of species *Leuconostoc, Weissella,* and some lactobacilli (AxelssonandAhrné, 2000).

Sources and importance aspects for probiotic selection

Probiotics can be isolated from several sources as demonstrated in Figure 1. Dairy and fermented products provide a complex community of LAB species, and are a good source of probiotic strains. Recent studies found that breast milk is not sterile and also considered a useful source of LAB as probiotics, which prefer the predominant species of lactobacilli and bifidobacteria, for constitution in intestine of pre-term and full-term infants. Several reports indicated that lactobacilli from breast milk are effective as alternative for antibiotics in treatment of mastitis on lactation period. Human and animal gastrointestinal tracts are a powerful source of probiotics. Many commercial probiotics available today are isolated from this source. However, a frequent misunderstanding concept is that probiotics must reside only in GI tract. Fecal samples of healthy subjects are also an acceptable source of probiotic strains such as *L. acidophilus* and *B. longum*. Markedly, the probiotic strains isolated from breast milk also found in feces of breast-fed infants (Fontana et al., 2013).

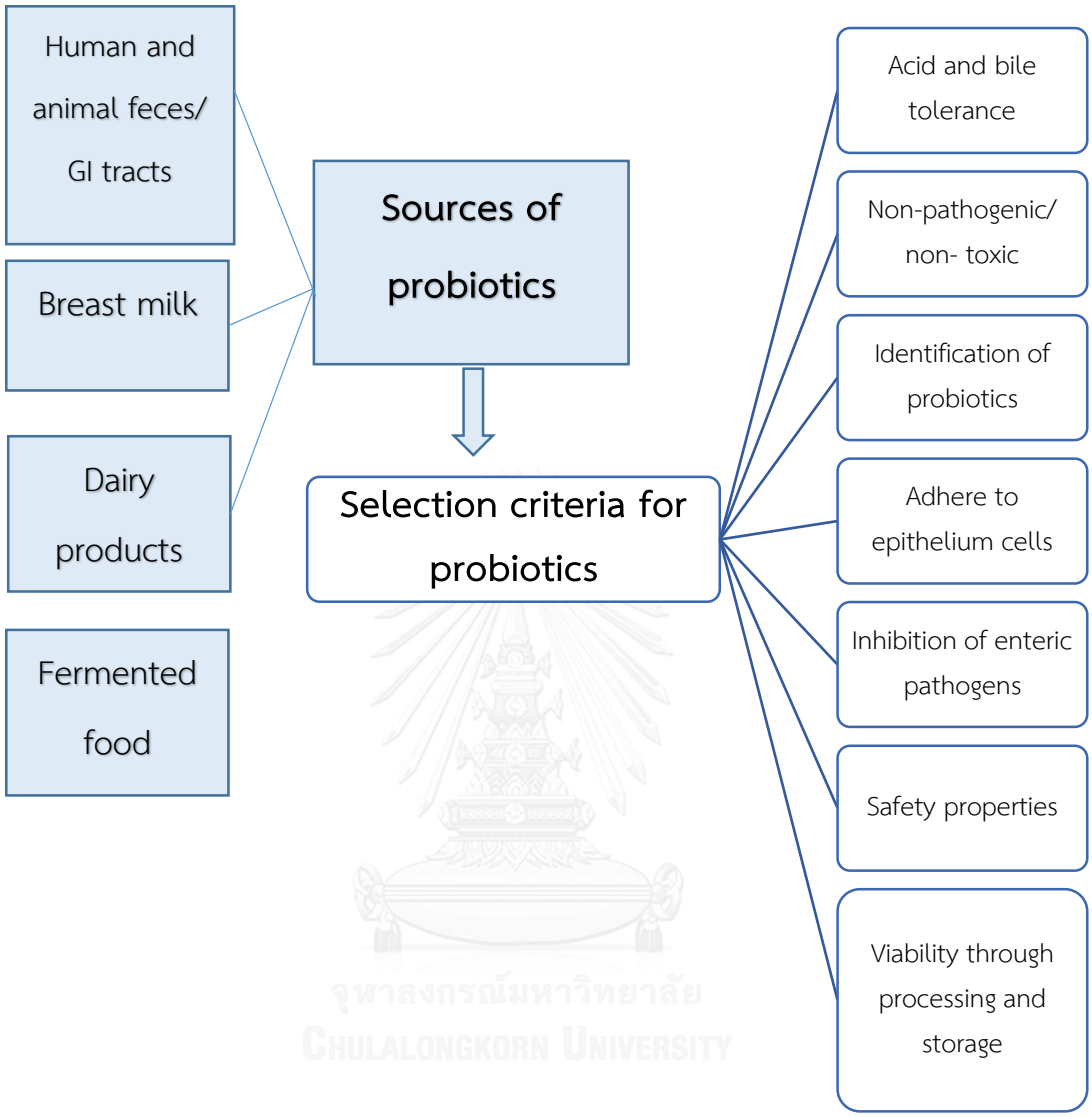


Figure 1 sources and selection criteria of probiotics

The main criteria for selection of probiotics include safety, functional and technological criteria (Figure 1). The primary requirement to select probiotic microorganisms is the safety aspects towards consumers (Sanders et al., 2010). The origin of probiotics should be from healthy and the same species of the target hosts as they could function better in similar GIT environments (Saarela et al., 2000). The strains must be identified before use due to the strain specificity according to WHO/FAO guidelines. A combination of phenotypic and genotypic characterizations is recommended for the species/strain identification (DonelliandVuotto, 2013). The strains selected as probiotics should not have history of being pathogenic and toxic. It would be preferable that they do not deconjugate bile salts as it would not benefit in the small intestine, and show no resistance to medical important antibiotics following the "guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance" by European Food Safety Authority (EFSA) (EFSA, 2012). Nevertheless, bacterial probiotics can naturally be multi-resistant, or susceptible to antibiotics. Therefore, they can be used as co-administration with antibiotics for treatment of enteric diseases and restore balance of gut microbiota (Courvalin, 2006). Since the interest in using LAB as probiotics, the antibiotic sensitivity and resistance of these bacteria are usually confirmed by non-transference of antibiotic resistant genes to consumers and patients before practice. These seem to be intrinsic resistance in genus level without genetic transferable phenomenon (DuttaandDevriese, 1981). The functional and desirable criteria require the use of *in vitro* methods to evaluate probiotic candidates before experimenting *in vivo* (Saarela et al., 2000). Acid tolerance is considered the first screening property when selecting the probiotic candidates (Tuomola et al., 2001). Probiotic bacteria must tolerate and survive the transit through the acidic condition of the stomach before reaching the intestinal tract. The ability to survive the GIT is varies between probiotic strains. While some strains are able to pass through the GIT and maintaining high bacterial concentration, others are easily killed in the low pH of the stomach (Dunne et al., 2001; SalminenandVon Wright, 2004). Simple *in vitro* examinations can be used to evaluate the ability to tolerate acidic condition since *in vivo* validation of the tolerance of probiotic strains through host's stomach is rather difficult to achieve (Tuomola et al., 2001). Moreover, probiotic candidates should also be able to withstand bile condition, and *in vitro* tests can also be used to observed the condition. The same as ability to tolerate acidic environments, the ability to bile tolerance is different between strains of the same bacterial species. Therefore, the *in vitro* tests can assist in selection the best probiotic candidates on the basis of these properties. Adhesion characteristic of the probiotic bacteria is also

important to evaluate the gut barrier effects since adhesion may relate to a reduction of diarrheal duration, immunogenic effects, competitive exclusion of pathogens (Elo et al., 1991; Isolauri et al., 1990; SALMINEN et al., 1996; Tuomola et al., 2001). Another impact of probiotics towards hosts is the antagonistic ability against pathogens by the production of antimicrobial substances and competitive exclusion. Bacteriocins are produced by probiotics to inhibit the closely related bacterial species. Whilst low molecular weight metabolites and secondary metabolites such as lactic acid, diacetyl and hydrogen peroxide present a wider range of inhibitory effects towards many pathogenic microorganisms for examples *Salmonella* and *E. coli.* The technological aspects of probiotics are to evaluate and deliver safe probiotics with good functionalities that answer the market's demands. The strains of probiotics must be able to withstand manufactured conditions and retain the functionalities and viability during the storage. (Dunne et al., 2001; Saarela et al., 2000).

Isolation, Identification and characterization of probiotic strains

The initial step to isolate probiotic bacteria is to preserve the obtained samples in appropriate conditions before laboratory culture. Culture media and culturing atmosphere are also important in isolation of these microorganisms; since LAB often show a delay growth and could easily be overgrown by other microorganisms if not culture selectively. There are several selective media for isolation of LAB such as de Man Rogasa and Sharpe (MRS) media, Briggs, M17, and Raka-Ray agar (Reuter, 1985). Normally, the incubation period for these microorganisms are ranging between 48 to 72 hours at 37 ^{o}C in the presence of CO_2 within microaerophilic/ anaerobic atmosphere (Fontana et al., 2013).

In the past, bacterial identification was relied heavily on phenotypic methods looking at both the types of sugar fermentations and the products from fermentation processes. These days, genotypic and molecular methods have been available for bacterial identification such as ribotyping, random amplified polymorphic DNA, 16S rRNA gene analysis, amplified fragment length polymorphism, and Pulse field gel electrophoresis. Amongst these methods, the analysis of 16S rRNA gene have been chosen as a method of choice. This method allows microbiologists to observe the relatedness between microorganisms through the construction of phylogenetic tree and through the sequence comparison with the databases such as GenBank and DDBJ (Fontana et al., 2013; YadavandShukla, 2015).

Among the probiotic bacteria, *Lactobacillus* and *Bifidobacterium* are the two most important genera of probiotics due to the ability to tolerate and persist within the GI tract. The most challenging for probiotics is the capability to survive and maintain certain viable number through extreme conditions such as low pH (1.5 to 3.0), bile salts, and intestinal enzyme. Therefore, several *in vitro* experiments were designed to imitate those stress conditions within the GI tract in order to assess probiotic ability of the chosen bacterial candidates. The assessment of acid (pH 2-4) and bile (0.3-0.7%) tolerance are the general criteria for selection of probiotic bacteria with the aim to guarantee the viability and functionality of those strain, since these characteristics are species and strain dependent (Fontana et al., 2013). Other assays for evaluation of probiotic strains include adherence ability, antimicrobial activity against pathogens (OhandJung, 2015), and antimicrobial susceptibility as a safety evaluation according to European Food Safety Authority (EFSA) (EFSA, 2012).

Probiotics: Mechanisms of actions

The beneficial effects particularly the actions related to the antagonistic effects of probiotics towards many pathogens are still unclear; however, are most likely to be the mechanisms that involve multi factors (Bermudez-Brito et al., 2012; VernaandLucak, 2010). The main mode of actions of probiotics include the enhancement and increase adhesion of epithelial barrier and intestinal mucosa, competitive exclusion of pathogens, inhibition of pathogens adhesion, production of antimicrobial substances, and immunomodulation (Figure 2).

The epithelium of the GI tract is always in contact with luminal environments. The maintaining of intestinal integrity is the primary defense mechanism and major protection against enteric microbiota and environments. When the intestinal barrier has been disrupted, food and bacterial antigens can cross the submucosa and cause inflammatory responses. The probiotic consumption could provide and retain the effectiveness of intestinal barriers by regulation the genes encoding adherence junction proteins, preventing mucosal disruption by enteropathogenic *E. coli*, or restoring integrity and initiation the repairing of intestinal barrier (Bermudez-Brito et al., 2012). In addition, probiotics may help strengthening the mucosal barrier in inflammatory intestinal diseases by immunomodulation of cytokine-induced epithelial damage (Hardy et al., 2013), and promote the mucous secretion which support mucosal barrier functions and pathogen exclusion (Bron et al., 2017; Van TassellandMiller, 2011).

Bacterial adhesion is the main requirement for colonization and interaction between probiotics and host. The study by Collado et al. demonstrated that the acid-resistant bifidobacteria show better adhesion ability to intestinal mucus than acid-sensitive bifidobacteria which possibly be a strategy to select only the strains those able to improve intestinal stability and surface properties against enteric pathogens (M Carmen Collado et al., 2006).

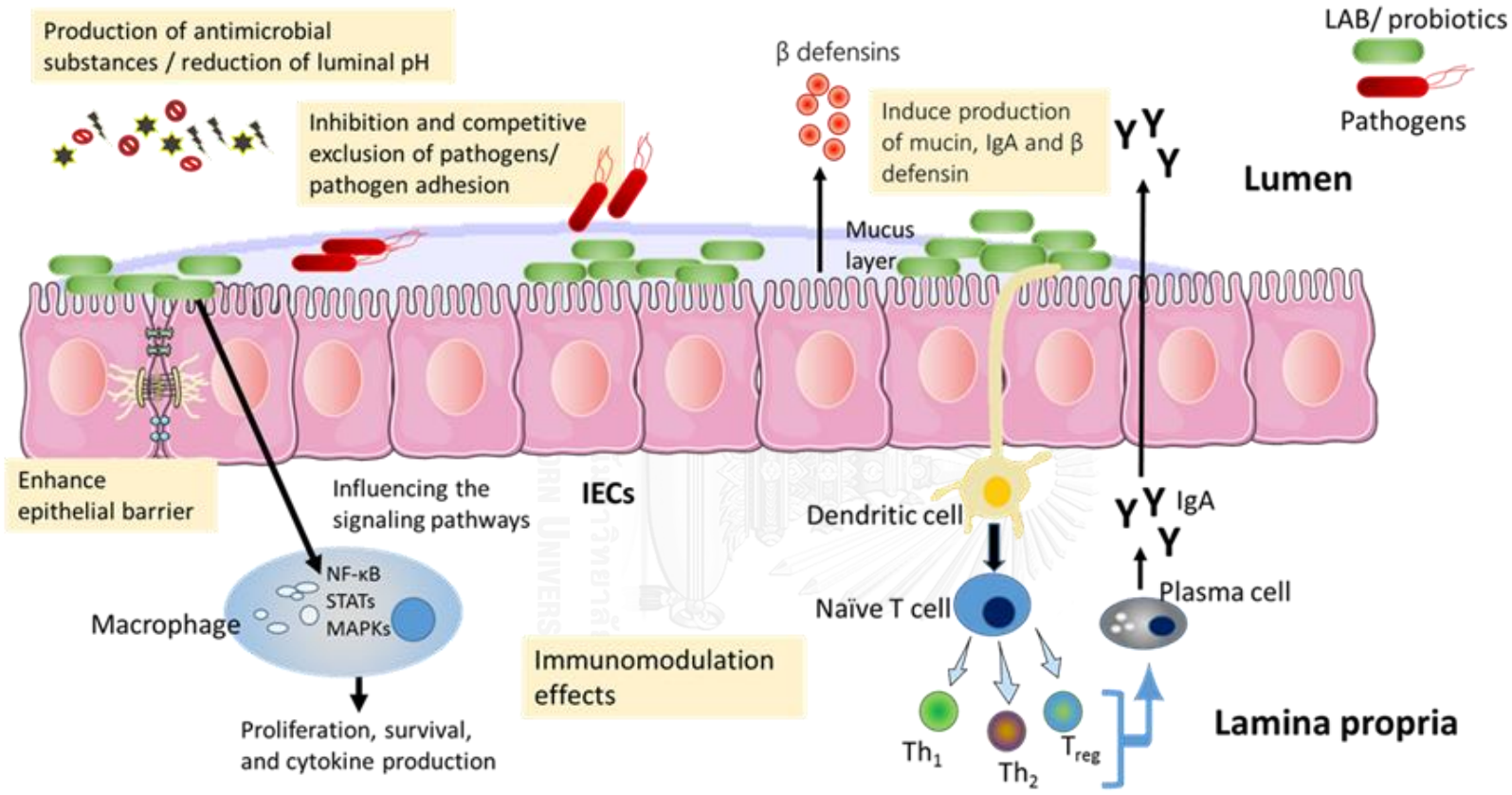


Figure 2 Modes and mechanisms of action of probiotics (Adapted from (Bermudez-Brito et al., 2012; ThomasandVersalovic, 2010)

Many researches showed that several surface proteins of LAB are able to interact with intestinal epithelial cells and induce mucin secretion which can subsequently inhibit pathogen adhesion (Bermudez-Brito et al., 2012; Lebeer et al., 2010). For instance, the induction of mucus-binding protein by *L. plantarum* has been reported to prevent the adhesion of enteropathogenic *E. coli.* Moreover, probiotic can induce a secretion of defensins from epithelial cells which active against wide range of bacteria, viruses and fungi. The interaction of defensins against microbial organisms is non-specific which mainly target the membrane integrity and disruption by a pore formation, and induce lysis (Bermudez-Brito et al., 2012; Kagan et al., 1990)

The competitive exclusion is referred to a situation when one bacterial species is more capable in competing for available nutrients and mucosal receptor sites than another species. Some bacteria can gain a competitive advantage by modification the environments to make it less appropriate for the competitive species. Generally, probiotics prevent pathogenic attachment by steric hindrance at pathogen receptors. The sharing of specific carbohydrate binding receptors in some strains of lactobacilli and enteric pathogens can make it possible for the lactobacilli to compete with the pathogens for receptor sites (Mukai et al., 2002; Neeser et al., 2000; SchiffrinandBlum, 2002). For example, a study reported that *L. rhamnosus* strain could inhibition the internalization of enterohemorrhagic *E. coli* (EHEC) *in vitro* (Hirano et al., 2003).

Several species of LAB are capable of producing antimicrobial peptides such as organic acids (less than 1000 Da), bacteriocins (more than 1000 Da), and other small antimicrobial compounds. Organic acids especially acetic acid and lactic acid are considered the key antimicrobial compounds in inhibition of against Gram-negative bacteria by entering and lowering the intracellular pH or accumulation of ionized form of organic acid intracellularly which eventually destroy the pathogens. Unlike organic acids, bacteriocins produced by Gram-positive probiotic bacteria have a narrower inhibitory spectrum and mostly showed inhibitory effects against only closely related bacteria species by inhibition of cell wall synthesis or formation of a pore at target cell (Hassan et al., 2012). Moreover, some of LAB strains are able to produce fatty acids (with health-promoting effects such as conjugated linoleic acid which possesses anti-carcinogen and anti-obesity effect) and metabolites with antifungal activity such as benzoic acid, phenyllactic acid and short-chain fatty acids (Bermudez-Brito et al., 2012).

Probiotic bacteria have also known to be able to modulate immune effects mostly by the interaction with intestinal epithelial cells (IECs), dendritic cells (DCs), and macrophages through pattern recognition receptors (PRRs). Both commensal and probiotic bacteria build the immune tolerance state through the action of TLRs on DCs

which mediate for anti-inflammatory responses. Moreover, the downregulation of TLR expression by probiotics could help suppress the intestinal inflammation (Hardy et al., 2013; Plaza-Diaz et al., 2014). Stimulation and activation of TLR2 is also important for enhancement of transepithelia resistance against pathogen invasion. Several reports showed that TLR2 requires recognition of peptidoglycan of some *Lactobacillus* strains in order to exert immunodulatory effects. For instance, some authors proposed that the inhibition of IL-12 production was the result from signaling of lactobacilli's peptidoglycan via TLR2. Furthermore, the experiment conducted in healthy mice administered with probiotics revealed the increase in expression of TLRs (such as TLR2, TLR4, and TLR9), and the secretion of tumor necrosis factor-α (TNF-α), IFN-γ and IL-10 (Bermudez-Brito et al., 2012).

Lactic acid bacteria as probiotics in pigs

LAB have been used as probiotics in pigs to replace the antibiotics growth promotor. The purpose of using LAB as probiotics is difference depending on the age of pigs. The neonatal period is the most critical and vulnerable to diseases and pathogens because of the not fully development of gastrointestinal tract and immune system. The purpose of probiotic supplementation in neonatal piglets is to support development of GIT and establishment of beneficial gut microflora. Previous studies found that oral administration of LAB in piglets could reduce the expression of inflammatory cytokine, strengthen the intestinal integrity and barrier, and decrease the number of enteric pathogens such as pathogenic *E. coli, Salmonella spp.* and *Clostridium spp.* The changes in diets and environments during weaning period results in tremendous stress in piglets. LAB supplementation in weaned piglets can help reduce stress by increasing the protein levels associated with lipid and energy metabolism, intestinal cell structure, and protein synthesis, and decrease the occurrence of diarrhea, and increase growth rate and feed conversion by improving the beneficial gut microbiota providing the protective layer against enteric pathogens along with the promotion of mucous secretion (F. Yang et al., 2015).

