EVALUATION OF THE EFFICACY OF LACTIC ACID BACTERIA ISOLATED FROM CHICKEN GASTROINTESTINAL TRACT FOR USE AS PROBIOTIC IN BROILER DIETS

MERISA SIRISOPAPONG

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การประเมินคุณสมบัติของแบคทีเรียกรดแลคติกที่ได้จากท่อทางเดินอาหารของไก่เพื่อใช้เป็นโพรไบโอติกในอาหารไก่เนื้อ

นางสาวเมริษา ศิริโสภาพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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เมริษา ศิริโสภาพงษ์: การประเมินคุณสมบัติของแบคทีเรียกรดแลคติกที่ได้จากท่อทางเดินอาหารของไก่เพื่อใช้เป็นโพรไบโอติกในอาหารไก่เนื้อ (EVALUATION OF THE EFFICACY OF LACTIC ACID BACTERIA ISOLATED FROM CHICKEN GASTROINTESTINAL TRACT FOR USE AS PROBIOTIC IN BROILER DIETS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร. สุทิศา เข็มผะกา, 106 หน้า.

คำสำคัญ: แบคทีเรียกรดแลคติก/โพรไบโอติก/สมรรถนะการผลิต/การตอบสนองทางภูมิคุ้มกัน/สัตว์ปีก

การศึกษานี้มีวัตถุประสงค์เพื่อคัดแยกแบคทีเรียกรดแลคติกจากท่อทางเดินอาหารของไก่ และประเมินคุณสมบัติในการเป็นโพรไบโอติก โดยทำการทดสอบแบคทีเรียโพรไบโอติกที่คัดแยกได้ในไก่เนื้อทั้งภายใต้สภาวะการเลี้ยงดูปกติ และสภาวะที่ถูกกระตุ้นให้เกิดการอักเสบด้วยไลโปโพลีแซคคาร์ไรด์ (Lipopolysaccharide, LPS)

การทดลองที่ I การคัดแยกเชื้อจุลินทรีย์กรดแลคติกจากท่อทางเดินอาหารของไก่ และประเมินคุณสมบัติความเป็นโพรไบโอติก โดยทำการคัดแยกเชื้อแบคทีเรียกรดแลคติกที่ได้จากท่อทางเดินอาหารไก่ จากนั้นนำไปตรวจวิเคราะห์ลำดับนิวคลีโอไทด์ในส่วนของ 16S rRNA ทดสอบความสามารถในการทนทานต่อกรดและน้ำดี ฤทธิ์ต้านเชื้อก่อโรค ความสามารถในการยึดเกาะลำไส้ และคุณลักษณะเพิ่มเติมในการกำจัดคอเลสเตอรอล พบว่าแบคทีเรียที่มีคุณสมบัติเป็นโพรไบโอติก มี 5 สปีชีส์ดังนี้ *Lactobacillus acidophilus, L. ingluviei, L. reuteri, L. salivarius* และ *L. saerimneri* จากนั้นทำการคัดเลือก *L. ingluviei* และ *L. salivarius* เพื่อทดสอบความคุณสมบัติการเป็นโพรไบโอติกเพิ่มเติมในไก่เนื้อเนื่องจากยังมีข้อมูลการศึกษาน้อย พบว่าจุลินทรีย์ทั้ง 2 ชนิด ช่วยปรับปรุงสุขภาพทางเดินอาหาร โดยเพิ่มจำนวนประชากร *Lactobacillus* และ *Bifidobacterium* อีกทั้งยังสามารถเพิ่มปริมาณการผลิตกรดวาเลอริกในซีกัม โดย *L. ingluviei* มีคุณสมบัติที่ดีกว่า *L. salivarious* ในการลดปริมาณเชื้อก่อโรค

การทดลองที่ II ศึกษาผลของ *L. ingluviei* C37 (LIC37) ในการตอบสนองต่อการอักเสบและการแสดงออกของยีนที่เกี่ยวข้องกับความแข็งแรงของผนังลำไส้ ในไก่ที่ถูกกระตุ้นด้วย LPS ซึ่งเป็นสารพิษที่ได้จาก *Escherichia coli* กลุ่มการทดลองมี 4 กลุ่มประกอบด้วย 1) กลุ่มควบคุม (ป้อนฟอสเฟตบัฟเฟอร์ซาไลน์, PBS) 2) ป้อนโพรไบโอติก LIC37 ปริมาณ 10^8 CFU /ตัว/วัน 3) ป้อนโพรไบโอติก LIC37 ปริมาณ 10^9 CFU /ตัว/วัน และ 4) กลุ่มควบคุมลบ (ป้อน PBS) เป็นระยะเวลา 14 วัน เมื่อไก่อายุ 14 วัน ทำการฉีด LPS (1 มิลลิกรัมต่อกิโลกรัม) เข้าที่ช่องท้องของไก่กลุ่มการทดลองที่ 2 3 และ 4 ส่วนกลุ่มควบคุมฉีดด้วย PBS ผลการศึกษาพบว่าโพรไบโอติก LIC37 สามารถลดการอักเสบในตับ และเพิ่มความแข็งแรงของผนังลำไส้ โดยเพิ่มการแสดงออกของยีนในกลุ่มโปรตีนไทด์จังก์ชั่น

(JAM2, occludin, ZO1) และยีนที่เกี่ยวข้องกับการหลั่งเยื่อเมือก (MUC2) เพิ่มการทำงานของไลโซไซม์ในซีรัมของไก่ที่อยู่ในสภาวะถูกกระตุ้นด้วย LPS

การทดลองที่ III ศึกษาผลของการเสริม LIC37 ในอาหารไก่เนื้อทั้งในรูปแบบเซลล์มีชีวิตและเซลล์ตาย ต่อสมรรถนะการเจริญเติบโต คุณภาพซาก คอเลสเตอรอลในเนื้อ ประชากรจุลินทรีย์ การสร้างภูมิคุ้มกัน ปริมาณการผลิตกรดไขมันสายสั้น และแอมโมเนียในซีกัม กลุ่มการทดลองมี 4 กลุ่ม ประกอบด้วย 1) อาหารพื้นฐาน (กลุ่มควบคุม) 2) อาหารพื้นฐานเสริม zinc bacitracin 50 มิลลิกรัมต่อกิโลกรัม (กลุ่มควบคุมบวก) 3) อาหารพื้นฐานเสริม LIC37 ในรูปแบบเซลล์มีชีวิตระดับ 10^8 CFU/กิโลกรัมอาหาร และ 4) อาหารพื้นฐานเสริม LIC37 ในรูปแบบเซลล์ตายระดับ 10^8 CFU/กิโลกรัมอาหาร ผลการศึกษาพบว่าจุลินทรีย์โพรไบโอติก LIC37 ทั้งสองรูปแบบ สามารถเพิ่มน้ำหนักตัว และปริมาณการกินได้เมื่อเทียบกับกลุ่มควบคุม ($P<0.05$) และเทียบเท่ากลุ่มควบคุมบวกที่เสริมยาปฏิชีวนะ ($P>0.05$) นอกจากนี้จุลินทรีย์โพรไบโอติก LIC37 ทั้งสองรูปแบบ สามารถเพิ่มประชากรจุลินทรีย์ *Lactobacillus* และ *Bifidobacterium* ลดจุลินทรีย์ก่อโรค *Enterobacteria* และ *E. coli* ลดปริมาณคอเลสเตอรอลในเนื้อ เพิ่มการผลิตกรดไขมันสายสั้น และลดการผลิตแอมโมเนียได้

โดยภาพรวมการศึกษาครั้งนี้สามารถคัดแยกแบคทีเรียกรดแลคติก ที่มีคุณสมบัติเป็นโพรไบโอติก 5 สปีชีส์ คือ *L. acidophilus, L. ingluviei, L. reuteri, L. salivarius* และ *L. saerimneri* โดย LIC37 เป็นโพรไบโอติกชนิดใหม่ที่มีศักยภาพสำหรับใช้เป็นสารเสริมในอาหารไก่เนื้อทั้งในรูปแบบเซลล์มีชีวิต และเซลล์ตาย

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์
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ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
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MERISA SIRISOPAPONG: EVALUATION OF THE EFFICACY OF LACTIC ACID BACTERIA ISOLATED FROM CHICKEN GASTROINTESTINAL TRACT FOR USE AS PROBIOTIC IN BROILER DIETS. THESIS ADVISOR: ASSOC. PROF. DR. SUTISA KHEMPAKA, Ph. D., 106 PP.

Keyword: Latic acid bacteria/Probiotic/Productive performance/Immune responses/ Poultry

This research aimed to isolate Lactic acid bacteria (LAB) from the gastrointestinal tract of chickens and to evaluate the efficacy of isolated LAB in broilers under normal conditions and the lipopolysaccharide (LPS) challenge.

Experiment I caracterized LAB chicken digestive tract strains and evaluate probiotic properties. LAB was isolated from the chicken gastrointestinal tract and identified by sequencing the 16S rDNA gene, followed by acid and bile tolerance, antimicrobial activity, adhesion to epithelial cells and additional characteristics on the removal of cholesterol. It revealed that five isolated strains can attain all probiotic property measurements, including *Lactobacillus acidophilus*, *L. ingluviei*, *L. reuteri*, *L. salivarius* and *L. saerimneri*. Two strains of *L. ingluviei* and *L. salivarious* were then selected for testing in chickens, since there is still a lack of information in previous studies. It was found that the two isolated strains can improve gut health by increasing the population of *Lactobacillus* and *Bifidobacterium* with an associated increase of valeric acid in the cecum. However, *L. ingluviei* exhibited greater properties than *L. salivarious* in reduction of pathogenic populations.

Experiment II studied the effects of *L. ingluviei C*37 (LIC37) on modulation of host inflammatory responses and expression of gut barrier gene in broilers induced with LPS endotoxin from *Escherichia coli* Chickens were randomly allocated into four treatment groups: 1) control (orally administered phosphate buffered saline, PBS) 2) orally administered probiotic LIC37 10^8 CFU/bird/day 3) orally administered probiotic LIC37 10^9 CFU/bird/day and 4) negative control (orally administered PBS) for 14 days. At 14 days of age. Treatments 2 3 and 4 chicken groups were injected intraperitoneally with LPS (1 mg/kg body weight), and control chickens were treated with a sterile PBS

injection. The results indicated that probiotic LIC37 has a beneficial protective effect on broiler chickens by modulating hepatic cytokine expression and strengthening the intestinal wall through improvement of the expression of intestinal tight junction protein (JAM2, occludin, ZO1) and mucin, and upregulation serum lysozyme during the LPS-mediated immunological challenge.

Experiment III studied the potential effects of live and heat killed LIC37 in broiler diets on growth performance, carcass quality, meat cholesterol, cecal microbial population, SCFAs and ammonia production. Broiler chickens were allocated into 4 groups in 6 replicate pens with 10 chicks each. Four treatments were as follows: 1) the basal diet, control; 2) basal diet supplemented with 50 mg/kg diet of zinc bacitracin, positive control (PC); 3) basal diet supplemented with 1×10^8 CFU/kg diet of live LIC37 and 4) basal diet supplemented with 1×10^8 CFU/kg diet of heat killed LIC37. The results showed that LIC37 in both live and heat killed cells can improve body weight gain and feed intake of broilers when compared to control ($P<0.05$) and similar as positive control that are supplemented with antibiotic ($P> 0.05$). In addition, *L. ingluviei* C37 in both forms can increase *Lactobacillus* and *Bifidobacterium* and decrease *Enterobacter* and *E. coli* in cecal content, reduce meat cholesterol, improve cecal SCFAs concentration and reduce ammonia production.

Overall, this present study can isolate five strains of LAB with probiotic properties, including *L. acidophilus*, *L. ingluviei, L. reuteri*, *L. salivarius* and *L. saerimneri*. LIC37 is a novel probiotic with great potential as a feed additive in broiler diets, both live and heat killed cells.

School of Animal Technology and Innovation
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LIST OF ABBREVIATIONS

%	=	Percentage
℃	=	Degree Celsius
ADG	=	Average Daily Gain
AGPs	=	Antibiotic Growth Promoter
ANOVA	=	Analysis of Variance
BHI	=	*Brain Heart Infusion*
BSH	=	Bile Salt Hydrolase
BW	=	Body Weight
BWG	=	Body Weight Gain
CFU	=	Colony Forming Unit
CP	=	Crude Protein
FCR	=	Feed Conversion Ratio
FI	=	Feed Intake
g	=	Gram
GIT	=	Gastrointestinal Tract
h	=	Hour
IL	=	Interleukin
L	=	Liter
LAB	=	Lactic Acid Bacteria
LIC37	=	*Lactobacillus ingluviei* C37
LPS	=	Lipopolysaccharide
M	=	Molar
m	=	Meter
mg	=	Milligram

LIST OF ABBREVIATIONS (Continued)

min	=	Minute
mL	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
MRS	=	De Man, Rogosa And Sharpe
NF-**K**B	=	Nuclear Factor Kappa Beta
nm	=	Nanometer
PBS	=	Phosphate Buffered Saline
PI	=	Productive Index
qPCR	=	Quantitative Polymerase Chain Reaction
rDNA	=	Ribosomal Deoxy Ribonucleic Acid
SCFAs	=	Short Chain Fatty Acids
SEM	=	Standard Error of Mean
TAE	=	Tris Acetate EDTA
TJ	=	Tight Junction
UV	=	Ultraviolet
v/v	=	Volume/Volume
µg	=	Microgram
µL	=	Microliter
µm	=	Micrometer
µmol	=	Micromole

CHAPTER I
INTRODUCTION

1.1 Introduction

The use of antibiotics as a growth promoter (AGPs) is now banned, leading to increase the risks of pathogen infections, health issues, and growth reduction in broilers. As a reason, a novel natural source has been investigated for further use as animal feed additives. Probiotics are one of the alternative feed additives that can improve immune and gastrointestinal health. Health-promoting probiotics not only contribute to healthy gut microflora, but also modulate the immune response of the host to prevent pathogen infection, resulting in an enchantment growth performance. The most well-known probiotics are Gram-positive lactic acid bacteria (LAB), such as Enterococcus, Lactobacillus, Pediococcus, Streptococcus, Lactococcus, Vagococcus, Leuconostoc, Oenococcus, Weissella, Carnobacterium and Tetragenococcus, which are natural microflora in the gastrointestinal tract (GIT) of humans and animals (Hill et al., 2014; Musikasang et al., 2009; Vaughan et al., 2005).

LAB are the main source of probiotics used in animal feed, which have several health benefits for the host, including gut microbiota modulation, immunomodulation, anti-inflammatory and antimicrobial effects. LAB possesses a wide range of beneficial and health promoting properties which influence the intestinal microbial balance of the host to contribute to the regulation of innate intestinal immunity and homeostasis. LAB also produces metabolites such as lactic acid, antioxidants and antimicrobial compounds, especially bacteriocins and short chain fatty acids (SCFAs) that contribute inhibiting the growth of pathogenic bacteria. Many probiotics have been introduced through the screening of the natural intestinal microflora of farm animals. Consideration of the original habitat of the bacteria should be considered when selecting them for use as probiotics. Strains belonging to bacterial species which are generally present in the intestinal flora of the animal species, which is to be targeted, have been generally selected. These bacteria have a better chance of

supplanting resident bacteria and establishing themselves at a numerically significant level in their new host (Morelli, 2000). In order to qualify as probiotics, candidate bacterial strains must be able to tolerate acid and bile, coaggregation with pathogens, antimicrobial activity, adhesion to intestinal mucosa, antibiotic resistance, and modulation of intestinal barrier functions (Hugas et al., 1998; Ehrmann et al., 2000; Klaenhammer et al., 2008; Walter, 2008). Probiotic strains of the same ecological origin can be more compatible with animal gut microbes, which makes it possible to optimize productive performance (Krysiak et al., 2021). For this reason, native and species-specific probiotics should be considered, in which LAB with health promoting properties are primarily the major components of the intestinal microflora of chicken.

Besides the consequence of the health problem of the forbidden of the use of AGPs in broilers, immunological and oxidative stresses damage have received a significant attention in recent years (Xie et al., 2019). In poultry production, intestinal health and function play a crucial role in the efficient feed utilization and growth, and the overall profitability of the farm. The GIT microbiota mainly consists of bacteria, fungi, and protozoa. Microbiota population varies across the compartment, with maximum at the distal segments of the GIT. Intestinal epithelial in response to commensal bacteria produce oxidative substances, which serve as a second messenger and participates in cellular signaling. Tight junctions between the intestinal epithelial cells act as a barrier, preventing invasion of the microorganism. Many studies have suggested the interaction between the mucosa and microbes or their toxin triggers oxidative stress. Particularly, immunological stress alters nutrient distribution, decreases production performance, induces various diseases, and results in a high mortality rate. Environmental stress affects the intestinal epithelial cells and further stimulates intestinal bacteria and bacterial lipopolysaccharide (LPS). As LPS is known to induce apoptosis and injury in various cell types (Kaiser et al., 2012; Tong et al., 2022). Therefore, research into novel and functional probiotics has been conducted based on the ability of bacterial isolates to module intestinal barrier function and anti-inflammatory properties.

However, effects of probiotic supplementation may not be consistent, due to differences in preparation methods, feed storage, and their ability to survive the

passage through the stomach and proliferate in the intestine. In addition, the animal production industry is also concerned about the increase in the number of probiotics used which may introduce live bacteria into the environment. Although many probiotic cultures consist of live organisms, some researchers have reported the benefits from administering inactivated or killed organisms. Various microbiological components, such as cell-free supernatants, exopolysaccharides (EPS), teichoic acid, lipoteichoic acids, peptidoglycans and other metabolites (Nataraj et al., 2020) have anti-inflammatory and immunomodulatory properties by stimulating the innate immune system (Adams, 2010), adaptive responses, and the integrity of the intestinal mucous membrane. Heat-killed probiotics are also able to antagonize pathogens with antimicrobial compounds and in competition with pathogens for adhesion and colonization. However, there is little research on the effects of non-viable or heat-treated microorganisms on broiler chickens.

Therefore, this study aims to isolate and evaluate LAB from the gastrointestinal tract of chickens for a future use as highly stable probiotics in poultry diets. The findings of this study would also provide valuable and highly probiotic sources appropriate for the poultry industry.

1.2 Research objective

1.2.1 To isolate and evaluate LAB from the gastrointestinal tract of poultry for a future use as highly stable probiotics in broiler diets.

1.2.2 To evaluate the efficacy of the LAB isolated from poultry gastrointestinal tract on liver inflammatory, immunity organ index and gene expression related to gut health and inflammation during LPS-mediated immunological challenge states.

1.2.3 To evaluate the efficacy of the LAB isolated from poultry gastrointestinal tracts both live and heat killed cells on growth performance, carcass quality, meat cholesterol, cecal microbial population, SCFAs concentration, immune responses and ammonia production of broilers.

1.3 Research hypothesis

1.3.1 LAB isolated from the poultry gastrointestinal tracts can be used as highly stable probiotics in broiler diets.

1.3.2 LAB isolated from poultry gastrointestinal tract can alleviate liver damage, improve intestinal immunity, and alter the expression of mRNA tight junction protein during LPS-mediated immunological challenge states.

1.3.3 Supplementation of LAB probiotics isolated from poultry gastrointestinal tracts in broiler diets can improve growth performance, carcass quality, meat cholesterol, immune responses, cecal microbial population, SCFAs concentration, and ammonia production of broilers.

1.4 Scope of this study

This study aimed to isolate and evaluate LAB from the gastrointestinal tract of poultry for future use as highly stable probiotics in broiler diets. The strains were characterized based on various factors, including their tolerance to low pH and bile, adhesion to intestinal mucous, antibacterial activity, antibiotic susceptibility and cholesterol removal ability isolate and evaluate LAB from poultry gastrointestinal tract. The novel probiotics were selected and evaluated for their functional properties associated with gut health by oral administration in broiler chickens (0-14 days of age) induced with LPS endotoxin. In addition, properties of selected LAB strains were also assessed based on their efficacy in terms of growth performance, meat cholesterol, cecal microbial population, SCFAs, ammonia production and immune response in broilers 0-42 days of age.

1.5 Expected results

1.5.1 LAB isolated from poultry gastrointestinal tracts can be used as highly stable probiotics.

1.5.2 LAB isolated from poultry gastrointestinal tracts has beneficial effects in broiler chickens in terms of improvement of gut health, immune responses, growth performance, carcass quality, as well as reduction of meat cholesterol.

1.5.3 Obtain an alternative probiotic substitute with antibiotics, especially the heat-killed cell (postbiotics) which is more stable and easier to incorporate into poultry diets.

1.5.4 This novel probiotic would improve production efficiency and food safety through the ability to create a competitive floor for Thailand for the poultry production industry.

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CHAPTER II
LITERATURE REVIEWS

2.1 Probiotics

Probiotics, according to the revised definition of the Food and Agriculture Organization (FAO)/World Health Organization (WHO), are considered as non-pathogenic live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Probiotics are now widely used in many countries in clinical practice and, frequently, they are acquired by consumers with or without a prescription. The concept of a balanced intestinal microbiota enhancing resistance to infection and reduction in resistance when the intestinal microbiota is disturbed is important in understanding the microbe-host relationship. The proposed mechanisms by which the intestinal microbiota inhibits pathogens include competition for nutrients, the production of toxic conditions and compounds such as short-chain fatty acids (SCFAs), low pH, and bacteriocins, competition for binding sites on the intestinal epithelium, and stimulation of the immune system. A variety of microbial species have been used as probiotics, including species of *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus, Streptococcus*, a variety of yeast species, and undefined mixed cultures. Lactic acid bacteria (LAB) have been widely used in the animal feed industry and they have several benefits for the host health including gut microbiota modulation, immunomodulation, anti-inflammatory and antimicrobial effects (Corcionivoschi et al., 2010; Ashaolu, 2020). LAB have been reported to possess a broad spectrum of beneficial and health promoting properties which influence the intestinal microbial balance of the host to contribute to the regulation of innate intestinal immunity and homeostasis (Chen et al., 2022). LAB also produces metabolites such as lactic acid, antioxidants and antimicrobial compounds, especially bacteriocins and organic acids that contribute to the inhibition of the growth of pathogenic bacteria (Riaz et al., 2015; Kumar et al., 2021). The main candidate strain introduced for probiotic purposes belongs to the genus

Lactobacillus which is a major genus of LAB and accommodates more than 200 species (Noohi et al., 2021). In poultry, feeding *Lactobacillus* probiotic strains improves not only the digestion of feed, but also the absorption of nutrients. In addition, probiotics increase the growth performance, neutralizing various enterotoxins and enhancing the immune responses of poultry (Al-Khalaifa et al., 2019). Additionally, probiotics reduce the risk of gastrointestinal colonization by foodborne pathogens, such as *E. coli*, *Campylobacter* (Awad et al., 2009; Neal-McKinney et al., 2012), *Clostridium* (Li et al., 2017) and *Salmonella* (Kizerwetter-Świda and Binek, 2009; Tellez et al., 2012), and increase the safety of poultry-based foods (Gaggia et al., 2010). Due to their diverse advantages, LAB has been chosen as the best candidate for probiotics.

