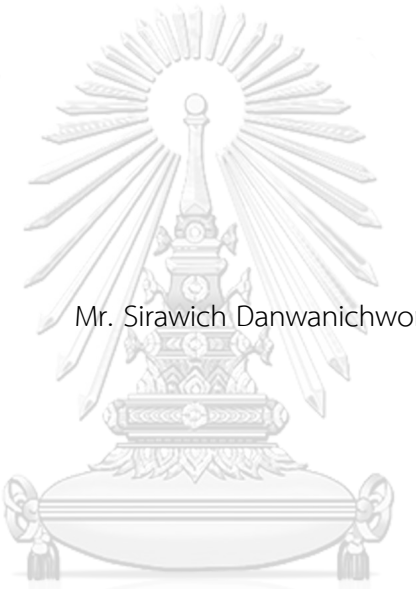
SELECTION OF CANDIDATE PROBIOTIC BACTERIA FROM CHICKENS WITH ANTAGONISM AGAINST *CAMPYLOBACTER*

Mr. Sirawich Danwanichwong



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การคัดเลือกแบคทีเรียที่มีคุณสมบัติในการต้านแคมไพโลแบคเตอร์จากไก่เพื่อใช้เป็นสารเสริมชีวนะ

นายสิรวิชญ์ ด่านวณิชวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	SELECTION OF CANDIDATE PROBIOTIC BACTERIA FROM CHICKENS WITH ANTAGONISM AGAINST *CAMPYLOBACTER*
By	Mr. Sirawich Danwanichwong
Field of Study	Veterinary Public Health
Thesis Advisor	Assistant Professor TARADON LUANGTONGKUM, D.V.M., Ph.D.

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สิรวิชญ์ ด่านวณิชวงศ์ : การคัดเลือกแบคทีเรียที่มีคุณสมบัติในการต้านแคมไพโลแบคเตอร์จากไก่เพื่อใช้เป็นสารเสริมชีวนะ. (SELECTION OF CANDIDATE PROBIOTIC BACTERIA FROM CHICKENS WITH ANTAGONISM AGAINST *CAMPYLOBACTER*) อ.ที่ปรึกษาหลัก : ผศ. น.สพ.ดร.ธราดล เหลืองทองคำ

แคมไพโลแบคเตอร์เป็นแบคทีเรียซึ่งเป็นหนึ่งในสาเหตุหลักที่ทำให้เกิดโรคอาหารเป็นพิษในมนุษย์ การศึกษาที่ผ่านมาพบว่าประสิทธิภาพในการควบคุมป้องกันการติดเชื้อแคมไพโลแบคเตอร์ในไก่ด้วยสารเสริมชีวนะ ส่วนใหญ่มักให้ผลที่ไม่แน่นอน ดังนั้นจึงมีความจำเป็นในการศึกษาเกี่ยวกับสารเสริมชีวนะที่สามารถยับยั้งหรือควบคุมการติดเชื้อแคมไพโลแบคเตอร์ในไก่ได้ การศึกษานี้มีวัตถุประสงค์เพื่อคัดเลือกสายพันธุ์ของเชื้อแบคทีเรียในกลุ่ม lactobacilli *Bacillus* หรือ *Enterococcus* ที่มีคุณสมบัติในการต้านเชื้อแคมไพโลแบคเตอร์และคุณสมบัติอื่นๆ ที่เหมาะสมสำหรับการนำไปใช้เป็นสารเสริมชีวนะ ในการศึกษาครั้งนี้ เชื้อแบคทีเรียที่อาจนำไปพัฒนาเป็นสารเสริมชีวนะจำนวน 602 ไอโซเลท เพาะแยกได้จากทางเดินอาหารของไก่ที่มีความทนทานต่อการติดเชื้อแคมไพโลแบคเตอร์ ได้ถูกนำมาทดสอบคุณสมบัติต่างๆ ในการเป็นสารเสริมชีวนะ อันได้แก่ ความสามารถในการยับยั้งเชื้อแคมไพโลแบคเตอร์ คุณสมบัติในการทำให้เม็ดเลือดแดงแตกตัว ความสามารถในการทนกรดและน้ำดี การดื้อต่อยาปฏิชีวนะ ความสามารถในการยึดเกาะกับเยื่อเมือกของระบบทางเดินอาหาร รวมไปถึงการตรวจหาการปรากฏและตำแหน่งของยีนดื้อยาในเชื้อดังกล่าวโดยใช้เทคนิค Whole genome sequencing เพื่อค้นหาเชื้อแบคทีเรียที่มีคุณสมบัติเหมาะสมที่จะนำไปพัฒนาเป็นสารเสริมชีวนะต่อไป ผลการศึกษาพบว่าเชื้อ *Limosilactobacillus reuteri* จำนวน 2 ไอโซเลท (i 24.1/2 และ i 24.2/2) มีคุณสมบัติในการเป็นสารเสริมชีวนะที่ดี กล่าวคือ ไอโซเลทเหล่านี้มีความสามารถในการยับยั้งการเจริญเติบโตของเชื้อแคมไพโลแบคเตอร์ โดยมีเส้นผ่านศูนย์กลางของบริเวณการยับยั้งเชื้อแคมไพโลแบคเตอร์ขนาด 16 มิลลิเมตร (i 24.1/2) และ 15 มิลลิเมตร (i 24.2/2) ใน agar well diffusion assay นอกจากนี้ เชื้อทั้ง 2 ไอโซเลทดังกล่าวยังไม่ทำให้เกิดการแตกตัวของเม็ดเลือดแดง อีกทั้งยังมีความทนทานต่อกรด (มีอัตราการอยู่รอด 91.12% และ 99.58% สำหรับไอโซเลท i 24.1/2 และ i 24.2/2 ตามลำดับ) และความทนทานต่อน้ำดี (มีอัตราการอยู่รอด 99.47% และ 102.95% สำหรับไอโซเลท i 24.1/2 และ i 24.2/2 ตามลำดับ) ยิ่งไปกว่านั้น ไอโซเลททั้งคู่ยังมีความสามารถในการยึดเกาะกับเยื่อเมือกจากระบบทางเดินอาหารของไก่ โดยมีค่าเฉลี่ยอัตราการยึดเกาะ 80.10% (i 24.1/2) และ 70.35% (i 24.2/2) นอกจากนี้ ถึงแม้ว่าเชื้อทั้ง 2 ไอโซเลทจะมีการตรวจพบการดื้อต่อยา vancomycin และ ampicillin แต่การดื้อต่อยาดังกล่าวในเชื้อเหล่านี้มีความเสี่ยงต่ำที่จะทำให้เกิดการถ่ายทอดยีนดื้อยาแบบ horizontal ได้ เนื่องจากการดื้อต่อยา vancomycin ในแบคทีเรียจำพวก lactobacilli ส่วนใหญ่นั้น ถือเป็นการดื้อยาตามธรรมชาติ (intrinsic resistance) และการดื้อต่อยา ampicillin ใน *L. reuteri* นั้น คาดว่าน่าจะเกิดจากการกลายพันธุ์เฉพาะจุด (point mutations) ของยีนที่ทำหน้าที่ในการสร้าง penicillin-binding proteins อย่างไรก็ตามเนื่องจากเชื้อทั้ง 2 ไอโซเลท มีการตรวจพบยีนดื้อยา *lnuA* อยู่บนพลาสมิด ทำให้เชื้อเหล่านี้ยังไม่เหมาะสมที่จะนำไปใช้เป็นสารเสริมชีวนะในทันที แต่ถ้าสามารถกำจัดพลาสมิดที่มียีนดื้อยาออกจากเชื้อจุลินทรีย์ดังกล่าวได้ ซึ่งเคยประสบผลสำเร็จมาแล้วในการศึกษาก่อนหน้านี้ เชื้อทั้งสองไอโซเลทก็เป็นตัวเลือกที่น่าสนใจที่จะนำไปพัฒนาเป็นสารเสริมชีวนะต่อไป ยิ่งไปกว่านั้น เชื้อเหล่านี้ควรถูกนำไปทดลองในไก่เพื่อทดสอบประสิทธิภาพต่างๆ ในการเป็นสารเสริมชีวนะที่ดี ก่อนที่จะทำการพัฒนาเป็นสารเสริมชีวนะต้นแบบในอนาคต

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: Probiotics, Limosilactobacillus reuteri, Campylobacter, Chickens

Sirawich Danwanichwong : SELECTION OF CANDIDATE PROBIOTIC BACTERIA FROM CHICKENS WITH ANTAGONISM AGAINST *CAMPYLOBACTER*. Advisor: Asst. Prof. TARADON LUANGTONGKUM, D.V.M., Ph.D.

Campylobacter is one of the most common causes of bacterial foodborne disease in humans worldwide. The effect of probiotics on *Campylobacter* control in poultry is inconsistent or rarely observed. For this reason, research on probiotics for *Campylobacter* control in poultry is required. Therefore, the objective of this study was to identify lactobacilli, *Bacillus* or *Enterococcus* strains isolated from *Campylobacter*-negative chickens which have the ability to inhibit the growth of *Campylobacter in vitro* and demonstrate favorable probiotic characteristics. A total of 602 bacterial isolates from *Campylobacter*-negative broiler chickens were assessed for their probiotic properties including *Campylobacter* inhibiting activity test, hemolytic activity test, acid and bile tolerance test, antimicrobial susceptibility test, and chicken intestinal mucus adhesion test. Additionally, determination of antimicrobial resistance genes and their location on the genome by whole genome sequencing was performed on the candidate probiotic bacteria. We identified 2 *Limosilactobacillus reuteri* isolates (isolate i 24.1/2 and i 24.2/2) that showed good probiotic properties. These isolates demonstrated *Campylobacter* inhibiting activity with *Campylobacter* inhibition zone diameter of 16 mm (i 24.1/2) and 15 mm (i 24.2/2) in agar well diffusion assay and they were negative for hemolytic activity test. In addition, the two isolates exhibited excellent acid tolerance (91.12% and 99.58% survival rates for i 24.1/2 and i 24.2/2, respectively) and bile tolerance (99.47% and 102.95% survival rates for i 24.1/2 and i 24.2/2, respectively). Furthermore, these isolates also showed the ability to adhere to chicken intestinal mucus with 80.10% (i 24.1/2) and 70.35% (i 24.2/2) adhesion efficiency. Even though vancomycin and ampicillin resistance was found in both isolates, it presents a minimal risk for horizontal resistance genes transfer because resistance to vancomycin is considered intrinsic resistance in most lactobacilli and ampicillin resistance in *L. reuteri* is probably caused by point mutations in the genes encoding penicillin-binding proteins. However, both candidate probiotic isolates still harbored plasmids that carried *lnuA* resistance gene. Thus, these candidates were not suitable to be used as a probiotic at present. But, if the plasmid carrying the antimicrobial resistance gene is removed from the candidate probiotic bacteria, which had been accomplished in the past, these candidates could be suitable for being used as a probiotic. Furthermore, in order to develop a new probiotic product, these candidates should be further evaluated for their efficacy as probiotics by *in vivo* experiments in chickens.

Field of Study:	Veterinary Public Health	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

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Sirawich Danwanichwong

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion
bp	base pair (s)
°C	degree Celsius
CFU	Colony Forming Unit
CLSI	Clinical & Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
et al.	et alia (and others)
EU	European Union
FAO	Food and Agriculture Organization
g	gram
GRAS	Generally Recognised as Safe
HCCA	alpha-Cyano-4-hydroxycinnamic acid
hr	hour (s)
LAB	Lactic acid bacteria
LSM	Lactic acid bacteria susceptibility test medium
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
mCCDA	Modified Charcoal-Cefoperazone-Deoxycholate agar
mg	milligram
MHA	Muller-Hinton agar

MIC	Minimum inhibitory concentration
min	minute (s)
ml	milliliter (s)
MRS	De Man, Rogosa and Sharpe
MYP	Mannitol Egg Yolk Polymyxin
NaCl	sodium chloride
NCBI	National center for Biotechnology Information
NCTC	National Collection of Type Cultures
NSS	normal saline solution
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
pH	potential of hydrogen
QPS	Qualified Presumption of Safety
rRNA	Ribosomal Ribonucleic acid
s	second (s)
spp.	species
TAE	Tris-acetate-EDTA
US FDA	United States Food and Drug Administration
UV	Ultraviolet
WHO	World Health Organization
µg	microgram (s)
µl	microliter (s)

CHAPTER I

INTRODUCTION

Campylobacter is one of the most common causes of bacterial foodborne disease in the world (Kaakoush et al., 2015). European food safety authority (EFSA) reported that *Campylobacter* has been the leading cause of gastrointestinal bacterial infection in humans in the European Union (EU) since 2005. In 2017, the number of *Campylobacter* infections in humans exceeded that of *Salmonella* by almost 3-fold (EFSA, 2018a). In Thailand, acute bacterial diarrhea in children under 5 years of age is mostly associated with *Campylobacter* (Samosornsuk et al., 2015; Okada et al., 2020). Campylobacteriosis in humans is most frequently caused by *Campylobacter jejuni*, followed by *Campylobacter coli* (EFSA, 2018a). The symptoms of human campylobacteriosis include watery or bloody diarrhea, acute abdominal pain and fever. Long term complications such as Guillain-Barré Syndrome (GBS) can also occur (Humphrey et al., 2007).

Campylobacter is a commensal organism in many animals including chickens. Chickens are considered to be an important reservoir of *Campylobacter* (Hermans et al., 2012). Broiler chicken meat is often contaminated with *Campylobacter* and is the main source of *Campylobacter* infection in humans (Skarp et al., 2016). Therefore, reduction of *Campylobacter* in poultry can reduce the risk of human campylobacteriosis.

Controlling *Campylobacter* in primary broiler production will greatly reduce the public health risk (Andreoletti et al., 2011). There are many strategies to control *Campylobacter* at the farm level such as vaccination, bacteriocins, bacteriophages, organic acids, medium-chain fatty acids, prebiotics and probiotics. However, to date,

there is no effective approach to control *Campylobacter* in broiler farms (Hermans et al., 2011; Meunier et al., 2016; Umaraw et al., 2017).

Antimicrobial resistance has become an increasing concern for public health and livestock industry. Many countries have banned the use of antimicrobials as growth promoter. In consequence, it becomes necessary to find an alternative approach to promote growth and reduce bacterial pathogens in livestock animals without the use of antimicrobial agents. Probiotics, by definition, are "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotics can eliminate pathogenic microbes through several proposed mechanisms such as secretion of antibacterial substance (organic acids, bacteriocins and hydrogen peroxide), competition with pathogenic organisms for nutrients and adhesion sites, and activation of the immune system against pathogenic bacteria (Jadhav et al., 2015). Bacterial strains that will be used as probiotics should be considered as safe by standard regulatory guidelines, for instance the Generally Recognized as Safe (GRAS) status by The United States Food and Drug Administration (US FDA) and the Qualified Presumption of Safety (QPS) list by EFSA. Microorganisms that have been used as feed additives in poultry include lactobacilli, *Bacillus*, *Enterococcus*, etc (Lutful Kabir, 2009).

Probiotics have been used in poultry industry for many decades to improve growth rate and feed conversion efficiency, and to control or prevent enteric pathogenic microbes (Bajagai et al., 2016). Several studies have identified probiotic microorganisms with inhibiting activity against *Salmonella* colonization in poultry, and these selected microorganisms have been successful in control or prevention of *Salmonella* at commercial level (Pascual et al., 1999; Tellez et al., 2012; Neveling et al., 2019). However, the use of probiotics to reduce colonization of *Campylobacter* in poultry have limited success and inconsistent results in previous studies (Santini et

al., 2010; Ghareeb et al., 2012; Aguiar et al., 2013). For this reason, research on probiotics for *Campylobacter* control in poultry is required. Probiotics with the ability to control or reduce *Campylobacter* colonization in chickens at the farm level can consequently reduce the contamination of *Campylobacter* in poultry meat, thus lower the risk of human campylobacteriosis. Therefore, this study will be conducted to identify candidate probiotic bacteria from gastrointestinal tract of *Campylobacter*-negative chickens and evaluate their probiotic properties with a specific purpose of inhibiting *Campylobacter*.

