ISOLATION IDENTIFICATION AND CHARACTERIZATION OF PROBIOTIC BACTERIA FROM INFANT' S FECES FOR POTENTIAL USE AS VACCINE DELIVERY VEHICLE

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Researcher

บทคัดย่อ

เรื่อง	:	การคัดแยก จำแนก และศึกษาคุณสมบัติของโพรไบโอติกจากอุจจาระเด็กแรกเกิดเพื่อใช้เป็นตัวนำส่งวัคซีน
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คำสำคัญ	:	*Lactobcillus rhamnosus*, โพรไบโอติก, วัคซีนชนิดกิน

การศึกษานี้มีวัตถุประสงค์เพื่อคัดแยก จำแนก และศึกษาคุณสมบัติของโพรไบโอติกของแบคทีเรียที่คัดแยกจากอุจจาระของเด็กแรกเกิดที่เกิดโดยวิธีธรรมชาติและดื่มน้ำนมแม่ คุณสมบัติของโพรไบโอติกที่ศึกษา ได้แก่ การย่อยสลายเม็ดเลือดแดง การทนต่อกรดและเกลือน้ำดี การสร้างสารยับยั้งจุลินทรีย์ก่อโรค การทดสอบความไวต่อยาปฏิชีวนะ การเกาะติดเซลล์ลำไส้ และการทดสอบความเป็นพิษต่อเซลล์ลำไส้ ผลการศึกษาพบแบคทีเรียโพรไบโอติกจำนวน 4 ไอโซเลต ได้แก่ LW01 LW02 LW09 และ LW10 การจัดจำแนกสปีชีส์ของแบคทีเรียโดยวิธี 16S rRNA sequence analysis พบว่าทั้ง 4 ไอโซเลต เป็นเชื้อ *Lactobacillus rhamnosus* จึงกำหนดเป็น *L. rhamnosus* LW01 *L. rhamnosus* LW02 *L. rhamnosus* LW09 และ *L. rhamnosus* LW10 การทดสอบอิเล็กโตรทรานฟอร์เมชั่นด้วยเวกเตอร์ pRCEID-LC13.9 พบว่าเวกเตอร์สามารถเพิ่มจำนวนและควบคุมการเรืองแสงสีเขียวในเซลล์แบคทีเรีย *L. rhamnosus* 10 ได้ ดังนั้น แบคทีเรียทั้ง 4 ไอโซเลต นี้ จึงเหมาะสำหรับนำไปทดสอบคุณสมบัติโพรไบโอติกในขั้นสูงต่อไป เช่น การทดสอบคุณสมบัติโพรไบโอติกในระดับสัตว์ทดลอง และการทดสอบใช้ในงานด้านพันธุวิศวกรรมเพื่อพัฒนาตัวนำส่งวัคซีนชนิดกิน

ABSTRACT

TITLE : ISOLATION IDENTIFICATION AND CHARACTERIZATION OF PROBIOTIC BACTERIA FROM INFANT'S FECES FOR POTENTIAL USE AS VACCINE DELIVERY VENHICLE
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This study aimed to isolate, identify and characterize probiotic bacteria from breastfed-infant's feces who was born by vagina delivery. These properties include hemolysis, tolerate to acid and bile salt, antimicrobial activity, antibiotic susceptibility, adhesion and cytotoxicity to intestinal cell. It was found that there were four bacterial isolates include LW01, LW02, LW09 and LW10, showed good probiotic properties. Bacterial species identification by 16S rRNA sequence analysis of demonstrated that four isolates LW01, LW02, LW09 and LW10 were identified as *Lactobacillus rhamnosus* LW01, *Lactobacillus rhamnosus* LW02, *Lactobacillus rhamnosus* LW09 and *Lactobacillus rhamnosus* LW10. Electrotransformation of plasmid pLC13.9:pLDH-PRO1:GFPuv to *L. rhamnosus* LW10 isolate found that this plasmid was able to replicate and control the expression of express green fluorescent protein (GFP) gene in all *L. rhamnosus* isolates. For these results, these candidate probiotic bacteria should be considered for the further advance study of probiotic properties in animal model and determination of genetic engineering for development of these bacterial isolates as vaccine delivery vector.

CONTENTS

CONTENTS (CONTINUED)

CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Bp	Base pairs
CFU	Colony forming
ºC	Degree Celsius
Cont.	continue
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates

DW	Distilled water
EDTA	Ethylene diamine tetraacetic acid disodium salt
et al	Et. Aalii (Latin), and other
g	gram
FBS	Fetal bovine serum
HCl	Hydrochloric acid
KCl	Potassium chloride
LB	Luria-Bertani medium
m	Milli (10^{-3})
M	Molar
$MgCl_2$	Magnesium chloride
mg	Milligram
ml	Milliliter
mm	Millimeter
PEG	polyethylene glycol
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TAE	Tris-acetate EDTA
TE	Tris-EDTA buffer
Tris	Tris-Hydroxyethyl amino
w/v	Weight by volume

LIST OF ABBREVIATIONS (CONTINUED)

µ	Micro (10^{-6})
µF	microfarads
µl	Microliter
Ω	Ohm

CHAPTER 1
INTRODUCTION

1.1 Background and rational of the study

According to the Food and Agriculture Organization/World Health Organization (FAO/WHO), probiotics are live microorganism that upon administrated in adequate amounts can contribute health benefits to the host (FAO/WHO, 2006). Several probiotic strains belonged to bacterial genera that include *Lactobacillus* sp. *Bifidobacterium* sp. *Lactococcus* sp. *Leuconostoc* sp. and *Enterococcus* sp. (Fijan, 2014). Probiotics are member of Lactic acid bacteria (LAB). LAB are heterogenous group of Gram-positive bacteria that produce lactic acid as major end product during fermentation of carbohydrate. They can be isolated from various environments including, fermentation products, breast milk, gastrointestinal tract (GI) of animal and human.

There are many beneficial effects in human that are exerted by *Lactobacillus* sp. and *Bifidobacterium* sp. These benefits include contribute to intestinal microbial balance, protect against pathogen infection, prevent and treat of inflammatory bowel disease (IBD)(Bermudez-Humaran et al., 2013), reduce allergies, cancer prevention and promote immune system stimulating (Ouwehand, Salminen and Isolauri 2002 ; Nagpal, 2012). The properties of probiotic bacteria, such as stability in gastric juice and bile salt, adhere and colonize in the gastrointestinal (GI) tract, produce antimicrobial against the pathogens and are safe for the host. In additional, probiotic strain especially *Lactobacillus* and isolated from human's intestine had ability to resist gastrointestinal condition better than those of strain isolate from another source (Tulumoglu et al., 2013). These properties are important in the application of probiotics.

Recently, probiotics has been increasing interest in the use as mucosal delivery vehicles for development oral live vaccine to promote specific immune system response and for therapeutic and prophylactic molecules, which is used microorganism for the antigen or DNA delivery vehicle which have immunogen properties (Wells and Mercenier, 2008).

Therefore, it is necessary to isolate and characterize the properties of probiotics for applications in genetic engineering.

For the above reason, it is interesting to isolate identify and characterized of probiotic bacteria from infant' s feces for potential use as vaccine delivery vehicle. As well as studying the properties and applications of probiotics. If receiving probiotics have good properties will be able to further develop the experiment to take these probiotic bacteria in the benefit of using vaccine delivery vehicle.

1.2 Research question

1.2.1 Can *Lactobacillus* sp. and *Bifidobacterium* sp. isolate from infant's feces?

1.2.2 *Lactobacillus* sp. and *Bifidobacterium* sp. isolate can have probiotic.

1.3 Hypothesis

1.3.1 *Lactobacillus* sp. and *Bifidobacterium* sp. can isolate from infant's feces.

1.3.2 *Lactobacillus* sp. and *Bifidobacterium* sp. isolated are probiotic strain.

1.4 Objective

1.4.1 To isolated *Lactobacillus* sp. and *Bifidobacterium* sp. from infant's feces.

1.4.2 To determine probiotic properties of *Lactobacillus* sp. and *Bifidobacterium* sp. isolated from infant's feces.

1.4.3 To study probiotic applications in genetic engineering.

1.5 Anticipated outcome

1.5.1 Received *Lactobacillus* sp. and *Bifidobacterium* sp. with good probiotic properties for further application.

1.6 Scopes and Limitation

1.6.1 Isolate probiotic from breastfed- infant's feces who was born by vagina delivery.

1.6.2 Identify and characterize of probiotics isolated from infant's feces

CHAPTER 2
LITERTURE REVIEW

2.1 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are heterogenous group of non-sporulating, catalase negative and Gram-positive bacilli or cocci (Axelsson, 2004). They can produce lactic acid as major end product during fermentation of carbohydrate which include *Lactobacillus,* Bifidobacterium, *Lactococcus*, *Leuconostoc, Pediococcus, Carnobacterium, Streptococcus* and *Enterococcus* sp. LAB that can be found in diverse environments, such as yogurt, milk product, wine, plan, fermented food and gastrointestinal tract of animal and human (Michon et al., 2016*).*

Lactic acid bacteria can be divided into two groups. The first group, homofermentative LAB convert sugars almost quantitatively to lactic acid. The second group, heterofermentative LAB produce not only lactic acid but ethanol or acetic acid and carbon-dioxide (Klaenhammer, 1993). The optimal growth temperature rang 18 to 45 ºC, sodium chloride concentration 6.5% and 8.0% and pH of 4.4 to 9.3

Cell wall of lactic acid bacteria contain of thick peptidoglycan (PG) that surrounds the inner cytoplasmic membrane. This membrane is decorated with lipopolymers such as teichoic acids (TAs), lipoteichoic acids (LTAs) or polysaccharides (PSs) and proteins such as mucus binding protein (Chapot-Chartier and Kulakauskas, 2014).

Bacterial cell surface components such as adhesins that bind to lectin, polysaccharides and proteins like mucus binding proteins play major roles in adherence of LAB to surface such as the intestinal epithelial cells.

Species of LAB such as *Lactobacillus acidophyrus*, *Lactobacillus plantarum, Lactobacillus delbruckii* and *Lactobacillus bulgaricus,*etc were use as food technology and can be produce growth factor such as amino acid, vitamin and nucleic acid precursors.

2.2 *Lactobacillus* species

Lactobacilli are a diverse group of Gram-positive bacilli or rod (Figure 2.1, A) , non-sporulating (Calasso and Gobbetti, 2011) and non-hemolytic on blood agar (Figure 2.1, B), lactic acid producing anaerobic rods with varying oxygen tolerance. They are acid-tolerant and may grow at pH as low as 3.5. More than 150 species have been identified within the *Lactobacillus* genus, with substantial genetic and phenotypical differences between different groups. Lactobacilli are ubiquitous where carbohydrate substrates are available, i.e. on mucous membranes of man and animals, on plant and plant materials, in manure, sewage and in fermented or spoiled food (Rogosa and et al., 1961). Lactobacillus-containing food produced through fermentation includes e.g. sourdough, cheese, yoghurt, marinated fish and meat, fermented vegetables and wine. Lactobacilli are generally considered nonpathogenic or even health-promoting. Lactobacilli are often found in carious lesions and were previously believed as being one of the main cariogenic bacterial groups. Today, however, they are mainly regarded as secondary invaders without a causative role in the caries process.

(A) **(B)**

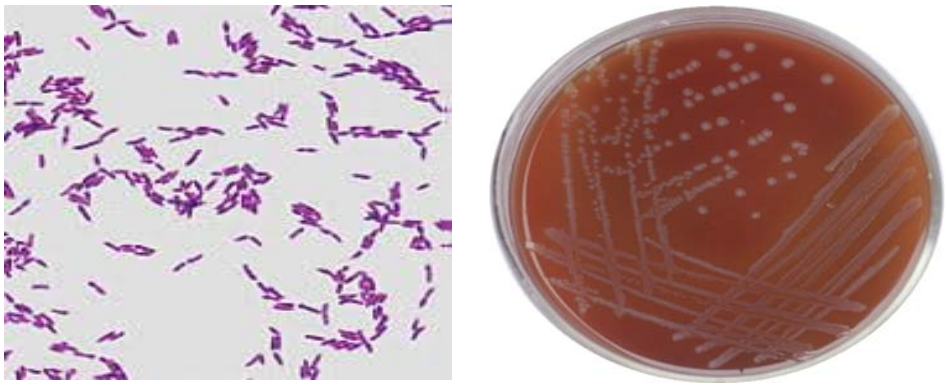

Figure 2.1 A) Morphological characteristics of Lactobacilli is Gram positive bacilli
B) Non-hemolysis of Lactobacilli on blood agar

2.3 *Bifidobacterium* species

Bifidobacterium is a genus of bacteria with various forms; nonmotile they are gram-positive irregular bacilli (Figure 2.2, A), nonsporulating, anaerobic branched non-hemolytic on blood agar (Figure 2.2, B). These bacteria were first isolated from infant feces and attracted attention because of their important physiological significance to the host organism. Species that are important human gut bacteria include *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium adobacterium* and *Bifidobacterium longum*. The percent GC in Bifidobacterium DNA ranges from 55% to 67% (Caballero, 2012).