2.2 Criteria of the selection of probiotic strain

LAB characteristics and its safety profile also need to be assessed. In order to qualify as probiotics, candidate bacterial strains must be able to tolerate acid and bile, coaggregation with pathogens, antimicrobial activity, adherence to intestinal mucosa, antibiotic resistance, and modulation of intestinal barrier functions (Ehrmann et al., 2002; Hugas et al., 1998; Klaenhammer et al., 2008; Walter, 2008).

2.2.1 Survival in the gastrointestinal tract (resistance to gastric acid and bile acids)

Acid and bile salt tolerances are the most important criteria for selecting strains of probiotics capable of survival in the gastrointestinal tract. Therefore, microorganisms must be able to tolerate gastric acid (pH 2-3) and tolerate to bile salts within the small intestine (pH 5-7). Microorganisms isolated from the chicken gastrointestinal tract were highly tolerant in the gastrointestinal environment (Noohi et al., 2021) because these bacteria are well known to be an indigenous flora of the gastrointestinal tract of animals and in this ecological niche, these LAB encounter bile salts (Pringsulaka et al., 2015). These properties helped them survive in the gastrointestinal tract of chickens and they adhered to the intestinal cells while exerting beneficial effects.

2.2.2 Antimicrobial activity

Infections by zoonotic and foodborne enteric pathogens cause high morbidity and mortality with significant economic loss in the poultry industry. Also, these pathogens are often transmitted to humans either via occupational exposure or through the food chain, which is of immense public health concern. Antimicrobial activity against these pathogens is a major requirement of potential probiotics. Antagonistic activity by LAB is sustained by the secretion of different antimicrobial substances including SCFAs, bacteriocins, hydrogen peroxide and antimicrobial peptides (Vieco-Saiz et al., 2019). They are present in high concentrations with acid pH and exert antimicrobial effects. These acids are lipophilic and they penetrate the bacterial cell wall and produce H^+ ions which in turn destroy the internal physiology of bacterial cells.

2.2.3 Adherence to epithelial cells

Adhesion to mucosal surfaces has been used as a criterion for the selection of probiotic bacteria because this characteristic plays a major role in the colonization of the GIT by these bacteria (Gusils et al., 2002; Zhang et al., 2013). Caco-2 cells have been accepted as an in vitro model for determining bacterial adhesion, which correlates with adherence to the intestinal epithelium in vivo (Ohland and Macnaughton, 2010). The ability of probiotic bacteria to adhere to the intestinal epithelium enables them to colonize the gut and to resisting the gut peristaltic movements. Additionally, mucosal adhesion is important for pathogenic antagonism, modulation of the immune system and healing of damaged gastric mucosa (Monteagudo-Mera et al., 2019; Šikić Pogačar et al., 2020).

2.2.4 Antibiotic susceptibility

It is important that probiotic organisms are not inhibited by antibiotics, because strains with intrinsic antibiotic resistance could in fact be useful for restoring gut microbiota after antibiotic therapy (Gueimonde et al., 2013; Sharma et al., 2014). However, an important requirement of probiotics is that the isolated strain must be safe for animal and human consumption. Antimicrobial resistance is an increasingly serious global threat to human, animal and environmental health. As a result, with the advent and spread of antibiotic-resistant strains of bacteria, as well as the presence of low amounts of antibiotics in broiler meat, antibiotic supplementation

has recently aroused severe concerns (Manyi-Loh et al., 2018; Tang et al., 2022) . Therefore, a crucial parameter for evaluating prospective probiotics is the antimicrobial susceptibility profile. Probiotic microbial strains should not act as a reservoir for antibiotic resistance genes that could be passed on to gut pathogens (Li et al., 2020; Noohi et al., 2021; Tang et al., 2022).

2.2.5 The cholesterol removal ability

Recently, the incidence of cardiovascular disease in humans has increased, which has a strong correlation with the serum cholesterol level. As a result, much attention has been given to the screening of probiotics that can increase the removal of cholesterol. From several in vitro studies, a number of mechanisms have been proposed for the purported cholesterol-lowering action of probiotic bacteria. They include the assimilation of cholesterol, cholesterol binding to the bacterial cell wall, microbial transformation of cholesterol to coprostanol, and enzymatic deconjugation of bile salt (Kriaa et al., 2019; Ma et al., 2019; Hameed et al., 2022).

Probiotic strains of the same ecological origin can be more compatible with animal gut microbes, which makes it possible to optimize productive performance (Krysiak et al., 2021). For this reason, native and species-specific probiotics should be considered, in which LAB with health promoting properties are the major components of the chicken intestinal microflora (Noohi et al., 2014; Kizerwetter-Świda and Binek, 2016).

2.3 Role of probiotics on poultry

Probiotics exert their effectiveness through diverse mechanisms. Probiotics inhibit and control enteric pathogens along with improving the functioning and production capacity of animals. The basic probiotic mode of action includes inhibition of pathogen adhesion; production of antimicrobial components; competitive exclusion of pathogenic microorganisms; enhancement of barrier function; reduction of luminal pH; and modulation of the immune system (Fig 2.1).

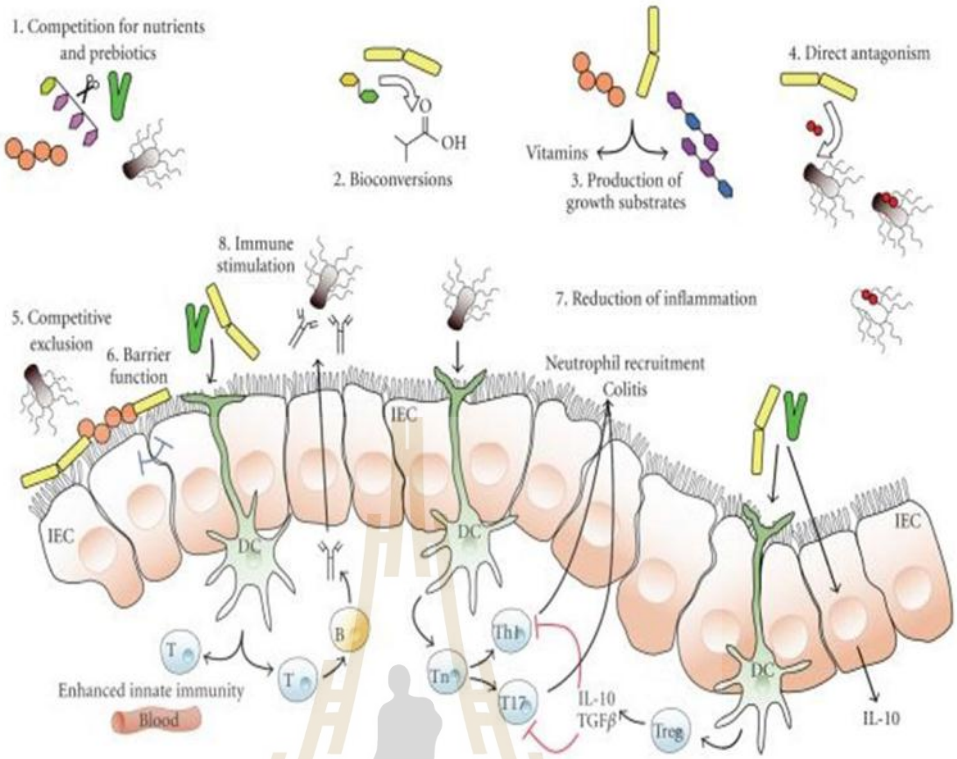

Figure 2.1 The potential impact of probiotic bacteria on microbiota (Yaqoob et al., 2022).

2.3.1 Probiotics on growth performance and meat quality

Improving growth performance in chickens has long been one of the most important goals in poultry research. Although microbiota influence chicken productivity, there are other factors which influence microbiota composition and these may in turn influence performance. Several studies found that the growth performance of broilers is improved by using single and multiple strains of *Lactobacillus* (Jahromi et al., 2016; Gao et al., 2017; Elbaz et al., 2021)(Table 2.1). Feeding multiple strains of probiotics significantly improved WG, FCR, and production efficiency (Elbaz et al., 2021). Variable results are found in the literature about the effect of probiotics on meat quality in broilers. Improvement in meat flavor was observed by feeding broilers on *B. subtilis* but no significant effect of probiotics was found on sensory attributes of meat in another study (Bai et al., 2017). These contradictions in the results might be due to the use of different types or doses of probiotics, duration of the experiment or the age of the birds at which the probiotics were fed to the birds.

Table 2.1 Effect of probiotics on the productive performance of broilers.

Probiotic strains	Effects	References
L. plantarum	Improved feed efficiency	Gao et al. (2017)
Lactobacillus spp.	Probiotics positively affected final body weight and improved the fatty acid profile in meat.	Jahromi et al. (2016)
B. subtilis	Probiotics significantly improved the average daily gain, average daily feed intake and feed conversion ratio of broilers.	Bai et al. (2017)
Multi-strain probiotic	Dietary supplements improved growth performance and decreased abdominal fat and serum cholesterol.	Elbaz et al. (2021)

2.3.2 Probiotic effects on gut health and microbiota

Probiotics promote health conditions by inhibiting harmful bacteria. They can influence pathogenicity by modifying the communication process in pathogenic bacteria and they produce antibacterial substances which impede bacterial adherence and translocation (Raheem et al., 2021; Zhang et al., 2023). Also, probiotic microbes can withstand pathogenic bacteria by lowering luminal pH. They can shape the cell–cell communication of bacteria and host and maintain cellular consistency by strengthening the intestinal barrier function. Such consistency is achieved through the modulation of cytoskeletal and tight junctional protein phosphorylation. *Lactobacillus* can reside in the epithelial cells of the ileum of chickens (Huyghebaert et al., 2011; Hernández-González et al., 2021). Probiotics can competitively exclude pathogenic microorganisms from the host intestine by strengthening this intestinal communication system (Mookiah et al., 2014). This anti-pathogenic mechanism (competitive exclusion) demonstrates that bacterial species strongly battle for attachment to receptors at particular binding sites in the GIT and they might integrate antimicrobial substances secretion and competition for accessible nutrients (Kizerwetter-Świda and Binek, 2009; Zhang et al., 2013; Dec et al., 2014;). The gut microbiota is one of the main defense components in the GI tract against

enteric pathogens. Disturbance of the gut microbiota–host interaction plays a crucial role in the development of intestinal disorders. Short chain fatty acids (SCFAs) play a major role in the physiology of the intestinal mucosa. The majority of SCFAs are absorbed from the gut and metabolized in various body tissues. Butyrate exerts a wide variety of effects on intestinal function (Qing et al., 2019; Ma et al., 2022). Early intestinal colonization with beneficial bacteria not only prevents pathogenic bacteria but also improves maturation of the gut and its integrity. Development of the broiler intestinal microbiota starts with hatching. Therefore, the type of microbes provided in the initial days of chickens helps in establishing the gut microbial community . Also, there is a lifetime stable community of microbiota from the first inoculum that leads to a developed immune system (Ohland and Macnaughton, 2010; Rescigno, 2011).

LAB culture has shown accelerated development of healthy and beneficial microflora in broiler chickens which provides increased resistance against *Salmonella* infections (Higgins et al., 2010), (Table 2.2). The time point of administration may influence the beneficial effects of probiotic. *L. reuteri* are administrated to broiler chickens only during the first weeks of age. There was no effect at 3 weeks of age, however, at 6 weeks of age, there was a greater diversity and abundance of *Lactobacillus* and a significant reduction in the presence of chicken pathogens when compared with the control group (Nakphaichit et al., 2011). Probiotics can prevent Salmonellosis in chickens. Hatched chicks that were inoculated with 1×10^9 CFU *L. plantarum* were not affected by Salmonellosis. *L. plantarum* can inhibit the gut colonization of harmful bacteria and stabilize the expression of tight junction genes in the gut epithelial cells, resulting in more resistance to infection (Wang et al., 2018).

Physiological alterations in broilers have been observed as a result of gut microbiota manipulation. These include changes in the integrity of the gut wall and the rate at which cells undergo apoptosis. Maintaining the integrity of the gut barrier is thus critical to prevent the translocation of pathogens into the intestinal lumen. Probiotic bacteria regulate epithelial permeability by modulating tight junction (TJ) proteins (occludin, zonula occludens, claudins and junction adhesion molecules), which in turn inhibit pathogen colonization, modulate cell proliferation and apoptosis, and control mucin production. Increased permeability causes mucosal

barrier dysfunction; however, probiotics can restore barrier integrity by normalizing TJ protein production and localization (Wibowo et al., 2019; Leon-Coria et al., 2021). According to the study conducted by Chang et al. in 2020, it was observed that *Salmonella* infection led to a decrease in the expression of occludin and claudin genes in the ileum and jejunum of broiler chickens. This reduction indicated that the tight junction structures in the intestinal lining were disrupted due to the invasion of *Salmonella enterica* subsp. enterica. This suggests that the probiotics helped enhance the integrity of the tight junctions, potentially restoring the disrupted barrier function caused by *Salmonella* invasion.

Mucin-2 proteins play a crucial role in the chemical barrier of the intestinal mucosa. They contribute to the inhibition of bacterial infections and the neutralization of toxins, while also serving as a lubricant in the small intestine, maintaining the integrity of the mucosal barrier function. Research conducted by Zhen et al. (2018) suggests that probiotics could enhance mucin synthesis. By promoting mucin production, probiotics can increase the resistance of the broiler intestines against infections, including those caused by enteric bacteria. This implies that probiotics can positively influence the mucosal barrier function, providing protection against pathogens. Furthermore, Siddiqui et al. (2017) found that probiotics have the potential to stimulate gut epithelial cells to produce an increased amount of mucin. This stimulation helps restore the normal level of the mucus layer in the intestine. By enhancing mucin production, probiotics contribute to maintaining the integrity and function of the mucosal barrier, which is essential for protecting against pathogens and maintaining gut health.

Table 2.2 Effects of probiotics on chicken gut health and immune responses.

Probiotic strains	Effects	References
Lactobacillus cultures	Develop normal microflora in chicken gut and reduce incidence of *Salmonella*	Higgins et al. (2010)
L. reuteri	Probiotics can increase the diversity and abundance of *Lactobacillus* and reduce pathogenic bacteria	Nakphaichit et al. (2011)

C. butyricum HJCB998	Decrease cecal *Salmonella* and *C. perfringens* population, increase *Lactobacillus* and *Bifidobacterium* populations in the cecum.	Yang et al. (2012)
B. amyloliquefaciens (BA)	Probiotics decreased IL-1β and increased IL-10 transcriptional levels in jejunum under LPS challenges condition	Li et al. (2015)
B. coagulans	*B. coagulans* supplementation appears to be effective at reducing *S. enteritidis* prevalence in broilers by regulating intestinal mucosal immune responses	Zhen et al. (2018)
P. acidilactici	The addition of probiotics to the diet of broilers infected with *E. coli* can modulate the intestinal inflammatory responses induced by *E. coli* infection and minimize inflammation-induced damage.	Ateya et al. (2019)
Mix strains (*L. acidophilus*, *L. fermentum*, *P. acidilactici*, and *L. casei*)	Probiotics can increase cecal *Lactobacillus* and reduce *Enterobacteria.* Probiotics increased the mRNA expression of TJs in ileal, downregulated the mRNA levels of proinflammatory cytokines and upregulated *IL-10* expression in the cecal tonsil.	Chang et al. (2020)
B. subtilis and L. acidophilus	Probiotics can regulate the expression of IL-2, IL-10, and TLR-4 mRNA in broiler chickens during *E. coli* infection	Liang et al. (2021)

2.3.3 Probiotics on immune response

Besides the consequences of health problem from the prohibition of the use of AGPs in broilers, the damage caused by immunological and oxidative stresses has received significant attention in recent years (Reuben et al., 2021). Particularly, immunological stress can alter nutrient distribution, decrease production performance, induce various diseases, and result in a high mortality rate. Chickens challenged with LPS are an excellent model for the study of immunological stress, and these models have

been extensively studied (Kaiser et al., 2012; Tong et al., 2022). Indeed, the LPS found in the cell wall of gram-negative bacteria can induce the activation of the transcription factor NF-**K** B signaling pathway and the induction of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukins (IL-6, IL-1β, IL-8, and IL-12), interferon (IFN), and co-stimulatory molecules. IL-10 is involved in reducing chronic gastrointestinal problems and is considered a major anti-inflammatory cytokine that maintains the balance of the immune responses during infection and inflammation. The administration of multi-strain probiotics modulated intestinal microbiota, gene expression of tight junction proteins, and immunomodulatory activity in broiler chickens challenged with *Salmonella enterica* subsp. enterica (Chang et al., 2020) (Table 2.2). Moreover, *B. coagulans* supplementation appears to be effective at reducing *S. enteritidis* prevalence by regulating intestinal mucosal immune responses as well as inhibiting harmful intestinal bacterial colonization and invasion in chickens (Zhen et al.,2018). This is consistent with the findings of Liu et al. (2015) who found that the supplementation of *B. amyloliquefaciens* at 1.0 g/kg in the broiler diet decreased IL-1β, whereas IL-10 increased both protein and transcriptional levels and attenuated the elevated mRNA expression of TLR-4 induced by LPS. Liang et al. (2021) also revealed that probiotics can improve the indicators of diarrhea and regulate the expression of IL-2, IL-10, and TLR-4 mRNA in broiler chickens during *E. coli* infection.

However, their use still has some limitations with respect to inconsistencies in the preparation methods, feed processing and storage, and durability to survive in the gastrointestinal tract. These concerns prompt consideration of alternative agents such as postbiotics (products of microbial fermentation), specific components of probiotic strains and heat-killed probiotic strains.

2.4 Heat- killed or dead cell probiotics

Despite the general definition that probiotics are live micro- organisms, a variety of biological responses have been reported from administering dead, frequently heat-killed, probiotics to various mammalian and avian species. The concept of postbiotic ingredients is not new but has been recently defined by the International Scientific Association of Probiotics and Prebiotics. The association's definition describes postbiotics as "preparations of inanimate microorganisms and/or

their components that confer a health benefit on the host". This concept has been thoroughly studied for almost 20 years, and is often referred to as heat-killed, tyndallized, or non-viable bacteria. Heat-inactivated probiotics, on the other hand, can extract bacterial components, including peptidoglycans and lipoteichoic acids, which are important in immunomodulation and pathogen suppression (Piqué et al., 2019). The preparation of dead cells has also been fractionated and various cellular components shown to produce a range of biological responses. Different methods of inactivation may affect structural components of the cell differently, and influence their biological activities. Heat treatments of bacterial suspensions can use a range of temperatures between 70 and 100°C. Different strains, including LAB and *Bifidobacterium*, are able to produce beneficial effects in their heat-inactivated form.

There are also considerable data showing that not only dead cells, but also metabolites, cell fractions, and culture supernatants of probiotic bacteria can exert biological effects. Their use is based on the evidence suggesting that individual effector molecules interacting with host cells may underlie probiotic effects (Table 2.3). These specific components are usually active on Toll-like and other signal transduction receptors in the intestinal epithelium, dendritic cells, and other immune intestinal cells. Early intestinal colonization with beneficial bacteria not only prevents pathogenic bacteria but also improves maturation of the gut and its integrity (Qin et al., 2022).

Therefore, the selection of new probiotic organism strains demonstrates the most beneficial or the most specific effects. Assessment focuses mostly on safety and the benefit-to-risk ratio associated with the use of a given probiotic strain. To qualify as probiotics, candidate bacterial strains must be able to tolerate acid and bile salt, coaggregation with pathogens, antimicrobial activity, adherence to intestinal mucosa and have antibiotic resistance. In addition, some beneficial features related to gut health should also be assessed, such as modulation of gut barrier functions, reduction in intestinal leakage, alleviation of oxidative stress and anti- inflammatory properties.

Table 2.3 The efficacy of heat killed probiotic in chickens.