Objective of study

To identify lactobacilli, *Bacillus* or *Enterococcus* strains isolated from *Campylobacter*-negative chickens which have the ability to inhibit the growth of *Campylobacter in vitro* and demonstrate favorable probiotic characteristics

Research question

Do lactobacilli, *Bacillus* and *Enterococcus* isolated from *Campylobacter*-negative chickens have the ability to inhibit the growth of *Campylobacter in vitro* and can be used as probiotics ?

CHAPTER II

LITERATURE REVIEW

1. General characteristics of *Campylobacter*

Campylobacter are small (0.2–0.8 μm. × 0.5–5 μm.) gram negative, spirally-curved rods bacteria in *Campylobacteriaceae* family. They have a single polar flagellum at one or both ends of the cell, which makes them motile with a cork-screw like motility (Smibert, 1978). The optimal growth of *Campylobacter* is observed at 42℃ under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) (Garenaux et al., 2008). *Campylobacter* species are commensal organisms normally found in wide range of animals including livestock animals such as swine, sheep, cattle and avian species, with avian as the most common host because they have high body temperature (Skirrow, 1977). Chickens pose greater risk of *Campylobacter* infection to humans than all other commercial poultry because they are consumed in large quantities (Humphrey et al., 2007).

2. *Campylobacter* in poultry

Campylobacter colonization in broiler flocks generally occurs around 2 to 3 weeks of age (Evans and Sayers, 2000; Newell et al., 2011). Colonized chickens are usually asymptomatic. The infectious dose of *C. jejuni* for successful colonization of chickens has been reported to be as low as 35 CFU (Stern et al., 1988). *Campylobacter* resides in the lower part of chicken intestines, predominantly in the ceca. Colonized broilers carry a large number of *Campylobacter* in their ceca, commonly around 10^6 to 10^8 CFU/g (Beery et al., 1988). Once established in a flock, transmission of *Campylobacter* is very rapid between chickens by fecal-oral route. The external environment around the broiler house, contaminated water supply and domestic or wild animals can also be the sources of infection (Newell and Fearnley,

2003). Chickens in *Campylobacter* positive flocks can remain infected until slaughter (Newell et al., 2011). During transportation of chickens to slaughter house, transportation vehicles and the environment can be contaminated with *Campylobacter* from chicken feces. At slaughter and processing, intestinal contents may leak from chicken intestines onto the skin of carcasses (Berrang et al., 2001; Stern and Robach, 2003). *Campylobacter* can then survive and persist through processing and storage, consequently causing illness to consumers (Chantarapanont et al., 2003; Scherer et al., 2006).

3. Campylobacteriosis

Campylobacteriosis is an infection in humans caused by *Campylobacter* spp., mainly *C. jejuni* and *C. coli*. *Campylobacter* is the leading cause of bacterial foodborne disease in humans, it is estimated to be responsible for 400–500 million cases of gastroenteritis worldwide per year (Olson et al., 2008). An infective dose of *C. jejuni* to cause illness in humans can be as low as 360 CFU (Hara-Kudo and Takatori, 2011). The symptoms of the disease include nausea, watery or bloody diarrhea, acute abdominal cramps, fever and weight loss (Humphrey et al., 2007). Post-infection complications such as peripheral neuropathies (Guillain-Barré Syndrome), reactive arthritis and irritable bowel syndrome can occur in some cases. Ten percent of campylobacteriosis patients need hospitalization (Bessell et al., 2010). Humans can get infected by *Campylobacter* through consumption of contaminated sources such as meat product of food animals (chickens, cattle, pigs, dairy cows, turkeys, ducks, lambs and shellfish), untreated water, raw or unpasteurized milk, fruits and vegetables (Humphrey et al., 2007; Silva et al., 2011). However, broiler meat is recognized as the primary source of human infections with *Campylobacter*. Handling of raw chickens, eating undercooked broiler meat and cross-contamination of raw to cooked foods are associated with human campylobacteriosis cases (Silva et

al., 2011). Reduction of the *Campylobacter* load in ceca of live chickens in the course of primary broiler production can significantly decrease the carcass contamination with *Campylobacter* during processing, thus reducing the risk of human campylobacteriosis (Lin, 2009).

4. Probiotics

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Most microorganisms used as probiotics are bacteria, but yeasts and fungi can be used in probiotic products as well. Probiotics can benefit the health of host animals through various mechanisms including promoting the growth of favorable gut microflora and inhibit the growth of pathogenic microorganisms by production of antimicrobial substances (organic acids, bacteriocins and hydrogen peroxide), competition for adhesion sites and nutrients with pathogenic bacteria, and stimulation of immune response and improvement of intestinal barrier function against pathogenic microbes. Probiotics can also increase activity of the digestive enzymes which improve digestion and absorption of nutrients (Lutful Kabir, 2009; Jadhav et al., 2015). Therefore, the major beneficial health effects from using probiotics in animals include improving animal growth, reduction in mortality, and improvement in feed conversion efficiency (Yeo and Kim, 1997; Lutful Kabir, 2009; Mousavi et al., 2018).

5. Microorganisms used as probiotics in poultry industry

Probiotics have been used in poultry industry for many purposes such as enhancement of growth rate, improvement of feed intake and feed efficiency, and control or prevention of enteric pathogens (Bajagai et al., 2016). Many probiotics have positive effects on the aforementioned purposes, however, previous studies show

that the results can be inconsistent among different probiotics. Microorganisms that have been used as probiotics in poultry industry include lactobacilli, *Bacillus* spp. and *Enterococcus* spp.

5.1 Lactobacilli

Recently, a re-evaluation of the taxonomy of genus *Lactobacillus*, which comprised of 261 species (as of 2020) that displayed immense diversity among species at phenotypic, genotypic and physiological levels, had been made. The study evaluated genetic relatedness and phylogeny of the species within the *Lactobacillus* genus on the basis of whole genome sequences. Several parameters were evaluated including average nucleotide identity (ANI), average amino acid identity (AAI), core genome phylogeny, physiological and ecological criteria, etc. Based on the analysis, the genus *Lactobacillus* was reclassified into 25 genera, including the emended genus *Lactobacillus* and *Paralactobacillus*, and 23 novel genera such as *Agrilactobacillus*, *Ligilactobacillus*, and *Limosilactobacillus* (Zheng et al., 2020). In the present study, the generic term 'lactobacilli' will be used to describe all the bacteria that were classified as genus *Lactobacillus* before the reclassification.

Lactobacilli are gram positive, facultative anaerobic or microaerophilic, rod bacteria in *Lactobacillaceae* family, which is a part of lactic acid bacteria group (LAB). Lactobacilli may be the most commonly used probiotic microorganisms in humans and animal feed (Pandey et al., 2015). They are also possibly the safest probiotic microorganisms, because they are natural inhabitants of gastrointestinal tract in humans and animals (Huse et al., 2012; Yeoman and White, 2014). Infections caused by these bacteria are extremely rare, and they have been traditionally used as probiotics in human food and animal feed for a long time (Bajagai et al., 2016). Most of the lactobacilli have intrinsic antimicrobial resistance to vancomycin, some species of lactobacilli also have natural resistance to bacitracin, cefoxitin, ciprofloxacin,

fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, and trimethoprim/sulphamethoxazole. The most frequently found antimicrobial resistance genes in lactobacilli are tetracycline resistance genes (*tet*) (Ammor et al., 2008; Gueimonde et al., 2013). Some antimicrobial resistance genes of lactobacilli are able to transfer horizontally via mobile genetic elements (Mathur and Singh, 2005; Thumu and Halami, 2019).

5.2 *Bacillus*

Bacillus are gram-positive, rod-shaped, spore-forming bacteria in *Bacillaceae* family, they can be either obligate aerobes or facultative anaerobes. The use of spore-forming bacteria as probiotic supplement in animal feed, especially *Bacillus*, are increasingly popular. They can withstand harsh food processing conditions such as high temperature, low water activity and starvation stress (Elshaghabee et al., 2017). Fourteen species of *Bacillus* have been qualified with Qualified Presumption of Safety (QPS) status by EFSA (EFSA, 2019) including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. coagulans*, these strains were qualified as safe because absence of toxin production such as cytotoxic and emetic toxins. Some *Bacillus* spp. are pathogenic to humans and animals, such as *B. anthracis, B. cereus,* and *B. thuringiensis* (Raymond et al., 2010). There have been reports on antimicrobial resistance in *Bacillus* spp. used as probiotics. Macrolide and tetracycline resistance genes have been found in mobile genetic elements of *B. subtilis* (Gueimonde et al., 2013).

5.3 *Enterococcus*

Enterococcus are gram positive, facultative anaerobic, cocci bacteria in *Enterococcaceae* family. Despite a long history of being used as probiotics in both humans and animals, in the last few decades, *E. faecalis* and *E. faecium* have been

associated with hospital acquired infections in human (Bajagai et al., 2016). There are reports of enterococcal opportunistic infection in humans that can caused urinary tract infection, endocarditis and bacteremia. *Enterococcus* spp. have intrinsic resistance to cephalosporins and aminoglycosides, the emergence of vancomycin resistant *Enterococci* (VRE) have been a major concern in hospitals worldwide (Franz et al., 2011). Although many strains of *Enterococcus* have been authorized by EFSA to use as probiotic microorgansms in animal feed, the *Enterococcus* spp. have not been listed in the QPS list, the applications to use *Enterococcus* strains as probiotics must be assessed and approved by EFSA on a case by case basis (Bajagai et al., 2016).

6. Selection of candidate probiotic bacteria

To select microorganisms suitable for use as probiotics, the microorganisms must be considered for both their probiotic functionality and safety (FAO/WHO, 2002). For their probiotic functionality, probiotic properties of the microorganisms must be assessed. First is the selection of the source of the bacteria, the most suitable source of bacteria for animal use are natural microflora from the animal's own intestinal tract (Sornplang and Piyadeatsoontorn, 2016). Then, the microorganisms need to be assessed for functional capabilities by several *in vitro* tests. Probiotic microorganisms should be able to survive through the gastrointestinal tract of the animals, this means the microorganisms must be resistant to gastric acid in the stomach and bile in the intestine (Musikasang et al., 2009), acid and bile tolerance test should be evaluated. Adhesion to intestinal mucosa is another essential property for probiotics to be able to colonize the intestines and exert its beneficial effects on the host. There are mainly 2 *in vitro* adhesion assay models, intestinal epithelial cell lines and intestinal mucus adhesion assay. Most available commercial intestinal epithelial cell lines are derived from human cell lines (Caco-2,

HT-29, and HT-29 MTX cell lines) (Ouwehand and Salminen, 2003; Dicks and Botes, 2010). Thus, intestinal epithelial cell adhesion assay from these human derived cell lines might not be the best model to assess the adhesion ability of probiotics intended for use in chickens. Intestinal mucus adhesion assay might be more suitable for probiotics used in chickens, because the process of chicken intestinal mucus preparation is relatively simple and most gut bacteria also adhere and grow in the mucus layer overlying the intestinal epithelium (Lertworapreecha et al., 2011; Sicard et al., 2017). Probiotic microorganisms should also be able to compete with or inhibit the growth of pathogenic bacteria, many *in vitro* evaluation of antimicrobial activity methods can be performed such as agar well diffusion method, and agar plug diffusion method, agar spot test (Ghareeb et al., 2012; Balouiri et al., 2016). After that, *in vivo* experiment in animals should be done to validate the results of *in vitro* tests.

For safety concern of probiotics, the Generally Recognized as Safe (GRAS) status and the QPS list by US FDA and EFSA, respectively, have assessed the safety and risks of microorganisms for use in food and feed, and provided lists of microorganisms that are considered to be safe for use as probiotics. The GRAS status and QPS list are based on genus and species of bacteria. Thus, the microorganisms considered for use as probiotics should be identified at species level at least. The major risk posed by probiotic bacteria is horizontal transfer of antimicrobial resistance to other bacteria. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) issued a guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, the guidance provide a systematic scheme to define the genetic basis of resistance (intrinsic or acquired resistance), acquired reisistance of probiotic bacteria due to acquisition of genetic determinants is not acceptable for use as feed additive (EFSA, 2012).

CHAPTER III

MATERIALS AND METHODS

This study consists of 2 phases. The first phase is sample collection, identification of *Campylobacter* status of broiler chickens, and isolation of candidate probiotic bacteria. For the second phase, probiotic properties of potential candidates were assessed. Research outline of this study is shown in Figure 1.

Phase 1 Sample collection, identification of *Campylobacter* status of broiler chickens, and isolation of candidate probiotic bacteria

In this study, a total of 300 ceca were collected from 30 broiler farms at a slaughterhouse. The cecal content was used for identification of *Campylobacter* status of broiler chickens. Then, cecal content from *Campylobacter*-negative chickens was used for isolation of candidate probiotic bacteria.

1.1 Sample collection

Three hundred ceca were collected from commercial broiler chickens at the age of 6 weeks. Ten ceca were collected from each farm. The ceca were properly packed in plastic bags and placed on ice during transportation.

Selection of candidate probiotic bacteria from chickens with antagonism against *Campylobacter*

Phase 1

Sample collection,
identification of *Campylobacter* status of broiler chickens,
and isolation of candidate probiotic bacteria

- **Sample collection from 30 broiler farms (n=300)**
  - 10 ceca per farm
- **Identification of *Campylobacter* status of broiler chickens**
  - Isolation of *Campylobacter* by direct plating method on Modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA)
  - Confirmation of *Campylobacter* isolates by multiplex PCR
- **Isolation of candidate probiotic bacteria (n=602)***
  - Lactobacilli: Pour plate method on de Man, Rogosa and Sharpe (MRS) agar (n=253)
  - *Bacillus* spp.: Spread plate method on Mannitol Egg Yolk Polymyxin (MYP) agar (n=169)
  - *Enterococcus* spp.: Spread plate method on m Enterococcus agar (n=180)

***** Candidate probiotic bacteria were isolated from *Campylobacter*-negative broiler chickens.

Phase 2

Assessment of probiotic properties of potential candidates

- ***Campylobacter* inhibiting activity test (n=602)**
- **Hemolytic activity test (n=194)**
- **Acid and bile tolerance test (n=194)**
- **Identification of candidate probiotic bacteria (n=56)**
- **Antimicrobial susceptibility test (n=56)**
- **Mucus adhesion assay (n=6)**
- **Whole genome sequencing (n=4)**

Goal

Candidate probiotic bacteria with ability to inhibit the growth of *Campylobacter in vitro*

Figure 1. Research outline of this study

1.2 Identification of *Campylobacte*r status of broiler chickens

1.2.1 *Campylobacter* isolation

Ceca of broiler chickens were opened by sterile scissors using aseptic technique, and cecal contents were collected. Isolation of *Campylobacter* was performed on individual cecal content samples according to ISO 10272-1: 2017 (ISO, 2017). Cecal content was directly streaked onto modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA). The plates were incubated at 42°C for 48 hours under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2).

1.2.2 *Campylobacter* identification

Suspected colonies of *Campylobacter* were selected and confirmed by multiplex PCR using three primers specific for *Campylobacter* genus, *C. jejuni* and *C. coli* (Table 1). Multiplex PCR was performed according to the previously published protocol with some modifications (Wang et al., 2002). Briefly, presumptive *Campylobacter* colony from each sample was transferred into 100 µl of sterilized distilled water and boiled for 10 min. The DNA was placed on ice immediately. After that, the DNA samples were centrifuged at 13,000 rpm for 5 min and supernatant was used as DNA template. The reaction mixture (25 µl) containing 12.5 µl of KAPA Taq ReadyMix (Kappa Biosystems, Wilmington, Massachusetts, USA), 0.5 µl of each 0.2 µM 23S rRNA forward and reverse primers, 1.25 µl of each 0.5 µM *C. jejuni* forward and reverse primers, 2.5 µl of each 1 µM *C. coli* forward and reverse primers, 2.5 µl of DNA template, and 1.5 µl of nuclease-free water was used for DNA amplification in a thermocycler. The conditions for DNA amplification was carried out as follows: an initial denaturation step at 95°C for 6 min, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds (s), annealing at 57°C for 30 s, extension at 72°C for 30 s, and a 7 min final extension step at 72°C. Aliquots (5 µl) of the amplified

products were separated by electrophoresis in 1.5% agarose gel in TAE buffer. The gels were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc., Gyeonggi, Republic of Korea) and visualized under UV light for analysis of PCR product size. The PCR product sizes are shown in Table 1.