The bacterial cells are short and thin, with pointed ends, and are irregular. They also appear as long cells with many branches and slightly branching spoon-shaped cells. Cells are arranged as single cells, chains, polymer-shaped, V-shaped, or palisade-shaped. Their distinct cell morphology can be helpful in differentiating bacteria belonging to this genus. For example, *B. bifidum* appear as flask-shaped cells, while *Bifidobacterium asteroides* are star-shaped. All members of this genus are gram-positive. Some strains are resistant to oxygen in the presence of CO_2. The optimal temperature for the growth of this bacterium is 37–41 °C, and the optimal pH value ranges from 6.5 to 7.0.

(A) **(B)**

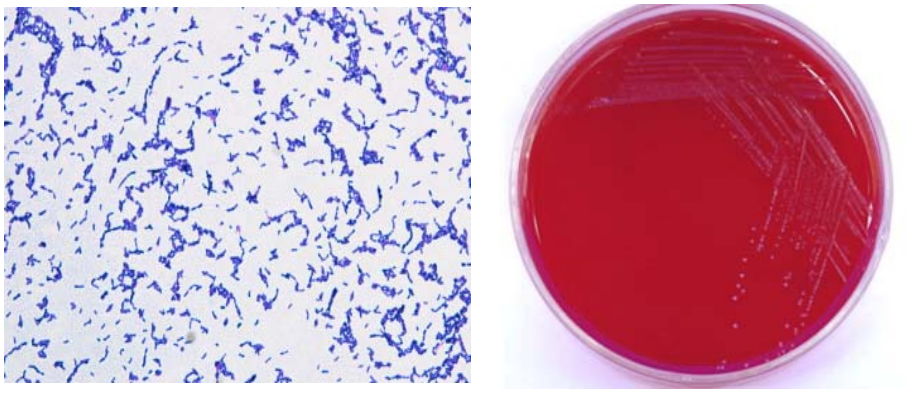

Figure 2.2 A) Morphological characteristics of Bifidobacterium is Gram positive irregular bacillus

B) Non-hemolysis of Bifidobacterium on blood agar (Pereira and Gibson, 2002).

2.4 Identification of LAB

Many LAB have similar nutritional and growth requirements, biochemical-based methodologies for identification are not conclusive in many cases.

2.4.1 Phenotypic identification of LAB

The conventional microbiological methods for bacterial identification are based on morphological and physiological characteristics such as Gram staining, cell shape, spore formation, enzyme production and the fermentation of different carbohydrates. Considering these methods, the API system and Biolog (Biolog, are both widely used). Both methodologies are based on the fermentation patterns presented by the microorganisms. The API strips used for LAB identification are 50 CHL, as the results are given as a combination of the fermentation of 49 carbohydrates and esculin hydrolysis. Biolog is a unique plate used for Gram-positive and Gram-negative bacteria and it analyses the fermentation of 96 carbohydrates. However, these methods are non-reliable for identifying of LAB.

Some LAB species such as *L. rhamnosus*, *L. casei* and *L. paracasei* are difficult to identify by phenotypic features and so, need molecular methods for genotypic identification of LAB (Holzapfel et al., 2001).

2.4.2 Molecular identification of LAB

16S ribosomal (rRNA) gene sequencing is one of the most commonly used techniques for identification of LAB species (Temmerman et al., 2004; Elmaci and et al., 2015).

2.5 LAB as probiotic agent

Probiotics are defined by the Food and Agriculture Organization/World Health Organization (FAO/WHO) as “live microorganism that upon administrated in adequate amounts can contribute health benefits to the host” (FAO/WHO, 2006). Many LAB such as *Bifidobacterium longum, Lactobacillus casei* and *Lactobacillus brevis* are reported probiotic. They are non-toxic and safe to animal and human, which probiotic properties such as resist to acid and bile, produce antimicrobial against pathogenic and adherence to intestinal cell.

Probiotics have been reported to provide a wide variety of beneficial health effects and such effects have shown to be strain-dependent. The most commonly used probiotic bacterial strains belong to the genera *Lactobacillus* and *Bifidobacterium.* These bacterial genera are regarded as a part of the normal human microbiota. Even though some species of these genera are “Generally Recognized As Safe” (GRAS) by the United States Food and Drug Administration (FDA) or have the “Qualified Presumption of Safety” (QPS) status by the European Food Safety Authority (EFSA), the working groups of the WHO/FAO recommend further studies to demonstrate that a particular probiotic strain is safe (Papizadeh et al., 2016).

Some LAB can be found in food industry for making yoghurt, cheese, sourdough bread, sauerkraut, pickles, beer, wine and other fermented foods and animal feeds like silage. LAB can also produce a variety of functional oligosaccharides that have applications as prebiotics. They can elevate the nutritional value of foods by the manufacture and development of flavor in fermented food products. In addition, degradation of various compound can be further converted to various such as alcohols, aldehydes, acids, esters and Sulphur compounds for specific flavor development in fermented food products. The three main pathways which are involved in the manufacture and development of flavor in fermented food products are the first glycolysis (fermentation of sugars). Second lipolysis (degradation of fat) and third proteolysis (degradation of proteins). Lactate is the main product generated from the metabolism of carbohydrates and a fraction of the intermediate pyruvate can alternatively be converted to diacetyl, acetoin, acetaldehyde or acetic acid

Besides probiotic LAB, certain strains of LAB are able to produce specific beneficial compounds in foods include. LAB producing folate such as

Bifidobacterium animalis Also they can produce vitamin B-group thiamine (B_1), riboflavin (B_2), niacin (B_3), pyridoxine (B_6), pantothenic acid (B_5), biotin (B_7 or H) such as *Lactococcus lactis*, *Lactobacillus fermentum* and *Streptococcus thermophilus* (Gu and Li, 2016)

Probiotic foods containing live microorganisms such as *Lactobacillus* and *Bifidobacterium* strains are growing in popularity. These probiotics foods and probiotics LAB can promote health by maintaining the gut microflora balance, aiding in the treatment and prevention of infectious diseases such as acute GI tract infections.

The majority of the microorganisms used for probiotic purposes belong to the genus *Lactobacillus* and *Bifidobacterium*. In the oral cavity, lactobacilli usually comprise fewer than 1% of the total cultivable microbiota, but no species specific to the oral cavity has been found. In contrast, some species are found in both oral and fecal samples include *L. paracasei, L. plantarum, L. rhamnosus,* and *L casei.* In addition both *lactobacilli* and *bifidobacteria* can be found in breast milk, suggesting early exposure of the oral cavity to these bacteria. Bifidobacterial species isolated from oral samples include *B. bifidum, B. dentium,* and *B. longum.*

2.6 Properties of probiotic bacteria

The properties of probiotic include testing of the following important criteria such as plasmid stability, carbohydrate and protein utilization patterns, acid and bile tolerance and survival and growth, intestinal epithelial adhesion properties, production of antimicrobial substances, antibiotic resistance patterns, ability to inhibit known pathogens, spoilage organisms, or both, and immunogenicity (Harzallah and Belhadj, 2013). The ability to adhere to the intestinal mucosa is one of the more important selection criteria for probiotics because adhesion to the intestinal mucosa is considered to be a prerequisite for colonization.

2.6.1 Acid and bile tolerance

Viability and activity of probiotics during storage and when passing through the gastrointestinal tract (GIT) is also essential. Stomach and the surroundings of the GIT have the highest acidity; therefore, it is critical to establish the behavior and fate of the microorganism during the passage through this condition. In vitro tests typically resembling the conditions in the GIT are commonly used as a screening tool to identify potential probiotics. This is because colonization and potential health benefits can only be anticipated when these viable cells are able to survive through the natural barriers that exist in the GIT such as low pH conditions and degradation by digestive enzymes as well as by bile salts (Kailasapathy and Chin, 2000).

2.6.2 Antimicrobial activity

Some bacterial of the Bifidobacterium and Lactobacilli are used in commercial probiotic products which confer beneficial effects to the host by improving intestinal microbial balance. The ability of probiotics to decrease and inhibit the infectious diseases in the gut is one of the most important health claims. It is well documented that probiotics promote many aspects of human health such as innate immunity and healing of gastrointestinal disorders via different approaches. They can compete with bacterial pathogens by producing antimicrobial compounds such as organic acids and bacteriocins

The antimicrobial metabolites produced by probiotics can be divided into two groups: (1) low-molecular mass compounds (below 1.000 Da) such as organic acids, which have a broad spectrum of action and (2) antimicrobial proteins, termed bacteriocins (> 1.000 Da), which have a relatively narrow specificity of action against closely related organisms and other gram-positive bacteria.

The organic acids secreted in the fermentative metabolism of carbohydrates by probiotics have been considered to be the main antimicrobial compounds responsible for their inhibitory activity against pathogens. In addition, in the presence of oxygen, some probiotics are able to generate hydrogen peroxide that has oxidizing effect on bacterial cell surface. Carbon dioxide is produced by heterofermentative bacteria and its antimicrobial activity is due to inhibition of enzymatic decarboxylation and its accumulation in the membrane causing dysfunction in the permeability of the membrane. Bacteriocins are compounds with potential antimicrobial activity

synthesized by many bacterial species, including lactic acid bacteria (Chen and Hoover, 2003). Bacteriocins have been subdivided into four classes: class I, the antibiotics, comprising small (< 5 kDa) heat-stable peptides that contain post translationally modified amino acids; class II, the nonantibiotic peptides, comprising small (< 10 kDa) heat-stable proteins; class III, comprising large (> 30 kDa) heat-labile proteins; and class IV, comprising an undefined mixture of proteins, lipids, and carbohydrates. In addition, the term bacteriocin-like compound has been coined to refer to antagonistic substances that are incompletely defined or do not fit the typical criteria defining bacteriocins and tend to have a broader spectrum of activity. Some bacteriocin-like compounds have also been described for probiotics and a unique bacteriocin from *Bifidobacterium bifidum* NCFB 1454 has been purified. Probiotics have exhibited antagonistic effects against different pathogenic species, including *Salmonella*, *Listeria*, and *Helicobacter* among others. Owing to the potential interest of these antimicrobial proteins in novel therapeutic developments, further studies should be carried out on their genetics, biochemistry, and mechanisms of action.

2.6.3 Adhesion to human cell

The ability to adhere to the intestinal mucosa is considered one of the main selection criteria for potential probiotics as it prolongs their persistence in the intestine and thus allows the probiotic to exert its healthful effects longer bacteria have a greater impact when able to adhere to the intestinal mucosal surface and thus maintain their presence in the gut longer (Apostolou et al., 2001). Mucus has a dual role in the management of the microflora. It can promote bacterial colonization by serving as an initial binding site,

2.7 LAB in human gastrointestinal tract (GI)

The gastrointestinal (GI) tract of humans and other mammals is inhabited by ~10^{14} bacteria from approximately 1000 or more different species, which continuously interact with their host as they grow and form a diverse microbiota of the human GI microbiota

The intestinal microbiota, plays an important role in the development of the gut immune system, digestion of food, production of short-chain fatty acids and essential vitamins, and resistance to colonization from pathogenic microorganism. The human

gut microbiome consists of many different species of bacteria, some of which are nonculturable and therefore not well known or characterized. Indeed, it has only been through the advent of deep sequencing, genomics, and metagenomics in the last decade that the complexity of the microbiota has been fully appreciated.

The distribution of the intestinal microbiota varies along three main locations in the digestive tract

2.7.1 The stomach, populated by $<10^2$ colony-forming units (CFU)/ml, including lactobacilli and streptococci.

2.7.2 The ileum and distal ileum, populated by 10^2–10^3 CFU/ml of bacteria, including *E. coli*, *Klebsiella*, *Enterococcus*, and *Bacteroides*.