Probiotic strains	Effects	References
L. sakei HS-1	*L. sakei* supplementation can increase BW gain and G:F compared with control	Khonyoung and

		Yamauchi (2019)
L. plantarum L-137	Heat killed L-137 fed birds showed a significant improvement in BW, weight gain, and FCR at 42 days old, but there were no significant differences in feed intake, survival rate, carcass characteristics, and visceral organs.	Incharoen et al. (2019)
L. plantarum L-137	Heat killed L-137 significantly influenced host defense gene expression in the intestine and enhanced organ index values, conferring a significantly improved host growth performance.	Thi et al. (2022)
B. subtilis and *L. acidophilus*	Heat killed probiotics can enhance feed efficiency, decrease plasma cholesterol and creatinine contents and modulate composition, diversity and functions of cecal microbiota.	Zhu et al. (2020)
Tsukamurella inchonensis	*T. inchonensis* in the broiler diet can improve intestinal morphology and humoral immune response, but it cannot affect FI and FCR.	Nofouzi et al. (2021)
Lactiplantibacillus plantarum	Dietary postbiotics can reduce abdominal fat, increase villus heights. Colon mucosa sIgA was significantly influenced by the dietary treatments.	Danladi et al. (2022)
L. acidophilus	Postbiotics can improve growth performance and enhance the humoral immune response	Abd El-Ghany et al. (2022)

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CHAPTER III

SCREENING OF LACTIC ACID BACTERIA ISOLATED FROM THE CHICKEN DIGESTIVE TRACT FOR POTENTIAL USE AS POULTRY PROBIOTICS

3.1 Abstract

The use of probiotics as an alternative to antibiotics in animal feed has received considerable attention in recent decades. LAB have remarkable functional properties promoting host health and are major microorganisms for probiotic purposes. The aim of this study was to characterize LAB strains of the chicken digestive tract and to determine their functional properties for further use as potential probiotics in poultry. A total of 2,000 colonies were isolated from the ileum and cecal contents of chickens based on their phenotypic profiles and followed by a preliminary detection for acid and bile tolerance. The selected 200 LAB isolates with exhibited well-tolerance in acid and bile conditions were then identified by sequencing the 16S rDNA gene, followed by acid and bile tolerance, antimicrobial activity, adhesion to epithelial cells and additional characteristics on the removal of cholesterol. Then, the two probiotic strains (*L. ingluviei* and *L. salivarious*) which showed the most advantages *in vitro* testing were selected to assess their efficacy in broiler chickens. It was found that 200 LAB isolates complied with all measurement criteria belonged to five strains, including *L. acidophilus* (63 colonies), *L. ingluviei* (2 colonies), *L. reuteri* (58 colonies), *L. salivarius* (72 colonies) and *L. saerimneri* (5 colonies). We found that *L. ingluviei* and *L. salivarius* could increase the population of *Lactobacillus* and *Bifidobacterium* while reduced *Enterobacteria* and *E. coli* in the cecal content of chickens. Additionally, increased concentrations of valeric acid and total SCFAs were also observed. This study indicates that all five *Lactobacillus* strains isolated from gut contents of chickens are safe and possess probiotic properties, especially *L. ingluviei* and *L. salivarius.* Future studies should evaluate the potential for growth improvement in broilers

Keywords: Chicken, Isolation, Lactic acid bacteria, *Lactobacillus*, Probiotic.

3.2 Introduction

The poultry industry is one of the fastest growing sectors of global livestock production. Various aspects (such as breed, nutrition, animal health, etc.) are used to develop all segment chains to improve potential production efficiencies (Mottet and Tempio, 2017). However, due to the high efficiency of meat or egg production, inputs for specific nutrients and health management require more attention. Among the aspects that should be taken into consideration for optimal poultry performance, overall health and proper functioning of the avian gastrointestinal tract are crucial. In addition, the intensive poultry production system has led to an increase in stress, which can lead to a decrease in immune function and allow colonization by pathogens. This may pose a serious health hazard to birds and consumers of poultry products as outbreaks of different diseases have resulted in huge economic losses. Therefore, finding alternative feed additives that can effectively control pathogens and retain growth promoting properties would help address these issues. Probiotics are defined as "living microorganisms that, when taken in sufficient quantities, provide a host with health benefits", which play a key role in the development of immunity against pathogens as well as the health and growth of broilers, resulting in safe and cost-effective production (Hill et al., 2014).

LAB are the main source of probiotics used in animal feeds, which have several benefits for the host health including gut microbiota modulation, immunomodulation, anti-inflammatory and antimicrobial effects (Ashaolu, 2020). LAB have been reported to possess a broad spectrum of beneficial and health promoting properties which influence the intestinal microbial balance of the host to contribute to the regulation of innate intestinal immunity and homeostasis (Chen et al., 2022). LAB also produces metabolites such as lactic acid, antioxidants and antimicrobial compounds, especially bacteriocins and short chain fatty acids (SCFAs) that contribute to the inhibition of the growth of pathogenic bacteria (Heravi et al., 2011). LAB including species *Enterococcus, Lactobacillus, Pediococcus*, *Streptococcus*, *Lactococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Carnobacterium* and *Tetragenococcus* are natural microflora in the gastrointestinal tract (GIT) of humans and animals (Hill et al.,

2014) characterized by the production of lactic acid. The main candidate strain introduced for probiotic purposes belongs to the genus *Lactobacillus* which is a major genus of LAB and accommodates more than 200 species (Noohi et al., 2021). In poultry, feeding *Lactobacillus* probiotic strains improves not only the digestion of feed, but also the absorption of nutrients. In addition, probiotics increase the growth performance, neutralizing various enterotoxins and enhancing the immune responses of poultry (Al-Khalaifa et al., 2019). Additionally, probiotics reduce the risk of gastrointestinal colonization by foodborne pathogens, such as *E. coli*, Campylobacter, *Clostridium* and *Salmonella* (Kizerwetter-Świda and Binek, 2016) and increase the safety of poultry-based foods. Due to their diverse advantages, LAB has been chosen as the best candidate for probiotics.

However, not all LAB are probiotics and their characteristics and safety profile also need to be assessed. In order to qualify as probiotics, candidate bacterial strains must be able to tolerate acid and bile, coaggregation with pathogens, antimicrobial activity, adherence to intestinal mucosa, antibiotic resistance, and modulation of intestinal barrier functions (Ehrmann et al., 2002). Probiotic strains of the same ecological origin can be more compatible with animal gut microbes, which makes it possible to optimize productive performance (Krysiak et al., 2021). For this reason, native and species-specific probiotics should be considered, in which LAB with health promoting properties are mostly the major components of the chicken intestinal microflora (Noohi et al., 2021).The findings of this study would also provide valuable sources of highly efficacy and appropriate probiotics for the poultry industry.

3.3 Objective

This study aimed to isolate and evaluate LAB from the gastrointestinal tract of chickens and to determine their functional properties for further use as potential probiotics in poultry. In addition, the most advantage strains *in vitro* testing were selected to assess their efficacy in broiler chickens.

3.3 Materials and methods

All experiments were conducted according to the principles and guidelines approved by the Animal Care and Use Committee of Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.4.1 Sample collection

Bacteria were isolated from the digestive tract of healthy broilers, slow-growing chickens (Korat chickens) and laying hens raised on the farm at Suranaree University of Technology. All birds were fed a standard diet without any antibiotic supplement. After birds were euthanized by chloroform inhalation, the blood was removed and the peritoneal cavity opened. The digesta contents of the ileum and cecum were separately removed under sterile conditions and transported to the laboratory immediately on ice for microbial analysis.

3.4.2 Isolation of LAB

Ten-fold serial dilutions of each sample (ileum and cecal digesta) were made by suspension in a phosphate-buffered saline (PBS, pH 7.4). For each dilution, 100 µL was spread plated on De Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstok, Hampshire, England) plates. The inoculated plates were incubated under anaerobic conditions using a gas pack at 37°C for 48 h. Isolated pure cultures were evaluated by the catalase test, Gram stain and bacterial morphology. The isolated bacteria with the characteristics of *Lactobacillus* specifications, such as creamy white colony, catalase negative reaction and Gram's positive rod shape were stored in MRS broth containing 20% (v/v) sterile glycerol and stored at –80°C for further analysis.

3.4.3 16S rRNA gene sequencing for identification

The bacterial isolates that passed the confirmatory tests for *Lactobacillus* were subsequently selected for molecular identification. The *Lactobacillus* strains were grown overnight and the genomic DNA was extracted from the culture using a bacterial genome extraction kit (KOD FX Neo™, Toyobo Inc. Osaka, Japan). The 16S rRNA gene was amplified using universal primers as follows: 27F (5'-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3'). The PCR amplification had initial DNA denaturation at 94°C for 5 min, followed by 35 denaturing cycles at 94°C for 1 min, annealing at 55°C for 1 min, elongating at 72°C

for 1 min, and followed by a final extension at 72°C for 5 min. The 5 µL PCR product was analyzed by electrophoresis in 1% agarose gel at 90 volts for 45 min, followed by staining with a 1% solution of ethidium bromide (50 µL /L) and distaining with TAE 1x for 10 min. Gel was visualized by UV transillumination and recorded by digital camera. The sequencing of 16S rRNA gene was conducted using a genetic analyzer. The 16S rRNA gene sequences of strains were automatically compared using BLAST against the sequences of bacteria available in databanks (http://www.ncbi.nlm.nih.gov/). The phylogenetic analysis was conducted using the neighbor-joining algorithm. After the *Lactobacillus* strains were identified by 16S rRNA sequencing, all strains were then screened for the probiotic properties mentioned below.

3.4.4 Screening of probiotic properties

3.4.4.1 Tolerance to acidic pH

The resistance of the examined strains under acid conditions was tested as previously described by Heravi et al. (2011). Isolated bacteria were grown in MRS broth at 37°C for18 h, then a subculture was added to fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 4000 × *g* for 5 min, the pellets washed twice in sterile PBS, pH 7.4, and resuspended in PBS. Each strain was diluted 1/100 in PBS at pH 2.0, 2.5, 3.0 and 3.5 then incubated for 4 h. The bacteria were then transferred to MRS agar incubated anaerobically at 37°C overnight and survival cell counts were determined by plating on MRS.

3.4.4.2 Bile tolerance

Bile tolerance was studied according to the method of Walker and Gilliland (1993). Briefly, isolated bacteria were grown in MRS broth at 37°C for 18 h, then the subculture was transferred into fresh MRS broth and incubated for another 24 h. MRS broth containing 0.3% and 1.0% of oxgall (Sigma-Aldrich, St. Louis, MO, USA) was inoculated with each strain, and incubated at 37°C. The control comprised MRS broth without bile salt. Bacterial growth was monitored by measuring absorbance with a spectrophotometer (Multiskan GO, Thermo Scientific, Finland) at 600 nm at hourly intervals for 6 h. All tests were carried out in triplicate.

3.4.4.3 Antimicrobial activity

A standard agar-well diffusion assay (Nami et al., 2014) was used to evaluate antagonistic activities against five common chicken pathogens including *Escherichia coli* (ATCC 43888), *Staphylococcus aureus* (ATCC 29213), *Campylobacter jejuni* (ATCC 33291), *Clostridium perfingen* (ATCC 3624) and *Samonella enteritidis* (ATCC 13076) obtained from the American Type Culture Collection (ATCC; Manassas, US). An overnight culture of each tested pathogen strain was inoculated (0.1%) in Brain-Heart Infusion agar (BHI; Conda-Pronadisa, Spain), incubated at 37°C for 16 h. Each of the examined *Lactobacillus* strains were also cultures in MRS broth incubated at 37°C for 18 h as previously described, then harvested by centrifugation (4000 × *g*, 10 min, 4°C). The first supernatant portion (cell-free supernatant) of each isolated strain was neutralized to a pH of 6.5 and the remaining portion was not adjusted for pH, and thereafter both portions were filtered with 0.22 µm membrane filter sterilization. Then normal cells and cell-free supernatant (adjusted or unadjusted pH) of each strain (100 µL) were pipetted into the agar holes (7 mm). The plates were then incubated at 37°C and examined after overnight incubation. Antimicrobial activity was recorded as growth free inhibition zones (mm) around the well. All tests were done in triplicate.

3.4.4.4 Testing antibiotic susceptibility

The testing of antibiotic susceptibility was conducted by isolating all the LAB using the Kirby-Bauer disk diffusion test (Hudzicki, 2009). Isolates strains were grown in MRS broth at 37°C for 18 h to obtain a density of 10^8 CFU/mL, then the culture suspension was plated on MRS agar. Antibiotic discs were placed aseptically on the inoculated plates and the agar plates were incubated at 37°C for 24 h. The diameters of the inhibition zones around the discs were measured (in triplicate) and the results were interpreted according to CLSI standard as sensitive (S), intermediate (I) and resistant (R). The antibiotics tested included ampicillin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg) and erythromycin (15 µg) (Oxoid, Basingstoke, Hampshire, England).

3.4.4.5 Cell adherence assay

An adhesion assay was conducted as previously reported (Pennacchia et al., 2006) by using Caco-2 cells. The Caco-2 cells were grown in

Dulbecco's modified Eagle medium (DMEM; Gibco, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (0.1 g/L; Gibco, USA) at 37°C in 5%CO_2. The culture medium was replaced every 48 h to maintain single-layered Caco-2 cells in the culture plates. Six well tissues were washed twice with PBS and 2 mL of DMEM was added to each well. Plates were incubated at 37°C for 1 h. After incubation, DMEM was removed from each well and replaced with 1 mL of bacterial suspension. After 90 min incubation at 37°C, the wells were washed 3 times with PBS to remove non-adherent bacteria. The washed monolayer was treated with 1 mL of 0.05% aqueous solution of Triton X-100 for 10 min for the lysis of the cells. The number of viable attached bacteria was determined by plating a 10-fold series dilution of a mixture consisting of denatured Caco-2 cells and bacterial cells on MRS agar after 48 h of incubation at 37°C. The adhesion capacity of the species on Caco-2 cells was calculated as the percentage of viable bacteria based on the initial population.

3.4.4.6 Cholesterol removal ability

Isolated *Lactobacillus* was used to remove cholesterol as previously reported by Liong and Shah (2005). Briefly, each isolated strain at 1.0% was inoculated into a freshly prepared MRS broth containing 0.3% oxgall and incubated at 37°C for 24 h. Subsequently, cells were harvested by centrifugation (5000 × *g*, at 4°C, 20 min) and washed twice with sterile water. To prepare heat-killed cells, cell pellets were suspended in 10 mL of sterile water and autoclaved at 121°C for 15 min. Heat-killed cells were further suspended in MRS broth supplemented with 0.3% oxgall acid and 100 µL/mL of water-soluble cholesterol and incubated for 24 h at 37°C. To prepare resting cells, cell pellets were suspended in 10 mL sterile phosphate buffer (0.05 M, pH 6.8) containing 0.3% oxgall acid and 100 µL/mL of water-soluble cholesterol and incubated for 24 h at 37°C. To prepare growing cells, the fresh MRS broth was supplemented with 0.3% oxgall as bile salt. Water-soluble cholesterol was then filter-sterilized and added to the broth at a final concentration of 100 µg/mL, inoculated with each isolated strain (at 1%) and anaerobically incubated at 37°C for 24 h. Subsequently, the mixtures were centrifuged and the cholesterol concentrations in the supernatants were measured using spectrophotometry. All tests were conducted in triplicate.

3.4.5 Evaluation of probiotics *L. ingluviei* and *L. salivarius* in broiler chickens

After the *in vitro* screening, the two isolated *Lactobacillus* strains (*L. ingluviei* and *L. salivarius*) which were the most stability to the treatments with high possible potential, but still a lack of information, were primarily efficacy studies in broiler chickens.

3.4.5.1 Broiler chicken care and management

Thirty, one-day-old broilers were used for the *in vivo* evaluation. The chicks were randomly divided into three groups of ten birds each. The birds were orally gavaged once daily with PBS, *L. ingluviei* or *L. salivarius* from day 1 to 14. The three treatments were: (1) control group (gavaged with PBS), (2) gavaged with *L. ingluviei* (1 mL/day, 1×10^8 CFU/mL), and 3) gavaged with *L. salivarius* (1×10^8CFU/mL). On day 14, the chicks were injected intraperitoneally with lipopolysaccharide (LPS) 1 mg/kg chicken. The chicks received continuous light for 23 hours per day for day 1 to 10 days which was reduced to 18 hours per day from day 11 onwards. Chickens were vaccinated against Newcastle disease and Infectious Bronchitis on day 7. All chickens in each treatment received the same basal diet. The diets were formulated to meet or to exceed the minimum nutrient requirements of broiler chickens as recommended by NRC (1994) and cobb 500 broiler nutrition specification (Cobb-Vantress, 2018) for starter (0-10 days) and grower (11-14 days) periods. Feed (mash form) and water were provided *ad libitum* throughout the experimental period. Nutrient composition of experimental diet is presented in Table 3.1.

3.4.5.2 Sample collection

After 24 hours of LPS injections (day 15), six chickens were randomly selected from each group, euthanized by exsanguination and their cecal content was immediately collected for further analysis of the cecal microbial population and SCFAs.

3.4.5.3 Cecal microbial population analysis by qRT- PCR

The contents of the cecal digesta were used to quantify *Lactobacillus*, *Bifidobacterium*, *Enterobacter* and *E. coli.* Bacterial DNA was isolated using the QIAamp Fast DNA Stool kit (Qiagen Inc., Hilden, Germany) following the manufacturer's instructions. The extracted DNA was quantified with a Nano Vue Plus

Nano Drop spectrophotometer (GE Healthcare, USA) to assess purity and concentration. The populations of cecal microbes were analyzed by quantitative real-time PCR (qPCR). The extracted DNA was used as DNA templates for PCR amplification. The qPCR assay was performed with a LightCycler® 480 Instrument II (Roche Diagnostics, GmbH, Mannheim, Germany). The PCR reaction was performed in a white LightCycler® 480 Multiwell Plate 96 plates (Roche, Mannheim, Germany) with a final volume of 10 µL using a LightCycler® 480 SYBR Green I Master. Each reaction included 5.0 µL of 2×SYBR Green Master Mix, 0.4 µL of 10 µM forward primer, 0.4 µL of 10 µM reverse primer, 2.0 µL of DNA samples and 2.2 µL of nuclease-free water. Each sample was analyzed with triplicate reactions. The reaction conditions for amplification of DNA were initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 20 seconds, then primer annealing at 50°C for *E. coli,* 58°C for *Lactobacillus*, 60°C for *Bifidobacterium* and *Enterobacter* for 30 seconds respectively, and extended at 72°C for 20 seconds (Samsudin et al., 2015). To confirm the specificity of amplification, a melting curve analysis was carried out after the last cycle of each amplification. Absolute quantification of the cecal microbial population was achieved using standard curves constructed by amplification of the known amount of target bacterial DNA.

3.4.5.4 SCFAs analysis

The concentration of SCFAs (acetic, propionic, isobutyric, butyric, isovaleric and valeric acid) was analyzed according to the method of Mookiah et al. (2014). The cecal digesta was treated with 24% meta-phosphoric acid in 1.5 M H_2SO_4 and vortexed to mix. The samples were left at room temperature overnight, then centrifuged at 10,000×g, at 4°C for 20 min, and the supernatant was used for the next step. The analysis was conducted with a gas chromatograph (Agilent 7890B; Agilent Technologies, USA), with flame ionization detection (FID).

3.4.6 Statistical Analysis

Data were analyzed using the one-way ANOVA of SPSS version 18.0 (SPSS, Inc., 2010). Significant differences among treatments were assessed by Tukey's post hoc test. A threshold level of $P < 0.05$ was used to determine the significance.

Table 3.1 Nutrient composition of the experimental diets (as-fed basis).

Item	Starter diets (1-10 days)	Grower diets (11-14 days)
Ingredients (%)		
Corn	53.00	55.30
Soybean meal, 44% CP	32.56	31.00
Full-fat soybean, 36% CP	6.80	5.00
Cassava starch	0.30	0.30
Rice bran oil	3.00	4.06
Calcium carbonate	1.45	1.25
Monocalcium phosphate	1.40	1.44
Sodium chloride	0.51	0.50
Premix[1]	0.50	0.50
L-lysine	0.14	0.23
DL-methionine	0.29	0.30
L-threonine	0.05	0.12
Calculated composition (%)		
Metabolizable energy (kcal/kg)	3008	3086
Calcium	0.92	0.84
Available phosphorus	0.42	0.42
Digestible lysine	1.17	1.17
Digestible methionine	0.57	0.57
Digestible methionine + cystine	0.87	0.86
Digestible threonine	0.76	0.79
Analyzed composition (%)		
Dry matter	90.61	90.67
Crude protein	21.12	20.09
Crude fat	6.32	7.12

[1]Premix (0.5%) provided the following per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 7 mg; vitamin B6, 4 mg; vitamin B12, 25 µg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15 µg; choline chloride, 250 mg; Cu, 1.6 mg; Mn, 60 mg; Zn, 45 mg; Fe, 80 mg; I, 0.4 mg; Se, 0.15 mg.

3.5 Results

3.5.1 Isolation of LAB

A total of 2,000 colonies were isolated from the ileum and cecal contents of the chickens based on the preliminary identification of LAB using the criteria of a creamy white colony and Gram's positive rod shape. These colonies were isolated from laying hens (450 isolates in cecum) and broilers (350 isolates in ileum and 1,200 isolates in cecum). Thereafter, these colonies were initial screened for acid and bile salt tolerance in order to eliminate some isolates that did not survive under these conditions. It was found that 200 colonies exhibited well-tolerance in both conditions. The selected 200 colonies of LAB were then identified by sequencing the 16S rRNA gene. The results revealed that 200 LAB isolates belonged to five strains, including *L. acidophilus* (63 colonies), *L. ingluviei* (2 colonies), *L. reuteri* (58 colonies), *L. salivarius* (72 colonies) and *L. saerimneri* (5 colonies).

3.5.1.1 Tolerance to acidic pH and bile salt

The tolerance of 200 LAB colonies to acidic pH and bile salt were again tested to select those with the best potential under such conditions. The results indicated that all the strains tested exhibited different survival rates under various acidic pH conditions (Table 3.2). They showed that *L. acidophilus* and *L. ingluviei* had the highest survival rate, followed by *L. reuteri*, *L. salivarius* and *L. saerimneri* ($P < 0.05$). All *Lactobacillus* strains were able to survive at pH levels of 3.0 and 3.5 for 4 h. While at pH 2.0 and 2.5, the four strains (*L. ingluviei, L. reuteri*, *L. acidophilus* and *L. salivarius*) were able to survive, except for *L. saerimneri* for which the survival rate was retained for only 4.25% and 5.78% after exposure to pH 2.0 and 2.5 for 4 h, respectively. Considerable resistance to low pH was observed in strains of *L. acidophilus*, whereas the highest sensitivity to an acidified environment was noted for *L. saerimneri* ($P < 0.05$).

In addition to acidic conditions, the bile salt tolerance of selected *Lactobacillus* strains was also examined for which the results are shown in Table 3.3. All the isolates tested were able to resist various bile salt concentrations of 0.3% to 1.0%. However, as the bile salt concentration increased, their growth rate

decreased while *L. acidophilus, L. ingluviei* and *L. reuteri* exhibited well resistance to bile salt at a concentration of 1.0% ($P < 0.05$).

