EFFICACIES OF THAI PROBIOTIC STRAINS AGAINST ANTIBIOTIC RESISTANCE BACTERIA AND MODULATE GUT MICROBIOME AND RESISTOME IN PIG MODEL

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ประสิทธิภาพของโปรไบโอติกสายพันธุ์ไทยต่อการต่อต้านเชื้อแบคทีเรียดื้อยา และการปรับสมดุลไมโครไบโอมและรีซิสโตมในสุกร

นายประเสริฐ อภิวัฒน์ศิริ

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ประเสริฐ อภิวัฒน์ศิริ : ประสิทธิภาพของโปรไบโอติกสายพันธุ์ไทยต่อการต่อต้านเชื้อแบคทีเรียดื้อยา และการปรับสมดุลไมโครไบโอมและรีซิสโตมในสุกร. (EFFICACIES OF THAI PROBIOTIC STRAINS AGAINST ANTIBIOTIC RESISTANCE BACTERIA AND MODULATE GUT MICROBIOME AND RESISTOME IN PIG MODEL) อ.ที่ปรึกษาหลัก : รศ.น.สพ. ดร.ณุวีร์ ประภัสระกูล, อ.ที่ปรึกษาร่วม : ดร.สิทธิรักษ์ รอยตระกูล,รศ. ดร.สัญชัย พยุงภร

เชื้อแบคทีเรียผลิตกรดแลคติก (lactic acid bacteria; LAB) ถูกนำมาใช้เป็นโปรไบโอติก (probiotics) กันอย่างแพร่หลายในอุตสาหกรรมการเลี้ยงสัตว์ปศุสัตว์เนื่องด้วยคุณสมบัติที่ดีหลายประการ ได้แก่ คุณสมบัติในการขัดขวางกระบวนการส่งผ่านของยีนดื้อยาปฏิชีวนะ และการสร้างฟิล์มชีวภาพ (biofilm) รวมไปถึงการปรับสมดุลของเชื้อจุลชีพในลำไส้ของสัตว์ จากงานวิจัยก่อนหน้าของทางทีมผู้วิจัยพบว่าเชื้อแบคทเรียผลิตกรดแลคติกสายพันธุ์ *Lactobacillus plantarum* สเตรน 22F, 25F (L22F and L25F) และ *Pediococcus acidilactici* สเตรน 72N (P72N) นั้นมีคุณสมบัติที่ดีหลายประการ ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของแบคทีเรียผลิตกรดแลคติกสายพันธุ์ไทยต่อการต่อต้านการส่งผ่านของยีนดื้อยาผ่านกระบวนการ conjugation และการสร้างฟิล์มชีวภาพจากเชื้อ *Escherichia coli* ที่มียีนดื้อยาชนิด *mcr*-1 ซึ่งทางผู้วิจัยพบว่าสารสกัดส่วนใสปราศจากเซลล์ (cell-free supernatant; CFS) ที่ความเข้มข้น 1 ต่อ 16 (pH ประมาณ 5.70-5.92) สามารถลดอัตราการส่งผ่านของยีนดื้อยาปฏิชีวนะชนิด *mcr*-1 ได้อย่างมีนัยสำคัญทางสถิติสูงสุดถึง 100 เท่าเมื่อเปรียบเทียบกับ CFS ของเชื้อแบคทีเรียผลิตกรดแลคติกที่มีการปรับความเข้มข้นเป็นกลางที่ pH เท่ากับ 6.5 นอกจากนี้ CFS จากเชื้อแบคทีเรียผลิตกรดแลคติกของเราสามารถลดการสร้างฟิล์มชีวภาพจากเชื้อ *E. coli* ได้สูงถึง 82% และ 60% สำหรับฟิล์มชีวภาพในระยะ planktonic และ sessile ตามลำดับ สำหรับ CFS ที่มีการปรับความเข้มข้นเป็นกลางนั้นพบว่ายังสามารถลดการสร้างฟิล์มชีวภาพจากเชื้อ *E. coli* ในระยะ sessile ได้มากถึง 60% อีกด้วย นอกจากนั้น งานวิจัยนี้ยังมีวัตถุประสงค์เพื่อสำรวจและติดตามประสิทธิภาพของเชื้อแบคทีเรียผลิตกรดแลคติกต่อข้อมูลสัดส่วนของเชื้อทั้งหมด (microbiome) และ ยีนดื้อยาทั้งหมด (resistome) ในลูกสุกรที่ได้รับและไม่ได้รับการป้อนเชื้อ enterotoxigenic *E. coli* (ETEC) ด้วยวิธี whole-genome shotgun metagenomics ซึ่งผลการทดลองพบว่าเชื้อแบคทีเรียผลิตกรดแลคติกหลายสายพันธุ์อันได้แก่ L22F L25F และ P72N นั้นสามารถเพิ่มจำนวนเชื้อจุลชีพที่มีประโยชน์เช่น *Lactobacillaceae*, *Lachnospiraceae* และ *Ruminococcaceae* อีกทั้งยังสามารถลดยีนดื้อยาปฏิชีวนะกลุ่ม beta-lactams การดื้อโลหะหนักชนิดคอปเปอร์ (copper) การดื้อสารชีวฆาตหลายชนิด (multi-biocide) และยังสามารถลดยีนดื้อยาปฏิชีวนะชนิด *tet*W and *tet*Q ได้อีกด้วยเมื่อเปรียบเทียบกับกลุ่มสุกรที่ได้รับยาปกฏิชีวนะ ยิ่งไปกว่านั้นพลาสมิดชนิด IncX4 ซึ่งมีความเกี่ยวข้องกับการดื้อต่อยาปฏิชีวนะโคลิสตินนั้น พบว่าลดลงอย่างเห็นได้ชัด รวมไปถึงไม่พบ integrase gene (*intI*) กลุ่มที่ 2 และ 3 ในกลุ่มสุกรที่ได้รับการป้อนด้วยแบคทีเรียผลิตกรดแลคติกหลายสายพันธุ์ แต่อย่างไรก็ตาม insertion sequence (IS) ชนิดที่ 3 และ 30 ซึ่งมีความเกี่ยวข้องกับการใช้สารอาหารนั้น พบว่ามีการเพิ่มขึ้นในสุกรกลุ่มนี้อีกด้วย ยิ่งไปว่านั้นกระบวนการจำกัดพิษ (detoxification) และภาวะเครียดจากออกซิเดชั่น (oxidative stress) ซึ่งมีความเกี่ยวข้องกับกระบวนการต้านสารอนุมูลอิสระ (antioxidant activity) เช่นเดียวกับการใช้กรดอะมิโน (amino acid metabolism) และการใช้คาร์โบไฮเดรต (carbohydrate metabolism) พบว่ามีการเพิ่มขึ้นในกลุ่มสุกรที่มีการป้อนด้วยแบคทีเรียผลิตกรดแลคติกหลายสายพันธุ์ ดังนั้นผลการศึกษานี้แสดงให้เห็นว่าแบคทีเรียผลิตกรดแลคติกได้แก่ L22F L25F และ P72N สามารถยับยั้งกระบวนการส่งผ่านของยีนดื้อยาปฏิชีวนะและ การสร้างฟิล์มชีวภาพรวมไปถึงสามารถปรับสมดุลของเชื้อจุลชีพและ ยีนดื้อยาทั้งหมดในลำไส้โดยอ้างอิงผลจากการศึกษาสารพันธุกรรมทั้งหมด (metagenomic analysis)

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ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
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Prasert Apiwatsiri : EFFICACIES OF THAI PROBIOTIC STRAINS AGAINST ANTIBIOTIC RESISTANCE BACTERIA AND MODULATE GUT MICROBIOME AND RESISTOME IN PIG MODEL. Advisor: Assoc. Prof. NUVEE PRAPASARAKUL, D.V.M., Ph.D. Co-advisor: Dr. Sittiruk Roytrakul, B.Sc., M.Sc, Ph.D.,Assoc. Prof. SUNCHAI PAYUNGPORN, B.Sc., Ph.D.

Lactic acid bacteria (LAB) have been widely used as probiotics in the livestock industry because of their high potential for antibacterial activity, anticonjugation, antibiofilm capacity, and gut microbiome regulation. *Lactobacillus plantarum* 22F, 25F (L22F and L25F), and *Pediococcus acidilactici* 72N (P72N) showed several promising in vitro and in vivo properties, according to our recent research. The objective of this study was to evaluate the Thai LAB efficacy on anticonjugation and antibiofilm activities in *E. coli* harboring the *mcr*-1 gene. When compared to the neutralizing cell-free supernatant (CFS), we discovered that the CFS derived from our LAB strains at 1:16 dilution (pH 5.70-5.92) significantly reduced the transfer frequency of the *mcr-1* gene between the donor and recipient *E. coli* by up to 100 times (pH 6.5). Furthermore, our non-neutralizing CFS has the potential to significantly reduce the production of *E. coli* biofilm by more than 82 % for planktonic biofilm and 60 % for sessile biofilm, respectively. It has the potential to inhibit biofilm development in the planktonic stage by up to 52 % when used to neutralize CFS. The effect of our LAB on the gut microbiota and resistome in weaned piglets with and without enterotoxigenic *Escherichia coli* (ETEC) infection was monitored and observed using whole-genome shotgun metagenomics. The findings revealed that a multi-strain LAB containing L22F, L25F, and P72N could help to expand beneficial bacteria families such as *Lactobacillaceae, Lachnospiraceae,* and *Ruminococcaceae*. In comparison to antibiotic pigs, it also lowered beta-lactam resistance, copper resistance, multi-biocide resistance, *tetW,* and *tetQ.* In piglets treated with our LAB strains, IncX4, a plasmid associated with colistin resistance, was reduced, and an integrase gene (*intI)* class 2 and 3 could not be detected. However, this group was also enriched in the insertion sequences (IS) 3 and 30, which are linked to nutrition use. Furthermore, in LAB supplemented piglets, the detoxification and oxidative stress response, which is connected to antioxidant activity, as well as amino acid and glucose metabolism, were all boosted. Our findings may highlight the LAB properties inhibiting antimicrobial resistance bacteria, positively affecting gut microbiome, and resistome modulation based on metagenomic research.

Field of Study:	Veterinary Pathobiology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....
		Co-advisor's Signature .....
		Co-advisor's Signature .....

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Prasert Apiwatsiri

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

%	Percent
℃	Degree Celsius
ADG	Average daily gain
AMA	Antimicrobial agents
AMC	Amoxicillin-clavulanic acid
AMK	Amikacin
AMP	Ampicillin
AMR	Antimicrobial resistance
AMX	Amoxicillin
ARB	Antibiotic resistance bacteria
ARG	Antibiotic resistant gene
ATCC	The American Type Culture Collection
B. longum	*Bifidobacterium longum*
BF	Biofilm formation
bp	Base pair
BTS	Bruker's bacterial test standard
CARD	Comprehensive Antibiotic Resistance Database
CEF	Ceftiofur
CFS	Cell free supernatant

CFU	Colony Forming Unit
CHL	Chloramphenicol
CLSI	Clinical and Laboratory Standards Institute
CLX	Cefalexin
CO_2	Carbon dioxide
CPD	Cefpodoxime
CPF	Charoen Pokphand Foods
CST	Colistin
CW	Control well
Cu	Copper
DFM	Direct-feed microbial
dpc	Day post challenge
DW	Distilled water
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	Ethylene diamine tetraacetic acid
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
ENRO	Enrofloxacin
EPS	Extracellular polysaccharide
erm	erythromycin RNA methylase

ERY	Erythromycin
ESBL	Extended-spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
EU	European Union
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FAO	The Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
G	Gram
GABA	Gamma-aminobutyric acid
GEN	Gentamicin
GI	Gastro-intestinal
GIT	Gastro-intestinal tract
hpc	Hour post challenge
hr	Hour
IL	Interleukin
Inc	Incompatibility
INN	Cefovecin
Intl	Integrase gene
IPM	Imipenem
IS	Insertion sequence

K. pneumoniae	*Klebsiella pneumoniae*
KAN	Kanamycin
KEGG	Kyoto Encyclopedia of Genes and Genomes database
L. acidophilus	*Lacobacillus acidophilus*
L. casei	*Lacticaseibacillus casei*
L. delbueckii	*Lactobacillus delbueckii*
L. plantarum	*Lactiplantibacillus plantarum*
L. rhamnosus	*Lacticaseibacillus rhamnosus*
L. salivarius	*Ligilactobacillus salivarius*
LAB	Lactic acid bacteria
LB	Luria-Bertani
LPS	Lipopolysaccharide
M	Molarity
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
mcr	Mobilized colistin resistance
MDR	Multi-drug resistance
MFS	Major facilitator superfamilies
MFX	Marbofloxacin
MGE	Mobile genetic element
MIC	Minimal Inhibitory Concentration
min	minute

ml	Milliliters
MLS	Macrolide-lincosamide-streptogramin
MG-RAST	Metagenome rapid annotation using subsystem technology
MRS	De Man, Rogosa and Sharpe
NA	No available
NaCl	Sodium Chloride
NaN_3	Sodium azide
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
ND	No determined
Neg	Negative
NIT	Nitrofurantoin
nm	Nanometer
NSS	Normal Saline Solution
OD	Optical density
OIE	The World Organisation for Animal Health
OS	Observed species
P. acidilactici	*Pediococcus acidilactici*
P. aeruginosa	*Pseudomonas aeruginosa*
P. pentosaceus	*Pediococcus pentosaceus*

P-value	Probability value
PBRT	PCR-based replicon typing
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCoA	Principal coordinate analysis
PDS	Peptone Dilution Saline
PEA	Phosphoethanolamine
PEDV	Porcine epidemic diarrhea virus
PGA	β-1,6- N-acetyl-D-glucosamine polymer
PIP	Piperacillin
POL	Polymyxin B
POS	Positive
PWD	Post-weaning diarrhea
QAC	Quaternary ammonium compound
R	Resistance
RCF	Relative centrifugal force
ROS	Reactive oxygen species
RPM	Round Per Minute
rRNA	Ribosomal ribonucleic acid
S	Susceptibility
S. aureus	*Staphylococcus aureus*

S. lactis	*Streptococcus lactis*
S. thermophilus	*Streptococcus thermophilus*
SCFA	Short chain fatty acid
sec	Second
SEM	Scanning electron microscopy
STR	Streptomycin
SXT	Trimethoprim/sulfamethoxazole
TBE	Tris-borate-EDTA
TET	Tetracycline
TOB	Tobramycin
TraW	Type IV traffic ATPase
TSA	Tryptic Soy Agar
TSB	Tryptic soy broth
TW	Test well
TYL	Tylosin
µg	Micrograms
µl	Microliters
USA	The United States of America
V. cholerae	*Vibrio cholerae*
WHO	World Health Organization
Zn	Zinc

CHAPTER I

INTRODUCTION

1.1 Importance and rationale

Antimicrobial resistance (AMR) is one of the critical global threats to both human and animal health (O'Neill, 2014). Colistin is used as the 'last-resort' indication for treating bacterial infection not only human medicine but also veterinary medicine, especially swine production (Gao et al., 2016; Liu et al., 2016; Ye et al., 2016). The plasmid-mediated colistin resistance, *mcr*-1 gene, is defined as one of the mechanisms for colistin resistance, which is much potential for wide-spread transmission via mobile genetic processes such as plasmid conjugation (Liu et al., 2016). Thus, a rapid widespread of colistin resistance has found in several countries and discovered at least ten more additional types of *mcr* gene among enteric bacteria and geographical difference (Liu et al., 2016; Olaitan et al., 2016; Ye et al., 2016; Wang et al., 2020a).

Biofilm is a crucial virulence factor of the pathogens leading to preferentially adherence to a variety of surface, microbial aggregation and tolerance to various stresses, e.g., disinfectants, host immunity, and antimicrobial substances (Jacques et al., 2010). Biofilm formation of Gram-negative bacteria is one of its mechanisms of antimicrobial resistance by more than 1000-fold with several mechanisms including, decreasing activity and efficacy of antimicrobial agents, increasing horizontal antibiotic-resistant gene transfer within the biofilm and upregulating efflux pump gene (Vasudevan, 2014; Rabin et al., 2015; Fleming and Rumbaugh, 2017).

Previous studies have been denoted that probiotics are one of the potential approaches to handle with them through several mechanisms. All of their mechanisms are strain-specific manner, and they involve either the production of

several antimicrobial compounds (organic acids, antimicrobial peptides, enzymes) or cell-cell interaction (competitive exclusion, nutrients competition) (O'Toole and Cooney, 2008; Hossain et al., 2017). Probiotics are defined by the World Health Organization (WHO) that 'Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (WHO/FAO, 2006). Lactic acid bacteria (LAB), the famous member of probiotic, have been broadly studied in the past few decades and widely utilized for numerous purposes in both humans and animals such as enhance nutrient utilization, elevate immune activities, inhibit the growth of numerous pathogens (Angmo et al., 2016). Besides, LAB strains are found as an inhibitor during horizontal transmission of mobile genetic elements (Moubareck et al., 2007; Nehal El-Deeb, 2015b) and anti-biofilm formation (Walencka et al., 2008; Fang et al., 2018b; Mahdhi et al., 2018; Kim et al., 2019).

Probiotics have also been considered as an alternative to in-feed antibiotics in the livestock production, especially weaning transition period with many beneficial effects, including modulate gut microbiota, improve gut health, enhance growth performance, and reduce pathogens. However, the study about probiotic effects on antibiotic resistance genes (ARGs) modulation in pig gut are still very rare (Liao and Nyachoti, 2017a; Ma et al., 2019; Shin et al., 2019; Wang et al., 2019b). Our previous studies proposed the approved LAB strains from healthy pigs in Thailand, including *L. plantarum* strain 22F and 25F, and *P. acidilactici* strain 72N, exhibiting proper tolerance properties, antibacterial activity, antiviral effect, and safety from antimicrobial-resistant genes (Sirichokchatchawan et al., 2017b; Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b). Moreover, they also *in vivo* proved to improve growth performance and enhance overall gut health along the production cycles of the pigs (Pupa et al., 2021a; Pupa et al., 2021b). Therefore, this study aims to determine the effects of Thai LAB against antibiotic-resistant bacteria in aspect of anti-conjugation, anti-biofilm activity, and to monitor and observe the efficacy of LAB fed in neonatal pigs on gut microbiota and ARGs.

1.2 Literature review

1.2.1 Colistin resistance

Antimicrobial resistance (AMR) is one of the critical global threats in the 21st century that threaten both animal and human health, and it also threatens the efficient treatment and prevention of an increased type of the infections. According to the estimation of antimicrobial resistance impacts, the deaths from antimicrobial-resistant infection has reached 10 million people annually, and its loss has raised to 100 trillion USD worldwide by 2050 (O'Neill, 2014; Prestinaci et al., 2015). AMR has extensively prescribed the phenomenon of microorganisms, including bacteria, fungi, viruses, and parasites turning to inferior susceptible to the antimicrobials used for controlling or eradicating them. Amongst microbial organisms, bacteria are an urgent problem regarding antibiotic resistance due to wide ranges of bacterial species and strains which provide the current drugs less effective as a result of evolution or acquisition the diverse characteristics (Prestinaci et al., 2015; Chandler, 2019).

Colistin

Colistin is one of the cationic polypeptide antibiotics in the polymyxins family, and it was primarily isolated in 1947 from soil bacterium named *Paenibacillus polymyxa* subsp. *colistinus* (Gao et al., 2016; Poirel et al., 2017). Polymyxins contain five different compounds, including polymyxins A, B, C, D and polymyxins E or colistin. There are only two polymyxins compounds that are commonly used in clinical practice and presently available on the marketplace, which is polymyxin B and polymyxin E or colistin (Ahmed et al., 2020). It has been known that colistin is a broad-spectrum antibiotic against Gram-negative bacteria, especially *Enterobacteriaceae* (Liu et al., 2016).

Moreover, some reports have elucidated that colistin is a potential antibiotic to combat the lethal bacterial infection from various pan-drugs resistant Gram-negative pathogens (Ye et al., 2016). The mode of colistin action for antibacterial

activity is associated with electrostatic interaction between the positive charge of the amino acid residue of colistin and negative charge of the phosphate group on lipid A locating on lipopolysaccharide (LPS) at the bacterial outer membrane (Gao et al., 2016; Poirel et al., 2017). Colistin penetrates inner membrane through the periplasm and increases the permeability of the bacterial cell membrane, consequently occurring the pore, leading to leakage of cell content and death of the bacterial cell (Gao et al., 2016; Rhouma et al., 2016a; Poirel et al., 2017). Though colistin possesses a past generation of antibiotics, their properties are defined as 'last-resort' for life-threatening multidrug resistant bacterial infection (Gao et al., 2016). Later, polymyxins, especially colistin, were re-introduced throughout the world at both clinical medicines and veterinary medicines for treating bacterial infection (Kempf et al., 2016; Olaitan et al., 2016).

Colistin usage

Colistin has been extensively used for a decade in livestock production for both therapeutic and prophylactic purposes (Sun et al., 2018). For therapeutic propose, colistin has the main indication for treating the bacterial infection, particularly *Enterobacteriaceae* infections in various animal species, including pigs, chickens, goats, sheep and cows (Kempf et al., 2016). For prophylactic propose, colistin also used as a growth promoter in food animals improving growth performances such as feed efficiency and body weight gain. Moreover, a previous study has reported that the worldwide demand for colistin utilization in the agricultural industry is approximately 12,000 tons annually (Rhouma et al., 2016b; Sun et al., 2018). Unfortunately, over- and misusage of colistin in humans and food animals is associated with the emergence of colistin resistance in several bacterial species of *Enterobacteriaceae* family (Gao et al., 2016; Ahmed et al., 2020).

Colistin resistance

Colistin resistance mainly occurs via two mechanisms comprise of chromosomally mediated colistin resistance and plasmid-mediated colistin resistance. For chromosome-mediated colistin resistance, this mechanism transfers the resistance characteristics with vertical transmission route and has low evolution rate. It can resist by modifying lipid A on LPS of the bacterial cell by 4'-phosphoethanolamine (PEA). Furthermore, the global prevalence of chromosome-mediated colistin resistance is about 10% amongst Gram-negative bacteria, and it is the highest in the Southeast Asian and Mediterranean countries (Al-Tawfiq et al., 2017). In November 2015, the mobilized colistin resistance or *mcr*-1 gene, which confers colistin resistance through plasmid-mediated mechanism had been found in the *Enterobacteriaceae* family in China (Liu et al., 2016). This gene encodes the phosphoethanolamine transferase enzyme and catalyzes the lipid A modification on LPS of the bacterial cell. After discovering of *mcr*-1 gene, a retrospective study also reveals that *mcr* gene has already been identified since the 1980s in China (Gao et al., 2016; Al-Tawfiq et al., 2017). Moreover, the plasmid harboring *mcr*-1 gene is much potential for wide-spread transmission via horizontal gene transfer (Liu et al., 2016).

Factor impacting on antibiotic resistance: Plasmid conjugation

Horizontal or lateral gene transfer is a mechanism of transporting strange DNA, and this DNA can be recognized as mobile genetic elements when they transfer to other bacteria (Gyles and Boerlin, 2014). Besides, previous studies have suggested that those transferred genes could encode the assorted performances, including virulence factors, metabolic characteristics, and antibiotic resistance (Lopatkin et al., 2016). For mobile genetic element transportation, three mechanisms have been proposed as transduction, transformation, and conjugation (Gyles and Boerlin, 2014). The transduction mechanism involved with the virus (bacteriophages) for transferring the DNA from one bacterial cell to other bacterial cells. These phages can be divided

into two types (lysogenic pathway or incorporating their genes into chromosomes, and lytic pathway or producing phage particles and destroying the recipient cells) (Gyles and Boerlin, 2014; Lindsay, 2014). The transformation associated with uptake free DNA in the environment by the bacterial cell, which is competence state (being capable of taking up DNA) (Thomas and Nielsen, 2005; Gyles and Boerlin, 2014). The conjugation generally transferred mobile genetic elements such as a plasmid, integrative or conjugative elements and pathogenicity island between the donor and recipient bacterial cells through connecting tubes (sex pilus, nanotubes), cell to cell adhesion or pores (Gyles and Boerlin, 2014; Lindsay, 2014).

By plasmid conjugation, colistin-resistant bacteria are successful in transferring *mcr* gene from strain to strain and also from species to others in *Enterobacteriaceae* family, resulting in colistin resistance in other susceptible bacteria (Liu et al., 2016). Thus, a rapid wide-spread of colistin-resistant bacteria have been found in several countries across at least five continents among isolates of humans, animals, and environments (Wang et al., 2018a). Nowadays, up to twenty-two functional genetic variants of *mcr*-1 (*mcr*-1.1 to *mcr*-1.22) have been indicated (Ahmed et al., 2020). Besides, more than ten additional *mcr*-like genes have already been identified, including *mcr*-2 (Xavier et al., 2016), *mcr*-3 (Yin et al., 2017b; Yin et al., 2017a), *mcr*-4 (Carattoli et al., 2017), *mcr*-5 (Borowiak et al., 2017), *mcr*-6 (AbuOun et al., 2017; Partridge et al., 2018), *mcr*-7 (Yang et al., 2018), *mcr*-8 (Wang et al., 2018b), *mcr*-9 (Carroll et al., 2019) and *mcr*-10 (Wang et al., 2020a).

The transferable plasmids are a severe concern for *mcr* gene dissemination, and the predominant plasmids distributing to the global spread of *mcr* gene in *Enterobacteriaceae* are IncI2, IncP, IncHI2 and IncX4 (Liu and Liu, 2018). Likewise, the first transferred plasmid (pHNSHP45; 64,105 bp) from *Escherichia coli* SHP45 harboring *mcr*-1 gene also belongs to IncI2 plasmid backbone (Liu et al., 2016). Moreover, the other plasmid replicon types have been reported that they have the ability to carry *mcr* gene belonging to IncK2, IncQ, IncF, IncFI, IncFIB, IncFII, IncN and IncY plasmids. These could indicate that *mcr*-like genes contributed worldwide by applying these diverse types of the plasmid (Ahmed et al., 2020).

The cross-resistance phenomenon between colistin and other antimicrobial drugs is another factor that maintains *mcr* genes in the variety of hosts, even without selective pressure from colistin use (Rhouma et al., 2016b). This phenomenon emphasizes the threat of these multi-drug resistance pathogens to global public health (Han et al., 2020). The worldwide screening of *mcr* gene from several previous studies indicated that the colistin resistance bacteria carries *mcr* gene together with one or several other antibiotic resistance genes, including beta-lactams (bla_{NDM-1}, bla_{NDM-5}, bla_{NDM-9}, bla_{OXA-48}, bla_{KPC-2}, bla_{VIM-1} $bla_{CTXM-1,}$ $bla_{CTXM-55}$, and $bla_{CTXM-15}$), trimethoprim (*dfr*A12), aminoglycosides (*aad*A1a and *aadA*2), sulfonamides (*sul*1, *sul*2 and *sul*3), tetracycline (*tet*A, *tet*B, *tet*D), quinolones (*qnr*S1, *qnr*B52, *qnr*B4 and *oqx*A), lincosamides [*inu*(F)], and phenicol (*cml*A1) (Rhouma et al., 2016b; Poirel et al., 2017; Ahmed et al., 2020). Of note, the integration of the *mcr* gene into bacterial chromosome is worrisome. It has been reported in some bacterial strains, indicating that the *mcr* gene might be more stable after integrating into their genome (Poirel et al., 2017).

Factor impacting on antibiotic resistance: Biofilm formation

Biofilm is a crucial virulence factor of the pathogen that initiated from bacterial communication via quorum sensing resulting and then forms a community encased by an extracellular matrix of protein, polysaccharide, and nucleic acids leading to preferentially adherence to a variety of surface, microbial aggregation, and tolerance to various stresses, e.g., disinfectants, host immunity and antimicrobial substances. Moreover, there are various critical bacterial pathogens for veterinary medicine which can produce biofilm, including *Campylobacter* spp., *Clostridium perfringens*, *Escherichia coli*, *Streptococcus* spp., *Salmonella* spp. and *Staphylococcus* spp. (Jacques et al., 2010; Miquel et al., 2016).