Table 1. Primer sequences, target genes and PCR product sizes of *Campylobacter* multiplex PCR

Primer	Sequence (5' to 3')	Target gene	Product size
Campylobacter genus			
23SF	TATACCGGTAAGGAGTGCTGGAG	23S rRNA	650 bp
23SR	ATCAATTAACCTTCGAGCACCG		
Campylobacter coli			
CCF	GTAAAACCAAAGCTTATCGTG	*glyA*	126 bp
CCR	TCCAGCAATGTGTGCAATG		
Campylobacter jejuni			
CJF	ACTTCTTTATTGCTTGCTGC	*hipO*	323 bp
CJR	GCCACAACAAGTAAAGAAGC		

1.3 Isolation of candidate probiotic bacteria

Candidate probiotic bacteria were isolated from cecal content of broiler chickens with *Campylobacter*-negative status. In brief, cecal contents from *Campylobacter*-negative chickens were serially diluted in normal saline solution (NSS). Then, the suspension of each dilution was plated on different culture media for isolation of candidate probiotic bacteria including lactobacilli, *Bacillus* spp., and *Enterococcus* spp.

1.3.1 Lactobacilli isolation

Isolation of lactobacilli was performed by pour plate method according to ISO 15214:1998 (ISO, 1998), 1 ml of cecal content suspension from the dilution series was pipetted onto sterile Petri dishes. Then, sterile molten de Man, Rogosa and Sharpe (MRS) agar was poured into the Petri dishes. Then, the dishes were swirled gently on the laboratory bench in order to mix the cecal content suspension and molten MRS agar thoroughly. The agar plates were allowed to solidify at room temperature and then incubated at 37°C for 48-72 hours under anerobic condition.

1.3.2 *Bacillus* isolation

Isolation of *Bacillus* spp. was performed by spread plate method according to ISO 7932: 2004 (ISO, 2004). Cecal content suspension (0.1 ml) from the dilution series was pipetted onto the center of the surface of Mannitol Egg Yolk Polymyxin (MYP) agar plate. Then, sterile glass spreader was used to spread the cecal content suspension evenly over the surface of MYP agar. The plates were incubated at 30°C for 24 hours under aerobic condition.

1.3.3 *Enterococcus* isolation

Isolation of *Enterococcus* spp. was performed by spread plate method according to Nordic Committee on Food Analysis Method No.68 (NMKL, 2004) with some modifications. Cecal content suspension (0.1 ml) from the dilution series was pipetted onto the center of the surface of m Enterococcus agar plate. Then, sterile glass spreader was used to spread the sample evenly over the surface of m Enterococcus agar. The plates were incubated at 37°C for 24-48 hours under aerobic condition.

Colonies of lactobacilli, *Bacillus* and *Enterococcus* were subcultured until pure colony was obtained. At least 1 colony of each bacterial genus per

Campylobacter-negative broiler chicken was selected and transferred into Brain Heart Infusion (BHI) broth with 20% glycerol. These bacterial suspension samples were kept at -80°C for further study.

Phase 2 Assessment of probiotic properties of potential candidates

Candidate probiotic bacteria from the first phase were assessed for their probiotic properties. To select the best candidate probiotic bacteria, assessment of *Campylobacter* inhibiting activity, hemolytic activity test, acid and bile tolerance test, antimicrobial susceptibility test, mucus adhesion assay, and whole genome sequencing were performed.

2.1 *Campylobacter* inhibiting activity test (n=602)

Assessment of *Campylobacter* inhibiting activity was performed by agar well diffusion method. Briefly, a culture of *Campylobacter jejuni* NCTC 11168 was suspended in normal saline solution and adjusted to 0.5 McFarland standard by densitometer (Grant Instruments Ltd., United Kingdom). The *Campylobacter* suspension was spread over the surface of Mueller-Hinton agar (MHA) with a sterile cotton swab. A well of 5 mm in diameter was bored in the agar plate using sterile cork borer. Each well was filled with 50 µl of cell-free supernatant from candidate probiotic bacteria, which was prepared by centrifuging overnight cultures of candidate probiotic bacteria grown in BHI broth (*Bacillus* and *Enterococcus*) or MRS broth (lactobacilli) at 14,000 rpm for 20 min. After the cell free supernatant was filled in the wells, the MHA plates were incubated at 42°C for 48 hr under microaerobic conditions. After incubation, the inhibition zone around the wells was observed and measured.

2.2 Hemolytic activity test (n=194)

Hemolytic activity test was carried out by streaking the bacterial isolates onto Columbia agar with 5% sheep blood and incubated at 37°C for 24-48 hours under anaerobic condition for lactobacilli and aerobic condition for *Bacillus* and *Enterococcus*. After incubation, hemolytic reaction was examined. The characteristics of hemolytic activity were recorded as beta hemolysis or complete hemolysis (clear zone in blood agar underlying the colonies), alpha hemolysis or partial hemolysis (green zone in blood agar underlying the colonies), and gamma hemolysis or no hemolysis (no zone in blood agar underlying the colonies).

2.3 Acid and bile tolerance test (n=194)

For acid and bile tolerance test. Culture of candidate probiotic bacteria was suspended in normal saline solution and adjusted to 0.5 McFarland standard. The bacterial suspension was transferred into MRS (for lactobacilli) or BHI (for *Bacillus* and *Enterococcus*) broth adjusted to pH 2.5 with hydrochloric acid for acid tolerance test, and into MRS or BHI broth with 2% oxgall bile for bile tolerance test. Then, the bacterial suspension was incubated at 37°C. Samples were taken at 0 and 2 hr for acid tolerance test and at 0 and 3 hr for bile tolerance test to check for viable bacteria (Jin et al., 1998; Gotcheva et al., 2002; Musikasang et al., 2009). For lactobacilli, bacterial suspension taken before and after the acid and bile tolerance test was used for enumeration of viable cells by pour plate method using MRS agar. Unlike lactobacilli, enumeration of *Bacillus* and *Enterococcus* viable cells was performed by spread plate method on MYP and m Enterococcus agar, respectively. After incubation in appropriate conditions for each bacterial genus, colonies on the plates were counted to determine the number of bacteria in colony forming unit/ml (CFU/ml.). The survivability of each bacterial isolate was calculated by the following formula.

$$Survival\ rate(\%) = \frac{\log CFUN}{\log\ CFUN_0} \times 100$$

(N is the viable count of bacterial isolate after acid and bile tolerance test and N_0 is the initial viable count before the test)

2.4 Identification of genus and species of potential candidate probiotic bacteria (n=56)

After acid and bile tolerance test was performed, bacterial isolates that demonstrated favorable probiotic properties in *Campylobacter* inhibiting activity test, hemolytic activity test, and acid and bile tolerance test were chosen for antimicrobial susceptibility test. Genus and species of selected candidate probiotic bacteria were identified by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and further confirmed by multiplex PCR prior to antimicrobial susceptibility testing.

Identification of candidate probiotic bacteria by MALDI-TOF MS was performed at the Department of Microbiology, Faculty of Veterinary Science, Chulalongkorn University. The bacterial isolates were incubated in appropriate condition for 20-24 hours. A small amount of colony from each isolate was smeared directly onto a spot on MALDI target plate. Then, the bacterial colony on the plate was overlaid with 1 µl of 70% formic acid for peptide extraction. After that, alpha-Cyano-4-hydroxycinnamic acid (HCCA) was used as matrix for co-crystallization, the sample was overlaid with HCCA and allowed to dry at room temperature. Next, the samples were ionized by laser beam and the signals were detected in TOF mass analyzer. Peptide mass fingerprint (PMF) of the sample was generated and used for bacterial identification by comparing the sample PMF to PMFs of bacterial strains within the database using MALDI Biotyper 3.0 software.

After genus and species of candidate probiotic bacteria were identified by MALDI-TOF MS, they were further confirmed by multiplex PCR using specific primers. Since the candidate probiotic bacterial isolates selected for antimicrobial susceptibility test in this study were identified as either *Limosilactobacillus reuteri* or *Ligilactobacillus salivarius* by MALDI-TOF MS, a multiplex PCR protocol with specific primers for *L. reuteri* and *L. salivarius* was chosen for confirmation of candidate probiotic bacterial genus and species (Song et al., 2000). For the isolates that could not be identified by MALDI-TOF database, 16S rRNA gene sequencing was implemented for identification of bacterial genus and species.

Selected candidate probiotic bacterial isolates identified by MALDI-TOF MS as *L. reuteri* or *L. salivarius* were confirmed by multiplex PCR with specific primers for *L. reuteri* and *L. salivarius* (Table 2). DNA extraction of lactobacilli was performed by using Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) following the manufacturer's instructions. Twenty-five µl of a reaction mixture consisted of 12.5 µl of TopTaq Master Mix (Qiagen, Düsseldorf, Germany), 2.5 µl of CoralLoad Concentrate (Qiagen), 0.75 µl of each 0.3 µM *L. reuteri* forward and reverse primers, 0.75 µl of each 0.3 µM *L. salivarius* forward and reverse primers, 2 µl of DNA template, and 5 µl nuclease-free water. DNA amplification was carried out in a thermocycler with initial denaturation step for 2 min at 95°C, followed by 35 cycles of amplification (denaturation at 95°C for 20 s, annealing at 56°C for 20 s, extension at 74°C for 20 s), ending with a final extension at 74°C for 5 min. Aliquots (5 µl) of the amplified products were separated by electrophoresis in 1.5% agarose gel in TAE buffer. The gels were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc., Gyeonggi, Republic of Korea) and visualized under UV light for analysis of PCR product size.

Table 2. Primer sequences and PCR product sizes of multiplex PCR for *L. reuteri* and *L. salivarius*

Primer	Sequence (5' to 3')	Product size
L. reuteri		
Lreu-1	CAGACAATCTTTGATTGTTTAG	303 bp
Lreu-4	GCTTGTTGGTTTGGGCTCTTC	
L. salivarius		
Lsal-1	AATCGCTAAACTCATAACCT	411 bp
Lsal-2	CACTCTCTTTGGCTAATCTT	

The bacterial isolate that could not be identified by MALDI-TOF was subjected to 16S rRNA gene sequencing. DNA extraction was performed by using Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan) as manufacturer's instructions. Universal primers (forward primer 5'- AGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGTACCTTGTTACGACTT-3') were used for amplification of 16S rRNA gene sequences according to the previously published protocol (Gee et al., 2003). The PCR reaction mixture of 25 µl contained 12.5 µl of TopTaq Master Mix (Qiagen, Düsseldorf, Germany), 2.5 µl of CoralLoad Concentrate (Qiagen), 1.5 µl of each 0.5 µM forward and reverse primers, 2 µl of DNA template, and 5 µl of nuclease-free water. DNA amplification was performed in a thermocycler with initial denaturation step for 5 min at 95°C, followed by 35 cycles of amplification (denaturation at 94°C for 15 s, annealing at 60°C for 15 s, extension at 72°C for 90 s), ending with a final extension at 72°C for 5 min. PCR product was purified with Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany) and then submitted for DNA sequencing at Bionics Co., Ltd. (Seoul, Republic of Korea). The obtained 16S rRNA gene sequence was compared with those available in the

National Center for Biotechnology Information (NCBI) database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 Antimicrobial susceptibility test (n=56)

All candidate probiotic bacterial isolates selected for antimicrobial susceptibility test in this study were all lactobacilli. The minimum inhibitory concentration (MIC) of 10 antimicrobial agents including ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, linezolid, streptomycin, tetracycline and vancomycin was examined using broth microdilution method according to the protocol previously published by Klare et al. (Klare et al., 2005) as recommended by European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). Briefly, each well in the microdilution plate was filled with 50 µl of different antimicrobial concentrations in Lactic acid bacteria Susceptibility test Medium (LSM) broth. Concentration ranges of 10 antimicrobials tested in this study are shown in Table 3. Then, inocula of lactobacilli isolates were prepared by suspending bacterial colonies in 0.85% NaCl and adjusted to 0.5 McFarland standard (equivalent to 10^8 CFU/ml). Subsequently, 1:100 dilution of bacterial suspension was performed by using LSM broth. After that, 50 µl of adjusted bacterial suspension (approximately 10^6 CFU/ml) was transferred into each well of the microdilution plate containing 50 µl of antibiotic solution, resulting in a final bacterial concentration of approximately 5 x 10^5 CFU/ml. The plates were incubated at 37°C for 24 hours under anerobic condition and the MIC results were determined. A panel of antimicrobial agents tested in this study and their microbiological cut-off values (µg/ml) are shown in Table 3. List of antimicrobial agents and their microbiological cut-off values were chosen according to the guidance on the characterization of microorganisms used as feed additives or as production organisms by EFSA (EFSA, 2018b) and the criteria for assessing the safety

of microorganisms resistant to antibiotics of human clinical and veterinary importance by SCAN (SCAN, 2003). *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used as quality control strains for antimicrobial susceptibility test in this study.

Table 3. Concentration ranges of antimicrobial agents and Microbiological cut-off values for lactobacilli tested in this study

Antimicrobials	Concentration range (µg/mL)	microbiological cut-off values			References
		Limosilactobacillus reuteri	*Ligilactobacillus salivarius*	*Limosilactobacillus alvi*	
Ampicillin	0.5-256	>2	>4	>2	EFSA, 2018
Clindamycin	0.125-64	>4	>4	>4	EFSA, 2018
Chloramphenicol	0.5-256	>4	>4	>4	EFSA, 2018
Erythromycin	0.25-128	>1	>1	>1	EFSA, 2018
Gentamicin	1-512	>8	>16	>8	EFSA, 2018
Kanamycin	1-512	>64	>64	>64	EFSA, 2018
Streptomycin	1-512	>64	>64	>64	EFSA, 2018
Tetracycline	1-512	>32	>8	>32	EFSA, 2018
Vancomycin	1-512	n.r.*	n.r.*	n.r.*	EFSA, 2018
Linezolid	0.125-64	>4	>4	>4	SCAN, 2003

*n.r., not required: Vancomycin is recommended by EFSA for susceptibility test of microorganisms used as feed additives including lactobacilli. However, resistance to vancomycin is considered intrinsic resistance in *L. reuteri*, *L. salivarius*, and *L. alvi*. Therefore, MIC determination for vancomycin is not required in these microorganisms.

2.6 Mucus adhesion assay (n=6)

After antimicrobial susceptibility test was performed, candidate probiotic isolates with no antimicrobial resistance or the isolates that were resistant to the least number of antimicrobial agents were chosen for the mucus adhesion assay. The method for chicken mucus preparation was performed as described by Nitisinprasert et al. (2006). Segments of ileum from 42 days old broiler chickens were opened and the surface of intestinal lumen was scrapped off by spatula. The material from the scrapping was collected in phosphate-buffered saline (PBS). To remove epithelial cells, cellular debris, and other contaminants, the mucus suspension was centrifuged at 5,500 rpm for 10 min and then centrifuged again at 13,000 rpm for 15 min. Subsequently, the mucus preparation was lyophilized and stored at -20 ºC until further use (Nitisinprasert et al., 2006).