2.7.3 The large intestine, which constitutes the largest microbial population of the body, with 10^{10}–10^{12} CFU/ml

Each individual organism presents a specific "bacterial fingerprint," which is influenced by a variety of factors including the maternal environment, host genotype, diet, and antibiotic treatment. but even though the composition of the microbiota differs from person to person, its clusters in three distinct groups, so-called enterotypes. These human enterotypes are enriched in *Bacteroides*, *Prevotella*, or *Ruminococcus* and use different routes to generate energy from fermentable substrates available in the colon: *Bacteroides* uses carbohydrates; *Prevotella*, mucins; and *Ruminococcus*, mucins and sugars.

Enterotypes have also been associated with long-term diets. Despite this heterogeneity, Firmicutes and Bacteroidetes are the most common intestinal phyla across all vertebrates, representing > 90% of the microbiota, followed by Actinobacteria and Proteobacteria. Members of the microbiota from phylum Bacteroidetes are represented by a variety of species, including *Bacteroides fragilis*, which was recently shown to possess immunomodulatory capabilities via its polysaccharide capsule. In contrast, phylum Firmicutes is mainly represented by species belonging to class Clostridia, which are known for their abilities to metabolize fiber and produce butyrate, a short-chain fatty acid with immunomodulatory activity. Bacteria belonging to class Bacilli, including *Lactobacillus acidophilus* and *Enterococcus faecalis*, constitute the rest of the Firmicutes phylum.

2.8 LAB from infant newborn

LAB some species, especially Lactobacilli and Bifidobacterium, colonize the new-born as soon as the infant is breast-fed.

Bacterial colonization of the human gut is a complex process that seems to start, at a small scale, during the fetal period. Contact with microorganisms belonging to the vaginal, intestinal and mammary microbiota of the mother, and to the surrounding environment of the neonate, leads to a notable intensification of this process after birth. As a consequence, factors such as composition of the maternal microbiota, place and way of birth and/or feeding pattern, play key roles in a process that exerts a strong influence on host functions so important as nutrient absorption, formation of host barriers against pathogens, or maturation of the immune system.

Breastfeeding is the best way to provide newborns with the nutrients that they need for healthy growth and constitutes a continuous source of bacteria to the infant gut, including staphylococci, streptococci, bifidobacterial and lactic acid bacteria.

The World Health Organization (WHO) and The United Nations Children's Fund (UNICEF) recommended exclusive breastfeeding for the first six months of life, and its pursuit up to two years associated with appropriate complementary feeding (Jeurink et al., 2012). Human milk educates the infant immune system and confers protection against gastrointestinal infections, respiratory infections, allergic diseases and it is also associated with a reduced long-term risk of diseases such as inflammatory bowel disease (IBD) (Duijts and et al., 2010 ; Turin and Ochoa, 2014), obesity or diabetes as reviewed by the American Academy of Pediatrics. The protective role of breast milk seems to be the result of the synergistic action of several bioactive molecules such as carbohydrates, oligosaccharides, nucleotides, fatty acids, immunoglobulins, cytokines, immune cells, lysozyme, lactoferrin and other immunomodulating factors (Scholtens et al., 2012). Human milk oligosaccharides (HMOs) are nondigestible carbohydrates that are fermented in the colon, stimulating the growth and/or activity of specific fecal bacteria that impact health positively in infants receiving breast milk (Scholtens et al., 2012).

2.9 LAB from Thai infant newborn

Lactic acid bacteria and Bifidobacterium isolated from feces of healthy Thai Infants from Prince of Songkla University, Thailand (Nakpheng et al., 2012). The human isolated LAB and Bifidobacterium were identified by using 16S ribosomal DNA sequencing as he human isolates LAB and bifidobacterial identified as *L. rhamnosus, L. casei, L. plantarum, B. longum* subsp. *longum* and *B. bifidum* exhibited high acid and bile tolerance and antibacterial activity against food-borne pathogens. These strains suggest the great potentiality of probiotics in controlling infection by food-borne pathogens in humans. The inhibition of pathogens by LAB was mainly caused by the secretion of antibacterial substances while bifidobacterial effectively inhibited pathogens through their highly competitive exclusion activity of mucin adhesion. The combination of these multiple strains in the presence of gut microflora challenged with food-borne pathogens should be further investigated.

In additional, five *L. rhamnosus* strain include UBU03, UBU06, UBU09, UBU034 and UBU37 isolated from breast-feed from Ubon Ratchathani University, Thailand (Thaw et al., 2017), which have good probiotic properties. All five *L. rhamnosus* strains exhibited high capacity to adhere to Caco-2 cells.

There are three strain, UBU09, UBU34 and UBU37 showed higher percentages of adhesion and all tested strains except UBU03 could competitively inhibit the adhesion of enteropathogenic bacterium, *E. coli* EPEC O157:H7 to Caco-2 cells.

In assays of their immunomodulatory activity using the THP-1 cell line, it was found that the most potent inducers of IL-6, IL-10 and TNF-α production were UBU03, UBU37 and UBU34, respectively. None of the tested strains could induce IL-12 production. Induction of multiple cytokines occurred in some cases: UBU06 induced production of IL-6 and IL-10, UBU34 induced IL-6 and TNF-α and UBU37 induced IL-6, IL-10 and TNF-α. Our findings on the adhesive and immunomodulatory properties of five strains of probiotic *L. rhammosus* set the scene for further studies on animal models before translation to proper clinical applications.

2.10 The interaction of probiotic with intestinal epithelial cell

The intestinal mucosa is the body's first line of defense against pathogenic and toxic invasions from food. After ingestion, orally administered antigens encounter the GALT (gut associated lymphoid tissue), which is a well-organized immune network that protects the host from pathogens and prevents ingested proteins from hyper stimulating the immune response through a mechanism called oral tolerance (O'flaherty et.al, 2010).

Probiotics interact with intestinal epithelial cells (IECs), mucosal dendritic cells (DCs) and macrophages through diverse way. Pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) play essential roles in recognition and delivery of signaling cascades, which mediate different gene expression profiles. DCs in lamina propria (LP) could contact with probiotic in gut lumen through the dendrites and then importing them into the lumen by M-cell mediated transcytosis. Depending on types of probiotic strains, they can either induce immune activation signaling by producing IL-12, IL-1b and TNF-a or trigger tolerance signaling by stimulating anti-inflammatory cytokines such as IL-10 and TGF-b levels. Under the IL-10/TGF-b enriched cytokine milieu, DCs and macrophages can enhance the generation of induced regulatory T cells (iTregs) that play key roles in maintaining peripheral immune tolerance by balancing the ratio of effector and regulatory T cell (Figure 2.3) (Lebeer et al., 2010).

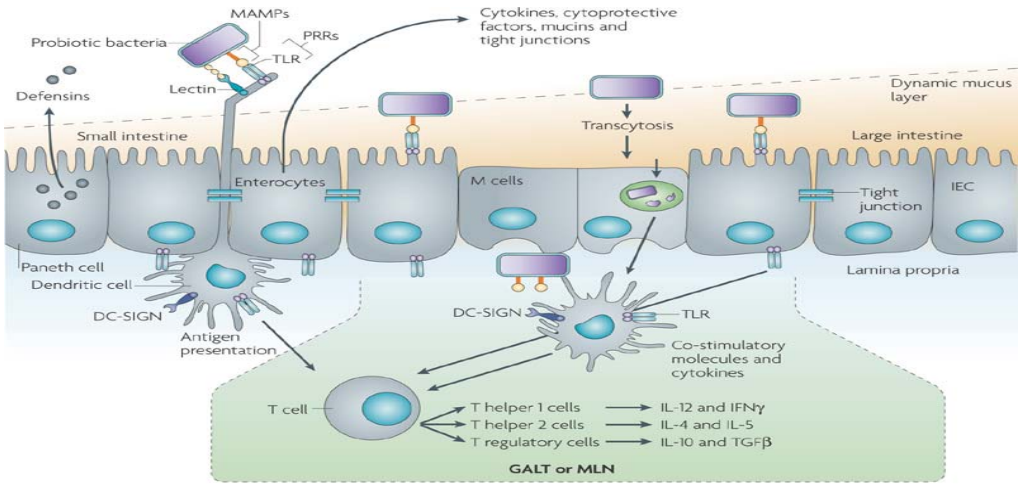

Figure 2.3 The interaction of probiotic with intestinal epithelial cell

Source: Harzallah and Belhad (2013)

2.11 Clinical uses of probiotic

L rhamnosus strain GG (ATCC 53103) is by far the most thoroughly explored of all lactic acid bacteria so far correctly taxonomically identified. It has been clinically tried and is extensively regarded as being suitable for microbial interference treatment (MIT)to the prevention and treatment of infections include Diarrhea disease, Inflammatory bowel disease (IBD), Colorectal cancer and etc. (Bengmark, 1998).

2.11.1 Diarrhea disease

Use of probiotic to treat or prevent acute diarrhea disease of chidden caused by rotavirus. *L. rhamnosus* strain GG use combined with *B. bifidum* and *S. thermophilus* found that probiotic can reduce symptoms of diarrhea from rotavirus (Shornikova et al., 1997). Besides, probiotic also help prevent and treat diarrhea in tourists and the elderly caused by *Clostridium difficile* in colon.

2.11.2 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) affects millions of people worldwide and refers to two chronic diseases that cause inflammation of the intestines: Crohn's disease (CD) and ulcerative colitis (UC) (Bermúdez-Humarán et al., 2013). Previous studies have shown that probiotic, especially *L rhamnosus* strain GG can reduce inflammation by reduce the amount of microorganisms in the intestines that cause inflammation.

2.11.3 Colorectal cancer

Intestinal bacteria could play a part in initiation of colon cancer through production of carcinogens, cocarcinogens, or procarcinogens. In healthy people, diets rich in fat and meat but poor in vegetables increase the fecal excretion of N-nitroso compounds, a group of genotoxic substances that are known initiators and promoters of colon cancer. probiotic such as lactobacillus and bifidobacteria prevent tumorigenesis. modulates risk of colonic cancer in human beings.

2.12 Live bacteria as vaccine delivery vehicle

Vaccination is one of the most successful immunology applications that has considerably improved human health (Yurina, 2018). Several studies found that One of the best DNA vaccine delivery systems use a live bacterial as the carrier. The live bacterial vector induces a robust immune response due to its natural characteristics that are recognized by the immune system. Moreover, the route of administration used by the live bacterial is through the mucosal route that beneficially induces both mucosal and systemic immune responses. The mucosal route is not invasive, making the vaccine easy to administer, increasing the patient's acceptance. Lactic acid bacterium is one of the most promising bacteria used as a live bacterial for vaccine delivery vehicle

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Ethical approval

The experiment was approved by the Human Ethical Committee of Ubon Ratchathani University, Ethics Committee Ref. No UBU – REC – 14/2561, Ubon Ratchathani University, Thailand, during on 30 Much to October 2018.

3.2 LAB isolation from infant's feces

The study was performed on 30 Macrh to January to October 2018 and site of sample collection was done in Ubon Ratchathani, Thailand. Fecal sample were collected from 10 healthy infants which have inclusion criteria and exclusion criteria are as follow.

3.2.1 Inclusion criteria

3.2.1.1 Healthy infants aged of 10 day to 5 months

3.2.1.2 Born by vagina delivery

3.2.1.3 Not received antibiotics

3.2.1.4 Breastfed

3.2.2 Exclusion criteria

3.2.2.1 Unhealthy infants and aged over aged 5 months

3.2.2.2 Not born by vagina delivery.

3.2.2.3 Received antibiotics

3.2.2.4 Not breastfed

One gram of fecal samples was homogenized in 9 ml of normal saline. The homogenized were diluted by the 10-fold serial dilution method. 100 µl of sample were spread plate on de Man, Rogosa and Sharpe agar (MRS)plate (LAB M, United Kingdom) containing 0.5% (w/v) Calcium Carbonate ($CaCO_3$) and incubated at 37 ºC for 48 h. under aerobic condition to selected *Lactobacillus* strain. For *Bifidobacterium* strain 100 µl of sample were spread plate on Bifidus Selective agar (BSM) plate (LAB

M, United Kingdom) containing 0.5% (v/v) L-cysteine and incubated at 37 ºC for 48 h. under anaerobic condition

A single colony on plate from each condition was investigated morphology and Gram strain. Rod shape morphology, gram positive and catalase negative of each isolate were selected and incubated in MRS broth at 37 ºC for 48 h. The stock culture of LAB was collected in MRS broth containing 30% glycerol and stored at -20 ºC

3.3 Hemolysis activity

The each of LAB isolates were incubated in MRS broth. 10 µl of LAB was dropped on blood-base agar with 5.0% human blood and incubated at 37 ºC for 24 h with aerobic condition and then observed hemolysis activity. There are three hemolysis type, beta-hemolysis (clear zone around bacterial colony) , and alpha-hemolysis (green zone and partial clear zone around bacterial colony) and grammar-hemolysis (no clear zone around bacterial colony) . LAB isolates with grammar-hemolysis were collected for use further study of probiotic properties.