Biofilm formation of Gram-Negative bacteria is one of its mechanisms for antimicrobial persistence which is responsible for elevating the resistance of antibiotics more than 1,000 times by several mechanisms (Fleming and Rumbaugh,

2017). The extra polymeric matrix decreases the activity of antimicrobial agents by trapping and preventing them from reaching the target site. Also, most of the bacteria within the biofilm are slow-growing cells which could reduce the efficacy of antimicrobial agents since they exhibit the best properties on only active growing cells. The horizontal gene transfer can be occurred within the biofilm by distributing the antibiotic-resistant genes to other bacterial cells in the same environment, and the frequencies of horizontal gene transfer was higher than the planktonic cells at outside of biofilm. Moreover, the efflux pump genes are also upregulated when the planktonic cultures persist in biofilm which allows the bacterial cells to pump the antibiotics out of biofilm (Vasudevan, 2014; Rabin et al., 2015).

It has been known that Gram-negative bacteria, particularly *E. coli* can form a biofilm, and it utilizes several extracellular factors to colonize and form the biofilm (Vasudevan, 2014). The biofilm development involves many processes as follows.

(i) Primary contact to the surface

(ii) Reversible adhesion

(iii) Irreversible adhesion

(iv) Biofilm maturation and biofilm detachment

The initial stage of biofilm formation is the primary contact to the surface involving with passive movement (Gravitational forces or Brownian movement), and the surface adhesins (flagellum or curli) can assist this primary interaction between bacterial cells and the surface for biofilm formation. For reversible adhesion, motile bacterial cells or planktonic cultures have to overcome the repulsive forces (electrostatic and hydrodynamic forces) in this stage. Moreover, the environment factor such as ionic forces, pH, temperature, and type of surfaces are also important. Hydrophobic surfaces allow planktonic cells to a better adhesion than hydrophilic surfaces. In irreversible attachment, there are a variety of components that are crucial for this process, including autotransporter adhesins (Ag43, AIDA, TibA), curli fimbria, bacterial flagellum, and conjugative pili. The biofilm maturation at sessile stage is the final step of biofilm development. The several components could be found such as water, microbial cells, lipid, proteins, ions, nucleic acids, and enzymes. Moreover, various exopolysaccharide polymers, including cellulose,

β-1,6- N-acetyl-D-glucosamine polymer (PGA) and colonic acid, have also been detected. Once the number of microbial cells within biofilm achieve, detachment of sessile biofilm occurs and released cells to start a new cycle of biofilm formation (Jacques et al., 2010; Vasudevan, 2014; Hobley et al., 2015).

As above mentioned, they are the results of overuse and misuse of antibiotic, leading to antibiotic resistance, or factors for maintaining the antibiotic resistance phenomenon. Moreover, antibiotic utilization in livestock has been restricted in several countries across the world, including Thailand (Heo et al., 2013; Poolperm et al., 2020). Therefore, an alternative strategy to combat this trouble associated with antibiotic resistance should be determined.

1.2.2 Probiotic characteristics against antibiotic resistance

For the past two decades, several studies have concentered on discovering the potential alternative strategies for reducing antibiotic utilization and maintain host health. There are numerous researched alternatives, including acidifiers, essential oils, prebiotics, and probiotics. Amongst these non-antibiotic alternatives, probiotics have a high potential for combating pathogenic microorganisms. Moreover, they are also approved as generally recognized as safe (GRAS) (Wang et al., 2019b).

Probiotics

Probiotics are derived from the Greek language, which means “for life” or “in favor of life” (Liao and Nyachoti, 2017a). Probiotics have been proposed the new definition by the United Nations Food and Agriculture Organization/World Health Organization or FAO/WHO joint working group as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (WHO/FAO, 2006). Most probiotics are bacteria; however, some yeasts and mold can also be employed as probiotic (Hossain et al., 2017). For decades, probiotics have been extensively utilized in both humans and animals for their health benefits with the main reason for offering an alternative to antibiotics. Generally, it has been suggested to contain the concentration of viable cells more than 10^6 colony forming unit (CFU) per gram or milliliter of the probiotic product, indicating as functional probiotic for host health benefits (Angmo et al., 2016; Sornplang and Piyadeatsoontorn, 2016).

Lactic acid bacteria (LAB) are a common group of probiotics which have been generally studied in the past decades (Angmo et al., 2016). There are various microbial genera which commonly apply to animal fields, including *Lactiplantibacillus, Pediococcus, Saccharomyces,* and *Bacillus* (Yirga, 2015). Recently, the *Lactobacillus* genus has been reclassified into 25 genera by employing a bacterial whole-genome sequence. Moreover, *Lactobacillus plantarum* was also reclassified into the novel genus “*Lactiplantibacillus plantarum*” to enable us to better understand of general mechanisms of probiotics (Zheng et al., 2020).

These microorganisms have been anticipated as beneficial microorganisms due to providing a variety of beneficial properties, including balancing of intestinal microbiota, enhancing of nutrient utilization, inhibition the growth of pathogenic microorganisms, stimulating of immune responses, reducing of antibiotic application, and exhibiting antimicrobial activities (Angmo et al., 2016; Hossain et al., 2017).

In vitro antibacterial activity of probiotic

The group of LAB strains have been reported that they can produce a variety of antimicrobial substances to inhibit the growth of pathogens, including organic acids (lactic acid, acetic acid and formic acid), ethanol, hydrogen peroxide, carbon dioxide, fatty acids, acetaldehyde, acetoin, diacetyl, reutericyclin, reuterin, exopolysaccharides and bacteriocins (Arena et al., 2016; Chen et al., 2019; Silva et al., 2020). Several studies in both animals and humans have shown the beneficial effects of probiotics against numerous pathogens and multi-drug resistance (MDR) bacterial strains, including *Escherichia coli*, *Clostridium difficile*, *Pseudomonas aeruginosa, Staphylococcus aureus*, *Streptococcus mutans*, *Klebsiella pneumoniae*, *Salmonella* spp. and *Shigella* spp. (Chen et al., 2019; Saud et al., 2020).

From previous studies, CFS of probiotic strains in *Lactiplantibacillus* and *Bifidobacterium* genus showed the potential antibacterial activities against multidrug resistant *E. coli* isolates which resisted at least five antibiotic drugs, including ampicillin, amoxicillin plus clavulanic acid, ceftriaxone, ceftazidime, and clarithromycin. Nonetheless, some *Lactiplantibacillus* isolates could not inhibit the growth of some tested MDR *E.coli* strains (Abdelhamid et al., 2018). The CFS of *Lactiplantibacillus* isolates exhibited vigorous antibacterial activities against carbapenem-resistant *E. coli* and carbapenem-resistant *K. pneumoniae* isolates, but the antagonistic effects displayed in several degrees (Chen et al., 2019). Moreover, CFS of lactobacilli isolates also displayed the high antibacterial activities against

extended-spectrum beta-lactamase (ESBL)-producing *P. aeruginosa* and *K. pneumoniae* isolates in variable levels (El-Mokhtar et al., 2020).

Apart from antibacterial activity, several studies have been reported other remarkable activities of probiotic, including anti-cancerous and antimutagenic activities (Gorska et al., 2019; Legesse Bedada et al., 2020), anti-toxin and anti-sporulation activities (Valeria De Las et al., 2020) and antifungal and anti-mycotoxigenic activities have been mentioned in their indications (Sadiq et al., 2019). Additionally, anti-plasmid transfer activity and anti-biofilm activity have also been accounted in the controlling of the antibiotic resistance.

In vitro anti-plasmid transfer activity of probiotic

For anti-plasmid transfer activity, a few publications have exhibited antagonistic effects on horizontal transfer, for example, thermostable metabolites produced by *Bifidobacterium* strains illustrated effect to significantly reduce in various antibiotic resistance transfer (beta-lactam, kanamycin, and tetracycline) amongst *Enterobacteriaceae* which these substances might impact on conjugation process by affecting donor pili or recipient cell surfaces (Moubareck et al., 2007). The cellular fractions of *Enterococcus faecium* were potential to increase susceptibility pattern for numerous antibiotics (beta-lactam, aminoglycosides, and quinolones) in enteropathogenic *E. coli* and the possible mechanisms for this phenomenon might occur from decrease enzyme expression associated with antibiotic resistance and affect the cell wall structure of *E. coli* targeting the antibiotic activities (Ditu et al., 2011). Extracts from diverse probiotics (*B. longum*, *S. thermophilus*, *S. lactis*, *L. casei*, *L. plantarum*, *L. acidophilus*, *L. rhamnosus* and *L. delbueckii*) could thoroughly eradicate the plasmid- encoded antibiotic resistance (chloramphenicol, doxycycline, erythromycin, lincomycin, penicillin, and gentamycin) or called plasmid curing activity (Nehal El-Deeb, 2015b). In addition, low pH and anaerobic conditions can influence transconjugant production in the conjugation process (Viljanen and Boratynski, 1991; Hossain et al., 2017). For carbon dioxide (CO_2) production, the probiotic as CO_2

producer also involved with plasmid curing functions (Viljanen and Boratynski, 1991; Angmo et al., 2016).

In vitro anti-biofilm activity of probiotic

For anti-biofilm activity, it has been proposed that probiotics can influence the formation of biofilm by several possible substances or mechanisms, including extracellular substance (Fang et al., 2018b), biosurfactant (Walencka et al., 2008; Zakaria Gomaa, 2013; Sharma et al., 2015; Giordani et al., 2019), exopolysaccharides (Kim et al., 2009; Mahdhi et al., 2018), bacteriocins (Mathur et al., 2018; Kim et al., 2019), different enzymes (Nijland et al., 2010; Thallinger et al., 2013; Barraud et al., 2015) or quorum quenching activity (Park et al., 2014; Kim et al., 2018). They involve the inhibition of the growth biofilm-producing microorganisms, hindering bacterial attachment, aggregation and quorum sensing (Barzegari et al., 2020).

Previously, the biofilm production of multidrug resistant *E. coli* isolates was reduced over 64% by using CFS of *L. plantarum*. Whereas, the CFS of *B. longum* could decrease biofilm formation of multidrug resistant *E. coli* isolates up to 57% (Abdelhamid et al., 2018). Cui et al. (2018) have reported that neutralizing CFS of LAB isolates could inhibit biofilm obtained from *E. coli* and *S. aureus* by more than 50%. However, they found that some LAB isolates could not reduce biofilm formation in both tested strains, and they also indicated that the anti-biofilm ability of LAB was strain-specific dependence (Cui et al., 2018).

Moreover, the previous study of Kaur and colleagues have reported that non-neutralizing CFS of lactobacilli isolates could decrease biofilm of *Vibrio* spp. up to 96%, and it could disperse the sessile biofilm formed by *Vibrio* spp. up to 85%. For neutralizing CFS of lactobacilli isolates, it still reduced biofilm production of *Vibrio* spp. by more than 90%, and it showed a maximum of mature biofilm interference up to 75%. Nevertheless, some lactobacilli isolates did not show an anti-biofilm effect on some *Vibrio* spp. (Kaur et al., 2018). These outcomes are contrary to that of Chapman et al. (2014) who found that neutralizing CFS of

lactobacilli strains showed no anti-biofilm activity on *E. coli* NCTC 9001 and *E. faecalis* NCTC 00775 (Chapman et al., 2014).

The specific manner of probiotic characteristics

The probiotic characteristics are considered as specific manners even there are within the same species, but they are not necessarily applicative to other strains (Bermudez-Brito et al., 2012; Hill et al., 2014). As above mentioned, the probiotic effects of *in vitro* antibacterial and anti-biofilm activities demonstrate the diverse ranges of their properties (Chapman et al., 2014; Abdelhamid et al., 2018; Cui et al., 2018; Kaur et al., 2018; Chen et al., 2019; El-Mokhtar et al., 2020). Thus, the isolated probiotics approving the species identification and safety aspects need to be further determined their other potential characteristics (Sornplang and Piyadeatsoontorn, 2016).

From our previous study, a total of 204 of probiotic isolates from 60 Thai healthy fattening indigenous and commercial pigs in antibiotic-free farm locating at Nan and Chai-nart province. A 34 of acid and bile tolerant isolates were performed the species identification by using whole-cell protein patterns and 16S rDNA sequencing analysis before examining antimicrobial susceptibility determination. Only five isolates comprise of *Pediococcus pentosaceus* 77F, *Lactiplantibacillus plantarum* (22F, 25F and 31F) and *Pediococcus acidilactici* 72N displayed the susceptibility to the eight antibiotics (vancomycin, erythromycin, ampicillin, gentamicin, chloramphenicol, tetracycline kanamycin and streptomycin) followed the criteria of European Food Safety Authority (EFSA) (Sirichokchatchawan et al., 2017b).

Those five probiotic isolates were further determined *in vitro* on functional and safety properties, including antibacterial activities against the common swine enteric pathogens, including *Salmonella* Choleraesuis, *Streptococcus suis* and pathogenic *Escherichia coli*, and antiviral activity against porcine epidemic diarrhea virus (PEDV) as well as antimicrobial resistance genes detection. Ultimately, only

L. plantarum 22F, *L. plantarum* 25F and *P. acidilactici* 72N were the most three potential probiotics from all tested properties, and they are also suitable as probiotic candidates for the further investigations in the aspect of probiotic characteristics against antibiotic resistance. (Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b).

1.2.3 Gut microbiome and resistome in pigs

For the past decades, the rapid development of high-throughput sequencing approaches had enabled the analysis of metagenomic fields for understanding microbial diversity, ecology, and evolution (Quince et al., 2017; Zaheer et al., 2018). Metagenomic studies can provide the knowledge of complex microbial communities in several areas including gastro-intestinal (GI) tract of both humans and animals rendering to understand the association between microbial communities and hosts (Zaheer et al., 2018). Shotgun metagenomics is the method for untargeted sequencing of all microbial genome within the sample. It had been employed in a variety of aspects including the composition of taxonomic profiles, determination of unique features (mobilome, virulome and resistome), the functionality of microbial communities and recovery of whole genome sequences (Quince et al., 2017; Lanza et al., 2018; Zaheer et al., 2018).

Gut microbiota in pigs

The porcine gastro-intestinal tract (GIT) harbors complicated and various microbial communities, and most of them are bacteria. The estimation of total microbiota inhabiting within the gut is probably 10^{10}-10^{14} bacteria (Kim and Isaacson, 2015; Guevarra et al., 2019). The gut microbiota of pigs performs several beneficial roles associated with sustaining physiological, nutritional, and immunological functions (Fouhse et al., 2016; Guevarra et al., 2019).

The swine GIT has been believed that it is sterile organ before birth, and microorganisms colonize it after parturition through vertical contact (vagina, feces, and skins of a sow) and horizontal contact (surrounded environment) (Nowland et al., 2019; Knecht et al., 2020). The colonization of microbes can be called that “microbial succession” process which completely established within weeks after the birth (Liao and Nyachoti, 2017a; Guevarra et al., 2019). When the microbial community is in balanced co-existence or symbiosis, the gut of pigs will be healthy, and it can perform function efficiently. Whereas, once the microbial community is dysbiosis or overgrowth of pathogens, it can cause clinical abnormalities (diarrhea,

ulcer, constipation, poisoning, or gas bloating), leading to reduction of nutrient utilization and retardation growth performance (Isaacson and Kim, 2012; Liao and Nyachoti, 2017a).

The utilization of pigs as an alternative animal model for the study about gut microbiota in human has been suggested according to various similarities of anatomy and physiology (Heinritz et al., 2013). Several studies have reported the gut microbiota in diverse niches of swine GIT, including stomach, duodenum, jejunum, ileum, cecum, proximal colon, distal colon, and rectum. The high beta diversity and low alpha diversity are observed in the foregut, whereas low beta diversity and high diversity are noticed in the hindgut (Crespo-Piazuelo et al., 2018; Gresse et al., 2019). The gut microbiota composition in feces collected from rectum seems to be stable, and it shows the same pattern with hindgut regions, indicating that it can be used as a representation of gut microbiota in the large intestine of the pigs (Zhao et al., 2015; Gresse et al., 2019).

The composition of the intestinal microbiota of pigs involves several factors such as host genetics, dietary change, feed additives (Liao and Nyachoti, 2017a; Guevarra et al., 2019). During the weaning transition, it is one of the critical periods of the pigs' life due to exposing to many stressful factors, including environmental alterations, psychological challenges, nutritional and physiological changes. The most crucial factor is nutritional changes to plant-based solid feed instead of sow's milk, leading to the physiological modification of morphology and functions in piglet's intestine. This nutritional change can also shift the gut microbiota of piglets for supporting nutrient utilization and absorption. Moreover, weaned piglets have been reported that they are one of the significant reservoirs of ARGs in the farm which elevated the antibiotic resistance by disseminating ARGs to other pathogens or environment, and the immune system of piglets in this period is still an immature stage, leading more chances to get infections. Consequently, it precedes to increased susceptibility of harmful microorganisms that disrupt the gut microbiota and cause post-weaning diarrhea (PWD). PWD is commonly caused by enterotoxigenic *Escherichia coli* (ETEC), and it is the major concern in the pig industry due to high

mortality rate of piglets (Liao and Nyachoti, 2017a; Guevarra et al., 2018; Li et al., 2018; Lugsomya et al., 2018b; Guevarra et al., 2019).

The modulation of the gut ecosystem is one of the general strategies for preventing diarrhea, enhancing health status and growth performance in pigs by applying feed additives, including administration of an in-feed antibiotic, supplementation of prebiotic, probiotic and symbiotic or utilization of botanical products, enzymes, organic acids and inorganic acids (Liao and Nyachoti, 2017a).

Antibiotic administration impacting on the gut microbiome in pigs

The antibiotics have been employed for past decades in worldwide swine production with several indications such as prophylactic use, therapeutic use and subtherapeutic use (Liao and Nyachoti, 2017a). The use of antibiotic with subtherapeutic indication can promote piglet growth performance by modulation of gut microbiota. (Guevarra et al., 2019). For example, tylosin administration to the pigs could cause the shift of gut microbiota composition by increasing the genus of *Acetanaerobacterium*, *Eggerthella*, *Lactiplantibacillus* and *Sporacetigenium*, indicating it elevated the microbial development and maturation to adult-like microbiota which resulted in improvement of growth performance (Kim et al., 2012).

Nonetheless, the modes of action of antibiotics demonstrate that they may impact on both susceptible commensals and pathogenic microorganisms in the gut. The study of Ghanbari and colleagues showed that administration of oxytetracycline reduced not only bacterial diversity but also bacterial richness in the gut microbiota of piglets, and the diversity of bacterial community could not be fully recovered even the withdrawal of oxytetracycline was performed for two weeks (Ghanbari et al., 2019). Piglets exposing to antibiotic may elevate colonization of pathogenic microorganisms and increase the susceptibility of diseases by suppressing the host's innate immune responses (Fouhse et al., 2016). These data are correlated with the findings of Wang and colleagues that piglets receiving apramycin sulfate revealed the high relative abundance of phylum *Proteobacteria* and family of *Spirochaetae* and

Campylobacteraceae. They are recognized as pathogens, leading to cause disturbance of gut microbiota and gastroenteritis (Wang et al., 2019b). Moreover, antibiotic usages have been concerned associated with the emergence of antibiotic-resistant microorganisms and residues of used antibiotics in animal products and manures. Antibiotic resistance bacteria (ARBs), antibiotic resistance genes (ARGs) and antimicrobial agents (AMAs) can distribute into the environment and may transfer to human through the food chain and water cycle which critically impact on public health (Thanner et al., 2016; Liao and Nyachoti, 2017a; Guevarra et al., 2019; Joyce et al., 2019).

The use of antibiotics as feed additives has been reported that it can enrich the abundance and diversity of ARGs and mobile genetic elements (MGEs) in pig's gut. After investigation of ARGs and MGEs in pig receiving in-feed antibiotic, 146 ARGs conferring the resistance to multidrug, aminoglycoside, beta-lactam, tetracycline, MLSB, vancomycin, sulfonamide, chloramphenicol, and others, and 10 MGEs associated with class 1 integron–integrase genes and transposon-transposase genes could be detected from all samples. Antibiotic deactivation, efflux pumps and cellular protection were the main mechanisms found in that study (Zhao et al., 2018). Another study also shows the consistent results that pigs acquiring oxytetracycline exhibited high diversity and abundance of ARGs, especially tetracycline, beta-lactam and multi-drugs through ribosomal protection proteins, class A beta-lactamases and multidrug efflux pumps mechanisms (Ghanbari et al., 2019).

According to several adverse effects of antibiotic use, numerous countries have banned antibiotic usages in livestock production such as European Union (EU), Canada, the USA, and Thailand (Guevarra et al., 2019; Poolperm et al., 2020) Therefore, another potential feed additive like probiotic has been raised attention to use as alternative to in-feed antibiotic for controlling the enteric pathogens and modulating gut microbiota (Hossain et al., 2017; Liao and Nyachoti, 2017a).

Probiotic supplementation affecting gut microbiome in pigs

Probiotics have been considered extensively as an alternative to in-feed antibiotics in the livestock industry as direct-feed microbial (DFM) (Fouhse et al., 2016). Probiotics, especially LAB group which are commonly used in animal feeds comprise numerous genera of *Pediococcus* spp., *Lactiplantibacillus* spp., *Bacillus* spp., *Enterococcus* spp., *Lactococcus* spp. and *Leuconostoc* spp. (Liao and Nyachoti, 2017a). The previous studies have reported that supplementation of probiotic to animals and humans provides several beneficial effects such as modulation of gut microbiota, improvement of gut immunity, the elevation of disease resistance, reduction of pathogen shedding, a decrease of disease symptoms and increase of health status, enhancement of nutrient utilization, alleviation of oxidative stress and alteration of gene expression of bacteria and host. (O'Toole and Cooney, 2008; Hossain et al., 2017; Liao and Nyachoti, 2017a).

There are many modes of probiotic action effecting on gut microbiota including, competing adhesion site within intestinal epithelium for hindering attachment from harmful microorganisms, competing the nutrients and energies which commonly are carbon sources by rapidly using nutrients, energy sources and enzymes (*e.g.* arginine dehydrogenase) to suppress the growth of undesirable bacteria, trapping pathogenic microorganisms by coaggregation action, producing antimicrobial compounds (*e.g.* lactic acid, acetic acid, hydrogen peroxides, diacetyl, carbon dioxide and antimicrobial peptides) for inhibiting the growth of pathogens, enhancing gut barrier function by overproduction of mucin to prevent the adhesion of pathogens, inducing innate immunity such as phagocytic activities to deal with these harmful bacteria, stimulating immune response such as producing antibody against pathogenic bacteria (O'Toole and Cooney, 2008; Hossain et al., 2017; Kassaa, 2017; Liao and Nyachoti, 2017a).

The supplementation of probiotics to the piglets can expand the diversity of the gut microbial community, leading to the exclusion of pathogenic microorganisms from colonization of pathogens and cause of diseases (Fouhse et al., 2016). In a study by Riboulet-Bisson and colleagues, the pigs supplemented with

L. salivarius UCC118 showed the decreased abundances of *Lactonifactor*, *Anaerostipes* and *Treponema*. In contrast, the relative abundance of *Hallella*, *Oribacterium* and *Sudboligranulum* were increased. However, it could not improve growth performance and productivity of pigs (Riboulet-Bisson et al., 2012).

The supplementation of *L. plantarum* PFM105 could increase the relative abundance of beneficial microbes such as *Bifidobacteriaceae* and *Prevotellaceae*, which are associated with nutrient utilization and anti-inflammatory activity. Moreover, it could enhance the small intestine development and growth performance in piglets, decrease the mortality rate and diarrhea incidence, enhance the metabolic capacity of the gut microbiota by increasing the expression of critical metabolic genes (Wang et al., 2019b).

The study of Shin and colleagues has demonstrated that *L. plantarum* JDFM LP11 could increase the richness and diversity of gut microbiota, and family *Ruminococcaceae* was increased in the probiotic supplemented group by more than 25 % when compared to the control group. This family was related to short chain fatty acid (SCFA) production and subsequently, modulation of gut health, inhibition of pathogenic growth and providing the anti-inflammatory effects (Shin et al., 2019).

The supplementation of the *Bifidobacterium longum* subsp. *infantis* EVC001 to the breastfed infants could significantly reduce the relative abundance of ARGs by more than 90% when compared to the control group. This phenomenon might occur from the modulation of gut microbiota by decreasing the ARGs carriers, especially *Enterobacteriaceae* family such as *Escherichia coli* (Casaburi et al., 2019).

Albeit, several studies exhibit the effect of probiotic on the modulation of gut microbiota in pigs and reduction of ARGs in human gut, but to the best of our knowledge, no study has yet determined the effect of probiotic supplementation on the modulation of antimicrobial resistance genes in GI tract of pigs (Ma et al., 2019).

Although those studies have shown that probiotic supplementation can modulate gut microbiota of the pigs, the inconsistent responses to the gut microbiome can be found, indicating that each probiotic strain has an individual effect (Fouhse et al., 2016) Generally, the application of probiotics isolated from the same host origin is ideally advantageous according to their efficient function in the

same gut environment of the host (Sirichokchatchawan et al., 2017b). Therefore, this study aims to monitor and observe the effect of probiotic prototype fed in neonatal pigs on gut microbiota and antibiotic resistance gene (ARGs) in weaned piglets and weaned piglets challenging with enterotoxigenic *Escherichia coli*.

1.3 Research hypotheses

1.3.1 The selected lactic acid bacteria can reduce the capability of horizontal transfer of plasmid containing *mcr*-1 gene in *Escherichia coli*.

1.3.2 The selected lactic acid bacteria can interfere with biofilm production of *Escherichia coli*.

1.3.3 The probiotic prototype fed in neonatal pigs can affect gut microbiome and antibiotic resistance gene (ARGs) in weaned piglets and the weaned piglets infected with enterotoxigenic *Escherichia coli*.

1.4 Research objectives

1.4.1 To evaluate anti-conjugation activity of the selected lactic acid bacteria against *Escherichia coli* harboring *mcr*-1 gene.

1.4.2 To examine the anti-biofilm activity of the selected lactic acid bacteria on biofilm formation produced by *Escherichia coli*.

1.4.3 To monitor and observe the efficacy of probiotic prototype fed in neonatal pigs on gut microbiome and antibiotic resistance genes (ARGs) modulation in weaned piglets and the weaned piglets infected with enterotoxigenic *Escherichia coli*.

1.5 Advantages of Study

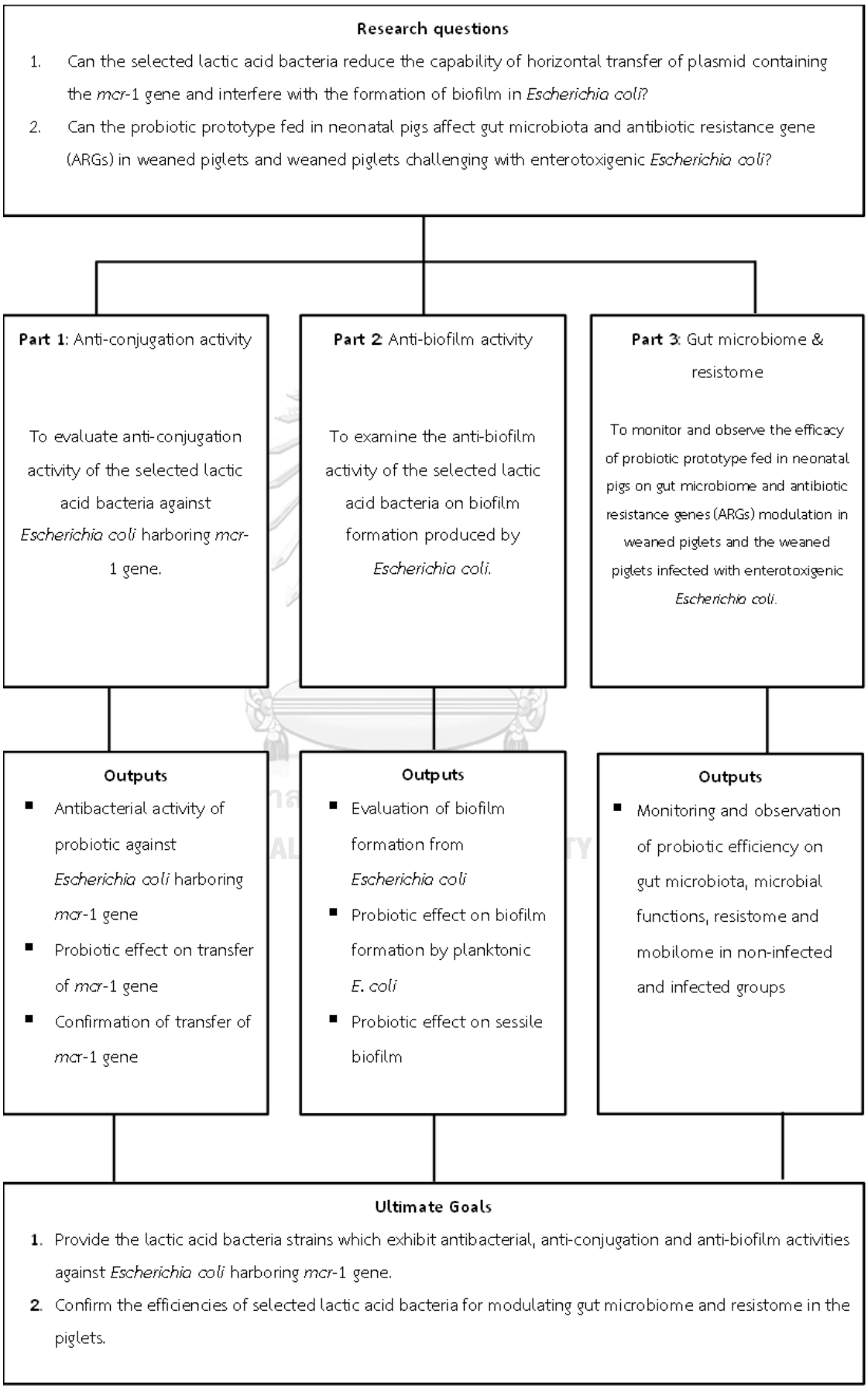
1.5.1 This study is able to propose the lactic acid bacteria strains which exhibit antibacterial, anti-conjugation and anti-biofilm activities against *Escherichia coli* harboring *mcr*-1 gene.