Adhesion assay was performed according to the modified method described previously (Sanchez et al., 2010). Lyophilized mucus was used to prepare mucus solution at a concentration of 10 mg/ml in PBS. One hundred µl of mucus solution was transferred into a well in the 96-well polystyrene microtiter plate and the plates were incubated overnight at 4 ºC. After incubation, the wells were washed twice with 200 µl of PBS. Candidate probiotic bacteria were grown in MRS broth at 37 ºC for 18 hr. After incubation, the bacterial suspension was centrifuged at 5,000 rpm for 10 min, the bacterial pellets were washed twice with PBS and adjusted to 0.5 McFarland standard by densitometer. One hundred µl of bacterial suspension was added into each well of the 96-well microtiter plate that was prepared for mucus adhesion assay, then the plates were incubated at 37 ºC for 1 hr. After incubation, the wells were washed with 200 µl of PBS for 5 times to remove the bacteria that were unattached to mucus. Subsequently, 200 µl of a 0.05% (v/v) Triton X-100 solution was added to the wells for the removal of attached bacteria. The plates were

incubated at room temperature for 2 hr. The suspension in each well was thoroughly mixed with a micropipette. One hundred µl of candidate probiotic bacterial suspension from before and after the adhesion assay were sampled and pipetted onto MRS agar and incubated at 37 ºC for 24 hr under anaerobic condition. After incubation, colonies on the plates were counted to determine the number of CFU/ml. Adhesion assay of each bacterial isolate was performed in triplicate. *Lacticaseibacillus rhamnosus* GG was used as a positive control. The adhesion efficiency of each bacterial isolate was calculated by the following formula.

$$\text{Adhesion efficiency (\%)} = \frac{\log CFUN}{\log CFUN_0} \times 100$$

(N is the viable count of adhered bacteria after adhesion assay and N_0 is the initial viable count of bacteria before adhesion assay)

2.7 Whole genome sequencing (n=4)

Finally, bacterial isolates with the best overall results were selected and examined for their antimicrobial resistance determinants by whole genome sequencing. DNA extraction of candidate probiotic isolates was performed by using ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corp., California, U.S.A.) following the manufacturer's instructions. The extracted DNA was cleaned up using magnetic beads (Beckman Coulter Inc., California, U.S.A.). Purified DNA was used for sequencing by Oxford nanopore technologies at Siriraj Medical Research Center (SiMR), Faculty of Medicine Siriraj Hospital, Mahidol University. DNA sequencing was performed on the MinION nanopore DNA sequencer using a MinION Flow cell (R9.4.1). The preprocessed data from Oxford nanopore sequencing was used for bioinformatics analysis of candidate probiotic isolates including taxonomic assignment, *de novo* assembly of bacterial genome, and identification of antimicrobial resistance genes with their location on the genome.

Taxonomic assignment of candidate probiotic bacteria was done by read based method using Kraken2 program with Standard-8 database (release 2021). *De novo* assembly of bacterial genome was performed by flye v2.8.3 and the assembly graphs of candidate probiotic isolates were visualized by Bandage v0.1.8. Polishing of assembly data was performed by medaka v1.3.4 and QUAST v5.0.2 was used for evaluation of assembly quality. Confirmation of chromosomal and plasmid contigs was performed by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the sequence similarity of chromosomal or plasmid contigs in this study with chromosomal or plasmid DNA data in the NCBI database. Identification of antimicrobial resistance genes and their location on the genome was performed by using ResFinder v4.1 (https://cge.cbs.dtu.dk/services/ResFinder/).

Data analysis

Descriptive statistics was used to describe the probiotic properties of potential candidates such as *Campylobacter* inhibiting activity rates, acid and bile tolerance rates, and antimicrobial resistance rates. One-way analysis of variance (ANOVA) test and post hoc test (Dunnett's test) were used to compare the adhesion efficiency of candidate probiotic strains to the positive control strain. SPSS version 28.0 software (IBM Corporation) was used for statistical analysis. A p-value <0.05 was considered statistically significant.

CHAPTER IV
RESULTS

1. Isolation of candidate probiotic bacteria from *Campylobacter*-negative chickens

Among 30 broiler farms where 300 ceca were collected, 20 farms (66.67%) were infected with *Campylobacter*. Only 10 broiler farms (33.33%) showed *Campylobacter*-negative status. Details of *Campylobacter* status of broiler chickens in each farm are shown in Appendix C. A total of 602 isolates of candidate probiotic bacteria were isolated from *Campylobacter*-negative chickens. At least 1 colony of lactobacilli, *Bacillus*, and *Enterococcus* was selected from each broiler chicken. The candidate probiotic bacterial isolates were comprised of 253 lactobacilli, 169 *Bacillus* spp. and 180 *Enterococcus* spp. These isolates were further assessed for their probiotic properties.

2. Assessment of probiotic properties of potential candidates

2.1 *Campylobacter* inhibiting activity test

A total of 602 isolates of candidate probiotic bacteria were tested for their ability to inhibit the growth of *Campylobacter in vitro*. The results obtained from agar well diffusion method showed that 228 bacterial isolates demonstrated *Campylobacter* inhibiting activity. Among these 228 isolates, 223 isolates (97.8%) were lactobacilli and the other 5 isolates (2.2%) were *Enterococcus*. No *Bacillus* isolates exhibited inhibitory effect against *Campylobacter*. The largest diameter of *Campylobacter* inhibition zone was 25 mm and the smallest was 7 mm. More than 50% of isolates (122 isolates or 53.51%) showed inhibition zone between 11-13 mm in diameter. A total of 194 isolates (190 lactobacilli and 4 *Enterococcus* isolates) with inhibition zone diameter greater than or equal to 11 mm were chosen for hemolytic

activity test, acid tolerance test, and bile tolerance test. *Campylobacter* inhibition zone diameter of candidate probiotic bacterial isolates is shown in Table 4.

Table 4. *Campylobacter* inhibition zone diameter of candidate probiotic bacterial isolates

Diameter of *Campylobacter* inhibition zone (mm)	No. of isolates (%)
25	1 (0.44%)
21	3 (1.32%)
20	1 (0.44%)
19	2 (0.88%)
18	5 (2.19%)
17	4 (1.75%)
16	5 (2.19%)
15	35 (15.35%)
14	16 (7.02%)
13	46 (20.18%)
12	18 (7.89%)
11	58 (25.44%)
10	22 (9.65%)
9	11 (4.82%)
7	1 (0.44%)
Total	**228**

2.2 Hemolytic activity test

For hemolytic activity test, 79 out of 194 isolates showed no hemolytic activity (75 lactobacilli and 4 *Enterococcus* isolates), while the other 115 isolates demonstrated alpha hemolysis.

2.3 Acid and bile tolerance test

The acid tolerance test evidenced that 102 out of 194 isolates (52.58%) were able to survive at pH 2.5 for 2 hr in varying levels, while the other 92 isolates (47.42%) did not show viability after testing in the acidic condition. The isolates that demonstrated acid tolerance showed varying degrees of survival rates. The survival rates range from 36.32 % to 99.74%. Among the 102 acid-tolerant isolates, 98 isolates (96.07%) demonstrated more than 50% survival rate and 51 isolates (50%) showed survival rates in the 90%-100% range. All 4 *Enterococcus* isolates were not able to survive in the acid tolerance test. The survival rates of 194 candidate probiotic bacterial isolates in acid tolerance test are shown in Table 5.

Table 5. Survival rates of candidate probiotic bacteria isolates in acid tolerance test

Survival rates	No. of isolates	
	Lactobacilli	*Enterococcus* spp.
>90-100%	51	0
>80-90%	22	0
>70-80%	14	0
>60-70%	5	0
>50-60%	6	0
>40-50%	2	0
>30-40%	2	0
0	88	4
Total	**190**	**4**

The results from bile tolerance test indicated that almost all of the isolates (183 isolates or 94.33%) demonstrated viability in 2% bile for 3 hr. Only 11 isolates (5.67%) were not able to survive the bile tolerance test. Among the bile- tolerant isolates, the survival rates range from 59.28% to 110.35%. The majority of the isolates (166 isolates or 90.71%) exhibited more than 80% survival rates. Only 17 isolates (9.29%) demonstrated survival rates between 59.28% to 79.45%. All 4 *Enterococcus* isolates survived well in the bile tolerance test with more than 90% survival rates. The survival rates of 194 candidate probiotic bacterial isolates in bile tolerance test are shown in Table 6.

Table 6. Survival rates of candidate probiotic bacterial isolates in bile tolerance test

Survival rates	No. of isolates	
	Lactobacilli	*Enterococcus* spp.
>90-≥100%	122	4
>80-90%	40	0
>70-80%	9	0
>60-70%	7	0
>50-60%	1	0
>40-50%	0	0
>30-40%	0	0
0	11	0
Total	**190**	**4**

The results from *Campylobacter* inhibiting activity test, hemolytic activity test, and acid and bile tolerance test identified 44 isolates that demonstrated *Campylobacter* inhibition zone diameter greater than or equal to 11 mm, did not cause hemolytic reaction, and showed at least 85% survival rate in acid and bile tolerance tests. These isolates were selected for antimicrobial susceptibility test. In addition, 12 more isolates with large *Campylobacter* inhibition zone diameter (13 mm or more) but demonstrated less than 85% survival rate in acid and bile tolerance test, were also included for antimicrobial susceptibility testing. The details of *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates are shown in Appendix D.

2.4 Identification of genus and species of potential candidate probiotic bacteria

Among 56 bacterial isolates that showed good preliminary probiotic properties, 55 isolates were identified as either *Limosilactobacillus reuteri* (51 isolates) or *Ligilactobacillus salivarius* (4 isolates) by MALDI-TOF MS, and 1 isolate could not be identified by the database. The bacterial identification results from MALDI-TOF MS were concurrent with the results from multiplex PCR using specific primers for *L. reuteri* and *L. salivarius*. For the isolate that could not be identified by MALDI-TOF MS, the results of 16S rRNA gene sequence analysis showed that the isolate had highest sequence similarity with *Limosilactobacillus alvi*. Details of genus and species identification of potential candidate probiotic bacteria are shown in Table 7.

2.5 Antimicrobial susceptibility test

The MIC of 10 antimicrobial agents determined by broth microdilution method showed that all 56 isolates tested in this study were resistant to vancomycin, 82.14% of the isolates were resistant to ampicillin and tetracycline, 80.36% to clindamycin and erythromycin, 46.43% to kanamycin, 33.93% to streptomycin, 26.79% to chloramphenicol, 10.71% to gentamicin, and 1.79% to linezolid (Table 7). Although most isolates were resistant to 4-7 antimicrobial agents and multidrug resistance was observed in 89.29% of the isolates (50 out of 56 isolates), 1 isolate (i 33.6/2) was resistant to only one antimicrobial agent (vancomycin) and 5 isolates (i 24.1/2, i 24.2/2, i 24.5/2, i 35.3/2, and i 35.7/2) were resistant to 2 antimicrobial agents (vancomycin and ampicillin). These six isolates were all identified as *L. reuteri*. Since resistance to vancomycin is considered intrinsic resistance in most lactobacilli (Gueimonde et al., 2013) and ampicillin resistance in *L. reuteri* is probably caused by point mutations in the genes encoding penicillin-binding proteins (Pbp) (Rosander et al., 2008; Egervärn et al., 2009), these six isolates were selected for further evaluation of their adhesion property by mucus adhesion assay.

Table 7. Genus and species of candidate probiotic bacterial isolates and their resistance patterns

No.	Isolate code	Genus and species	Resistance patterns*
1	i 33.6/2	*Limosilactobacillus reuteri*	VAN
2	i 24.1/2	*Limosilactobacillus reuteri*	AMP, VAN
3	i 24.2/2	*Limosilactobacillus reuteri*	AMP, VAN
4	i 24.5/2	*Limosilactobacillus reuteri*	AMP, VAN
5	i 35.3/2	*Limosilactobacillus reuteri*	AMP, VAN
6	i 35.7/2	*Limosilactobacillus reuteri*	AMP, VAN
7	i 13.8	*Limosilactobacillus reuteri*	AMP, TET, VAN
8	i 33.2/2	*Limosilactobacillus alvi*	AMP, KAN, VAN
9	i 27.2	*Limosilactobacillus reuteri*	AMP, KAN, TET, VAN
10	i 27.9	*Limosilactobacillus reuteri*	ERY, CLI, TET, VAN
11	i 29.1	*Limosilactobacillus reuteri*	ERY, CLI, TET, VAN
12	i 33.3/2	*Limosilactobacillus reuteri*	ERY, CLI, TET, VAN
13	i 33.4/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, VAN
14	i 33.5/2	*Limosilactobacillus reuteri*	AMP, GEN, KAN, VAN
15	i 34.6/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, VAN
16	i 5.2	*Limosilactobacillus reuteri*	KAN, ERY, CLI, TET, VAN
17	i 13.10	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
18	i 20.9	*Limosilactobacillus reuteri*	AMP, KAN, TET, CMP, VAN
19	i 22.3	*Limosilactobacillus reuteri*	STR, ERY, CLI, TET, VAN
20	i 22.6	*Limosilactobacillus reuteri*	STR, ERY, CLI, TET, VAN
21	i 22.8	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
22	i 22.10	*Limosilactobacillus reuteri*	KAN, ERY, CLI, TET, VAN
23	i 27.5	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN

Table 7. (Continued)

No.	Isolate code	Genus and species	Resistance patterns*
24	i 27.8	*Limosilactobacillus reuteri*	ERY, CLI, TET, CMP, VAN
25	i 29.9	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
26	i 22.10/2	*Limosilactobacillus reuteri*	ERY, CLI, TET, CMP, VAN
27	i 24.3/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
28	i 24.6/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
29	i 24.10/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, VAN
30	i 4.1	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
31	i 4.8	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
32	i 5.6	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, CMP, VAN
33	i 5.10	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
34	i 8.5	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
35	i 8.7	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
36	i 11.3	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, VAN
37	i 22.4	*Limosilactobacillus reuteri*	AMP, STR, ERY, CLI, TET, VAN
38	i 29.4	*Ligilactobacillus salivarius*	AMP, STR, ERY, CLI, TET, VAN
39	i 21.1/2	*Limosilactobacillus reuteri*	AMP, STR, ERY, CLI, TET, VAN
40	i 24.4/2	*Limosilactobacillus reuteri*	AMP, ERY, CLI, TET, CMP, VAN
41	i 4.3	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, VAN
42	i 4.6	*Limosilactobacillus reuteri*	AMP, STR, ERY, CLI, TET, CMP, VAN
43	i 4.9	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, VAN
44	i 5.4	*Limosilactobacillus reuteri*	AMP, KAN, ERY, CLI, TET, CMP, VAN
45	i 8.4	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, VAN
46	i 8.8	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, VAN
47	i 13.1	*Limosilactobacillus reuteri*	AMP, STR, ERY, CLI, TET, CMP, VAN

Table 7. (Continued)

No.	Isolate code	Genus and species	Resistance patterns*
48	i 23.4/2	*Ligilactobacillus salivarius*	AMP, KAN, ERY, CLI, TET, CMP, VAN
49	i 4.7	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, CMP, VAN
50	i 4.10	*Limosilactobacillus reuteri*	AMP, KAN, STR, ERY, CLI, TET, CMP, VAN
51	i 13.5	*Limosilactobacillus reuteri*	AMP, GEN, KAN, STR, ERY, CLI, TET, VAN
52	i 13.7	*Limosilactobacillus reuteri*	AMP, GEN, KAN, STR, ERY, CLI, TET, VAN
53	L 22.7	*Ligilactobacillus salivarius*	AMP, KAN, STR, ERY, CLI, TET, CMP, VAN
54	i 13.9	*Limosilactobacillus reuteri*	AMP, GEN, KAN, STR, ERY, CLI, TET, CMP, VAN
55	i 23.8/2	*Ligilactobacillus salivarius*	AMP, GEN, KAN, STR, ERY, CLI, TET, CMP, VAN
56	i 13.4	*Limosilactobacillus reuteri*	AMP, GEN, KAN, STR, ERY, CLI, TET, CMP, LIN, VAN

*AMP, ampicillin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CMP, chloramphenicol; LIN, linezolid; VAN, vancomycin

2.6 Mucus adhesion assay

The results of mucus adhesion assay showed that the adhesion efficiency of candidate probiotic isolates ranges from 64.75±0.35% to 80.10±1.10%. *L. rhamnosus* GG, which was used as a positive control showed an adhesion efficiency of 82.66±3.11%. The isolate that demonstrated highest adhesion efficiency in this study was i 24.1/2, which had adhesion efficiency of 80.10±1.10%. Although the adhesion efficiency of the other 5 isolates was significantly different from that of the positive control ($p < 0.05$), these isolates still demonstrated the ability to adhere to intestinal mucus with close to 70% adhesion efficiency. Adhesion efficiency of candidate probiotic isolates and *L. rhamnosus* GG is shown in Table 8.