3.4 Acid and bile tolerance

A single colony of LAB isolates were grown in 10 ml MRS broth and incubated at 37 ºC for 18 hours with aerobic condition and then centrifugation at 3,000 rpm for 10 min and removed supernatant. The bacterial cells were washed twice in PBS pH 7.4 and was then adjusted of bacterial cells to $1.5x10^8$ CFU/ml by comparing with McFarland No. 0.5. In this study *Lactobacillus rhamnosus* GG (ATCC53103) was used as reference strain

To determine tolerance of LAB in acid solution. 100 µl of each LAB which has amount cell bacterial $1.5x10^8$ CFU/ml was added into acid solution (NaCl 12 g, pepsin 3.2 g, dissolved in 1 L of distill water, pH 2 and 3) and pH 7 (control). All 10 LAB isolates in each of acid solution were incubated at 37 ºC for 3 hours with aerobic condition. After incubation, 100 µl of each LAB solution was prepared 10-fold serial dilution and dropped 10 µl on MRS plate. The number cell bacterial was used to calculate the percentage (%) of cell survival by form as. follow.

$$\text{percentage (\%) of cell survival} = (\log CFU_T / \log CFU_c) \times 100$$

Where CFU_T and CFU_C represent the total to viable count of LAB before and after incubated in acid and bile solution

The determine tolerance of LAB in bile solution. 100 µl of each LAB which has amount cell bacterial $1.5x10^8$ CFU/ml was added into 0.3 % (w/v) bile salt solution pH 8 (Sigma, USA) and incubated at 37 ºC for 4 hours with aerobic condition. After incubation, 100 µl of each LAB solution was prepared 10-fold serial dilution and drop 10 µl on MRS plate. The number cell bacterial was used to calculate the percentage (%) of cell survival as previously described.

3.5 Antimicrobial activity

The antimicrobial activity of 10 LAB were examined by Agar well-diffusion method. Three pathogens were used indicator strain that include *Escherichia coli* O157:H7, *Salmonella thyphimurim* and *Vibrio cholerae*. The indicator strain was grown in 5 ml Brain heart infusion (BHI) (HiMedia) broth at 37 ºC for 18-24 hours. 200 µl of each pathogenic mixed with 20 ml warmed 0.8% (w/v) BHI agar. The mixture was poured into Pedti dish plate. When the BHI medium became solid, BHI media were made to well with diameter 5 mm.

LAB isolates were grown in 10 ml MRS broth at 37 ºC for 18-24 hours. After incubation, the cell-free supernatant (CFS) was collected by centrifugation 3000 rpm for 10 min. The CFS was filtered by 0.45 µm of syring membrane filter and then 50 µl of the CFS of LAB was added into well prepared as above and incubated at 37 ºC for 24 hours.

3.6 Antibiotic susceptibility test

To determine to antibiotic susceptibility of the all ten LAB isolates. Antibiotic susceptibility test was performed by using Disk diffusion method. A single colony of each LAB was grown in 10 ml MRS broth and incubated at 37 ºC for 18 hours with aerobic condition. The bacterial culture was adjusted to $1.5x10^8$ CFU/ml by comparing McFarland No. 0.5. One ml of adjusted bacterial culture was supplemented in 20 µl warmed MRS agar medium and pour into Pedti dish plate. After the MRS become solid. Each antibiotic dick was placed on MRS plate and incubated at 37 ºC for 24 hours. The diameter of zone of inhibition was measured as millimeter (mm). The results obtained were expressed in terms of susceptibility, moderate susceptibility, or resistance. Results were compared with interpretative zone diameters described by of the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards (CLSI, 2017). Eight antibiotics used in this study were Ampicillin (Amp10) Chloramphenicol (Ch30) Ciprofloxacin (Cip5) Erythromycin (E15) Gentamycin (Gen10) Penicillin G (PG10) Tetracycline (T30) Vancomycin (V30) (Table 3.1)

Table 3.1 Antimicrobial disk diffusion zone interpretation guide for LAB

Antimicrobial Agent	Concentration (µg)	Zone Diameter Interpretive Criteria (mm)		
		R	I	S
Ampicillin (Amp10)	10	<21	22–28	>29
Chloramphenicol (Ch30)	30	<12	13–17	>18
Ciprofloxacin (Cip5)	5	<15	16–20	>21
Erythromycin (E15)	15	<11	14–20	>22
Gentamycin (Gen10)	10	<12	13-14	>15
Penicillin G (PG10)	10	<14	15-17	>18
Tetracycline (T30)	30	<11	12-14	>15
Vancomycin (V30)	30	<9	10-11	>12

3.7 Cell adhesion assay

For the bacterial adhesion assay, the human intestinal Caco-2 cells line were used in this study. The frozen Caco-2 cells were thawed once. Night milliliter were transferred into complete media which prepared from Dulbecco' Modified Eagle's Medium: Nutrient Mixture F-12(D-MEM/F12) (Gibco,USA) supplemented 20% (v/v) Fetal bovine serum (FBS) and 1x Pen-Strep and then centrifugation at 3,500 rpm for 5 min at room temperature and decade supernatant. The Caco-2 cells were resuspended with 5 ml complete media (D-MEM/F12 supplemented 10% FBS) and transferred to tissue culture flask and incubated at 37 ºC for 5 day with 5% CO_2 to cell adherence. Monolayer of Caco-2 cells showed compete morphological and functional differentiation or 80-90% confluence. The cell culture was trypsinizated with 4 ml of 0.25 (w/v) trypsin EDTA (Corning, USA) and incubated 37 ºC for 5 min with 5% CO_2. After incubation, 4 ml of complete media were added to stop trypsin activity. For bacterial adhesion assay, the cell was calculated at a density of $2x10^5$ cell/ml and were seeded 1 ml/well into 24-well tissue culture plates and incubated at 37 ºC for 24 with 5% CO_2 to keep cells adherence. The culture medium was removed and the cells were washed twice with 1 ml PBS (pH 7.4) before preformed of bacterial adhesion assay.

3.8 Bacterial adhesion assay

The cells of LAB were harvested by centrifugation at 3,000 rpm for 10 min and washed twice with PBS (pH 7.4) Then LAB was adjusted amount of cell bacterial was $1.5x10^8$ CFU/ml by comparing McFarland No. 0.5 and then suspended in D-MEM/F12

One ml LAB suspension was added to Caco-2 monolayers prepared as above, after incubated at 37 ºC for 2 and 5 hours with 5% CO_2, the Caco-2 monolayers were washed twice with PBS. Then 0.2 ml PBS contain 1% Trion X-100 were added into the Caco-2 monolayers. After ten minutes, 0.2 ml PBS was added and 10-fold serial dilution to count bacterial cells by viable plate count using MRS agar. 10 µl each dilution was dropped to MRS agar, after incubated at 37 ºC for 48 hours, the adhesion was used to calculate the percentage (%) of viable bacterial as the formulation described below, compared with *L. rhamnosus* GG was used reference strain. This experiment was done in triplicate (Fang, 2015).

$$\% \text{ of cell adherence} = (\log CFU_T/\log CFU_c) \times 100$$

Where CFU_T and CFU_T represent the total to viable count of LAB before and after incubated on Caco-2 cells

3.9 Cytotoxicity assay

Caco-2 cells were cultivated on 96-well tissue culture plates for 24 hours. LAB was adjusted amount of cell bacterial was $1.5x10^8$ CFU/ml in D-MEM/F12. were added on cell culture monolayer in wells and incubated for 24 h at 37 °C with 5% CO_2. After incubation, supernatants were removed and the Caco-2 cells monolayer was washed twice and incubated with DMEM medium containing 2% Pen-strep for 3 hours. The supernatant was removed. MTT assay based on the reduction of MTT dye by active mitochondria was used to assess cell viability of the treated Caco-2 cells. 90 µL D-MEM/F12 containing 10 µL of MTT solution were added in each well and cells were incubated for 4 hours. After incubation, 100 µl DMSO was add into well to dissolve purple formazan. Plates were then read at 540 nm in a microplate reader spectrophotometer (Belguesmia, 2016). This study *L. rhamnosus* GG was used reference strain. The percentage (%) cell survival was calculated as the formulation described below:

$$\% \text{ of cell viable} = (A540_T/A540_c) \times 100$$

Where $A540_T$ represent the total to viable of Caco-2 cells without LAB and $A540_c$ represent the total to viable of Caco-2 cells after incubated with LAB

3.10 Genomic DNA extraction of Gram positive bacterial

One ml of bacterial culture for 24 hours was added into microtube on ice and bacterial culture was centrifugated at 13,000 rpm for 1 min and remove supernatant. The cell bacterial was washed twice in PBS and resuspended with 300 µl cell suspension solution (50 mg/ml lysozyme in STE). Add 1.5 µl lytic Enzyme solution (Protease K) and invert tube 25 time and then incubated at 37°C for 30 min to digest cell wall, centrifuge at 13,000 rpm for 1 min and remove supernatant. A 300 µl cell lysis solution was added into the cell pellet to lyse the cells. RNase treatment, 1.5 µl RNase A solution was added and mix by inverting the tube 25 time. Then on ice 1 min, Add 100 µl sodium acetate pH 4.8 to protein precipitate, vortex 20 sec and centrifuge at 13,000 rpm for 3 min. The supernatant was removed to the new microtube and was add 300 µl isopropanol. The solution was mix by inversion for 50 times and was centrifuged at 13,000 rpm for 1 min and remove supernatant. The DNA pellet was washed with 300 µl of 70% ethanol and invert tube. After centrifuge at 13,000 rpm for 2 min remove supernatant and air dry 30-45 min. 100 µl TE buffer were added into genomic DNA to rehydrate by incubating sample for 1 hour at 65°C. The sample of DNA were storage at -20°C or -80°C

3.11 Molecular identification

Lactobacillus strain were selected on the basis of probiotic properties and confirmed by 16S ribosomal RNA (16S rRNA) amplification and sequencing. Polymerase chain reaction (PCR) was used to amplify the target 16S rRNA with the universal primer, 27F(5'-agagtttgatcctggctcag-3') and 1492R (5'-ggttaccttgttacgact -3') The PCR components for the amplification were done in 50 µl of PCR mixture and consisted of 5 µl (2-50 ng/µl),36.5 µl PCR-grade water, 1.0 forward primer (10µM), 1.0 reverse primer (10µM), 5.0 µl 10x PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 2.0 µl dNTP solution (1.25 mM) and 1.0 µl *Taq* DNA polymerase (50 units/µl). The PCR conditions were show in Table 3.2.

The PCR products derived from PCR were purified by using Gel/ PCR DNA Fragments Extraction Kit (RBC, Bioscience Taiwan) according to the manufacturer's instruction. In briefly, one volume (one-hundred microliters) of PCR product was added with 5 volumes of DF buffer and mixed by vortex mixer. The sample mixture was applied into the DF column and centrifuged at 10,000 rpm for 30 sec. The flow-through was discarded. The DF column was added with 600 μL wash buffer and centrifuged at 10,000 rpm for 30 sec. After centrifugation, the column matrix was dried by centrifugation for 2 minutes at 10,000 rpm. The column matrix was added with 20-50 μL elution buffer at the center of the column and stand for 2 min until elution buffer or water was absorbed by the matrix. The purified DNA was eluted after centrifugation for 2 min at 10,000 rpm. The eluted DNA was stored at -20 °C until used.