1.5.2 This study is able to confirm the efficiencies of selected lactic acid bacteria for modulating gut microbiome and resistome in piglets.

1.6 Keywords

antibiotic-resistant bacteria, microbiome, pig, probiotics, resistome

1.7 Conceptual framework



CHAPTER II

Anti-conjugation and anti-biofilm evaluation of probiotic strains *Lactobacillus plantarum* 22F, 25F and *Pediococcus acidilactici* 72N against *Escherichia coli* harboring *mcr*-1 gene

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2.1 Abstract

Several species of lactic acid bacteria (LAB) are commonly used as probiotics and as an alternative to antibiotics in various industries, especially in the livestock industry. This study aimed to investigate the anti-conjugation and anti-biofilm activity of cell-free supernatant (CFS) of Thai LAB strains (*Lactiplantibacillus plantarum* 22F, 25F, and *Pediococcus acidilactici* 72N) against colistin-resistant *E. coli* isolates. A total of 6 colistin-resistant *E. coli* strains were isolated from different sources, including pigs, farmers, and farmhouse environments. The *E. coli* were characterized by plasmid profiling, PCR detection of *mcr*-1 gene, and antibiotic susceptibility patterns. The CFS at the dilutions ≥1:16 was chosen as the proper dilution for anti-conjugation assay. Besides, it could significantly reduce the transfer frequencies of resistance gene *mcr*-1 up to 100 times compared to the neutralizing CFS (pH 6.5). The biofilm production in the planktonic stage was reduced by non-neutralizing and neutralizing CFS determining with crystal violet staining assay up to 82% and 60%, respectively. Moreover, the non-neutralizing CFS also inhibited the biofilm formation in the sessile stage up to 52%. The biofilm illustration was confirmed by scanning electron microscopic (SEM). These results agreed with the findings of the crystal violet technique, which showed a significant reduction in the cell density, aggregation, and extracellular polysaccharide (EPS) matrix. The application of Thai LAB may serve as an attractive alternative to antibiotics for reducing biofilm formation and limiting the proliferation of antibiotic-resistant genes.

2.2 Introduction

Antimicrobial resistance (AMR) is one of the serious global health concerns that threaten both animal and human survival. The increase in resistance has made challenging to treat such type of infections caused by antibiotic-resistant bacteria. Such bacterial infections can lead to ineffective treatments, higher treatment costs, and mortality in humans and animals. By 2050, antimicrobial-resistant pathogens could cause 10 million deaths annually with an expected cost of $100 trillion (O'Neill, 2014). Antibiotics are widely used as a feed additive in livestock production to improve growth performance and combat several infections. Colistin is used as a last resort for the treatment of multi-resistant bacterial infections not only in humans but also in animals, especially in swine (Gao et al., 2016; Liu et al., 2016; Ye et al., 2016). The emergence of plasmid-mediated colistin resistance encoded by the *mcr*-1 gene in *E. coli* isolates of pigs, chickens, and humans has raised global concern about the potential horizontal transfer of this gene between humans and animals (Liu et al., 2016). Therefore, the rapid spread of colistin-resistant *E. coli* has been found in many countries, and more than ten additional gene homologs of *mcr* have been identified since then (Liu et al., 2016; Wang et al., 2020a). Worldwide, there are more reports of *mcr*-mediated resistance in animals when compared to human isolates, suggesting that plasmid-mediated colistin resistance is more prevalent in livestock (Luo et al., 2020).

Bacterial biofilms, the polymeric substances secreted by microbes, are one of the main resistance mechanisms that bacteria use to survive against various stresses, including antibiotics, disinfectants, and host defenses (Jacques et al., 2010). Biofilms decrease the activity of antimicrobial agents by trapping and preventing them to reach the target sites. Besides, most of the biofilm-forming bacteria are less active metabolically, which could reduce the efficacy of antibiotics, which are effective against active dividing cells (Vasudevan, 2014). Consequently, microbial biofilms

present a severe medical problem and contribute to the development of chronic and recurrent infections in both humans and animals. Therefore, there is an urgent need to find alternative therapies that can overcome these challenges.

Recently, food-based probiotics have assumed great significance for their nutritional and therapeutic potential (Hossain et al., 2017). Probiotics are defined by the World Health Organization (WHO) as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (WHO/FAO, 2006). Probiotics have been categorized by genus, species, and strain, for example, *Lacticaseibacillus rhamnosus* GG. Studies have shown that physiological benefits of probiotics are strain-specific since different strains of the same species can have different health effects (O'Toole and Cooney, 2008; Hossain et al., 2017). During the past few decades, Lactic acid bacteria (LAB), a popular member of probiotic, have been extensively used in humans and animals for various purposes to enhance nutrient utilization, to modulate both the innate and the adaptive immune systems, and to inhibit the growth of numerous pathogenic microorganisms (Angmo et al., 2016; Liao and Nyachoti, 2017a). Besides, LAB strains have been shown to limit the emergence of bacterial resistance by inhibiting the horizontal transmission of resistance genes (Moubareck et al., 2007; El-Deeb et al., 2015) and biofilm production (Fang et al., 2018b; Mahdhi et al., 2018). The LAB produces several active metabolites, including organic acids, bacteriocins, hydrogen peroxides, exopolysaccharides and, biosurfactants, all of which may prevent the formation of biofilms (Hossain et al., 2017). Generally, most of the metabolites are secreted into broth medium during the propagation of bacteria and known as supernatant. The LAB supernatant exhibits the anti-conjugation, and anti-biofilm activity against various pathogens, as mentioned above. Based on our previous studies, *L. plantarum* 22F, 25F and, *Pediococcus acidilactici* 72N showed a promising performance and strong antibacterial activity against enteric pathogens (Sirichokchatchawan et al., 2017b; Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b). However, their

anti-biofilm and anti-conjugation potentials were not determined yet. To the best of our knowledge, this is the first report on the anti-biofilm and anti-conjugation activity of LAB using cell-free supernatant (CFS) against colistin-resistant *E. coli*.

In line with that, the objective of this study was to evaluate the anti-conjugation, and anti-biofilm activities of CFS of different LAB species (*L. plantarum* 22F, 25F, and *P. acidilactici* 72N) against *E. coli* harboring *mcr*-1 gene from human, pig, and environmental origins.

2.3 Materials and methods

2.3.1 Bacterial strains

All of the methods, LAB isolates and E. coli isolates used in this experiment were approved by Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University under Biosafety Use Protocol number IBC 1931004.

In our previous studies, *Lactiplantibacillus plantarum* 22F, 25F, and *Pediococcus acidilactici* 72N were isolated in Thailand from antibiotic-free healthy commercial fattening and indigenous pigs (Sirichokchatchawan et al., 2017b). The LAB isolates displayed attractive probiotic properties, and their *in vitro* features make them potential candidates for probiotic applications (Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b). In this study, six *mcr*-1 positive colistin-resistant isolates of *E. coli* was employed based on antimicrobial sensitivities, plasmid replication, biofilm formation, and the source (Table 1). These isolates were collected from feces or wastewater at swine farm in central part of Thailand. The biohazard execution control was approved by Institutional Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (IBC 1731021). The samples were collected directly into a sterile container and transferred to laboratory at 4°C. All samples were ten-fold diluted in sterile normal saline, and the dilutions at 10^7-10^8 were spread on Eosin Methylene blue agar (Oxoid, Hampshire,

England, UK) supplemented with colistin (2 μg/ml: Sigma, St Louis, MO, USA) for selecting colistin-resistant *E. coli*. A representative pure colony was selected randomly to detect the *mcr*-1 gene by using a specific primer as described previously (Liu et al., 2016). In addition, wild-type *E. coli* J53 was used as the recipient strain to examine bacterial conjugation (Matsumura et al., 2018). This strain is negative for fertility factors and resistant to sodium azide (MIC >512 μg/ml), and sensitive to colistin (MIC <2 μg/ml). All isolates used in this study were affirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) Biotyper (Bruker Daltonics, MA, USA) according to the manufacturer's recommendation with a high-confidence identification score. The colony of Gram-negative bacteria was thin-film smeared on the target plate before adding 1 μl of the matrix which contains CHCA (α-cyano-4-hydroxycinnamic acid) dissolved in 2.5% TFA (trifluoroacetic acid) and 50% acetonitrile, whereas the colony of Gram-positive bacteria was smeared on the target plate as a thin layer before adding 1 μl of 70% formic acid and 1 μl of the matrix after completely air-drying. The target plate was placed into the MALDI-TOF Biotyper machine after air drying at room temperature, and the extracted molecules were analyzed and compared with the reference database. Only a high-confidence identification score values by more than 2.00 was accepted. Moreover, BTS (Bruker's bacterial test standard) was used as quality control for calibrating the MALDI-TOF machine before each identification (Singhal et al., 2015).

2.3.2 Plasmid replicon typing

All colistin-resistant *E. coli* strains were extracted the bacterial DNA by employing GeneJET Genomic DNA Purification Kit (catalogue no. K0721; ThermoFisher Scientific, MA, USA) according to manufacturer's recommendations. They were determined the PCR-based replicon typing (PBRT) by using multiplex and simplex PCR with over 18 replicon types, including IncF (FIA, FIIA, FIB, FIC, and Frep), I1-Iγ, N, P, W, HI1, HI2, L/M, T, A/C, K, B/O, X, and Y. The PBRT was carried out using specific primers and conditions as previously described (Carattoli et al., 2005). Table 2 shows the 18 pairs of the specific primer for different plasmid replicon types that used in this study. For all PBRT amplification, except IncF type, it was started by initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute with final extension at 72°C for 5 minutes. For IncF type, the PCR was performed with the same amplification condition except with the temperature of annealing at 52°C for 30 seconds. PCR positive replicons identified in our previous studies were used as positive controls (Lugsomya et al., 2018a; Lugsomya et al., 2018b).

Table 1 Profiles of lactic acid bacteria and *Escherichia coli* strains used in this study.

Bacteria	Isolate	Accession number	Antibiogram	Plasmid replicon	Colistin MIC (μg/ml)	*mcr*-1 gene	Biofilm formation	Origin	Reference
Lactobacillus plantarum	22F	LC035101	CST	ND	ND	ND	ND	pig	(Sirichokchatchawan et al., 2017)
	25F	LC035105	CST	ND	ND	ND	ND	pig	
Pediococcus acidilactici	72N	LC035107	CST	ND	ND	ND	ND	pig	
Escherichia coli	P01	NA	AMP-AMX- CEF- CHL-CLX-CPD- CST-ENRO-INN-MFX-NIT-PIP-SXT-TET	FIB, Frep, W	8 (R)	+	strong	pig	This study
	P02	NA	AMP-AMX-CLX-CHL-CST-PIP-SXT	Frep	16 (R)	+	strong	pig	
	H01	NA	AMP-AMX- CLX-CST-PIP-TET	FIB	4 (R)	+	strong	human	
	H02	NA	AMP-AMX-CEF-CHL-CLX-CPD-CST-INN-GEN-MFX-PIP-SXT-TET	FIB, Frep	8 (R)	+	strong	human	
	E01	NA	AMP-AMX-CHL-CLX-CST-ENRO-GEN-MFX-PIP-SXT-TET	FIB, Frep, Y	4 (R)	+	strong	environment	
	E02	NA	AMC-AMP-AMX-CEF-CHL-CLX-CPD -CST-ENRO-INN-GEN-MFX-PIP	FIB, Frep	4 (R)	+	moderate	environment	
	J53	NA	AMP-AMX	not detected	<2 (S)	-	ND	NA	

R represents resistance to colistin (MIC values are more than 2) and S represents susceptibility to colistin. Isolates of E. coli which showed positively to mcr-1 gene by using PCR are expressed as + (presence) or – (no presence). AMC, amoxicillin–clavulanic acid; AMP, ampicillin; AMX, amoxicillin; CEF, ceftiofur; CHL, chloramphenicol; CLX, cefalexin; CPD, cefpodoxime; CST, colistin; ENRO, enrofloxacin; ERY, erythromycin; KAN, Kanamycin; INN, Cefovecin; IPM, Imipenem; GEN, gentamicin; MFX, marbofloxacin; NA, no available; ND, no determined; NIT, nitrofurantoin; PIP, piperacillin; STR, Streptomycin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TYL, Tylosin.

Table 2 Specific primers for different replicon types used in this study.

No.	Target sites	Product size (bp)	Primers	Nucleotide sequences (5' – 3')
1	iterons	462	FIA-F	ccatgctggttctagagaaggtg
			FIA-R	gtatatccttactggcttccgcag
2	repA	270	FIIA-F	ctgtcgtaagctgatggc
			FIIA-R	ctctgccacaaacttcagc
3	repA	702	FIB-F	ggagttctgacacacgattttctg
			FIB-R	ctcccgtcgcttcagggcatt
4	repA2	262	FIC-F	gtgaactggcagatgaggaagg
			FIC-R	Ttctcctcgtcgccaaactagat
5	RNAI/repA	270	Frep-F	tgatcgtttaaggaattttg
			Frep-R	gaagatcagtcacaccatcc
6	RNAI	139	I1-F	cgaaagccggacggcagaa
			I1-R	tcgtcgttccgccaagttcgt
7	repA	559	N-F	gtctaacgagcttaccgaag
			N-R	gtttcaactctgccaagttc
8	iterons	534	P-F	ctatggccctgcaaacgcgccagaaa
			P-R	tcacgcgccagggcgcagcc
9	repA	242	W-F	cctaagaacaacaaagccccccg
			W-R	ggtgcgcggcatagaaccgt
10	parA-parB	471	HI1-F	ggagcgatggattacttcagtac
			HI1-R	tgccgtttcacctcgtgagta
11	iterons	644	HI2-F	tttctcctgagtcacctgttaacac
			HI2-R	ggctcactaccgttgtcatcct
12	repA,B,C	785	L/M-F	ggatgaaaactatcagcatctgaag
			L/M-R	ctgcaggggcgattctttagg
13	repA	750	T-F	ttggcctgtttgtgcctaaaccat
			T-R	cgttgattacacttagctttggac
14	repA	465	A/C-F	gagaaccaaagacaaagacctgga
			A/C-R	acgacaaacctgaattgcctcctt
15	RNAI	160	K-F	gcggtccggaaagccagaaaac
			K-R	Tctttcacgagcccgccaaa

No.	Target sites	Product size (bp)	Primers	Nucleotide sequences (5’ – 3’)
16	RNAI	159	B/O-F	gcggtccggaaagccagaaaac
			B/O-R	tctgcgttccgccaagttcga
17	ori γ	376	X-F	aaccttagaggctatttaagttgctgat
			X-R	tgagagtcaatttttatctcatgttttagc
18	repA	765	Y-F	aattcaaacaacactgtgcagcctg
			Y-R	gcgagaatggacgattacaaaacttt

2.3.3 Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of antimicrobial for all *E. coli* isolates were performed by the Vitek®2 compact automated Identification/Antimicrobial sensitivity testing instrument (BioMe´rieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. The overnight culture of all *E. coli* isolates was measured the bacterial concentration to McFarland 0.50 (approximately $1.5x10^8$ CFU/ml) by using Vitek DensiCheck plus™ in 0.45% sterile normal saline. The AST-GN 65 test kit was added into Vitek tube containing bacterial suspension before placing into the cassette. It was loaded into the filler station and transferred into Vitek®2 compact cassette loading station, respectively. The MIC results were reported within 4 to 18 hr. The AST-GN 65 test kit card contains several tested antimicrobial agents including AMK (Amikacin), AMC (amoxicillin-clavulanic acid), AMP (ampicillin), AMX (amoxicillin), CEF (ceftiofur), CHL (chloramphenicol), CLX (cefalexin), CPD (cefpodoxime), ENRO (enrofloxacin), INN (cefovecin), IPM (imipenem), GEN (gentamicin), MFX (marbofloxacin), NIT (nitrofurantoin), PIP (piperacillin), POL (Polymyxin B), SXT (trimethoprim/sulfamethoxazole), TET (tetracycline) and TOB (tobramycin). In addition, the existence of ESBL (Extended Spectrum Beta-Lactamase) is also included in this test kit card. Table 3 exhibits the interpretation of the MIC breakpoints that are reported according to several organizations, including CLSI (Clinical and Laboratory Standards Institute), EUCAST (The European Committee on Antimicrobial Susceptibility Testing) and FDA (Food and Drug Administration) (Dell'Orco et al., 2019; Khine et al., 2020).

Table 3 The MIC breakpoints of antimicrobial agents containing in AST-GN 65 test kit card.

No.	Antimicrobial agents	Concentration range (µg/ml)	MIC Breakpoints	
			Intermediate	Resistance
1	Amikacin	2 - 64	32	≥ 32
2	Amoxicillin–clavulanic acid	2-1/32-16	16	≥ 32
3	Ampicillin	2-32	16	≥ 32
4	Ceftiofur	1-8	4	≥ 8
5	Chloramphenicol	2-64	16	≥ 64
6	Cefalexin	4-64	16	≥ 64
7	Cefpodoxime	0.25-8	2-4	≥ 8
8	Enrofloxacin	0.12-4	1-2	≥ 4
9	Cefovecin	0.5-8	4	≥ 8
10	Imipenem	1-16	8	≥ 16
11	Gentamicin	1-16	1-2	≥ 16
12	Marbofloxacin	0.5-4	2	≥ 4
13	Nitrofurantoin	16-512	64	≥ 128
14	Piperacillin	4-128	64	≥ 128
15	Polymyxin B	0.25-16	-	≥ 16
16	Trimethoprim/Sulfamethoxazole	20-320	-	≥ 320
17	Tetracycline	1-16	8	≥ 16
18	Tobramycin	1-16	8	≥ 16
19	Extended Spectrum Beta-Lactamase	NEG (negative) or POS (positive)		

2.3.4 Preparation of LAB cell-free supernatants (CFS)

Cell-free supernatants (CFS) were prepared as described previously with minor modifications (Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b). Briefly, each LAB isolates at 10^8 CFU/mL concentration were inoculated into 30 mL of MRS (de Mann Rogosa Sharpe) broth (Becton, Dickinson, and Company, MD, USA) left incubated at 37℃ for 24 h. Subsequently, all CFS were obtained by centrifugation for 10 min at 4500 rpm and 4 ℃. The collected supernatants were separated into two groups, a cell-free fraction and neutralizing fraction, where the latter was obtained by adjusting pH to 6.5±0.1 using 1 M NaOH (Carlo Erba Reagents, Val de Reuil, France). Both fractions were filter-sterilized by 0.22 µm surfactant-free cellulose acetate filters (Corning, NY, USA).

2.3.5 Preparation of CFS dilution

In our previous study, three LAB strains had shown strong antibacterial activity against enteric pathogens (Sirichokchatchawan et al., 2018a). Therefore, the minimal bactericidal concentrations of CFS of LAB against *E. coli* strains were evaluated before performing an anti-plasmid conjugation assay. The *E. coli* strains were overnight grown at 37℃ on Luria-Bertani (LB) agar which contains Yeast extract 5 g/L, Tryptone 10 g/L (Becton, Dickinson, and Company, MD, USA), and NaCl 10 g/L (Carlo Erba Reagents, Val de Reuil, France). The pH of LB media was adjusted to 7.5. The harvested colonies were resuspended in LB broth and adjusted to $1.5x10^8$ CFU/ml. CFS of *L. plantarum* 22F, 25F, and JCM1149 as reference strain were two-fold serially diluted (non-diluted, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64), where the diluted CFS at 1:64 reflected the same pH value with the neutralizing CFS. A 600 µl of CFS was added to equal amount of bacterial inoculum and incubated overnight at 37℃. The viable cells were then analyzed by measuring colony forming units (CFUs/ml) on the LB agar plates (Sanders, 2012). The highest dilution without bactericidal effect

was used to determine the plasmid conjugation rate. The experiments were performed in triplicates.

2.3.6 Anti-plasmid conjugation

To investigate the mechanism of action of LAB on gene transfer, experiments were performed on donor and recipient strains as previously described (Moubareck et al., 2007; Lugsomya et al., 2018b) using *in vitro* broth mating. The donor and recipient strains were cultured in LB broth and incubated at 37°C for overnight. At an equal quantity, the donor and recipient strains were mixed in a sterile tube with the final concentration at log 7.5 CFU/ml. The bacterial suspension was added with CFS (1:16 dilution), neutralizing CFS, or CFS of *E. coli* ATCC 25922 as internal control, while sterile LB broth was used as a negative control. Each assay was performed in triplicate. After incubation of 24 h, the suspensions were serially ten-fold diluted in sterile normal saline. Transconjugants were selected on LB agar plates supplemented with NaN_3 (200 µg/ml: Oxoid, Hampshire, England, UK) and colistin (2 µg/ml) (Sigma, St Louis, MO, USA). This condition was also used in our preliminary study for examining the growth of both colistin-resistant and recipient *E. coli*. The results demonstrated that they could not grow on this selected medium. Therefore, only recipient *E. coli* (J53) receiving colistin- resistant gene from the donor *E. coli* could grow on this medium (data not shown). Transfer frequencies were determined by dividing the number of transconjugants by the number of donor colonies (log of transconjugants on selective media/ log of the donor).

2.3.6 Confirmation of anti-conjugation activity

The presence of *mcr*-1 gene in transconjugants was also screened using PCR, broth microdilution assay, and plasmid replicon typing. For PCR assay, at least 3 colonies of transconjugants were selected randomly and individually detected the *mcr*-1 gene as described previously (Liu et al., 2016). The colistin-resistant phenotypes of the transconjugants were determined by the broth dilution method, while *E. coli* ATCC 25922 was used as a control strain (CLSI, 2015).

The plasmid replicon types were also confirmed in the transconjugants using PBRT (Carattoli et al., 2005).

2.3.7 Evaluation of biofilm formation

This experiment was carried out followed the protocol from the previous studies with slight modification (Wasfi et al., 2012; Kaur et al., 2018). All six *E. coli* isolates were cultured on Tryptic soy agar (TSA; Becton, Dickinson and Company, MD, USA) and incubated at 37°C for overnight. They were transferred into sterile Tryptic soy broth (TSB; Becton, Dickinson and Company, MD, USA) and further incubated for 16-18 hr. The bacterial suspension was measured to an optical density (OD) at 600 nm. equal to 1 ($1x10^8$ CFU/ml.) using UV/Vis spectrophotometer (BlueStar A, Labtech, MA, USA) and diluted with sterile TSB broth to final concentration at 10^6 CFU/ml. A 200 µl of final bacterial concentration was added into a sterile flat-bottom microtiter plate (Corning, NY, USA) and incubated at 37°C for 24 hr., while sterile TSB was used as control. After incubation, the incubated plate was washed with sterile distilled water (DW) and fixed with 200 µl of methanol (RCI Labscan, Bangkok, Thailand) for 15 mins. Adhesive biofilm was stained with 200 µl of 0.1% crystal violet (Carlo Erba Reagents, Val de Reuil, France) in distilled water for 5 min before washing with sterile DW. The stain from fixed cells were determined at OD_{570} nm by using AMR-100 microplate reader (Allsheng Co, Ltd, Hangzhou, China) after distributing with 160 µl of absolute ethanol (Merck, Darmstadt, Germany).

The extent of biofilm formation (BF) was examined by the absorbance of the test well (TW) minus the absorbance of the control well (CW). Where TW indicates the OD_{570} nm of stained adherent bacterial cells and CW represents the OD_{570} nm of stained control wells containing only sterile TSB. Interpretation of the biofilm formation was divided into four levels: strong biofilm formation (BF> 0.3), moderate biofilm formation (BF <0.3 and >0.2), weak biofilm formation (BF <0.2 and >0.1), and negative biofilm formation (BF <0.1) (Wasfi et al., 2012).

2.3.8 Effects of CFS on biofilm formation

Biofilm forming abilities were determined in microtiter plates using a crystal violet binding assay with minor amendment (Kaur et al., 2018). In brief, a 200 µl of 10^6 CFU/ml of the overnight culture *E. coli* was thoroughly mixed with 100 µl of CFS and 100 µl of NCFS of LAB in a sterile microtiter plate (Corning, NY, USA) and incubated at 37°C for 24 h, whereas sterile MRS broth was used as control. The non-adherent cells were then gently removed by washing twice with sterile distilled water (DW) and fixed with 200 µl of methanol (RCI Labscan, Bangkok, Thailand) for 15 mins. The fixed cells were stained with 200 µl of 0.1% crystal violet (Carlo Erba Reagents, Val de Reuil, France) in distilled water for 5 min. Following treatment with 160 µl of absolute ethanol (Merck, Darmstadt, Germany), the stained cells were determined by AMR-100 microplate reader (Allsheng Co, Ltd, Hangzhou, China) at OD_{570} nm. The test was performed in triplicates. The percentage inhibition of biofilm (%) was calculated by 100-(OD_{570} of wells in the treatment group x 100/ OD570 of wells in the control group).

2.3.9 Effects of CFS of lactic acid bacteria on dispersal of biofilm

The effect of CFS of lactic acid bacteria was determined on the dispersion of preformed biofilm of *E. coli*. Biofilm was developed in microtiter plate by adding 200 µl of 10^6 CFU/ml of *E. coli* suspension and incubated at 37°C for 24 h. Following incubation, non-adherent cells were removed gently without disrupting the biofilm construction and washed with sterile DW before adding 200 µl of non-neutralizing CFS of lactic acid bacteria. The microtiter plate was incubated at 37°C for 2 h before performing a crystal violet staining assay as described above. The experiments were carried out in triplicates.

2.3.10 Scanning electron microscopy (SEM) for biofilm production

All biofilm specimens of the planktonic stage and sessile stage were examined with a scanning electron microscope as described elsewhere, with minor modifications (Costa et al., 2014). For the planktonic stage, *E. coli* P01 was mixed with non-neutralizing or neutralizing CFS of P72N, while for the mature stage, it was mixed with non-neutralizing CFS of L25F in a sterile 24-well microtiter plate (Corning, NY, USA) with 12 mm round cover glass (no.1 thickness; Electron Microscopy Sciences, PA, USA) and left incubated for 24 h at 37°C. *E. coli* P01 in a sterile MRS broth was used as a control for both stages. After incubation, the microtiter plate was gently washed to remove the non-adherent cells before fixation with glutaraldehyde. Dehydration of cover glass was performed by ethanol before drying with a critical point dryer (Leica EM CPD300, Leica Microsystems, Wetzlar, Germany). The cover glass was coated with gold in Balzers SCD 040 sputter coater (Balzers Union Ltd., Balzers, Germany) before photographing with a scanning electron microscope (JSM-IT500HR, JEOL, Akishima, Japan).

2.3.11 Statistical Analysis

The Mann-Whitney U test was performed to compare the transfer frequencies of each treatment, and an independent t-test was conducted to analyze the relation of biofilm formation between control and CFS of lactic acid bacteria by using SPSS version 22 for Windows (IBM, NY, USA). The significant difference was defined at $P < 0.05$.