Table 8. Adhesion efficiency of candidate probiotic bacterial isolates and positive control

Isolate code	Bacteria	Adhesion efficiency (%)*			
		1st test	2nd test	3rd test	Mean±SD
i 33.6/2	*L. reuteri*	68.58	70.35	71.37	70.1±1.41
i 24.1/2	*L. reuteri*	81.06	78.9	80.34	80.1±1.1
i 24.2/2	*L. reuteri*	71.11	69.81	70.14	70.35±0.67
i 24.5/2	*L. reuteri*	69.64	67.13	65.33	67.37±2.16
i 35.3/2	*L. reuteri*	73.65	70.82	71.15	71.87±1.55
i 35.7/2	*L. reuteri*	64.68	65.13	64.44	64.75±0.35
Control	*L. rhamnosus* GG	86.18	81.51	80.29	82.66±3.11

* mucus adhesion assay was performed in triplicate

The aforementioned six *L. reuteri* isolates that could inhibit the growth of *Campylobacter*, showed no hemolytic activity, demonstrated acid and bile tolerance, were least resistant to antimicrobial agents, and exhibited mucus adhesion ability, were chosen to be the candidate probiotics. However, 2 isolates (i 24.5/2 and i 35.7/2) were not able to grow after subculturing from the stock. So, only 4 candidate probiotic isolates were selected for whole genome sequencing. Details of probiotic properties of 4 candidate probiotic isolates are shown in Table 9.

Table 9. Summary of probiotic properties of selected candidate probiotic isolates

Isolate code	*Campylobacter* inhibition zone diameter (mm)	Survival rates in acid tolerance test	Survival rates in bile tolerance test	Mucus adhesion efficiency (%)	Resistance patterns
i 33.6/2	13	78.01	102.09	70.10 ± 1.41	VAN
i 24.1/2	16	91.12	99.47	80.10 ± 1.10	AMP, VAN
i 24.2/2	15	99.58	102.95	70.35 ± 0.67	AMP, VAN
i 35.3/2	16	99.67	103.36	71.87 ± 1.55	AMP, VAN

2.7 Determination of antimicrobial resistance genes and identification of their location on the genome by whole genome sequencing

Bioinformatics analysis of preprocessed data from Oxford nanopore sequencing indicated that all four candidate probiotics isolates were identified as *Limosilactobacillus reuteri*. *De novo* assembly graphs of 4 *L. reuteri* isolates are shown in Figure 2.

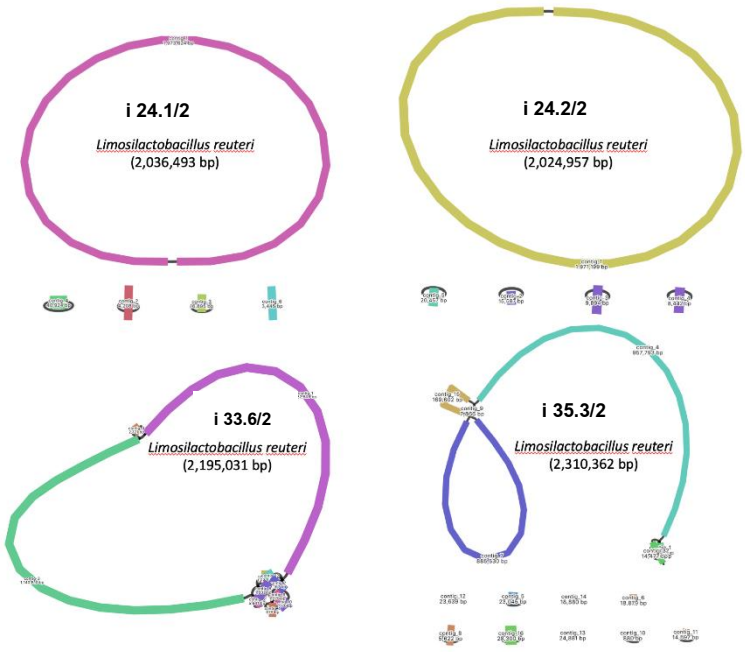

Figure 2. *De novo* assembly graphs of 4 candidate probiotic isolates

The genome of isolate i 24.1/2 assembled into a single completed circular chromosomal contig of 1,972,709 base pair (bp) with 3 circular (40,125 bp, 16,070 bp, and 4,171 bp) and 1 linear (3, 418 bp) plasmid contigs. The genome of isolate i 24.2/2 was also assembled into a single circular chromosomal contig of 1,971,257 bp with 4 circular plasmid contigs (20,454 bp, 15,562 bp, 9,271 bp, and 8,413 bp). As for isolate i 33.6/2, the genome assembly resulted in 4 linear contigs. One contig was chromosomal DNA (2,139,628 bp) and 3 contigs were identified as plasmids (28,764 bp, 13,446 bp and 13,193 bp). Lastly, the assembled genome of isolate i 35.3/2 resulted in 6 linear chromosomal contigs (967,799 bp, 897,240 bp, 177,447 bp, 61,531 bp, 23,639 bp, and 20,392 bp) with 4 circular (27,576 bp, 23,005 bps, 14,562 bp, and 5,613 bp) and 4 linear (30,542 bp, 24,148 bp, 18528 bp, and 18,388 bp) plasmid contigs (Table 10).

Antimicrobial resistance genes were identified in both chromosome and plasmids of candidate probiotic isolates. For isolate i 24.1/2 and i 24.2/2, *lnuA* which confers resistance to lincosamides was located on the plasmid. As for isolate i 33.6/2 and i 35.3/2, *tetW* (conferring tetracycline resistance) was located on the chromosome of both isolates. Moreover, *lnuA* was located on the plasmid of isolate i 35.3/2, and *ermB* which confers macrolide resistance was located on the plasmid of isolate i 33.6/2. Details of antimicrobial resistance genes identification and their location on the genome of candidate probiotics are shown in Table 10.

Table 10. Antimicrobial resistance genes identification and their location on the genome of 4 candidate probiotic isolates

Isolate	sequence name	Length	Chromosome/ Plasmid	Resistance genes		
				tetW	*lnuA*	*ermB*
i 24.1/2	contig_1	1,972,709	Chromosome			
	contig_2	40,125	Plasmid		✓	
	contig_3	16,070	Plasmid			
	contig_4	4,171	Plasmid			
	contig_5	3, 418	Plasmid			
i 24.2/2	contig_1	1,971,257	Chromosome			
	contig_2	20,454	Plasmid		✓	
	contig_3	15,562	Plasmid			
	contig_4	9,271	Plasmid			
	contig_5	8,413	Plasmid			
i 33.6/2	scaffold_1	2,139,628	Chromosome	✓		
	contig_1	28,764	Plasmid			
	contig_2	13,446	Plasmid			✓
	contig_3	13,193	Plasmid			✓
i 35.3/2	contig_1	967,799	Chromosome			
	contig_2	897,240	Chromosome			
	contig_3	177,447	Chromosome	✓		
	contig_4	61,531	Chromosome			
	contig_5	30,542	Plasmid			
	contig_6	27,576	Plasmid		✓	
	contig_7	24,148	Plasmid			
	contig_8	23,639	Chromosome			
	contig_9	23,005	Plasmid			
	contig_10	20,392	Chromosome			
	contig_11	18528	Plasmid			
	contig_12	18,388	Plasmid			
	contig_13	14,562	Plasmid			
	contig_14	5,613	Plasmid			

CHAPTER V
DISCUSSION

1. *Campylobacter* inhibiting activity test

Detection of anti-*Campylobacter* activity is an essential step in the *in vitro* screening of potential candidate probiotics with antagonistic activity against *Campylobacter*. The cell-free supernatants of candidate probiotic bacteria can be tested by agar well diffusion assay to examine the diameter of *Campylobacter* inhibition zone. Agar well diffusion method is relatively uncomplicated to perform and does not require advanced or expensive laboratory equipment. Thus, this method can be used to perform on large number of bacterial strains to detect their *Campylobacter* inhibiting activity. In this study, a total of 602 bacterial isolates were tested for their ability to inhibit the growth of *Campylobacter jejuni* NCTC 11168. These isolates include 253, 169 and 180 isolates of lactobacilli, *Bacillus* spp. and *Enterococcus* spp., respectively.

The results indicated that the majority of lactobacilli isolates were able to inhibit *Campylobacter jejuni* growth (223 out of 253 isolates or 88.14%), only a few isolates of *Enterococcus* spp. demonstrated *Campylobacter* inhibiting activity (5 out of 169 isolates or 2.96%), and all *Bacillus* isolates did not exhibit *Campylobacter* inhibition ability. These findings were in agreement with the results from previous studies (Robyn et al., 2012; Dec et al., 2018; Erega et al., 2021). Several studies have shown that many lactic acid bacteria isolated from chickens, especially lactobacilli, demonstrated the ability to inhibit the growth of *Campylobacter* spp. *in vitro* (Messaoudi et al., 2011; Kobierecka et al., 2017). Dec et al. (2018) isolated several species of lactobacilli from fresh feces or cloaca of chickens. Cell-free supernatant from these lactobacilli isolates was used in agar well diffusion method and

Campylobacter inhibition zone was observed in many lactobacilli species including *L. salivarius*, *L. johnsonii*, *L. crispatus*, *L. reuteri*, *L. ingluviei* and *L. oris*. The diameter of *Campylobacter* inhibition zone ranged from 11-23 mm in diameter (the diameter of the well was 8 mm), with *L. salivarius* and *L. reuteri* exhibited the largest inhibition zone when compared with other lactobacilli (Dec et al., 2018). Similarly, 3 strains of *L. salivarius* (*L. salivarius* SMXD51, MMS122, and MMS151) isolated from chicken ceca demonstrated inhibiting activity against *C. jejuni* and *C. coli* in agar well diffusion assay (Messaoudi et al., 2011). Furthermore, *Campylobacter* inhibitory effects of *L. salivarius*, *L. plantarum*, *L. crispatus*, and *L. agilis* isolated from feces or cloaca of chickens were observed (Kobierecka et al., 2017). Moreover, lactobacilli isolated from other sources such as human feces and cheese, showed the ability to inhibit the growth of *Campylobacter* as well (Santini et al., 2010; Nishiyama et al., 2014; Deng et al., 2020). Unlike lactobacilli, fewer studies have reported evidence of *in vitro Campylobacter* inhibiting activity in *Enterococcus* strains, such as *E. faecium* THT (Dubois-Dauphin et al., 2011) and *E. faecalis* MB5259 (Robyn et al., 2012). Similar to *Enterococcus* spp., *in vitro* inhibitory effects against *Campylobacter* of *Bacillus* spp. have been indicated in only few previous studies. For instance, two strains of *Bacillus* (*B. subtilis* 3 and *B. licheniformis* 31) from human commercial probiotic product (Biosporin®) demonstrated antagonistic activity against *C. jejuni* and *C. coli* by agar blocks method (Sorokulova et al., 1997). In another study, *B. subtilis* PS-216 strain from laboratory collections showed antagonism against *Campylobacter* in agar diffusion assay and co-culture assay (Erega et al., 2021).

Campylobacter growth inhibition in agar well diffusion assay was the results of antibacterial substances produced by probiotic bacteria. Antibacterial substances from probiotic bacteria include organic acids, hydrogen peroxide, bacteriocins, etc. (Mohan, 2015; Saint-Cyr et al., 2016). Lactic acid bacteria including lactobacilli and

Enterococcus can produce different organic acids such as lactic acid, acetic acid, and butyric acid (Özcelik et al., 2016; Tachedjian et al., 2017). Lactic acid produced by *L. acidophilus*, *L. crispatus*, *L. gallinarum*, and *L. helveticus* could inhibit the growth of *C. jejuni in vitro* and reduce *Campylobacter* colonization in chickens (Neal-McKinney et al., 2012). Hydrogen peroxide is a metabolic byproduct of many microorganisms including lactic acid bacteria especially when they were grown under aerobic condition. High level of hydrogen peroxide can be toxic to bacterial cells (Erttmann and Gekara, 2019). Bacteriocins are antimicrobial peptides produced by many bacteria including lactobacilli (e.g., nisin, lactocin, gassericin), *Enterococcus* (enterocins) and *Bacillus* (e.g., subtilin, lichenicidin, coagulin) (Pavan et al., 2000; Arakawa et al., 2009; Abriouel et al., 2011; Lü et al., 2014; Hanchi et al., 2018). Reuterin, an antimicrobial compound produced by *L. reuteri*, demonstrated antimicrobial activity against various pathogens including *C. jejuni* (Arqués et al., 2004; Asare et al., 2020).

2. Hemolytic activity test

According to the FAO and WHO (FAO/WHO, 2002), one of the safety considerations for bacterial strains to be used as probiotics is the absence of hemolytic activity. Hemolysis is a known virulence factor. Lack of hemolytic activity ensures that such bacterial strains are non-virulent and do not produce hemolysins (Yasmin et al., 2020). In this study, 79 out of 194 isolates showed no hemolytic activity (gamma hemolysis), while alpha hemolysis was observed in the other 115 isolates. All 4 *Enterococcus* isolates were non hemolytic strains. Different *Enterococcus* strains can demonstrate beta, alpha, or gamma hemolysis (Semedo et al., 2003). Lactobacilli isolates in this study exhibited both gamma (no hemolysis) and alpha hemolysis. There were previous reports of alpha hemolytic activity on blood agar by lactobacilli (Goldstein et al., 2015; Aristimuño Ficoseco et al., 2018), but the

strains used as probiotics in most studies were non hemolytic (Sieladie et al., 2011; Ji et al., 2015; Padmavathi et al., 2018).

3. Acid and bile tolerance test

Probiotics are normally given to poultry orally via feeds or water. Thus, probiotics must be able to survive a passage through harsh acidic condition in proventriculus and high concentration of bile in small intestines. Ingested microorganisms can be damaged and destroyed by acid and bile. Probiotic microorganisms that exhibit weak acid and bile tolerance may survive in low or inadequate amount and consequently cannot exert its beneficial effects on the host.

The pH in different parts of chicken gastrointestinal tract varies from acidic to neutral and mildly alkaline. The most acidic part of the gastrointestinal tract are the proventriculus and gizzard, which function as true stomach in poultry. The pH in these compartments range from 2.5 to 3.5 (Ravindran, 2013; Svihus, 2014). The passage time of food through the entire gastrointestinal tract of chickens ranges from 2-4 hours, with the retention time in proventriculus and gizzard around 30-120 minutes, depending on factors such as breeds, digestion efficiency, and type and amount of feeds (Svihus et al., 2002; Rougière and Carré, 2010; Ravindran, 2013). Therefore, acid tolerance test in this study was performed at pH 2.5 for 2 hours.

In the present study, more than 50% (102 out of 194 isolates) of the selected bacterial isolates were able to survive acid tolerance test at pH 2.5 for 2 hours. Among the tested 190 lactobacilli isolates, 102 isolates showed survivability against acid tolerance test. The results were in concordance with previous studies which demonstrated that numerous strains of lactobacilli were able to survive in low pH condition (Goldin et al., 1992; Jacobsen et al., 1999; Matijasic and Rogelj, 2000; Fontana et al., 2013) including lactobacilli strains isolated from gastrointestinal tract

of chickens (Jin et al., 1998). One of the characteristics of lactic acid bacteria, particularly lactobacilli, is their tolerance to acidity (van de Guchte et al., 2002). However, acid tolerance is highly specific to species and strains in a very low pH environment, especially at pH values below 3.0 (Jin et al., 1998; Rönkä et al., 2003; Corcoran et al., 2005). The acid tolerance of lactic acid bacteria including lactobacilli is attributed to several mechanisms. F_0F_1-ATPase enzyme is involved in the expulsion of protons out of the cell when the extracellular pH is low (Nannen and Hutkins, 1991; van de Guchte et al., 2002; Corcoran et al., 2005). Arginine deiminase pathway produces ammonia that can react with protons, which help alkalize the pH value (Cunin et al., 1986; van de Guchte et al., 2002; Rollan et al., 2003). Other known mechanisms contributed to acid tolerance of lactic acid bacteria include transmembrane pH gradient established by K^+ ATPase (van de Guchte et al., 2002), production of urea by urease enzyme (Quivey et al., 2000), and decarboxylation reactions and electrogenic transport (Konings et al., 1997; Sanders et al., 1999).