Table 3.2 PCR condition used the 16s rDNA gene amplification

Target gene	Primer		DNA template	Amplification condition (Temperature)	Time (sec or min)	Number of cycle	Amplicon sized (bp)
	Forward	Reverse					
16s rDNA	27F	1492R	Genomic DNA of *Lactobacillus* strain	Pre-denaturation (94 ºC)	4 min	1	1,500
				Denaturation (94 ºC)	1 min	35	
				Annealing (54 ºC)	30 sec		
				Extension (72 ºC)	2 min		
				Final extension (72 ºC)	10 min	1	

3.12 Plasmid extraction

For plasmid extraction, 1.5 ml of bacterial culture were centrifuged at 13,000 rpm for 10 min and wash twice with STE. The bacterial cells were resuspended in 200 µl STE containing 50 mg/ml lysozyme, 1 µl mutanolysin (15 U/µl), 1µl RNase (50 mg/ml) and incubated at incubated at 37°C for 3 h. After incubation, the cells suspension were added with 400 µl of solution II and inversion at room temperature for 7 min and added 300 µl of solution III, on ice 5 min and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred into new microtube and added with 200 µl of Phenol: Chloroform: Isoamyl Alcohol (1:1:24) and were centrifuged at 10,000 rpm for 10 min. A 500 µl of Upper phase was transferred into new microtube and was added with 500 µl isopropanol and incubated at -20 ºC for 30 min and centrifuged at 10,000 rpm for 10 min. Plasmid were washed in 1 ml 70 % ethanol and centrifuged at 10,000 rpm for 10 min, resuspend plasmid in 15-20 µl of TE buffer, 2 µl were used for gel electrophoresis. For *Lactobacillus casei* TISTRI 1341 were used positive control

3.13 Agarose gel electrophoresis

The molecular size of DNA was determined by gel electrophoresis. The DNA or plasmid was mixed with the 6X gel-loading buffer in a ratio 1:2. The mixture was then loaded into the wells covered by electrophoresis buffers. The electrophoresis was carried through appropriate voltage (V) until the bromophenol blue have migrated the appropriate distance through the gel. The agarose gel was stained with ethidium bromide and destained with water. Finally, the gel was visualized under UVP's ChemiDoc-ItTS2 Imagers. The molecular size of DNA was determined by comparing with bands of DNA ladder (K plus marker).

3.14 Preparation competent cell

3.14.1 *Escherichia coli*

A single colony of *E. coli* DH5 alpha on LB agar plate was inoculated into 2 ml of LB medium and incubated at 37 ºC with shaking 250 rpm. The 1.5 ml of overnight bacterial culture was added into the flask containing 150 ml of LB medium and incubated with shaking 250 rpm at 37 ºC until the OD_{600} of the reached 0.6. the bacterial cell was harvested by centrifugation at 12,000 x g for 15 minutes, washed twice in cold sterile distilled water, the first wash with the volume of 150 ml and the second wash with the volume 75 ml. After washing, the bacterial pellet was washed with 6 ml of ice-cold sterile 10% glycerol and the bacterial pellet was finally resuspended in 750 µl of ice-cold 10% glycerol. Seventy microliters of bacterial suspension were aliquoted in ice-cold sterile 1.5 microcentrifuge tube and stored at -80 ºC until use.

3.14.2 *Lactobacillus* sp.

A single colony were grown in MRS broth at 37 ºC for 24 h and bacterial cell of LAB were harvest by centrifugation at 3,000 rpm for 10 min and placed on ice. The cell suspension was resuspended in 100 ml MRS broth and incubated at 37 ºC until as 1 of optical density (OD600). The bacterial culture was centrifugated at 3,000 rpm for 10 min and 3 time washed in deionize water and dissolved cell suspension in 1 ml of 30% PEG800 on ice.

3.15 Electroporation

3.15.1 *Escherichia coli*

One µg of pLC13.9: LDH-PRO1: GFPuv were mixed with 75 µl of competent cell. The DNA-competent cell mixture were add to cuvette (1 mm-width gap) was then subjected to high voltage electric pulse of 1.8 kV and contrasts time 4-5 milliseconds. Then the electroporated cell were added 1 ml soc medium and incubated with shaking 150 rpm at 37 ºC for 1 hours. After incubation, the electroporated cell were spread on 2xYT agar plate containing 100 µg/ml Ampicillin and incubated at 37 ºC for 24 hours.

3.15.2 *Lactobacillus* sp.

One µg of pLC13.9: LDH-PRO1: GFPuv is show in Figure 3.1 and were Mixed with 50 µl of competent cell. The DNA-competent cell mixture was added to cuvette (2 mm-width gap) was then subjected to high voltage electric pulse, 25 uF of capacitance, 400 ohms of parallel resistance, 2.5 kV of peak voltage for 8-10 milliseconds. Then the electroporated cell were added with 450 µl fresh MRS medium and incubated at 37 ºC for 3 hours After incubation, the electroporated cell were centrifugated at 3,000 rpm for 10 min and resuspended in 100 µl MRS broth. 100 µl cell suspensions were spread plate on MRS agar supplement 2.5 µg/ml and were and incubated at 37 ºC for 24 hours.

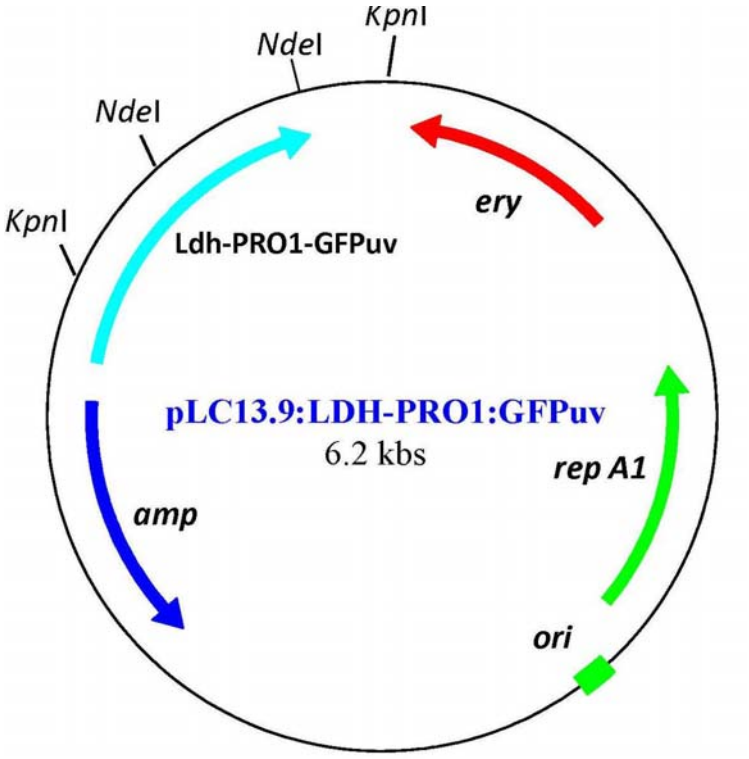

Figure 3.1 Schematic diagram of expression vector containing pLC13.9:LDH-PRO1: GFPuv, *Amp* ampicillin resistant gene, *Ery* Erythromycin resistant gene, origin of replication, gene encoding replication protein A1

3.16 Expression of Green fluorescent protein (GFPuv)

For determination expressing green fluorescent protein from transformed probiotic bacteria. A single colony of transformed probiotics which have pLC13.9: LDH-PRO1:GFPuv were pick up colony to 10 ml of MRS broth supplement 2.5 µg/ml erythromycin and incubated at 37 ºC for 18 hours. The bacterial cells were harvested by centrifugation at 3,000 rpm for 5 min and washed twice with PBS (pH 7.4) and resuspended in 500 µl PBS. Ten µl of cells were spread on glass slide and cover with cover slide and observed fluorescence by fluorescence microscopy. The fluorescence was detected with 395-nm excitation filter.

3.17 Site for conducing the study

Microbiology Laboratory, Collage of Medicine and Public Health, Ubon Ratchatani University and Department of Microbiology, Faculty of Medicine, Khon Kaen University

CHAPTER 4
RESULTS

4.1 Isolation of LAB from infant's feces

Totally, ten fecal samples from healthy infants in Ubon Ratchathani Province were collected for LAB isolation. For *Lactobacillus* spp. isolation, one hundred and seven colonies surrounded with clear zone on MRS agar plates supplemented with 0.5 % (w/v) $CaCO_3$ (Figure 4.1, A), indicated the lactic acid producing bacteria (LAB), were randomly selected for further bacterial identification. Based on Gram 'staining method, it was found that all selected colonies exhibited gram positive bacilli (Figure 4.1, B). All selected bacterial colonies gave negative result for catalase test. Thus, all these bacterial colonies, designated as LW isolates, were further used for probiotic properties evaluation.

For *Bifidobacterium* spp. isolation, nighty-eight colonies from two fecal samples were selected from BSM agar supplemented with 0.5 % (w/v) L-cysteine. Gram's staining revealed that all colonies, designated as LAB-Bifi, were gram positive and branched rod-shaped bacteria as shown in Figure 4.2, B.

Therefore, totally 205 bacterial colonies were purified by re-streaked on agar media and then grown in MRS broth (For *Lactobacillus* spp.) or in MRS both supplemented with 0.5 % (w/v) L-cysteine (For *Bifidobacterium* spp.). After incubation, bacterial cells were collected, suspended in MRS supplemented with 30% glycerol and stored at -80 ºC until use

(A) (B)

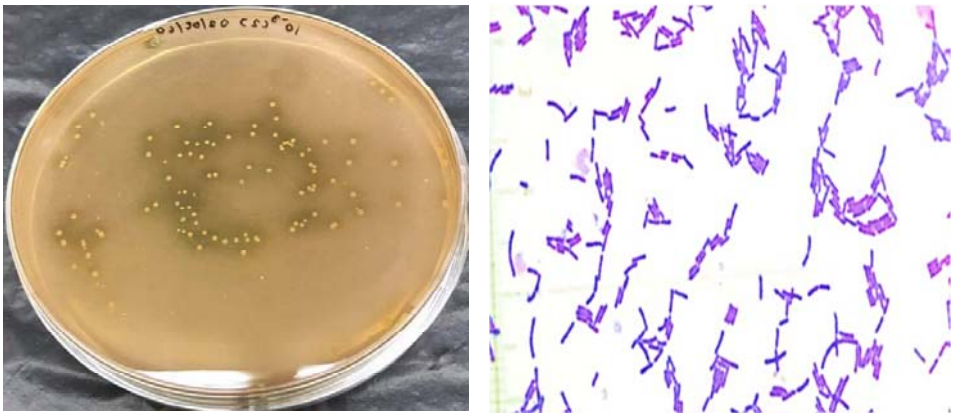

Figure 4.1 Colony and cell morphology of bacteria selected from BSM agar plates supplement 0.5 % (w/v) L-cysteine. (A) Bacterial colonies with clear zone. (B) Gram's staining of selected bacterial colony shows gram positive bacilli.

(A) (B)

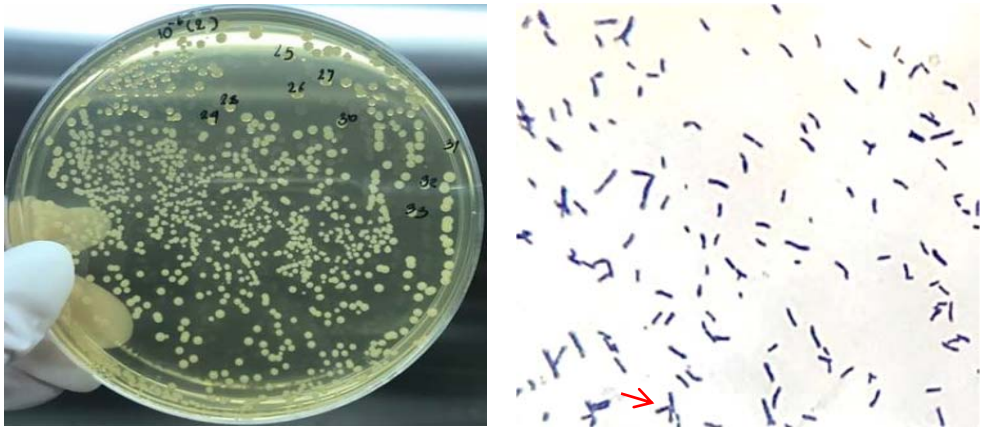

Figure 4.2 Colony and cell morphology of bacteria selected from BSM agar plates supplement 0.5 % (w/v) L-cysteine. A) Bacterial colonies B) Gram's staining of selected bacterial colony shows gram positive with branched rod-shaped as indicated by arrow.

4.2 Hemolysis activity

For determination of hemolysis activity, 107 bacterial isolates of LW showed γ-hemolysis or non-hemolysis on human blood agar as shown in Figure 4.3. Thus, all bacterial isolates were selected for further evaluating of probiotic properties.