2.4 Results

2.4.1 Preparation of CFS dilution

To determine the proper non-toxic CFS concentration that inhibits the conjugation, the bactericidal activity of serial diluents of LAB-CFS was evaluated against donor and recipient *E. coli* strains using the microdilution method. The proper dilution that allowed the growth of donor and recipient E. coli are shown in Figure 1A and 1B, representatively. The CFS at dilutions of ≥1:16 showed no bactericidal activity against the tested strains, while the strong inhibition was still observed with lower dilutions at 1:4 and 1:8, proposing that 1:16 dilution was a good candidate for further experiments.

2.4.2 Anti- conjugation effect of CFS

The CFS were evaluated for their anti-conjugation effect on six colistin-resistant *E. coli* strains. A significant decrease in the transfer frequencies of colistin resistance gene *mcr*-1 was observed in the presence of non-neutralizing CFS (Table 4). The CFS (1:16) of *L. plantarum* 22F, 25F, and *P. acidilactici* 72N decreased the gene transfer frequency up to 100 times compared to the control. Interestingly, *L. plantarum* 22F significantly reduced the transfer frequencies in all colistin-resistant *E. coli* isolates (P <0.05). The transconjugants or recipient *E. coli* J53 receiving colistin-resistant gene from donor *E. coli* strains were confirmed by the presence of *mcr*-1 gene using PCR, broth microdilution assay, and plasmid replicon typing. The results showed that transconjugants acquired the *mcr*-1 gene, colistin resistance, and three plasmid replicon types of FIB, Frep, and Y (Table 5).

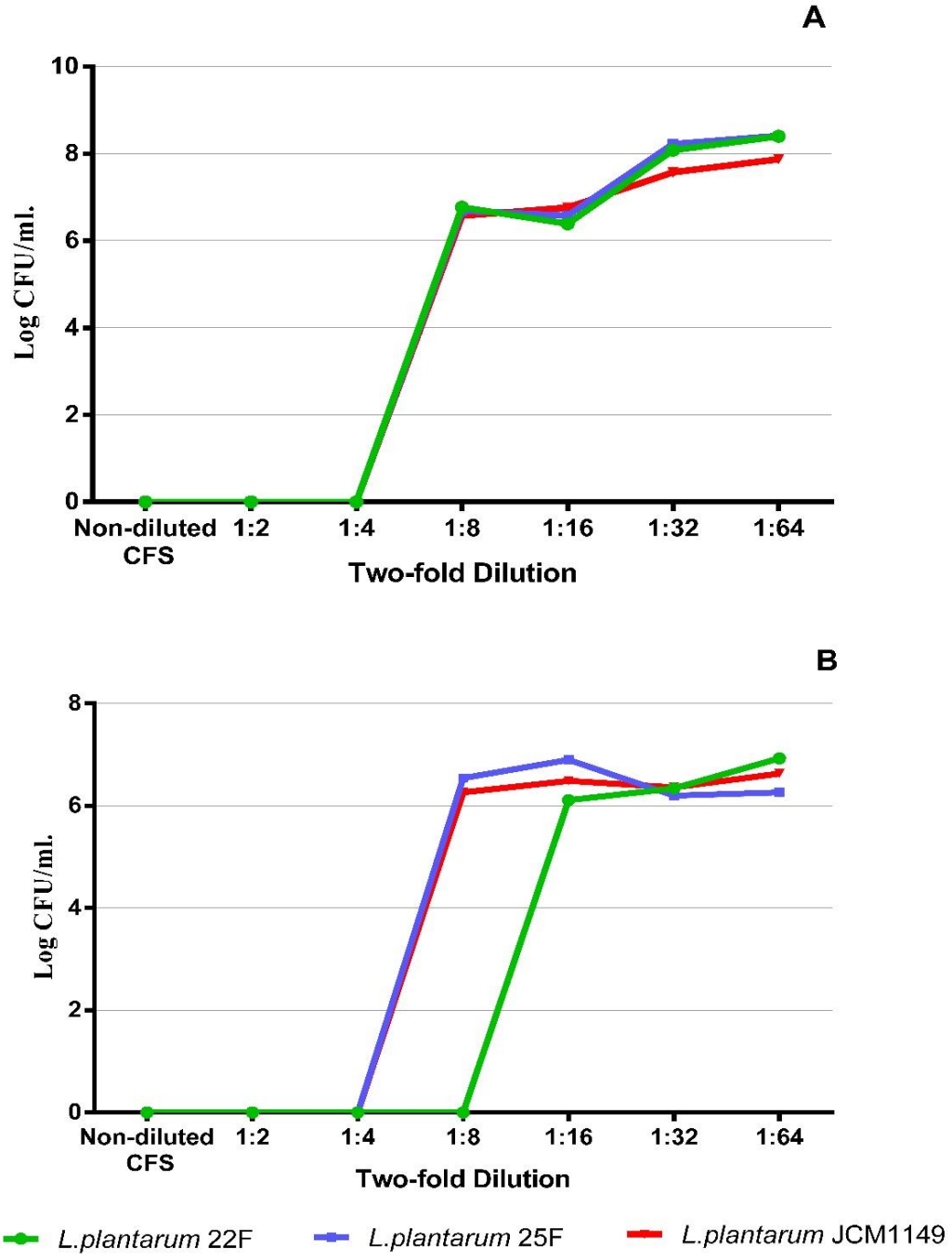

Figure 1 Bacterial survival of representative donor strain **(A)** and recipient strain **(B)** after culture with non-diluted and diluted CFS (non-diluted to 1:64) produced from selected LAB.

Table 4 Effects of CFS of LAB on transfer frequency. The different lowercase letters within the row indicate significant differences between treatments (P< 0.05) determined by Mann-Whitney U test.

E. coli strains	Treatments							
	Control	L22F (di1ution at 1:16)	L22F (neutralize condition)	L25F (di1ution at 1:16)	L25F (neutralize condition)	P72N (di1ution at 1:16)	P72N (neutralize condition)	*E. coli* ATCC 25922
P01	8.67×10^{-4b}	3.90×10^{-5a}	9.56×10^{-4b}	4.38×10^{-5a}	1.04×10^{-3b}	4.95×10^{-5a}	1.05×10^{-3b}	1.10×10^{-3b}
P02	8.63×10^{-5d}	2.86×10^{-5a}	2.41×10^{-4b}	4.95×10^{-5ad}	2.53×10^{-4bc}	3.24×10^{-5a}	4.48×10^{-4b}	2.92×10^{-4b}
H01	4.28×10^{-4c}	4.86×10^{-5a}	6.92×10^{-4b}	1.37×10^{-4a}	6.01×10^{-4bc}	3.24×10^{-4abc}	7.27×10^{-4b}	7.38×10^{-4b}
H02	3.37×10^{-4cd}	1.62×10^{-5a}	3.14×10^{-4cd}	1.29×10^{-4ace}	8.36×10^{-4bd}	2.95×10^{-5e}	3.96×10^{-4d}	7.21×10^{-4d}
E01	4.48×10^{-4d}	1.42×10^{-4a}	1.04×10^{-3c}	1.86×10^{-4a}	9.75×10^{-4bc}	1.29×10^{-4a}	9.93×10^{-4c}	1.01×10^{-3c}
E02	1.05×10^{-3b}	1.71×10^{-4a}	1.30×10^{-3b}	2.11×10^{-4a}	1.28×10^{-3b}	3.05×10^{-4a}	1.18×10^{-3b}	1.42×10^{-3b}

Table 5 The characteristics of transconjugants after treatment with CFS of LAB.

Donor *E. coli* strains	Recipient *E. coli* J53 (transconjugants)		
	colistin MIC (µg/ml)	*mcr*- 1 gene	Plasmid replicon
P01	8	+	Frep
P02	16	+	Frep
H01	4	+	FIB
H02	8	+	FIB, Frep
E01	4	+	FIB, Y
E02	4	+	FIB

2.4.3 Assessment of the anti-biofilm Activity of LAB-CFS against planktonic and sessile stages of *E. coli*

During the planktonic stage, all of our non-neutralizing CFS significantly decreased (P <0.05) the biofilm formation of all tested *E. coli* strains (Figure 2A). *P. acidilactici* 72N demonstrated the highest reduction in biofilm formation; however, the percentage of inhibition induced by other LAB-CFS were ranged between 50.20 and 82.28% (Table 6). For the neutralizing CFS (pH 6.5), CFS of *L. plantarum* 25F exhibited the highest potential towards the anti-biofilm activity of the tested *E. coli* isolates (Figure 2B). Nevertheless, the maximum percentage inhibition (52.59%) was observed after the treatment with *P. acidilactici* 72N CFS, while other LAB-CFS showed variable degrees of inhibition ranged between 0 and 51.03% (Table 7).

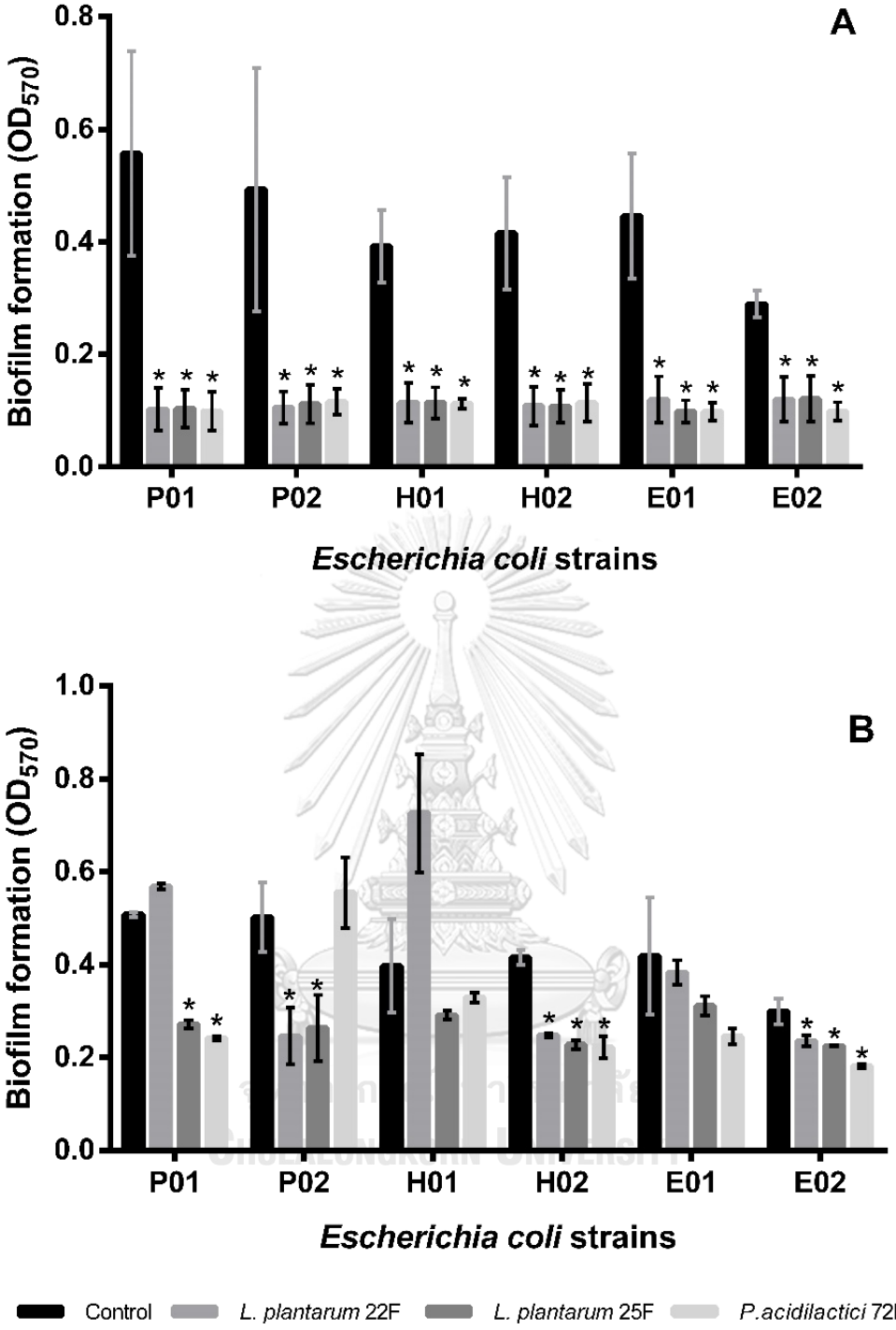

Figure 2 Effects of non- neutralizing CFS **(A)** and neutralizing CFS **(B)** of LAB on biofilm of *E. coli* evaluated by crystal violet assay. A significant difference (*, P <0.05) was calculated by an independent t-test when compared with the control group.

Table 6 Percentage inhibition of planktonic biofilm formation of *E. coli* by non-neutralizing CFS.

Lactic acid bacteria	*Escherichia coli*					
	P01	P02	H01	H02	E01	E02
L. plantarum 22F	81.83±1.78	77.29±6.71	77.22±6.71	74.36±6.71	73.58±3.42	50.54±2.95
L. plantarum 25F	81.29±3.93	76.31±6.11	71.18±4.40	74.22±1.82	77.69±2.19	50.20±3.99
P. acidilactici 72N	82.28±1.41	74.95±1.41	71.02±3.78	72.65±3.93	77.48±2.51	57.68±6.91

The results express as means ± the standard errors. The experiment was performed in triplicates.

Table 7 Percentage inhibition of planktonic biofilm formation of *E. coli* by neutralizing CFS producing by lactic acid bacteria.

Lactic acid bacteria	*Escherichia coli*					
	P01	P02	H01	H02	E01	E02
L. plantarum 22F	0	51.03±12.20	0	40.24±1.10	8.37±6.13	21.18±3.58
L. plantarum 25F	46.49±1.72	47.51±14.10	26.62±2.39	45.14±2.38	25.60±5.02	24.86±0.39
P. acidilactici 72N	52.59±0.97	0	17.21±2.72	46.67±5.58	41.31±3.87	39.46±1.53

The results express as means ± the standard errors. The experiment was performed in triplicates. Zero indicates that CFS of tested LAB showed no biofilm inhibition.

Regarding the sessile stage, the anti-biofilm activity of different neutralizing CFS of LAB against *E. coli* strains is presented in (Figure 3). As indicated, *P. acidilactici* 72N CFS significantly decreased the sessile biofilms formation against most of the tested *E. coli* strains. Similarly, *L. plantarum* 25F CFS induced a substantial reduction in *E. coli* adherence and biofilm production. The percentage inhibition of biofilm was 60.10%, in the case of *L. plantarum* 25F CFS, while ranged from 8.38 to 56.34% in other LAB-CFS (Table 8).

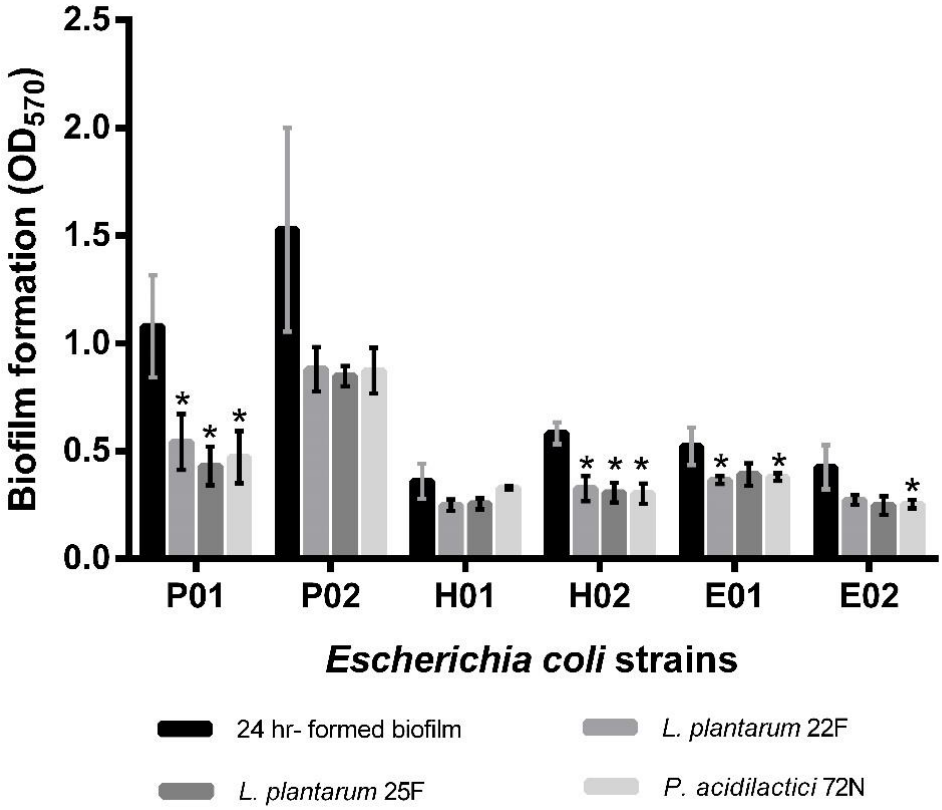

Figure 3 Effects of non-neutralizing CFS of LAB on sessile biofilm of by *E. coli* evaluated by crystal violet assay. A significant difference (*, P <0.05) was calculated by an independent t-test when compared with the control group.

Table 8 Percentage inhibition of sessile biofilm formation of *E. coli* by non-neutralizing CFS producing by lactic acid bacteria.

Lactic acid bacteria	*Escherichia coli*					
	P01	P02	H01	H02	E01	E02
L. plantarum 22F	49.76±12.02	42.41±6.79	31.04±7.25	43.99±10.79	29.92±3.68	35.63±5.36
L. plantarum 25F	60.10±8.29	44.51±3.10	29.21±7.12	47.30±7.93	25.15±9.94	41.62±10.19
P. acidilactici 72N	56.34±11.18	42.82±6.95	8.38±2.20	48.04±8.04	27.41±3.44	40.34±4.59

The results express as means ± the standard errors. The experiment was performed in triplicates.

Further, the biofilm illustration produced by *E. coli* P01 during the planktonic stage was confirmed by SEM. It was observed that the control sample (*E. coli* P01 cultured into sterile MRS broth) showed high cell density, aggregation (Figure 4A), and extracellular polysaccharide (EPS) matrix (Figure 4B). Compared to the control, the non-neutralizing CFS of P72N substantially reduced the adherence and aggregation of tested *E. coli* strain after 24 h of incubation (Figure 4C). Moreover, the neutralizing CFS of P72N also demonstrated the low cell density and EPS matrix against the tested *E. coli* strain (Figure 4D). On the other hand, the scanning electron micrographs of biofilm formed by *E. coli* P01 during the sessile stage are illustrated in (Figure 5). It appeared that the low cell density, aggregation of the tested strain, and the EPS matrix were obviously reduced in which grew in non-neutralizing CFS of L25F for 2 hours (Figure 5B), while the *E. coli* P01 cultured into sterile MRS broth for 24 hours. (Figure 5A). These results are in agreement with the findings of the crystal violet technique, which showed a significant reduction in bacterial adherence and biofilm formation (Figure 2 and 3).

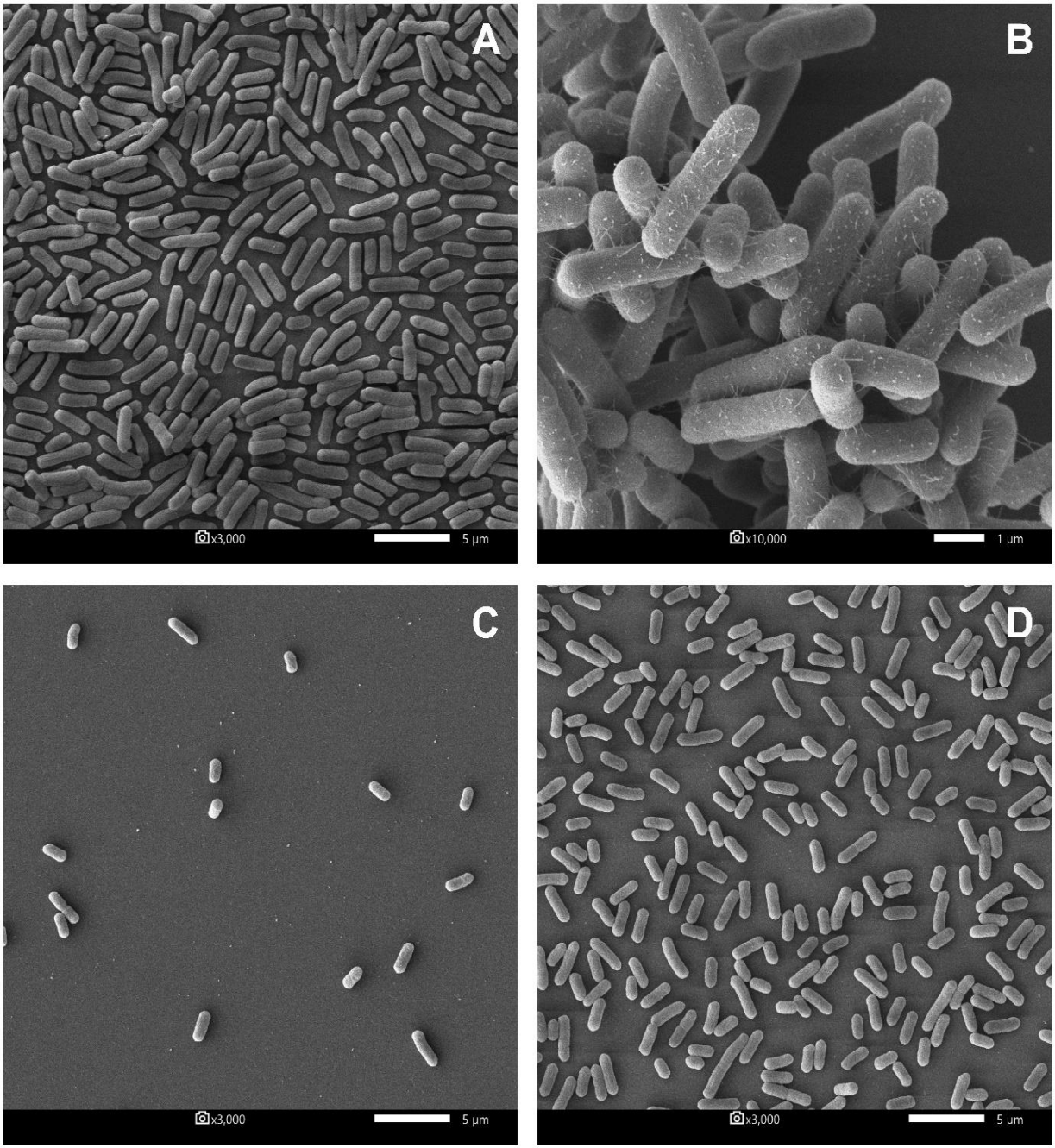

Figure 4 Scanning electron micrographs of biofilm formed by *E. coli* P01 in planktonic stage with different conditions. **(A-B)** represent *E. coli* grew towards sterile MRS broth (magnification: 3000X and 10,000X, respectively). **(C-D)** represent *E. coli* cultured in non-neutralizing and neutralizing CFS of P72N, respectively (magnification: 3000X). Scale bars are 1µm or 5µm.

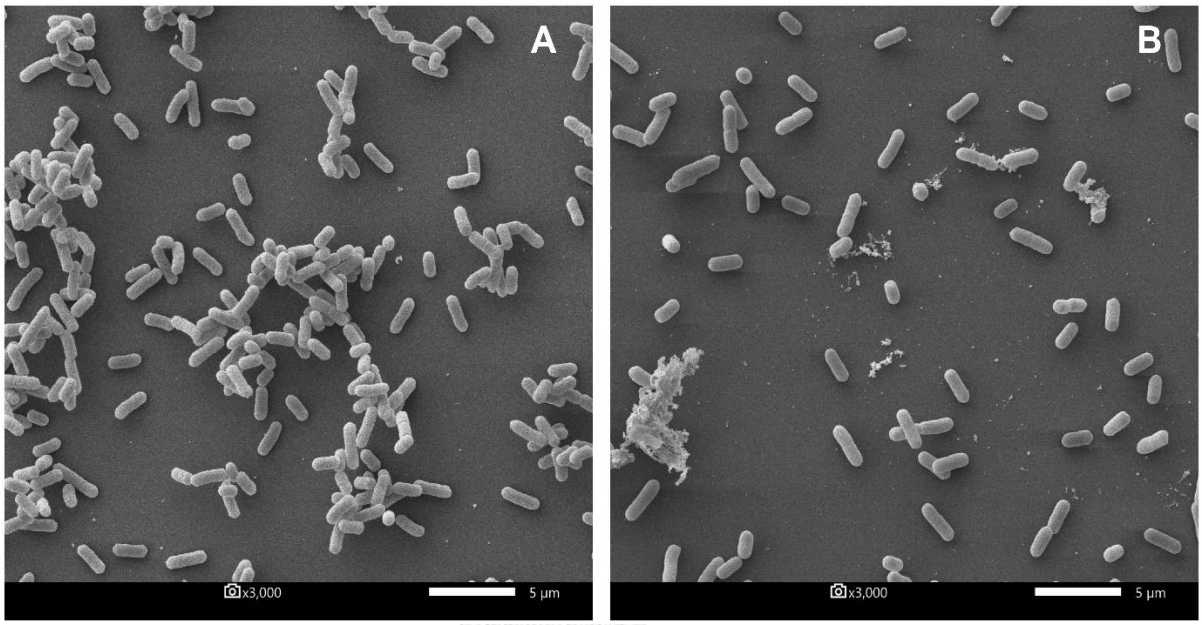

Figure 5 Scanning electron micrographs of biofilm formed by *E. coli* P01 in sessile stage with different conditions. **(A)** represents *E. coli* cultured into sterile MRS broth for 24 hours. **(B)** represents 24 h-biofilm of *E. coli* after exposure to non-neutralized CFS of L25F for 2 hours. Scale bar is 5µm, and magnification is 3000X.

2.5 Discussion

Probiotics, especially lactobacilli, have received significant attention because of the growing evidence of health benefits associated with their use. In our previous studies, *Lactiplantibacillus plantarum* 22F, 25F and, *Pediococcus acidilactici* 72N were characterized based on acid, bile, and temperature tolerance, good survivability, and absence of antibiotic-resistant genes (Sirichokchatchawan et al., 2017b). Furthermore, they displayed promising bactericidal capacity against several bacterial pathogens as well as antiviral activity against PEDV (Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b). The emergence of plasmid-mediated colistin resistance and biofilm formation among different pathogens has increased global awareness and concerns. In the present study, we reported for the first time the beneficial role of LAB strains on antibiotic resistance gene transfers and biofilm formation in six *E. coli* strains. Our results clearly supported the anti-biofilm, and anti-conjugation role of the LAB strains against *E. coli* harboring the *mcr*-1 gene. *E. coli* isolated from different origins (animal, farmer, and farm environment) with different characteristics such as variable degrees of colistin resistance, antibiotic susceptibility, plasmid replicon types, and biofilm formation. Thus, *E. coli* isolates in this study may be good and representative candidates for antimicrobial study.

Lactic acid bacteria generally secrete many inhibitory substances such as bacteriocins, fatty acids, and organic acids (lactic and acetic acids). These inhibitory compounds can directly disrupt the bacterial outer membrane leading to cell death (Alakomi et al., 2000; Arena et al., 2016; Ozcelik et al., 2016). Therefore, the optimum non-bactericidal dilution of CFS was determined prior to anti-conjugation and anti-biofilm experiments. We found optimum dilution of CFS (1:16) that was non-inhibitory to bacterial growth, however, yet maintained the strong anti-conjugation and anti-biofilm activity. Colistin resistance encoded by the *mcr*-1 gene is mostly harbored on a conjugative plasmid, which facilitates its transfer to other bacteria through horizontal gene transfer (Liu et al., 2016). Conjugation generally transfers mobile genetic elements such as a plasmid, integrative and conjugative element,

or pathogenicity islands between donor and recipient cells through direct physical contact via sex pilus or nanotubes (Gyles and Boerlin, 2014).