In poultry, the average concentration of bile in duodenum is approximately 0.3% (w/v), but may elevated to 2% during feed digestion (Bakari et al., 2011). Thus, 2% bile concentration was used in the bile tolerance test of this study. Exposure to bile can cause the destruction of bacterial cells (Begley et al., 2005). This is due to the fact that cell membranes of bacteria consist of lipids and fatty acids. Bile at high concentration can cause the dissociation of lipid bilayer and integral protein in cell membranes, which lead to the breakdown of bacterial cell membranes and eventually cell death (Jin et al., 1998; Musikasang et al., 2009; Hassanzadazar et al., 2012). For this reason, candidate probiotic bacteria need to possess bile tolerance ability in order to survive in the small intestine and able to colonize there. The results from bile tolerance test in this study revealed that almost all of the isolates (94.33%) were able to survive in 2% bile for 3 hours. Nearly all of the lactobacilli

isolates (179 from 190 isolates) and all 4 *Enterococcus* isolates showed survivability in the bile tolerance test. Bile tolerance of lactic acid bacteria including lactobacilli (Jin et al., 1998; Musikasang et al., 2009; Singh et al., 2012; Shehata et al., 2016; Dec et al., 2018) and *Enterococcus* (Lertworapreecha et al., 2011; Nami et al., 2019) have also been reported in previous studies.

Bile tolerance in gram-positive microorganisms is not well understood (Begley et al., 2005). Similar to acid tolerance, bile tolerance ability is different among genus, species, and even strains of lactic acid bacteria (Begley et al., 2005; Menconi et al., 2014). It has been suggested in several reports that bile tolerance in members of lactic acid bacteria including many species of lactobacilli and some *Enterococcus* spp., is related to activity of bile salt hydrolase (BSH) enzyme (De Smet et al., 1995; Šušković et al., 2000; Begley et al., 2005; Singh et al., 2012). BSH enzyme deconjugates bile acids by catalyzing the hydrolysis of the amide bond between glycine or taurine amino acid side chain and steroid core of bile acids, which resulted in the alteration of bile properties and consequently reduce its detergent activity, thus decreasing its bactericidal effect (du Toit et al., 1998; van de Guchte et al., 2002; Begley et al., 2005; Menconi et al., 2014). The activity of BSH enzyme has been found most frequently in microorganisms isolated from intestinal tract of animals (Tanaka et al., 1999).

4. Antimicrobial susceptibility test

Antimicrobial resistance is a major public health concern. Antimicrobial-resistant bacteria from livestock animals can be introduced to humans via food production chain (Rossi et al., 2014). Therefore, microorganisms intended to be used as probiotics in animal feeds should not be able to transfer antimicrobial resistance genes to other bacteria, particularly gastrointestinal pathogenic bacteria.

According to EFSA (EFSA, 2018b), the MIC of 9 antimicrobial agents including ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin should be determined as a basic requirement for bacterial strains intended for use as feed additives in animal feeds. In addition, this study selected linezolid, recommended by SCAN (SCAN, 2003), for antimicrobial susceptibility test as well. In this study, the 56 bacterial isolates selected for antimicrobial susceptibility test were *L. reuteri* (51 isolates), *L. salivarius* (4 isolates), and *L. alvi* (1 isolate). Among these candidates, one isolate was resistant to only 1 antimicrobial agent, which was vancomycin. Five isolates were resistant to 2 antimicrobial agents, which were ampicillin and vancomycin. Multidrug resistance was observed in the other 50 isolates.

We selected the aforementioned 6 isolates (all *L. reuteri*) that were resistant to the least number of antimicrobial agents for further assessment. Although vancomycin and/or ampicillin resistance were found in selected *L. reuteri* isolates, it presents a minimal risk for horizontal transfer of antimicrobial resistance genes. In fact, most lactobacilli are intrinsically resistant to vancomycin. The reason for this is that vancomycin normally binds with high affinity to peptidoglycan precursors terminating in D-alanyl-D-alanine (D-Ala-D-Ala), consequently inhibit cell wall synthesis. However, the terminus of peptidoglycan precursors in many species of lactobacilli is D-alanyl-D-lactate (D-Ala-D-Lac), in which vancomycin has low-affinity binding, resulting in vancomycin resistance (Gueimonde et al., 2013; Zhang et al., 2018). Therefore, vancomycin resistance is not a cause for concern in lactobacilli selected to be candidate probiotics. Moreover, even though ampicillin resistance mechanisms in most lactobacilli have remained largely unclear, there were evidences that ampicillin resistance in many strains of *L. reuteri* was attributed to point mutations in the genes encoding penicillin-binding proteins (Pbp). A study by

Rosander et al. (2008) aimed to characterize the nature of ampicillin resistance in *L. reuteri* ATCC 55730, they did not find any genes associated with β-lactam resistance in the genome of *L. reuteri* ATCC 55730. Instead, point mutations in the genes encoding Pbp1a, Pbp2a, and Pbp2x were identified. Alterations in the genes encoding Pbp have been reported to cause β-lactam resistance in *Streptococcus* (Coffey et al., 1995; Hiramatsu et al., 2004). Since point mutations of the Pbp encoding genes in *L. reuteri* ATCC 55730 were located on the chromosome, β-lactam resistance in *L. reuteri* ATCC 55730 was non-transferable (Rosander et al., 2008). The results from a study by Egervarn et al. (2009) also confirmed this phenomenon in different *L. reuteri* strains (Egervärn, 2009). Additionally, in many studies, no β-lactam resistance genes (e.g., β-lactamase-encoding genes and *mecA*) were detected in ampicillin-resistant lactobacilli isolates (Liu et al., 2009; Mayrhofer et al., 2010; Dec et al., 2017).

The other 50 multidrug-resistant isolates present a potential for horizontal dissemination of antimicrobial resistance genes. Several antimicrobial resistance determinants associated with other antimicrobial agents tested in this study have been reported in lactobacilli. Tetracycline resistance genes are the most common resistance genes found in lactobacilli (Gueimonde et al., 2013; Campedelli et al., 2019). Several tetracycline resistance genes including *tetW*, *tetM*, *tetS*, *tetO*, *tetQ*, *tetK*, and *tetL* have been identified on plasmids and transposons in many species of lactobacilli (Klare et al., 2007; Chang et al., 2011; Devirgiliis et al., 2013; Gueimonde et al., 2013). Numerous genes responsible for resistance to macrolides (e.g., erythromycin) have also been detected on plasmids and transposons of several lactobacilli species. Among such genes, *ermB* is the most frequently found resistance determinant (Gueimonde et al., 2013; Dec et al., 2017). Other macrolide resistance genes such as *ermA*, *ermC*, and *ermT* have also been reported in lactobacilli (Cauwerts et al., 2006; Mayrhofer et al., 2010; Thumu and Halami, 2012). Moreover,

detection of *lnuA*, a gene conferring resistance to lincosamides have been reported in *L. reuteri* isolated from chickens (Cauwerts et al., 2006) and *L. reuteri* ATCC 55730, a probiotic strain (Kastner et al., 2006). The *cat* gene which confers resistance to chloramphenicol have been identified in many lactobacilli species as well (Hummel et al., 2007; Mayrhofer et al., 2010). Aminoglycoside resistance genes are less commonly found in lactobacilli, but some of those genes such as *aac(6′)-Ie-aph(2′)-Ia*, *aph(2″)-Ic*, *aph(3′)-IIIa*, and *ant(6)-Ia* have been detected (Rojo-Bezares et al., 2006; Dec et al., 2017). To our knowledge, linezolid resistance determinants such as *cfr* and *optrA* have not been reported in lactobacilli and resistance to linezolid in lactobacilli is extremely rare. Only 1 isolate in this study demonstrated linezolid resistance and exhibited MIC value of 8 µg/ml (microbiological cut off value for *L. reuteri* against linezolid is >4 µg/ml). However, this isolate was also resistant to the other 9 antimicrobial agents tested in this study.

5. Mucus adhesion assay

Adhesion to intestinal mucus is considered to be one of the important criteria for probiotic selection because adhesion ability is essential for bacterial colonization. Probiotics must be able to colonize the intestine in order to exert its beneficial effects on the host. Most gut bacteria adhere and grow in the mucus layer overlying the intestinal epithelium (Sicard et al., 2017). Therefore, candidate probiotic bacterial isolates in this study was tested for chicken mucus adhesion assay. The results indicated that isolate i 24.1/2 demonstrated the highest adhesion efficiency (80.10±1.10%), which was similar to the adhesion efficiency of *L. rhamnosus* GG (82.66±3.11%). And even though the adhesion efficiency of other 5 isolates (64.75±0.35% to 71.87±1.55%) including i 24.2/2, i 24.5/2, i 33.6/2, i 35.3/2, and i 35.7/2 was significantly different from that of the positive control ($p < 0.05$), these isolates still demonstrated the ability to adhered to intestinal mucus with around

70% adhesion efficiency. Mucus adhesion properties of lactobacilli are varied among different strains. However, adhesive characteristics in many strains of lactobacilli are considered to be associated with mucus-binding proteins on the bacterial cell wall. Several mucus-binding proteins have been identified in numerous lactobacilli species, MUB and MapA proteins have been demonstrated to promote mucus adhesion in *L. reuteri* (Roos and Jonsson, 2002; Boekhorst et al., 2006; Miyoshi et al., 2006). *L. rhamnosus* GG which was used as positive control displayed higher adhesion efficiency than the tested candidate probiotic isolates because they possess a unique feature for mucus adhesion among lactobacilli. A study by Kankainen et al. (2009) demonstrated that intestinal mucus adherence ability of *L. rhamnosus* GG was mediated by pili on the surface of bacterial cells. SpaC, a pilin subunit found throughout the pili structure was demonstrated to be a crucial part of mucus binding in *L. rhamnosus* GG (Kankainen et al., 2009). Genes encoding pili and SpaC are uncommon in most lactobacilli (Van Tassell and Miller, 2011).

6. Determination of antimicrobial resistance genes and identification of their location on the genome by whole genome sequencing

Whole genome sequencing was performed on 4 candidate probiotic isolates. One isolate showed resistance to only vancomycin and 3 other isolates were resistant to vancomycin and ampicillin. The data from Oxford nanopore sequencing was used for identification of antimicrobial resistance genes and their location on the genome. No vancomycin or ampicillin resistance genes were identified in these candidates. This outcome confirmed that the selected isolates were intrinsically resistant to vancomycin and ampicillin resistance in these isolates present a minimal risk for horizontal dissemination of antimicrobial resistance. The results also showed that *tetW*, which confers resistance to tetracycline, was located on the chromosome of 2 candidate probiotic isolates (i 33.6/2 and i 35.3/2). Since *tetW* was located on

the chromosome, the risk for horizontal gene transfer may be minimal but still possible due to the fact that *tetW* is frequently associated with conjugative transposons (Barbosa et al., 1999; Roberts, 2005; Egervärn, 2009; Botelho and Schulenburg, 2021). The identification of *InuA* (conferring lincosamide resistance) and *ermB* (conferring macrolide resistance) on the plasmid of 3 and 1 candidate probiotic isolates, respectively, was a concern. This is because plasmids that carry antimicrobial resistance genes present a great risk for horizontal dissemination of antimicrobial resistance.

Notably, phenotypic resistance based on MIC determination was not in concurrent with the results of genotypic resistance from whole genome sequencing data. *tetW* was located on the chromosome of isolates i 33.6/2 and i 35.3/2 and *ermB* was identified in plasmids of isolate i 33.6/2, even though none of these isolates demonstrated phenotypic resistance to tetracycline or erythromycin. Similar occurrences have also been reported in some other studies. A study by Egervarn et al. (2009) found one *L. reuteri* isolate that was positive for *tetW* but the isolate was not resistant to tetracycline (Egervärn et al., 2009). Another study detected *ermB* by PCR method in some lactobacilli isolates that were not resistant to erythromycin (Guo et al., 2017). However, the reasons behind these occurrences remained unexplained. Nevertheless, the ability of lactobacilli in transferring *tetW* and *ermB* resistance genes to foodborne pathogens has been reported. A study by Thumu and Halami (2019) demonstrated that some strains of *L. reuteri* and *L. salivarius* were able to transfer *ermB* and multiple *tet* genes (including *tetW*) to foodborne pathogenic bacteria such as *Enterococcus faecalis*, *Listeria monocytogenes*, and *Yersinia enterocolitica* via conjugation (Thumu and Halami, 2019). Both resistance genes have also been found in other pathogens. *tetW* has been identified in *Clostridium*, *Staphylococcus*, *Streptococcus*, etc., and *ermB* has been detected in

Campylobacter, *Escherichia*, *Pseudomonas*, etc. (van Hoek et al., 2011; Zhang et al., 2016; Kecerova et al., 2019). Since *ermB* has been identified in plasmids (Li et al., 2016; Thumu and Halami, 2019) and *tetW* is frequently associated with conjugative transposons located in plasmid and chromosome (Barbosa et al., 1999; Roberts, 2005; Egervärn, 2009; Botelho and Schulenburg, 2021), the isolates i 33.6/2 and i 35.3/2 in the present study that harbored *tetW* and *ermB* resistance genes should not be used as probiotics.

Although no bacterial isolates selected for whole genome sequencing in this study were resistant to clindamycin which is an antibiotic in a class of lincosamides, *lnuA* was detected in 3 isolates. Other studies showed similar results with our finding because *lnuA* appears to confer lincomycin resistance but not clindamycin resistance. Rosander et al. (2008) found that *L. reuteri* ATCC 55730 harbored a plasmid that carried *lnuA*. However, the *L. reuteri* strain was resistant to lincomycin (MIC > 16 µg/ml) but not to clindamycin (MIC < 0.125 µg/ml) (Rosander et al., 2008). This observation has also been reported in *Staphylococcus* and *Streptococcus* (Achard et al., 2005; Lüthje et al., 2007). The reason for this phenomenon is not well understood. Achard et al. (2005) hypothesized that clindamycin might have better affinity for the ribosomes than lincomycin (Achard et al., 2005).

According to a guideline by EFSA, bacterial strains with acquired resistance due to acquisition of antimicrobial resistance genes should not be used as probiotics in animal feed. Only acquired resistance of probiotic bacteria due to chromosomal mutation is generally acceptable (EFSA, 2012). Since all four candidate probiotic isolates harbored plasmids that carried either *lnuA* or *ermB* resistance genes, and *tetW* was also located on the chromosome of two isolates, these candidates are not appropriate to be used as probiotics at the moment. However, if the isolates i 24.1/2 and i 24.2/2 which demonstrated favorable probiotic properties (*in vitro*

Campylobacter inhibiting activity, no hemolytic activity, tolerance to acid and bile, and adhesion ability to chicken intestinal mucus) but harbored only *lnuA* resistance gene-carrying plasmids can be cured of the plasmids that carried *lnuA*, they will be suitable to be used for further development of a probiotic product to control or reduce *Campylobacter* colonization in chickens.

Removal of plasmids carrying antimicrobial resistance genes from potential probiotic bacteria had been accomplished before. For instance, *L. reuteri* ATCC 55730 which was a commercially available probiotic strain was found to harbor 2 plasmids that carried antimicrobial resistance genes. One plasmid carried *lnuA* and the other carried *tetW* resistance gene. Rosander et al. (2008) successfully removed the 2 plasmids carrying resistance genes by a method of protoplast formation and regeneration. After removal of the 2 plasmids, the resulting daughter strain (*L. reuteri* DSM 17938) lost the lincomycin and tetracycline resistance but still preserved all of its probiotic properties (Rosander et al., 2008).

Since *L. reuteri* isolates i 24.1/2 and i 24.2/2 harbored only *lnuA* resistance gene-carrying plasmids, which are possible to be removed by plasmid curing technique such as protoplast formation and regeneration, they were selected as the final candidate probiotics. The isolates i 33.6/2 and i 35.3/2 on the other hand were not selected as final candidates because even if resistance gene-carrying plasmids can be removed from these isolates by plasmid curing technique in the future, they still possess *tetW* on the chromosome. Since *tetW* is often associated with conjugative transposons that have been located in both plasmid and chromosome (Barbosa et al., 1999; Roberts, 2005; Egervärn, 2009; Botelho and Schulenburg, 2021), *tetW* that was located on the chromosome of isolate i 33.6/2 and i 35.3/2 may present a potential for horizontal transfer of resistance gene. Moreover, detection of

antimicrobial resistance genes in the chromosome of bacterial strains intended to be used as probiotics is not acceptable according to EFSA as mentioned earlier.

After the removal of plasmids carrying antimicrobial resistance genes in the isolates i 24.1/2 and i 24.2/2, these isolates should be further evaluated for their efficacy as candidate probiotics by *in vivo* experiments in chickens. If these candidates show favorable results from *in vivo* experiments, they will be a promising candidate to be used for further development of a probiotic product to control or reduce *Campylobacter* colonization in broiler chickens.