From the beneficial effects and characteristics of LAB, they have been received considerable attention from researchers in many field of studies including pig feeds and companies. The use of probiotic administration in pig generally aims to improve growth performance and to prevent enteric diseases (R FULLER, 1989; Le Bon et al., 2010). The focus is on weaning piglets since they are facing with many stresses ranging from mixing with piglets from other herds, separating from sows, ending of the lactational immunity, to changing in the environments and diets. These contribute to

an imbalance of the gut microbiota which allow the growth and colonization of enteric pathogens such as pathogenic *E.coli, Salmonella,* and PEDV; causing post-weaning diarrhea syndrome (Kyriakis et al., 1999). Administrations of probiotic supplementation at this period can control the diseases and improves health performances which may result from LAB's ability to restore the balance of gut microbiota and to maintain the epithelial cells from pathogens by strengthen the communication between epithelial cells (Meddings, 2008). For examples, the study on *Bacillus cereus* var. *toyoi* suggested an ability to stimulate host T cells against pathogenic invasion by allowing dendritic cells to flag the part of itself via MHC class II and induce naïve T cells to mature to adaptive T cells and fight against pathogens (Kyriakis et al., 1999; Le Bon et al., 2010; Simon et al., 2001; Zani et al., 1998). Furthermore, ability to induce fermentation and digestion of *Lactobacillus* and *Bacillus* by producing acetic acid and enzymes to help digest and absorb nutrients in GIT which contributes to the improvement in growth rate and performance in suckling, weaning, growing and fattening pigs (Abe et al., 1995; Mathew et al., 1998; Ouwehand et al., 2002; Yu et al., 2008). The study of Meng and colleagues (2010) suggested that pigs receiving probiotic supplementation showed better digestion of proteins than those receiving normal feed (Meng et al., 2010).

How probiotics are given as feed supplementations in pigs

Probiotics can be administered as time of diseases (curative) and/or after birth (preventive) by continuously supply in food and water or oral injection; in the form of pelleted or ground. The form of probiotics such as:

Fermented liquid feeds

Fermented liquid feed is a combination of probiotics with fermented liquid. A commonly use LAB for this process is *Lactobacilli* due to the ability to produce acetic acid; making them able to ferment natural feeds. The studies suggested that fermented liquid feeds can reduce pathogens in feeds and in environments, and decrease the chance to pass on pathogens between pigs comparing to pigs receiving normal feed. Nevertheless, fermented liquid feed is limited to only some strains of probiotics that are able to withstand the process of feed's production (Boesen et al., 2004; Van der Wolf et al., 2001; van Winsen et al., 2002).

Direct fed microbials

Direct fed microbial is usually given as a supplementation with normal feed to sows and newborn piglets till four weeks old after wean. This method is suitable for farmers since there is no complicated direction for use (Huys et al., 2006; Marcobal et al., 2008). The study on *B. cereus* var. *toyoi* given to sows showed the reduction of *E.coli* passage to the piglets, reduction of the diarrhea incidences in the herds, and increasing of the average daily gain (Taras et al., 2005; Scharek et al., 2007).

Effects of enteric pathogens in pigs

Weaning period poses a threat on farmers due to a high risk of decreasing in health performances and mortality from post-weaning diarrhea caused by enteric pathogens. Common causatives in pigs are pathogenic *Escherichia coli*, *Salmonella,* and coronavirus.

Effects of enteric pathogenic bacteria and antibacterial activity of probiotics in pigs

Pathogenic *Escherichia coli* and *Salmonella* strains *are* the cause of enteric diseases worldwide such as severe watery diarrhea especially in piglets and calves. The common symptoms caused by both pathogens include depression, dehydration, reduction of feed intake, weight loss, and mortality. Although mortality rate is only approximately 10-15%, they still cause significant economic losses due to reduction of growth performances and sudden death in affected pigs (Nabuurs et al., 1996; Kyriakis et al., 1999; Letellier et al., 1999; Bergeron et al., 2010). Several studies on antagonistic effects of LAB against pathogenic *E. coli* and *Salmonella* suggested that lactic acid and bacteriocins produced by LAB could inhibit the growth of these pathogens. Moreover, LAB might also be responsible for activation of macrophages and enhancing host systemic immune responses (Hudault et al., 1997; Schiffrin et al., 1997; Forestier et al., 2001).

Effects of coronavirus and antiviral activity of probiotics in pigs

Coronavirus such as transmissible gastroenteritis (TGE) and porcine epidemic diarrhea virus (PEDV) is an agent responsible for highly contagious enteric diseases includes viral enteritis and fetal diarrhea with almost 100% mortality rate. They both cause watery diarrhea, but PEDV mostly affects on suckling pigs less than 4 to 5 weeks old (Pan et al., 2012; Song and Park, 2012). Since the emergence in Thailand on 1995, PED became one of the most sources of concerns in Thailand due to the more severity and acuteness of the disease than the observed PED in Europe. The majority of the

outbreaks were observed in less than 10 day-old piglets showing acute watery diarrhea, vomiting, anorectic depression, and dehydration. Mortality rate can range from 30 to 100% (Walsh et al., 2008;Kumar et al., 2010; Olanratmanee et al., 2010; Pan et al., 2012; Song and Park, 2012). The studies on antiviral effects of probiotics against TGEV suggested that the cells treated with supernatants produced by probiotics showed no cytopathic effects from the virus, and moderate to complete protection of the studied monolayer against the virus could also be observed (Kumar et al., 2010). However, from the reviews, the study of antiviral effect of live probiotics against PEDV is not found.

1.3 Research Hypothesis

1.3.1 Lactic acid bacteria (LAB) selected from Thai indigenous and commercial pigs are able to develop as candidate probiotics.

1.3.2 Selected lactic acid bacteria have the ability of to inhibit pathogenic bacteria and porcine epidemic diarrhea virus.

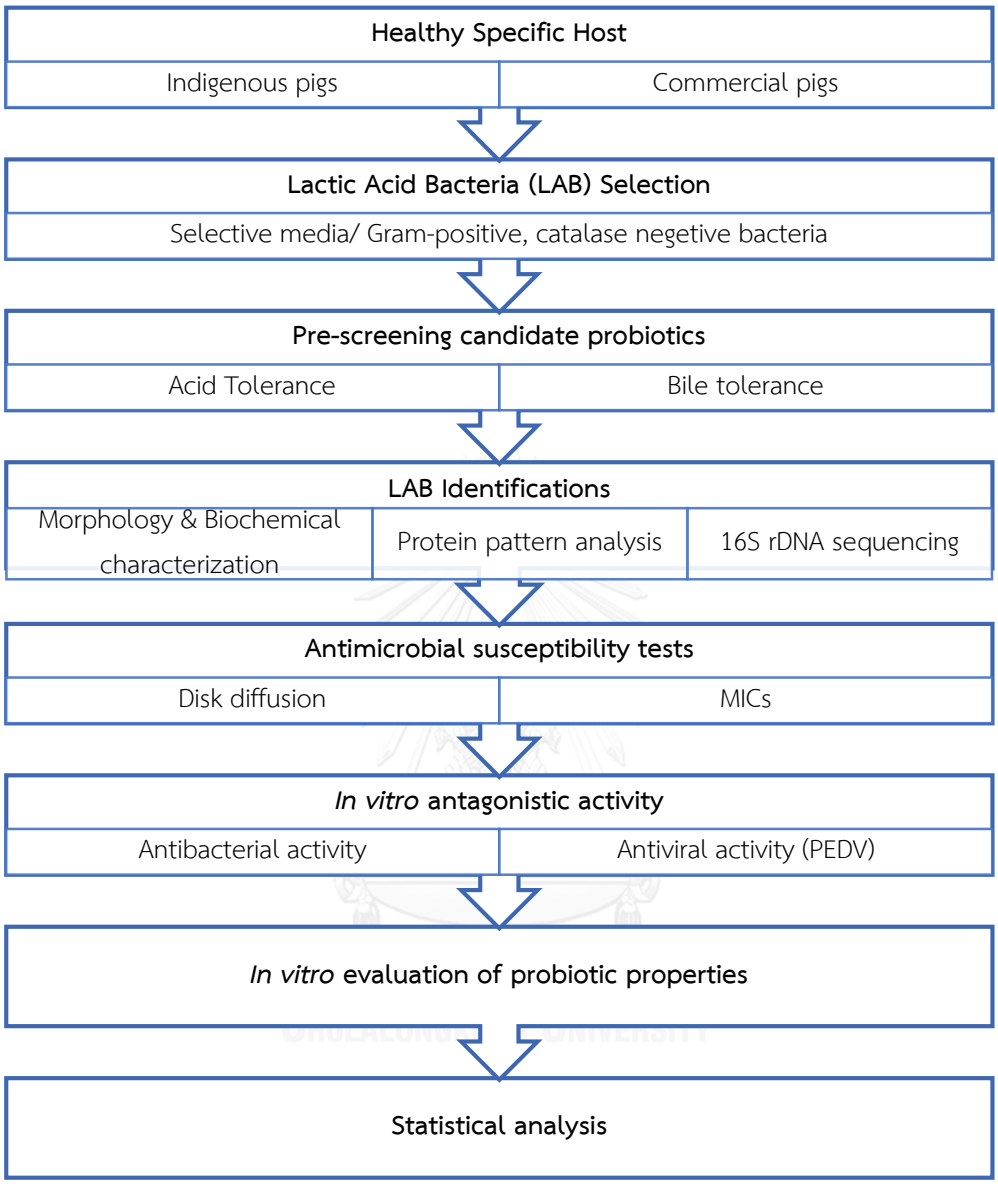
1.4 Research Objectives

1.4.1 To select lactic acid bacteria that can be potential candidate probiotics used in pig industry.

1.4.2 To characterize and compare the abilities of selected lactic acid bacteria from Thai indigenous and commercial pig and between our candidate and a commercial probiotic.

1.4.3 To evaluate antimicrobial and anti-viral activities of the selected lactic acid bacteria against pathogenic *E. coli*, *Salmonella*, and PED virus.

1.5 Conceptual framework



1.6 Advantages of Study

The main benefit of this study will be the obtainment of local lactic acid bacteria from healthy, antibiotic-free indigenous and commercial pigs that are well-characterized with antibacterial effects and evaluated probiotic properties, that can be further developed as promising probiotic candidates. Therefore, the results obtained will help to achieve the ultimate purpose on improving the pig's performances, and subsequently benefit Thai pig industry. From the scientific point of view, apart for this main benefit, this study will also provide useful information on antivral effects of cell-free supernatant and live candidate probiotics against porcine epidemic diarrhea virus which has never been studied before.

1.7 Keywords

Lactic acid bacteria, pathogenic bacteria, pig, porcine epidemic diarrhea virus, probiotics

CHAPTER II

IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY OF LACTIC ACID BACTERIA FROM FECAL SAMPLES OF INDIGENOUS AND COMMERCIAL PIGS

Wandee Sirichokchatchawan[1], Somboon Tanasupawat[2], Waree Niyomtham[1] and Nuvee Prapasarakul[1]*

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.
[2]Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

***Corresponding author:**
Nuvee Prapasarakul
Tel: +66 2 218 9582
Fax: +662 251 1656.
E-mail address: Nuvee.P@chula.ac.th

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2.1 Abstract

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

2.2 Introduction

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

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

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

can be incorporated in identification process reflected the dominance of LAB species from all GIT sources (Klose et al., 2010).

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

2.3 Materials and Methods

2.3.1 Sample collection and LAB isolation

A total of 60 fecal samples were collected directly from the rectum of indigenous pigs in Nan province and commercial pigs in Chai-nart province. All samples were collected from eight month-old healthy, antibiotic-free pigs weight around 120 to 130 kg with no evidence of clinical sign of enteric diseases and have perfect body condition score (cannot observe ribs, hips and backbone). The LAB were isolated by dilution and plating. Amount 10 g of the sample was 10-fold serially diluted and inoculated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) supplemented with 0.3% (w/v) calcium carbonate. Three to five bacterial colonies presented clear zone were selected, and re-streaked on MRS agar to ensure purity. The bacterial isolates were subjected to Gram's stain and catalase test to identify as presumptive LAB. Only the isolates which were Gram-positive and catalase negative rods and cocci were selected for further studies (Maragkoudakis et al., 2006). Approval

for use of the experimental animals was obtained from the ethical committee of Faculty of Veterinary Science, Chulalongkorn University (No. 1531011).

2.3.2 Acid and bile tolerance of LAB

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

2.3.3 Phenotypic characterizations

Based on cell morphologies and ability to tolerate acidic and bile condition, 34 isolates were selected for this study. A set of 26 tests including cell morphology, CO_2 production from glucose, ability to grow at 45 ºC and 50 ºC, and acid production from 21 types of carbohydrates (amygdalin, L-arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-Xylose), was used to classify and characterize the isolates (Ricciardi et al., 2005; TanasupawatandKomagata, 1995).

2.3.4 Whole-cell protein profiling by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

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

2.3.5 16S rDNA sequence analysis

A pure culture of each selected isolate was grown for 24 h in MRS broth and prepared for DNA extraction. The cells were harvested and washed twice with 0.85% physiological saline and centrifuged at 8,000 × g for 2 min. The bacterial DNA was extracted using a Nucleospin® tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. The 16S rDNA of selected isolates were amplified by PCR using the universal 16S ribosomal gene primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), as reported previously (Angmo et al., 2016). The reference strains used for the whole-cell protein pattern analysis were also used as control strains for the PCR amplification,

while DNase free water was used as a negative control. The PCR products were purified using a QIAquick purification kit (Qiagen, Tokyo, Japan) prior to submission for commercial sequencing (WardMedic, Bangkok, Thailand). The obtained 16S rDNA sequences were compared with the sequences of type strains available in the GenBank database on the National Center for Biotechnology Information (NCBI) for species identification and nucleotide identity using the online BLASTn algorithm. A phylogenetic tree was constructed from the aligned 1,400-1,500 bp sequences (after removal of indels) using the neighbor-joining (NJ) distance method with bootstrap resampling of 1,000 replicates in the MEGA6 software program (Tamura et al., 2007). The nucleotide sequences of all the analyzed isolates were deposited in the DDBJ gene databank (Shizuoka, Japan), with the accession numbers presented in Table 2.

2.3.6 Antimicrobial susceptibility

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

Clinical and Laboratory Standards Institute (CLSI, 2012; Jorgensen et al., 2007). Breakpoints for the studied MICs followed the microbiological cut-off values proposed by the EFSA FEEDAP Panel (EFSA, 2012). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as standard controls.

2.3.7 Confirmation of antimicrobial resistance genes

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

2.3.8 Statistical analysis

The phenotypic characteristics were coded as 0 = negative, or 1= positive for the ability to produce acid from the 21 types of carbohydrates. For cell morphology, two variables were applied: C/R (0 = cocci, 1 = rods) and C/ST (0 = chains, 1 = single cells or tetrads). A similarity matrix was built using Jaccard coefficient (Sj). Hierarchical clustering of the phenotypic tests was performed using Unweighted Pair-Group Average Linkage Analysis (Ricciardi et al., 2005; TanasupawatandKomagata, 1995). Statistical analyses were performed using Systat 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

2.4 Results

2.4.1 Isolation and selection of LAB for further identification

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

2.4.2 Identification of acid and bile tolerant LAB

The 34 selected isolates were characterized using a set of 26 phenotypic tests (Table 2). They were statistically grouped into eleven clusters at 80% similarity level. Most strains were identified as *E. faecium* (cluster 4, 6 isolates), *E. hirae* (cluster 1, 5 isolates) and *L. plantarum* (cluster 5, 4 isolates), whereas the 19 isolated strains could not classify into a species (Figure 3). To confirm the identification of selected LAB obtained from phenotypic tests, a near full-length 16S rDNA sequence was obtained for all 34 acid and bile tolerant LAB and depicted as a phylogenetic relationship as inferred by neighbor-joining analysis (Figure 4). The isolates were placed into six clustered (designated as I to VI), and were identified as genus *Enterococcus* (11 isolates as *E. faecium* and 9 isolates as *E. hirae*), 7 isolates were assigned to the genus *Lactobacillus* (4 isolates as *L. plantarum* and 3 isolates as *L. agilis*) and 7 isolates to genus *Pediococcus* (6 isolates as *P. pentosaceus* and 1 isolate as *P. acidilactici*) (Table 1). From the mirror image, the isolate number 80N which identified as *E. hirae* by phenotypic dendrogram was identified as *E. faecium* by 16S rDNA sequencing and also later identified as *E. faecium* by whole-cell protein pattern analysis (Figure 3).

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

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

Table 1 Source, isolate number and identification results of the 34 selected acid and bile tolerant lactic acid bacteria from pig feces in Thailand (continue).

Sample origins	[a] Strain and accession numbers	[b] 16 rDNA sequencing	[c] Phenotypic cluster	[d] Whole-cell protein cluster	[e] Species identification (≥99% similarity to type strain)
Commercial pig	31F (LC035106)	IV	5	E	*L. plantarum*
Commercial pig	25F (LC035105)	IV	5	E	*L. plantarum*
Commercial pig	22F (LC035101)	IV	5	E	*L. plantarum*
Commercial pig	44F (LC035111)	IV	5	E	*L. plantarum*
Commercial pig	56F (LC035108)	III	11	D	*L. agilis*
Commercial pig	74F (LC035116)	III	10	D	*L. agilis*
Commercial pig	75F (LC035127)	III	10	D	*L. agilis*

[a] Accession number: sequences determined in this study were deposited in the DDBJ gene databank in Japan.

[b] Group: isolates were grouped and identified by 16S rRNA gene

[c] Cluster: isolates were clustered and analysed by hierarchical clustering of a set of 26 phenotypic tests

[d] Cluster: isolates were clustered and analysed by SDS-PAGE according to whole-cell protein profiles

[e] Type strains: *E. hirae* ATCC 9790^T; *E. faecium* ATCC 19434 ^{T}; *P. pentosaceus* DSM 20336 ^{T}; *P. acidilactici* DSM 20284 ^{T}; *L. plantarum* JCM 1149 ^{T}; and *L. agilis* JCM 1187 ^{T}

The analysis of whole-cell protein patterns classified the isolates on provisional species level into six clusters (A to F) (Table 2 and Figure 5) of *E. faecium* ATCC 19434 (82% similarity), *E. hirae* ATCC 9790 (82% similarity), *P. acidilactici* DSM 20284 (91% similarity), *P. pentosaceus* ATCC 25745 (96% similarity), *L. agilis* DSM 20509 (82% similarity) and *L. plantarum* JCM 1149 (80% similarity), and were confirmed by the mirror image with the phylogenetic relationships as shown in Figure 5. The distinctions on the acid production from carbohydrates of *E. faecium, L. agilis, L. plantarum, P. acidilactici* and *P. pentosaceus* isolates in this study were the ability to produce acid from mannitol, mannose, trehalose and D-Xylose as shown in Table 3.

2.4.3 Determination of antimicrobial susceptibility and resistance gene detection

The antimicrobial susceptibilities by disk diffusion of the 34 selected isolates are summarized in Table 4. All isolates were susceptible to amoxicillin but resistant to colistin sulfate, gentamicin, kanamycin and streptomycin and 94.1%, 79.4%, 58.9%, and 52.9% of the isolates were susceptible to ampicillin, chloramphenicol, tetracycline, and vancomycin, respectively. Furthermore, over 75% of the tested strains showed resistance to erythromycin and clindamycin, especially among *E. hirae, E. faecium* and *L. agilis*. From the MIC values (Table 5), the final five selected LAB strains (*P. pentosaceus* 77F, *P. acidilactici* 72N, *L. plantarum* 22F, 25F, and 31F) were susceptible to ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, vancomycin and tylosine; while the two strains of *E. faecium* (79N and 40N) were only susceptible to tetracycline and vancomycin. With respect to the antimicrobial resistance gene profile, none of the genes studied were detected in all three *L. plantarum* strains (22F, 25F, and 31F), whereas genes associated with resistance to four antibiotics were detected in *E. faecium* (*erm(B), aac(6')aph(2"), aph(3")-III* and *aadE*) and to three and two antibiotics in *P. acidilactici* (*erm(B), aac(6')aph(2")* and *aph(3")-III*) and *P. pentosaceus* (*erm(B)* and *aph(3")-III*), respectively (Table 6).

Table 2 Phenotypic characteristics of the 34 selected acid and bile tolerant LAB isolated from indigenous and commercial pig feces as shown in Figure 4.