In this study, all non-neutralizing LAB-CFS significantly decreased the transfer frequencies of colistin resistance gene *mcr*-1; however, neutralizing CFS failed to show any anti-conjugation activity. These findings were consistent with the results described in the previous report of *Bifidobacteria* in decreasing β-lactam resistance gene transfer (*bla* genes) amongst *Enterobacteriaceae*(Moubareck et al., 2007). Inhibition of conjugation has been described with the agents that affect the formation of sex pili or allow plasmid curing of donor strains (Buckner et al., 2018). Deeb et al (2015) (El-Deeb et al., 2015) reported plasmid curing activity of *B. longum*, *L. plantarum*, and *S. thermophilus* against multidrug-resistant bacterial isolates (MDR). It was assumed that certain chemicals present in CFS may interfere with plasmid DNA replication via blocking DNA gyrase activity (Spengler et al., 2006). Unsaturated fatty acids, including linoleic, oleic, and stearic acid secreted by LAB, have also been proposed as one of the inhibitory compounds of conjugation. These fatty acids inhibited the activity of the plasmid-encoded type IV traffic ATPase (TraW). TraW regulates the switching between DNA translocation and pilus biogenesis through the conjugation machinery(Fernandez-Lopez et al., 2005; Ripoll-Rozada et al., 2016). Even though an increasing number of *mcr*-like genes (*mcr*-2 (Xavier et al., 2016), *mcr*-3 (Yin et al., 2017b; Yin et al., 2017a), *mcr*-4 (Carattoli et al., 2017), *mcr*-5 (Borowiak et al., 2017), *mcr*-6 (AbuOun et al., 2017; Partridge et al., 2018), *mcr*-7 (Yang et al., 2018), *mcr*-8 (Wang et al., 2018b), *mcr*-9 (Carroll et al., 2019) and *mcr*-10 (Wang et al., 2020a)) have been identified yet, however, given the common mechanism of conjugation, our LAB trains may decrease the transfer frequencies of other plasmid-encoded colistin resistance genes.

Bacterial biofilms are known as sessile microbial communities that are attached to the surface and mostly embedded in a self-produced matrix of organic polymers. Bacteria in biofilms are more resistant to antibiotics, disinfectants, dying, and dynamic environments. Antibiotics are of limited use against biofilms as most of the antibiotics are only active against planktonic microorganisms and cannot disperse

biofilms. Targeting biofilm formation is a promising target for therapeutic intervention, which has gained significant attention in the last few decades and encouraged the discovery of biofilm inhibitors (Rabin et al., 2015; Sharma et al., 2019). Indeed, several anti-biofilm compounds do not have any antimicrobial properties against planktonic cells. Hence, it is necessary to evaluate the potential effects of our LAB strains on biofilm formation in both planktonic and sessile stages (Jacques et al., 2010). The results showed that the tested LAB strains were able to reduce the biofilm formation in both planktonic and sessile states of *E. coli*. Biofilm formation in contact with non-neutralizing CFS reduced about 82.2% compared to the control. Interestingly, the neutralizing CFS also reduced the biofilm formation up to 52%, despite having no bactericidal activity (Sirichokchatchawan et al., 2018a). Similar findings of biofilm reduction have been reported in other bacterial pathogens, where 50 to 57% reduction in the biofilm formation of *Vibrio cholerae*, *E. coli*, and *S. aureus* was observed by the neutralizing CFS of lactobacilli isolates (Cui et al., 2018; Kaur et al., 2018). In contrary, Chapman et al (Chapman et al., 2014) reported no anti-biofilm activity of neutralizing lactobacilli-CFS against *E. coli* NCTC 9001 and *E. faecalis* NCTC 00775. Thus, it could be attributed to the different origins and characteristics of LAB isolates and tested pathogens.

Indeed, there is no specific mechanism by which LAB prevents the biofilms formation; however, several studies have proposed that probiotics can influence the expression of genes involved in quorum sensing, cell adhesion, virulence factors, and the formation of biofilms (Barzegari et al., 2020). LAB also secretes a variety of extracellular inhibitory substance, which includes extracellular substance (Fang et al., 2018b), exopolysaccharides (Mahdhi et al., 2018), biosurfactants (Walencka et al., 2008; Zakaria Gomaa, 2013), bacteriocins (Kim et al., 2019), different enzymes (Thallinger et al., 2013), and anti-quorum compounds (Park et al., 2014; Kim et al., 2018). Specifically, several studies have reported that bacteriocin may decrease the formation of biofilm due to growth inhibition. However, the neutralizing CFS of our LAB strains showed no antibacterial activity, proposing that bacteriocin may not have caused biofilm inhibition in this study (Sirichokchatchawan et al., 2018a).

The average pH in the pig's intestine ranges from 6.0 to 6.7, but LAB could acidify the intestine conditions by producing different organic acids (Merchant et al., 2011; Ozogul and Hamed, 2018). In this study, the non-neutralizing CFS (pH in the range 3.70-3.98) markedly reduced biofilm formation up to 82%, whereas the anti-biofilm activity of neutralizing CFS (pH: 6.5) was also decreased (up to 60%); moreover, the significant inhibition was still observed when compared to control. Similar findings have been reported earlier, where the lactobacilli-CFS dispersed the sessile biofilm of *Vibrio cholerae* between 62% and 85% in the non-neutralizing form between 50–75% in the neutralizing form (Kaur et al., 2018). However, their results showed a non-significant difference between the neutralizing and pH neutralizing CFS to biofilm dispersal effect suggesting that the inhibition of biofilm formation by lactobacilli CFS was not due to its antimicrobial activity, but CFS component such as certain disintegrative enzymes needed to prove in the further study. Simultaneously, SEM analysis in our study showed low aggregation of *E. coli* cells in biofilm after treatment with LAB-CFS. This suggests the active role of certain metabolites such as enzymes, or dispersal signal molecules that may have contributed to biofilm inhibition (Barraud et al., 2015; Fleming and Rumbaugh, 2017). Overall, the current study gave insight into the potential role of LAB-CFS on the biofilm reduction and growth inhibition of *E. coli*. However, further study is still urgently needed to fully understand the molecular mechanisms responsible for the anti-conjugation and anti-biofilm activity of LAB.

In conclusion, the present study showed the ability of LAB isolates to produce antimicrobial compounds that inhibit bacterial conjugation and limit the dissemination of antibiotic resistance genes. The biofilm formed by colistin-resistant *E. coli* was successfully removed by the cell-free supernatants of LAB, proving that LAB can serve as a potential alternative to antibiotics.

CHAPTER III

Metagenomic analysis of the gut microbiota in piglets either challenged or not with enterotoxigenic *Escherichia coli* reveals beneficial effects of probiotics on microbiome composition, resistome, digestive function and oxidative stress responses

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Status: During submission process

3.1 Abstract

This study used metagenomic analysis to investigate the gut microbiota and resistome in piglets that were or were not challenged with enterotoxigenic *Escherichia coli* (ETEC) and had or had not received dietary supplementation with microencapsulated probiotics. The 72 piglets belonged to six groups that were either non-ETEC challenged (groups 1-3) or ETEC challenged (receiving 5ml of 10^9 CFU/ml pathogenic ETEC strain L3.2 one week following weaning at three weeks of age: groups 4-6). On five occasions at 2, 5, 8, 11, and 14 days of piglet age, groups 2 and 5 were supplemented with 10^9 CFU/ml of multi-strain probiotics (*Lactiplantibacillus plantarum* strains 22F and 25F, and *Pediococcus acidilactici* 72N) while group 4 received 10^9 CFU/ml of *P. acidilactici* 72N. Group 3 received 300mg/kg chlortetracycline in the weaner diet to mimic commercial conditions. Rectal faecal samples were obtained for metagenomic and resistome analysis at 2 days of age, and at 12 hours and 14 days after the timing of post-weaning challenge with ETEC. The piglets were all euthanized at 42 days of age. The piglets in groups 2 and 5 were enriched with several desirable microbial families, including *Lactobacillaceae*, *Lachnospiraceae* and *Ruminococcaceae*, while piglets in group 3 had increases in members of the *Bacteroidaceae* family and exhibited an increase in *tetW* and *tetQ* genes. Group 5 had less copper and multi-biocide resistance. Mobile genetic elements IncQ1 and IncX4 were the most prevalent replicons in antibiotic-fed piglets. Only groups 6 and 3 had the integrase gene (*intI*) class 2 and 3 detected, respectively. The insertion sequence (IS) 1380 was prevalent in group 3. IS3 and IS30, which are connected to dietary intake, were overrepresented in group 5. Furthermore, only group 5 showed genes associated with detoxification, with enrichment of genes associated with oxidative stress, glucose metabolism, and amino acid metabolism compared to the other groups. Overall, metagenomic analysis showed that employing a multi-strain probiotic could transform the gut microbiota, reduce the resistome, and boost genes associated with food metabolism.

3.2 Introduction

The gut microbiota of the pig plays a critical role in maintaining health and productivity through supporting optimal nutritional, physiological and immunological functions (Fouhse et al., 2016; Guevarra et al., 2018). Piglets in the weaning transition period are exposed to a variety of stressful factors that may disrupt their newly acquired gut microbiome, resulting in poor growth and health (Guevarra et al., 2018). Infection with enterotoxigenic and verotoxigenic *Escherichia coli* (ETEC and VTEC) are known to cause post-weaning diarrhoea, which results in increased morbidity and mortality, decreased average daily gain (ADG), and the need for increased administration of antibiotics, which all contribute to financial losses for the pig sector (Guevarra et al., 2019; Sun et al., 2019). In response, feed additives such as antibiotics, prebiotics, and probiotics have been used to manipulate the piglet gut micro-ecosystem in order to boost growth, improve health status, and prevent diarrhoea after weaning (Liao and Nyachoti, 2017a).

Antibiotics have been utilized worldwide in the swine industry for many years in order to increase pig productivity while lowering morbidity and mortality (Liao and Nyachoti, 2017a; Tunsagool et al., 2021). However, administration of in-feed antibiotics impacts both pathogenic and commensal microbes in the gut, leading to decreased alpha-diversity and causing a microbial shift in the animal gut (Wang et al., 2019b). For example, oxytetracycline treatment may diminish bacterial diversity and richness in the gut microbiota of piglets, moreover subsequent removal of oxytetracycline for 2 weeks does not completely restore bacterial diversity (Ghanbari et al., 2019). Several studies have found that pigs exposed to in-feed antibiotics are more likely to develop infections from members of the *Enterobacteriaceae*, *Spirochaetae,* and *Campylobacteraceae* families (Ghanbari et al., 2019; Wang et al., 2019b; Tunsagool et al., 2021).

Antibiotic-treatment of piglets also can increase the diversity and abundance of antibiotic-resistant genes (ARGs) and mobile genetic elements (MGEs) in the porcine gut: these include genes conferring resistance to aminoglycosides, beta-

lactams, chloramphenicol, macrolide-lincosamide-streptogramin B (MLS_B), sulfonamides, tetracycline, and vancomycin, as well as class 1 integrons and transposons (Zhao et al., 2018). Antibiotic usage has negative consequences that may affect public health, and, as a result many countries including Thailand have banned the use of antibiotics in livestock agriculture (Tunsagool et al., 2021). Consequently, the use of non-antibiotic alternatives for stimulating growth and altering the gut microbiome has received considerable attention in the livestock industries (Sirichokchatchawan et al., 2021).

Probiotics are live microorganisms that are a non-antibiotic option for maintaining gut health, and they have been thoroughly researched over the years (Wang et al., 2019b). Probiotic supplementation has been shown to have various benefits for humans and animals, including altering the gut microbiota, enhancing food utilization, strengthening gut immunity, and reducing enteric disease (O'Toole and Cooney, 2008; Hossain et al., 2017; Liao and Nyachoti, 2017a). The intestinal microbiota of pigs that were supplemented with *Lactiplantibacillus plantarum* PFM105 was found to be enriched by desirable bacterial families such as *Prevotellaceae* and *Bifidobacteriaceae*, which improve nutrient absorption and have anti-inflammatory activity (Wang et al., 2019b). Pigs supplemented with 2.5×10^7 CFU/ml of *Lactiplantibacillus plantarum* JDFM LP11 showed significantly increased gut microbial richness and diversity, and an increased *Ruminococcaceae* relative abundance of up to 25% compared to a control group (Shin et al., 2019). The effects of probiotics on decreasing the human gut resistome have been studied (Casaburi et al., 2019). For example, infants who received *Bifidobacterium longum* subsp. *infantis* EVC001 had a 90% reduction in ARG abundance when compared to a control group (Casaburi et al., 2019). Unfortunately, to date there have been relatively few comparable studies on the effect of probiotics on modulating the pig gut resistome (Ma et al., 2019). Importantly, studies on the pig resistome may provide better insight into antimicrobial resistance (AMR) issues that impact on AMR transmission from pigs to pork consumers.

In our previous studies, several probiotic strains, including *Lactiplantibacillus plantarum* strains 22F and 25F (L22F and L25F) and *Pediococcus acidilactici* strain 72N (P72N), showed excellent safety features, including lack of antimicrobial-resistance genes based on the European Food Safety Authority (EFSA) criteria (Sirichokchatchawan et al., 2017a). Furthermore, they demonstrated promising antibacterial, antiviral, anticonjugation, and antibiofilm action *in vitro* (Sirichokchatchawan et al., 2018a; Sirichokchatchawan et al., 2018b; Apiwatsiri et al., 2021). In addition, we previously created a method for preserving our probiotic strains in the form of double-coated microencapsulation for use in pig farms. In an *in vivo* investigation, these probiotic strains used at a final concentration at 10^9 CFU/ml improved intestinal health and growth development in pigs during the rearing cycle (Pupa et al., 2021a; Pupa et al., 2021b). The purpose of the current study was to undertake whole-metagenome shotgun sequencing on faecal samples to investigate how feeding microencapsulated single-strain and multi-strain probiotics to neonatal pigs influenced their gut microbiota and modulated carriage of ARGs. The study also examined changes in the microbiota that were associated with feeding chlortetracycline or that resulted from ETEC challenge after weaning.

3.3 Materials and methods

3.3.1 Animals and housing

The experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the Thai Food Research Center, Thai Foods Group (TFG) Public Company Limited (PLC.) under protocol no. 6112-01, and the Feed Research and Innovation Centre, Charoen Pokphand Foods (CPF) Public Company Limited (PLC.) under protocol no. FRIC-ACUP-1707013. All animal usage and procedures were performed in compliance with the International Guiding Principles for Biomedical Research Involving Animals. The euthanasia procedures were performed following the guidelines for the euthanasia of animals, in compliance with the American Veterinary Medical Association (AVMA). The piglets were rendered unconscious by administering intravenous sodium pentobarbital anaesthesia followed by potassium

chloride to induce cardiac arrest and death. The use of all bacterial strains, including lactic acid bacteria (LAB) and ETEC, was approved by the Institutional Biosafety Committee, Chulalongkorn University under Biosafety Use Protocol numbers IBC1831044 and IBC1831045, respectively.

A total of 72 two-day-old healthy neonatal piglets (Large White × Landrace × Duroc) were recruited into the study. The production and health data for 60 of the pigs has been published elsewhere (Pupa et al., 2022). In the current study an additional 12 piglets were included as a positive control group that were administered with chlortetracycline, with these being reared and handled in an identical fashion to the previously described piglets. The 72 piglets were randomly allocated into six experimental groups with male and female replicate pens per group (6 pigs per pen) at the CPF Feed Research and Innovation Centre. At 21 days of age, piglets in all experimental groups were weaned and transferred to the TFG Research Center. Each experimental group was raised in separate rooms with controlled humidity under an evaporative cooling system at 80 %. The environment within the building was temperature-controlled at 32 ± 2°C and 27 ± 1°C for neonatal and weaned piglets, respectively. All piglets were allowed to independently suck the milk from their sows in the neonatal period. They were allowed *ad libitum* access to a basal diet and water in the weaning period. The ingredient composition and nutrient concentration of the weaner diet is presented in Table 9.

Table 9 Ingredient composition and nutrient concentration of the experimental basal diet.

Ingredient Composition	Percent of dry matter
Corn (7.7 % CP)	26.75
Broken rice (7.7 % CP)	22.97
Dehulled-soybean meal (48.9 % CP)	18.47
Full fat soybean (36.0 % CP)	10.00
Fish meal (60 % CP)	6.00
Rice bran, full fat (13.6 % CP)	5.00
Whey powder sweet	5.00
Soybean oil	2.67

Mono-Dicalcium Phosphate (MDCP; P 18.0 %, Ca 21.8 %)	1.29	
SP Premix (vitamin A 18,000 IU, vitamin D_3 2500 IU, vitamin E 250 IU, vitamin K_3 0.60 mg, vitamin B_1 3.2 mg, vitamin B_2 9.4 mg, vitamin B_6 5 mg, vitamin B_{12} 80 µg, biotin 80 µg, choline 550 mg, folic acid 2.15 mg, *D*-pantothenic acid 25 mg, nicotinic acid 75 mg, Ca 55 mg, Co 148 mg, Fe 148 mg, I-4.3 mg, Mn 80 mg and Se 0.60 mg)	0.50	
Pellet binder	0.30	
L-Lysine HCl	0.27	
Sodium chloride	0.23	
Limestone (Ca 36.4%)	0.20	
DL-Methionine	0.15	
L-Threonine	0.15	
L-Tryptophan	0.05	
Total	100.00	
Nutrient concentration		**Unit**
Crude protein	20.40	%
Crude fat	7.50	%
Ashes	6.89	%
Crude fiber	4.23	%
Standardized ileal digestible Lysine:Metabolisable Energy (SID Lysine:ME)	3.83	g/Mcal
Digestible Lysine	1.33	%
Digestible Threonine	0.83	%
Calcium	0.82	%
Digestible Methionine + Cysteine	0.76	%
Phosphorus	0.54	%
Digestible Methionine	0.49	%
Digestible Tryptophan	0.27	%

3.3.2 Experimental designs and sample collection

Information about the treatments received by the six experimental groups is summarized in Table 10 and Figure 6. The three groups supplemented with probiotics received these on five occasions, when the piglets were 2, 5, 8, 11, and 14 days of age, followed our previous study (Pupa et al., 2021a).

Following weaning at 21 days of age, pigs in groups 1-3 were not challenged with ETEC, but received 3 ml of sterile peptone water (Becton, Dickinson and

Company, Maryland, USA) at the same time that the ETEC groups (groups 4-6) were challenged. Piglets in the negative control group (group 1) were fed with a basal diet without probiotic and antibiotics. Piglets in the probiotic control group (group 2) were orally supplemented with a 3 ml double-coated multi-strain LAB mixture (L22F, L25F, and P72N) suspended in sterile peptone water at a final concentration at 10^9 CFU/ml through sterile syringe, receiving this on the five occasions mentioned above. Following weaning, piglets in the antibiotic group (group 3) were fed with a basal diet mixed with antibiotic (chlortetracycline at 300mg/kg), as previously described (Pupa et al., 2021a).

In the ETEC challenged groups (groups 4-6), piglets in all groups were fed with a basal diet after weaning. Those in the single strain group (group 4) as neonates previously had been orally supplemented with 3 ml of double-coated single-strain LAB (P72N) suspended in sterile peptone water at a final concentration at 10^9 CFU/ml via sterile syringe, whilst those in the multi-strain group (group 5) had been orally supplemented with 3 ml of double-coated multi-strain LAB mixture (L22F, L25F, and P72N) suspended in sterile peptone water at a final concentration at 10^9 CFU/ml through sterile syringe. The piglets in the ETEC control group (group 6) only received 3 ml of sterile peptone water. All piglets in the three ETEC challenged group were orally inoculated with ETEC strain L3.2 at a final concentration at 5×10^9 CFU/ml at 28 days of age (7 days after weaning).

Faeces samples were obtained from individual piglets through digital stimulation of the rectum. Approximately five grams of faeces were collected from four of the piglets (2 male and 2 female) in each group on Day 2, 12 hours post-challenge (hpc) and 14 days post-challenge (dpc), with different pigs sampled at each collection. For each group and each collection time, the four faecal samples were combined into one pooled sample before genomic DNA extraction. Faeces were collected into sterile containers and stored at -20°C until processed within a week of collection.

Table 10 Summary of the experimental groups.

No.	Experimental group	Probiotic supplementation			ETEC infection	Antibiotic administration
		P. acidilactici 72N (P72N)	*L. plantarum* 22F (L22F)	*L. plantarum* 25F (L25F)		
			Non-ETEC infection			
1	Negative control	-	-	-	-	-
2	Probiotic control	+	+	+	-	-
3	Antibiotic	-	-	-	-	+
			ETEC infection			
4	Single-strain	+	-	-	+	-
5	Multi-strain	+	+	+	+	-
6	ETEC control	-	-	-	+	-

+ and – indicate with or without probiotic supplementation, antibiotic administration or ETEC infection.

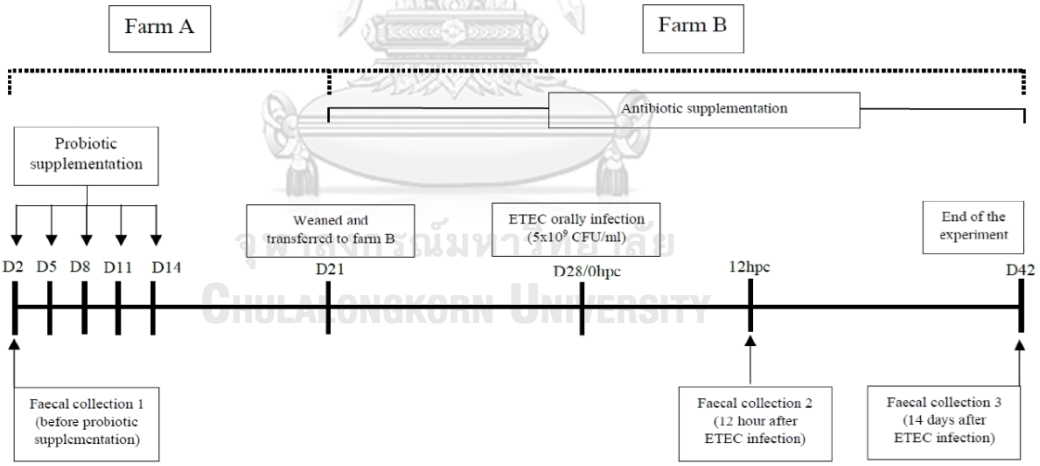

Figure 6 Schematic of experimental design and sample collection. D indicates day after birth and hpc refers to hours post ETEC challenge.

3.3.3 DNA extraction and shotgun metagenomic sequencing

Total genomic DNA was extracted from each pooled faecal sample from four piglets per treatment per timepoint using the Quick-DNA/soil microbe microprep kit (Zymoresearch, CA, USA) according to the manufacturer's recommendation. The extracted DNA was checked for purity by A_{260}/A_{280} comparison using the OneDrop TOUCH lite micro-volume spectrophotometer (Biometrics Technologies, Wilmington, DE, USA). DNA degradation was checked by 2% agarose gel electrophoresis (Vivantis, Selangor Darul Ehsan, Malaysia) and visualized under UV in the Syngene™ Ingenius 3 Manual Gel Documentation System (SynGene InGenius, Cambridge, UK). In addition, the total DNA concentration was measured using a Qubit™ 4 fluorometer with the dsDNA broad-range assay kit (Invitrogen™, Thermo Fisher Scientific, Waltham, USA). Shotgun metagenomic sequencing was undertaken using the Illumina Novaseq 6000 on the Illumina HiSeq-PE150 platform at 10-GB data output according to the manufacturer's instructions (Novogene Bioinformatics Technology Co. Ltd., Beijing, China).

3.3.4 Quality control

The paired-end raw sequence reads were quality filtered in several steps for removing sequencing adapters and low-quality sequences with quality scores <30 using Trimmomatic v.0.36.5 (Bolger et al., 2014). Finally, any sequences mapped to the pig genome (*Sus scrofa*, NCBI accession no. NC010443) were filtered out using Bowtie2 v.2.3.4.32 (Langmead and Salzberg, 2012). All the bioinformatic analyses were performed on the European Galaxy server (https://usegalaxy.eu/).

3.3.5 Taxonomic annotation

The taxonomic classifications of the metagenome datasets were identified by Kraken2 (Galaxy Version 2.0.85) (k=35, ℓ=31). The Kraken2 database, the complete genomes in RefSeq for the bacterial, archaeal, and viral domains, the human genome and a collection of known vectors were all retrieved from NCBI (Wood et al., 2019). Alpha diversity (Species richness, Shanon and Simpson diversity index) and

beta-diversity (Bray-Curtis dissimilarity matrix) were analyzed with the QIIME2 platform version 2021.4 (https://qiime2.org/) (Bolyen et al., 2019).

3.3.6 Antibiotic resistance, metal resistance and biocide resistance gene annotation

The clean raw reads after the quality filtering processes were used for similarity searches against the antimicrobial resistance, metal resistance and biocide resistance MEGARes database (Doster et al., 2020) by using NCBI BLAST+ blastn (Galaxy Version 2.10.1)(Cock et al., 2015). The MEGARes database contains the sequences of approximately 7,868 nucleotide sequences of antimicrobial resistance genes (ARGs) based on a nonredundant compilation of sequences contained in ResFinder, ARG-ANNOT, the Comprehensive Antibiotic Resistance Database (CARD, the National Center for Biotechnology Information (NCBI) Lahey Clinic beta-lactamase archive and BacMet was accessed on 14-10-2019.

3.3.7 Mobile genetic elements (MGEs) annotation

The clean raw reads after the quality filtering processes were used for similarity searches for plasmids using the PlasmidFinder database (Carattoli et al., 2014) and for class 1, 2, and 3 integron integrase genes in the INTEGRALL database (Moura et al., 2009; Stalder et al., 2019) by using NCBI BLAST+ blastn (Galaxy Version 2.10.1) (Cock et al., 2015). The PlasmidFinder database contains approximately 469 nucleotide sequences accessed on 13-07-2020, whereas the INTEGRALL database contains 11 nucleotide sequences related to class 1, 2, and 3 integron integrase genes. After the quality filtering processes, the clean raw reads were used for similarity searches against insertion sequences in the ISFinder database (Siguier et al., 2006) by using Diamond (Galaxy Version 0.9.21.0) (Buchfink et al., 2015). The ISFinder database contains approximately 8,836 amino acid sequences and was accessed on 6-10-2020.

Additionally, the confidence match to those databases associated with antibiotic resistance genes and mobile genetic elements was set by considering both

percent identity cutoff at 90% and minimum query coverage at 80%, as suggested elsewhere (Ghanbari et al., 2019; Stalder et al., 2019). Moreover, the results of taxonomic profiles, antibiotic resistance, and mobile genetic elements were illustrated in the form of relative abundance by the total count method, which was performed as previously described (Pereira et al., 2018).

3.3.8 Functional annotation

The clean raw reads from each sample were *de novo* metagenomic assembled with default settings using MEGAHIT (Galaxy Version 1.1.3.43) (Li et al., 2015). The assembled contigs were examined for genome assembly quality using Quast (Galaxy Version 5.0.24) (Gurevich et al., 2013). Functional annotation was determined through metagenome rapid annotation using subsystem technology server version 4 (MG-RAST) (Meyer et al., 2019). The assembled contigs were submitted to MG-RAST and functional annotation was performed against the Kyoto Encyclopedia of Genes and Genomes database (KEGG) and SEED subsystem database applying the following thresholds: >60% identity, 15 amino acids for a minimum alignment length, and e-value <1e-5: The investigated markers of stress response were catalase, fumarate and nitrate reduction regulatory protein, iron-binding ferritin-like antioxidant protein, redox-sensitive transcriptional regulator, superoxide dismutase and transcriptional regulator. In addition, the functional results were presented in normalized abundance which was generated by MG-RAST using DESeq analysis, as suggested elsewhere (Guevarra et al., 2018).

3.3.9 Data availability

The datasets of raw metagenomic sequences were deposited in NCBI Sequence Read Archive (SRA) and are available in the BioProject under the accession number PRJNA769425 (https://www.ncbi.nlm.nih.gov/sra/PRJNA769425).

3.4 Results

3.4.1 Overall sequencing data and microbial diversity of the piglet faecal samples

DNA extracted from piglet faeces was sequenced with Illumina Hi-seq, obtaining 1.4 billion reads with read counts ranging from 68.9 to 115.2 million. After quality filtering, 1.2 billion high-quality readings were acquired, resulting in an 89.47 percent clean-read rate (Table 11). After *de novo* metagenomic assembly by MEGAHIT, there were 133,927 to 624,196 assembled contigs (Table 12). The species richness and diversities (Shanon and Simpson) of gut microbial alpha diversity were lower in the probiotic control group than in the negative control and antibiotic groups within the non-ETEC challenged groups at 12-hours and 14-days after the time of ETEC challenge. However, amongst the ETEC challenged groups, the multi-strain group tended to have higher alpha diversity than the single-strain and ETEC control groups, not just in terms of species richness but also in terms of species diversity (Table 13). The principal coordinate analysis (PCoA) plot on Day 2 (two days of age; before probiotic treatment), at hour 12 (12-hour post-ETEC infection, 12 hpc), and at day 42 (14 days post-ETEC infection, 14 dpc) demonstrated three different clusters, as shown in Figure 7.