CHAPTER VI
CONCLUSION AND SUGGESTIONS

The present study assessed the probiotic potential of 602 bacteria isolated from *Campylobacter*-negative broiler chickens. Assessment of probiotic properties including *Campylobacter* inhibiting activity test, hemolytic activity test, acid and bile tolerance test, antimicrobial susceptibility test, chicken intestinal mucus adhesion test, and determination of antimicrobial resistance genes and their location on the genome by whole genome sequencing was performed on the candidate probiotic bacteria. We identified the final 2 candidate probiotic isolates that can inhibit the growth of *Campylobacter in vitro*, showed no hemolytic activity, exhibited good acid and bile tolerance, and demonstrated the ability to adhere to chicken intestinal mucus. Both isolates are *L. reuteri*. Despite their favorable probiotic characteristics, the two candidate probiotics still harbored plasmids that carried *lnuA* resistance gene. Plasmid carrying resistance genes presents a potential for horizontal spread of antimicrobial resistance. Thus, these candidate probiotics are not appropriate to be used as probiotics at the moment. However, if these candidate probiotics can be cured of plasmids carrying antimicrobial resistance genes, they will be suitable to be used for further development of a probiotic product to control or reduce *Campylobacter* colonization in chickens. Therefore, further studies including removal of plasmids carrying antimicrobial resistance genes from candidate probiotic bacteria by plasmid curing technique such as protoplast formation and regeneration should be carried out. Moreover, prior to application in broiler chickens, *in vivo* experiments in chickens must be conducted to evaluate the efficacy of candidate probiotics in various aspects such as the ability to control or reduce *Campylobacter* colonization, stimulation of the immune system, modulation of gut microbiota, effects on intestinal histomorphology, and enhancement of growth performance. If the

candidate probiotics showed promising *in vivo* results, they will be excellent candidates to be used as probiotics in poultry for control or reduction of *Campylobacter* colonization in chickens at the farm level, which consequently decreases the contamination of *Campylobacter* in chicken meat, thus lowering the risk of human campylobacteriosis. In case that the candidate probiotics demonstrate the ability to improve growth performance and overall health of chickens, they may be used as growth promoter in poultry farms instead of antimicrobial agents, which will help reduce the occurrence of antimicrobial resistance in poultry production due to the fact that the use of antimicrobials as growth promoter has contributed to the occurrence and spread of antimicrobial resistance.

In addition to the candidate probiotic bacteria identified in this study, antimicrobial substances produced by these bacteria should also be further investigated. The nature and characteristics of the produced substances need to be elucidated. In case that bacteriocins or other antimicrobial compounds were identified, they can be used for further development of a product for control or reduction of enteric pathogens in livestock animals.

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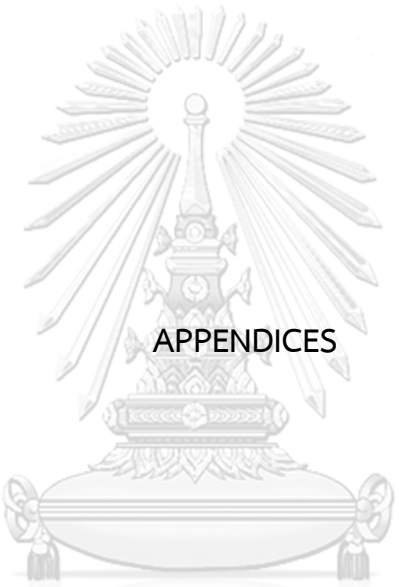
APPENDICES



APPENDIX A

Culture Media

1. modified Charcoal-Cefoperazone-Deoxycholate Agar (mCCDA) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom)

Typical formula	**(g/litre)**
- Nutrient Broth No.2	25.0
- Bacteriological charcoal	4.0
- Casein hydrolysate	3.0
- Sodium desoxycholate	1.0
- Ferrous sulphate	0.25
- Sodium pyruvate	0.25
- Agar	12.0
- Cefoperazone	32 mg
- Amphotericin B	10 mg

2. Muller Hinton Agar (MHA) (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Acid digest hydrolysate of casien	25.0
- Beef extract powder	4.0
- Strach	3.0
- Agar	1.0

3. Lactobacilli MRS Agar (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Proteose Peptone No. 3	10.0
- Beef extract	10.0
- Yeast extract	5.0
- Dextrose	20.0
- Polysorbate	80.0
- Ammonium Citrate	2.0
- Sodium Acetate	5.0
- Magnesium Sulfate	0.1
- Manganese Sulfate	0.05
- Dipotassium Phosphate	2.0
- Agar	15.0

4. Mannitol Egg Yolk Polymyxin (MYP) Agar (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Beef extract	1.0
- Peptone	10.0
- L, D-Mannitol	10.0
- Sodium Chloride	10.0
- Phenol red	25.0
- Agar	15.0
- Egg yolk emulsion	100.0
- Polymyxin B	10^6 IU

5. m Enterococcus Agar (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Tryptose	20.0
- Yeast extract	5.0
- Dextrose	2.0
- Dipotassium Phosphate	4.0
- Sodium Azide	0.4
- 2,3,5-Triphenyl Tetrazolium Chloride	0.1
- Agar	10.0

6. Lactobacilli MRS Broth (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Proteose Peptone No. 3	10.0
- Beef extract	10.0
- Yeast extract	5.0
- Dextrose	20.0
- Polysorbate	80.0
- Ammonium Citrate	2.0
- Sodium Acetate	5.0
- Magnesium Sulfate	0.1
- Manganese Sulfate	0.05
- Dipotassium Phosphate	2.0

7. Brain Heart Infusion Broth (Difco™, MD, USA)

Typical formula	**(g/litre)**
- Calf brain (infusion from 200g)	7.7
- Beef heart (infusion from 250g)	9.8
- Proteose Peptone	10.0
- Dextrose	2.0
- Sodium Chloride	5.0
- Disodium Phosphate	2.5

8. LAB Susceptibility test Medium (LSM) Broth

The LSM broth consists of 90 % IST broth and 10 % MRS broth, the formula for MRS broth is the same as Lactobacilli MRS Broth (Difco™, MD, USA) mentioned above.

8.1 Iso-Sensitest (IST) Broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom)

Typical formula	**(g/litre)**
- Hydrolysed casein grams per litre	11.0
- Peptones	3.0
- Glucose	2.0
- Sodium chloride	3.0
- Soluble starch	1.0
- Disodium hydrogen phosphate	2.0
- Sodium acetate	1.0
- Magnesium glycerophosphate	0.2
- Calcium gluconate	0.1
- Cobaltous sulphate	0.001
- Cupric sulphate	0.001
- Zinc sulphate	0.001

8.1 Iso-Sensitest (IST) Broth (Continued)

Typical formula	**(g/litre)**
- Ferrous sulphate	0.001
- Manganous chloride	0.002
- Menadione	0.001
- Cyanocobalamin	0.001
- L-Cysteine hydrochloride	0.02
- L-Tryptophan	0.02
- Pyridoxine	0.003
- Pantothenate	0.003
- Nicotinamide	0.003
- Biotin	0.0003
- Thiamine	0.00004
- Adenine	0.01
- Guanine	0.01
- Xanthine	0.01
- Uracil	0.01

APPENDIX B

Table B. Solvents, diluents, and concentration ranges for antimicrobial agents

Antimicrobials	Solvents	Diluents	Concentration range (μg/mL)
Ampicillin	Distilled water	Distilled water	0.5-256
Clindamycin	Distilled water	Distilled water	0.125-64
Chloramphenicol	95% ethanol	Distilled water	0.5-256
Erythromycin	95% ethanol	Distilled water	0.25-128
Gentamicin	Distilled water	Distilled water	1-512
Kanamycin	Distilled water	Distilled water	1-512
Streptomycin	Distilled water	Distilled water	1-512
Tetracycline	70% ethanol	Distilled water	1-512
Vancomycin	Distilled water	Distilled water	1-512
Linezolid	95% ethanol	Distilled water	0.125-64

APPENDIX C

Table C. *Campylobacter* status of broiler chickens from 30 broiler farms

Farm No.	No. of *Campylobacter*-positive samples/ Total samples (%)	No. of specific *Campylobacter* sp. -positive samples/Total *Campylobacter*-positive samples (%)	
		Campylobacter jejuni	*Campylobacter coli*
1	10/10 (100)	10/10 (100)	0/10 (0)
2	10/10 (100)	10/10 (100)	0/10 (0)
3	10/10 (100)	10/10 (100)	0/10 (0)
4	0/10 (0)	n/a	n/a
5	0/10 (0)	n/a	n/a
6	10/10 (100)	10/10 (100)	0/10 (0)
7	10/10 (100)	10/10 (100)	0/10 (0)
8	0/10 (0)	n/a	n/a
9	10/10 (100)	0/10 (0)	10/10 (100)
10	10/10 (100)	10/10 (100)	0/10 (0)
11	0/10 (0)	n/a	n/a
12	10/10 (100)	10/10 (100)	0/10 (0)
13	0/10 (0)	n/a	n/a
14	7/10 (70)	7/7 (100)	0/7 (0)
15	10/10 (100)	10/10 (100)	0/10 (0)
16	10/10 (100)	10/10 (100)	0/10 (0)
17	10/10 (100)	10/10 (100)	0/10 (0)
18	0/10 (0)	n/a	n/a
19	10/10 (100)	10/10 (100)	0/10 (0)
20	0/10 (0)	n/a	n/a

Table C. *Campylobacter* status of broiler chickens from 30 broiler farms (continued)

Farm No.	No. of *Campylobacter*-positive samples/ Total samples (%)	No. of specific *Campylobacter* sp. -positive samples/Total *Campylobacter*-positive samples (%)	
		Campylobacter jejuni	*Campylobacter coli*
21	9/10 (90)	9/9 (100)	0/9 (0)
22	0/10 (0)	n/a	n/a
23	10/10 (100)	10/10 (100)	0/10 (0)
24	10/10 (100)	10/10 (100)	0/10 (0)
25	8/10 (80)	8/8 (100)	0/8 (0)
26	10/10 (100)	10/10 (100)	0/10 (0)
27	0/10 (0)	n/a	n/a
28	10/10 (100)	10/10 (100)	0/10 (0)
29	0/10 (0)	n/a	n/a
30	10/10 (100)	0/10 (0)	10/10 (100)

APPENDIX D

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
Lactobacilli					
1	i 34.9/2	25	Alpha hemolysis	0	101.3
2	L 8.4	21	Alpha hemolysis	71.96	0
3	i 34.6/2	21	No hemolysis	0	100.45
4	i 24.9/2	21	Alpha hemolysis	67.05	97.81
5	i 23.9/2	20	Alpha hemolysis	80.29	93.2
6	i 8.2	19	Alpha hemolysis	0	97.37
7	i 24.8/2	19	Alpha hemolysis	92.31	101.83
8	i 4.5	18	Alpha hemolysis	92.34	90.35
9	i 8.8	18	No hemolysis	90.91	99.32
10	i 24.7/2	18	Alpha hemolysis	99.04	99.94
11	i 23.4/2	18	No hemolysis	58.95	97.37
12	L 18.2	17	Alpha hemolysis	94.5	104.78
13	i 5.3	17	Alpha hemolysis	81	88.15
14	i 4.6	17	No hemolysis	0	93.57
15	i 23.5/2	17	Alpha hemolysis	77.05	97.67
16	i 24.1/2	16	No hemolysis	91.12	99.47
17	i 35.2/2	16	Alpha hemolysis	87.05	97.88
18	i 23.6/2	16	Alpha hemolysis	74.68	89.77
19	i 23.3/2	16	Alpha hemolysis	73.37	87.23
20	i 23.8/2	16	Alpha hemolysis	0	110.35
21	i 35.3/2	16	No hemolysis	99.67	103.36
22	i 4.7	15	No hemolysis	99.74	93.21
23	i 4.10	15	No hemolysis	99.48	98.98

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
24	i 29.7	15	Alpha hemolysis	98.87	104.48
25	i 4.9	15	No hemolysis	97.46	97.26
26	i 29.6	15	Alpha hemolysis	95.26	97.33
27	L 20.8	15	Alpha hemolysis	94.78	107.06
28	i 8.3	15	Alpha hemolysis	93.27	98.86
29	i 13.7	15	No hemolysis	92.01	91.21
30	i 29.2	15	Alpha hemolysis	90.43	96.24
31	L 11.9	15	Alpha hemolysis	90.08	103.44
32	i 18.9	15	Alpha hemolysis	82.7	92.87
33	i 4.8	15	No hemolysis	84.1	107.55
34	L 27.10	15	Alpha hemolysis	81.13	65.85
35	i 18.2	15	Alpha hemolysis	82.48	103.91
36	L 8.8	15	Alpha hemolysis	78.74	88.88
37	L 22.8	15	Alpha hemolysis	77.86	89.56
38	L 11.1	15	Alpha hemolysis	74.37	79.29
39	i 20.10	15	Alpha hemolysis	72.73	0
40	L 4.1	15	Alpha hemolysis	61.53	82.83
41	L 8.6	15	Alpha hemolysis	50.54	89.08
42	i 18.1	15	No hemolysis	0	0
43	L 18.5	15	Alpha hemolysis	0	104.56
44	L 5.9	15	Alpha hemolysis	0	71.21
45	i 5.8	15	Alpha hemolysis	0	0
46	i 33.2/2	15	No hemolysis	0	100.35
47	i 24.2/2	15	No hemolysis	99.58	102.95
48	i 24.4/2	15	No hemolysis	97.84	99.96
49	i 24.6/2	15	No hemolysis	96.54	99.37

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
50	i 35.6/2	15	Alpha hemolysis	96.3	96.77
51	i 24.11/2	15	Alpha hemolysis	95.21	107.45
52	i 23.7/2	15	Alpha hemolysis	82	87.35
53	i 35.4/2	15	Alpha hemolysis	79.34	87.44
54	i 36.10/2	15	Alpha hemolysis	76.58	92.8
55	i 23.2/2	15	Alpha hemolysis	67.4	94.3
56	i 22.10	14	No hemolysis	98.07	100.53
57	i 29.4	14	No hemolysis	94.95	102.85
58	i 5.2	14	No hemolysis	92.34	93.07
59	i 18.4	14	Alpha hemolysis	90.69	105.01
60	i 18.5	14	Alpha hemolysis	57.92	99.04
61	i 18.7	14	Alpha hemolysis	52.81	99.29
62	L 27.11	14	Alpha hemolysis	0	89.17
63	L 27.7	14	Alpha hemolysis	0	85
64	i 33.4/2	14	No hemolysis	0	97.69
65	i 22.6/2	14	Alpha hemolysis	0	102.14
66	i 24.5/2	14	No hemolysis	99.11	103.74
67	i 35.1/2	14	Alpha hemolysis	86.09	86.95
68	i 36.1/2	14	Alpha hemolysis	81.36	91.92
69	i 36.9/2	14	Alpha hemolysis	74.2	92.57
70	i 23.1/2	14	No hemolysis	55.52	94.44
71	i 36.7/2	14	Alpha hemolysis	0	86.71
72	i 4.3	13	No hemolysis	98.23	96.12
73	i 20.3	13	No hemolysis	82.24	87.82
74	L 20.9	13	Alpha hemolysis	56.46	60.08
75	i 5.1	13	No hemolysis	0	91.3