Table 3.2 Acid tolerance of *Lactobacillus* strains isolated from the digestive tract of chickens in different pH.

Strain	Survival rate (%)			
	pH 2.0	pH 2.5	pH 3.0	pH 3.5
L. salivarius	18.00^{c}	16.04^{c}	21.20^{c}	39.20^{c}
L. reuteri	22.56^{c}	33.75^{b}	57.14^{b}	67.70^{b}
L. acidophilus	45.50^{a}	59.00^{a}	78.40^{a}	89.45^{a}
L. ingluviei	34.00^{b}	45.70^{a}	68.50^{a}	78.54^{ab}
L. saerimneri	4.25^{d}	5.78^{c}	27.09^{c}	30.45^{c}
Pooled SEM	2.91	3.11	2.85	9.17
P-value	0.023	0.024	< 0.001	0.032

Pooled SEM, standard error of the means.

^{a,b,c,d} Means with different superscripts in a column are significantly different ($P < 0.05$).

3.5.1.2 Antimicrobial activity

The five isolated strains were tested for their antimicrobial activities against various bacterial pathogens which constitute the main problem in the intestinal tract of poultry. All five strains exhibited antibacterial activity against pathogenic bacteria including *E. coli, S. aureus*, *C. jejuni*, *C. perfingen* and *S. enteritidis* under normal conditions (Table 3.2), in which *L. ingluviei, L. acidophilus* and *L. salivarius* showed strong inhibitory effects. The cell free supernatants of *L. ingluviei, L. acidophilus* and *L. salivarius* exhibited inhibitory activities against all the pathogens. However, *L. saerimneri* did not show any effect on *C. jejuni* and *C. Perfringen*, where as *L. reuteri* did not show any effect on *C. perfringen*. When the supernatant of the five strains was treated with NaOH to achieve a pH of 6.5, it found that the *L. ingluviei, L. acidophilus* and *L. salivarius* strains showed inhibitory activities against all pathogens. Overall, *L. ingluviei* and *L. acidophilus* demonstrated the most beneficial effects on antimicrobial activity under various conditions.

Table 3.3 The bile salt tolerance of *Lactobacillus* strains isolated from the digestive tract of chickens in different bile salt.

Strain	Survival rate (%)	
	0.30% bile salt	1.00% bile salt
L. salivarius	70.00[b]	59.45[b]
L. reuteri	89.45[a]	79.55[a]
L. acidophilus	93.34[a]	88.56[a]
L. ingluviei	84.46[a]	78.44[a]
L. saerimneri	69.54[b]	55.43[b]
Pooled SEM	8.75	6.33
P-value	0.024	< 0.001

Pooled SEM, standard error of the means.
[a,b] Means with different superscripts in a column are significantly different ($P < 0.05$).

3.5.1.3 Antibiotic susceptibility assay

The sensitivity of *Lactobacillus* strains to the selected antibiotics is presented in Table 3.5. This table demonstrates that all the tested strains were interpreted as resistant to ampicillin and erythromycin. On the other hand, all the strains exhibited intermediate susceptibility to tetracycline and sensitivity to chloramphenicol.

3.5.1.4 Caco-2 cell adhesion

The adhesion of *Lactobacillus* isolates to intestinal cells was investigated using Caco-2 cells (Table 3.6). The *L. ingluviei* strain exhibited the strongest adhesion to Caco-2 cells followed by *L. salivarius* and *L. acidophilus*, whereas *L. saerimneri* and *L. reuteri* expressed less strength of adherence.

3.5.1.5 Cholesterol removal ability

The ability of five isolated bacteria, either growing or non-growing (resting or dead cells) to remove cholesterol was assessed. The removal of cholesterol varied significantly amongst growing, resting and dead cells, ranging from 63–72%, 45–60% and 29–42%, respectively. *L. reuteri, L. ingluviei* and *L. acidophilus* were more effective in cholesterol removal than the other probiotics (Figure 3.1).

Table 3.4 Inhibitory effects of *Lactobacillus* strains isolated from the digestive tract of chickens against pathogenic bacteria[1].

Treatment	Strain	Pathogenic bactheria[2]				
		S. aureus	*C. jejuni*	*E. coli*	*C. perfringens*	*S. enteritidis*
Normal cell	*L. saerimneri*	+++	+	+++	+	+++
	L. salivarius	+++	+++	+++	++	+++
	L. reuteri	+++	++	+++	+	+++
	L. ingluviei	+++	+++	+++	+++	+++
	L. acidophilus	+++	+++	+++	+++	+++
Cell free supernatant	*L. saerimneri*	++	-	++	-	+
	L. salivarius	+++	+	++	+	+
	L. reuteri	++	+	++	-	+
	L. ingluviei	+++	+	+++	++	+++
	L. acidophilus	+++	++	+++	++	++
Cell free supernatant neutralized[3]	*L. saerimneri*	-	-	+	-	+
	L. salivarius	+	+	+	+	+
	L. reuteri	+	-	+	-	-
	L. ingluviei	+	+	+	+	++
	L. acidophilus	++	+	+	+	++

[1]Inhibition zone (mm): no inhibition (-); weak (+) < 14); ++, good (15–19); +++, strong (> 20).
[2]The pathogenic groups that almost possess the problem towards the intestinal tract of poultry.
[3] Supernatant treated with NaOH to obtain a pH 6.5.

Table 3.5 Antibiotic susceptibility of *Lactobacillus* strains isolated from the digestive tract of chickens.

Strain	Ampicillin	Erythromycin	Tetracycline	Chloramphenicol
L. saerimneri	R	R	I	S
L. salivarius	R	R	I	S
L. reuteri	R	R	I	S
L. ingluviei	R	R	I	S
L. acidophilus	R	R	I	S

S, sensitive; I, intermediate and R, resistant.

Table 3.6 Adhesion ability of the *Lactobacillus* strains isolated from the digestive tract of chickens to Caco-2 cell.

Strain	Adhesion capacity (%)
L. salivarius	47.77[b]
L. reuteri	36.00[c]
L. acidophilus	48.78[b]
L. ingluviei	56.80[a]
L. saerimneri	34.55[c]
Pooled SEM	1.02
P-value	0.035

Pooled SEM, standard error of the means.
[a,b,c] Means with different superscripts in a column are significantly different ($P < 0.05$).

3.5.2 Evaluation of probiotics *L. ingluviei* and *L. salivarius* in broiler chickens

3.5.2.1 Cecal microbial populations

The effects of probiotics *L. ingluviei* and *L. salivarius* on the cecal microbial population of broilers are shown in Table 3.7. It was found that the oral administration of both *L. ingluviei* and *L. salivarius* can increase *Lactobacillus* and *Bifidobacterium* population in cecal content. In addition, these probiotics can also decrease the number of *Enterobacter* and *E. coli* compared to the negative control group (Table 3.7).

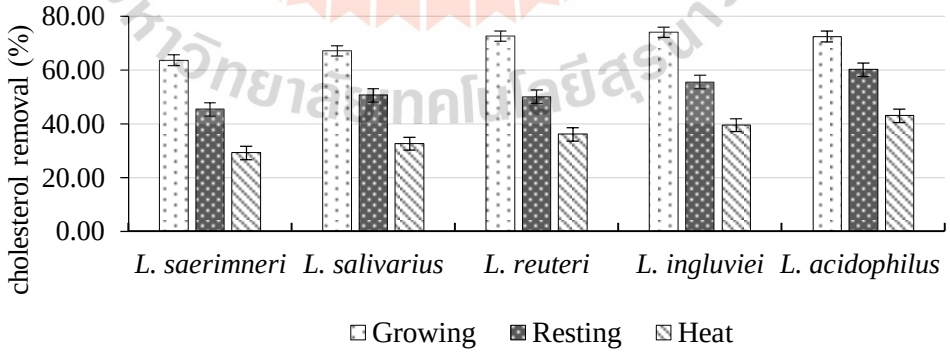

Figure 3.1 The percentage of cholesterol removal of growing, resting and dead cells of *Lactobacillus* strains isolated from the digestive tract of chickens cultured in De Man, Rogosa and Sharpe supplemented with 100 g/mL water-soluble cholesterol and 0.3% oxgall.

Table 3.7 Effects of probiotics *L. ingluviei* and *L. salivarius* administration on cecal microbial population of broiler chickens at 14 days of age (log10 of copy number/g DNA extract).

Item	Control	*L. ingluviei*	*L. salivarius*	Pooled SEM	P-value
Lactobacillus	7.80[b]	9.86[a]	9.97[a]	0.11	0.016
Bifidobacterium	6.70[b]	8.86[a]	8.87[a]	0.13	0.017
Enterobacter	8.79[a]	5.78[c]	7.10[b]	0.25	0.029
E. coli	8.25[a]	7.44[b]	6.66[c]	0.17	0.013

Pooled SEM, standard error of the means.

[a,b,c] Means with different superscripts in a row are significantly different (P < 0.05)

3.5.2.2 SCFAs analysis

The effects of *L. ingluviei* and *L. salivarius* on cecal VFA concentrations of broilers are shown in Table 3.8. These showed that the administration of *L. ingluviei* and *L. salivarius* can increase valeric acid and SCFA concentrations in the cecal content (P < 0.05). However, there were no significant differences in the concentrations of acetic acid, propionic acid, butyric acid or branched SCFA.

Table 3.8 Effects of probiotics *L. ingluviei* and *L. salivarius* administration on cecal SCFA concentrations (µmol/g of digesta) of broiler chickens at 14 days of age.

Item	Control	*L. inguluviei*	*L. sarivarius*	Pooled SEM	P-value
Acetic acid	30.88	39.11	38.83	3.51	0.569
Propionic acid	4.99	5.46	3.20	0.50	0.216
Butyric acid	4.39	6.73	4.35	0.73	0.346
Valeric acid	40.31[b]	116.58[a]	107.37[a]	11.52	<0.001
Branched SCFA[1]	0.52	0.94	0.66	0.21	0.728
Total SCFA	81.08[b]	168.82[a]	154.40[a]	13.39	<0.001

[1] Branched SCFA = isobutyric acid + isovaleric acid

Pooled SEM, standard error of the means.

[a,b] Means with different superscripts in a row are significantly different (P < 0.05)

3.6 Discussion

Probiotics are a potential alternative feed additive to improve the gut health of animals and address issues related to the intensive animal rearing system and the ban on the use of antibiotics as growth promoters. It is well known that LAB is the main probiotic used in animal feed, and that their function is associated with conductive properties for the host health, gut acidification, elimination of unfavorable microflora, improvement of digestive and metabolic processes, stimulation of immunological response, enhancement of intestinal barrier function and maintenance of natural microbial balance (Dowarah et al., 2018). Although several LAB such as *Lactobacillus, Lactococcus, Bifidobacterium, Pediococcus, Enterococcus* and *Propionibacterium* are already established and widely used as probiotics in animal feed (Mountzouris et al., 2007; Dowarah et al., 2018). Unfortunately, application in the practical field may vary depending on several factors such as the animal host, diet, hygiene conditions, antibiotic treatment, and stress factors (Piqué et al., 2019). Therefore, there is still a need to search for new probiotic strains with the greatest potential and benefits for the poultry industry. Probiotics are likely to function efficiently depending on their source and the specificity of their host (Morelli, 2000; Mountzouris et al., 2007) . In the present work, the study of the functional properties of LAB strains from various types of poultry (broilers, slow-growing chickens and laying hens) were conducted according to acid and bile tolerance, antimicrobial activity, adhesion to epithelial cells and additional characteristics on cholesterol removal. In this study, the 200 LAB isolates belong to five strains, including *L. acidophilus* (63 isolates), *L. ingluviei* (2 isolates), *L. reuteri* (58 isolates), *L. salivarius* (72 isolates) and *L. saerimneri* (5 isolates).

Acid and bile salt tolerances are the most important criteria for selecting strains of probiotic capable of survival in the gastrointestinal tract. The pH of the GIT in chicken varies from 2.5 to 6.5. Retention times and pH in GIT of the chickens have been recorded as follows: crop pH 4.8 and 30 min; proventriculus pH 4.4 and 15 min; gizzard pH 2.6 and 90 min; small intestine pH 6.2 and 90 min; and large intestine pH 6.3 and 15 min (Svihus, 2014). In this study acidic conditions were tested at pH 2.0, 2.5, 3.0 and 3.5 in order to cover the pH values in the gizzard, as well as the tolerance of *Lactobacillus* strains to bile salt (at levels of 0.3% and 1%). It was found

that all the five *Lactobacillus* strains tested showed resistance to pH 3.0 at 90 min, but their viability declined to pH 2.0. In particular, *L. acidophilus* and *L. ingluviei* exhibited the strong acid resistance followed by *L. salivarius*. The findings of this study are consistent with those of other previous studies, Ehrmann et al. (2002) indicated that *Lactobacillus* strains isolated from ducks can survive for 4 h when incubated at pH 2.0 and 3.0, and few of them can even survive for an hour at pH 1.0. In addition, Hutari et al. (2011) reported that *L. salivarius* and *L. fermentum* isolated from chickens were able to survive at pH 2.5 for 3 h. Our findings also revealed that all *Lactobacillus* strains can resist various bile salt levels (0.3 and 1%), although the survival rate decreased as the bile salt concentration increased (average survival rate of 81% and 72% in bile salt levels of 0.3% and 1%, respectively). These results are similar to those result obtained by Erkkilaè & Petaè, (2000) with the strains of *Pediococcus acidilactici, L. curvatus* and *L. sake* being the most resistant to 0.3% bile salt at pH 6.0. Pennacchia et al. (2004) reported *Lactobacillus* strains (*L. plantarum* and *L. brevis*) were able to grow in a MRS agar supplemented with 0.3% bile salt. This study indicated that five strains of *Lactobacillus* isolated from the cecum and ileum of chickens have good resistance to acid pH and bile salt, as these properties helped them survive in the gastrointestinal tract of chickens and they adhered to the intestinal cells while exerting beneficial effects.

Probiotics with antibacterial activity against pathogens are a promising alternative to antibiotics (Vieco-Saiz et al., 2019). Interestingly, the *Lactobacillus* isolates in this study were highly detectable in cases of *L. salivarius*, *L. ingluviei* and *L. acidophilus* which showed significant antibacterial activities against all the tested pathogenic bacteria (*E. coli, S. aureus*, *C. jejuni*, *C. perfingen* and *S. enteritidis*). Antagonistic activity by LAB is sustained by the secretion of different antimicrobial substances including short chain fatty acids, bacteriocins, hydrogen peroxide and antimicrobial peptide (Vieco-Saiz et al., 2019). Once the pH of the cell free supernatant was neutralized (pH 6.5), all the *Lactobacillus* isolates lost their antagonistic activity against the pathogens tested, with the exception of *L. ingluviei* and *L. acidophilus* which demonstrated weak and moderate antagonistic activity against pathogenic bacteria. In addition, LAB strains from poultry also showed efficacy on antimicrobial activity in the pH range of 1.0 to 4.0, but complete loss of activity at

5.0 to 11.0 pH. The benefit of *Lactobacillus* isolates as shown by our study on antimicrobial activity are likely attributable to the function of organic acid secretion, bacteriocins and other antimicrobial substances (Vieco-Saiz et al., 2019). The secretion of bacteriocin by LAB is highly affected by temperature, pH, incubation time and certain other environmental factors. It was also reported that there is optimum secretion of bacteriocin when LAB remains in the pH range of 5.0 and 6.0 (Onwuakor et al., 2014). In the present study, all five isolated strains showed antibacterial activity against various bacterial pathogens, including *E. coli, S. aureus*, *C. jejuni*, *C. perfingen* and *S. enteritidis* under normal conditions, which almost possess the problem towards the digestive tract of poultry.

An important requirement of probiotics is that the isolated strain must be safe for animal and human consumption. Antimicrobial resistance is an increasingly serious global threat to human, animal and environmental health. Antibiotic resistance properties in various *Lactobacillus* species appeared to be associated with drug-resistant genes which are mainly located on the chromosome. In the current study, a group of drugs (such as ampicillin, erythromycin, tetracycline and chloramphenicol), which are commonly used to treat the disease in poultry, have been tested for susceptibility to the five *Lactobacillus* strains. Our study found that all the *Lactobacillus* strains were resistant to a broad range of antibiotics related to various modes of action, such as ß-lactam antibiotics (ampicillin) and macrolide antibiotic (erythromycin). In addition, all strains showed intermediate susceptibility and susceptibility to broad-spectrum antibiotics (tetracycline and chloramphenicol). It has been reported that *Lactobacillus* strains can produce ß-lactamase which is resistant to ß-lactam antibiotics including ampicillin (Dec et al., 2017). Dowarah et al. (2018) also reported high sensitivity to penicillin, ampicillin and chloramphenicol by LAB strains isolated from pigs and poultry. Nevertheless, it has also been documented that *Lactobacillus* are generally susceptible to ampicillin (Dec et al., 2017). Jose et al. (2015) reported that LAB strains isolated from milk, animal rumen and most commercial probiotics exhibited intrinsic resistance to streptomycin, gentamicin and vancomycin, which are aminoglycosides and glycopeptides. The intrinsic antibiotic resistant nature of LAB probiotics suggests their application for both therapeutic and preventive purposes in the treatment and control of intestinal

infections, especially when administered concurrently with antibiotics and that GIT microflora recovery can be enhanced by this probiotic.

Adhesion of probiotic strains to the intestinal mucosa is considered as a prerequisite characteristic for potential probiotic microorganisms. As probiotics adhere to the intestinal mucosa, their function can have several beneficial effects on the host gut, such as the prevention of pathogenic colonization, the maintenance of gut mucosal immunity and the healing of damaged mucous membranes (Monteagudo-Mera et al., 2019). In this study we found that *L. ingluviei* exhibited the strongest adhesion to Caco-2 cells followed by *L. salivarius* and *L. acidophilus*, whereas *L. saerimneri* and *L. reuteri* expressed less strength of adherence. This may indicate that the good adhesiveness of *L. ingluviei* and *L. salivarius* suggest beneficial functions for the health of the host in comparison to other isolated strains. A previous study demonstrated that single or multi-strain LAB probiotics showed excellent adhesion to COLO 205 cells (an epithelial colorectal adenocarcinoma), which could indicate their ability to colonize intestinal epithelial cells and act as a barrier to protect intestinal mucosa from pathogens (Pringsulaka et al., 2015). Noohi et al. (2021) reported that *L. brevis* and *L. reuteri* strains showed significant attachment to Caco-2 cells and a high capacity for biofilm formation. In general, LAB adhesion is a complex process initiated from the foremost bacterial contact with the cell membrane of the host enterocytes, followed by various surface interactions. Most LAB can produce cell surface proteins that aid bacteria in binding to intestinal epithelial cells and activate immunoregulation to protect pathogens.

Recently, the incidence of cardiovascular disease in humans has increased, which has a strong correlation with the serum cholesterol level. As a result, much attention has been given to the screening of probiotics that can increase the removal of cholesterol. In this study we found that all five *Lactobacillus* strains have the potential of cholesterol removal either growing or non-growing (resting or dead cells) in which *L. reuteri, L. ingluviei* and *L. acidophilus* were more effective in cholesterol removal than the other strains. This additional function of *Lactobacillus* strains could be useful in applications to improve the quality of production with low meat or egg cholesterol. The function of removing cholesterol is probably due to the bile salt hydrolase (BSH) in probiotics particularly in the strains *Lactobacillus* and

Bifidobacterium, in which this enzyme is responsible for the hydrolysis of conjugated bile acids into deconjugated bile acids and amino residues, whose deconjugated forms are less soluble and less absorbed by the intestine, leading to the elimination of excreta (Liong and Shah, 2005). As a result, cholesterol is now used to synthesize new bile acids in a homeostatic response, leading to a reduction in serum cholesterol in animals (Liong and Shah, 2005). The highest cholesterol removal properties of growing cells found in this study indicate that the degree of bound cholesterol may depend on cell growth. It is interesting to note that the resting and dead cells of *Lactobacillus* isolates still maintain a function in cholesterol removal, which is probably due to their cell membrane still having the ability to bind cholesterol. This is in accordance with the report of Lye et al. (2010) who stated that the membrane bilayer of probiotic cells (*Lactobacillus* and *Bifidobacterium*) have the ability to incorporate cholesterol, especially in the areas of the phospholipid tail, upper phospholipids and polar heads.

In this study, the two strains (*L. ingluviei* and *L. salivarius*) were selected to assess their efficacy in broiler chickens. Since these two probiotic strains have shown the greatest advantages in the *in vitro* test, although previous studies still lack information about broilers. Probiotics *L. ingluviei* and *L. salivarius* were found to increase the cecal population of *Lactobacillus* and *Bifidobacterium* while reducing *Enterobacteria* and *E. coli* relative to the control group. This is consistent with the results of the *in vitro* tests in the present study, which showed that the *L. ingluviei* and *L. salivarius* were against all tested bacterial pathogens (*E. coli, S. aureus*, *C. jejuni*, *C. perfingen* and *S. enteritidis*). Angelakis et al. (2012) reported that in mice inoculated with *L. ingluviei*, the number of *Lactobacillus* and Firmicutes in the feces increased significantly. This is in accordance with the findings of Shokryazdan et al. (2021) who reported that supplementation of the three *L. salivarius* strains at levels of 0.5 or 1 g/kg diet can increase the populations of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* and decrease harmful bacteria such as *E. coli* and total aerobes. Sureshkumar et al. (2021) also reported that the oral administration of *L. salivarius* can increase the population of beneficial bacteria and reduce pathogenic bacteria in the fecal microbiota.