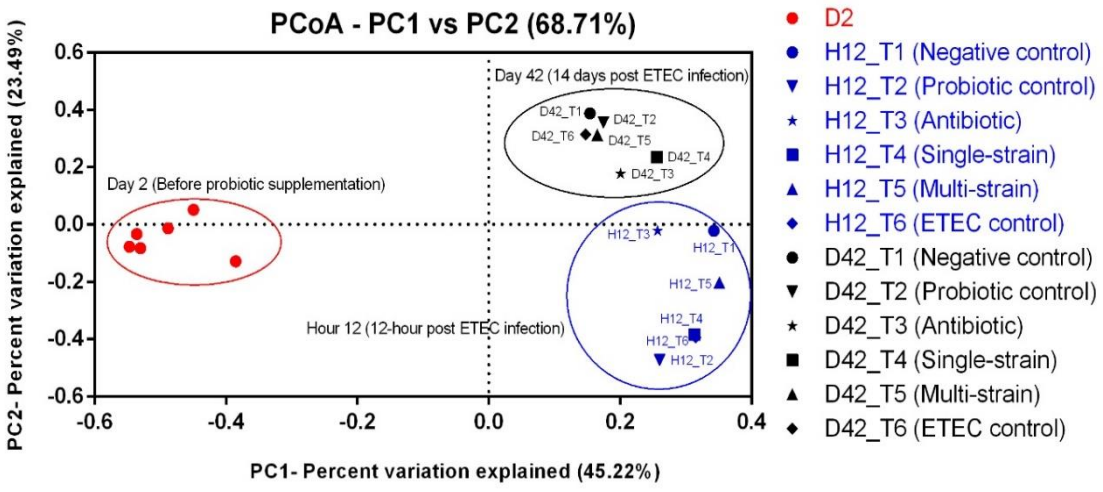

Figure 7 Principal coordinate analysis (PCoA) plot based on Bray-Curtis dissimilarity index of microbial taxonomic profile at the species level from piglet faecal samples across treatments in each time-point. The geometric shapes demonstrate the group of samples in each time-point. D2 refers to 2 days of age, before probiotic treatment.

Table 11 Summary of overall sequencing data.

Groups	Raw reads (bp)	Filter reads (bp)	Clean reads (%) ǂ
D2	74,120,140	62,794,856	84.72
	72,871,648	60,319,200	82.77
	73,524,896	59,669,194	81.16
	74,116,640	65,053,102	87.77
	72,232,588	60,155,428	83.28
	78,739,346	64,092,260	81.40
12-hours post ETEC challenging			
Non-ETEC infection			
Negative control	71,742,904	67,131,364	93.57
Probiotic control	68,918,602	64,408,060	93.46
Antibiotic	71,224,186	66,434,034	93.27
ETEC infection			
Single-strain	72,708,420	68,426,454	94.11
Multi-strain	71,052,434	66,982,406	94.27
ETEC control	88,132,182	82,394,492	93.49
14-days post ETEC challenging			
Non-ETEC infection			
Negative control	76,764,072	69,651,048	90.73
Probiotic control	115,242,034	109,361,858	94.90
Antibiotic	77,712,366	67,600,042	86.99
ETEC infection			
Single-strain	77,700,814	74,171,530	95.46
Multi-strain	82,498,506	74,089,966	89.81
ETEC control	86,952,230	77,731,060	89.40

ǂ Clean reads were calculated as (Filter reads/Raw reads) x 100. D2 refers to 2 days of age, before probiotic treatment.

Table 12 The summary of all *de novo* assembled metagenomic sequence data by using MEGAHIT and determining by QUAST.

Groups	Assembled contigs	Total sequence length (bp)	N50
D2	347,741	196,215,439	749
	228,884	154,065,378	840
	257,749	166,668,686	821
	242,934	166,548,412	848
	219,197	155,302,907	1,169
	259,329	166,280,013	830
12-hours post ETEC challenging			
Non-ETEC infection			
Negative control	355,198	448,048,080	3,885
Probiotic control	133,927	153,868,780	3,707
Antibiotic	624,196	668,618,351	2,419
ETEC infection			
Single-strain	207,869	268,616,577	3,588
Multi-strain	280,248	347,364,359	3,762
ETEC control	214,206	283,634,843	3,889
14-days post ETEC challenging			
Non-ETEC infection			
Negative control	323,166	411,426,419	3,779
Probiotic control	323,707	504,325,326	7,425
Antibiotic	358,033	300,817,974	2,618
ETEC infection			
Single-strain	430,156	528,248,353	3,403
Multi-strain	443,347	566,818,604	3,686
ETEC control	318,388	416,422,550	4,425

D2 refers to 2 days of age, before probiotic treatment.

Table 13 Alpha diversity of gut microbial communities from piglet fecal samples across treatments and each time-point.

Groups	Species richness	Shannon	Simpson
D2	5473	4.567	0.838
12-hours post ETEC challenging			
Non-ETEC infection			
Negative control	5410	5.218	0.875
Probiotic control	4882	2.471	0.549
Antibiotic	5433	6.259	0.914
ETEC infection			
Single-strain	5193	2.404	0.482
Multi-strain	5345	3.734	0.758
ETEC control	5243	2.430	0.481
14-days post ETEC challenging			
Non-ETEC infection			
Negative control	5511	7.894	0.969
Probiotic control	5489	7.364	0.956
Antibiotic	5500	7.956	0.977
ETEC infection			
Single-strain	5505	6.837	0.954
Multi-strain	5529	8.123	0.982
ETEC control	5499	7.295	0.972

D2 refers to 2 days of age, before probiotic treatment.

3.4.2 Taxonomic abundance and composition of the piglet gut microbiota

The abundance and composition of bacterial taxonomy at the phylum, family, and genus level are depicted in Figure 8-10. The most prevalent phyla at 2 days of age (Day 2) were Proteobacteria and Bacteroidetes (Figure 8). *Enterobacteriaceae* and *Bacteroidaceae* were the top two families identified at the family level (Figure 9). Furthermore, at Day 2, piglet faeces samples were enriched in the genera *Escherichia* and *Bacteroides* (Figure 10).

The average relative abundance of the Firmicutes, Bacteroidetes, and Proteobacteria phyla was approximately 97% of the total abundance at 12 hpc (Figure 8). In the non-ETEC infected groups, the probiotic control group had a higher proportion of members of the Firmicutes phylum and *Lactobacillaceae* family, while Proteobacteria were found in the highest abundance in the antibiotic group (Figure 8 and 9). The antibiotic group had a higher percentage of *Bacteroidaceae* than the other groups. Furthermore, the probiotic control group had an increased quantity of *Lactiplantibacillus* genus (Figure 10). In the ETEC challenged groups, Firmicutes were found to be the most abundant in all experimental groups, at more than 92% (Figure 8). Firmicutes phylum members *Lachnospiraceae*, *Veillonellaceae* and *Ruminococcaceae* were significantly increased in the multi-strain group (Figure 9). In addition, when compared to the single-strain and ETEC control groups, the relative abundance of *Megasphaera*, *Blautia* and *Ruminococcus* was significantly higher in the multi-strain group (Figure 10).

At 14 dpc, the dominating phyla showed a similar trend as at 12 hpc, with Firmicutes, Bacteroidetes, and Proteobacteria enriched across the experimental groups (Figure 8). In the non-ETEC infection groups, members of the Firmicutes phylum and *Ruminococcaceae* family were found in greater abundance in the probiotic control group than in the other groups, while the *Bacteroidetes* phylum and *Bacteroidaceae* family were still prominent in the antibiotic group (Figure 8 and 9). Furthermore, piglets in the probiotic control group showed higher levels of the genera *Faecalibacterium*, *Megasphaera* and *Ruminococcus* (Figure 10). All the ETEC

challenged groups exhibited a high proportion of members of the Firmicutes phylum (Figure 8). At the family level, *Lachnospiraceae* and *Clostridiaceae* were markedly increased in the multi-strain group. In contrast, a high abundance of *Bacteroidaceae* was also observed in the ETEC control group (Figure 9). Furthermore, the genera *Clostridium* and *Bacillus* were enriched in the multi-strain group. At the same time, the ETEC control group had a higher number of *Bacteroides* genus than the other groups (Figure 10).

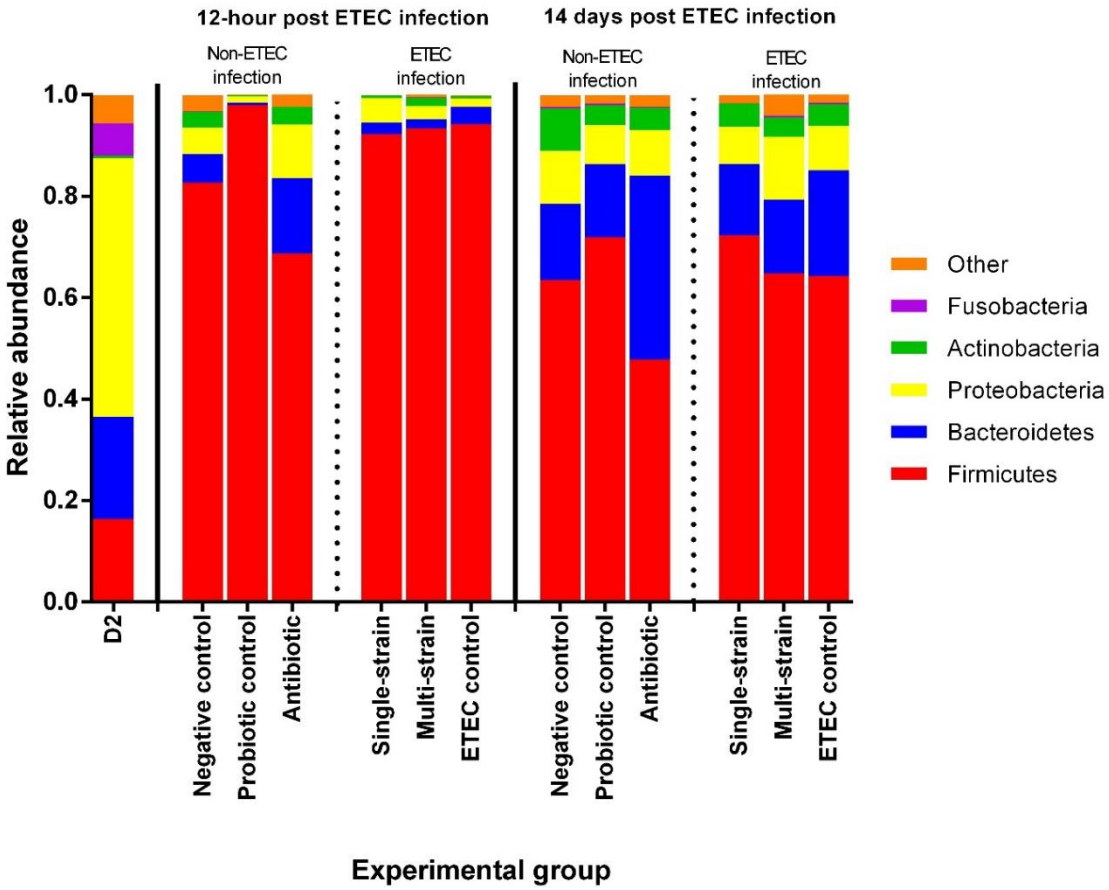

Figure 8 The relative abundance of fecal taxonomic classification across treatments in each time-point at the phylum level according to annotation with Kraken2 database. D2 refers to 2 days of age, before probiotic treatment.

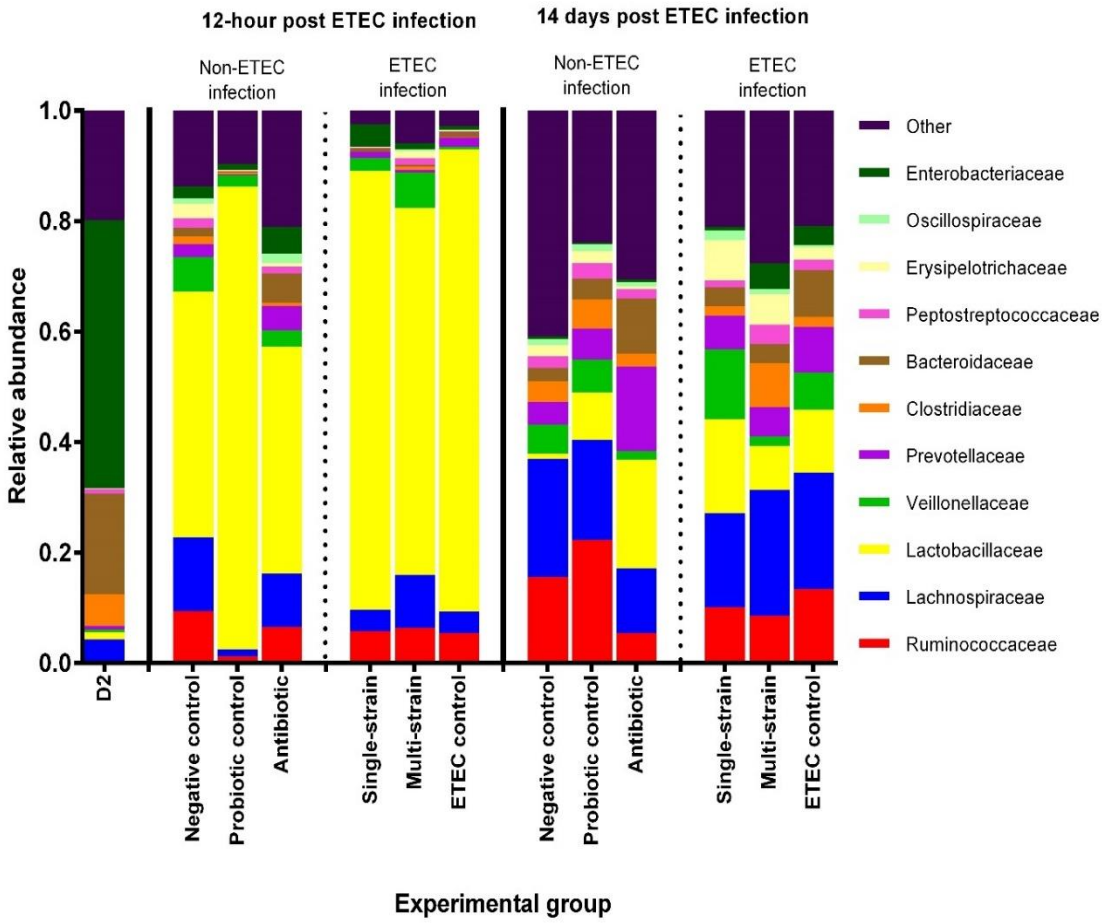

Figure 9 The relative abundance of fecal taxonomic classification across treatments in each time-point at the family level according to annotation with Kraken2 database. D2 refers to 2 days of age, before probiotic treatment.

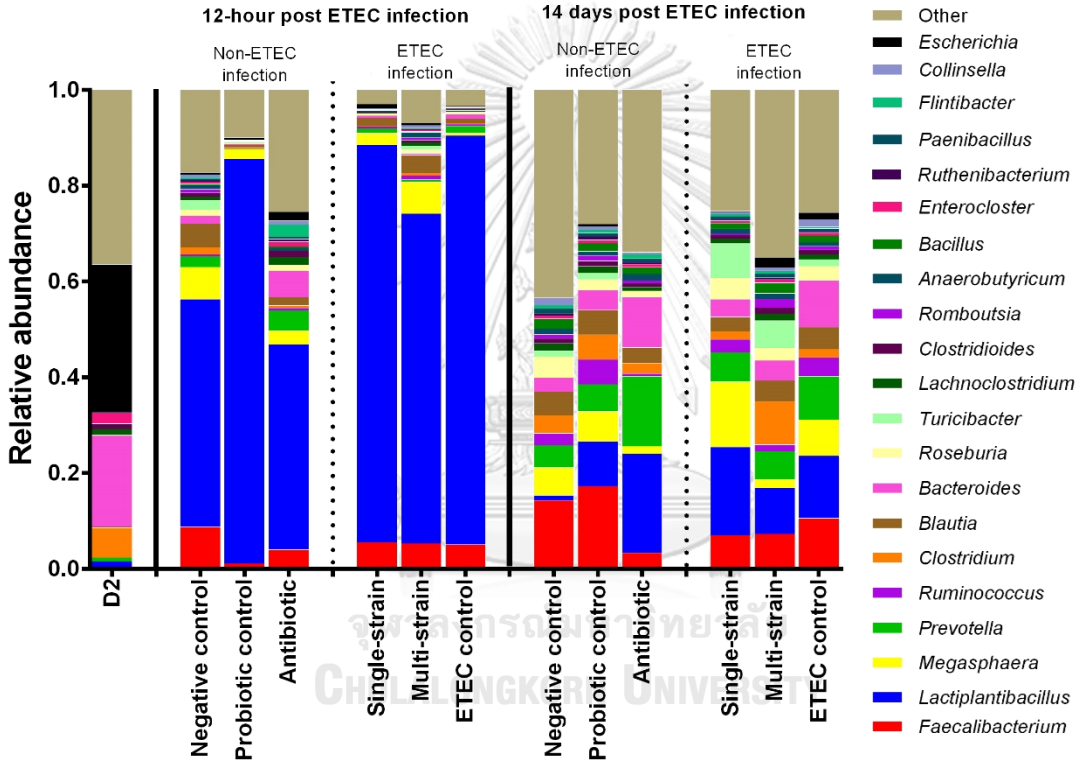

Figure 10 The relative abundance of fecal taxonomic classification across treatments in each time-point at the genus level according to annotation with Kraken2 database. D2 refers to 2 days of age, before probiotic treatment.

3.4.3 Abundance and composition of the piglet gut resistome

At Day 2, TEM genes associated with beta-lactam resistance were the most prominent antimicrobial resistance (AMR) determinants (Figure 11 and 12). The beta-lactam resistance class was enriched in the negative control and antibiotic groups of the non-ETEC infected groups at 12 hpc (Figure 11). In addition, the *tetW* and *tetQ* genes were overrepresented in those groups (Figure 12). Beta-lactam resistance in the ETEC challenged groups was lower in the multi-strain group than in the single-strain and ETEC control groups (Figure 11). Furthermore, the single-strain and ETEC control groups had more TEM and *tetQ* genes than the multi-strain group (Figure 12).

At 14 dpc, amongst the non-ETEC infected groups beta-lactam resistance was dominant in the antibiotic group (Figure 11). In the antibiotic group, the *tetQ*, *mefA* and *tetM* genes were all found in abundance (Figure 12). Furthermore, in the ETEC challenged groups, the *tetQ*, *mefA* and *tetM* genes were less frequent in the multi-strain group than in the single-strain and ETEC control groups (Figure 11 and 12).

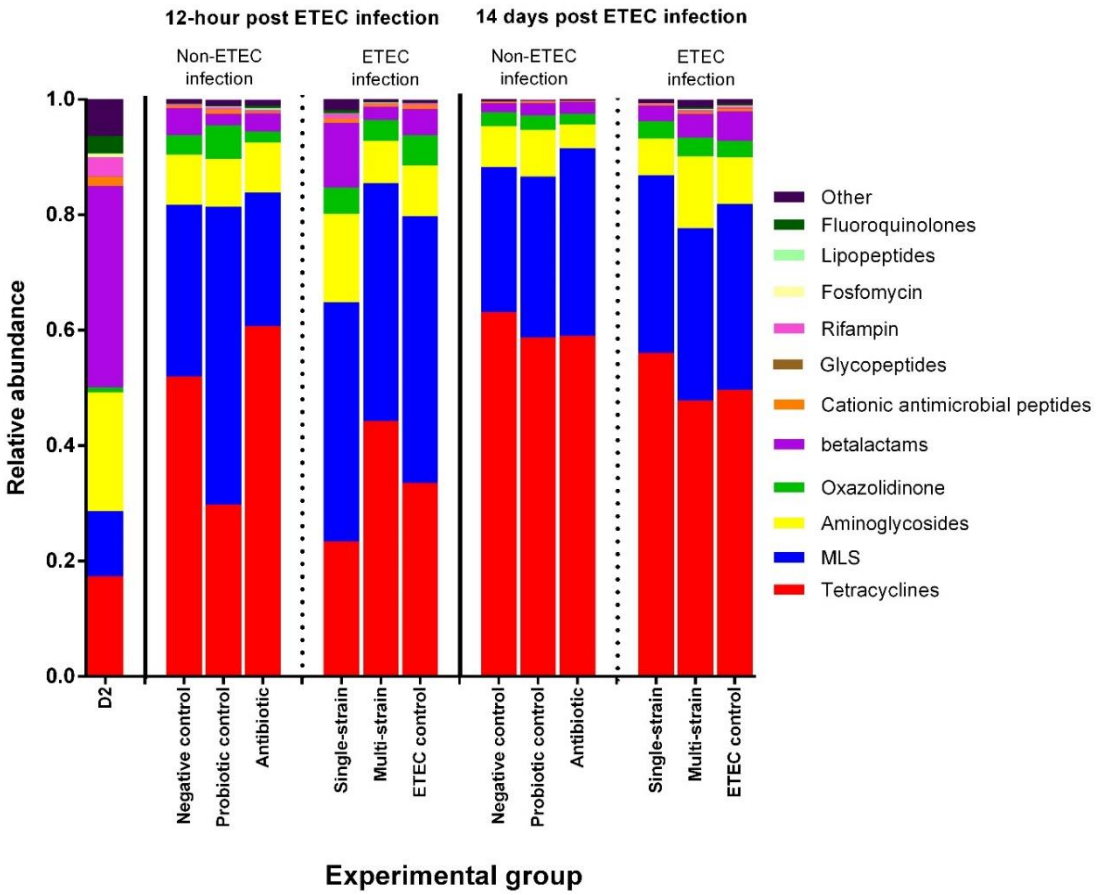

Figure 11 The relative abundance distribution of faecal antimicrobial resistance classes across treatments at each time-point based on annotation with MEGARes database. D2 refers to 2 days of age, before probiotic treatment.

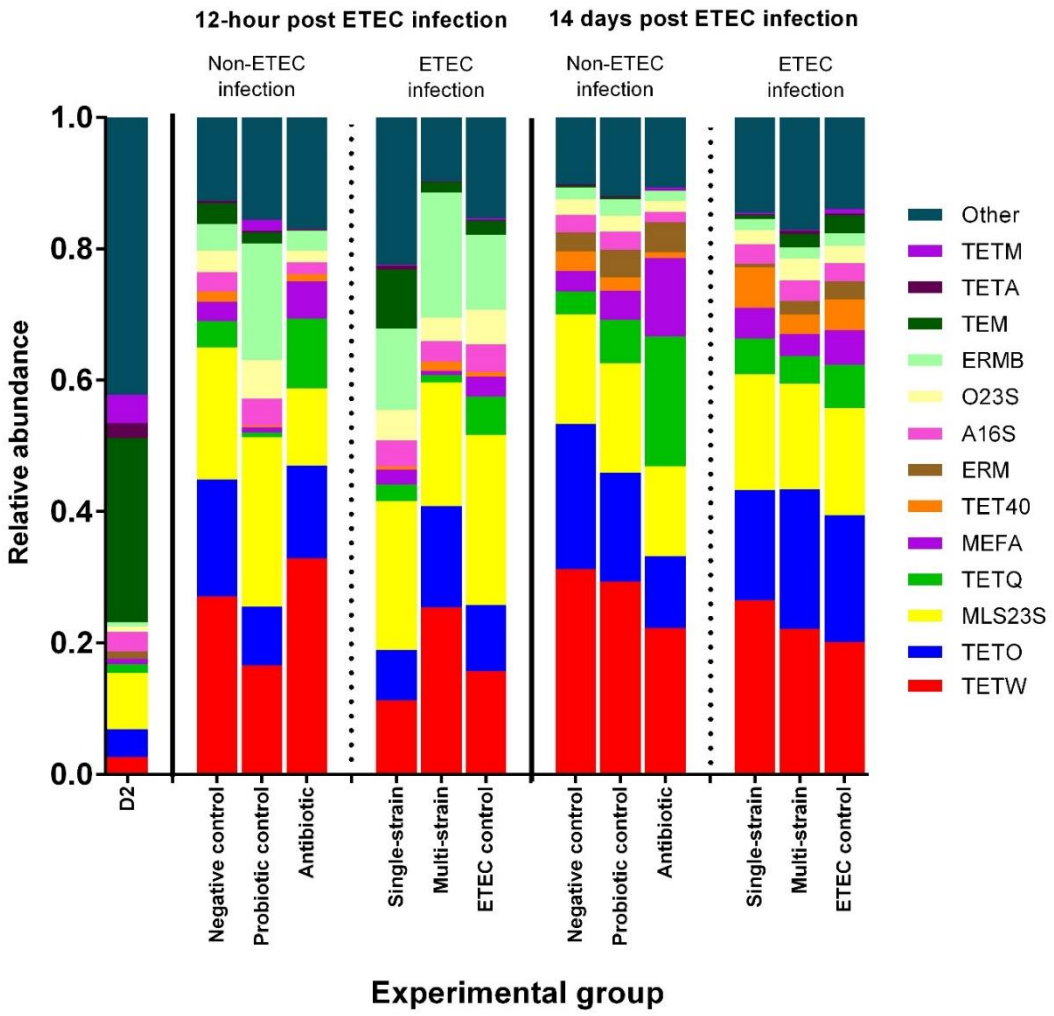

Figure 12 The relative abundance distribution of faecal antimicrobial resistance groups across treatments at each time-point based on annotation with MEGARes database. D2 refers to 2 days of age, before probiotic treatment.

3.4.4 Abundance and diversity of metal and biocide resistance

According to the metal resistance analysis, multi-metal resistance was the most common type identified, followed by copper (Cu) and zinc (Zn) resistance (Table 14). At 12 hpc and 14 hpc, the Cu and Zn resistances were more abundant in the antibiotic group than in the negative control and the probiotic control groups. Moreover, Cu resistance in the single-strain group was higher than in the multi-strain and the ETEC control groups (Table 14).

Multi-biocide resistance was the most common biocide resistance, followed by acid and acetate resistance. At 12 hpc and 14 hpc, amongst the non-ETEC infected groups the multi-biocide resistance in the probiotic control group was lower than in the negative control and antibiotic groups. The multi-strain group had lower multi-biocide resistance and more abundant peroxide resistance than the single-strain and ETEC control groups in the ETEC infection groups (Table 15).

3.4.5 Mobile Genetic Elements (plasmid replicons, integron integrase genes and insertion sequences) within the piglet gut microbial community

The antibiotic group had higher levels of several plasmid replicons, including IncQ1, IncX4, IncHI2, and IncHI2A than the other groups (Figure 13). Integrase gene (*intI*) class 1 was the most common integron in all experimental groups, accounting for more than 97 % of all detected integrons. Furthermore, an *intI* class 2 was found in the ETEC control group at 14 dpc, whereas an *intI* class 3 was only found in the antibiotic group (Figure 14). At 12 hpc and 14 dpc, insertion sequence (IS) 1380 was enriched in the negative control and antibiotic groups (Figure 15). IS1380 was prominently detected in the ETEC infected groups, while IS3 and IS30 were prominently detected in the single-strain and multi-strain groups (Figure 15).

Table 14 The percentage of relative abundance of metal resistance group based on metal resistance genes in piglet fecal samples.

Metal resistance group	D2	12-hours post ETEC challenging						14-days post ETEC challenging					
		Non-ETEC infection			ETEC infection			Non-ETEC infection			ETEC infection		
		Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control	Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control
Multi-metal	57.11	67.27	67.50	59.07	47.83	66.15	55.56	55.62	61.78	57.14	60.77	56.79	55.88
Copper	13.31	10.12	8.93	14.65	35.75	10.76	12.59	11.23	15.53	42.86	12.10	12.00	19.80
Nickel	8.96	7.00	7.02	8.22	5.37	6.31	9.35	7.40	7.05	0.00	9.86	9.24	7.98
Zinc	8.52	6.38	6.84	7.77	4.17	7.17	9.45	12.05	7.38	0.00	6.57	8.42	7.54
Arsenic	5.41	5.65	4.91	5.36	3.36	5.42	5.59	6.58	4.96	0.00	6.64	6.89	4.90
Sodium	4.93	3.15	3.80	4.22	2.98	3.63	5.25	1.92	2.64	0.00	3.08	5.12	3.27
Iron	0.66	0.02	0.23	0.03	0.01	0.12	0.02	1.64	0.00	0.00	0.00	0.00	0.00
Chromium	0.62	0.41	0.54	0.43	0.50	0.41	0.64	1.10	0.00	0.00	0.56	0.62	0.49
Mercury	0.46	0.00	0.20	0.00	0.01	0.03	0.28	2.47	0.66	0.00	0.00	0.00	0.15
Tellurium	0.01	0.00	0.03	0.25	0.01	0.00	1.27	0.00	0.00	0.00	0.42	0.91	0.00

Table 15 The percentage of relative abundance of metal resistance group based on biocide resistance genes in piglet fecal samples.