Characteristics	Phenotypic cluster										
	1	2	3	4	5	6	7	8	9	10	11
	[a] (*E. faecium*/ *E. hirae*)	(*E. hirae*)	(*E. faecium*)	(*E. faecium*)	(*L. plantarum*)	(*P. pentosaceus*)	(*P. pentosaceus*)	(*P. pentosaceus*)	(*P. acidilactici*)	(*L. agilis*)	(*L. agilis*)
No. of isolates	6	2	4	8	4	2	1	4	1	2	1
Cell morphology[b]	CC	CC	CC	CC	T	T	T	RC	RC	RC	CC
CO_2 from glucose	-	-	-	-	-	-	-	-	-	-	-
Growth at											
45 °C	+	+	+	+	+	+	+	+	+	+	+
50 °C	-	-	-	-	-	-	+	-	-	-	-
Esculin hydrolysis	-	-1	-	-4	+	+	+	+	-	-	
Acid from:											
Amygdalin	+	+	-	+	-	-1	+	+	+	-	+
L-arabinose	-1	-	+	-	+	+	+	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	-	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	-	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	+	-	-	+	+	+

Table 2 Phenotypic characteristics of the 34 selected acid and bile tolerant LAB isolated from indigenous and commercial pig feces as shown in Figure 4. (continue)

Characteristics	Phenotypic cluster										
	1	2	3	4	5	6	7	8	9	10	11
	[a] (*E. faecium*/ *E. hirae*)	(*E. hirae*)	(*E. faecium*)	(*E. faecium*)	(*L. plantarum*)	(*P. pentosaceus*)	(*P. pentosaceus*)	(*P. pentosaceus*)	(*P. acidilactici*)	(*L. agilis*)	(*L. agilis*)
Maltose	+	+	+	+	+	-	+	+	-	-	-
Mannitol	+	-	+	-1	-	-	-	+	-	-	-
Mannose	+	+	+	+	+	+	+	+	-	-	-
Melezitose	-	-	-	-	-	-	+	-	+	-	+
Melibiose	-	-	-1	-3	+	-	+	-	+	+	+
Raffinose	-	-	-	-	-	-	+	-2	-	+	-
Rhamnose	-	-	-	-	-	-	-	-2	+	-	+
D-Ribose	+	+	+	+	+	+	+	+	+	-	+
Salicin	+	+	+	+	-	-1	+	+	-	-	-
Sorbitol	-2	-1	-	-	-	-	+	+	-	+	-
Sucrose	-3	+	+	+	-	-	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	-	-	-
D-Xylose	-	-	-	-	-	-	+	-	-	-	-

[a] showed the species identification of the members in the cluster by 16S rDNA sequencing (Table 1). +, positive; -, negative; Numbers in parentheses indicate the number of strains; [b] CC: cocci in chains; T: tetrads; RC: rods in chains

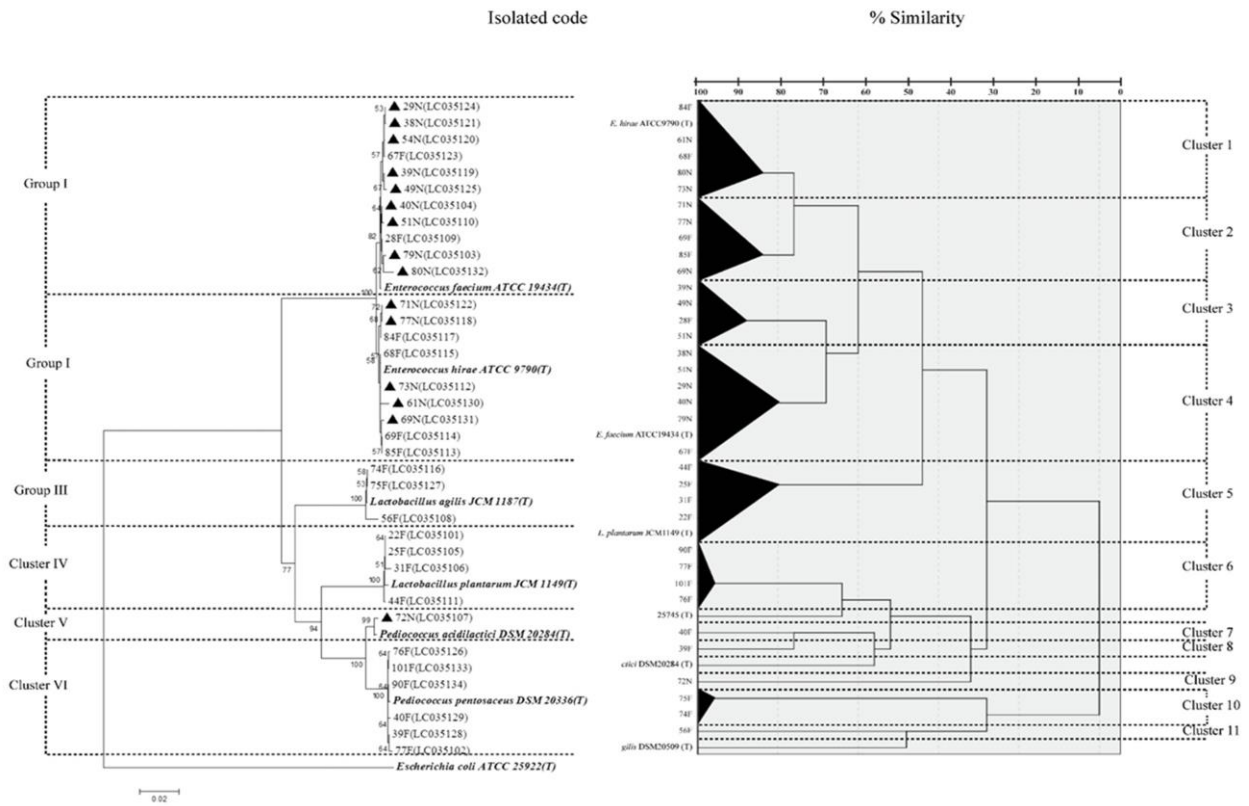

Figure 3 A mirror image comparing dendrograms depicting similarities based on phenotypic tests (right- hand side) and a phylogenetic relationship of 16S rDNA sequencing (left-hand side) of 34 selected acid and bile tolerant LAB isolated from feces of indigenous and commercial pigs in Thailand. Similarities in phenotypic characterization were calculated by the Jaccard coefficients and grouping with hierarchical clustering analysis by Unweighted Pair-Group Average Linkage analysis [23]. A neighbor-joining phylogenetic tree of the isolates and the reference strains based on 16S rRNA gene sequences. Accession numbers are given in parentheses. Bootstrap replicate values (1000 replicates) of > 50% are shown above the node. Escherichia coli is used as the outgroup. Scale represents 0.02 substitutions per site. ▲ represents isolates from indigenous pig feces, isolates without a symbol are from commercial pig feces and the reference strains are shown in bold italic with (T).

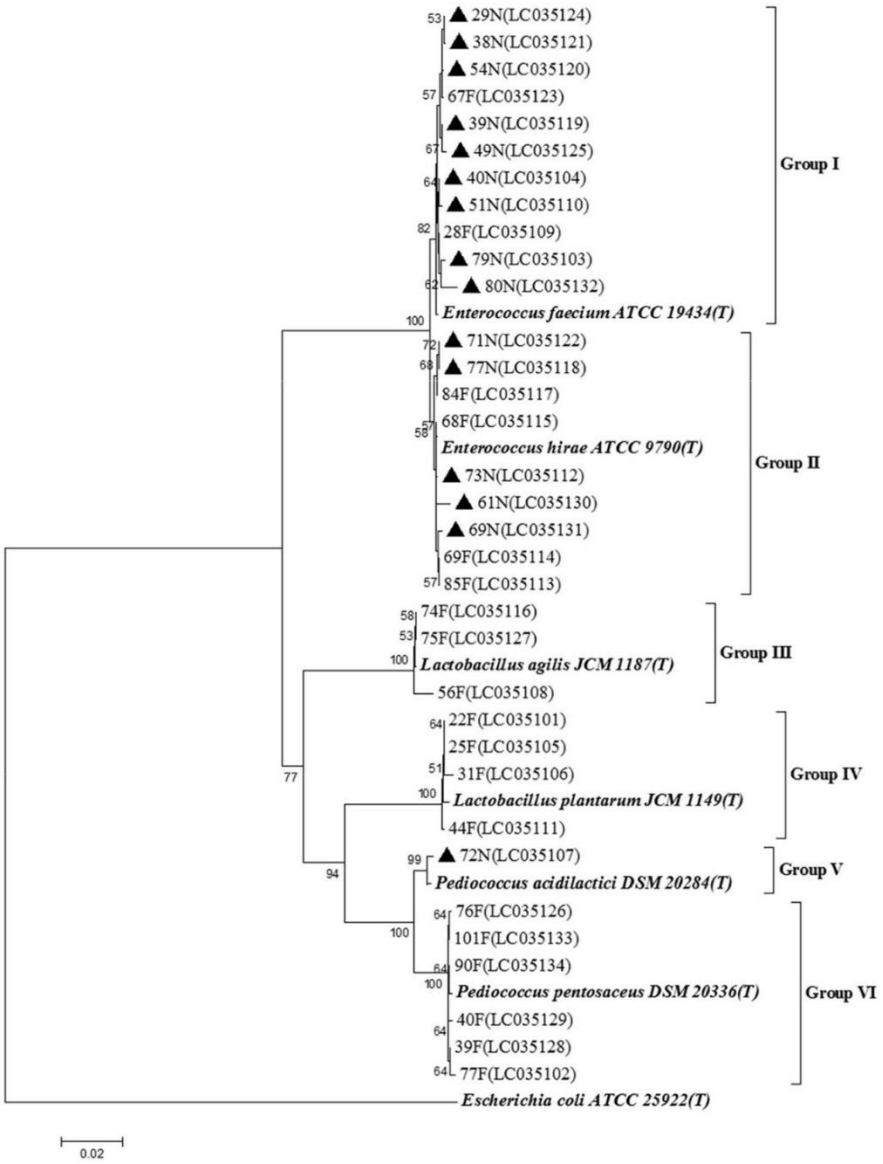

Figure 4 A phylogenetic relationship of the 34 selected LAB isolates and the reference strains based on 16S rRNA gene sequences. Accession numbers are given in parentheses. Bootstrap replicate values (1000 replicates) of > 50% are shown above the node. Escherichia coli is used as the outgroup. Scale represents 0.02 substitutions per site. ▲ represents isolates from indigenous pig feces, isolates without a symbol are from commercial pig feces and the reference strains are shown in bold italic with (T).

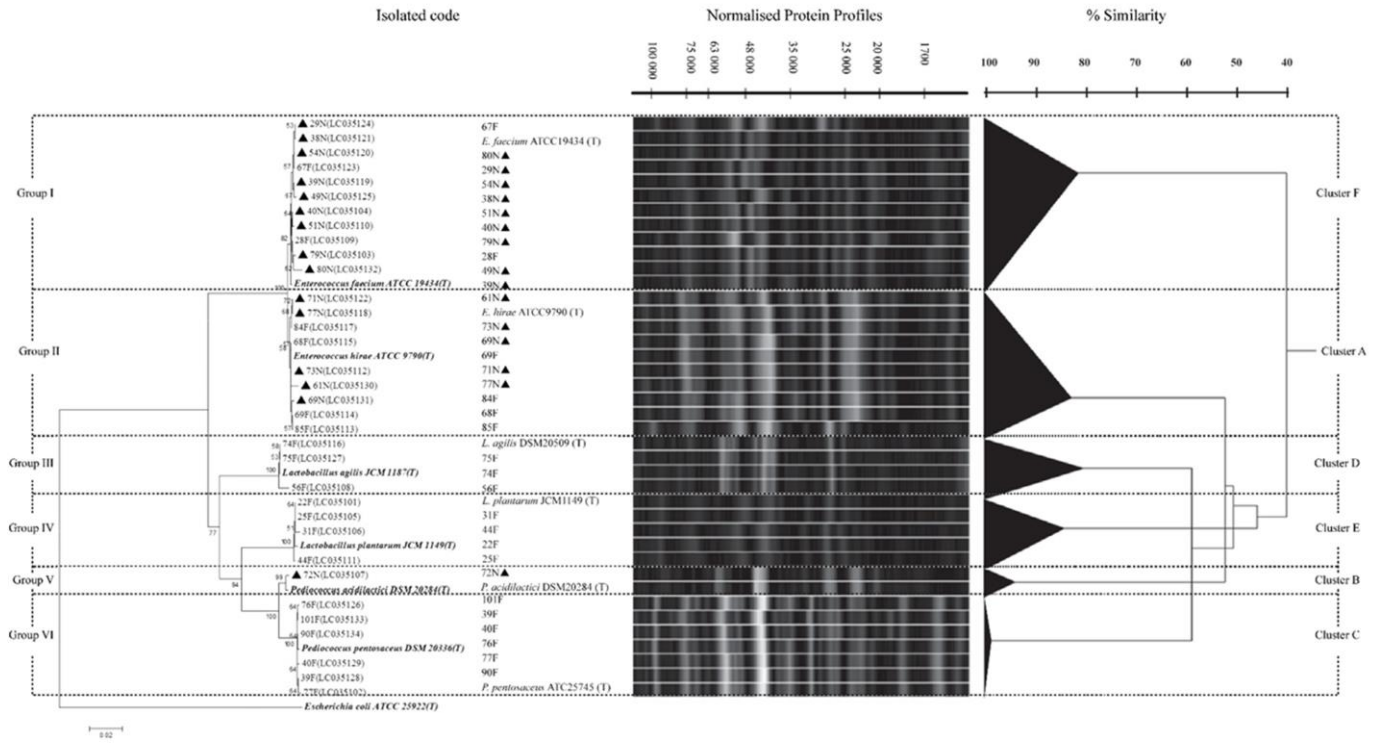

Figure 5 A mirror image comparing dendrograms depicting similarities based upon the whole-cell protein profiles (1D-SDS-PAGE analysis) (right- hand side) and a phylogenetic relationship of 16S rDNA sequencing (left-hand side) of 34 selected acid and bile tolerant LAB isolated from feces of indigenous and commercial pigs in Thailand. The whole-cell protein profiles derived from comparison with the shown reference strains. The mean correlation coefficient (R2 x100) represented as the UPGMA dendrogram. A neighbor-joining phylogenetic tree of the isolates and the reference strains based on 16S rRNA gene sequences. Accession numbers are given in parentheses. Bootstrap replicate values (1000 replicates) of > 50% are shown above the node. Escherichia coli is used as the outgroup. Scale represents 0.02 substitutions per site. ▲ represents isolates from indigenous pig feces, isolates without a symbol are from commercial pig feces and the reference strains are shown in bold italic with (T).

Table 3 Consensual agreement between phenotypic and genotypic characterizations for identification purpose of the six lactic acid bacteria species.

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

[a)] By phenotypic characteristics, profile of *E. hirae* is identical to *P. pentosaceus*

Table 4 Susceptibility of the 34 selected acid and bile tolerant LAB strains to eleven antibiotics [a)] as determined using the disk diffusion method

Species	Strain numbers	AM	AP	C	TC	VA	CS	G	K	ST	ER	CM
E. faecium	28F	S	S	R	S	S	R	R	R	R	R	R
E. faecium	29N	S	S	R	S	S	R	R	R	R	R	R
E. faecium	38N	S	S	R	S	S	R	R	R	R	R	R
E. faecium	39N	S	S	R	S	S	R	R	R	R	R	R
*E. faecium**	40N	S	S	S	S	S	R	R	R	R	S	R
E. faecium	49N	S	S	R	S	S	R	R	R	R	R	R
E. faecium	51N	S	S	S	S	S	R	R	R	R	R	R
E. faecium	54N	S	S	R	S	S	R	R	R	R	R	R
E. faecium	67F	S	R	R	S	R	R	R	R	R	R	R
*E. faecium**	79N	S	S	S	S	S	R	R	R	R	S	R
E. faecium	80N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	61N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	68F	S	S	S	R	S	R	R	R	R	R	R
E. hirae	69F	S	S	S	R	S	R	R	R	R	R	R
E. hirae	69N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	71N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	73N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	77N	S	S	S	R	S	R	R	R	R	R	R
E. hirae	84F	S	S	S	R	S	R	R	R	R	R	R
E. hirae	85F	S	R	S	R	R	R	R	R	R	S	R
L. agilis	56F	S	S	S	R	R	R	R	R	R	R	R
L. agilis	74F	S	S	S	R	R	R	R	R	R	R	R
L. agilis	75F	S	S	S	R	R	R	R	R	R	R	R
*L. plantarum**	22F	S	S	S	S	R	R	R	R	R	S	S
*L. plantarum**	25F	S	S	S	S	R	R	R	R	R	S	S
*L. plantarum**	31F	S	S	S	S	R	R	R	R	R	S	S
L. plantarum	44F	S	S	S	R	R	R	R	R	R	S	S
*P. acidilactici**	72N	S	S	S	S	R	R	R	R	R	S	S
P. pentosaceus	101F	S	S	S	S	R	R	R	R	R	R	R
P. pentosaceus	39F	S	S	S	S	R	R	R	R	R	R	R
P. pentosaceus	40F	S	S	S	S	R	R	R	R	R	R	R
P. pentosaceus	76F	S	S	S	S	R	R	R	R	R	R	R
*P. pentosaceus**	77F	S	S	S	S	R	R	R	R	R	S	S
P. pentosaceus	90F	S	S	S	S	R	R	R	R	R	R	R
Total percentage of susceptibility		100	94.1	79.4	58.9	52.9	0	0	0	0	17.7	23.5

[a)] R = resistant and S = susceptible for: AM=Amoxycillin, AP=Ampicillin, C=Chloramphenicol, TC=Tetracycline, VA=Vancomycin, CS=Colistin sulfate, G=Gentamicin, K=Kanamycin, ST=Streptomycin, ER=Erythromycin, CM=Clindamycin. *The selected LAB strain.

Table 5 Confirmation of antimicrobial susceptibility of seven selected acid and bile tolerant lactic acid bacterial strains by minimum inhibitory concentration (MIC) values.

Antimicrobials	MIC (µg/mL)						
	Enterococcus		*Lactobacillus*			*Pediococcus*	
	E. faecium 79N	*E. faecium* 40N	*L. plantarum* 22F	*L. plantarum* 25F	*L. plantarum* 31F	*P. acidilactici* 72N	*P. pentosaceus* 77F
Ampicillin	4 (R)	4 (R)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)
Chloramphenicol	64 (R)	64 (R)	8 (S)	4 (S)	8 (S)	4 (S)	2 (S)
Erythromycin	> 32 (R)	> 32 (R)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)	< 0.125 (S)
Gentamicin	> 32 (R)	> 32 (R)	0.125 (S)	0.125 (S)	0.25 (S)	0.125 (S)	0.125 (S)
Kanamycin	> 1024 (R)	> 1024 (R)	8 (S)	4 (S)	16 (S)	8 (S)	4 (S)
Streptomycin	> 256 (R)	> 256 (R)	n.r	n.r	n.r	4 (S)	2 (S)
Tetracycline	0.25 (S)	0.25 (S)	16 (S)	16 (S)	16 (S)	4 (S)	4 (S)
Vancomycin	0.25 (S)	0.25 (S)	n.r	n.r	n.r	n.r.	n.r.
Tylosine	> 16 (R)	>1 6 (R)	n.r	n.r	n.r	n.r.	n.r.

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

Table 6 Positive PCR for 13 investigated resistance genes from four antibiotics in selected acid and bile tolerant lactic acid bacterial strains.

Species	Positive PCR for resistance genes
P. pentosaceus 77F	*erm(B), aph(3")-III*
P. acidilactici 72N	*erm(B), aac(6')aph(2"), aph(3")-III*
E. faecium 79N	*erm(B), erm(C), aac(6')aph(2"), aph(3")-III, aadE*
E. faecium 40N	*erm(B), aac(6')aph(2"), aph(3")-III, aadE*
L. plantarum 22F	No genes detected
L. plantarum 25F	No genes detected
L. plantarum 31F	No genes detected

2.5 Discussion

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

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

Although 16S rDNA sequencing analysis is generally recommended for LAB identification, it is still costly, inconvenient and time-consuming in case of a routine laboratory service (Moraes et al., 2013), whereas the whole-cell protein analysis is less

expensive and reduces time consuming in the case of available databases or/and of the reference strains (Leisner et al., 1999).

The antimicrobial resistance phenotype and genotype is also the essential selection criterion for screening candidate probiotics (EFSA, 2012). We excluded the isolates that showed resistance toward clindamycin, erythromycin, chloramphenicol and gentamicin, as they pose a high risk of harboring transferable resistance genes (Muñoz-Atienza et al., 2013). The confirmation of antimicrobial susceptibility on these thirty-four acid and bile tolerant LAB revealed that only five isolates (three *L. plantarum, P. pentosaceus* and *P. acidilactici*) were found to be secure from being a possible source of antimicrobial resistance gene transmission, and were acceptable as potential candidates for further studies on probiotics followed the European Food Safety Authority (EFSA) recommendations (EFSA, 2012).