L. ingluviei and *L. salivarius* were observed to improve the production of valeric acid and total SCFAs. In general, SCFAs are metabolites of bacteria in the gut of which the concentration may vary depending on the prevailing microbiota, the type of fermentation substrate and the period of fermentation. In this study, a significant increase in valeric acid and total SCFAs in cecal digesta may be associated with an increase in the population of *Lactobacillus* and *Bifidobacterium*, which were more abundant in chicken groups administered with *L. ingluviei* and *L. salivarius.* SCFAs have been reported to decrease cecal pH and indirectly inhibit pathogenic microorganisms susceptible to pH changes, as well as passing into the cells of pathogens causing a change of positive and negative ions resulting in cells becoming unbalanced and inhibiting the growth of pathogens (Deleu et al., 2021). In addition, Tsukagoshi et al. (2020) reported that *L. ingluviei* C37 exerted anti-inflammatory effects by modulating cytokine profiles in a mice model. It is interesting to note that the *L. ingluviei* and *L. salivarius* increased the concentration of valeric acid approximately three times more than the control group. The valeric acid is mostly produced by certain members of gut microbiota belonging to Firmicutes bacteria (Qing et al., 2019). In addition, valeric acid was identified as a potential therapeutic target for a variety of disease pathologies. The findings of Onrust et al. (2018) revealed supplementation of valeric acid glyceride esters can improve feed efficiency, gut morphology and the density of glucagon-like peptide-2-producing enteroendocrine cells and reduce the incidence of necrotic enteritis.

This suggests that *L. ingluviei* and *L. salivarious* could be beneficial in improving gut health and preventing disease. Future studies will be necessary to investigate their efficacy on the growth performance of broilers.

3.7 Conclusion

In this study, the *Lactobacillus* strains isolated from the gut contents of chickens are safe and possess probiotic properties including tolerance to acid and bile salt, antibacterial activity, adhesion activity, antibiotic resistance and cholesterol removal. *L. ingluviei*, *L. acidophilus* and *L. salivarius* exhibited a strong resistance to acid and bile salt, antibacterial activity, antibiotic tolerance and high adherence to intestinal epithelial cell. *In vivo* evaluation of the efficacy of both selected probiotics

L. ingluviei and *L. salivarius* in broiler chickens was found to improve gut health by increasing the population of *Lactobacillus* and *Bifidobacterium* with an associated increase in valeric acid. The results indicate that all five *Lactobacillus* strains, especially *L. ingluviei* and *L. salivarius* have probiotic properties. Future studies should assess their potential for antibiotic replacement and improvement of broiler growth performance.

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CHAPTER IV

LACTOBACILLUS INGLUVIEI C37 ALLEVIATES IMMUNOLOGICAL STRESS AND IMPROVES INTESTINAL BARRIER GENE EXPRESSION IN LIPOPOLYSACCHARIDE CHALLENGED BROILER CHICKENS

4.1 Abstract

This study aimed to investigate the effects of *L. ingluviei* 37 (LIC37) on modulation of host inflammatory responses and intestinal barrier gene expression of broilers induced with LPS. Chickens were randomly allocated to four treatments: 1) control (orally administered PBS) 2) orally administered probiotic LIC37 10^8 CFU/bird/day 3) orally administered probiotic LIC37 10^9 CFU/bird/day and 4) negative control (orally administered PBS). At 14 days of age, the chickens in treatments 2, 3 and 4 were injected intraperitoneally with *E. coli* O55:B5 LPS (1 mg/kg body weight), and control chickens were injected with sterile PBS. At 24 h after the LPS injection, serum was collected to analyze the total Ig and lysozyme activity. After decapitation, liver, spleen and bursa were removed and the relative weight of the organs was measured. The liver and intestinal mucosa were also collected for the measurement of proinflammatory cytokine and tight junction (TJ) proteins, respectively. It was found that LIC37 can attenuate the increase relative weight of spleen, reduce LITAF, IL-17F and TNF-α, and increase IL-10 expression in LPS-induced chickens. The LPS-challenged LIC37 chicken groups showed a significant increase in intestinal TJ proteins and MUC2 mRNA. This finding indicates that LIC37 can improve mRNA expression of intestinal immune and TJ protein during LPS-mediated immunological challenge state.

Keywords: *L. ingluviei*, Broiler, LPS, Probiotic, Tight junction

4.2 Introduction

The use of antibiotics as growth promoters (AGPs) is now prohibited, which increases the risk of pathogenic infections, health problems and decreases the growth of broilers. Several novel natural feed additives have been identified to address the issues. Probiotics are one of the alternative feed additives which has the potential to improve immune and health of gastrointestinal tract (GIT) (Jeni et al., 2021). Health-promoting probiotics not only contribute to a healthy gut microflora, but also modulate the host's immune response to prevent pathogenic infection, thus improving growth performance. The most popular probiotics are gram-positive lactic acid bacteria such as species *Enterococcus, Lactobacillus, Pediococcus*, *Streptococcus*, *Lactococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Carnobacterium* and *Tetragenococcus*, which are natural microflora in the GIT of humans and animals characterized by the production of lactic acid (Huyghebaert et al., 2011; Li et al., 2020). However, not all LAB are probiotics, their characteristics and safety profiles also need to be assessed. To qualify as probiotics, candidate bacterial strains must be able to tolerate acid and bile salt, coaggregation with pathogens, antimicrobial activity, adherence to intestinal mucosa and antibiotic resistance. In addition, some beneficial features related to gut health should also be assessed, such as modulation of gut barrier functions, reduce intestinal leakage, alleviate oxidative stress, anti-inflammatory properties, etc (Wang and Li, 2022).

Lactobacillus ingluviei is a species of lactobacillus not widely known in poultry. It has been reported that *L. ingluviei* can modulate intestinal microbiota and increases body weight (BW) in new-born ducks and chickens (Angelakis and Raoult, 2010), as well as improves BW and liver weight in mice (Angelakis et al., 2012). In addition, *L. ingluviei* also has beneficial health-effects, such as microbiota alteration, anti-*Salmonella* activity and antioxidant activity in animal models (Angelakis and Raoult, 2010; Angelakis et al., 2012; Thomas et al., 2019). *L. ingluviei* C37 (LIC37) is the *Lactobacillus* strain isolated from the cecal content of chickens are safe and possess probiotic properties including tolerance to acid and bile salt, antibacterial activity,

adhesion activity, antibiotic resistance and cholesterol removal (Sirisopapong et al., 2023). Assessment of *L. ingluviei* efficacy in broiler chickens was found to improve gut health by increasing the population of *Lactobacillus* and *Bifidobacterium* with an associated increase in valeric acid and total SCFAs (Sirisopapong et al., 2023). Previously, we tested LIC37 in mice challenged with lipopolysaccharide (LPS) and found its beneficial effects on in vitro anti-inflammatory properties by suppressing the production of pro-inflammatory cytokines (tumor necrosis factor-α (TNF- α) and interleukin (IL)-6) and anti-inflammatory cytokines (IL-10) (Tsukagoshi et al., 2020).

Besides the consequence of health problem of the prohibition of the use of AGPs in broilers, the damage caused by immunological and oxidative stresses has received significant attention in recent years (Bai et al., 2017). Particularly, immunological stress can alter nutrient distribution, decrease production performance, induce various diseases, and result in a high mortality rate. Chickens challenged with LPS are an excellent model to study immunological stress, and these models have been extensively studied (Kaiser et al., 2012; Tong et al., 2022). Indeed, the LPS found in the cell wall of gram-negative bacteria can induce the activation of nuclear factor **K**B (NF-**K**B), a transcription factor presents in macrophages and mononuclear cells that induces the expression of pro-inflammatory cytokines, including TNF-α. Additionally, LPS also acts to reduce gut tight junctions (TJ) proteins. In general, intestinal permeability and barrier function are regulated by TJ proteins and downregulated by LPS, as well as TNF-α (Park et al., 2010). The damaged intestinal barrier is usually caused by changes in the expression of TJ proteins (Slifer and Blikslager, 2020). TJ regulates the transport of substances, connect intestinal epithelial cells, and affects the gut permeation of external substances in the cell complex. The TJ is comprised of tight junction proteins such as occludin, claudin, and zonula occludens-1 (ZO1). These proteins play an important role in regulating intestinal permeability and barrier function. The gastrointestinal system is divided into two layers and is mainly covered by a mucous membrane. The inner layer is laminated, thick, and tightly adhered to the epithelial cells, preventing bacteria from invading. While the outer layer is the first line of defense of the host innate immune system. Mucin 2 (MUC2) described as the intestinal mucin gene and the abnormalities

in its expression indicate the occurrence of certain gastrointestinal diseases. MUC2 gene expression is essential for the maintenance of gel layer structure on the intestinal surface and has been used as a marker for gut health. Decreased expression of MUC2 indicates more extensive tissue damage and injuries that lead to inflammation, oxidation, apoptosis, and autophagy (Wibowo et al., 2019; Leon-Coria et al., 2021). Intestinal inflammation can also raise the temperature of animal body, which reduces feed intake and ultimately leads to lower growth performance.

However, few studies have examined the probiotic properties of *L. ingluviei* and no studies have focused on the immune effects of this strain in chickens.

4.3 Objective

This study aimed to investigate the efficacy of LIC37 on modulation of host inflammatory responses and gene expression of the intestinal barrier of chickens induced with LPS endotoxin from *E. coli* O55:B5.

4.4 Materials and methods

All experiments were conducted according to the principles and guidelines approved by the Animal Care and Use Committee of Suranaree University of Technology, Nakhon Ratchasima, Thailand (SUT-IACUC-0014/2022).

4.4.1 Bacterial strain and culture conditions

The probiotic bacteria used in this study was LIC37 isolated from the cecal content of chickens. LIC37 was kept in stock solution with sterile glycerol (50% v/v), and the stock culture was stored at -20°C in sterile screw cap tubes. To activate, LIC37 was pre-cultured overnight at 37°C in de Man Rogosa Sharpe (MRS) broth, then culture of LIC37 was inoculated into MRS broth for 18 h at 37°C. The bacterial cells were pelleted by centrifugation at 5000 × g for 15 min at 4°C and washed twice with sterile PBS. The cells were then resuspended in PBS and adjust the concentration of LIC37 to 1.0×10^9 CFU/mL.

4.4.2 Animal management, experimental design and LPS challenge

A total of forty, one-day-old male broilers was used in this experiment. All chicks were provided with continuous light for 23 hours per day from day 1 to day 10 and were reduced to 18 hours per day from day 11 onwards. Birds were vaccinated against Newcastle disease and Infectious Bronchitis on day 7. All chickens in each treatment received the same basal diet. The diets were formulated to meet or to exceed the minimum nutrient requirements of broiler chickens as recommended by NRC (1994) and cobb 500 broiler nutrition specification (Cobb-Vantress, 2018) for starter (0-10 days) and grower (11-14 days) periods. Feed (mash form) and water were provided *ad libitum* throughout the experimental period. The calculated and analyzed values for the experimental diets are presented in chapter III, Table 3.1. The chicks were weighed and randomly allocated to 4 treatments: 1) control (orally administered PBS) 2) negative control (orally administered PBS) 3) orally administered probiotic LIC37 10^8 CFU/bird/day and 4) orally administered probiotic LIC37 10^9 CFU/bird/day. At 14 days of age, the chickens in group 2, 3 and 4 were injected intraperitoneally with *E. coli* O55:B5 LPS (Sigma Aldrich, St. Louis, MO, USA) at 1 mg/kg body weight, while birds in control group were injected with sterile PBS.

4.4.3 Sample collection

On day 15, six chickens were randomly selected from each group at 24 h post- LPS injection and used to collect samples. Blood samples were collected by jugular vein immediately following euthanasia. The liver, spleen and bursa were removed from each bird. The organ weights were immediately measured after dissection and expressed based on relative to body weight (g of organ/kg of BW). The liver was immediately stored at −80°C until it was used for RNA isolation and gene expression analysis. Approximately 10 cm of ileal segments were opened longitudinally and the contents were flushed with ice-cold PBS. Mucosa was scraped and rapidly frozen in liquid nitrogen and stored at −80°C until the gene expression was tested.

4.4.4 Serum total Ig and lysozyme activity

The blood sample collected in tube was taken by centrifugation at 2,000 × g for 15 min at 4°C to obtain serum and stored at −80°C until analysis. Serum samples were used for total immunoglobulin (Ig) analysis using a total protein kit,

(Micro Lowry, Peterson's Modification, Sigma) and were measured for lysozyme activity using a lysozyme assay kit. The methodology used in the kit was described by Kreukniet et al. (1994) using *Micrococcus lysodeikticus* cells as substrate. A series of concentrations of crystalline lysozyme dissolved in the phosphate buffer was used as the standard curve. The standard dilution series of the crystalline lysozyme and plasma samples was analyzed for their lysozyme activity during the lysis of *Micrococcus lysodeikticus.*

4.4.5 RNA extraction and qRT-PCR

Total RNA was isolated from intestinal tissue and liver using a commercial RNA isolation kit (NucleoSpin RNA, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purified total RNA was reverse transcribed using 5 µg of total RNA to cDNA using Quantinova reverse transcription kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Oligonucleotide primer sequences used for chicken pro-inflammatory cytokines, including interleukin (IL)-17F, IL-10, lipopolysaccharide (LPS)-induced tumor necrosis factor-α factor (LITAF), induced tumor necrosis factor-α (TNF-α) and intestinal tight junction (TJ) proteins junctional adhesion molecule 2 (JAM2), occludin, ZO1, and MUC2 are shown in Table 4.1. The qPCR assay was performed with LightCycler® 480 Instument II (Roche Diagnostics, GmbH, Mannheim, Germany). The PCR reaction was performed in white LightCycler® 480 Multiwell Plate 96 plates (Roche, Mannheim, Germany) in a final volume of 10 µL using LightCycler® 480 SYBR Green I Master. Each reaction included 5.0 µL of 2×SYBR Green Master Mix, 0.4 µL of 10 µM forward primer, 0.4 µL of 10 µM reverse primer, 2.0 µL of DNA samples and 2.2 µL of nuclease-free water. Each analysis was performed in triplicate. The average gene expression level relative to GAPDH of each sample was calculated using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

4.4.6 Statistical Analysis

Data were analyzed using the one-way ANOVA of SPSS version 18.0. Significant differences among treatments were assessed by Tukey's post hoc test. A threshold level of $P < 0.05$ was used to determine the significance.

Table 4.1 Primers for real-time quantitative PCR assay

Type	Target gene	Primer sequence (5'-3')
Reference	GAPDH	F: GGTGGTGCTAAGCGTGTTAT
		R: ACCTCTGCCATCTCTCCACA
	B-actin	F: AGACCACCTTCAACTCCATCATG
		R: TCCGATCCAGACAGAGTATTTACGC
Proinflammatory	IL17F	F: TGAAGACTGCCTGAACCA
		R: AGAGACCGATTCCTGATGT
	LITAF	F: TGTGTATGTGCAGCAACCCGTAGT
		R: GGCATTGCAATTTGGACAGAAGT
	TNF- α	F: GAGCGTTGACTTGGCTGTC
		R: AAGCAACAACCAGCTATGCAC
Anti- inflammatory	IL-10	F: GCTGTCACCGCTTCTTCACCT
		R: GGCTCACTTCCTCCTCCTCATC
TJ proteins	Occludin	F: ACGGCAGCACCTACCTCAA
		R: GGGCGAAGAAGCAGATGAG
	ZO1	F: CTTCAGGTGTTTCTCTTCCTCCTC
		R: CTGTGGTTTCATGGCTGGATC
	JAM2	F-AGCCTCAAATGGGATTGGATT
		R-CATCAACTTGCATTCGCTTCA
Mucin	MUC2	F: TTCATGATGCCTGCTCTTGTG
		R: CCTGAGCCTTGGTACATTCTTGT

F, forward; R, reverse

4.4.7 Experimental location

The experiment was conducted at Suranaree University of Technology's poultry farm, the Center for Scientific and Technological Equipment Building 11 and 14, Suranaree University of Technology.

4.4.8 Experimental period

The experiment was done from December 2022 to March 2023.

4.5 Results

4.5.1 Effects of *L. ingluviei* C37 on relative immune organ weight in broiler chickens

LPS challenge significantly increased the relative weight of the liver and spleen ($P < 0.05$) (Table 4.2). A reduction in spleen weight was observed in chicken injected with LPS and administered orally with LIC37 at a level of 10^8 CFU/bird/day, but it remained heavier than the positive control (PBS injection). However, there was no significant difference in the relative weight of bursa among groups ($P > 0.05$).

4.5.2 Effects of *L. ingluviei* C37 on total serum Ig and lysozyme activity in broiler chickens

The effects of LIC37 on total serum Ig and lysozyme activity are shown in Table 4.3. Compared with the control, the LPS challenge decreased lysozyme activity ($P < 0.05$), whereas oral administration of LIC37 at both levels (10^8 and 10^9 CFU/bird/day) increased serum lysozyme activity ($P < 0.05$) with higher than the control (PBS injection) and negative control (LPS injection only). However, there was no significant effect on serum total Ig.

Table 4.2 Effects of *L. ingluviei* C37 on the relative weight of immune organs (g/kg of body weight) of broiler chickens challenged with lipopolysaccharide at 14 d of age[1].

Item	Control	Negative control	*L. ingluviei* C37		Pooled SEM
			10^8 CFU	10^9 CFU	
Liver	2.53[b]	3.30[a]	3.43[a]	3.18[a]	0.095
Spleen	0.062[c]	0.105[a]	0.086[b]	0.098[ab]	0.004
Bursa	0.20	0.19	0.20	0.19	0.006

a, b, c Means with different superscripts in a row are significantly different at $P < 0.05$.
[1]Control: orally administered PBS; negative control: orally administered PBS (injected LPS); orally administered probiotic LIC37 at 10^8 and 10^9 CFU/bird/day (injected LPS), respectively.

Table 4.3 Effects of *L. ingluviei* C37 on serum total Ig and lysozyme activity in broiler chickens challenged with lipopolysaccharide at 14 d of age[1].

Item	Control	Negative control	*L. ingluviei* C37		SEM
			10^8 CFU	10^9 CFU	
Total Ig (mg/ml)	7.82	7.27	8.12	7.96	0.31
Lysozyme (µl/ml)	7.7^b	6.37^c	8.87^a	8.86^a	0.13

a, b, c Means with different superscripts in a row are significantly different at $P < 0.05$.
[1]Control: orally administered PBS; negative control: orally administered PBS (injected LPS); orally administered probiotic LIC37 at 10^8 and 10^9 CFU/bird/day (injected LPS), respectively.

4.5.3 Effect of *L. ingluviei* C37 on cytokine transcript levels

The expression levels of cytokine transcripts in the liver are shown in Fig 4.1. At 24 h post LPS injection, significant differences in pro-inflammatory cytokine including LITAF, IL-17F and TNF-α involved in pathological inflammatory process were observed. As compared to control, the level of expression of pro-inflammatory cytokines in LPS-injected chickens was significantly higher ($P < 0.05$). However, the lower expression levels of LITAF, IL-17F and TNF-α were observed in chicken groups injected with LPS and administered orally with LIC37 compared to the negative control (LPS injection only). In addition, a significant increase in the expression level of IL-10 mRNA was observed in the LPS-chicken groups administered with LIC37 compared to the negative control ($P<0.05$), which expression was also similar to the control ($P > 0.05$).

4.5.4 Effect of *L. ingluviei* C37 on intestinal tight junction proteins and mucin expression in ileum

The relative mRNA expression of different intestinal TJ proteins (JAM, occludin and ZO1) and the MUC2 gene is shown in Fig. 4.2. LPS challenge significantly reduced the expression levels of the junction protein and MUC2 in the ileum ($P < 0.05$). It is interesting to note that in chicken groups injected with LPS administered with LIC37 showed a significant increase in intestinal TJ proteins and MUC2 mRNA

post-infection similar to the control ($P > 0.05$) This indicates the potential effect of LIC37 for maintaining normal expression of TJ proteins. Interestingly, LIC37 can upregulate the occludin mRNA expression higher than the control ($P < 0.05$).

4.6 Discussion

Immunological stress is a critical issue that threatens poultry industry, leading to poor performance and high mortality. This had led to numerous studies examining novel probiotics to reduce the deleterious consequences of immunological stress. *Lactobacillus*, an important member of probiotic bacteria, plays an essential role in the immunomodulation of the intestinal mucosa (Suissa et al., 2022), which can attach intestinal epithelial cells and modulate their function, directly triggering immune responses by M cells, macrophages, or dendritic cells (Rescigno, 2011). In the present study, LIC37 can attenuate the intestinal damage of LPS-challenged chickens by increasing in intestinal TJ proteins and MUC2 mRNA expression. In addition, LIC37 can also decrease the relative weight of spleen and reduce liver inflammation in LPS-induced chickens.

In this study we found the potential of LIC37 to attenuate the negative effect on spleen weight in LPS-challenged chickens. Increased spleen weight in the LPS-challenged chickens was associated with simultaneous increase in pro-inflammatory cytokine production. This indicates the elevated activity of this organ during the systemic inflammatory response for an urgent need to cytokine synthesis (Liu et al., 2015). In general, spleen and bursa function as lymphoid tissues where lymphocytes are activated with antigens derived from an infection (Härtle et al., 2022). Attenuation increases the relative weight of spleen in chicken injected with LPS and administered orally with LIC37 at a level of 10^8 CFU/bird/day compared to the negative control (LPS injection only) indicates a beneficial effect of LIC37 on the homeostatic mechanisms of this immune organ which could be attributed to the anti-inflammatory function of LIC37.

A similar result was also observed in the report of Li et al. (2015) which revealed that the relative weight of spleen in LPS-challenged broilers was reduced by *Bacillus amyloliquefaciens* supplementation in diet. In the same way Chen et al.

(2012) also found that oral administration of LAB can recover the spleen from *Salmonella* invasion in broilers.

The liver plays an important physiological role in detoxification of various small molecules, in particular hepatocytes are involved in the clearance of intestinal-derived endotoxin (Jirillo et al., 2002). Increased relative liver weight in LPS-challenged chickens found in this study can indicate the liver injury. Huang et al. (2017) revealed that LPS is involved in acute liver damage and significantly altered liver structure and function in young chickens. Dose-related increases in liver weight are also commonly observed in repeated-dose toxicity studies (Greaves, 2012).