Biocide resistance group	D2	12-hours post ETEC challenging						14-days post ETEC challenging					
		Non-ETEC infection			ETEC infection			Non-ETEC infection			ETEC infection		
		Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control	Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control
Acid	34.427	32.567	29.961	37.233	31.562	32.426	32.642	27.723	35.223	0	29.978	30.665	30.196
Multi-biocide	34.392	34.602	26.800	32.058	37.785	33.557	36.889	38.614	27.935	100	40.940	36.569	37.444
Acetate	18.453	18.394	20.185	14.820	16.721	17.470	19.358	27.723	12.955	0	16.555	21.628	18.806
Peroxide	8.905	10.969	9.922	11.755	10.628	13.322	7.556	3.960	21.053	0	11.186	7.948	10.655
Phenolic compound	3.799	3.468	3.210	4.134	3.304	3.226	3.556	1.980	2.834	0	1.342	3.191	2.899
Biguanide	0.007	0	0	0	0	0	0	0	0	0	0	0	0
Quaternary ammonium compounds	0.016	0	9.922	0	0	0	0	0	0	0	0	0	0
Paraquat	0.001	0	0	0	0	0	0	0	0	0	0	0	0

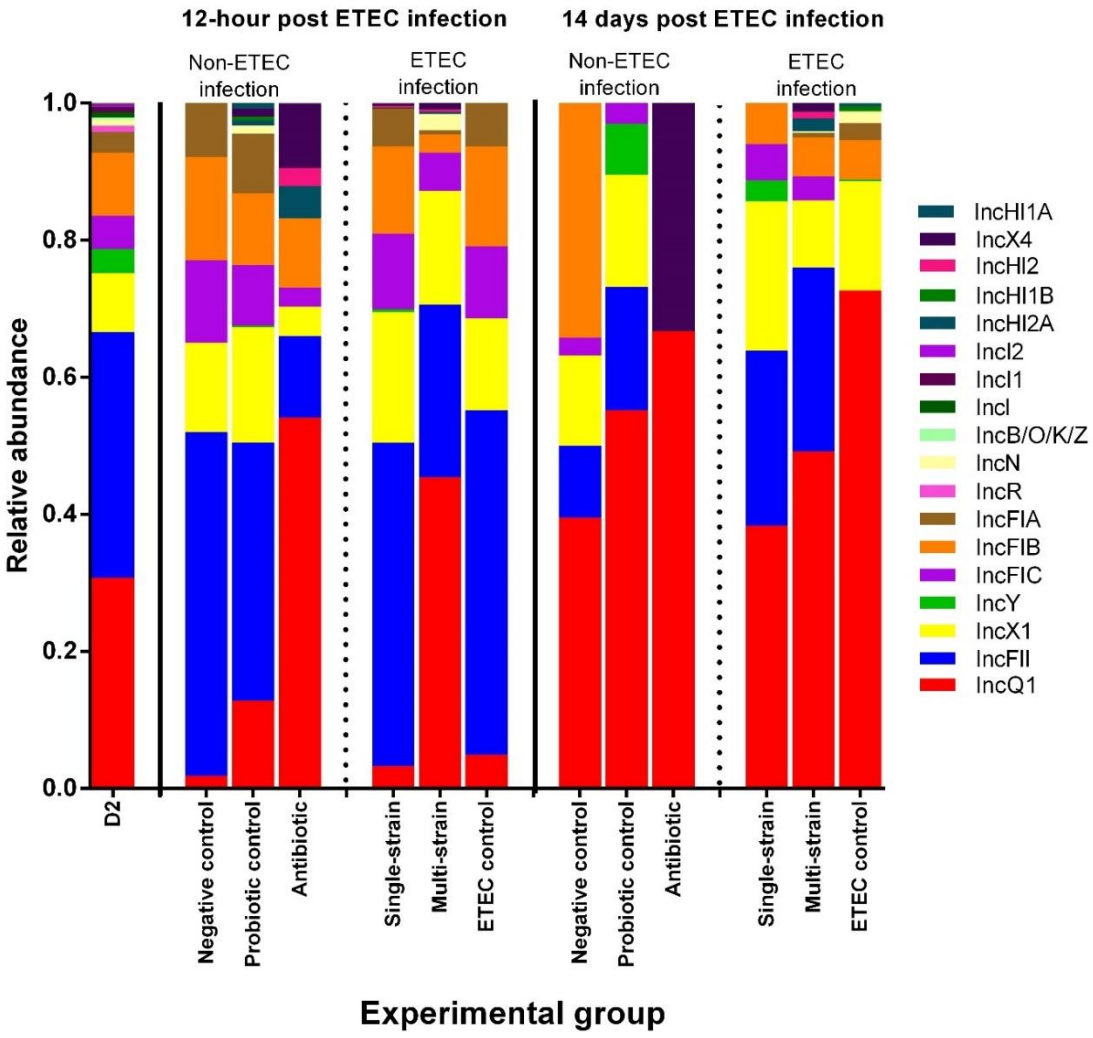

Figure 13 The relative abundance distribution of the classified plasmid replicons from PlasmidFinder database across treatments in each time-point. D2 refers to 2 days of age, before probiotic treatment.

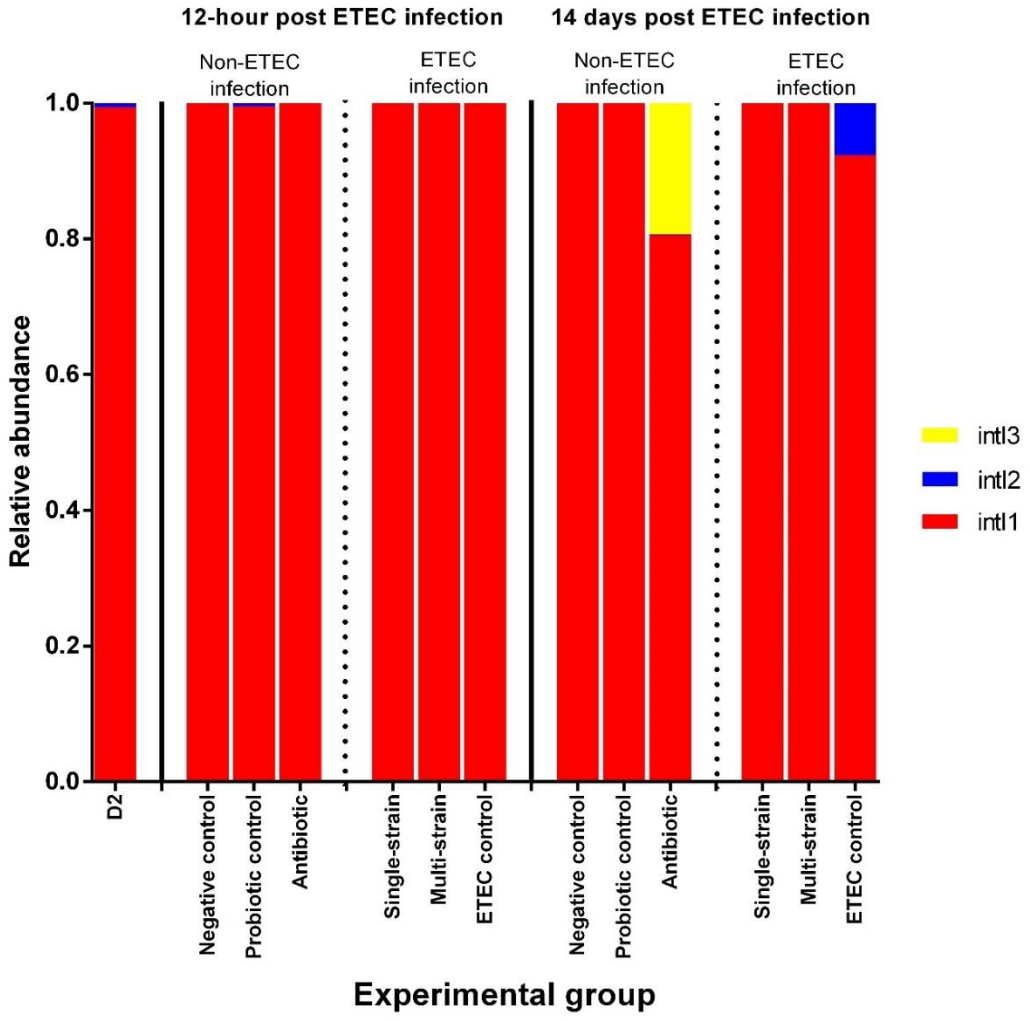

Figure 14 The relative abundance distribution of the aligned integron integrase genes from INTEGRALL database across treatments in each time-point. D2 refers to 2 days of age, before probiotic treatment.

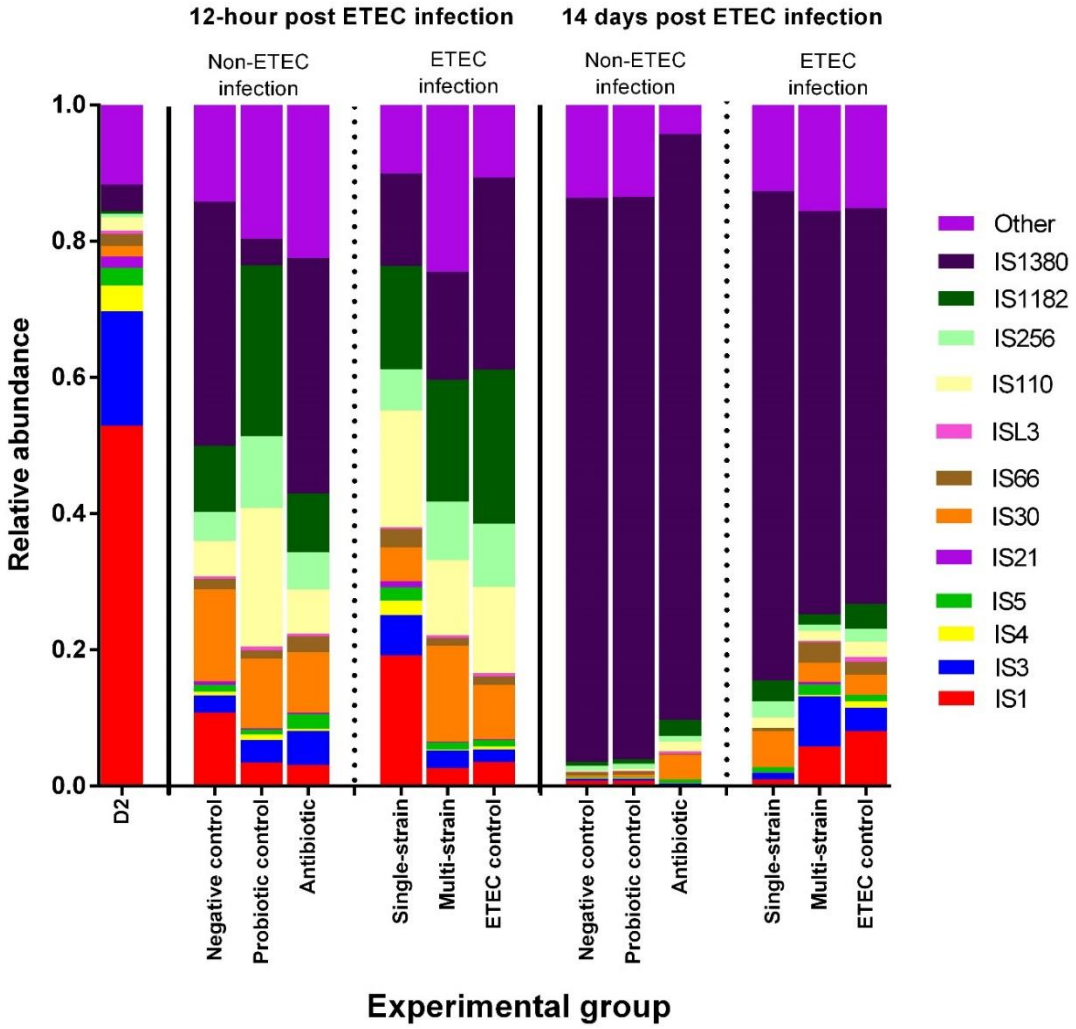

Figure 15 The relative abundance distribution of the sorted insertion sequences from ISFinder database across treatments in each time-point. D2 refers to 2 days of age, before probiotic treatment.

3.4.6 Microbial functional diversity of the gut metagenome related to stress response in ETEC and non-ETEC infected piglets.

As shown in Figure 16 and Figure 17. the stress response was analyzed using the SEED subsystem database within the MG-RAST server. In all experimental groups, oxidative stress was the most prevalent, ranging from 33.20 to 45.63% in the stress response at level 2. Surprisingly, the multi-strain group had the highest stress response associated with detoxification at 12 hpc, accounting for more than 19% of the total (Figure 16). The transcriptional and redox-sensitive transcriptional regulators, which were the main markers of oxidative stress responses in this study, were found in approximately 80% of the total sequences in the probiotic control and multi-strain groups. In addition, compared to the other groups, the multi-strain group had more catalase and superoxide dismutase (Figure 17).

3.4.7 Microbial functional diversity of the gut metagenome associated with nutrient metabolism in ETEC and non-ETEC infected piglets.

The relative abundance of functional genes at level 1 KEGG related to metabolism is shown in Figure 18. Amino acid and carbohydrate metabolism were dominant in roughly 60% of the total nutrient metabolism sequences (Figure 18). Most amino acid metabolism pathways involved alanine, aspartate, and glutamate metabolism, followed by glycine, serine and threonine metabolism, and cysteine and methionine metabolism (Table 16). Furthermore, glycolysis/gluconeogenesis, amino sugar and nucleotide sugar metabolism, and galactose metabolism were the top three carbohydrate metabolisms, respectively (Table 17). Among the non-ETEC infected groups, amino acid and carbohydrate metabolism pathways were less represented in the probiotic control group than in the antibiotic group at 12 hpc. The multi-strain group, on the other hand, had more genes related to amino acid metabolism than did the other ETEC infected groups (Table 16). The probiotic control and multi-strain groups had significantly more genes associated with amino acid and carbohydrate metabolism at 14 dpc than the other groups (Table 16 and Table 17).

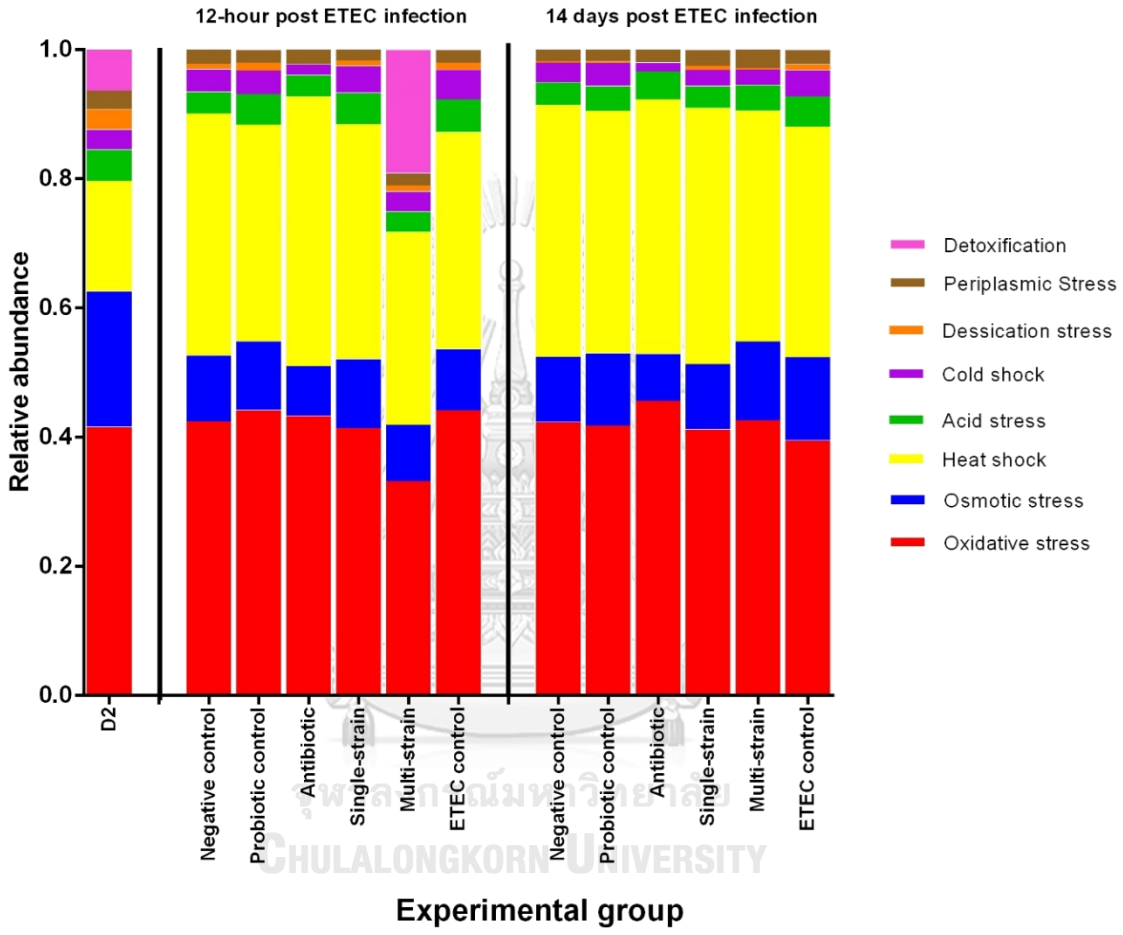

Figure 16 Relative abundance of the level 2 SEED subsystem aligned genes associated with stress response from piglet faecal samples in ETEC or non-ETEC infected piglets. D2 refers to 2 days of age, before probiotic treatment.

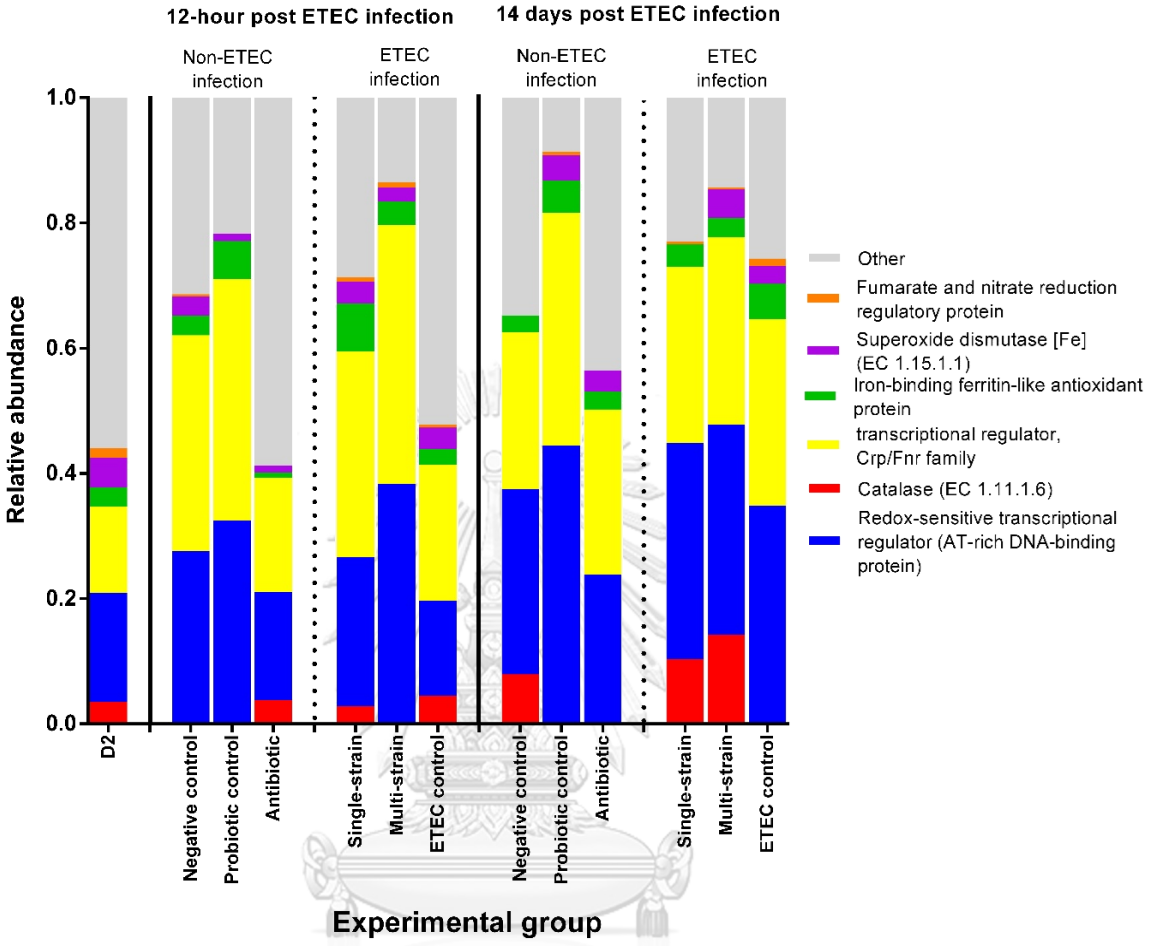

Figure 17 Relative abundance of the level 4 SEED subsystem classified reads associated with oxidative stress from piglet faecal samples in ETEC or non-ETEC infected piglets. D2 refers to 2 days of age, before probiotic treatment.

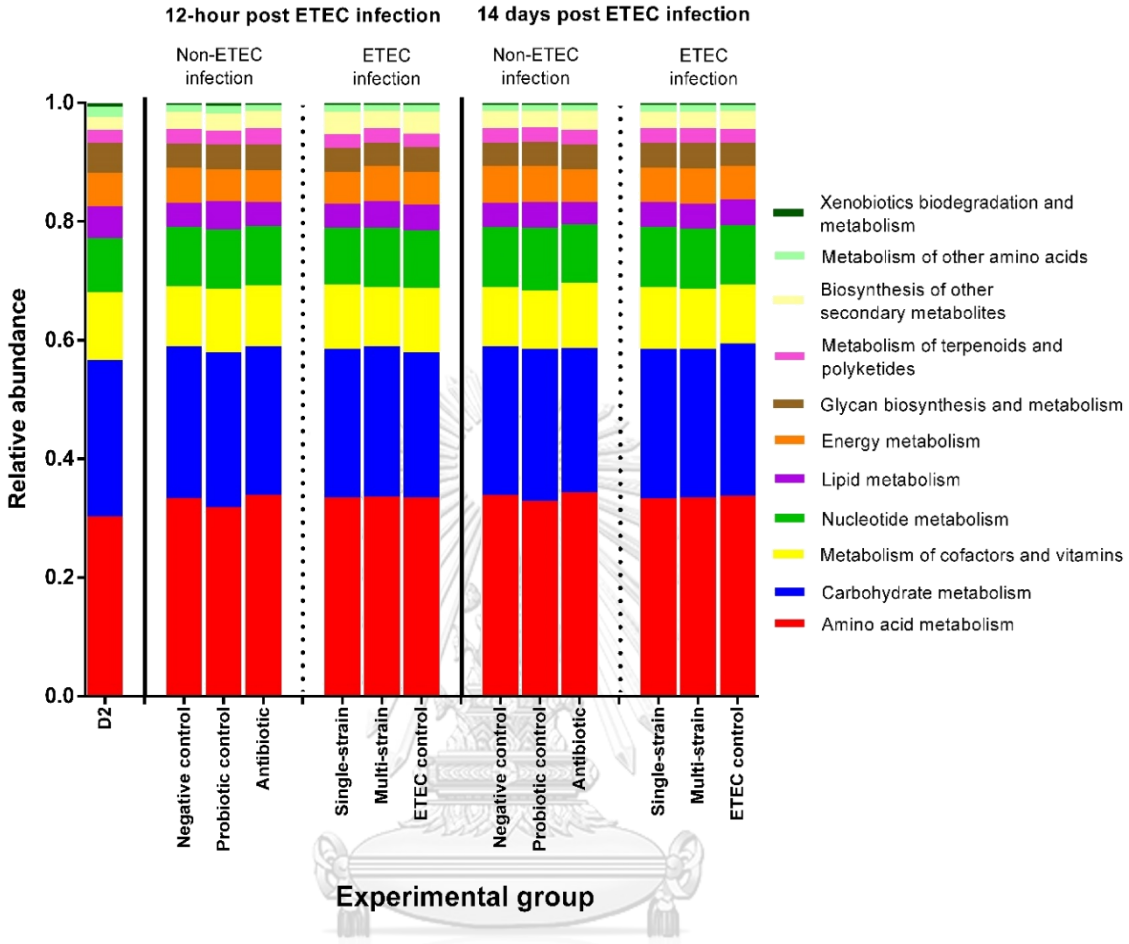

Figure 18 The relative abundance of the level 1 KEGG functional genes related to metabolism from piglet faecal samples in ETEC or non-ETEC infected piglets. D2 refers to 2 days of age, before probiotic treatment.

Table 16 Normalized abundance of the level 3 KEGG functional reads related to amino acid metabolism from faecal samples in ETEC or non-ETEC challenging piglets.

Amino acid metabolism	D2	12-hours post ETEC challenging						14-days post ETEC challenging					
		Non-ETEC infection			ETEC infection			Non-ETEC infection			ETEC infection		
		Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control	Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control
Glycine, serine, and threonine metabolism	1310	1144	3799	5668	2054	2732	2004	3382	3715	2852	4428	4668	2511
Alanine, aspartate, and glutamate metabolism	1390	1576	4677	6664	2775	3241	2511	4107	4512	3936	5647	5694	2004
Arginine and proline metabolism	837	792	2405	3453	1377	1767	1239	2213	2369	1914	2751	2948	1918
Cysteine and methionine metabolism	825	1018	3487	4642	1933	2521	1918	3135	3188	2629	3843	4213	1239
Lysine biosynthesis	639	740	2136	2937	1222	1505	1131	2011	2160	1573	2557	2761	1131
Phenylalanine, tyrosine, and tryptophan biosynthesis	504	515	1648	2320	1014	1129	909	1549	1667	1241	2029	1975	966
Valine, leucine, and isoleucine biosynthesis	494	444	1446	2191	965	1108	966	1515	1596	1268	1779	2018	919
Histidine metabolism	501	542	1493	2222	847	1040	919	1435	1595	1226	1835	1914	909
Valine, leucine, and isoleucine degradation	328	194	626	1146	334	460	314	569	648	525	775	813	314
Phenylalanine metabolism	300	109	430	566	193	303	173	375	424	249	407	497	173
Tyrosine metabolism	194	157	352	427	186	284	163	341	345	209	401	469	163
Lysine degradation	129	36	121	191	54	94	42	94	127	93	129	160	42
Tryptophan metabolism	41	23	50	95	14	41	21	35	38	40	57	72	21

Table 17 Normalized abundance of the level 3 KEGG functional reads associated with carbohydrate metabolism from faecal samples in ETEC or non-ETEC infected piglets.