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

CHAPTER III

Protective Effects of Cell-free Supernatant and Live Lactic Acid Bacteria Isolated from Thai Pigs Against a Pandemic Strain of Porcine Epidemic Diarrhea Virus

Wandee Sirichokchatchawan[1], Gun Temeeyasen[1], Dachrit Nilubol[1], and Nuvee Prapasarakul[1]*

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.

***Corresponding author:**
Nuvee Prapasarakul
Tel.: +662-218-958
Fax: +662-251-1656
E-mail: Nuvee.P@chula.ac.th

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3.1 Abstract

Porcine epidemic diarrhea virus (PEDV) is a coronavirus which causes severe diarrhea and fatal dehydration in piglets. In general, probiotic supplements could enhance recovery and protect piglets against enteric pathogens. Seven local lactic acid bacteria (LAB), (*E. faecium* 79N and 40N, *L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) from pig feces were well-characterized as high potential probiotics. Cell-free supernatants (CFS) and live LAB were evaluated for antiviral activities by co-incubation on Vero cells and challenged with a pandemic strain of PEDV isolated from pigs in Thailand. Cell survival and viral inhibition were determined by cytopathic effect (CPE) reduction assay and confirmed by immunofluorescence. At 1:16, CFS dilution (pH 6.3 – 6.8) showed no cytotoxicity in Vero cells and was therefore used as the dilution for antiviral assays. The diluted CFS of all *L. plantarum* showed the antiviral effect against PEDV; however, the same antiviral effect could not be observed in *E. faecium* and *Pediococcus* strains. In competitive experiment, only live *L. plantarum* 25F and *P. pentosaceus* 77F showed CPE reduction in the viral infected cells to <50% observed field area. This study concluded that the CFS of all tested lactobacilli, and live *L. plantarum* (22F and 25F) and *Pediococcus* strains 72N and 77F could reduce infectivity of the pandemic strain of PEDV from pigs in Thailand on the target Vero cells.

3.2 Introduction

Porcine epidemic diarrhea (PED) is one of the highly contagious and concerning viral diseases in the pig industry. The disease not only causes fatal watery diarrhea in piglets, but also significant weight loss in pigs of all ages. Porcine epidemic diarrhea virus (PEDV) is an RNA virus that belongs to the family *Coronaviridae.* The recent outbreaks and worldwide re-emergence of PEDV have been reported in several countries including the USA, China, Korea, and Thailand (C. Lee, 2015; Shen et al., 2015). Without the effective protective agents, the disease has led to great economic losses worldwide (Di-qiu et al., 2012).

Recent experiments and clinical studies show that gut microbiota plays an active role in serving as a primary barrier against food-borne pathogens including viruses (AchesonandLuccioli, 2004). Probiotic bacteria can promote the host defense mechanisms and modulation of immune system, with the potential to enhance the antiviral activity (Cross, 2002; Kaila et al., 1995). Among them, the group of lactic acid bacteria (LAB), including genera *Lactobacillus spp., Pediococcus spp*. and *Enterococcus faecium*, is generally used as probiotics in animal productions (Tannock, 1997). The failure in finding new antiviral substances without adverse side effects (Choi et al., 2009) and the benefits of probiotics treatment in patients with rotavirus (RV) and HIV-associated diarrhea have led to an increased interest in probiotic bacteria as antiviral inhibitors (Chang et al., 2003; Isolauri, 2003). Although many researches show the antiviral effects of LAB on several viral infections in humans and livestock (Botić et al., 2007; Chai et al., 2012; Maragkoudakis et al., 2010), few studies report on antiviral activity using only cell-free supernatant against a classical strain of PEDV (Choi et al., 2009). To the best of our knowledge, this is the first report on antiviral activity using both cell-free supernatant (CFS) and live LAB cells against a pandemic strain of PEDV.

This study investigated the cytotoxicity and potential antiviral activity of selected LAB from pig feces in Thailand as protective agents against a pandemic strain of PEDV isolated from pigs in Thailand, *in vitro*. To determine the antiviral ability and attachment ability of the LAB strains, as well as the cytotoxicity of cell-free

supernatants (CFS) to Vero cells, the study used both CFS and live LAB strains on Vero cell lines challenged with a pandemic strain of PEDV.

3.3 Materials and methods

3.3.1 Cell and virus

Vero cell line ATCC® CCL-81™ was maintained in Modified Eagle's Medium (MEM) (Gibco™, MA, USA), supplemented with 5% fetal bovine serum (FBS) (Gibco™, MA, USA), and 1% antibiotic-antimycotic (Gibco™, MA, USA) at 37 ºC in a humidified 5% CO_2 atmosphere. Porcine epidemic diarrhea virus (PEDV), a pandemic strain SBPED0211_1 (accession number: JQ966337), was propagated in Vero cells as described by Hofmann and Wyler (HofmannandWyler, 1988). For the antiviral assay, virus with 100 50% tissue culture infective dose (100 $TCID_{50}$/mL) was determined by the Reed and Muench method (ReedandMuench, 1938).

3.3.2 Bacterial strains and growth conditions

Experiments were carried out with three *L. plantarum* strains 22F (LC035101), 25F (LC035105) and 31F (LC035106), two *E. faecium* strains 79N (LC035103) and 40N (LC035104), *P. pentosaceus* 77F (LC035102), and *P. acidilactici* 72N (LC035107) selected from 60 fecal samples of antibiotic-free commercial and indigenous pigs base on *in vitro* probiotic properties. They were able to tolerate pH 2, pH 3, 0.3% ox gall, and grow at 45 °C with ≥ 10^4 CFU/mL, and were acceptable according to European Food Safety Authority on antimicrobial susceptibility. They were therefore characterized and identified by 26 phenotypic tests and 16S rDNA sequence analysis with ≥99% similarities towards the type strains (Table 7). Prior to experimentations, bacterial strains were grown in Man Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, England) for 48 h at 37 ºC under anaerobic condition (Wang et al., 2010).

Table 7 Characterization and identification of seven selected LAB strains on the basis of probiotic properties.

Isolates	Acid tolerance (3 h) [a]		Bile tolerance (0.3%) [a]	Growth at	Antimicrobial resistance	% Nucleotide identity with
	pH 2	pH 3	24 h	45 °C	profile [b]	type strain [d]
E. faecium 79N	80.86±1.89	88.7±6.31	78.5±6.89	+	Clindamycin-kanamycin	99.4
E. faecium 40N	81.25±5.21	90.35±2.63	79.21±3.67	+	Clindamycin-kanamycin	99
P. pentosaceus 77F[c]	87.8±3.49	93.31±1.93	83.74±1.93	+	Vancomycin-kanamycin	99.5
P. acidilactici 72N[c]	86.45±1.93	91.97±0.75	83.94±5.29	+	Vancomycin-kanamycin	99.5
L. plantarum 22F[c]	91.65±2.74	96.32±2.81	82±3.01	+	Vancomycin-kanamycin	99.3
L. plantarum 25F[c]	89.11±2.64	94.48±0.93	86.03±0.86	+	Vancomycin-kanamycin	99.5
L. plantarum 31F[c]	84.59±2.39	95.37±2.21	84.17±2.46	+	Vancomycin-kanamycin	99.6

[a] Survival percentage expressed as mean ± standard deviation

[b] Antimicrobial susceptibility by disk diffusion test against 8 antibiotics; amoxycillin, ampicillin, chloramphenicol, tetracycline, vancomycin, erythromycin, clindamycin, kanamycin

[c] Isolates are susceptible to the antibiotics by MICs confirmation according to European Food Safety Authority (EFSA) microbiological cut-off values

[d] Type strains: *E. faecium* ATCC 19434 [T]; *P. pentosaceus* DSM 20336 [T]; *P. acidilactici* DSM 20284 [T]; *L. plantarum* JCM 1149 [T]

3.3.3 CFS Preparation for cytotoxicity assay and measurement of antiviral activity

Bacterial culture supernatants were obtained from growing bacterial cultures (10^8 CFU/mL) in 30 mL MRS broth under anaerobic conditions for 24 h at 37 ºC. Supernatants were collected, measured pH values and two-fold serially diluted with MEM (1:2 to 1:64). The supernatants were then filtered with 0.22 µm filter (Milipore corp., Bedford, USA) to remove any remaining bacterial cells from interfering with the experiments (Choi et al., 2009).

3.3.4 Determination of adhering LAB strains

One hundred microliters of each LAB suspension in MEM (1 x 10^8 CFU/mL) was added in triplicate to confluent Vero cell monolayers in 24 well plates and incubated for 90 min at 37 ºC in a humidified 5% CO_2 atmosphere. After the incubation, the Vero cells were fixed and stained according to Lin et al. (2006). The number of adhered LAB cells per Vero cell was determined by counting LAB cells on 100 Vero cells, in 15 randomly selected microscopic fields (magnification fold, 100X) (Lin et al., 2006).

3.3.5 Determination of cytotoxicity by neutral red assay

The cytotoxicity of LAB CFS to Vero cells was determined by the neutral red assay modified from Borenfreund and Puerner (BorenfreundandPuerner, 1985). Briefly, Vero cell monolayers incubated with LAB CFS for 4 days were washed with PBS (pH 7.4) and added with 200 µL MEM containing 50 µg/mL neutral red dye. The plate was incubated, washed with formal-calcium, and added with 0.2 ml of an acetic acid-ethanol mixture. The plate was kept at room temperature until dissolution. The cell viability was determined by comparison the absorbance values at 540 nm obtained for control wells (without CFS) and tested wells (with CFS). The cytotoxicity assay and the quantitative colorimetric assay were carried out on the same cell culture plate.

3.3.6 Antiviral assays

3.3.6.1 Antiviral effect of bacterial cell-free supernatants (CFS)

The two-fold dilutions (1:16 to 1:64) of LAB CFS in MEM, and CFS adjusted to pH 7 by sodium hydroxide (NaOH) were added on the monolayer of Vero cell as

incubation medium (100 µL/well) followed by PEDV challenge (100 $TCID_{50}$/mL). CPE (indicated by syncytium cell formation and multiple nuclei) was determined using CPE scores after an additional 4-day incubation at 37 ºC in a humidified 5% CO_2 atmosphere. PEDV infected Vero cells without CFS treatment were used as a positive control. Untreated, non-infected Vero cells were used as a negative control (Botić et al., 2007; Choi et al., 2009). CPE scores were adjusted by CPE area observed under a microscope as; +++ (>75% of observed field area), ++ (50-75% of observed field area), + (<50% of observed field area) and – (no CPE) as modified from V J. et al. (V. J et al., 2010).

3.3.6.2 Co-incubation of bacteria and PEDV (Competition assay)

One hundred microliters of each live LAB strain at 10^8 CFU/mL to 10^4 CFU/mL in MEM were simultaneously added to pre-washed Vero cell monolayers in 96 well plates and subsequently challenged with 100 µL of 100 $TCID_{50}$/mL PEDV (the viral titer remained unchanged). The plates were further incubated at 37 ºC in a humidified 5% CO_2 atmosphere for 4 days and observed for CPE. All the controls and CPE scores were as described above (Botić et al., 2007; Maragkoudakis et al., 2010).

3.3.7 Immunofluorescence

Immunofluorescence was used to confirm viral infection in Vero cells, the monolayers of Vero cells were washed with PBS and air-dried. Cells were fixed with 80% acetone for 15 min. Fluorescein isothiocyanate (FITC) conjugated PEDV nucleoprotein (NP) monoclonal antibody (Medgene Lab, South Dakota, USA) against PEDV was added to each well. Plates were incubated for 30 min at 37 ºC in a humidified 5% CO_2 atmosphere, washed with PBS and examined under a fluorescent microscope BX51 with DP73 (Olympus, Tokyo, Japan).

3.3.8 Statistical analysis

The adhering LAB strains were done in triplicate and presented as means ± SD. One-way analysis of variance (ANOVA) with the Tukey-Kramer post-hoc comparison was performed to compare means among LAB strains using SPSS 14.0 for Windows. Differences with *P*-values less than 0.05 were considered significant.

3.4 Results

Seven LAB strains were preliminarily applied on the monolayers of Vero cell lines to ensure their adhesion ability (Figure 6). All LAB strains were able to adhere on the Vero cell, while *L. plantarum* strain 25F exhibited the highest adhesion ability (11.7%) (Figure 7). The cytotoxicity assay showed that at 1:16 to 1:64 CFS dilution of all LAB strains had no cytotoxicity to morphology of Vero cells (Figure 8), and were used for the further antiviral assays. The antiviral activity of LAB CFS against PEDV on Vero cells was investigated by co-incubation with serial dilutions of CFS, and CFS adjusted to pH 7. After the co-incubation, the reduction of viral infectivity could be observed using CPE reduction assays and confirmed by qualitative immunofluorescence. A reduction of immunofluorescence signal indicated a decrease in PEDV infectivity in Vero cell monolayers (Figure 9). The CFS of *L. plantarum* (22F, 25F, and 31F) at dilution 1: 16 were able to reduce the CPE and immunofluorescence signal to less than 50% observed field area (+) compared with only PEDV infected Vero cells (>75% observed field area, +++) (Table 8 and Figure 9). In contrast, the CFS of *E. faecium* (79N and 40N) at dilution 1:16 exhibited no reduction of CPE and immunofluorescence signal. Moreover, at the dilution of 1:32 and 1:64 and adjusted pH 7, no reduction of viral infectivity was observed from all LAB strains (Table 8 and Figure 9). Interestingly, at 10^6 CFU/mL in the competing experiment, only *L. plantarum* strains 25F exhibited CPE reduction to 50% of observed field area (++), and were able to reduce the viral infectivity to less than 50% observed field area (+) at 10^7 and 10^8 CFU/mL compared with the only PEDV infected Vero cells (+++). While both *P. pentosaceus* 77F and *L. plantarum* strains 22F at 10^7 CFU/mL were able to reduce the viral infectivity to 50% observed field area (++), only *P. pentosaceus* 77F at 10^8 CFU/mL displayed CPE reduction to less than 50% observed field area (+). In contrast, *P. acidilactici* 72N only exhibited CPE reduction at 10^8 CFU/mL to 50% of the observed field area. Furthermore, there were no reduction of CPE and immunofluorescence

signal observed in any of the bacterial concentrations from *E. faecium* (79N and 40N) and *L. plantarum* 31F (Table 8)

Table 8 Antiviral activity against PEDV by cell-free supernatant (CFS) and seven live LAB strains on Vero cell monolayers as measured by the presence of CPE

Bacterial strain	Supernatant dilution of LAB				Bacterial concentration					Negative control	Positive control
	1: 16 (~ pH 6.3)	1: 32 (~ pH 6.6)	1: 64 (~ pH 6.8)	Adjusted pH7	10^8	10^7	10^6	10^5	10^4		
E. faecium 79N	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	+++
E. faecium 40N	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	+++
P. pentosaceus 77F	+++	+++	+++	+++	+	++	+++	+++	+++	-	+++
P. acidilactici 72N	+++	+++	+++	+++	++	+++	+++	+++	+++	-	+++
L. plantarum 22F	+	+++	+++	+++	++	++	+++	+++	+++	-	+++
L. plantarum 25F	+	+++	+++	+++	+	+	++	+++	+++	-	+++
L. plantarum 31F	+	+++	+++	+++	+++	+++	+++	+++	+++	-	+++

Positive control: Virus only; Negative control: Vero cells only. CPE scores were adjusted by CPE area observed under a microscope as; +++ (>75% of observed field area), ++ (50-75% of observed field area), + (<50% of observed field area) and – (no CPE).

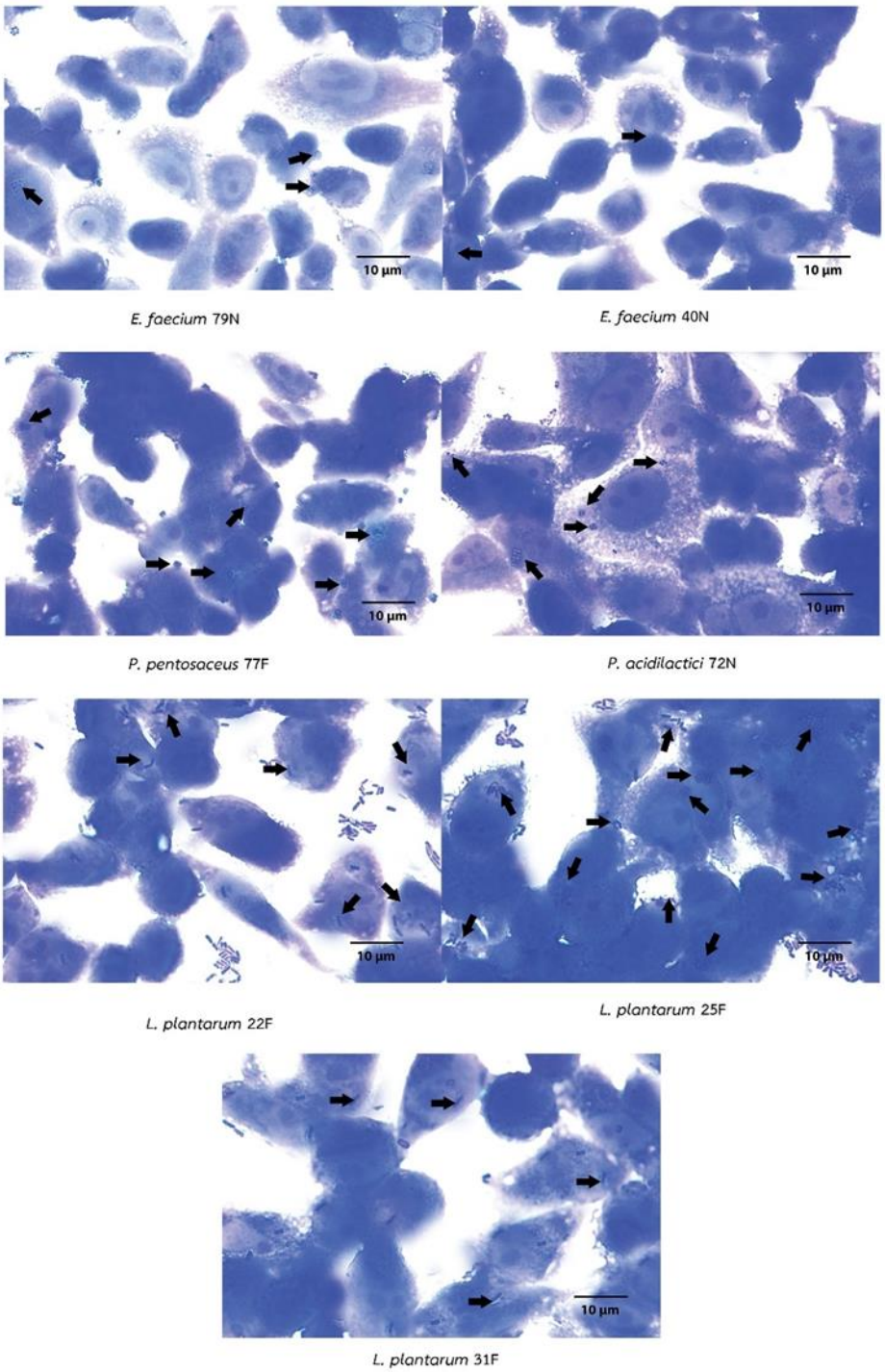

Figure 6 Adhesion ability of seven studied LAB strains on Vero cell monolayers. The observed magnification fold is 100X. The arrows indicate adhering bacterial cells.