Generally, LPS is transferred to the liver through the hepatic portal vein, and leads to induce acute liver injury and the complex mechanisms include activating Kupffer cells, which are known as resident hepatic macrophages by combining with TLR-4 complexes on the cell surface, and subsequently induce inflammatory cytokine production resulting in increased expression of LITAF, IL-17F and TNF-α RNA (Tsutsui and Nishiguchi, 2014; Li et al., 2017). However, the LIC37 did not attenuate the increase in liver weight in LPS-challenged chickens, although the positive effect was found in the decreased expression of inflammatory cytokine in the liver, the precise pathway remains poorly described. This is accordance with Angelakis et al. (2012) revealed a significantly increased in relative liver weight in mice inoculated with *L. ingluviei*, which was accompanied by an increased expression of key lipogenic markers, such as FAS and SREBP1c, which have been implicated in the development of steatosis. In addition, Awad et al. (2009) also reported that the supplementation of *Lactobacillus* in diets can increase the relative liver weight of broilers.

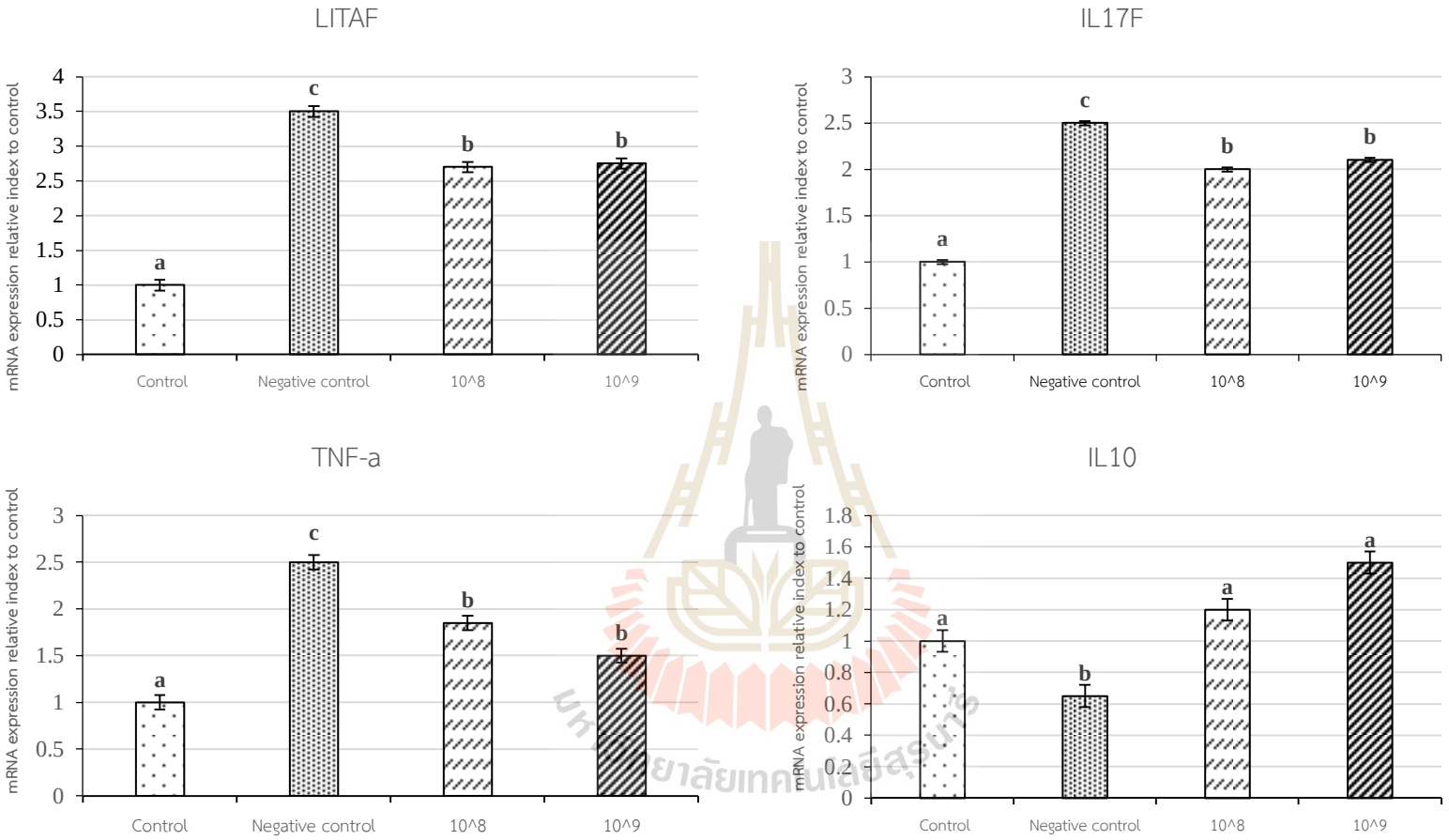

Figure 4.1 Effects of LIC37 on the levels of transcripts of pro-inflammatory cytokines (LITAF, IL17F, TNF-α) and anti-inflammatory (IL-10) cytokines expression in liver of chicken. [a, b, c] means with no common superscripts are significantly different at $P < 0.05$. Control: orally administered PBS; negative control: orally administered PBS (injected LPS); orally administered probiotic LIC37 at 10^8 and 10^9 CFU/bird/day (injected LPS), respectively.

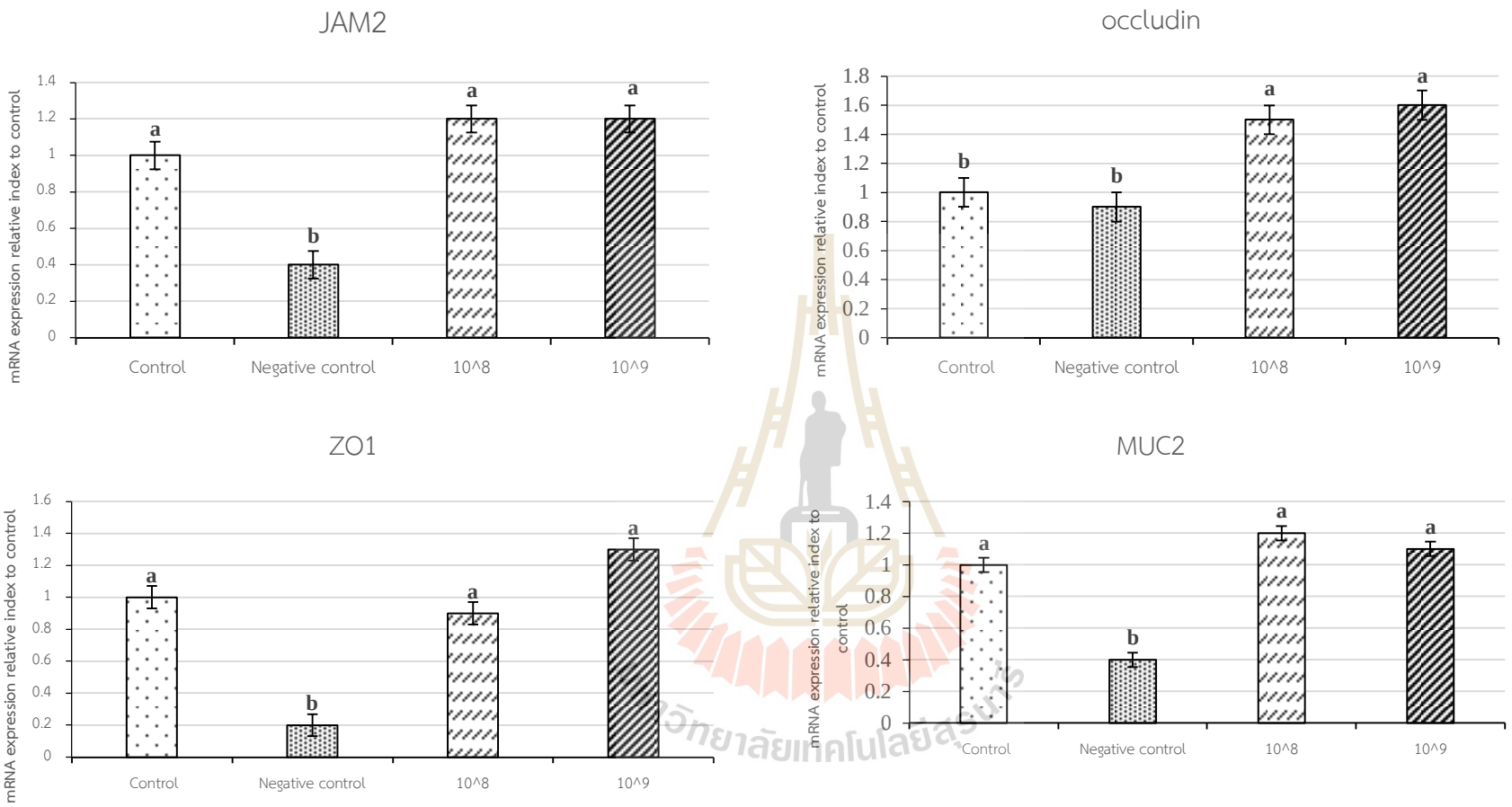

Figure 4.2 Effects of LIC37 on intestinal tight junction proteins (JAM2, occluding, ZO1) and mucin (MUC2) relative mRNA expression in ileum of chickens. [a,b] means with no common superscripts are significantly different at $P < 0.05$. Control: orally administered PBS; negative control: orally administered PBS (injected LPS); orally administered probiotic LIC37 at 10^8 and 10^9 CFU/bird/day (injected LPS), respectively.

The serum lysozyme activity decreased in LPS-challenged chickens, while the activity was improved by the LIC37. In addition, it is noteworthy that LIC37 can significantly activate the function of lysozyme higher than the control. Lysozymes are found in external secretions as well as in polymorphonuclear leukocytes and macrophages which exhibit effectiveness against bacteria. It was reported that the serum lysozyme can degrade glycosidic bonds in the cell walls of *E. coli* and *Staphylococcus* (Gong et al., 2017). The advantages of LIC37 over serum lysozyme activity observed in this study may indicate beneficial functions relative to antimicrobial ability, which is accordance with our previous study report that LIC37 can reduce pathogenic bacteria and improve innate immunity in broilers subjected to immunological stress (Sirisopapong et al., 2023). A similar result was also observed by Xie et al. (2019) who found that *L. reuteri* 22 increased the expression of lysozyme, which would be useful for protection against pathogenic infection in young chickens. Li et al. (2015) also reported that decreased lysozyme activity was observed in broiler chickens challenged chickens, wherease *Bacillus amyloliquefaciens* supplementation can increase lysozyme level.

In the present study, LPS induced upregulation of the LITAF, IL-17F and TNF-α expression in the liver. This evidence consistent with previous studied in animal model challenged with LPS which revealed several inflammatory mediators such as pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 and chemokines IL-8 were induced and released into tissues which lead to vasodilation, neutrophil migration, and an increase in vascular permeability (Kany et al., 2019). In this study we found the benefits of LIC37 on attenuate the expression of the genes LITAF, IL-17F and TNF- α. In general, TNF-α is the one of the most important inflammatory cytokines and is frequently used as an indicator of the inflammatory response. This indicates the beneficial function of LIC37 on inflammatory relief. Moreover, we also found that LIC37 can increase relative mRNA expression of the anti-inflammatory cytokines (IL-10) as similar to the control (without LPS injection). This phenomenon demonstrated that LIC37 has the potential to inhibit the expression of the inflammatory mediators, providing a negative feedback mechanism suggesting a decrease in inflammation (Moreira Lopes et al., 2020). IL-10 is involved in reducing chronic gastrointestinal

problems and is considered a major anti-inflammatory cytokine that maintains the balance of the immune responses during infection and inflammation (Subramanian and Cheng, 2012). This is consistent with the findings of Liu et al. (2015) who found that the supplementation of *Bacillus amyloliquefaciens* at 1.0 g/kg in broiler diet decreased IL-1ß, whereas IL-10 increased both protein and transcriptional levels and attenuated the elevated mRNA expression of TLR-4 induced by LPS. Hu et al. (2021) also revealed that *L. reuteri* BBC3 suppressed LPS-induced expression of pro-inflammatory genes (TNF-α, IL-1 ß, IL-6, IL-17 and IL-8), and improved the expression of anti-inflammatory genes (IL-10 and TGF- ß).

Intestinal permeability indicates the health and integrity of the alimentary tract, as the gut mucosa contains distinct lymphoid tissue known as gut-associated lymphoid tissue (GALT), interactions between intestinal bacteria and this immune system stimulate adaptive immunity against pathogen-associated molecular patterns (PAMPs) (Fooladi et al., 2013). The intestinal barrier is regulated by TJ proteins consists of several unique proteins including the junction adhesion molecule. The major TJ proteins, including occludin, claudin and ZO1 and junctional adhesion molecule (JAM) (Slifer and Blikslager, 2020) play an important role in maintaining intestinal permeability and the mucous barrier function by sealing the extracellular space between the epithelial cells and protecting the innate immune system of the host. This mechanical barrier plays an important role in the absorption of nutrients, electrolytes, and water, as well as the maintenance of the intestinal-barrier integrity and function, and the protection of the gut against enteric pathogen invasion. In this study, we found that LPS-challenged LIC37 chicken exhibited significant increases in intestinal TJ proteins and MUC2 expressions. This suggests that LIC37 could also maintain normal expression of TJ proteins and mucin. The increased expression of ZO1 may provide the necessary building blocks for the assembly of TJ proteins. Therefore, these observations of are probably responsible for the improvement of intestinal permeability under immunological stress. Interestingly, the occludin mRNA expression was also significantly higher in chickens administrated orally with LIC37 compared to the control, suggesting that LIC37 protected intestinal barrier function against intestinal pathogens possibly via the upregulation of occludin protein levels. Enhancement of TJ has also been shown to reduce gut permeability and pathogen

invasion in chickens (Lee et al., 2017). Recently, *L. reuteri* supplementation was found to improve intestinal mucosal barrier function by modulating tight junction-related protein expression in broiler chicks (Nii et al., 2020). In addition, Lokapirnasari et al. (2019) also reported that *Lactobacillus* positively improved the intestinal mucosa, strengthened gut barrier, competed with *E. coli* for colonization and modulated the inflammatory response.

4.7 Conclusion

Probiotic LIC37 has a beneficial protective effect on broiler chickens by enhancing the expression of intestinal tight junction protein and mucin, modulating liver cytokine expression, and upregulating serum lysozyme during LPS-mediated immunological challenge. Future research is required to further investigate the efficiency of production.

4.8 References

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CHAPTER V

INFLUENCE OF DIETARY PROBIOTIC *LACTOBACILLUS INGLUVIEI* C37 ON GROWTH PERFORMANCE, CARCASS QUALITY, IMMUNE RESPONSES, CECAL MICROBIAL POPULATION, SHORT CHAIN FATTY ACID AND AMMONIA PRODUCTION IN BROILERS

5.1 Abstract

This study aimed to investigate the potential effects of live and heat killed-cells of *L. ingluviei* C37 (LIC37) on growth performance, carcass quality, cecal microbial population, short chain fatty acid (SCFAs) and ammonia production in broilers. A total of 240 healthy one-day-old cobb 500 male chicks were allocated to 4 groups with 6 replicates per group and 10 chickens per replicate. Four dietary treatments were as follows: 1) the basal diet, control 2) basal diet supplemented with 50 mg/kg diet of zinc bacitracin, positive control (PC); 3) basal diet supplemented with 1×10^8 CFU/kg diet of live LIC37 and 4) basal diet supplemented with 1×10^8 CFU/kg diet of heat killed LIC37. The result showed that both live and heat killed cells of LIC37 can improve body weight gain and feed intake of broilers aged 1-42 days as compared to control ($P < 0.05$) and similar to a positive control with zinc bacitracin ($P>0.05$). There were no significant differences in feed conversion ratio (FCR), survival rate, carcass characteristics, and meat quality. The supplementation of LIC37 in both live and heat killed cells increasen *Lactobacillus* and *Bifidobacterium* (at 21 and 42 days) and decreasen *Enterobacter* and *E. coli* (at 21 days) in cecal content. This study indicates that probiotic LIC37 is effective in improving growth performance and beneficial cecal bacteria in broilers.

Keywords: Chicken, Heat killed, Productive performance, *Lactobacillus*, Probiotic.

5.2 Introduction

The use of antibiotics as growth promoter and for disease prevention is now banned, there are increased risks of microbial infections, health problems, and reduced growth performance of broilers. Nowadays, many researchers are seeking novel natural products to supplement in animal feeds. One of the most popular alternative feed additives with potential to improve immune and gastrointestinal systems is probiotics (Huyghebaert et al., 2011; Schilling et al., 2017). Health-promoting probiotics not only contribute to healthy gut microflora, but also modulate the host's immune response towards the prevention or reduction of infectious diseases. Thus, a positive improvement in growth performance. The most well-known group of probiotics are non-pathogenic Gram-positive lactic acid bacteria, such as *Bacillus*, *Bifidobacterium*, *Lactobacillus*, etc (Huyghebaert et al., 2011; Chen et al., 2022; Ma et al., 2022).

Lactobacillus ingluviei is a poorly understood LAB species in poultry. Several studies have shown that differences in the intestinal microbiota after inoculation with *L. ingluviei* can lead to weight increases in new-born ducks and chickens (Angelakis and Raoult, 2010) and *L. ingluviei* is associated with increased BW gain and enlarged liver after a single inoculation in mice (Angelakis et al., 2012). Several studies suggested that *L. ingluviei* has health-promoting effects, such as microbiota alteration, anti-*Salmonella* activity and immune response (Angelakis and Raoult, 2010; Angelakis et al., 2012; Thomas et al., 2019). Our previous study found that *L. ingluviei* C37 (LIC37) can improve gut health by increasing the population of *Lactobacillus* and *Bifidobacterium* with an associated increase in valeric acid and total SCFAs (Sirisopapong et al., 2023). In addition, we also tested LIC37 in mice challenged with lipopolysaccharide (LPS) and found its beneficial effects on *in vitro* anti-inflammatory properties by suppressing the production of pro-inflammatory cytokines (tumor necrosis factor-α (TNF-α) and interleukin (IL)-6) and anti-inflammatory cytokines (IL-10) (Tsukagoshi et al., 2020).

However, the effects of probiotic supplementation may not be consistent, due to differences in preparation methods, feed storage, and their ability to survive passage through the stomach and proliferate in the intestine and some concerns in

the animal production industry due to the increasing number of probiotics used that may introduce live bacteria into the environment (Piqué et al., 2019). Although many probiotic cultures consist of live organisms, some researchers have reported the benefits of administering inactivated or killed organisms. However, research into the effects of dietary non-viable or heat-treated microorganisms on broiler chicken is not well known (Khonyoung and Yamauchi, 2012).

5.3 Objective

This study aimed to evaluate the potential of live and heat killed LIC37 isolated from chickens in the diets on growth performance, carcass quality, immune responses, cecal microbial population, SCFAs production and ammonia production.

5.4 Material and Method

All experiments were conducted according to the principles and guidelines approved by the Animal Care and Use Committee of Suranaree University of Technology, Nakhon Ratchasima, Thailand (SUT-IACUC-0014/2022).

5.4.1 Bacteria and cultures

LIC37 was used in this study and was prepared similar as described in chapter IV, section 4.4.1. After that the cells were resuspended in PBS at a concentration of 1.0×10^9 CFU/mL and heat killed by incubation at 80°C for 30 min. The cell pellet was re-suspended in commercial soymilk and transferred into sterilized vials and frozen at -80°C for 4 h. Then, the samples were immediately freeze-dried for 18 h in a freeze-dryer (Alpha 1-2, Christ, Germany). After freeze-drying, the freeze-dried powders were mixed with dextrose at a concentration of 1.0×10^8 CFU/g.

5.4.2 Birds and housing

A total of 240, one-day-old male chicks (cobb 500) were purchased from a local commercial hatchery (Pakthongchai hatchery, CPF Public Co., Ltd., Thailand). The chicks were weighed and randomly divided into 4 groups of 60 birds, each group contained 6 replicates of 10 birds using Completely Randomized Design. The chicks were placed in floor pens on rice husk, equipped with one tray feeder

and 2 nipple drinkers. The housing temperature was maintained at 33 ℃ from day 1 to day 7 and then the temperature was gradually reduced and kept at 24 ℃ until the end of the trial. The chicks received almost continuous light of 23 h/day for 1-10 days of age, and this was reduced to 18 h/day from day 11 onwards. The birds were vaccinated for Newcastle disease and Infectious Bronchitis vaccines on day 7 and for Infectious Bursal Disease vaccine on day 14.

5.4.3 Experimental diets

Four dietary treatments included 1) the basal diet, control 2) basal diet supplemented with 50 mg/kg diet of zinc bacitracin, positive control (PC); 3) basal diet supplemented with 1×10^8 CFU/kg diet of live LIC37 and 4) basal diet supplemented with 1×10^8 CFU/kg diet of heat killed LIC37. All diets were formulated to contain similar levels of calculated ME and crude protein for each period. Other nutrients were calculated to meet or to exceed the minimum NRC (1994) and cobb 500 broiler nutrition specification (Cobb-Vantress, 2018) requirements of broiler chickens. Diet and water were provided *ad libitum* throughout the experimental period. All birds were fed with a starter, grower and finisher diet in mash form from day 1 to 10, day 11 to 21 and day 22 to 42, respectively. Feed ingredient and nutrient composition of experimental diets are presented in table 5.1.

5.4.4 Data collection and analyses

5.4.4.1 Growth performance

The body weight (BW) and feed intake (FI) were measured at 21 and 42 days of age and were used to calculate body weight gain (BWG), average daily gain (ADG), feed conversion ratio (FCR), and productive index (PI). Daily mortality was recorded, and FCR was adjusted by accounting for the BW of each dead bird. The PI was determined as per the formula: PI = [(viability (%) × BW) / (age of broiler × FCR)] × 100 (Zhang et al., 2021).