Carbohydrate metabolism	D2	12-hours post ETEC challenging						14-days post ETEC challenging					
		Non-ETEC infection			ETEC -infection			Non-ETEC infection			ETEC infection		
		Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control	Negative control	Probiotic control	Antibiotic	Single -strain	Multi-strain	ETEC control
Glycolysis / Gluconeogenesis	880	830	2373	3202	1250	1652	1209	2194	2506	1609	2773	2980	2094
Pyruvate metabolism	848	692	1801	2463	975	1243	977	1757	1937	1276	2201	2218	1655
Amino and nucleotide sugar metabolism	712	684	2274	3037	1245	1464	1136	1961	2213	1413	2593	2731	1994
Galactose metabolism	748	784	2134	2668	1329	1576	1171	1878	2057	1627	2384	2631	2006
Pentose phosphate pathway	703	632	1769	2377	923	1338	866	1602	1858	1162	2133	2152	1563
Starch and sucrose metabolism	569	673	1892	2653	1088	1493	1061	1679	1836	1458	2259	2382	1764
Fructose and mannose metabolism	567	535	1457	2360	894	966	820	1201	1337	1204	1742	1785	1289
Pentose and glucuronate interconversions	554	515	1392	1948	863	990	784	1048	1424	1182	1548	1507	1295
Citrate cycle (TCA cycle)	318	330	1146	1805	562	729	520	1031	1065	941	1362	1408	888
Glyoxylate and dicarboxylate metabolism	224	158	592	724	318	416	253	616	643	380	641	648	473
Ascorbate and aldarate metabolism	92	30	62	68	45	52	34	62	63	59	46	72	66
Butanoate metabolism	80	74	256	347	104	188	120	206	238	188	270	285	204
Inositol phosphate metabolism	60	28	103	106	38	77	43	82	90	42	90	117	71
Propanoate metabolism	58	21	69	80	35	36	36	43	63	34	75	65	44
C5-Branched dibasic acid metabolism	12	0	21	31	0	25	3	26	34	11	18	37	15

3.5 Discussion

In our previous study that used 60 of the pigs included in the current study, dosing the neonatal piglets with the multi-strain probiotic enhanced average daily gain and feed conversion ratio (FCR) of the piglets after ETEC challenge following weaning, whilst supplementing with the single-strain probiotic increased FCR (Pupa et al., 2022)The piglets receiving probiotics had an increase in lactic acid bacteria counts and a decrease in *E. coli* counts in the faeces, with lower levels of virulence genes being detected. Challenged piglets receiving probiotics had milder intestinal lesions with better morphology, including greater villous heights and villous height per crypt depth ratios, than pigs just receiving ETEC. This study demonstrated that prophylactic administration of microencapsulated probiotic strains may improve outcomes in weaned pigs with colibacillosis. The current study enlarged on these findings by examining the gut microbiota of these pigs in more detail. An additional group of pigs receiving chlortetracycline after weaning was included to help compare probiotics and antimicrobials in influencing the gut microbiota and supporting pig health after weaning. Whole-genome shotgun metagenomic sequencing of DNA extracted from faeces was used to investigate the gut microbiome, resistome, stress responses, and nutrient metabolism, and to examine how the probiotics cause beneficial changes in piglets infected with ETEC.

Faecal samples were used as a proxy for intestinal samples for examining the gut microbiota, as faeces can be obtained from live pigs which then can be sampled again at later stages. The gut microbiota composition in faeces collected from the rectum seems to be stable, and it shows the same pattern as the hindgut regions, indicating that the faecal microbiota can be used as a proxy for the microbiota in the large intestine of the pigs (Zhao et al., 2015; Gresse et al., 2019). Samples were pooled because it was not technically or financially possible to examine samples from all individual piglets in this study. It is acknowledged that this does not allow comparison of variations between pigs within a group, but this approach was necessary for practical purposes and does provide an overview of

group affects. The methodology used means that it was not appropriate to undertake statistical analysis between groups in this study.

Firmicutes and Proteobacteria were the most prevalent phyla found in piglet faeces at Day 2, which agrees with a previous study which found that these phyla were the most prevalent microbial components in early newborn piglets (Chen et al., 2018). In addition, the genus *Escherichia*, which belongs to the *Enterobacteriaceae* family, was found in abundance. Pathogenic strains of *Escherichia coli* can have an impact on human and animal health by acquiring and disseminating AMR and virulence genes through the food supply chain, and they act as a biomarker of diarrhoeal piglets in the lactation phase (Kang et al., 2018; Sun et al., 2019).

According to several studies, Firmicutes and Bacteroidetes are the most numerous phyla in the piglet faecal microbiota during the post-weaning phase (Li et al., 2017; Ghanbari et al., 2019; Shin et al., 2019; Sun et al., 2019). The probiotic control group had a larger proportion of Firmicutes phylum than the other groups in the current study. This result appears to be congruent with another study, which found that supplementing with *Enterococcus faecalis* UC-100 was associated with more than 85% of the total sequences enriched by the Firmicutes phylum (Li et al., 2017). In the current study the genera *Blautia, Lactiplantibacillus, Megasphaera, Ruminococcus, Clostridium* and *Faecalibacterium* were identified in the multi-strain and probiotic control groups. These genera are regarded as being beneficial microbes due to a variety of characteristics, including the ability to produce antibacterial substances (e.g., bacteriocins, organic acids) that inhibit growth of pathogens, the ability to increase carbohydrate metabolism by utilizing dietary starch and fiber, and the ability to produce short-chain fatty acids (SCFAs) that reduce gut inflammation (Niu et al., 2015; Jiang et al., 2016; Li et al., 2017; Guevarra et al., 2018; Shin et al., 2019; Wang et al., 2019b; He et al., 2021; Liu et al., 2021). The group receiving chlortetracycline showed a significant increase in Proteobacteria and Bacteroidetes,

which is consistent with prior research demonstrating that antibiotic administration could boost these phyla (Ghanbari et al., 2019; Wang et al., 2019b; Suriyaphol et al., 2021; Tunsagool et al., 2021). However, several studies have suggested that enhanced numbers of Bacteroidetes may promote host health by enhancing nutrient digestion and absorption (Guevarra et al., 2018; Wang et al., 2019b). Furthermore, it has been suggested that they act as a biomarker for gut dysbiosis in piglets given antibiotics (Zeineldin et al., 2019).

We found a variety of AMR determinants in neonatal piglets in this study, and the dominant antibiotic-resistant classes and genes discovered in this study appear to be linked to our previous research, which found that neonatal piglets in antibiotic-free farms had high levels of beta-lactam resistance and carriage of the *blaTEM* gene (Lugsomya et al., 2018c). The World Health Organization (WHO) classifies beta-lactams as critically important antimicrobials, meaning they have the potential to have a major impact on human health (WHO, 2019).

Previous studies have shown that tetracyclines and MLSs are the most common antibiotic resistance classes in weaned pigs receiving or not receiving in-feed antibiotics, and the findings of the current study are consistent with this (Ghanbari et al., 2019; Suriyaphol et al., 2021). High levels of beta-lactam resistance also were found in both the negative control and the antibiotic groups. This matches previous findings of dominant beta-lactam resistance in medicated and unmedicated piglets (Ghanbari et al., 2019). The antibiotic group had more *tetW, tetQ, tetM* and *mefA* genes, which are involved in tetracycline ribosomal protection proteins and MLS efflux pumps, on an AMR gene level (Iannelli et al., 2018; Ghanbari et al., 2019). These genes have been found on mobile genetic elements such as conjugative transposons, which can spread to other bacteria via horizontal transfer. Furthermore, previous research has found that the *Bacteroidaceae* family frequently carry such genes, suggesting that they could be a source of AMR genes for the gut microbial community (Ghanbari et al., 2019; Niestepski et al., 2019).

Piglets given probiotics in the current study had a lower proportion of AMR determinants like beta-lactam resistance, *mefA, tet,* and *tetW* genes than piglets given antibiotics. Probiotics may modify the gut microbial population by reducing the abundance of some antibiotic-resistant microorganisms through a variety of processes, including competition for food substrates and binding sites, production of antimicrobial compounds, and regulation of immune responses (O'Toole and Cooney, 2008). These data are consistent with prior research showing that probiotic treatment in infants can reduce ARG abundance by eliminating antibiotic-resistant carriers (Casaburi et al., 2019). To our knowledge, this is the first study to investigate the effects of probiotic supplementation on modulation of the pig gut resistome. However, since the existence of some antibiotic genes may not indicate phenotypic resistance, a weakness in the current study was the lack of comparison between AMR genotypic and phenotypic features. Phenotypic determinations should be performed on fresh faecal samples, and this was not possible with the frozen samples (Suriyaphol et al., 2021; Tunsagool et al., 2021).

Based on co-selection processes such as co-resistance, cross-resistance, and biofilm formation, there is evidence of a link between antibiotic, metal, and biocide resistances (Yu et al., 2017). Copper and multi-biocide resistances were found in abundance in the antibiotic group, which was linked to numerous antimicrobial drug resistances such as to beta-lactams, fluoroquinolones, macrolides and tetracyclines (Cheng et al., 2019; Paul et al., 2019; Zhang et al., 2019). This could explain why the antibiotic group had more Cu and multi-biocide resistance than the other groups. Biofilm production is critical for preserving metal and biocide resistances, protecting the population from metal and biocide toxicity, and increasing the lateral transfer of ARGs with co-selected metal resistant genes (Yu et al., 2017; Cheng et al., 2019). Our probiotic strains have been shown to minimize ARG transfer and biofilm development *in vitro* (Apiwatsiri et al., 2021). Taken together, this could be another reason why the probiotic supplemented groups had lower Cu and multi-biocide resistance genes detected.

The complete set of MGEs, and specifically the mobilome, are thought to hasten the spread of ARGs among members of the gut microbiota (Wang et al., 2020b). In the antibiotic group, IncQ1, IncX4, IncHI2, and IncHI2A plasmids were detected in abundance, which is of concern because it may allow multidrug resistance (MDR) in humans and animals, such as resistance to aminoglycosides, beta-lactams, and tetracycline (Rozwandowicz et al., 2018). Furthermore, they may be involved in colistin resistance where they contain the mobilized colistin resistance (*mcr*) gene (Sun et al., 2017; Rozwandowicz et al., 2018). Interestingly, the probiotic-supplemented groups had fewer plasmid replicons than the antibiotic-supplemented group. This finding supports the theory that probiotics can regulate the gut microbial community by lowering the proportion of microbiota carrying certain plasmids, or by blocking ARG transfer via a variety of pathways (O'Toole and Cooney, 2008; Apiwatsiri et al., 2021). In the current study, class 1 integrons were shown to be abundant in all groups. This finding is consistent with prior research that found it to be the most common integron type, accounting for about 80% of all types in enteric bacteria in humans and animals (Deng et al., 2015). The ETEC control group contained class 2 integron, which is involved in resistance to aminoglycosides, beta-lactams, and erythromycins (Deng et al., 2015; Pathirana et al., 2018). In addition, class 3 integron was found only in the antibiotic group, and it has been linked to beta-lactam resistance and the IncQ plasmid replicon (Deng et al., 2015). Furthermore, the antibiotic group had higher levels of IS1380, which can increase beta-lactam and nitroimidazole resistance in Bacteroidetes, the antibiotic group's predominant member (Vandecraen et al., 2017). In our study, the probiotic supplemented groups had more IS3 and IS30, which are involve with numerous metabolic modulations such arginine production and the use of acetate, citrate, and galactose (Vandecraen et al., 2017). This appears to be the first report to detail the effects of probiotic supplementation on MGE regulation in the pig gut microbial population.

An imbalance between reactive oxygen species (ROS) and antioxidant responses was typically seen in the weaning transition or after ETEC infection, which events are likely to be a source of oxidative stress (Guevarra et al., 2018). Excessive

exposure to ROS can have negative consequences on bacterial cells, resulting in protein activity dysfunction and bacterial cell death (Guevarra et al., 2018). In the probiotic groups, genes related to the oxidative response, particularly transcriptional and redox-sensitive transcriptional regulator contributing to antioxidant activity, were elevated (Zhu et al., 2012). Furthermore, antioxidant capacity was related to detoxification in the multi-strain group following ETEC challenge (Zhu et al., 2012). This finding agrees with previous studies suggesting that a variety of probiotic isolates may boost antioxidant defense mechanisms and reduce oxidative stress (Wang et al., 2012; Liao and Nyachoti, 2017a). Consequently, further research on the antioxidant activities of our probiotic strains (L22F, L25F, and P72N) is needed to improve understanding of the mechanism of stress response modulation.

The probiotic groups had increased numbers of amino acid metabolism genes, which agrees with previous work showing that many bacterial species, including *Lactiplantibacillus*, *Megasphaera* and *Veillonella* are involved in modulating amino acid metabolism (Liao and Nyachoti, 2017a; Wang et al., 2020c). Moreover, several amino acids e.g., alanine, arginine, glutamine, glycine, methionine and threonine have been shown to benefit pig gut health, including by altering the gut microbiota, maintaining intestinal shape, and increasing anti-inflammatory, and anti-oxidative stress functions (Yang and Liao, 2019). We also found that the probiotic supplemented groups had higher levels of genes involved in glucose metabolism. This result is consistent with previous studies that identified carbohydrate utilization via fermentation and hydrolysis pathways in a variety of gut bacteria, including *Faecalibacterium, Lactiplantibacillus* and *Ruminococcus,* (Liao and Nyachoti, 2017a; Wang et al., 2020c). SCFAs, which are readily available energy sources for pigs, are one of the bacterial metabolites produced following food digestion that may have anti-inflammatory and antagonistic properties (Shin et al., 2019; Wang et al., 2020c). However, additional investigations into the complete genomes of our probiotic strains are recommended to expand these findings. These data should be linked to global metabolomic and proteomic studies to better understand the mechanisms of the probiotic effects on the gut microbiome and resistome.

CHAPTER IV

GENERAL DISCUSSION AND CONCLUSION

This study used colistin-resistant *E. coli* containing the *mcr*-1 gene as a model to assess *in vitro* LAB effects on AMR parameters involving plasmid conjugation and biofilm formation. Our promising LAB strains showed anticonjugation and antibiofilm activity, confirming our assumptions that the chosen LAB can diminish the ability of plasmids harboring the *mcr*-1 gene to be horizontally transferred in *E. coli*. Furthermore, they may interfere with *E. coli* biofilm development.

The CFS of the LAB strains; *L. plantarum* 22F, 25F, and *P. acidilactici* 72N were assessed the anticonjugation activity. The optimized CFS dilution was first performed against chosen *E. coli* strains to exclude confounding variables that could raise from the bactericidal effect of our LAB strains (Sirichokchatchawan et al., 2018a). Then, *in vitro* broth mating was used to determine our LAB strains anticonjugation activity, and the results demonstrated that CFS with a final dilution of 1:16 (pH 5.70-5.92) could greatly reduce the *mcr*-1 gene transfer frequency. Furthermore, *L. plantarum* 22F has higher anticonjugation activity than other LAB strains. We hypothesized that the anticonjugation activity was due to probiotic metabolites that were well-functioning in weak acid conditions and could influence the bacterial conjugation process, such as eliminating plasmids containing the *mcr*-1 gene, hindering DNA replication and/or DNA translocation, and inhibiting the formation of sex pili (Fernandez-Lopez et al., 2005; Moubareck et al., 2007; El-Deeb et al., 2015; Ripoll-Rozada et al., 2016).

Our promising LAB strains, including *L. plantarum* 22F, 25F, and *P. acidilactici* 72N, were evaluated for antibiofilm capabilities in addition to anticonjugation activity. Biofilm formation was moderate to strong in all *E. coli* strains. The non-neutralizing CFS, especially *P. acidilactici* 72N and *L. plantarum* 25F, significantly reduced biofilm

growth in the non-sessile, planktonic, and sessile phases, respectively. Because the pH of non-neutralizing CFS was detected varying from 3.70-3.98, it may be concluded that the acidity condition within CFS is a significant reason for these phenomena. This could be due to organic acids (e.g., lactic acid, acetic acid) and fatty acids impacting bacterial development (Barzegari et al., 2020). Other inhibitory chemicals, such as biosurfactant, exopolysaccharides, extracellular proteins, and interference with bacterial aggregation and adhesion, have also been described (Walencka et al., 2008; Kim et al., 2009; Fang et al., 2018a; Mahdhi et al., 2018; Giordani et al., 2019). Surprisingly, neutralizing CFS (pH 6.5) was more effective in antibiofilm activity in the sessile stage, particularly against *P. acidilactici* 72N. Bacteriocins have been postulated as an antibiofilm material in previous investigations; however, our LAB strains have been shown to be incapable of producing bacteriocins (Mathur et al., 2018; Sirichokchatchawan et al., 2018a; Kim et al., 2019). Other antibiofilm elements, such as deoxyribonuclease or dispersal signal, which might destroy the mature biofilm structure or speed up the biofilm dispersion process, could thus be the major factors on intervening in biofilm establishment during the sessile phase (Nijland et al., 2010; Barraud et al., 2015). As a result, these data suggested that our native CFS exhibited the antibacterial activity and interfered with biofilm development in both planktonic and sessile stages. However, the CFS diluting up to 16 times still showed the promising effect in limiting ARG transmission, and neutralizing CFS from our LAB strains remained demonstrated the antibiofilm capacity.

According to our earlier research, those LAB strains exhibited potential *in vivo* properties as an antibiotic alternative in pigs throughout the rearing cycle (Pupa et al., 2021a). Furthermore, weaned pigs with ETEC infection may benefit from the administration of our LAB strains (Pupa et al., 2022). Another part of this study used a whole-genome shotgun metagenomic technique to access the fecal microbiome and resistome in weaned piglets and weaned piglets infected with ETEC. Table 18 displays the overall data analysis for the gut microbiota and gut resistome in this investigation. Our LAB strains efficiently modulated the gut microbiome and resistome in pigs by boosting the beneficial bacterial population, lowering the

problematic resistome, improving antioxidant response, and upregulating genes involved in food metabolism.

Gut dysbiosis could cause several important diseases in humans by considering the ratio between Firmicutes and Bacteroidetes phyla (F: B ratio). Several studies indicated that elevated or reduced F:B ratio correlated with some diseases such as obesity or intestinal bowel disease (IBD) (Ley et al., 2006; Stojanov et al., 2020). In humans, the F: B ratio has altered along with the lifespan depending on the age. Previous research suggested that F: B ratio for childhood, adulthood, and aging individuals were approximately 0.40, 10.90, and 0.60, respectively (Mariat et al., 2009). Firmicutes and Bacteroidetes were also the predominant phyla in the intestinal microbial community of the pigs (Li et al., 2017). However, the porcine and human gut microbial population was thoroughly different, where the proportion of Firmicutes was higher in the pigs than humans (Zhao et al., 2015). The F: B ratio has also changed over time in swine, associated with age determinants (Yang et al., 2021). Prior research revealed that the F: B ratio for weaner, grower and finisher pigs exhibited diverse ranges up to 4.48, 53.10, and 46.11, respectively (Zhao et al., 2015; Sarri et al., 2021). These variables involve several factors, including ages, diets, genetics, and environmental conditions. Hence, it seems to be complicated to compare the F: B ratio between distinct studies (Sarri et al., 2021).

In the current study, the F: B ratio on Day 2 (before probiotic supplementation) was 0.81. While the F: B ratio at 12 hours post-ETEC challenge ranged from 4.61 to 212.93; moreover, the F: B ratio at 14 days post-ETEC challenge was 1.32-5.13 (Table 18). Higher F: B ratio could be detected in the probiotic supplemented groups, which were consistent with previous studies that supplementation of probiotics could increase the abundance of Firmicutes (Shin et al., 2019; Mun et al., 2021). Enriched Firmicutes phylum could be observed in the fat pigs, which correlated with sustaining energy balance, and it is significantly involved with fat deposition (Zhao et al., 2015; Bergamaschi et al., 2020; Yang et al., 2021). Fat deposition is one of the major pathways for pig growth by utilizing glucose or acetate as the main carbohydrate or carbon precursors for fatty acid synthesis (Dunshea and

D'Souza, 2003). Therefore, a higher abundance of Firmicutes in this study might be one of the main reasons for improving growth performance which was consistent with our previous studies that probiotic supplementation could exhibit more significant average daily gain and feed conversion ratio (Pupa et al., 2021a; Pupa et al., 2022).

Pediococcus genus is one of the bacterial probiotic strains used in animal production (Yirga, 2015). The supplementation of *Pediococcus* spp. in the pigs could provide several beneficial properties, including improving growth performance, alleviating inflammation, regulating gut microbiota by enriching desirable microorganisms and inhibiting undesirable microorganisms, enhancing intestinal morphology, blood biochemical profile, and meat quality (Joysowal et al., 2018; Wang et al., 2019a; Morales-Partera et al., 2020). In this study, the *Pediococcus* genus was increased from the neonatal period (0.04%) to weaned period (0.18% to 0.29%). Although it was demonstrated in a low proportion, we believed that the supplementation at the early stage of pig life remained to provide various benefits to the pigs since it might act as a primary gut colonizer, which generated a suitable environment for other beneficial microbes to colonization as mentioned elsewhere (Pupa et al., 2021a; Pupa et al., 2022).

As shown in Table 18, our probiotic strains, particularly multi-strain probiotics, reduce some antimicrobial resistance, antimicrobial-resistant genes, and mobile genetic elements in the antimicrobial resistome and mobilome. It's possible that they could limit the growth of microbiota containing antimicrobial-resistant genes and mobile genetic elements through a variety of ways, as described elsewhere (O'Toole and Cooney, 2008; Liao and Nyachoti, 2017b). Furthermore, they may prevent the spread of resistant genes, delete mobile genetic elements, or interfere with biofilm formation, all of which are important components in the antimicrobial resistance phenomena (Viljanen and Boratynski, 1991; Moubareck et al., 2007; Jacques et al., 2010; Nehal El-Deeb, 2015a; Yu et al., 2017; Kunishima et al., 2019; Zhang et al., 2019).

Another important finding, as shown in Table 18, was that our LAB strains, particularly multi-strain LAB, demonstrated outstanding detoxification and oxidative responses during weaning transition and ETEC infection events, as these two events can produce excessive reactive oxygen species, which can harm animals (Guevarra et al., 2018; Sun et al., 2021). It's probable that our LAB strains' antioxidant activity originated as a result of their ability to scavenge free radicals by creating antioxidant molecules including superoxide dismutase, glutathione peroxidase, and catalase (Wang et al., 2012; Liao and Nyachoti, 2017b). In addition, they also increased nutrient metabolism in term of amino acid and carbohydrate utilization. These findings are likely due to an increase of beneficial microorganisms, which could provide variety digestive compounds (*e.g.*, amylase, cellulase, lactic acid, lactase, lipase, mannanase, phytase, protease, sucrase, and xylanase), ferment insoluble carbohydrate ingredients, dietary protein, and amino acids, as well as concurrently synthesize the amino acids (Liao and Nyachoti, 2017b; Guevarra et al., 2018; Wang et al., 2020c). Therefore, these findings could support our LAB strains' ability to promote symbiosis in the swine intestine microbial community. Their efficacies can improve human and environmental safety by lowering the alarming microbial resistome and mobilome.

In summary, our probiotic strains including *L. plantarum* 22F, 25F, and *P. acidilactici* 72N might inhibit the transfer of the *mcr-1* gene and impede the growth of *E. coli* biofilms, according to the results of our overall studies. Furthermore, supplementing piglets with our multi-strain probiotic may modify the gut microbiota and gut resistome.

Table 18 Summary of the overall data analyses regarding gut microbiome and gut resistome.

Data analyses/Groups	Negative control	Probiotic control	Antibiotic	Single-strain	Multi-strain	ETEC control
Gut microbiota						
Phylum levels	Bacteroidetes, Proteobacteria	Firmicutes	Bacteroidetes, Proteobacteria	Firmicutes	Firmicutes	Firmicutes, Bacteroidetes
Family levels	*Lachnospiraceae*, *Veillonellaceae*	*Ruminococcaceae*, *Lactobacillaceae*	*Prevotellaceae*, *Bacteroidaceae*	*Lactobacillaceae*, *Veillonellaceae*	*Lachnospiraceae*, *Clostridiaceae*	*Prevotellaceae*, *Bacteroidaceae*
Genus levels	*Faecalibacterium*, *Blautia*	*Faecalibacterium*, *Lactobacillus*	*Prevotella*, *Bacteroides*	*Lactobacillus*, *Megasphaera*	*Clostridium*, *Bacillus*	*Lactobacillus*, *Bacteroides*
Firmicutes: Bacteroidetes ratio	**H12:** 14.58; **D42:** 4.21	**H12:** 212.93; **D42:** 5.01	**H12:** 4.61; **D42:** 1.32	**H12:** 40.13; **D42:** 5.13	**H12:** 52.76; **D42:** 4.42	**H12:** 26.84; **D42:** 3.10
Antimicrobial resistome						
Antibiotic resistance classes	Tetracyclines, Betalactams	Aminoglycosides, MLS	Betalactams, MLS	Aminoglycosides, Betalactams	Aminoglycosides, Tetracyclines	Betalactams, MLS
Antibiotic resistance groups	*tet*W, *tet*O, TEM	MLS23S, *erm*B	*tet*Q, *mef*A, *tet*M	MLS23S, *erm*B	*tet*O, *erm*B	*tet*Q, *mef*A, *tet*M
Metal resistances	Multi-metal, zinc	Multi-metal, sodium	Multi-metal, copper	Multi-metal, copper	Multi-metal, sodium	Multi-metal, zinc
Biocide resistances	Multi-biocide, acid	Acetate, peroxide	Multi-biocide, acid	Multi-biocide, peroxide	Acid, acetate	Multi-biocide, acid
Mobile genetic elements						
Plasmid replicons	IncFII, IncFIB	IncFII, IncX1	IncQ1, IncX4	IncFII, IncX1	IncFII, IncQ1	IncFII, IncQ1
Integrons	Class 1	Class 1	Class 1, 3	Class 1	Class 1	Class 1, 2
Insertion sequences	IS1, IS1380	IS110, IS1182	IS1380, IS3	IS1, IS30	IS3, IS30	IS1182, IS1380
Microbial functionalities ǂ						
Stress responses	++	++++	+	+++	++++	+
Amino acid metabolisms	++	++++	+++	+++	++++	+
Carbohydrate metabolisms	++	++++	+++	+++	++++	+

ǂ the microbial functionalities were expressed as poor response (+), fair response (++), good response (+++), and excellent response (++++).

Conclusion remarks

Results of this dissertation have shown that

1. Our LAB strains containing *L. plantarum* 22F, 25F, and *P. acidilactici* 72N could reduce the capability of horizontal transfer of plasmid containing *mcr*-1 gene in *E. coli*. *L. plantarum* 22F exhibited the best anticonjugation compared to other LAB strains.
2. Our LAB strains comprising of *L. plantarum* 22F, 25F, and *P. acidilactici* 72N could interfere with the biofilm production of *E. coli*, not only the non-sessile stage but also the sessile stage. *L. plantarum* 25F and *P. acidilactici* 72N showed the most promising LAB for antibiofilm activity.
3. The supplementation of our multi-strain LAB to neonatal piglets could modulate gut microbiota, decrease the critical resistomes and mobilomes, as well as improve antioxidant activity and nutrient metabolism in weaned piglets and weaned piglets infected with ETEC.

Suggestions for further investigation

This study examined *in vitro* LAB efficacy on anticonjugation and antibiofilm and *in vivo* LAB efficiency on the gut microbiome and microbial resistome. Further research should be undertaken to investigate the mechanisms of anticonjugation and antibiofilm capacity, and it should also be conducted to determine the complete genome of our LAB strains, as well as comprehensive secretome component analyses. In addition, further investigation focusing on antioxidant activity is also recommended.

APPENDIX

MEDIA, BUFFER, AND SOLUTION PREPARATION

Luria-Bertani (LB) broth

Tryptone	10 g
Yeast extract	5 g
Sodium Chloride	5 g
Agar	15 g
Distilled water	1000 ml

Luria-Bertani (LB) broth

Tryptone	10 g
Yeast extract	5 g
Sodium Chloride	5 g
Distilled water	1000 ml

Normal Saline Solution (NSS; 0.85%)

Sodium Chloride	8.5 g
Distilled water	1000 ml

Phosphate Buffer Saline (PBS)

Sodium Chloride	8 g
Potassium Chloride	0.2 g
Sodium hydrogen phosphate	2.9 g
Potassium dihydrogen phosphate	0.2 g
Distilled water	1000 ml

Peptone Dilution Saline (PDS)

Peptone	1 g
Sodium Chloride	9 g
Distilled water	1000 ml

Sodium Hydroxide (NaOH; 1M)

Sodium Hydroxide	40 g
Distilled water	1000 ml

Tris-borate-EDTA (TBE) buffer (10X)

Tris base	108 g
Boric acid	55 g
Ethylene Diamine Tetra-Acetic Acid (EDTA)	7.5 g
Distilled water	1000 ml

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1. Wandee Sirichokchatchawan, Puwiya Pupa, Prasert Praechansri, Nutthee Am-in, Somboon Tanasupawat, Piengchan Sonthayanon and Nuvee Prapasarakul. 2018. Autochthonous lactic acid bacteria isolated from pig faeces in Thailand show probiotic properties and antibacterial activity against enteric pathogenic bacteria. Microb. Pathog. 119: 208–215. DOI: 10.1016/j.micpath.2018.04.031.
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