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
76	i 5.7	13	No hemolysis	0	87.83
77	i 13.5	13	No hemolysis	0	98.53
78	i 13.8	13	No hemolysis	0	96.41
79	L 22.7	13	No hemolysis	0	82.22
80	L 4.2	13	Alpha hemolysis	0	66.83
81	L 4.4	13	Alpha hemolysis	0	70.75
82	L 4.5	13	Alpha hemolysis	0	75.85
83	L 4.6	13	Alpha hemolysis	0	74.2
84	L 8.5	13	Alpha hemolysis	0	85
85	L 11.5	13	Alpha hemolysis	0	0
86	L 11.7	13	Alpha hemolysis	0	81.76
87	L 11.8	13	Alpha hemolysis	0	79.45
88	L 13.1	13	Alpha hemolysis	0	83.98
89	L 18.3	13	Alpha hemolysis	0	71.26
90	L 18.4	13	Alpha hemolysis	0	85.62
91	L 18.6	13	Alpha hemolysis	0	91.82
92	L 18.7	13	Alpha hemolysis	0	92.18
93	L 22.6	13	Alpha hemolysis	0	94.82
94	i 8.1	13	Alpha hemolysis	0	96.06
95	i 11.7	13	Alpha hemolysis	0	89.84
96	i 18.3	13	Alpha hemolysis	0	95.85
97	i 20.4	13	Alpha hemolysis	0	89.39
98	i 22.1	13	Alpha hemolysis	0	98.38
99	i 22.5	13	Alpha hemolysis	0	98.78
100	i 27.1	13	Alpha hemolysis	0	100.16
101	i 27.4	13	Alpha hemolysis	0	101.27

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
102	L 27.1	13	Alpha hemolysis	0	99.32
103	L 27.6	13	Alpha hemolysis	0	100.21
104	L 27.8	13	Alpha hemolysis	0	100.85
105	L 29.1	13	Alpha hemolysis	0	97.29
106	L 29.2	13	Alpha hemolysis	0	95.1
107	L 29.4	13	Alpha hemolysis	0	94.82
108	L 29.5	13	Alpha hemolysis	0	101.59
109	L 29.6	13	Alpha hemolysis	0	97.23
110	L 29.7	13	Alpha hemolysis	0	93.81
111	i 33.5/2	13	No hemolysis	0	68.26
112	i 33.6/2	13	No hemolysis	78.01	102.09
113	i 35.7/2	13	No hemolysis	97.66	100.68
114	i 24.3/2	13	No hemolysis	91.56	93.24
115	i 36.5/2	13	Alpha hemolysis	82.41	95.39
116	i 36.8/2	13	Alpha hemolysis	79.17	94.41
117	i 36.6/2	13	Alpha hemolysis	0	98.38
118	i 8.7	12	No hemolysis	93.64	104.04
119	i 13.10	12	No hemolysis	87	101.32
120	i 20.1	12	No hemolysis	84.14	86.25
121	i 29.10	12	No hemolysis	84.23	97.56
122	i 11.4	12	No hemolysis	74.44	99.54
123	i 13.6	12	No hemolysis	61.63	103.68
124	L 20.7	12	Alpha hemolysis	46.21	82.91
125	i 29.5	12	Alpha hemolysis	37.58	81.05
126	i 18.6	12	Alpha hemolysis	0	96.79
127	L 8.9	12	Alpha hemolysis	0	94.87

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
128	L 20.6	12	Alpha hemolysis	0	0
129	L 20.10	12	Alpha hemolysis	0	73.57
130	L 29.9	12	Alpha hemolysis	0	0
131	i 35.5/2	12	No hemolysis	36.32	99.01
132	i 22.9/2	12	No hemolysis	0	101.64
133	i 33.3/2	12	No hemolysis	0	100.63
134	i 36.2/2	12	Alpha hemolysis	0	67.21
135	i 36.4/2	12	Alpha hemolysis	0	86.46
136	i 29.1	11	No hemolysis	98.92	88.83
137	i 27.8	11	No hemolysis	98.41	97.39
138	i 22.8	11	No hemolysis	98.04	103.15
139	i 22.4	11	No hemolysis	97.92	95.65
140	i 5.6	11	No hemolysis	97.29	96.59
141	i 27.5	11	No hemolysis	96.76	97.45
142	i 4.1	11	No hemolysis	96.93	100.05
143	i 8.5	11	No hemolysis	95.82	108.19
144	i 22.3	11	No hemolysis	94.57	98.68
145	i 8.4	11	No hemolysis	93.85	91.72
146	i 5.4	11	No hemolysis	93.87	101.48
147	i 27.2	11	No hemolysis	93	0
148	i 20.9	11	No hemolysis	92.33	101.2
149	i 29.9	11	No hemolysis	92.22	81.3
150	i 13.4	11	No hemolysis	92.21	98.59
151	i 27.9	11	No hemolysis	91.99	95.43
152	i 13.1	11	No hemolysis	91.06	101.32
153	i 5.10	11	No hemolysis	89.92	95.27

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
154	i 13.9	11	No hemolysis	86.69	103.37
155	i 22.6	11	No hemolysis	86.25	103.37
156	i 11.3	11	No hemolysis	85.6	100
157	i 11.5	11	No hemolysis	84.5	99.48
158	i 29.8	11	Alpha hemolysis	83.04	80.82
159	i 5.9	11	No hemolysis	62.72	88.98
160	i 22.7	11	No hemolysis	45.86	99.18
161	i 18.8	11	No hemolysis	0	108.33
162	L 8.7	11	No hemolysis	0	100.44
163	L 22.3	11	No hemolysis	0	95.54
164	L 22.4	11	No hemolysis	0	92.12
165	L 22.5	11	No hemolysis	0	99.27
166	L 4.7	11	Alpha hemolysis	0	85.61
167	L 5.2	11	Alpha hemolysis	0	59.28
168	L 5.6	11	Alpha hemolysis	0	0
169	L 5.7	11	Alpha hemolysis	0	0
170	L 8.1	11	Alpha hemolysis	0	89.33
171	L 11.3	11	Alpha hemolysis	0	82.45
172	L 20.1	11	Alpha hemolysis	0	89.73
173	L 20.2	11	Alpha hemolysis	0	79.25
174	L 20.3	11	Alpha hemolysis	0	88.98
175	L 20.4	11	Alpha hemolysis	0	88.73
176	L 20.5	11	Alpha hemolysis	0	95.13
177	L 27.2	11	Alpha hemolysis	0	67.39
178	L 27.3	11	Alpha hemolysis	0	91.64
179	L 27.4	11	Alpha hemolysis	0	87.61

Table D. *Campylobacter* inhibition zone diameter, hemolytic activity, and survival rates in acid and bile tolerance test of 194 candidate probiotic isolates (continued)

No.	Isolate code	Diameter of *Campylobacter* inhibition zone (mm)	Hemolytic activity	Survival rates (%)	
				Acid tolerance test	Bile tolerance test
180	L 27.5	11	Alpha hemolysis	0	87.82
181	L 27.9	11	Alpha hemolysis	0	91.93
182	L 29.3	11	Alpha hemolysis	0	88.35
183	L 29.8	11	Alpha hemolysis	0	61.49
184	L 29.10	11	Alpha hemolysis	0	0
185	i 24.10/2	11	No hemolysis	93.97	97.64
186	i 22.10/2	11	No hemolysis	39.96	110.14
187	i 21.1/2	11	No hemolysis	0	104.95
188	i 22.1/2	11	No hemolysis	0	101.05
189	i 36.3/2	11	Alpha hemolysis	0	82
190	i 22.4/2	11	Alpha hemolysis	0	107.99
***Enterococcus* spp.**					
191	E 5.3	18	No hemolysis	0	105.28
192	E 11.1	11	No hemolysis	0	99.97
193	E 27.6	11	No hemolysis	0	107.69
194	E 27.9	11	No hemolysis	0	107.65

APPENDIX E

Table E. MIC values of 10 antimicrobial agents tested in 56 candidate probiotic isolates

No.	Isolate code	Genus and species	Minimum inhibitory concentration (µg/ml)*									
			AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CMP	LIN
1	i 4.1	*Limosilactobacillus reuteri*	8	512	2	128	64	>128	>64	512	4	2
2	i 4.3	*Limosilactobacillus reuteri*	16	512	<1	128	128	>128	>64	256	4	4
3	i 4.6	*Limosilactobacillus reuteri*	64	256	<1	16	256	>128	>64	256	64	2
4	i 4.7	*Limosilactobacillus reuteri*	>256	512	<1	128	512	>128	>64	128	64	1
5	i 4.8	*Limosilactobacillus reuteri*	16	512	4	128	64	>128	>64	512	4	4
6	i 4.9	*Limosilactobacillus reuteri*	8	512	2	128	128	>128	>64	512	4	4
7	i 4.10	*Limosilactobacillus reuteri*	256	512	2	128	>512	>128	>64	256	64	1
8	i 5.2	*Limosilactobacillus reuteri*	2	512	4	128	64	>128	>64	256	4	2
9	i 5.4	*Limosilactobacillus reuteri*	256	512	2	128	64	>128	>64	256	16	2
10	i 5.6	*Limosilactobacillus reuteri*	64	512	2	64	64	>128	>64	256	32	2
11	i 5.10	*Limosilactobacillus reuteri*	32	512	4	128	64	>128	>64	256	4	2
12	i 8.4	*Limosilactobacillus reuteri*	32	512	4	128	128	128	64	256	4	2
13	i 8.5	*Limosilactobacillus reuteri*	32	512	8	128	64	>128	>64	256	4	2
14	i 8.7	*Limosilactobacillus reuteri*	16	512	4	128	64	>128	>64	512	4	4
15	i 8.8	*Limosilactobacillus reuteri*	32	512	<1	128	128	>128	>64	256	4	2
16	i 11.3	*Limosilactobacillus reuteri*	16	512	4	128	64	>128	>64	256	4	2
17	i 13.1	*Limosilactobacillus reuteri*	32	512	4	64	> 512	>128	16	512	8	4
18	i 13.4	*Limosilactobacillus reuteri*	64	> 512	32	512	256	>128	>64	512	8	8

Table E. MIC values of 10 antimicrobial agents tested in 56 candidate probiotic isolates (continued)

No.	Isolate code	Genus and species	Minimum inhibitory concentration (µg/ml)*									
			AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CMP	LIN
19	i 13.5	*Limosilactobacillus reuteri*	64	> 512	64	512	512	>128	>64	512	4	2
20	i 13.7	*Limosilactobacillus reuteri*	64	> 512	64	512	> 512	>128	>64	512	4	4
21	i 13.8	*Limosilactobacillus reuteri*	8	256	2	64	64	0.5	0.5	512	4	4
22	i 13.9	*Limosilactobacillus reuteri*	64	512	32	512	256	>128	>64	512	8	2
23	i 13.10	*Limosilactobacillus reuteri*	32	256	2	64	32	>128	>64	259	2	1
24	i 20.9	*Limosilactobacillus reuteri*	32	512	2	128	32	0.5	0.5	128	8	4
25	i 22.3	*Limosilactobacillus reuteri*	1	256	2	64	> 512	>128	>64	128	4	4
26	i 22.4	*Limosilactobacillus reuteri*	4	512	2	64	> 512	>128	>64	128	4	2
27	i 22.6	*Limosilactobacillus reuteri*	1	256	2	64	> 512	>128	>64	128	4	2
28	i 22.8	*Limosilactobacillus reuteri*	8	256	<1	64	32	>128	>64	256	4	2
29	i 22.10	*Limosilactobacillus reuteri*	2	256	4	128	64	>128	>64	128	4	2
30	i 27.2	*Limosilactobacillus reuteri*	32	256	2	128	32	<0.25	0.25	512	4	2
31	i 27.5	*Limosilactobacillus reuteri*	16	256	<1	32	16	128	64	128	4	4
32	i 27.8	*Limosilactobacillus reuteri*	2	256	<1	16	16	>128	>64	256	8	4
33	i 27.9	*Limosilactobacillus reuteri*	2	256	<1	32	8	>128	>64	256	4	4
34	i 29.1	*Limosilactobacillus reuteri*	2	256	<1	32	8	>128	>64	256	4	2
35	i 29.4	*Ligilactobacillus salivarius*	16	512	<1	32	512	>128	>64	512	4	2
36	i 29.9	*Limosilactobacillus reuteri*	8	256	<1	32	16	>128	>64	256	4	2
37	L 22.7	*Ligilactobacillus salivarius*	256	512	16	512	128	64	>64	128	8	2
38	i 21.1/2	*Limosilactobacillus reuteri*	16	512	<1	16	256	>128	>64	256	4	2

Table E. MIC values of 10 antimicrobial agents tested in 56 candidate probiotic isolates (continued)

No.	Isolate code	Genus and species	Minimum inhibitory concentration (µg/ml)*									
			AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CMP	LIN
39	i 22.10/2	*Limosilactobacillus reuteri*	2	256	<1	32	16	>128	>64	128	256	4
40	i 23.4/2	*Ligilactobacillus salivarius*	128	512	8	128	64	>128	>64	128	8	2
41	i 23.8/2	*Ligilactobacillus salivarius*	128	512	32	256	128	>128	>64	256	8	2
42	i 24.1/2	*Limosilactobacillus reuteri*	64	512	<1	16	16	0.5	0.5	16	4	2
43	i 24.2/2	*Limosilactobacillus reuteri*	64	512	<1	32	16	0.5	0.5	16	4	4
44	i 24.3/2	*Limosilactobacillus reuteri*	16	512	<1	16	16	>128	>64	256	2	4
45	i 24.4/2	*Limosilactobacillus reuteri*	8	512	2	32	16	>128	>64	256	128	4
46	i 24.5/2	*Limosilactobacillus reuteri*	64	512	<1	16	16	0.5	1	16	4	4
47	i 24.6/2	*Limosilactobacillus reuteri*	32	512	<1	16	16	>128	>64	256	4	2
48	i 24.10/2	*Limosilactobacillus reuteri*	32	512	<1	16	16	>128	>64	256	2	2
49	i 33.2/2	*Limosilactobacillus alvi*	8	512	8	128	64	0.5	<0.25	4	4	4
50	i 33.3/2	*Limosilactobacillus reuteri*	2	512	<1	16	16	>128	>64	256	4	4
51	i 33.4/2	*Limosilactobacillus reuteri*	4	512	2	32	32	>128	>64	2	2	2
52	i 33.5/2	*Limosilactobacillus reuteri*	4	512	16	256	64	<0.25	<0.125	4	4	2
53	i 33.6/2	*Limosilactobacillus reuteri*	2	512	<1	32	32	0.5	0.5	4	4	4
54	i 34.6/2	*Limosilactobacillus reuteri*	32	512	4	32	32	>128	>64	2	2	2
55	i 35.3/2	*Limosilactobacillus reuteri*	16	256	<1	32	16	<0.25	0.25	2	2	2
56	i 35.7/2	*Limosilactobacillus reuteri*	32	512	<1	32	16	<0.25	0.5	2	2	4

*MICs of antimicrobials that were considered as resistance are highlighted in grey. AMP, Ampicillin; VAN, Vancomycin; GEN, Gentamicin; KAN, Kanamycin; STR, Streptomycin; ERY, Erythromycin; CLI, Clindamycin; TET, Tetracycline; CMP, Chloramphenicol; LIN, Linezolid

APPENDIX F

Table F. Adhesion efficiency of candidate probiotic bacteria and positive control (mucus adhesion assay was performed in triplicate)

Isolate code	Genus and species	1st adhesion assay			2nd adhesion assay			3rd adhesion assay			Adhesion efficiency mean (%)	Standard deviation
		Bacterial count before the test (log CFU/ml)	Adhered bacteria after the test (log CFU/ml)	Adhesion efficiency (%)	Bacterial count before the test (log CFU/ml)	Adhered bacteria after the test (log CFU/ml)	Adhesion efficiency (%)	Bacterial count before the test (log CFU/ml)	Adhered bacteria after the test (log CFU/ml)	Adhesion efficiency (%)		
i 33.6/2	*L. reuteri*	6.77	4.64	68.58	6.77	4.76	70.35	6.77	4.83	71.37	70.1	1.41
i 24.1/2	*L. reuteri*	7.07	5.73	81.06	7.07	5.58	78.9	7.07	5.68	80.34	80.1	1.1
i 24.2/2	*L. reuteri*	7.09	5.04	71.11	7.09	4.95	69.81	7.09	4.97	70.14	70.35	0.67
i 24.5/2	*L. reuteri*	7.11	4.95	69.64	7.11	4.77	67.13	7.11	4.64	65.33	67.37	2.16
i 35.3/2	*L. reuteri*	7	5.15	73.65	7	4.95	70.82	7	4.98	71.15	71.87	1.55
i 35.7/2	*L. reuteri*	6.85	4.43	64.68	6.85	4.46	65.13	6.85	4.41	64.44	64.75	0.35
Control	*L. rhamnosus* GG	7.46	6.43	86.18	7.46	6.08	81.51	7.46	5.99	80.29	82.66	3.11

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