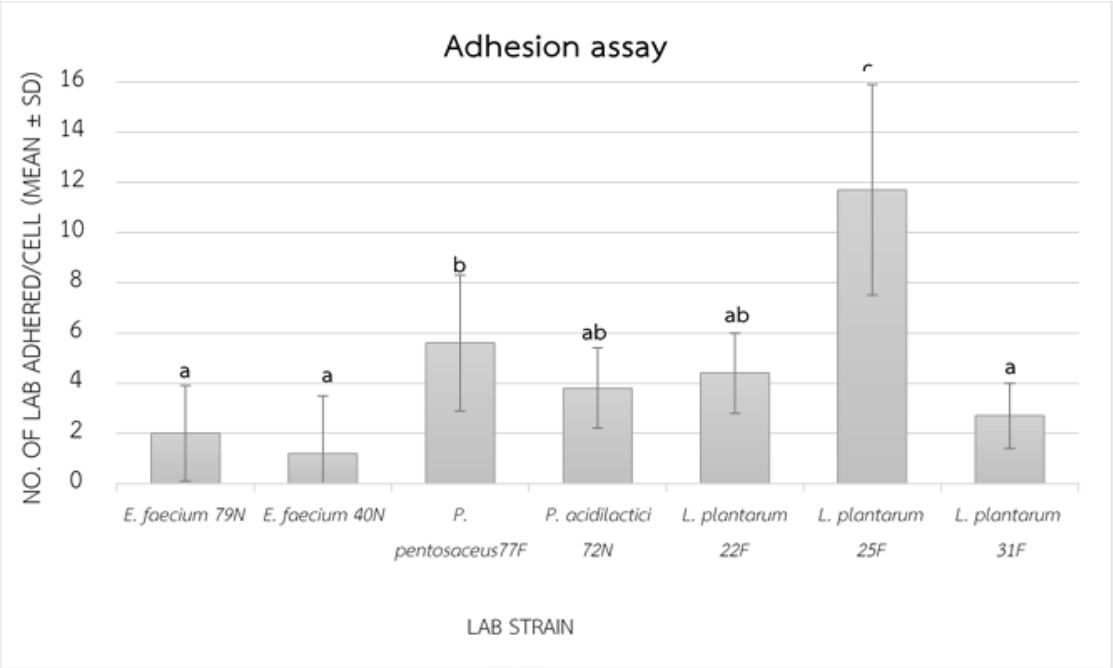

Figure 7 Number of bacterial cells adhered per Vero cell. The results are expressed as mean ± standard deviation. The different letters indicate statistically significant differences between LAB strains (P<0.05)

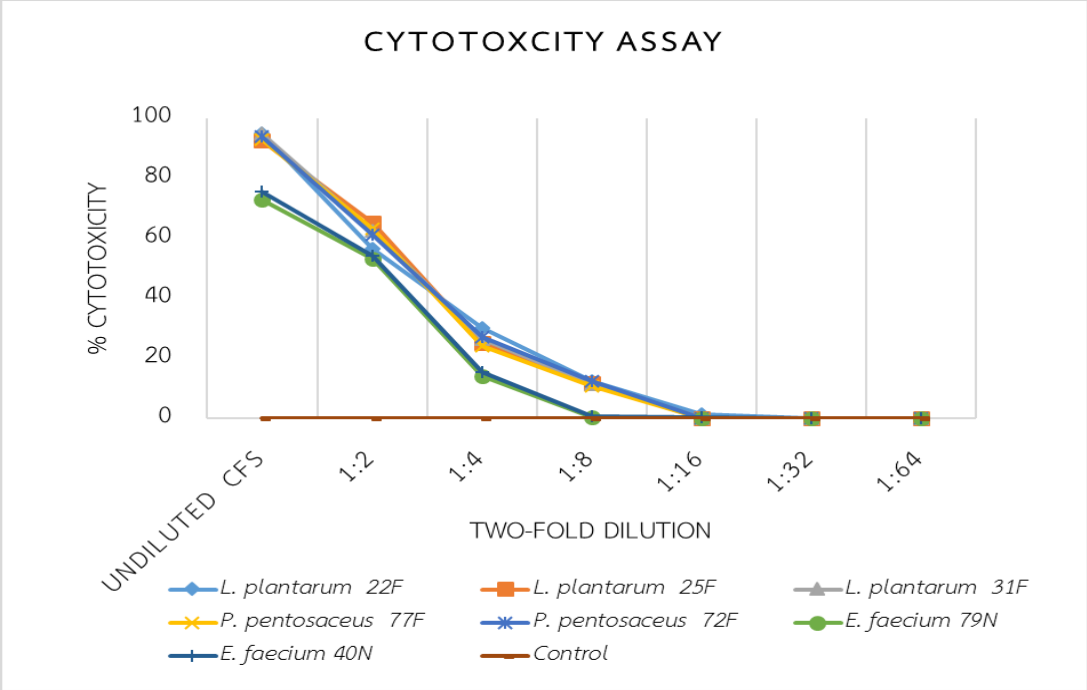

Figure 8 The cytotoxicity evaluation by neutral red assay on Vero cell monolayers after exposure to undiluted and serially two-fold dilutions (1:2 to 1:64) of CFS after day 4 of incubation

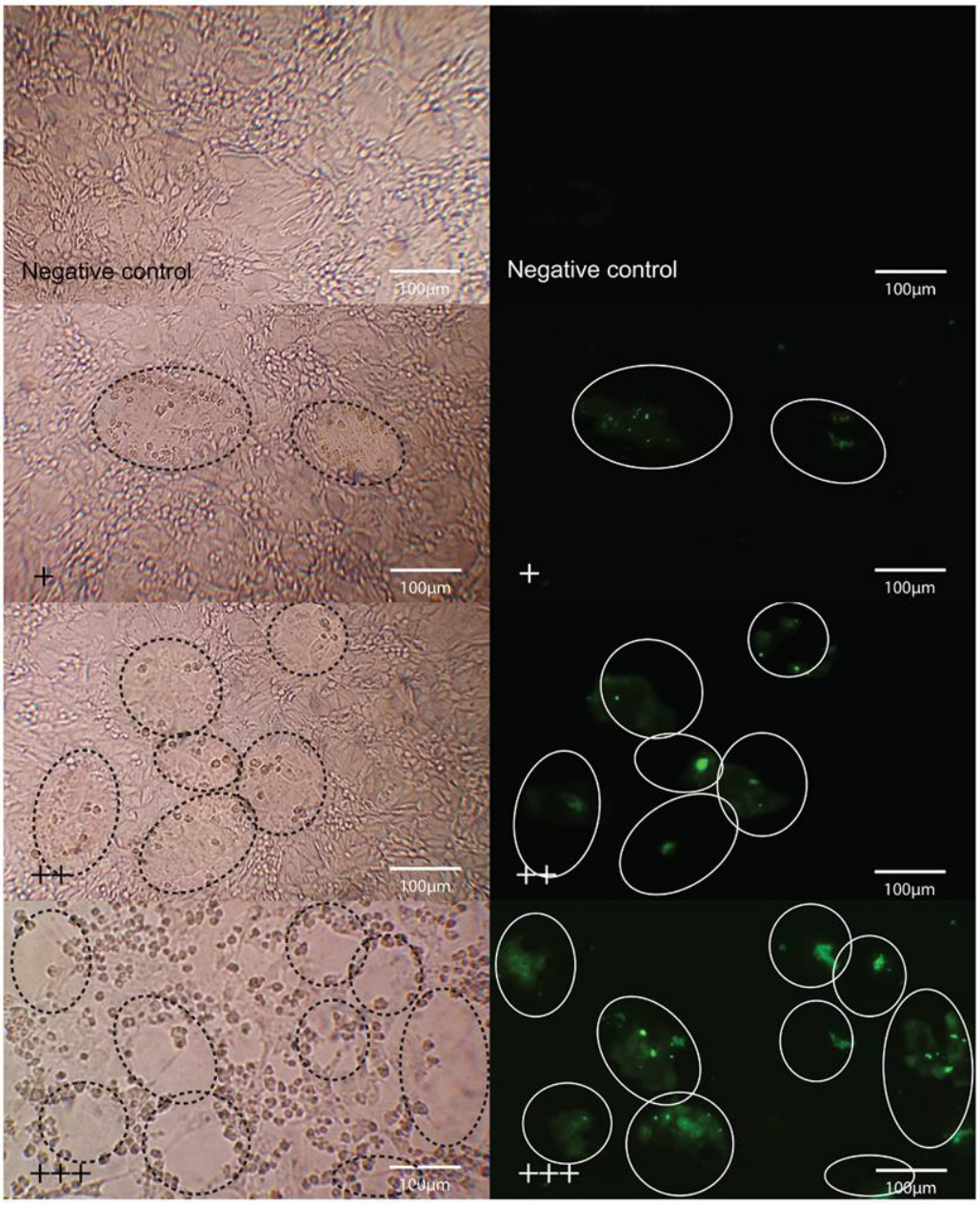

Figure 9 Antiviral activity of LAB against pandemic strain of PEDV on infected Vero cells. The CPE reduction was observed under a microscope at 100X. (a) no infection; (b) to (d) shows the CPE scores as; + (<50% of observed field area), ++ (50-75% of observed field area) and +++ (>75% of observed field area). The reduction of the fluorescent signals of the infected cells were observed under a fluorescent microscope as: (e) no infection; (f) to (g) shows the reduction signals scored as; + (<50% of observed field area), ++ (50-75% of observed field area) and +++ (>75% of observed field area). The circles indicate the infectivity area

3.5 Discussion

In this study, we observed the antiviral effects of CFS and live cells from seven local LAB strains against the pandemic strain of PEDV, *in vitro*. All LAB strains were well-characterized on the basis of probiotic properties including acid-bile tolerance, thermos-tolerance without a potential of forbidden antimicrobial resistant profile (Table 7). The pandemic strain of PEDV used in this study was propagated in Vero cell model. The viral strain was isolated from diseased pigs showing high morbidity and mortality; therefore, it would represent the true problematic issue in the field.

The low pH (pH 3.5 to 4.5) of LAB CFS (Fayol-Messaoudi et al., 2005) may directly impair the morphology of Vero cells. Our finding confirmed that at 1:16 dilution of CFS of all lactobacilli could reduce the CPE without cytotoxicity towards the Vero cells, but not with the higher dilutions. This might relate to the lower level of antiviral substances such as NO^-, hydrogen peroxide, fatty acid, lactic acid and acetic acid, in higher dilutions (Botić et al., 2007; Dembinski et al., 2014; Ermolenko et al., 2009; Maragkoudakis et al., 2010; Saarela et al., 2000). Furthermore, there were no antiviral effects observed from CFS adjusted to pH 7. Therefore, we assume that the inhibition of viral infectivity presented in this study might not be derived from bacteriocins, since it has been proven to also function at physiological pH (S.-C. Yang et al., 2014). Although, the accurate mechanisms of the antiviral effects from LAB CFS are still unclear, several studies have suggested possible explanations. Firstly, the acidity of CFS might help denaturing the viral capsid proteins and preventing them from cell attachment (Aboubakr et al., 2014). However, this might not apply for our study since we could not use the original pH of LAB CFS due to its toxicity towards the Vero cells. The more possible mechanisms maybe the hindering and blocking of viral adsorption into the cells by CFS metabolites (Allayeh et al., 2015). Therefore, the antiviral substances within CFS and their protective mechanisms will be investigated in further study.

The direct protective effect of live LAB cells to PEDV on Vero cells represented a possible scenario of probiotic feed supplements at the time of viral infection, without any cytotoxicity to normal Vero cell morphology. *L. plantarum* strain 25F showed the

most antiviral efficacy by reduction of CPE on Vero infected cells. The minimum viable LAB concentration required to observe antiviral effect at 10^6 CFU/mL was in agreement with previous studies against rotavirus and gastroenteritis coronavirus, while the strongest effect was shown at 10^8 CFU/mL (Aboubakr et al., 2014; Botić et al., 2007; Charteris et al., 1998; Y. Lee et al., 2000; Maragkoudakis et al., 2010). The decrease of viral infectivity in co-incubation assay could be explained by the competition for attachment to cell receptors between the bacterial cells and virus; the interference of viral attachment and cell entry, nonspecific or specific virus trapping; and the "cross-talk" signaling between LAB and the host cells which may alter the epithelial cells leading to antiviral responses (Aboubakr et al., 2014; Botić et al., 2007).

In conclusion, the CFS and live LAB in this study showed protective effects against the pandemic strain of PEDV in strain-specific manner. CFS of all tested lactobacilli could reduce viral infectivity in Vero cells, whereas other species lacked that ability. Live cells of *L. plantarum* strain 25F provided the greatest antiviral effects on reduction of CPE from the pandemic strain of PEDV. The preliminary study offered important findings for further studies for the extraction of antiviral compounds and the antiviral mechanisms of LAB with the virus and host.

CHAPTER IV

Probiotic Properties and Antibacterial Activity Against Enteric Pathogenic Bacteria of Autochthonous Lactic Acid Bacteria Isolated from Pig Feces in Thailand

Wandee Sirichokchatchawan[1], Puwiya Pupa[1], Prasert Praechansri[1], Nutthee Am-in[1], Somboon Tanasupawat[2], Piengchan Sonthayanon[3], and Nuvee Prapasarakul[1]*

Running Title: Probiotic Properties of Autochthonous LAB from Pig feces

[1]Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.
[2]Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand.
[3]Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

***Corresponding author:**
Nuvee Prapasarakul
Tel.: +662-218-958
Fax: +662-251-1656
E-mail: Nuvee.P@chula.ac.th

Status: During submission process

4.1 Abstract

Lactic acid bacteria (LAB) are often applied as probiotics for both human and animals. In general, probiotic supplements can benefit, protect and enhance recovery in individuals against enteric pathogens. Five autochthonous LAB, (*Lactobacillus plantarum* 22F, 25F and 31F, *Pediococcus acidilactici* 72N and *Pediococcus pentosaceus* 77F) from pig feces as potential probiotics were evaluated for their functional and safety aspects. *Lactobacillus plantarum* 22F and 25F showed better abilities in tolerance to low pH, simulated gastric, cell surface properties (hydrophobicity, auto- and co-aggregation), and antibacterial activity against the enteric pathogenic bacteria in pigs (such as pathogenic *Escherichia coli, Salmonella Choleraesuis* and *Streptococcus suis*). Even though, *Lactobacillus plantarum* 31F showed strong resistance to lysozyme, it was very susceptible towards 0.4% phenol. All five LAB strains showed production of diacetyl. Only *Pediococcus pentosaceus* 77F exhibited α-hemolytic and bile salts hydrolase activity; and was not recommended as probiotics. From the studied parameters, *Lactobacillus plantarum* 22F were selected as the most promising probiotic candidate by the analysis of a principal component analysis (PCA) demonstrated better abilities in tolerance of simulated gastrointestinal tract, cell surface properties, and antibacterial activity by cell-free supernatant without hemolytic and BSH activities. These results are useful for further development of the strains as feed additives for pig rearing.

4.2 Introduction

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” by FAO/WHO (Reid, 2016). “Probiotic” is often misused for beneficial commensal microbes; however, not until the bacterial strains are isolated, characterized, identified and fulfill requirements were then termed probiotics (Casarotti et al., 2017; Sanders, 2008). Nevertheless, these microbes especially the members of lactic acid bacteria (LAB) are the major source of probiotics. The important criteria in selection of probiotics includes the functional and safety aspects as these bacteria must survive, colonize, and exert health benefit without potential harm towards the host (Devi et al., 2015); therefore, the *in vitro* study is principally used to verify the probiotics properties such as resistance to gastric acid, bile, lysozyme and phenol compound, cell surface properties, antimicrobial activity, bile salt hydrolase activity, and blood hemolysis (Ren et al., 2014).

Because of their safety use and ability to prevent disease, benefit the host, and lessen the antimicrobial uses in feed additives, probiotics have gained rapid expansion of interest and knowledge in animal nutrition. Although, there are many commercial probiotic products available on the markets for veterinary practices and food animal; certain products have been proved to be inaccurate in the product’s labels, strain identity, spelling of the contents, and shelf-life, and insufficient viable number of microorganisms. In addition, most of the commercial strains were not well-characterized and not of the species-specificity (derived from the same species) as stated in safety criteria (Belicová et al., 2013; Sanders, 2008; WeeseandMartin, 2011). Even though, there have been many researches published on probiotics, the finding of new strains with promising probiotic properties are always desirable as probiotics are strain-specific possessing different beneficial properties and actions (Casarotti et al., 2017).

From the mentioned knowledge, the well identified five autochthonous LAB strains isolated from healthy pig feces in Thailand were previously selected following European Food Safety Authority (EFSA) criteria on antimicrobial susceptibility (EFSA, 2012) with antiviral activity against porcine epidemic diarrhea virus (PEDV)

(Sirichokchatchawan et al., 2017). To complete strain specific properties for probiotic use, the autochthonous LAB strains were *in vitro* evaluated for functional and safety properties, and antibacterial activity against important enteric pathogens in pigs to select the most promising strains as probiotics.

4.3 Materials and methods

4.3.1 Strains and culture conditions

Lactobacillus plantarum (L. plantarum) (22F, 25F and 31F), *Pediococcus pentosaceus (P. pentosaceus)* 77F, and *Pedicoccus acidilactici (P. acidilactici)* 72N used were isolated from feces of antibiotic-free pigs in Thailand based on EFSA antimicrobial susceptibility criteria, with antiviral activity against PEDV, and were proposed as probiotic candidates in a previous study (Sirichokchatchawan et al., 2017). *Lactobacillus plantarum* JCM1149, a commercial type strain with probiotic properties (Zago et al., 2011) and *Pediococcus acilactici* DSM20284 were used as reference strains. The strains were stored in MRS broth (Oxoid, Basingstoke, England) supplemented with 15% v/v glycerol at -80 °C. Five strains of *Enterotoxigenic Escherichia coli* (ETEC), five strains of *Enterohemorrhagic Escherichia coli* (EHEC), five strains of *Salmonella Choleraesuis (S. Choleraesuis), Salmonella Typhimurium (S. Typhimurium)* ATCC13311, *Escherichia coli (E. coli)* ATCC25922, *Staphylococcus aureus (S. aureus)* ATCC25923 were used for co-aggregation with enteric pathogen and antibacterial activity assays. These strains were periodically reactivated in tryptic soy broth (TSB) (Sigma-Aldrich, Munich, Germany), and stored in TSB supplemented with 15% v/v glycerol at -80 °C.

4.3.2 Evaluation of functional probiotic properties

4.3.2.1 Resistance to lysozyme

Overnight LAB cultures were harvested, washed twice in PBS and resuspended in Ringer solution (8.5 g/L NaCl, 0.4 g/L KCl, 0.34 g/L hydrated $CaCl_2$). The bacterial suspensions (10^8 CFU/mL) were inoculated in simulated saliva [sterile electrolyte solution (SES: 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) supplemented

with 100 mg/L of lysozyme (Sigma-Aldrich, Sydney, Australia)]. Survival percentage of CFU/mL was calculated after 30 and 120 min of incubation compared with the CFU/mL at 0 min. Assays were performed in triplicate (García-Ruiz et al., 2014).

4.3.2.2 Resistance to 0.4% phenol

Two percent of overnight LAB cultures (10^8 CFU/mL) were inoculated in 10 ml MRS broth (Oxoid, Wesel, Germany) with or without the presence of 0.4% phenol, and incubated at 37 °C for 24 h. Survival number of bacteria (CFU/mL) were determined at time 0 and 24 h. Survival rate (%) were calculated as: (viable cell count at 24 h / viable cell counts at 0 h) x 100 (Xanthopoulos et al., 2000).

4.3.2.3 Survival of LAB under low pH and simulated gastric juice

Overnight LAB cultures were harvested, washed and resuspened in sterile saline (0.85% NaCl, w/v). The bacterial suspensions (10^8 CFU/mL) was mixed in either simulated gastric juice [2X SES supplemented with 0.6% (w/v) porcine pepsin (Sigma-Aldrich, Sydney, Australia)] or MRS adjusted to pH 2.0 and pH 3.0 using 1M HCl. Bacterial suspensions were incubated at 37 °C with agitation, and collected for cell counts in MRS agar at 0, 1, 2, and 3 h. Assays were performed in triplicate (García-Ruiz et al., 2014; Z. Guo et al., 2009). Resistance percentage were calculated followed Feng et al. (Feng et al., 2017).

4.3.2.4 Survival of LAB in different concentration of bile

One mL of the LAB suspensions (10^8 CFU/mL) was inoculated into 9 mL of MRS broth supplemented with either 0.3%, 0.5% or 1% (w/v) bile salts (oxgall, Sigma-Aldrich, Sydney, Australia). The suspensions were further incubated at 37 °C for 24 h under anaerobic atmosphere. Assay was performed in triplicate trials (Ren et al., 2014). Resistance percentage were calculated followed Feng et al. (Feng et al., 2017).

4.3.2.5 Cell surface properties

4.3.2.5.1 Cell surface hydrophobicity

Microbial adhesion to hydrocarbons (MATH) was used to evaluate cell surface hydrophobicity. Overnight LAB cultures were harvested, washed twice with PBS, and resuspended in PBS to optical density (OD) 0.6 at 600 nm. 1 ml of either xylene and toluene was added to 3 mL of bacterial suspensions, and mixed for 90 s by vortex and incubated for 30 min. In two phase systems, the aqueous phase was collected to measure OD at 600 nm. The assay was performed in triplicate. The surface hydrophobicity was calculated into percentage by: $H\% = [(OD_{600\ \text{before mixing}} - OD_{600\ \text{after mixing}}) / (OD_{600\ \text{before mixing}})] \times 100$ (Ekmekci et al., 2009).