5.4.4.2 Sample collections

On day 21 and 42, two birds per pen were randomly selected and weighed. Blood samples without anticoagulants were collected from the jugular vein immediately, and then followed by euthanasia. The birds were killed by cervical dislocation, the digesta contents of the ceca (right side) were collected for microbial population evaluation. The digesta of the left ceca were collected for SCFAs and

ammonia concentration analysis. In addition, the liver and breast meat (on day 21 and 42) were collected and stored at −20°C until cholesterol was determined. At 42 days, two birds per pen were randomly selected for carcass characteristics and breast meat quality were measured.

5.4.4.3 Cholesterol analysis

The cholesterol content in breast meat and liver was analyzed according to the method of Rowe et al. (1999). Briefly, the 5 g sample was dissolved in ethanol, methanol and isopropanol (90:5:5 v/v/v) solution in an equivalent quantity of 20 mL and 5 mL 60% KOH. The flask containing the mixture was connected to a water-cooled condenser and refluxed for 1 hr. After cooling to room temperature, 100 mL of hexane was added and mixed for 10 min. Then, 25 mL of deionized water were added and the mixture stirred for 15 min. A 12.5 mL of hexane layer was dried off in a nitrogen flux . The residue was dissolved into 1 mL of hexane containing 0.1 mg of 5-α-cholestane internal standard (Sigma-Aldrich, St Louis, MO, USA) and transferred vial. Cholesterol was then analyzed by gas chromatography (Agilent 7890B; Agilent Technologies, USA) with cholesterol (Sigma-Aldrich, St Louis, MO, USA) as an external standard.

5.4.4.4 Cecal microbial population analysis

The contents of cecal digesta were used for quantification of *Lactobacillus*, *Bifidobacterium*, *Enterobacter* and *E. coli* using quantitative real time-PCR similar as described in chapter III, section 3.4.5.2.

5.4.4.5 SCFAs and ammonia analysis

Concentrations of SCFAs (acetic, propionic, isobutyric, butyric, isovaleric and valeric acids) were analyzed as in chapter III, section 3.4.5.3.

The ammonia contents of the cecal digesta were determined via a modified procedure from Willis et al. (1996). A total of 250 mg of sample was added to a polypropylene test tube, followed by 50 mL of 5% lithium carbonate (Li_2CO_3, Sigma-Aldrich, St Louis, MO, USA), vortexed to mix, and centrifuged at 10,000×g and 4 °C for 15 min. The supernatant up to 500 µL was then transferred to a 15 mL tube, added to 4 mL of salicylate reagent, followed by 1 mL of hypochlorite reagent and vortexed. The mixture was incubated at room temperature for 30 min, the absorbance was then measured at 685 nm using a microplate reader (Multiskan

GO, Thermo Fisher Scientific, Waltham, MA, USA) and compared to a standard ammonia calibration curve.

Table 5.1 Feed ingredient and nutrient composition of the basal diets (as-fed basis)

Item	Starter (1-10 d)	Grower (11-21 d)	Finisher (22-42 d)
Ingredients (%)			
Corn	55.72	54.76	58.62
Soybean meal, 44% CP	35.55	35.15	31.12
Meat meal, 58% CP	1.33	1.29	1.00
Palm oil	3.29	4.81	5.62
Calcium carbonate	1.22	1.16	1.08
Monocalcium phosphate (P21)	1.24	1.21	1.10
Sodium chloride	0.53	0.53	0.45
Premix[1]	0.50	0.50	0.50
L-lysine HCl 79%	0.19	0.17	0.15
DL-methionine	0.32	0.31	0.28
L-threonine	0.09	0.10	0.08
L-valine	0.02	0.01	0.00
Calculated composition (%)			
Metabolizable energy (kcal/kg)	3,010	3,100	3,200
Calcium	0.90	0.87	0.79
Available phosphorus	0.45	0.44	0.40
Digestible lysine	1.18	1.15	1.03
Digestible methionine	0.61	0.59	0.54
Digestible methionine + cystine	0.88	0.87	0.80
Digestible threonine	0.77	0.77	0.69
Analyzed composition (%)			
Dry matter	93.06	93.30	92.52
Crude protein	22.15	21.47	19.82
Crude fat	5.35	6.73	7.88

[1]Premix (0.5%) provided the following per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 IU; vitamin K3, 5 mg; vitamin B1, 2 mg; vitamin B2, 7 mg; vitamin B6, 4 mg; vitamin B12, 25 µg; pantothenic acid, 11.04 mg; nicotinic acid, 35 mg; folic acid, 1 mg; biotin, 15 µg; choline chloride, 250 mg; Cu, 1.6 mg; Mn, 60 mg; Zn, 45 mg; Fe, 80 mg; I, 0.4 mg; Se, 0.15 mg

5.4.4.6 Serum total Ig and lysozyme activity

The blood sample collected in tube was taken by centrifugation at 2,000 × g for 15 min at 4°C to obtain serum and stored at −80°C until analysis. Serum samples were used for analyzing total Ig and lysozyme activity similar as described in chapter IV, section 4.4.4.

5.4.5 Fourier transform infrared spectroscopy.

Fourier transform infrared spectroscopy (FTIR) was used to investigate the differences in structure and molecular composition changes in proteins, lipids, nucleic acids or carbohydrates between live and heat killed cells of LIC37. Infrared spectra were collected using Attenuated total reflectance (ATR)-FTIR spectroscopy with a single reflection ATR sampling module and coupled with a DTGS detector over the measurement range from 4,000 to 400 cm^{-1}. Measurements were performed with a spectral resolution of 4 cm^{-1} with 64 scans co-added (Bruker Optics Ltd, Ettlingen, Germany). OPUS software was used for data acquisition and the spectra evaluation. The spectral changes in functional groups were performed at the integral area of protein using OPUS software.

The FTIR spectra were exported to the Unscrambler X 10.5 (CAMO, Oslo, Norway) for PCA analysis. The spectral data were pre-processed by taking the second derivative with the Savitzky-Golay method, normalization with extended multiplicative signal correction and PCA were performed to determine a significant variation between spectra sets. In this study, PCA was used to compare the FTIR spectra of LIC37 as differences live and heat killed cells. The output of PCA were presented as sources of data variability which were concentrated into the principal component (PC). The resulting protein spectra were smoothed with a seven-point Savitzky-Golay function. Inverted second derivative spectra were used to estimate the number, position and relative contribution of individual elements composing Amide I band (1600–1720 cm^{-1}), and this information was considered to fit Amide I bands in protein spectra.

5.4.6 Statistical Analysis

Data were analyzed using one-way ANOVA of SPSS version 18.0 (IBM, Armonk, NY). Significant differences among treatments were assessed by Tukey's post-hoc test. A threshold level of $P < 0.05$ was used to determine significance.

5.4.7 Experimental location

The experiment was conducted at Suranaree University of Technology's poultry farm, the Center for Scientific and Technological Equipment Building 11 and 14, Suranaree University of Technology.

5.4.8 Experimental period

The experiment was done from December 2022 to March 2023.

5.5 Results

5.5.1 Effects of probiotic *L. ingluviei* C37 on growth performance and carcass characteristics

The results of probiotic LIC37 on growth performance in broiler chickens are presented in Table 5.2. From 1-21 day, it was found that dietary heat killed LIC37 could enhance body weight in broilers, when compared to control group ($P < 0.05$). At 42 days of age, body weight, weight gain and feed intake were higher in the LIC37 groups (both live and heat killed cells) than in control group ($P < 0.05$), these parameters also showed the same values as positive control ($P > 0.05$). While there were no significant differences in FCR, survival rate and PI among treatments ($P > 0.05$). In addition, the probiotic LIC37 did not show any significant effects on carcass, breast, thigh, drumstick and wing weights ($P > 0.05$). The breast meat quality included pH, meat color and water holding capacity of broilers fed the diets containing LIC37 was also not significantly different compared to the control diets ($P > 0.05$) (Table 5.3).

5.5.2 Effects of probiotic *L. ingluviei* C37 on cholesterol content in breast meat and liver

The effect of LIC37 in broiler diets on cholesterol content in breast meat and liver are shown in Table 5.4. Probiotic LIC37 exhibited lower cholesterol in breast meat than positive control in both periods ($P < 0.05$). Additionally, at 21 days of age, LIC37 could decease cholesterol content in liver when compared to control group. However, there were no significant differences among treatments on cholesterol content in liver at 42 days of age.

Table 5.2 Effects of probiotic *L. ingluviei* C37 on growth performance of broilers.

Item	Control	Positive control	*L. ingluviei* C37		SEM
			Live cell	Heat killed cell	
Body weight (BW), g/bird					
Initial weight	42	42	42	42	0.08
1 - 21 days	926[b]	968[a]	927[b]	976[a]	6.40
22 - 42 days	3036[c]	3270[a]	3213[b]	3261[a]	17.69
Body weight gain (BWG), g/bird					
1 - 21 days	894[ab]	926[a]	885[b]	934[a]	6.98
22 - 42 days	2094	2260	2291	2231	16.32
1 - 42 days	3029[b]	3228[a]	3172[a]	3220[a]	19.73
Feed intake (FI), g/bird					
1 - 21 days	1113[b]	1167[a]	1180[a]	1156[a]	4.29
22 - 42 days	3646	3704	3762	3744	16.12
1 - 42 days	4764[b]	4871[a]	4937[a]	4900[a]	18.25
Feed conversion ratio (FCR)					
1 - 21 days	1.26[a]	1.26[a]	1.38[b]	1.27[a]	0.01
22 - 42 days	1.72	1.68	1.67	1.69	0.01
1 - 42 days	1.56	1.55	1.55	1.54	0.01
Survival rate (%)					
1 - 21 days	100.00	100.00	96.30	98.15	0.60
22 - 42 days	100.00	97.62	95.24	97.62	0.89
1 - 42 days	100.00	97.62	95.24	97.62	0.89
Productive index (PI)					
1 - 21 days	333.41[a]	349.97[a]	289.12[b]	340.40[a]	4.19
22 - 42 days	290.52	319.99	309.44	299.11	3.06
1 - 42 days	463.28	496.15	463.39	472.73	4.03

[a, b, c] Means with different superscripts in a row are significantly different ($P < 0.05$).

Table 5.3 Effects of probiotic *L. ingluviei* C37 on carcass characteristics and meat quality of broilers (42 day of age).

Item	Control	Positive control	*L. ingluviei* C37		SEM
			Live cell	Heat killed cell	
Live weight (g)	3213	3268	3190	3222	26.57
Carcass weight (% of BW)	77.64	77.46	76.22	75.96	0.45
Carcass components (% of carcass weight)					
Breast meat	25.87	27.32	26.01	26.40	0.30
Thigh meat	14.20	14.41	15.42	14.80	0.19
Drumsticks	13.53	13.11	13.01	12.60	0.17
Wings	9.87	9.84	9.90	10.06	0.09
Breast meat pH[1]					
pH 45 min	6.50	6.63	6.66	6.61	0.04
pH 24 h	5.91	5.96	5.82	5.92	0.03
Breast meat color[2]					
L* (lightness)	48.86	46.76	46.48	46.18	0.60
a* (redness)	-1.80	-1.45	-1.36	-1.57	0.09
b* (yellowness)	2.11	1.55	2.85	2.28	0.22
Water holding capacity (%)					
Drip loss	4.44	4.15	4.10	4.12	0.08
Cooking loss	35.43	33.16	34.06	30.93	1.04
Shear force (N)	2139	2154	2031	2114	77.07

[1] pH 45 min: pH of breast and leg muscles measured 15 min postmortem; pH 24 h: pH of breast and leg muscles measured 24 h after postmortem and chilling the carcasses to 4°C.

[2] All color measurements were recorded on breast fillets 24 h after postmortem and chilling the carcasses to 4°C.

Table 5.4 Effects of probiotic *L. ingluviei* C37 on cholesterol content in breast meat and liver of broilers (mg/100 g).

Item	Control	Positive control	*L. ingluviei* C37		SEM
			Live cell	Heat killed cell	
Breast meat					
21 days	45.54[ab]	53.42[a]	44.83[b]	43.07[b]	8.25
42 days	77.50[a]	77.79[a]	59.44[b]	62.16[b]	9.55
Liver					
21 days	108.51[a]	108.28[a]	98.95[b]	96.11[b]	22.52
42 days	600.69	599.97	583.51	593.08	102.32

[a, b] Means with different superscripts in a row are significantly different ($P < 0.05$).

5.5.3 Effects of probiotic *L. ingluviei* C37 on cecal microbial population

The effect of supplementation of LIC37 on cecal microbial population is presented in Figure 5.1. It was found that the supplementation of LIC37 both live and heat killed cells could increase the *Lactobacillus* and *Bifidobacterium* DNA copy number in cecum content at 21 and 42 days of age ($P < 0.05$). Additionally, LIC37 could decrease the number of pathogenic bacteria (*Enterobacter* and *E. coli*) in cecum at 21 days of age when compared to control group ($P < 0.05$). However, no such significant difference was observed in broilers aged 42 days ($P > 0$ 05).

5.5.4 Effects of probiotic *L. ingluviei* C37 on cecal SCFAs and ammonia concentrations

The effects of dietary probiotic LIC37 on cecal SCFAs concentrations of broilers are shown in Table 5.5. At 21 days of age, significantly ($P < 0.05$) higher cecal valeric acid and branched SCFAs concentrations were observed in negative control group. In the other hand, at 42 days of age, the supplementation of LIC37 in both live and heat killed cells could produce cecal valeric acid concentration similar with positive control group. Although the concentrations of other SCFAs were not affected by dietary treatments.

In the same way, significant reduction in ammonia concentration was found in chicken fed with LIC37 in both live and heat killed cells at 42 days of age (P

< 0.05). However, concentrations of ammonia at 21 days of age were not affected by dietary treatments.

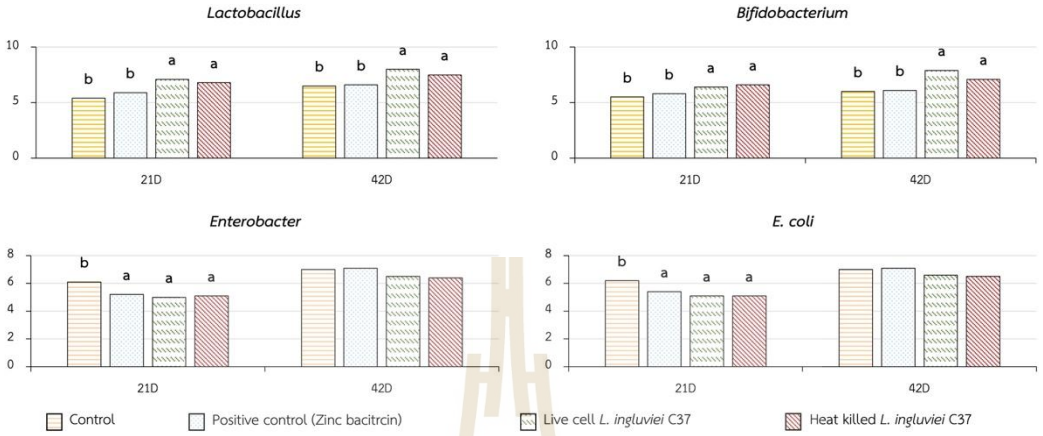

Figure 5.1 Effects of *L. ingluviei* C37 on cecal microbial population of broiler chickens at 21 and 42 days of age. (log10 of copy number/g DNA extract).
[a, b] Means with no common superscripts are significantly different (P < 0.05).

5.5.5 Effects of probiotic *L. ingluviei* C37 on serum total Ig and lysozyme activity

The effects of LIC37 on total serum Ig and lysozyme activity are shown in Table 5.6. There were no significant differences between control and positive control on serum lysozyme activity at 21 days of age. Whereas dietary LIC37 in both live and heat killed cells can increase serum lysozyme activity (P < 0.05) higher than control at 42 days of age. In addition, there was no significant effect on serum total Ig.

Table 5.5 Effects of probiotic *L. ingluviei* C37 on cecal SCFAs (% of total SCFA) and ammonia concentrations (mg/g of cecal digesta) of broiler chickens at 21 and 42 days of age.

Item	Control	Positive control	*L. ingluviei* C37		SEM
			Live cell	Heat killed cell	
At 21 days of age					
Short-chain fatty acids (% of total SCFA)					
Acetic acid	75.07	80.14	78.31	78.82	3.21
Propionic acid	6.61	9.25	8.00	6.30	0.95
Butyric acid	6.57	8.21	10.45	10.72	0.80
Valeric acid	6.62^{a}	1.07^{b}	1.48^{b}	1.96^{b}	0.75
Branched SCFA acid[1]	5.13^{a}	1.33^{b}	1.76^{b}	2.21^{b}	0.49
Ammonia concentration (mg/g of cecal digesta)					
	0.51	0.46	0.46	0.48	0.03
At 42 days of age					
Short-chain fatty acids (% of total SCFA)					
Acetic acid	72.86	65.06	61.18	65.50	2.73
Propionic acid	11.31	10.29	9.92	12.83	1.34
Butyric acid	9.43	9.98	15.13	15.04	2.03
Valeric acid	0.88^{b}	11.79^{a}	9.27^{a}	2.16^{ab}	0.43
Branched SCFA acid[1]	5.53	2.88	4.49	4.46	1.23
Ammonia concentration (mg/g of cecal digesta)					
	1.42^{c}	1.12^{b}	0.80^{a}	0.84^{a}	1.05

[1]Branched SCFA = isobutyric acid + isovaleric acid

$^{a, b}$ Means with different superscripts in a row are significantly different ($P < 0.05$).

Table 5.6 Effects of probiotic *L. ingluviei* C37 on total immunoglobulin and lysozyme activity in serum of broiler chickens at 21 and 42 days of age.

Item	Control	Positive control	*L. ingluviei* C37		SEM
			Live cell	Heat killed cell	
At 21 days of age					
Total Ig (mg/mL)	18.62	18.40	18.86	18.98	0.48
Lysozyme (μL/mL)	29.18	28.13	29.06	28.57	0.51
At 42 days of age					
Total Ig (mg/mL)	19.86	18.88	19.73	19.68	2.43
Lysozyme (μL/mL)	31.83^{c}	28.45^{d}	38.89^{b}	40.85^{a}	1.23

$^{a, b, c, d}$ Means with different superscripts in a row are significantly different ($P < 0.05$).

5.5.6 Characterization of secondary structure of *L. ingluviei* C37 by FTIR

In this study, structural changes in secondary proteins in both live and heat killed cells were investigated using the FTIR technique. The results of the analysis are presented in figure 5.2 which shows the observed changes, and figure 5.3 which displays the results of the PCA differential analysis. It was observed that heat application to LIC37 caused a change in the secondary structure of proteins present in the cell wall. as well as in the protein structure components of heat killed forms. In order to delve deeper into the analysis of secondary protein structure differentiation based on the amide I band, the obtained results were compared with the reference data presented in table 5.7. This comparison is illustrated in figure 5.4, which specifically highlights the differences between live and heat killed cells. The observation revealed that the absorbance units of the α-helix were highest in live cell LIC37. However, heat treatment resulted in a decrease in the percentage of α-helix while leaving unaffected to the percentage of β-sheet unaffected in LIC37.

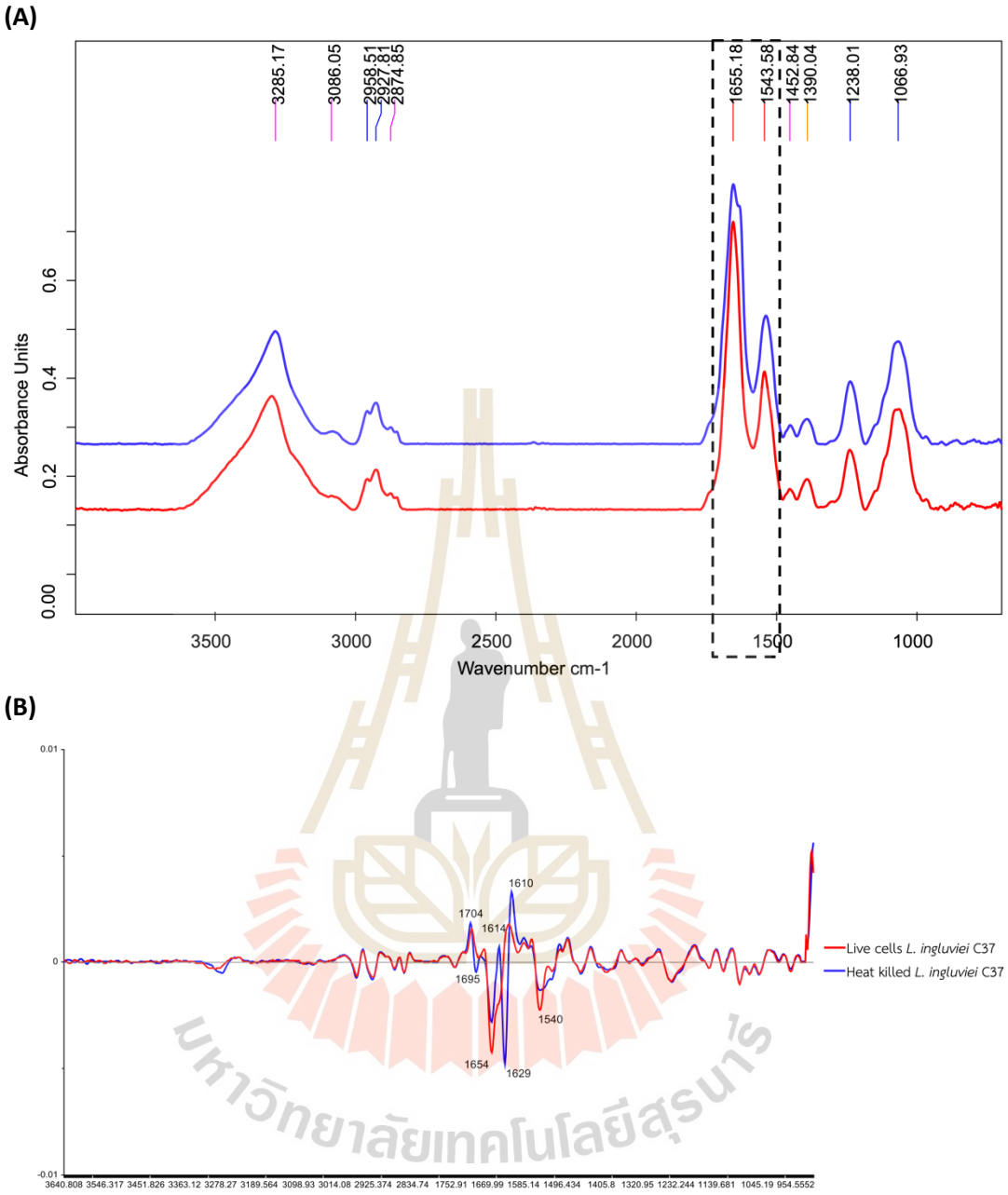

Figure 5.2 (A) Fourier transform infrared (FTIR) spectra of *L. ingluviei* C37, (B) The secondary derivative spectra of *L. ingluviei* C37.