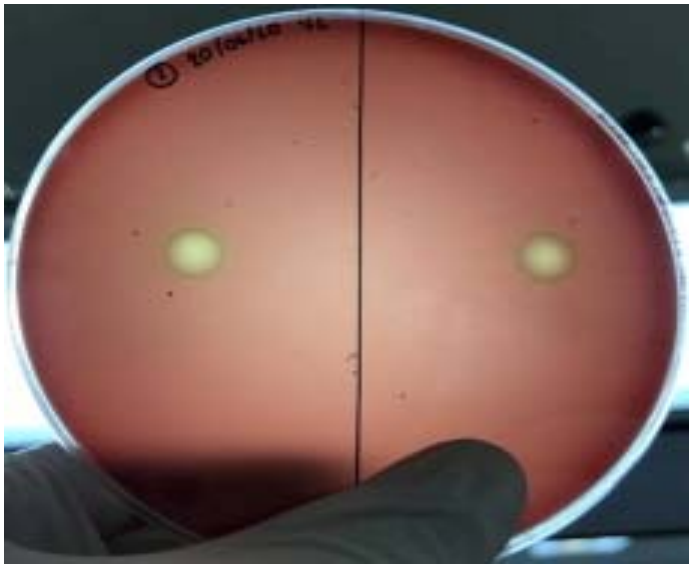

Figure 4.3 Hemolysis activity of 107 bacterial isolates on human blood agar.

4.3 Acid tolerance

To test the survival of bacteria in acid condition, 37 of 107 bacterial isolates of LW were cultured in the solution with pH 2.0 3.0 and 7.0 (control). It was found that 37 isolates did not survive at pH 2. At pH 3.0. 10 LW isolates, LW01,LW02, LW03, LW04, LW05, LW06, LW07, LW08, LW09, and LW10, showed the viability at more than 80% after 3 h exposure to acid solution (Figure 4.4, Table 4.1). This results demonstrated that 10 LW isolate show their high resistance to acid solution comparable to that of the reference strain, *Lactobacillus rhamnosus* GG (ATCC53103). Thus, 10 LW isolate were further selected for determination of bile tolerance.

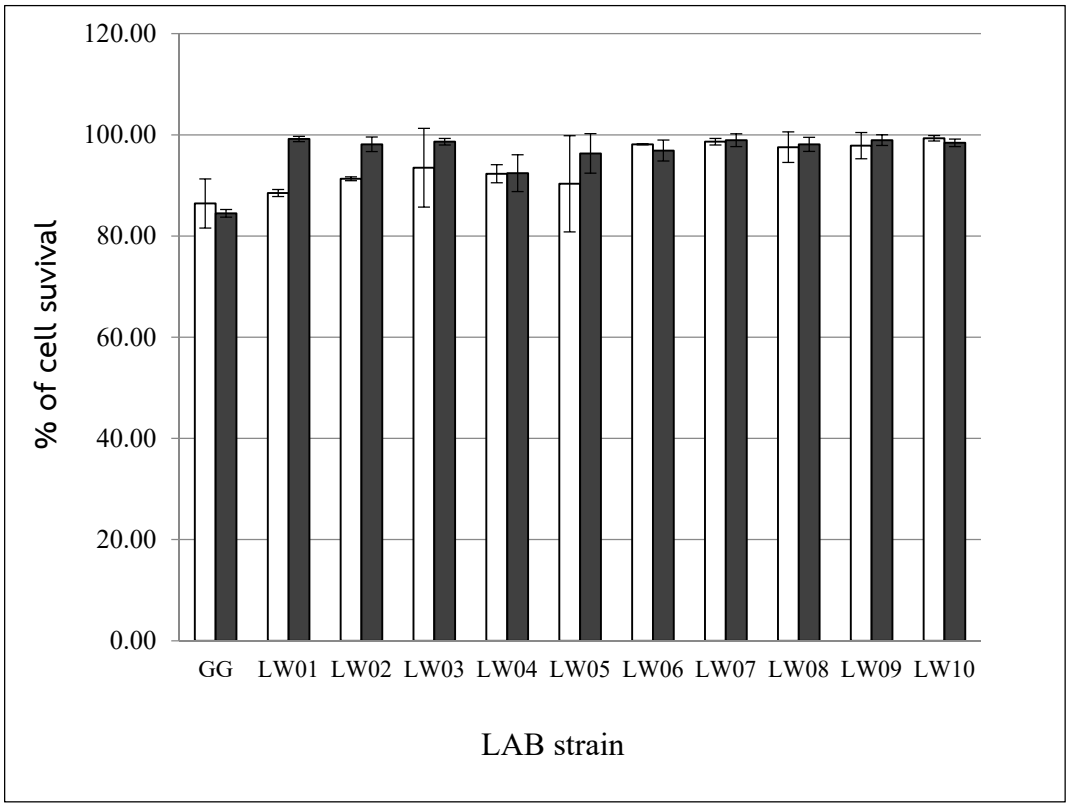

Figure 4.4 Percentage of survival of LW isolates after exposure to acid solution pH 3.0 (■) and pH 7.0 (□) for 3 h at 37 °C. The experiment was performed in duplicate. GG, *L. rhamnosus* ATCC53103.

4.4 Bile salt tolerance

Ten isolates of LW were tested for their viability in 0.3% bile solution at pH 8.0 for 4 h. All of 10 LW isolates showed the survival at more than 80% after exposure to 0.3% bile solution at pH 8.0 for 4 h. (Figure 4.5 and Table 4.1). The result could be comparable to that of the *L. rhamnosus* GG.

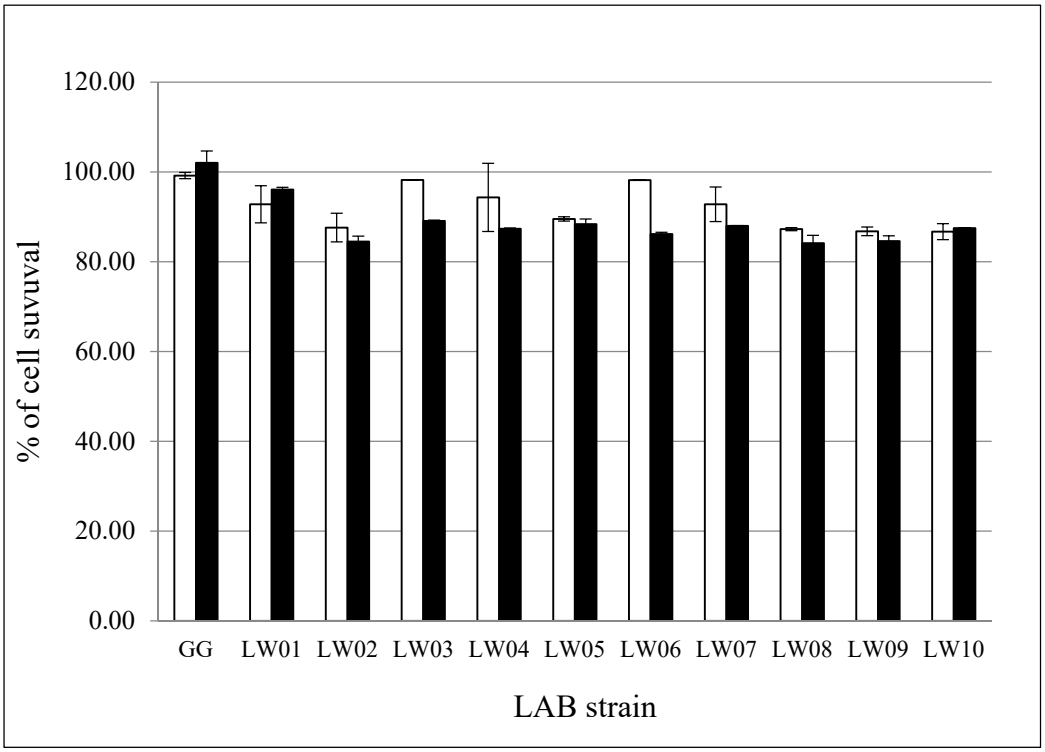

Figure 4.5 Percentage of survival of LW isolates after exposure to 0.3 % (w/v) bile solution pH8.0 (■) or MRS broth pH7.0 (□) for 4 h at 37 °C. The experiment was performed in duplicate. GG, *L. rhamnosus* ATCC53103.

Table 4.1 The percentage of survival of 10 LW isolates after exposure to acid and bile solution

Isolate	% cell survival (Mean ± SD) *		% cell survival (Mean ± SD) *	
	pH 3.0	pH 7.0	0.3 % (w/v) bile	MRS broth
GG	84.50±0.76	86.44±4.85	102.02±2.65	99.19 ±0.69
LW01	99.19±0.53	88.50±0.71	96.07±0.51	92.79±4.13
LW02	98.14±1.43	91.32±0.39	84.49±1.22	87.61±3.19
LW03	98.64±0.64	93.51±7.77	89.07±0.18	98.20±0.02
LW04	92.43±3.63	92.31±1.79	87.35±0.19	94.33±7.61
LW05	96.33±3.90	90.33±9.50	88.39±1.14	89.53±0.47
LW06	96.89±2.07	98.13±0.11	86.18±0.37	98.19±0.00
LW07	98.95±1.27	98.64±0.64	88.00±0.05	92.79±3.85
LW08	98.12±1.39	97.57±3.02	84.12±1.76	87.26±0.34
LW09	98.95±1.05	97.86±2.59	84.59±1.20	86.78±0.96
LW10	98.42±0.74	99.32±0.53	87.48±0.00	86.70±1.79

The experiment was performed in duplicate. GG, *L. rhamnosus* ATCC53103.

4.5 Antimicrobial activity

Non-adjusted pH and adjusted pH6.5 of cell-free supernatant (CFS) from 10 LW isolates were used for determining the antimicrobial activities by agar well-diffusion method. The inhibition zone around wells indicating antimicrobials activities of LW were recorded.

As shown in Figure 4.6 and Table 4.2, it was found that only non-adjusted pH CFS derived from 4 LW, LW01, LW02, LW09, and LW10 isolates showed antimicrobial effect against pathogenic bacteria. The bacterial code number of LW01, LW02, LW09, LW10 showed antimicrobial activities against to *Vibrio cholerae*. In addition, only LW01 isolate showed antimicrobial activities against to *Escherichia coli* O157:H7. All of 10 LW isolates did not show the antimicrobial effect to *Salmonella thyphimurim*. The adjust pH 6.5 Cell-free supernatant (CFS) from 10 LW isolates could not inhibit the growth of pathogenic bacteria.

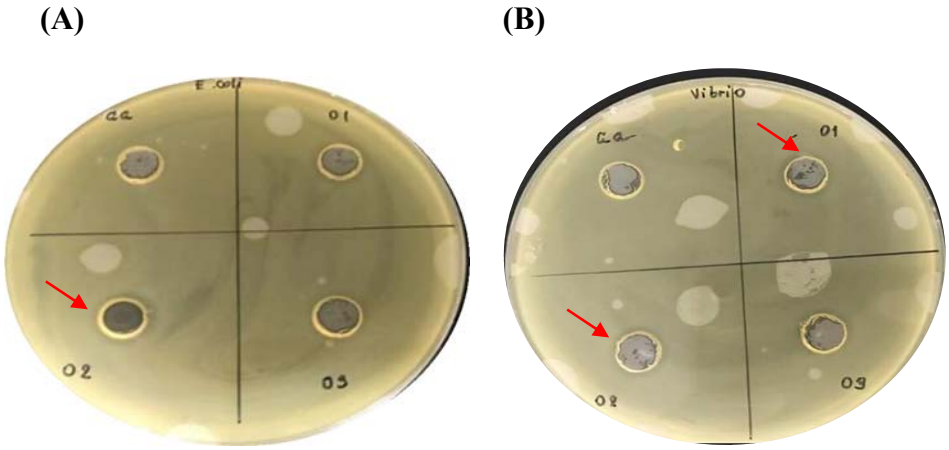

Figure 4.6 Antimicrobial activities as determined by agar well-diffusion method of on-adjusted pH supernatants. In panels A and B, *Escherichia coli* O157:H7 and *Vibrio cholerae* were used as the indicators, respectively. Key of the wells: GG, *L. plantarum* GG; 01, LW01; 02 LW02; and 03, LW03.

The table 4.2 Antimicrobials activities of ten LW isolates against pathogenic

Code No.	Original pH	Non-adjust pH (mm in diameter)			adjust pH (mm in diameter)		
		E. coli	*S. thyphimurium*	*V. cholerae*	*E. coli*	*S. thyphimurium*	*V. cholerae*
GG	4.52	-	-	-	-	-	-
LW01	3.89	+ (11)	-	+ (11)	-	-	-
LW02	4.22	-	-	+ (11)	-	-	-
LW03	4.42	-	-	-	-	-	-
LW04	4.28	-	-	-	-	-	-
LW05	4.30	-	-	-	-	-	-
LW06	4.66	-	-	-	-	-	-
LW07	4.39	-	-	-	-	-	-
LW08	4.23	-	-	-	-	-	-
LW09	4.24	-	-	+ (13)	-	-	-
LW10	4.02	-	-	+ (12)	-	-	-

(-) = Not show inhibition zone, (+) = Show inhibition zone

4.6 Antibiotic susceptibility test

The selected 10 LW isolates with acid and bile salt tolerance were used to evaluate for their antibiotic susceptibility. As shown in Table 4.3, they were susceptible to erythromycin and tetracycline, but resistant to vancomycin.