4.3.2.5.2 Auto-aggregation

The overnight LAB cultures were harvested, washed twice, and resuspended in PBS to an OD of 0.6 at 600 nm ($A_{0\ h}$). 3 ml of each bacterial suspension was vortexed at least 10 s and incubated at 37 °C. 1 ml of the supernatant was measured for the absorbance at 600 nm ($A_{\text{final h}}$) at 1 h, 2h, 3h, and 4h, serially. The assay was performed in triplicate. The auto-aggregation (%) was calculated as followed: $(1 - A_{\text{final h}} / A_{0\ h}) \times 100$ (Xu et al., 2009).

4.3.2.5.3 Co-aggregation

The overnight LAB cultures and pathogenic strains were harvested, washed twice, and resuspended in PBS to an OD of 0.6 at 600 nm. Equal volumes (2 mL) of LAB strain and pathogenic strain were mixed and vortexed for 10 s. After incubation at 37 °C for 4h, the supernatants were measured for the absorbance at 600 nm. The assay was performed in triplicate. The co-aggregation (%) was calculated as followed: $100 \times [(OD_{LAB} + OD_{pathogen}) - 2(OD_{mix})] / (OD_{LAB} + OD_{pathogen})$ (Ekmekci et al., 2009).

4.3.2.6 Screening for antibacterial activity

4.3.2.6.1 Antibacterial activity by CFS

Agar well diffusion assay was used to evaluate the antibacterial activity as described by Lin et al. (Lin et al., 2006). The cell-free supernatants of overnight LAB

cultures were tested against indicator pathogenic strains including *E. coli* ATCC25922, *S. aureus* ATCC25923, five strains of ETEC, five strains of EHEC, five strains of *S. Choleraesuis*, *S. Typhimurium* ATCC13311, *S. suis* type II. The diameters of inhibition zone were measured and interpreted as (+) weak inhibition (6 – 9 mm), (++) intermediate inhibition (10 – 13 mm), (+++) strong inhibition (14 – 16 mm) and (++++) very strong inhibition (> 17 mm).

4.3.2.6.2 Antibacterial activity by live cells

A single colony of each LAB strain was cultured in MRS broth to an OD of 0.2 at 600 nm, and 50 µl of each culture strain was spread in a 2 cm stripe across the MRS agar plate. The plates were incubated anaerobic atmosphere at 37 °C for 24 h. After overnight incubation, the plates were overlaid with 10 mL TSA agar. Overnight cultures of indicator pathogenic strains (one strain of ETEC, EHEC, *S. Choleraesuis*, *S. Typhimurium* ATCC13311, *S. suis* type II, *E. coli* ATCC25922, and *S. aureus* ATCC25923) were resuspended in sterile saline solution to 10^8 CFU/ml. Bacterial suspensions were streaked over the overlaid TSA surface by cotton swab, and incubated in aerobic atmosphere at 37 °C for 24 h. The antibacterial activity was examined as absent (-), weak (+), moderate (++), or strong (+++) growth inhibition which modified according to the inhibition zone (Presti et al., 2015).

4.3.2.6.3 Screening for diacetyl production

Overnight cultures of LAB strains were collected, and resuspened in peptone water to 10^8 CFU/mL. The bacterial suspensions (1% w/v) were inoculated in 10 mL whole UHT milk. After 24 h of incubation at 30 °C, 1 mL of the suspensions was added to 500 µL of **α**- naphthol solution (1% w/v) and KOH (16% w/v), and incubated for 10 min at 37 °C. Red ring formation was an indicator for diacetyl production (de Almeida Júnior et al., 2015).

4.3.3 Evaluation of safety probiotic properties

4.3.3.1 Screening for bile salt hydrolase activity

Bile salt hydrolase (BSH) activity of LAB strains was screened using sterile paper discs (6 mm diameter) method. Overnight LAB cultures were spotted on MRS agar supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA) (Sigma-Aldrich, Sydney, Australia) and 0.37 g/L $CaCl_2$. The colonies with precipitated bile acid presented as opaque halo were considered BSH-positive (X.-H. Guo et al., 2010).

4.3.3.2 Blood hemolytic activity

Overnight LAB cultures were streaked on the surface of tryptic soy agar (TSA) (Sigma-Aldrich, Munich, Germany) supplemented with 5% (w/v) sheep blood. After 48 h of incubation at 37 °C, the plates were examined for hemolytic reaction with *S. aureus* ATCC25923 as positive control for blood hemolysis (Adimpong et al., 2012).

4.3.4 Statistical analysis

The parameters in this study were presented as mean ± SD. All probiotic properties were compared among studied LAB strains using ANOVA. Repeated measurement ANOVA was used to compare the bile tolerance (0.3%, 0.5% and 1%), the survival rate in simulated gastric juice at pH 2 and 3 (at 1, 2 and 3h), and auto-aggregation (1, 2, 3 and 4h) of each LAB strains. Paired t-test was used to compare hydrophobicity (toluene and xylene), lysozyme tolerance (30 and 120 min), acid tolerance (pH2 and 3) and survival rate in simulated gastric juice at pH2 and 3. All above statistical analysis were performed by SAS® 9.3 (Cary, NC, USA) and limited significant difference at $p<0.05$. The quantitative descriptive analysis of functional and safety probiotic properties was performed by Principal Component Analysis (PCA) conducted with XLSTAT™ software (Addinsoft, Paris, France) (Angmo et al., 2016).

4.4 Results

4.4.1 Lysozyme and phenol tolerance

The resistance to lysozyme (100 mg/L) of studied LAB strains at 30 and 120 min were expressed as survival percentage (Table 9). At 30 min, five autochthonous LAB strains showed high lysozyme resistance (> 80% survival) with *P. pentosaceus* 77F and *L. plantarum* 31F possessed the most outstanding mean values of survival rate at 97.5% and 96.8%, respectively, compared with *L. plantarum* JCM1149 ($p<0.05$). At 120 min, all LAB strains demonstrated reduction of lysozyme tolerance with significant difference ($p<0.05$). Only *L. plantarum* 31F remained strong resistance (88.6%) towards lysozyme compared with all studied LAB included the reference strains ($p<0.05$).

All studied LAB showed good resistant towards 0.4% phenol after 24 h of incubation. Four autochthonous strains (*L. plantarum* 25F, *L. plantarum* 22F, *P. pentosaceus* 77F, *P. acidilactici* 72N) and *P. acidilactici* DSM20284 showed strong resistant with high survival percentages at 96.3%, 93.8%, 93.2, 95.4% and 95.3%, respectively. However, *L. plantarum* 31F and *L. plantarum* JCM1149 were more sensitive towards 0.4% phenol with survival rate at 86.8% and 83%, respectively ($p<0.05$) (Table 9).

Table 9 Survival ability of the LAB strains in the effects of simulated lysozyme and phenol compound

Isolate	Survival rate (%)		
	Lysozyme (100 mg/L)		0.4% Phenol
	t30 min	t120 min	24 h
L. plantarum 22F	90.4 ± 0.48[a,1]	79.6 ± 4.8[a,2]	93.8 ± 1[a]
L. plantarum 25F	83.3 ± 1.48[b,1]	72.8 ± 3.31[a,2]	96.3 ± 0.33[a]
L. plantarum 31F	96.8 ± 1.7[c,1]	88.6 ± 1.71[b,2]	83 ± 0.08[b]
P. acidilactici 72N	88.2 ± 1.03[d,1]	76.8 ± 0.93[a,2]	95.4 ± 0.06[a]
P. pentosaceus 77F	97.5 ± 1.64[c,1]	84.4 ± 2.63[c,2]	93.2 ± 0.37[a]
L. plantarum JCM1149	68.7 ± 0.79[e,1]	75.4 ± 6.54[a,2]	86.8 ± 0.3[b]
P. acidilactici DSM20284	87.7 ± 4.13[d,1]	80 ± 4 [ac,2]	95.3 ± 0.57[a]

[a, b, c, d, e] within a column indicated significant difference between strains ($p<0.05$)

[1, 2] within a row indicated significant difference between time ($p<0.05$)

4.4.2 Survival under low pH, simulated gastric juice and different bile concentrations

The survival percentage of studied LAB strains to pH 2 and 3 in MRS and simulated gastric juice are presented in Table 10. After 3 h of incubation in MRS adjusted to pH 2 and 3, the five autochthonous LAB strains showed high tolerance to both pH values when compared with the reference strains ($p<0.05$), with *L. plantarum* 22F demonstrated the highest survival percentage at 91.65% and 96.32, respectively. For simulated gastric juice, *L. plantarum* 25F exhibited the highest survival rate in pH 2 and 3 after 3h of incubation at 75.64% and 90.71%, respectively, with significant difference when compared with the reference strains ($p<0.05$). There was a significant difference ($p<0.05$) in viability of the LAB strains towards acid tolerance (in MRS) compared with the simulated gastric juice (Table 10).

The differences of bile concentration at 0.3% and 0.5% had no significant influence on the survival rate of LAB strains as shown in Table 10. The survival rate of the LAB strains was unaffected at 0.3% and 0.5%, with *L. plantarum* 25F demonstrated a better tolerance at 86.03% and 85.96%, respectively. Whereas, at 1% of bile, most of the LAB strains showed progressive decline in viability; except for *L. plantarum* 22F and 31F ($p<0.05$). *L. plantarum* 22F, 25F and 31F exhibited the highest survival percentage at 78.88%, 79.19% and 80.94%, respectively ($p<0.05$).

Table 10 Tolerance ability of the LAB strains in the effects of low pH, simulated gastric juice and different concentrations of bile

Isolate	Survival rate (%)										
	Acid tolerance (3 h)		Simulated gastric juice pH2			Simulated gastric juice pH3			Bile tolerance		
	pH2	pH3	1h	2h	3h	1h	2h	3h	0.3%	0.5%	1%
L. plantarum 22F	$91.65\pm2.74^{a,1,*}$	$96.32\pm2.81^{a,2,*}$	$88.24\pm1.22^{a,1}$	$84\pm1.5^{a,2}$	$71.84\pm2.31^{a,3,**}$	$95.5\pm0.52^{a,1}$	$90.47\pm0.97^{a,2}$	$81.28\pm2.08^{a,3,**}$	$82\pm3.01^{a,1}$	$80\pm4.25^{a,1}$	$78.88\pm1.66^{a,1}$
L. plantarum 25F	$89.11\pm2.64^{a,1,*}$	$94.48\pm0.93^{a,2,*}$	$85.89\pm1.67^{b,1}$	$80.39\pm0.23^{b,2}$	$75.64\pm2.64^{b,3,**}$	$95.9\pm1.71^{a,1}$	$94.2\pm1.92^{b,2}$	$90.71\pm1.17^{b,3,**}$	$86.03\pm0.86^{b,1}$	$85.96\pm3.01^{a,1}$	$79.19\pm2.89^{a,2}$
L. plantarum 31F	$84.59\pm2.39^{b,1,*}$	$95.37\pm2.21^{a,2,*}$	$80.76\pm1.12^{c,1}$	$75.02\pm2.24^{c,2}$	$73.02\pm2.08^{ab,2,**}$	$94.23\pm0.83^{a,1}$	$92.37\pm0.53^{c,2}$	$85.82\pm2.59^{c,3,**}$	$84.17\pm2.46^{ab,1}$	$82.14\pm1.45^{a,1}$	$80.94\pm2.51^{a,1}$
P. acidilactici 72N	$86.45\pm1.93^{ab,1,*}$	$91.97\pm0.75^{b,2,*}$	$85.40\pm2.71^{b,1}$	$75.7\pm1.7^{c,2}$	$71.12\pm1.72^{a,3,**}$	$92.44\pm0.07^{b,1}$	$88.86\pm1.67^{d,2}$	$81.21\pm1.27^{a,3,**}$	$83.94\pm5.29^{a,1}$	$79.59\pm2.67^{a,1}$	$74.22\pm2.89^{b,2}$
P. pentosaceus 77F	$87.8\pm3.49^{ab,1,*}$	$93.31\pm1.93^{ab,2,*}$	$84.61\pm1.96^{b,1}$	$74.46\pm4.58^{c,2}$	$73.87\pm4.72^{ab,2,*}$	$90.4\pm0.07^{b,1}$	$86.61\pm1.67^{d,2}$	$80.02\pm1.27^{a,3,**}$	$83.74\pm1.93^{a,1}$	$80.48\pm2.17^{a,1}$	$75.54\pm3.08^{b,2}$
L. plantarum JCM1149	$69.72\pm5.26^{c,1,*}$	$88.74\pm1.55^{c,2,*}$	$81\pm0.01^{bc,1}$	$72.67\pm0.85^{cd,2}$	$66.2\pm2.45^{c,3,*}$	$90.79\pm2.64^{b,1}$	$90.25\pm2.68^{cd,2}$	$82.7\pm2.29^{a,3,**}$	$79.69\pm2.57^{c,1}$	$78.46\pm0.76^{a,1}$	$69.97\pm0.49^{c,2}$
P. acidilactici DSM20284	$68.95\pm3.05^{c,1,*}$	$86.45\pm0.50^{c,2,*}$	$84.51\pm1.81^{b,1}$	$70.99\pm0.38^{d,2}$	$63.17\pm1.59^{c,3,*}$	$90.86\pm3.16^{b,1}$	$89.26\pm8.11^{cd,2}$	$78.39\pm1.57^{a,3,**}$	$80.78\pm1.33^{c,1}$	$79.9\pm3.09^{a,1}$	$69.76\pm2.1^{c,2}$

[a, b, c, d] within a column indicated significant difference between strains ($p<0.05$)

[1, 2, 3] within a row indicated significant difference ($p<0.05$) when compared in each variable (acid tolerance, simulated gastric juice pH2 and 3, and bile tolerance)

*,** within a row indicated significant difference ($p<0.05$) when compared with the survival rate among acid tolerance pH 2 and 3 and the simulated gastric juice pH 2 and 3

4.4.3 Cell surface properties

The cell surface hydrophobicity of the LAB strains was not significantly different ($p<0.05$) between xylene and toluene; except *P. acidilactici* 72N with better hydrophobicity percentage towards xylene (Figure 10A). The results of hydrophobicity percentages were greatly variable from 18% to 77%, depends on the bacterial strains. *L. plantarum* 22F and 25F noticeably exhibited the highest hydrophobicity percentage towards both xylene and toluene ($p<0.05$).

Based on the results of auto-aggregation from 1 h to 4 h demonstrated the improvement of aggregation ability over the experimental period. At 4 h, all of the strains showed the auto-aggregation percentage between 37% to 43%, with the highest percentage recovered from *L. plantarum* 22F and 25F (43.01% and 42.63%, respectively); while *L. plantarum* JCM1149 and *P. acidilactici* 72N and DSM20284 showed the least auto-aggregation ability (37.8%) with no significant difference between strains (Figure 10B).

Co-aggregation percentages were varied between each LAB strain and/with each indicator pathogenic strain from 15% to 32% as presented in Figure 10C. *L. plantarum* 25F showed the highest aggregation value towards EHEC, *S. Choleraesuis*, *S. Typhimurium* and *S. aureus* with 32.18%, 32.16%, 25.18% and 28.89%, respectively. Whereas, *P. pentosaceus* 77F and *L. plantarum* 22F exhibited the highest aggregation towards *E. coli* ATCC25922 and ETEC at 30.14% and 32.31%, respectively.

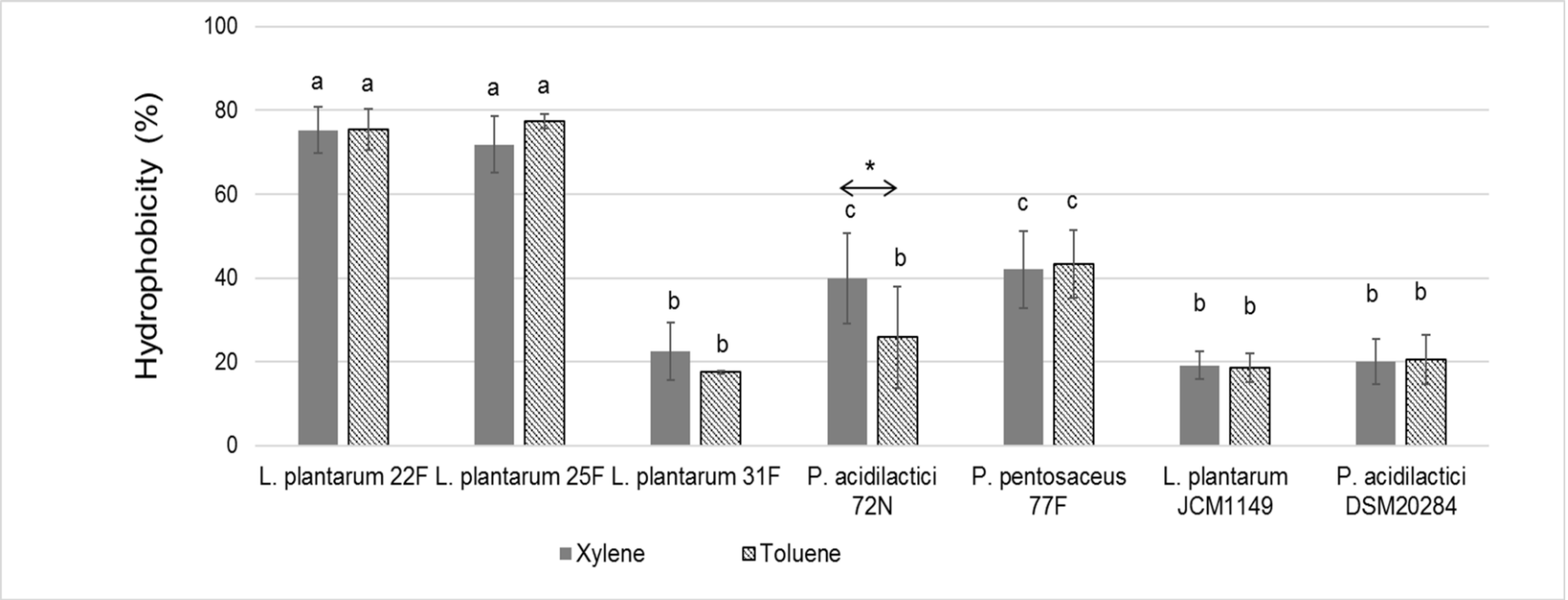

Figure 10A Hydrophobicity percentage of LAB strains towards tested hydrocarbons (xylene and toluene). The asterisk (*) shows significant difference of hydrophobicity % between tested hydrocarbons within the same strain. Error bars indicated ± SD value from three replications. The different lowercase letter with the similar color bars indicated significant difference ($p<0.05$).