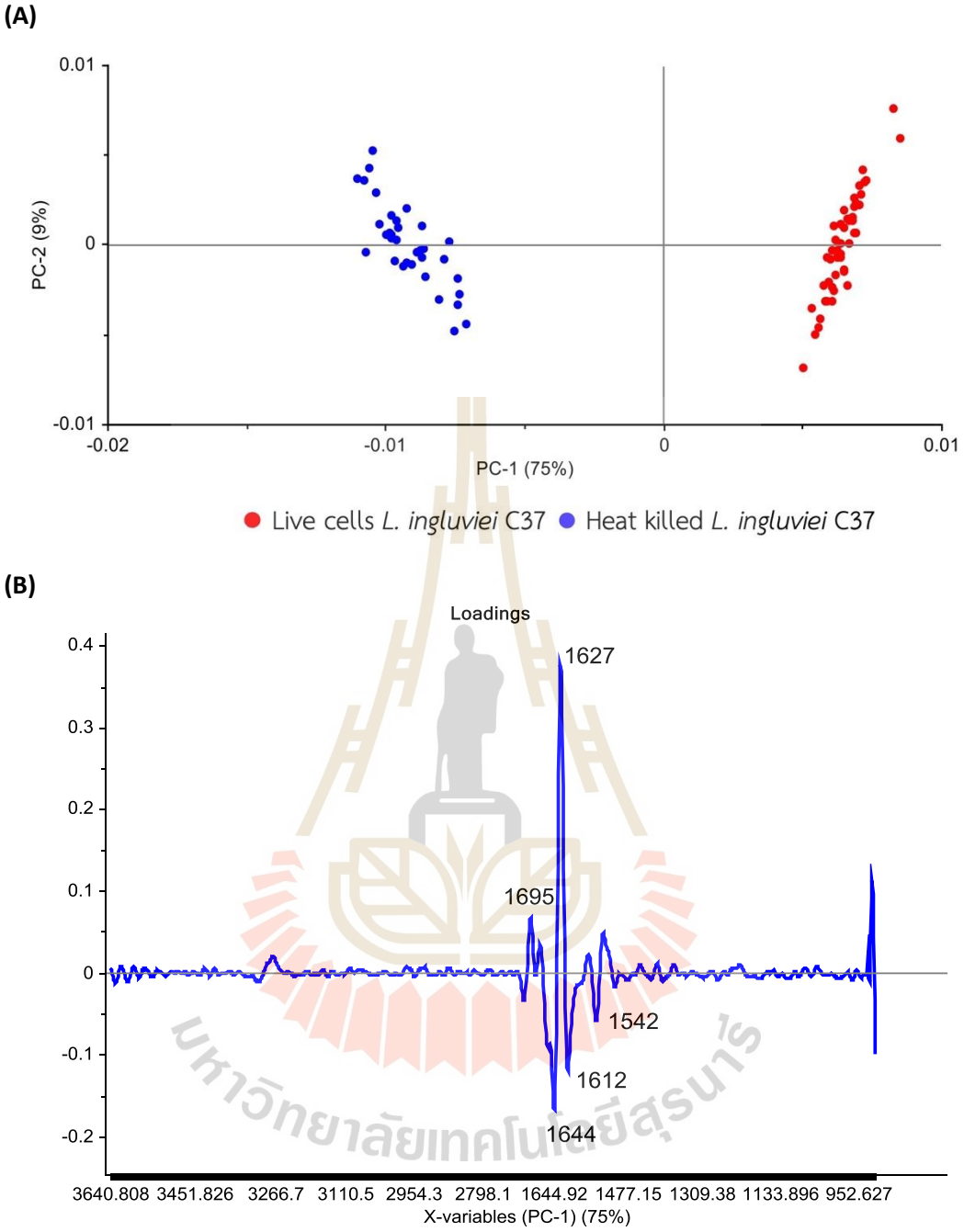

Figure 5.3 Principal component analysis (PCA) scores scatter plot of Fourier transform infrared (FTIR) spectra of live and heat killed cells *L. ingluviei* C37, (A) PCA scores plot, (B) PCA loading plot.

Table 5.7 Empirical assignment of secondary structure elements to different amide I band components in proteins.

Wavenumber (cm^{-1})	Assigned secondary structure
1620-1630	Intermolecular ß-sheet
1620-1640	Intramolecular ß-sheet
1640-1650	No order
1650-1660	α-helix
1660-1695	ß-turn
1675	Intramolecular ß-sheet
1690-1700	Intermolecular ß-sheet

Adapt from; Torii and Tasumi (1996); Murayama and Tomida (2004).

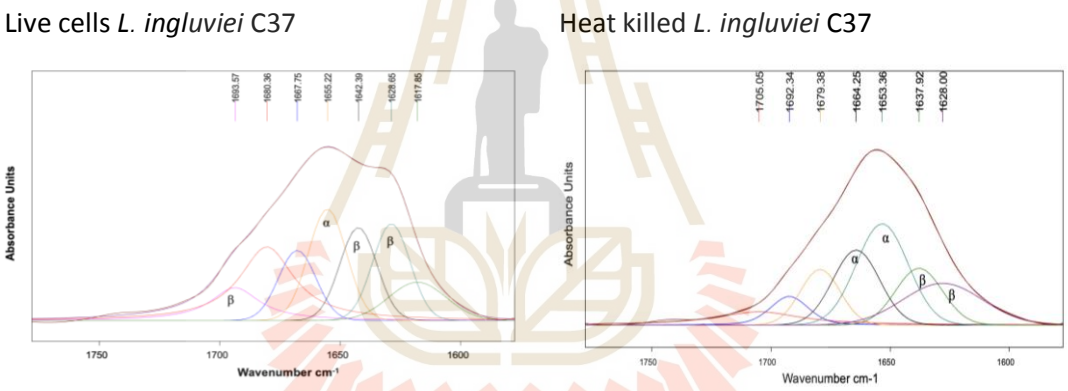

Figure 5.4 Unresolved FTIR spectra and curve fitting analysis in the Amide I region of *L. ingluviei* C37.

Table 5.8 Amide I position and secondary structure composition (%) of *L. ingluviei* C37, according to relative areas of component bands in peak fitting of amide I zone in original IR spectra.

Item	Amide I Maximum position (cm^{-1})	S-layer secondary structure (%)		
		ß-Sheet	α-Helix	Other[1]
Live cell LIC37	1662	38.6	44.0	17.4
Heat killed LIC37	1652	38.5	17.3	44.2

[1]Other = ß turn and non-ordered structures (side chain vibrations)

5.6 Discussion

Probiotics are widely used as dietary supplements and represent a non-antibiotic nutritional approach to modulating gut function and growth performance and enhancing immune response in chickens. These advantages are supplied via the interaction between probiotics, gut microbiota and the immune system (Markowiak and Śliżewska, 2018; Piqué et al., 2019). In this study we found that LIC37, both live and heat killed cells, can improve BW and FI in broilers. In addition, LIC37 can increase cecal *Lactobacillus* and *Bifidobacterium* population and decrease *Enterobacter* and *E. coli*, as well as enhanced SCFAs and decrease ammonia production.

It was found that in broiler groups received live and heat killed LIC37 had higher BW, BWG and FI compared to control ($P < 0.05$). While there were no significant differences in FCR, survival rate, carcass characteristics and breast meat quality ($P > 0.05$). Probably due to probiotic LIC37 could improve the balance of beneficial bacteria, which helps improving digestion and strengthen the immune system. It has been reported that probiotics colonies created a protective barrier to prevents harmful bacteria from damaging the intestinal lining, which can lead to disease and poor nutrient absorption (Onrust et al., 2018). LIC37 also helped to promote the production of SCFAs in the gut, which would provide energy and help maintaining a healthy intestinal pH levels. It has also been shown that SCFAs stimulate the growth of intestinal epithelial cells, which improve nutrient uptake and promote overall gut health (Deleu et al., 2021). Moreover, the study using mice models showed that the increase in *L. ingluviei* content in the intestinal microbiota

led to weight increase and caused metabolic changes (Angelakis et al., 2012). In addition, Incharoen et al. (2019) reported that the supplementation of heat killed *L. plantarum* L-137 in broiler diets could improve BW, BWG and FCR at 42 days of age , but there were no significant differences on FI , survival rate, carcass characteristic and visceral organs.

Furthermore, our research found that the supplementation of LIC37 in diets resulted in lowering cholesterol in breast meat compared to control. The function of removing cholesterol is probably due to the bile salt hydrolase (BSH) in probiotics particularly in the strains of *Lactobacillus* and *Bifidobacterium*, in which this enzyme is responsible for the hydrolysis of conjugated bile acids into deconjugated bile acids and amino residues, whose deconjugated forms are less soluble and less absorbed by the intestine, leading to the elimination of excreta (Rajan et al., 2019). As a result, cholesterol is now used to synthesize new bile acids in a homeostatic response, leading to a reduction in serum cholesterol in animals (Ooi and Liong, 2010). It is interesting to note heat killed LIC37 still maintain a function in cholesterol removal, which is probably due to their cell membrane still having the ability to bind cholesterol (Ma et al., 2019). This is in accordance with the report of Lye et al. (2010) who stated that the membrane bilayer of probiotic cells (*Lactobacillus* and *Bifidobacterium*) has the ability to incorporate cholesterol, especially in the areas of the phospholipid tail, upper phospholipids and polar heads.

Oral inoculation with probiotic bacteria is now recognized to reduce the risk of intestinal infection by pathogenic bacteria. In this study, probiotic LIC37 supplementation in diets reduced the population of *Enterobacteria* and *E. coli* and increased the number of *Lactobacillus* and *Bifidobacterium* in cecum compared to the control groups. Probiotic *Lactobacillus* can function against pathogens by producing antimicrobial compounds and decreasing pH (with SCFAs production) and competing with pathogens for adhesion and colonization, and for nutrients and other growth factors in the gut and suppressing the growth of pathogenic bacteria by directly binding to Gram-negative bacteria. In addition, some probiotic metabolites maintain the gut epithelial barrier by enhancing mucus secretion by goblet cells and increasing antimicrobial peptide synthesis in intestinal tract (Liu et al., 2020; Ma et al., 2022). Similarly, mice inoculated with 4×10^{10} *L. ingluviei* exhibited a significant

increase in weight gain and liver weight and significant increases in *Lactobacillus* and Firmicutes DNA copy numbers in their feces. This is in accordance with the findings of Mulder et al. (1997) who reported that *L. reuteri* significantly reduced the number of *Enterobacteria* in broiler chickens. Cao et al. (2013) reported that broiler chickens fed diets supplemented with *Lactobacillus* were more resistant to the pathogenic effects of *E. coli*. Heat killed probiotics are also able to antagonize pathogens. After inactivating probiotic bacteria by heat treatment, can result in the release of bacterial components such as cell wall components and surface layer proteins (SLPs) with immunomodulatory and pathogen-antagonistic activities (Piqué et al., 2019). The function of SLPs in bacterial adhesion and gut barrier protection can be attributed to SLPs' competition with pathogens for adhesion sites on the intestinal cell surface. Previous study reported that SLPs extracts from *L. helveticus* has prevented *E. coli* binding to epithelial cells *in vitro* (Sleytr et al., 2014). Exposure of epithelial cells with SLPs extracts decreased *E. coli* adherence and attaching-effacing lesions and preserved the epithelial barrier function (Johnson-henry et al., 2007). This evidence suggested that heat killed LIC37 has antagonizing characteristics against pathogenic bacteria.

In general, SCFAs are metabolites of bacteria in the gut of which the concentration may vary depending on the prevailing microbiota, the type of fermentation substrate and the period of fermentation. In this study, a significant increase in valeric acid and total SCFAs in cecal digesta may be associated with an increase in the population of *Lactobacillus* and *Bifidobacterium*, which were more abundant in chicken groups fed with LIC37 at finisher state. SCFAs have been reported to decrease cecal pH and indirectly inhibit pathogenic microorganisms susceptible to pH changes, as well as passing into the cells of pathogens causing a change of positive and negative ions resulting in cells becoming unbalanced and inhibiting the growth of pathogens (Deleu et al., 2021). The valeric acid is mostly produced by certain members of gut microbiota belonging to Firmicutes bacteria (Qing et al., 2019). In addition, valeric acid was identified as a potential therapeutic target for a variety of disease pathologies. The findings of Onrust et al. (2018) revealed supplementation of valeric acid glyceride esters can improve feed efficiency, gut morphology and reduce the incidence of necrotic enteritis.

Ammonia emission is a concern for the poultry industry from both an environmental and animal welfare point of view. Potential effects on birds include respiratory diseases, viral infections, decreased production and higher mortality (Han et al., 2021). NH_3 emissions from poultry are primarily produced because of the breakdown of uric acid by gut and manure microbiomes (Philippe et al., 2011). Therefore, manipulating the microbiome balance in the intestinal tract may significantly decrease NH_3 emissions from the gut and manure. In our current study, LIC37 can reduce cecal ammonia concentration when compared to control. Normally, probiotics contain lactic acid bacteria which can produce protease enzymes and inhibit uric acid conversion to ammonia by using it as a nutrient. In the same way, Zhang et al. (2012) reported that dietary *B. subtilis* can also reduce ammonia emission in poultry, by improving the activity of enzymes and N utilization. Moreover, the study of Chang and Chen (2003) reveal that environmental NH_3 levels in the broiler house were decreased by feeding *Lactobacillus*. Park et al. (2016) also reports that the supplementation of *E. faecium* in laying hens resulted in a change in the microbial composition of excreta fecal, which led to an increase in nutrient retention, a decrease in nutrient excretion, and ultimately improved nutrient digestibility and reduced excreta ammonia emission.

As a natural antibacterial enzyme, lysozyme exerts bacteriolytic activity directly by hydrolyzing glycosidic bonds in the cell walls of pathogenic bacteria and indirectly by stimulating macrophage phagocytic function (Xie et al., 2019). In the present study, broiler chickens fed with LIC37 had serum lysozyme activity higher than control group. Lysozyme is an important defense factor against microbial invasion (Saurabh and Sahoo, 2008). Higher lysozyme activity in LIC37 groups suggested that probiotics could enhance the antibacterial defense of chicken. A similar result was also observed by Gong et al. (2017) probiotic *Bacillus* showed higher serum lysozyme activity. Moreover, Xie et al. (2019) also found that *L. reuteri* 22 increased the expression of lysozyme, which would be useful for protection against pathogenic infection in young chickens.

After inactivating probiotic bacteria by heat treatment, can result in the release of bacterial components such as cell wall components and SLPs which exhibit immunomodulatory and pathogen-antagonistic activities (Piqué et al., 2019).

Arrangement of secondary structural elements within layer proteins can influence the surface properties of the strains they are associated with. These surface properties may include characteristics such as adhesion to other surfaces, biofilm formation, or interactions with the environment. This implies that variations in the secondary structure of surface proteins can affect the functional properties of the strains. FTIR is a well-established methodology widely utilized for analyzing and elucidating the secondary structure of proteins. In this study, the FTIR technique was employed to determine the position of the amide I band within the wavenumber range of 1600-1720 cm^{-1} , thereby evaluating the characteristics of the secondary structure of protein in cell wall component. Significant changes were observed in the secondary protein structure of LIC37 when it was subjected to heat treatment. In this study, the percentage of α-helix was observed to decline and change to others (further analysis is necessary to identify), while the percentage of ß-sheet remained unchanged. This change in protein structure can be attributed to the cellular adaptive response to the heat treatment. Furthermore, the unchanged levels of ß-sheet proteins suggest that it possess better resistance to high temperature compared to α-helix proteins. In a study conducted by Mobili et al. (2009), it was reported that the aggregative properties of *L. brevis* SLPs may be associated with its increased content of ß-sheet structures. The higher abundance of ß-sheet in the SLPs of *L. brevis* strains with aggregative behavior implies a potential role of these structural elements in promoting aggregation or clustering of cells. The study suggests that the levels of ß-sheet remain unchanged, indicating that heat killed LIC37 retains similar effects to the live cells LIC37. Therefore, the data obtained from this study supports the efficacy of heat killed LIC37, as it demonstrated beneficial effects in tissue inflammation, pathogen inhibition, gut health and immune stimulation in chickens. These findings are consistent with the established characterization and functional role of LIC37. However, since there is currently limited information on the specific roles of ß-sheet, α-helix and another protein structures in LIC37 cell wall components and their properties, further analysis and investigation are required to gain a comprehensive understanding of this relationship.

5.7 Conclusions

This present study indicates that both live and heat killed LIC37 can improve growth performance, gut health and stimulate immune response of broilers. Heat killed LIC37 can be easily implemented in practical application as it is less restrictive in use.

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CHAPTER VI
OVERALL CONCLUSION AND IMPLICATION

6.1 Conclusion

The use of probiotics as an alternative to antibiotics in animal diets has received considerable attention in recent decades. LAB are the main source of probiotics used in animal diets , which have several health benefits for the host, including gut microbiota modulation, immunomodulation, anti-inflammatory and antimicrobial effects. Therefore, this research aimed to isolate LAB from the gastrointestinal tract of chickens and to evaluate the efficacy of isolated LAB in broilers under normal condition and lipopolysaccharide (LPS) challenge. The main results are summarized as follows:

6.1.1 Five *Lactobacillus* strains namely *L. acidophilus*, *L. ingluviei, L. reuteri*, *L. salivarius* and *L. saerimneri* were isolated from the gut contents of chickens and passed all probiotic criteria, including tolerance to acid and bile salt, antibacterial activity, adhesion activity, antibiotic resistance and cholesterol removal. In addition, the evaluation of two selected strains (*L. ingluviei* and *L. salivarius*) in chickens demonstrated an improvement in gut health, with an associated increase in valeric acid and total SCFAs. As *L. ingluviei* exhibited greater properties than *L. salivarius* in reduction of pathogenic populations *(E. coli)*.

6.1.2 Probiotic LIC37 can modulate host inflammatory responses and gene expression of the intestinal barrier of chickens induced with LPS endotoxin from *E. coli* O55:B5. This is achieved through a reduction in the relative weight of the spleen, decrease in LITAF, IL-17F and TNF-**α**, and an increase in IL-10 expression in the liver. LIC37 also demonstrated an ability to strengthen the intestinal wall and improve the expression of intestinal tight junction protein (JAM2, occludin, ZO1) and mucin. Additionally, LIC37 has shown to upregulate serum lysozyme during LPS-mediated immunological challenge. This highlights the promising role of probiotic LIC37 in

enhancing the overall health and immunity of chickens, which can have positive consequences for the poultry industry.

6.1.3 The supplementation of LIC37 in both live and heat killed cells in broiler diets can improve growth performance and reduce cholesterol in breast meat and liver. In addition, LIC37 also increase *Lactobacillus* and *Bifidobacterium*, decrease *Enterobacter* and *E. coli*, as well as enhanced SCFAs and decrease ammonia production in cecum. Overall, the addition of LIC37 has potential benefits in improving the health and performance of broiler chickens, while also positively affecting the microbial balance and metabolite production in their digestive tract.

6.2 Implication

This study demonstrated that LIC37 has great potential to improve gut health, immune function and growth performance in broilers. Although, there are several potential advantages to using probiotics in poultry, it also has limitations of using including their susceptibility to environmental factors such as an instability during feed processing, in particular, the high temperatures and pressures involved in feed processing. This can weaken or even kill the live cells. Moreover, the addition of moisture to in diets can contribute to the breakdown of the probiotics cell walls.These factors can affect the probiotic viability and reduce their effectiveness. However, it is interesting to note that heat killed LIC37 showed a potential as similar as the live cell. A development of heat killed LIC37 as a feed additive for poultry nutrition which provides a sustainable and effective to enhance the overall health and well-being of chickens. In addition, from the 5 isolate strains as *L. acidophilus*, *L. ingluviei*, *L. reuteri*, *L. salivarius* and *L. saerimneri*, there is some strains (*L. salivarius* and *L. saerimneri*) that are still lack information on studies in chickens. Further research is needed to evaluate the safety and efficacy of these strains.

BIOGRAPHY

Merisa Sirisopapong was born on 22nd August 1993 in Chachoengsao, Thailand. In 2015, she obtained her Bachelor of Science in Animal Production Technology, Institute of Agricultural Technology, Suranaree University of Technology. In 2015, she was awarded got a scholarship by the Royal Golden Jubilee Ph.D. (RGJ-PHD) Program (Grant number PHD/0105/2558) for her Doctor of Philosophy (Ph.D. degree) study in Animal Production Technology at the School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima.

During her doctoral studies, she had the opportunity to conduct experiments abroad at the School of Science and Technology, Institute of Agriculture, Shinshu University in Japan for 8 months. As part of her doctoral studies, she published an article titled "Assessment of lactic acid bacteria isolated from the chicken digestive tract for potential use as poultry probiotics" in the journal Animal Bioscience. The citation for the article is as follows: Sirisopapong, M., Shimosato, T., Okrathok, S., & Khempaka, S. (2023). Assessment of lactic acid bacteria isolated from the chicken digestive tract for potential use as poultry probiotics. Animal Bioscience. 36(8), 1209–1220. https://doi.org/10.5713/ab.22.0455