Ten LW isolates were tested for antibiotic susceptibility. All ten isolates were susceptible to 5 antibiotics including chloramphenicol, ciprofloxacin, erythromycin, penicillin and tetracycline. All ten isolated were resistance to vancomycin. Some isolates including LW03, LW04 and LW07 were showed the results as intermediate resistance to ampicillin, LW06 and LW08 were showed the results as intermediate resistance to gentamycin.

The table 4.3 Antibiotic susceptibility test of LAB isolates.

Code No.	Inhibition zone (mm in diameter)							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	Amp	**Ch**	**Cip**	**E**	**Gen**	**PG**	**T**	**V**
LW01	35 (S)	40 (S)	36 (S)	43 (S)	20 (S)	40 (S)	43 (S)	0 (R)
LW02	32 (S)	36 (S)	42 (S)	36 (S)	18 (S)	41 (S)	46 (S)	0 (R)
LW03	28 (I)	32 (S)	37 (S)	41 (S)	19 (S)	37 (S)	41 (S)	0 (R)
LW04	25 (I)	37 (S)	31 (S)	40 (S)	19 (S)	46 (S)	40 (S)	0 (R)
LW05	36 (S)	35 (S)	30 (S)	40 (S)	19 (S)	38 (S)	40 (S)	0 (R)
LW06	36 (S)	31 (S)	30 (S)	39 (S)	14 (I)	36 (S)	39 (S)	0 (R)
LW07	28 (I)	31 (S)	34 (S)	41 (S)	17 (S)	36 (S)	41 (S)	0 (R)
LW08	38 (S)	42 (S)	35 (S)	45 (S)	13 (I)	43 (S)	45 (S)	0 (R)
LW09	37 (S)	40 (S)	29 (S)	39 (S)	13 (I)	37 (S)	39 (S)	0 (R)
LW10	31 (S)	37 (S)	31 (S)	38 (S)	19 (S)	42 (S)	38 (S)	0 (R)

1 = Aminopenicillin Amp = Ampicillin 5 = Aminoglycoside Gen = Gentamycin

2 = Other, Ch = Chloramphenicol 6 = Penicillin PG = Penicillin

3 = Fluoroquinolones, Cip = Ciprofloxacin 8 = Tetracycline T = Tetracycline

4 = Macrolide, E = Erythromycin 9 = Glycopeptide, V = Vancomycin

S = sensitive, I = Intermediate resistance, R = resistance

4.7 Bacterial adhesion to the Caco-2 cell lines

The human intestinal Caco-2 cells line were used for determination of cell adhesion ability of 10 LW isolates. *Lactobacillus rhamnosus* GG was used as reference strain. As shown Table 4.4, LW09 show that highest of percentage bacterial adhesion and all tested isolates showed high adhesive ability at more than 80 % after incubation 4 h.

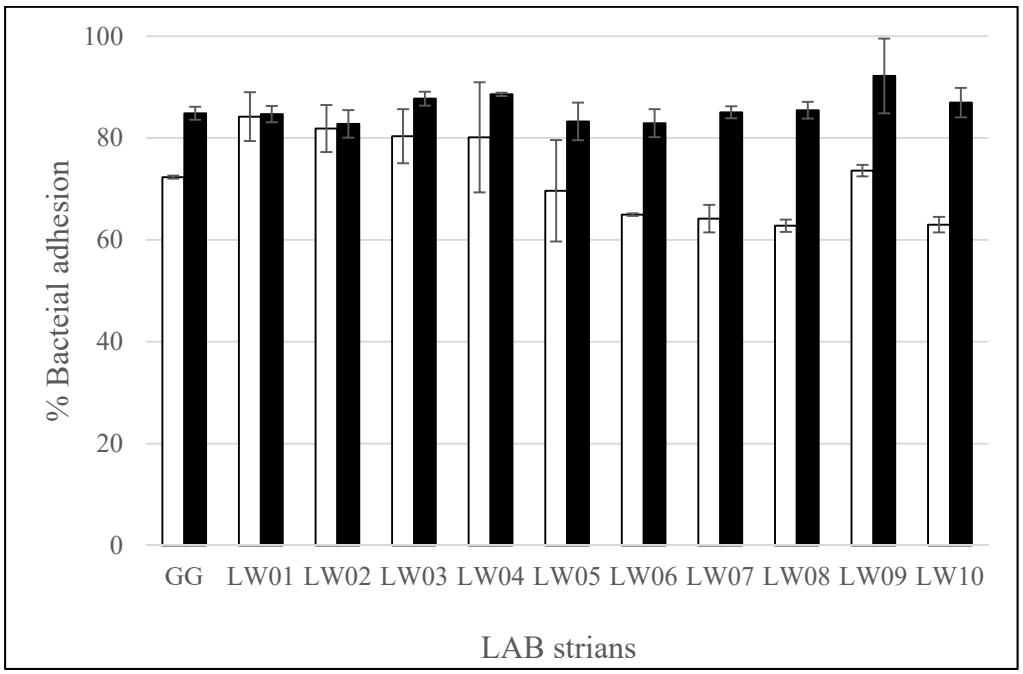

Figure 4.7 Percentage of adhesion of LW to Caco-2 cell line. (☐) after incubate 2 h (■) after incubate 4 h. The experiment was performed in triplicate. GG, *L. rhamnosus* ATCC53103.

Table 4.4 Percentage of Caco-2 cell line adhesion by LW isolates after incubation for 2 h and 4 h.

Isolates	Percentage of adhesion	
	2 hours (Mean ± SD)	**4 hours (Mean ± SD)**
GG	72.32±0.30	84.86±1.28
LW01	84.20±4.79	84.69±1.59
LW02	81.86±4.61	82.77±2.71
LW03	80.36±5.32	87.73±1.37
LW04	80.13±10.82	88.58±0.33
LW05	69.64±9.96	83.25±3.70
LW06	64.96±0.26	82.91±2.75
LW07	64.18±2.71	85.05±1.19
LW08	62.78±1.21	85.45±1.65
LW09	73.60±1.14	92.20±7.34
LW10	62.98±1.53	86.95±2.88

The experiment was performed in triplicated. GG, *L. rhamnosus* ATCC 53103

4.8 Cytotoxicity assay

All of 10 LW isolates were devoid of cytotoxic activity on the human intestinal Caco-2 cells line compared to *L. rhamnosus* GG as reference strain. The results were show in igure 4.8. Table 4.5 represents cells viable of Caco-2 cell line.

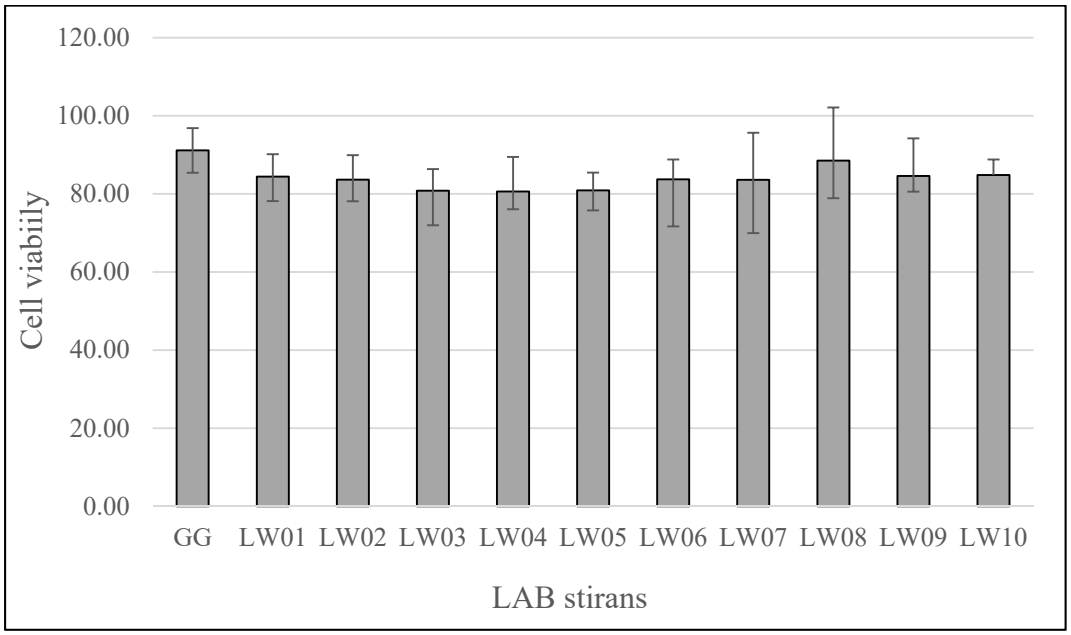

Figure 4.8 Cells viable of Caco-2 cell line after incubation with LW isolated. The experiment was performed in triplicate. GG, *L. rhamnosus* ATCC53103.

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Table 4.5 Percentage of cells viability of Caco-2 cell line

Isolates	% Cell viability (Mean ± SD)
GG	91.13±5.68
LW01	84.39±5.74
LW02	83.64±6.26
LW03	80.78±5.56
LW04	80.59±8.84
LW05	80.87±4.55
LW06	83.71±5.10
LW07	83.57±12.06
LW08	88.50±13.61
LW09	84.57±9.62
LW10	84.81±4.00

The experiment was performed in triplicated. GG, *L. rhamnosus* ATCC 53103

4.9 Molecular identification of *Lactobacillus* species

The fours LW isolates were identified by 16S ribosomal (rRNA) amplification and sequencing. The amplified 16S rRNA PCR product with the length of approximately 1,500 bp. were showed in Figure 4.9. The amplified product was purified by Gel/PCR DNA Fragments Extraction Kit, and sent for DNA sequencing. Based on nucleotide sequence analysis, it was demonstrated that *L. rhamnosus* (LW01, LW02, LW09 and LW10) could be identified as 97 to 100 % with 16S rDNA genes of several *L. rhamnosus* strains in database as shown in Table 4.6. Therefore, this result confirmed that fours isolates are belonged to *L. rhamnosus*.

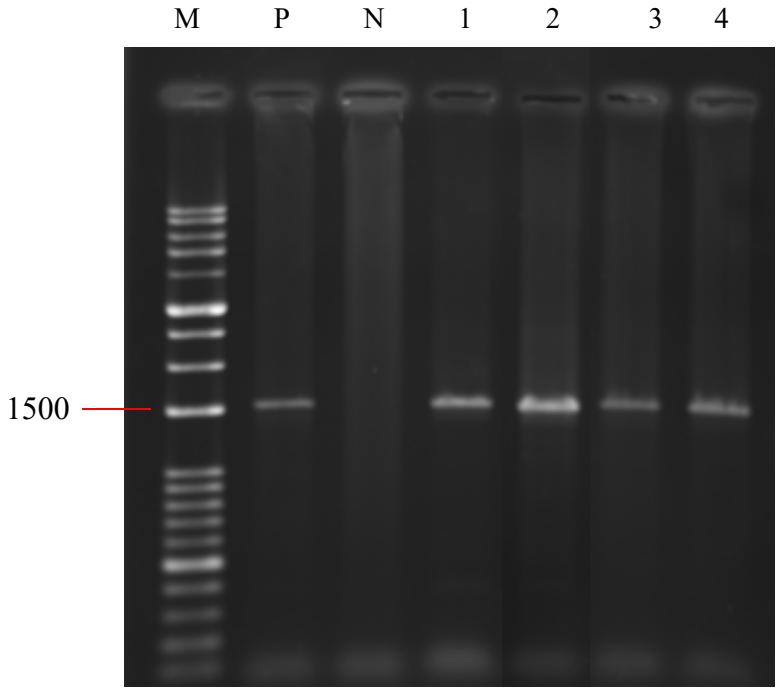

Figure 4.9 Ethidium bromide staining gel of amplify product of 16S rRNA of LW isolates, Lane P = positive control, GG, *L. rhamnosus* ATCC53103. Lane 1 = LW01, Lane 2 = LW02, Lane 3 = LW09, Lane4 = LW10) and Lane M = 1 kb plus DNA ladder

Table 4.6 The comparison of 16S rRNA sequence of *L. rhamnosus* strains with those of *L. rhamnosus* hold in NCBI database

Isolates	Gene similarity in NCBI dstabase	% of identity	Accession no.
LW01	*L. rhamnosus* strain LMEM 33 16S ribosomal RNA gene partial sequence	98 %	MK418585.1
LW02	*L. rhamnosus* strain SCH 16S ribosomal RNA gene partial sequence	100 %	MK246001.1
LW09	*L. rhamnosus* strain S16 16S ribosomal RNA gene partial sequence	97 %	KY780506.1
LW10	*L. rhamnosus* strain LR-B1 chromosome complete, genome	100 %	CP025428.1

4.10 Plasmid profile

Ten LW isolates were tested for plasmid profile. Plasmid DNA was extracted from each isolate. *Lactobacillus casei* TISTR1341, a strain containing four plasmids, was used as positive control. As shown in Figure 4.10, it was demonstrated that all LW isolates shown single plasmid DNA bands with the size of approximately of more than 13.0 kb, compared to that of *L. casei* TISTR1341.