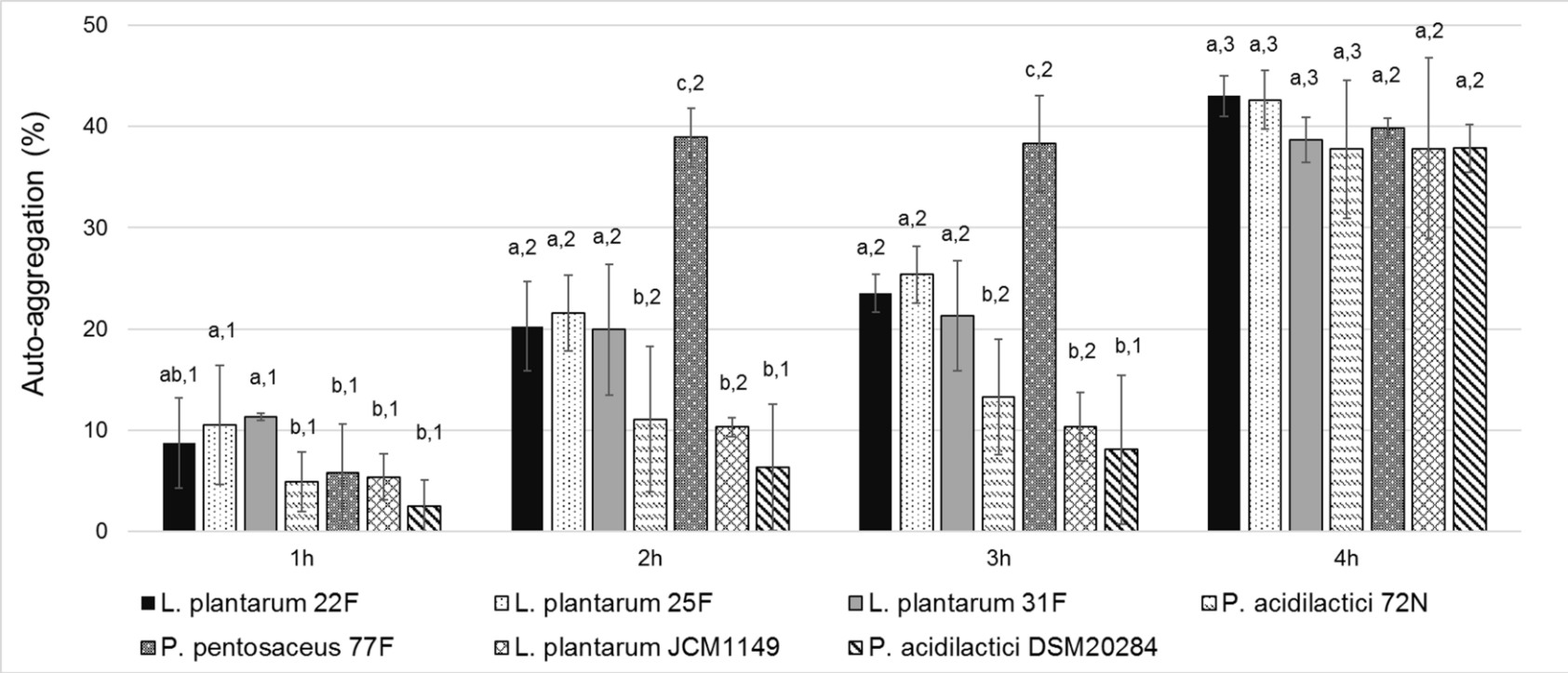

Figure 10B Auto-aggregation percentage of LAB strains after 1 to 4 h of incubation. Error bars indicated ± SD value from three replications. The different lowercase letter with the same time indicated significant difference (p<0.05) and the different lowercase number with the similar color bars differ (p<0.05)

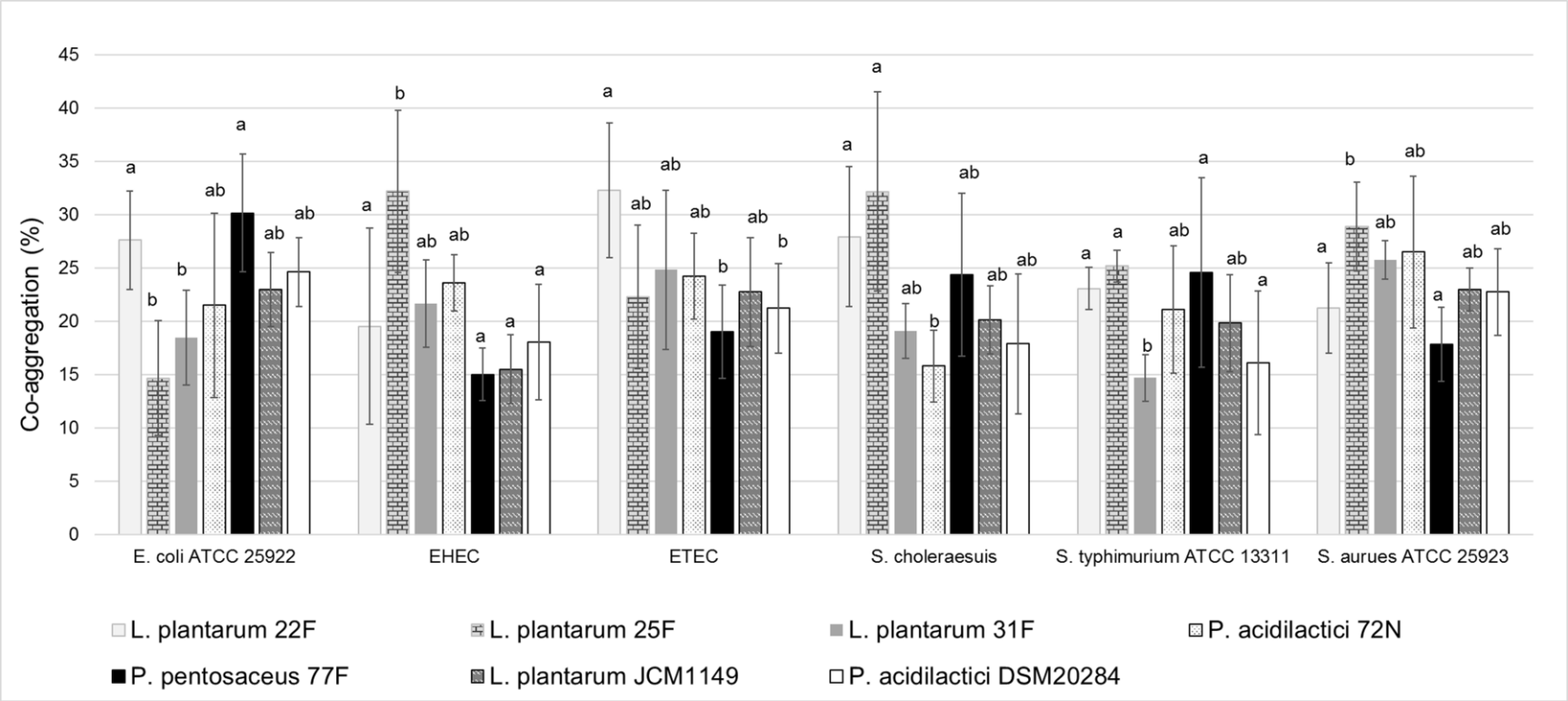

Figure 10C Co-aggregation percentage of LAB strains to indicator pathogenic bacteria after 4 h of co-incubation. Error bars indicated ± SD value from three replications. The different lowercase letter with the same pathogenic bacteria indicated significant difference (p<0.05)

4.4.4 Antibacterial activity against enteropathogenic bacteria

The antibacterial ability by CFS and live cells of the LAB strains against indicator pathogenic strains were shown in Table 11. The non-neutralized CFS of the reference strains only showed very strong inhibition against *S. Choleraesuis*; while intermediate inhibition was observed against other enteric pathogens. Interestingly, the non-neutralized CFS of all autochthonous strains demonstrated very strong inhibition against both *S. Choleraesuis* and *S. Typhimurium*. While, only *L. plantarum* 22F and 31F showed strong inhibition towards all indicator pathogenic *E. coli,* other LAB strains only exhibited intermediate inhibition. In contrast, the neutralized CFS (to pH 7) of all the LAB strains lost inhibitory effects towards all the tested indicator pathogens (data not shown).

To confirm the antibacterial activity of the LAB strains simulated *in vivo*, the live cells of LAB were overlaid by the pathogenic strains. The results showed that live cells of both reference strains were weakly inhibited EHEC, ETEC, and *S. suis* type II; whereas *L. plantarum* 25F exhibited the best antibacterial activity with strong inhibition towards all of the enteric pathogenic strains and *S. suis* type II (Table 11). While *L. plantarum* 22F showed moderate to strong inhibition against indicator pathogens, strong inhibition was not observed from *P. pentosaceus* 77F and *P. acidilactici* 72N.

Table 11 Antibacterial activity of cell-free supernatants (CFS) and live cells of LAB against enteric pathogenic bacteria in pig

Indicator strains	*L. plantarum* 22F		*L. plantarum* 25F		*L. plantarum* 31F		*P. pentosaceus* 77F		*P. acidilactici* 72N		*L.plantarum* JCM1149		*P. acidilactici* DSM20284	
	CFS*	Live cells	CFS*	Live cells	CFS*	Live cells	CFS*	Live cells	CFS*	Live cells	CFS*	Live cells	CFS*	Live cells
EHEC1 (T2R2-26-HB2)	++	+++	++	+++	+++	++	++	+	++	+	++	+	++	+
EHEC2 (T2R2-26-HB1)	+++	ND	++	ND	+++	ND	++	ND	+++	ND	++	ND	++	ND
EHEC3 (T2R2-14-26-HB1)	++	ND	++	ND	++	ND	++	ND	++	ND	++	ND	++	ND
EHEC4 (T1R2-8-HB3)	++	ND	++	ND	+++	ND	++	ND	++	ND	++	ND	++	ND
EHEC5 (T1R2-8-HB2)	+++	ND	++	ND	++	ND	++	ND	++	ND	++	ND	++	ND
ETEC1 (VP1Ltb+)	+++	ND	++	ND	+++	ND	++	ND	++	ND	++	ND	++	ND
ETEC2 (VP3Ltb+)	+++	ND	++	ND	+++	ND	++	ND	++	ND	++	ND	++	ND
ETEC3 (VP4Ltb+)	+++	ND	++	ND	+++	ND	++	ND	++	ND	++	ND	++	ND
ETEC4 (VP8Ltb+)	+++	ND	++	ND	+++	ND	++	ND	++	ND	++	ND	++	ND
ETEC5 (VP10Ltb+)	+++	++	++	+++	+++	+++	++	+	++	++	++	+	++	+
E.coli ATCC 25922	+++	++	++	+++	+++	++	++	++	++	+	++	++	++	++
S. aureus ATCC 25923	++	++	++	+++	++	++	+	++	++	+	++	+	++	+
S. Typhimurium ATCC 13311	++++	+++	+++	+++	+++	++	++	++	+++	+	+++	++	+++	+
S. Choleraesuis 86-1	++++	++	++++	+++	++++	++	++++	++	++++	+	++++	++	++++	+
S. Choleraesuis 88-1	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND
S. Choleraesuis 93-1	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND
S. Choleraesuis 97-1	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND
S. Choleraesuis 98-1	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND	++++	ND
S. suis type II	++	++	++	++	+++	++	++	+	++	+	++	+	++	+

* non-neutralized CFS; ND: not determine; The antibacterial activity by CFS was expressed as (+) weak inhibition (6 – 9 mm), (++) intermediate inhibition (10 – 13 mm), (+++) strong inhibition, (14 – 16 mm), and (++++) very strong inhibition (> 17 mm). The antibacterial activity by live cells expressed as absent (-), weak (+), moderate (++), or strong (+++) growth inhibition.

4.4.5 Screening for diacetyl production, and blood hemolytic and bile salt hydrolase activity

All studied LAB strains demonstrated the production of diacetyl compound (Table 12). Furthermore, *P. pentosaceus* 77F was the only strain exhibited BSH activity and partial (α hemolysis); while the *L. plantarum* 22F, 25F, 31F and JCM1149, and *P. acidilactici* 72N and DSM20284 showed no BSH activity and were γ-hemolysis (Table 12).

Table 12 Screening for diacetyl production, and BSH and blood hemolytic activity of the LAB strains

Isolate	Diacetyl production	BSH activity	Hemolytic activity
L. plantarum 22F	+	-	γ
L. plantarum 25F	+	-	γ
L. plantarum 31F	+	-	γ
P. acidilactici 72N	+	-	γ
P. pentosaceus 77F	+	+	α
L. plantarum JCM1149	+	-	γ
P. acidilactici DSM20284	+	-	γ

4.4.6 Principal component analysis (PCA)

The analysis of PCA presented low pH (pH2 and 3), simulated gastric juice pH2 at 3h, 1% bile, antibacterial activity against *S. Choleraesuis*, xylene, toluene, auto-aggregation, co-aggregation to ETEC and *S. Choleraesuis* were correlated to F1 (Table 13); while, 0.4% phenol, EHEC, ETEC, *E. coli, S. aureus, S. Typhimurium, S. suis* type II and co-aggregation to *S. Typhimurium* were correlated to F2 (Table 13). It suggested that variables correlated to F1 and F2 are contributing to the selection of the most suitable strains with regards to functional and safety properties as also shown in Figure 11A for the distribution of the variables plotted on the plane of the first two principal components. Figure 11**B** is the projection of LAB strains in two-dimensional space related to F1 and F2 loading factors (variables). It can be interpreted that LAB strains presented in quadrant I and IV (Figure 11B) were significantly correlated with the variables in Figure 11A, with *L. plantarum* 22F related to the positive values of variables in quadrant I and IV as shown in Figure 11B.

Table 13 Correlation of variables to the factors of the analysis of PCA on the basis of factor loading

Variables	F1	F2	F3	F4	F5
Ly (Lysozyme 100 mg/l)	0.012	0.241	0.133	**0.528**	0.013
Phenol (0.4%)	0.022	**0.536**	0.002	0.195	0.142
pH2 (in MRS at 3 h)	**0.704**	0.015	0.026	0.069	0.179
pH3 (in MRS at 3h)	**0.794**	0.028	0.021	0.112	0.030
GJ 2,3h (simulated gastric juice pH2 at 3 h)	**0.630**	0.035	0.007	0.246	0.042
GJ 3,3h (simulated gastric juice pH3 at 3 h)	0.403	0.000	**0.476**	0.028	0.074
1% (bile)	**0.743**	0.060	0.005	0.169	0.019
EHEC	0.048	**0.787**	0.045	0.023	0.096
ETEC	0.336	**0.482**	0.032	0.000	0.015
E. coli	0.379	**0.459**	0.006	0.095	0.061
S. aureus	0.000	**0.763**	0.000	0.197	0.020
S. Typhimurium	0.182	**0.478**	0.065	0.188	0.060
S. Choleraesuis	**0.477**	0.144	0.262	0.014	0.097
S. suis type II	0.282	**0.416**	0.020	0.193	0.088
Xylene (H%)	**0.781**	0.123	0.021	0.061	0.011
Toluene (H%)	**0.738**	0.168	0.023	0.045	0.009
A%	**0.830**	0.060	0.035	0.014	0.033
Co% E. coli	0.081	0.027	**0.888**	0.000	0.003
Co% EHEC	0.377	0.023	**0.536**	0.013	0.037
Co% ETEC	**0.336**	0.306	0.116	0.226	0.015
Co% *S. Choleraesuis*	**0.644**	0.183	0.003	0.001	0.159
Co% *S. Typhimurium*	0.287	**0.571**	0.044	0.007	0.001
Co% *S. aurues*	0.080	0.028	**0.825**	0.041	0.025

Values in bold correspond for each variable to the factor for which the squared cosine is the largest

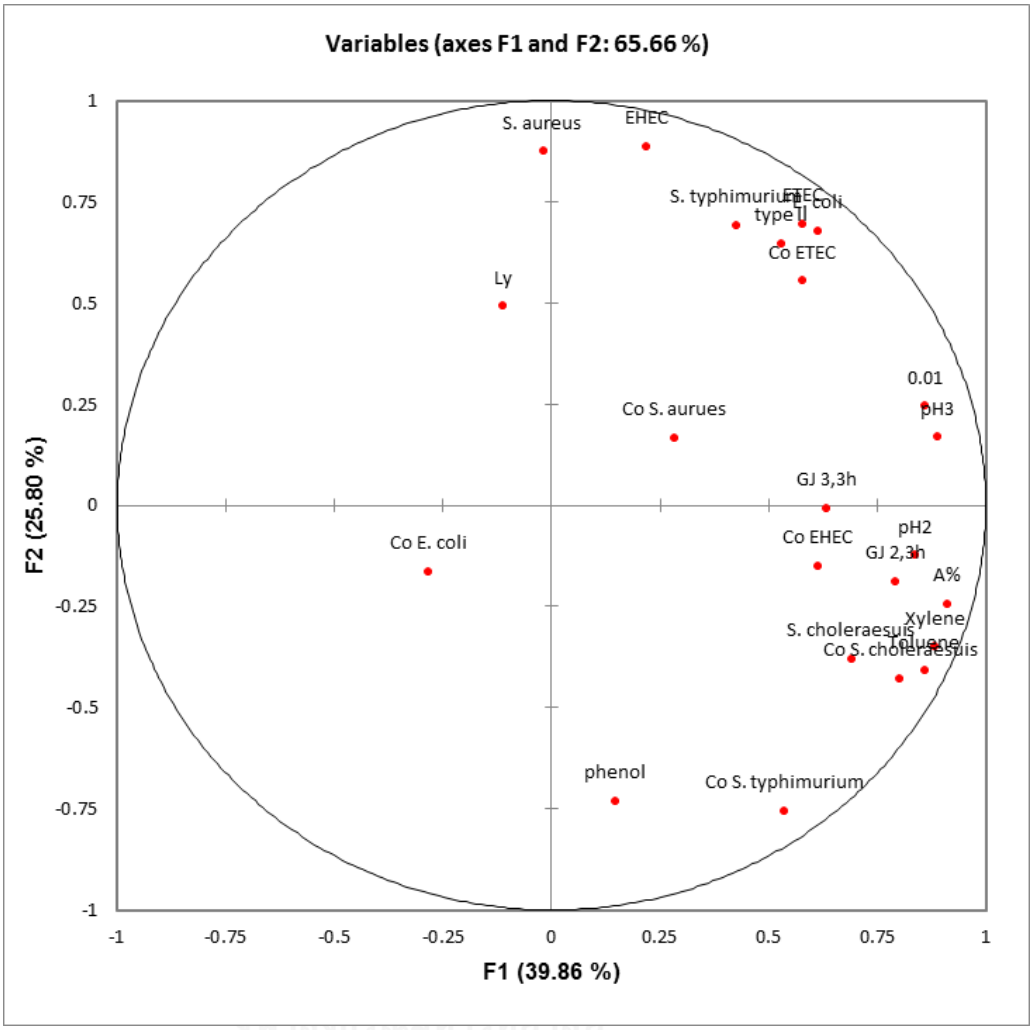

Figure 11A The analysis of Principal Component Analysis**.** Projection the chosen variables on the plane created by FC1 and FC2 after analyzed by PCA.

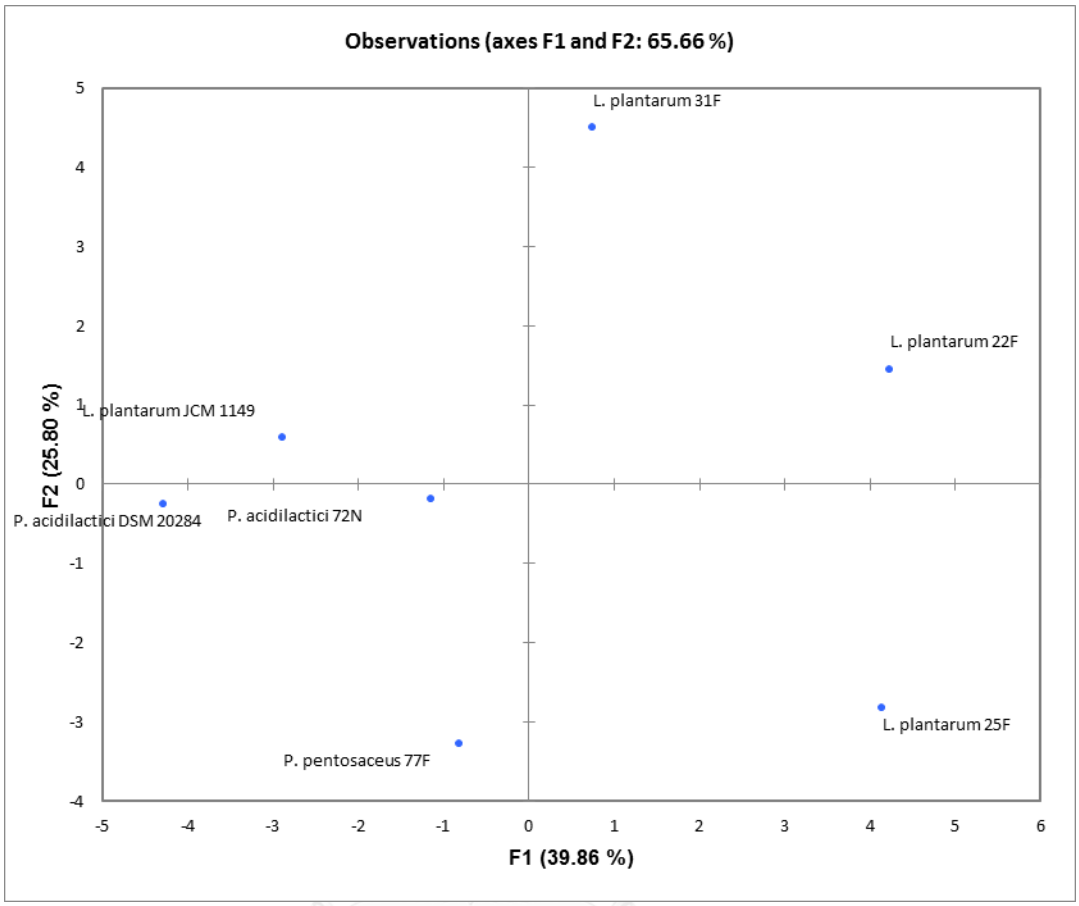

Figure 11B The analysis of Principal Component Analysis**.** Projection of the LAB strains in the observational plane.

4.5 Discussion

The autochthonous LAB strains isolated from healthy pig feces are the most suitable as probiotic for pigs in order to improve and protect the gastrointestinal environment since they are well adapted to pig's GI tract and safer than LAB from other sources (species-specificity criteria) (Ouwehand et al., 2002). In this study, the *in vitro* investigation was performed to assess probiotic properties according to evaluation guidelines for potential probiotics by FAO/WHO (FAO/WHO, 2002). Five autochthonous LAB strains were previously identified by 16S rDNA sequencing analysis and selected based on the MIC profile on antimicrobial susceptibility (EFSA, 2012) with antiviral activity against a pandemic strain of PEDV (Sirichokchatchawan et al., 2017), and compared with two reference strains, *L. plantarum* JCM1149 and *P. acidilactici* DSM20284. The results highlight that most of these strains showed the probiotic properties to develop as pig feed additive in the future.

The ability to withstand harsh environments is the prerequisite for probiotics, start from inside the oral cavity (saliva with lysozyme) to the present of low pH of gastric juice in the stomach and bile in the duodenum. The high resistance to lysozyme (100 mg/L) of *L. plantarum* 22F and 31F, and *P. pentosaceus* 77F demonstrated viability through simulated oral cavity (Zago et al., 2011). LAB must employ several mechanisms to survive acidic and bile environments, such as maintaining the functionality of cell membrane, intracellular pH, and maintain stability of DNA, RNA and proteins. However, these abilities vary within species and/or influenced by outside environments (Begley et al., 2006; Madureira et al., 2011; Wu et al., 2014). In our study, *L. plantarum* 22F and 25F showed good performance in tolerance to both acidic and bile conditions, as mentioned in previous studies that Lactobacillus could exhibited high tolerance in these conditions (Argyri et al., 2013; Jensen et al., 2012).

Cell surface properties can demonstrate the ability of bacterial strains for binding, colonization, biofilm formation, and adhesion (Kotzamanidis et al., 2010; Ren et al., 2014). *L. plantarum* 22F and 25F showed high percentages in hydrophobicity for both xylene and toluene, and auto-aggregation (along with good adhesion results on Vero cells showed in the previous study (Sirichokchatchawan et al., 2017). Strains with

high hydrophobicity also exhibited good auto-aggregation and adhesion ability (Ren et al., 2014). Furthermore, *L. plantarum* 22F and 25F showed good co-aggregation ability with indicator pathogenic *E. coli* and *Salmonella,* which indicated the ability to aggregate with pathogens leading to the lessen of possibility of pathogen adhesion to intestinal epithelial cells (Ferrando et al., 2016).

The antibacterial activity of LAB in this study was strongly confirmed by non-neutralized CFS and live cells especially the inhibition of indicator *Salmonella,* but the inhibitory effects could not be detected in any neutralized CFS. This might conclude that organic acids and low pH of the CFS were responsible for the observed antibacterial activity (Arena et al., 2016), and was not the effect of bacteriocins or diacetyl compound (Casarotti et al., 2017; Naidu et al., 1999; Ren et al., 2014).

Absence of hemolytic and BSH activities are essential safety properties for probiotic selection (FAO/WHO, 2002; Ruiz-Moyano et al., 2009). The results confirmed that all LAB strains were secured without BSH activity and α/β-hemolysis and suitable as probiotic candidates; except *P. pentosaceus 77F* that displayed α-hemolytic and BSH activities and would not recommended as probiotics. Even though BSH activity may increase the survival and persistence of the LAB strains by detoxification of bile salts, it could induce malabsorption in GI tract, and promotion of colon cancer. Moreover, LAB strains with no BSH activity were also able to tolerate bile salts, demonstrating that BSH and bile tolerant activity may not relate (Feng et al., 2017) Therefore, BSH activity is not desirable regarding safety criteria (Saarela et al., 2000; Vankerckhoven et al., 2008).

In conclusion, the multiple variables used in this study was analysis by PCA in order to help select the most outstanding strain with respect to studied probiotic properties. From the analysis of PCA, *L. plantarum* 22F is the most promising probiotic strain because it demonstrated both functional and safety properties included high tolerance in simulated GI tract and good cell surface properties without hemolytic and BSH activities, as well as strong inhibition from CFS against enteric pathogens. These findings could benefit the selection of LAB strains for further use involving production and manufacture processes to apply as feed additives suitable for pig of all ages.

CHAPTER V

GENERAL DISSCUSSION AND CONCLUSION

Based on the species-specificity criteria, probiotics should derive from the same host species (Ouwehand et al., 2002); therefore, the autochthonous LAB strains isolated healthy pigs are the most suitable as probiotics use for pig and safer to use than LAB from other sources. Moreover, it has been hypothesized that indigenous LAB from the same host species especially those originated in the same ecology were better- and well-adapted to the host's GI environment than LAB from other sources (Walter, 2008). In this dissertation, the selection of the most promising LAB strains as probiotics against enteric pathogenic bacteria and PEDV for pig feed additives was performed with three main purposes. The first purpose was to select LAB as potential probiotics with the ability to tolerate to pH2 and 0.3% bile salts as pre-screening criteria, and characterize and identify the selected LAB. The second purpose was to perform antimicrobial susceptibility of the previously selected LAB. The last purpose was to evaluate *in vitro* probiotic properties, and the antiviral and antimicrobial activity of the selected LAB against PEDV, pathogenic *E. coli,* and *Salmonella* compared to the commercial probiotic strain. Overall, significant efforts were made in selection of most suitable autochthonous lactic acid bacteria as probiotics from these fecal samples following the important criteria and requirements from European food safety authority (EFSA) and FAO/WHO on evaluation of probiotics (EFSA, 2012; FAO/WHO, 2002), to ensure their safety as probiotics for pigs and human consumption.

Since the source of LAB and acid/bile tolerace are the important criteria for probiotic selection (Tuomola et al., 2001), the fecal samples of antibiotic-free healthy Thai indigenous and commercial pigs were collected. The presumptive LAB isolates those tolerate pH 2 and 0.3% bile after 12 h of incubation with viability $\geq 10^4$ CFU/mL were isolated and pre-selected from the collected feces. These isolates were then

identified by phenotypic characterizations, whole-cell protein profiles, and 16S rDNA sequencing analysis. We found 34 acid/bile tolerance isolates (from 204 isolates) belonged to *E. faecium* (11 isolates), *E. hirae* (9 isolates), *L. plantarum* (4 isolates), *L. agilis* (3 isolates), *P. pentosaceus* (6 isolates), and *P. acidilactici* (1 isolate), which are commonly found LAB species in pig GI tract (Leser et al., 2002; Looft et al., 2014). The 16S rDNA sequencing analysis and protein pattern analysis could identify LAB to species level. Meanwhile, the analysis of biochemical profiles gave different results from the other two methods. This might be because LAB show great variety in ability to ferment different types of sugars within the same species (Khedid et al., 2009). Nevertheless, we found that LAB species identified in this study could be classified into genus or possible species level by using cell morphology with acid production ability of five sugars (fructose, mannitol, mannose, trehalose, and D-Xylose). The fecal samples of commercial pigs showed a more diversity of acid and bile tolerant LAB species than the fecal samples from indigenous pigs. This might be the results from the differences in diets of these pigs. We found that varieties of vegetables and grasses as fiber diets were given to the commercial pigs which might act as prebiotics influcing the growth of different LAB species (Praznik et al., 2015).

The antimicrobial resistance phenotype and genotype are also the essential selection criterion for screening probiotic candidates. The antimicrobial susceptibility of 34 acid- and bile- tolerant LAB strains were investigated by disk diffusion assay to eleven antimicrobials included amoxicillin, ampicillin, chloramphenicol, tetracycline, vancomycin, colistin sulfate, gentamicin, kanamycin, streptomycin, erythromycin, and clindamycin. The isolates that resistant to clindamycin, erythromycin, chloramphenicol and gentamicin were removed as they pose a high risk of harboring transferable resistance genes towards those antimicrobials (Muñoz-Atienza et al., 2013). The antimicrobial susceptibility by disk diffusion revealed that seven LAB strains (*E. faecium* 49N and 70N, *L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) displayed susceptibility towards the highest numbers of tested antimicrobials, and were selected for further confirmation on antimicrobial susceptibility by MICs using EFSA microbiological cut-off values (EFSA, 2012) and studied for their antiviral activity

against PEDV. Five strains (*L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) were acceptable as potential candidates for further investigations.

The antiviral effects of cell-free supernatant (CFS) and live bacterial cells of seven LAB strains (*E. faecium* 49N and 70N, *L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) against pandemic strain of PEDV isolated from diseased pigs compared with a commercial probiotic strain (*L. acidophilus*), *in vitro,* were investigated. Five LAB strains (*L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) exhibited different levels of antiviral effects against pandemic strains PEDV. The LAB strains, especially *L. plantarum* strain 25F*,* showed stronger or equivalent antiviral effects against PEDV compared with the commercial strain. We hypothesized that the antiviral effects from LAB CFS may come from LAB metabolites such as hydrogen peroxide, fatty acid, and/or organic acids (Botić et al., 2007; Dembinski et al., 2014; Maragkoudakis et al., 2010). However, LAB CFS adjusted to pH7 showed no antiviral effects; therefore, we concluded that the antiviral effects would not be from bacteriocins since bacteriocins were proved to function at wide range of pH value included physiological pH (S.-C. Yang et al., 2014). Nevertheless, the possible mechanisms underlying antiviral activity of LAB CFS might be from prevention of cell attachment and blocking viral adsorption into the cells (Aboubakr et al., 2014; Allayeh et al., 2015). Furthermore, the reduction of CPE by live LAB cells was assumed to be from the direct protection by the competition for cell receptors between LAB and PEDV, and/or the interfering of LAB cells in viral cell entry (Aboubakr et al., 2014; Botić et al., 2007). However, 10^6 CFU/mL was the lowest viable LAB concentration required to notice antiviral effects in this study and in other previous enteric viral infections such as against rotavirus and gastroenteritis coronavirus (Aboubakr et al., 2014; Botić et al., 2007; Maragkoudakis et al., 2010). In addition, LAB strains showed different levels of antiviral effects demonstrated that this ability ought to be strain-specific (Maragkoudakis et al., 2010; Martín et al., 2010).

Five LAB strains (*L. plantarum* 22F, 25F and 31F, *P. acidilactici* 72N and *P. pentosaceus* 77F) with antiviral effects were selected for further *in vitro* evaluation of functional and safety aspects of probiotic properties, and antibacterial activity against important enteric pathogenic bacteria in pigs (ETEC, EHEC, *S. Choleraesuis, S.*

Typhimurium, and *S suis* type II). The ability to tolerate simulated gastrointestinal tract of these LAB strains were investigated by examination the survival rate in simulated saliva (tolerance to lysozyme), simulated gastric juice at different pH values, and bile and phenol tolerance. The strains *L. plantarum* 22F and 25F displayed outstanding ability in tolerate to simulated gastric juice and bile tolerance. Moreover, these two strains also exhibited good cell surface properties included hydrophobicity and auto- and co- aggregation. *P. pentosaceus* 77F was the only strain showing β- hemolytic and BSH activity which considered unsuitable as probiotic candidates. For the antibacterial activity of LAB in this study, the activity was strongly confirmed by non-neutralized CFS and live cells especially the inhibition of indicator *Salmonella* by LAB CFS, but the inhibitory effects could not be detected in any neutralized CFS. This concluded that bacteriocins were not produced by our strains as also found in the previous studies (Casarotti et al., 2017; Ren et al., 2014). Moreover, when observed the activity by live LAB cells, *L. plantarum* 25F exhibited very strong antibacterial activity against all of the studied pathogens, except *S. suis* type II. The previous study also observed greater antimicrobial activity by live cells than CFS (Arena et al., 2016). We found that *L. plantarum* 22F was the most outstanding strain possessing most of the studied *in vitro* probiotic properties after analysis by principal component analysis (PCA). Therefore, results showed that among five LAB strains, the *L. plantarum* strains (specially *L. plantarum* 22F) demonstrated higher ability in simulated GI tract's tolerance, cell surface properties, and antibacterial activity by cell-free supernatant without blood hemolytic and BSH activity. These indicated that they were suitable for further develop as probiotics.

In summary, taken together all the experiments in this research, two selected LAB strains which are *L. plantarum* 22F and 25F were the most suitable/promising candidates for development as probiotics use in pig feed additives. Since *L. plantarum* 22F demonstrated better functional probiotic properties compared to other selected strains; while live cells of *L. plantarum* 25F possessed the best protective effects against most indicator enteric pathogenic bacteria and PEDV.

Conclusion remarks

Results of this dissertation have shown that

1. The autochthonous lactic acid bacteria suitable to use as probiotic candidates could be isolated from Thai indigenous and commercial pig feces.
2. The standard method used to identify LAB strain is the 16S rDNA sequencing analysis. The classification into genus or possible species level of LAB from investigation of cell morphology combined with acid production of five sugars (fructose, mannitol, mannose, trehalose, and D-Xylose) may be possible.
3. The autochthonous LAB strains isolated in this study exhibited stronger or equivalent antibacterial and antiviral against enteric pathogenic *E. coli, Salmonella*, and PED virus, respectively, compared with a commercial probiotic strain.
4. Two autochthonous LAB strains (*L. plantarum* 22F and 25F) showed the most promising as probiotic candidates for development as pig feed additives. *L. plantarum* 22F demonstrated better functional probiotic properties compared to other selected strains; while live cells of *L. plantarum* 25F possessed the best protective effects against ETEC, EHEC, *S. Choleraesuis*, and PEDV.

Suggestions for further investigation

In this research, main functional and safety aspects of probiotic properties of LAB strains from pig feces had been investigated and selected the best promising probiotic candidates. Further studies on technological aspects should be investigated to determine wheter the selected LAB strains would maintain their viability and properties after undergo manufacturer processes. Moreover, *in vivo* studies in pigs are also recommended.

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APPENDIX

Media, buffer and solution preparation

Phosphate buffer saline (PBS) (1000 mL)

Sodium chloride	8	g
Potassium chloride	0.2	g
Sodium hydrogen phosphate	2.9	g
Potassium dihydrogen phosphate	0.2	g

Normal saline

Sodium chloride	8.5	g
Distilled water	1000	mL

NaOH [0.1N]

Sodium hydroxide	6	g
Distilled water	1500	mL

Luria broth (1000 mL)

Tryptone	10	g
Yeast extract	5	g
Sodium chloride	5	g

(For Luria agar added 17 g of agar)

Peptone dilution saline (PDS)

Peptone	1	g
Sodium chloride	9	g
Distilled water	1000	mL

Bis-acrylamind (30:08)

Acrylamind	30	g
Bis-acrylamind	0.8	g
Distilled water	100	mL

Dye buffer

Trisma base	0.15	g
Sodium dodecyl sulfate (SDS)	0.4	g
2-Mercaptoethanol	1	mL
Glycerol	2	mL
Distilled water	7	mL
Bromophenol blue	0.02	g

APS (10%)

Ammoniumpersulfate	1	g
Distilled water	10	mL

Running buffer (10X)

Glycine	69.75	g
Trisma base	15	g
SDS	5	g
Distilled water (added to)	500	mL

SDS stock solution (10%)

SDS	10	g
Sterial water (added to)	100	mL

Saturated butanol

Butyl alcohol : distilled water	1 : 1	

Use the upper part after vigerously shaking

Tris-glycine buffer (5X)

Trisma base	15.1	g
Glycine	94	g
10% SDS	50	mL
Distilled water (added to)	1	L

Comassie brilliant blue (CBB)

Methanol	900	mL
Acetic acid	180	mL
Distilled water	900	mL
CBB (R250)	4.95	g

Mixed by magnetic stirrer for 24 hours and filter with filter paper No.2

Destaining buffer

Methanol	50	mL
Acetic acid	75	mL
Distilled water	875	mL

Tris-HCl 1.125M pH 8.8

Trisma base	13.62	g
Distilled water	70	mL

Dissolve the solution and cool to 25°C. Adjust to pH 8.8, and adjusted the final volumn to 100 mL with distilled water. Sterile by autoclave.

Tris-HCl 0.625M pH 6.8

Trisma base	7.56	g
Distilled water	70	mL

Dissolve the solution and cool to 25°C. Adjust to pH 6.8, and adjusted the final volumn to 100 mL with distilled water. Sterile by autoclave.

Preparation of 5% stacking gel

Components	volume	
Distilled water	3.4	mL
30% Bis acrylamide	0.83	mL
Tris-HCl pH 6.8	0.63	mL
10% SDS	0.05	mL
10% APS	0.05	mL
TEMED	0.005	mL

Preparation of 12% separating gel

Components	volume	
Distilled water	4.9	mL
30% Bis acrylamide	6.0	mL
Tris-HCl pH 8.8	3.8	mL
10% SDS	0.15	mL
10% APS	0.15	mL
TEMED	0.006	mL

Broth sugar

Peptone	10	g
Beef extract	3	g
NaCl	5	g
Distilled water	1,000	mL
Bromthymol blue (0.2%)	15	mL

Adjusted to pH7.2 and sterile by autoclave. Cool down below 55 °C and added 10% or 20% sugar.

Alpha-Napthal

Alpha-Naphal	5	g
Ethanol 95%	100	mL

List of publications and conference proceedings

1. Wandee Sirichokchatchawan, Thongchai Chalermchaikit, and Nuvee Prapasarakul. 2012. บทบาทของโปรไบโอติกในอุตสาหกรรมการเลี้ยงสุกร. J Thai Vet Med Assoc. 63: 1-3

2. Wandee Sirichokchatchawan, Gun Temeeyasen, Dachrit Nilubol, and Nuvee Prapasarakul. 2017. Protective Effects of Cell-Free Supernatant and Live Lactic Acid Bacteria Isolated from Thai Pigs Against a Pandemic Strain of Porcine Epidemic Diarrhea Virus. Probiotics & Antimicro. Prot. In Press. DOI 10.1007/s12602-017-9281-y

3. Wandee Sirichokchatchawan, Somboon Tanasupawat, Waree Niyomtham and Nuvee Prapasarakul. 2017. Identification and antimicrobial susceptibility of lactic acid bacteria from fecal samples of indigenous and commercial pigs. In Press. Thai J Vet Med.

4. W. Sirichokchatchawan, S. Tanasupawat, N Prapasarakul. Characteristics of lactic acid bacteria intended for probiotic use from indigenous and commerical pig feces in Thailand. The 24th International Pig Veterinary Society Congress (IPVS) / 8th European Symposium of Porcine Health Management (ESPHM) 07-10 June 2016. Dublin, Ireland. (Poster presentation)

5. W. Sirichokchatchawan, N Prapasarakul. Antimicrobial activity of putative probiotics isolated from feces of indigenous and commercial pigs in Thailand. The 24th International Pig Veterinary Society Congress (IPVS) / 8th European Symposium of Porcine Health Management (ESPHM) 07-10 June 2016. Dublin, Ireland. (Poster presentation)

6. W. Sirichokchatchawan, S. Tanasupawat, N Prapasarakul. Genetic and Phenotypic Identifications of Putative Probiotic Candidate Bacteria Derived from Pigs in Thailand. 14th Chulalongkorn University Veterinary Conference. 20-22 April 2015. Thailand. (Oral presentation)

7. W. Sirichokchatchawan, S. Tanasupawat, N Prapasarakul. Genetic and Phenotypic Identifications of Putative Probiotic Candidate Bacteria Derived from Pigs in Thailand. 14th Chulalongkorn University Veterinary Conference. 20-22 April 2015. Thailand. (Poster presentation)

8. W. Sirichokchatchawan, N Prapasarakul. *In vitro* assessment of cell surface characteristics of presumptive lactic acid bacteria from pig feces. 16th Chulalongkorn University Veterinary Conference. 22-24 March 2017. Thailand. (Poster presentation)

9. P. Praechansri, W. Sirichokchatchawan, N Prapasarakul. *In vitro* antibacterial activities of Thai presumptive probiotics against common enteric pathogens in pigs. 16th Chulalongkorn University Veterinary Conference. 22-24 March 2017. Thailand. (Poster presentation).

10. P. Pupa, N. Muangsin, N. Pirarat, W. Sirichokchatchawan, N. Prapasarakul. Microencapsulation in alginate/chitosan linkage to enhance viability of porcine lactic acid bacteria. 16th Chulalongkorn University Veterinary Conference. 22-24 March 2017. Thailand. (Poster presentation).

VITA

Ms. Wandee Sirichokchatchawan was born on the 12th of January 1987 in Bangkok province, Thailand. She graduated high school from St. Stephen International School (SIS), continued with her Bachelor of Science (BSc) at Mahidol University International College (MUIC), and graduated with 1st degree of honor in 2008. She then pursued and received her Master degree (MSc) in Molecular Biology and Pathology of Viruses from Faculty of Medicine, Imperial College London, United Kingdom in 2010. After graduation, she continued her PhD at the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her field of interest was lactic acid bacteria and probiotics which involved probiotic properties and antimicrobial activities against porcine pathogenic bacteria and virus.