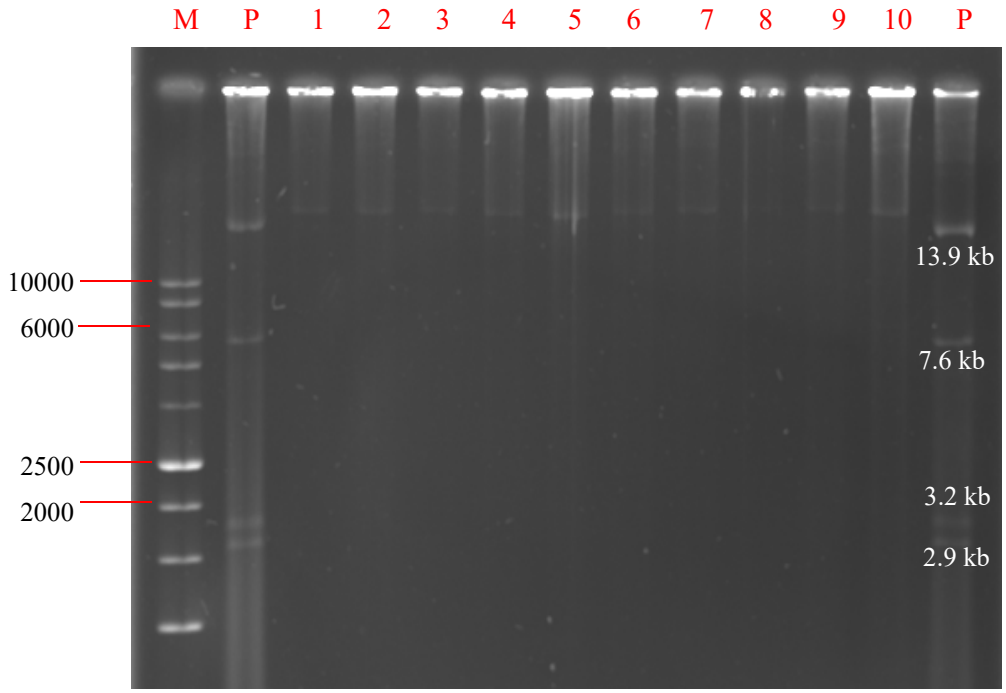

Figure 4.10 Ethidium bromide staining gel of plasmid DNA of LW isolates, Lane P = positive control, *L. casei* TISTR1341 containing pRCEID13.9, pRCEID7.6, pRCEID3.2, pRCEID2.9. Lane 1 to 10 = LAB isolates (LW01 to LW10) and Lane M = 1 kb plus DNA ladder

4.11 Verification of the plasmid constructs

To verify the plasmid constructs, the plasmid pLC13.9: LDH-PRO1: GFPuv was extracted from *E. coli* (Figure 4.11). Digestion of pLC13.9: LDH-PRO1: GFPuv with *Eco*RI or *Ncol* resulted in different digested DNA fragments (Figure 4.12) After *Eco*RI digestion, three fragments with the size of 1,019 bp., 2,185 bp., and 3,102 bp (Figure 4.12, Lane). were obtained. Digestion of plasmid with *Nco*l showed a band of 6,200 bp.

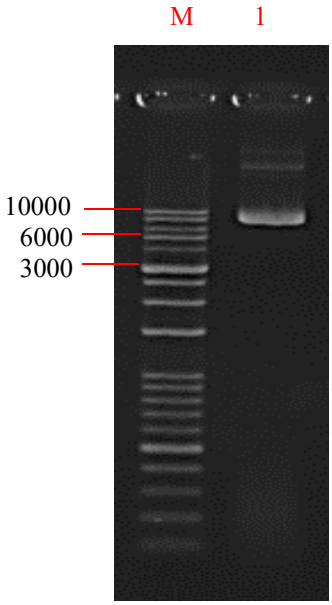

Figure 4.11 Ethidium bromide staining gel of amplified product of pLC13.9: LDH- PRO1: GFPuv from *E.coli* , Lane 1= pLC13.9: LDH-PRO1: GFPuv and Lane M = 1 kb plus DNA ladder

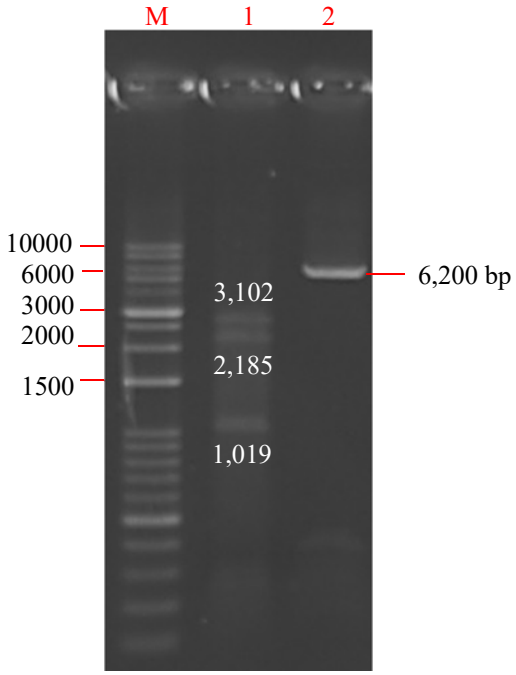

Figure 4.12 Ethidium bromide staining gel of restriction enzyme-digested pLC13.9:LDH- PRO1:GFPuv from *E.coli*, Lane 1= *Eco*RI - digested pLC13.9:LDH-PRO1:GFPuv, Lane 2 = *Nco*l - digested pLC13.9:LDH-PRO1: GFPuv and Lane M = 1 kb plus DNA ladder

4.12 The expression of GFPuv

4.12.1 *Escherichia coli* DH5α

The domination ability to express GFPuv protein in *E. coli*. After electrotransformation, the transformed *E.coli* DH5α could be control the expression GFPuv protein (Figure 4.13).

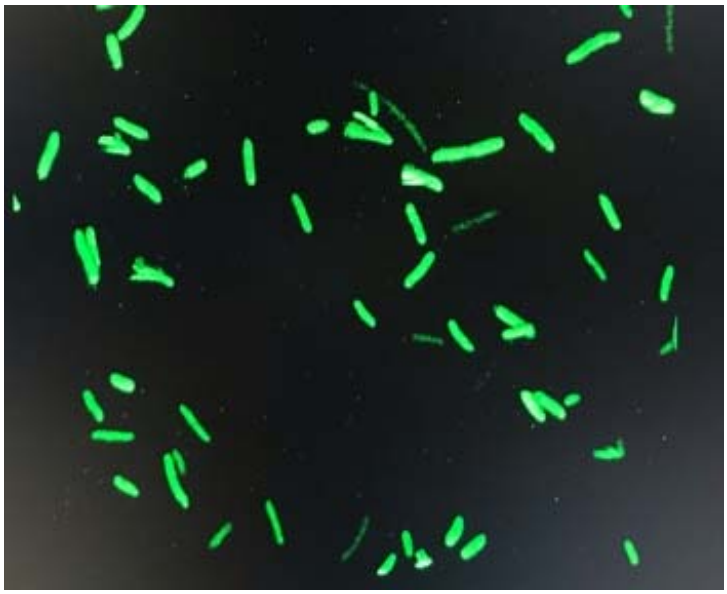

Figure 4.13 The transformed *E. coli* DH5α:GFPuv+ cells showed fluorescent under fluorescent microscopy

4.12.2 *Lactobacillus* species

The domination ability to expression GFPuv protein in *Lactobacillus* species including *L. rhamnosus* LW01, *L. rhamnosus* LW02, *L. rhamnosus* LW03 and *L. rhamnosus* LW04 After electrotransformation, the fours transformed *L. rhamnosus* could be control the expression GFPuv protein comparing positive control is *L casei* TISTR1341 and negative control is plasmid free *L casei* TISTR1341. (Figure 4.14).

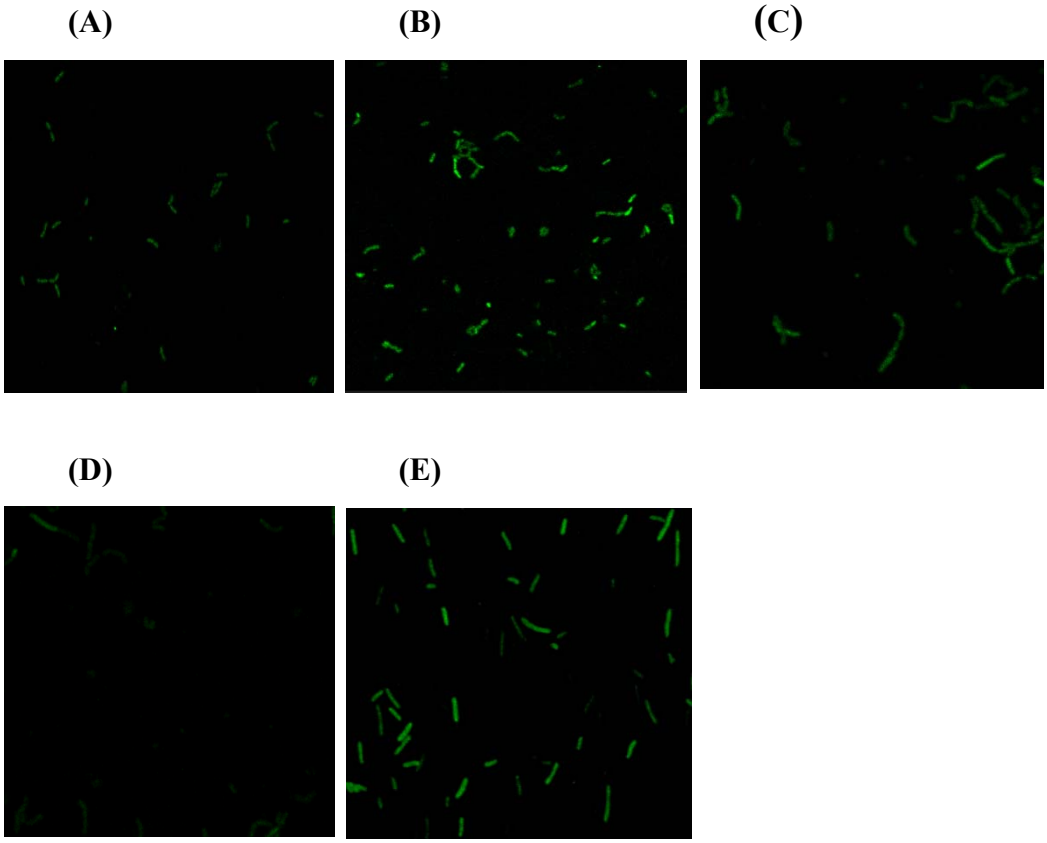

Figure 4.14 The transformed *L. rhamnosus* : GFPuv+ cells showed fluorescent under fluorescent microscopy. A) *L casei* RCEID02 (positive control), B) *L. rhamnosus* LW01, C) *L. rhamnosus* LW02, D) *L. rhamnosus* LW09, E) *L. rhamnosus* LW10

4.13 Verification of pLC13.9: LDH-PRO1 in *Lactobacillus* species

To verify the able to replicate and express fluorescent protein (GFP) gene in *Lactobacillus* species. pLC13.9: LDH-PRO1 were done extracted plasmid from *Lactobacillus* species (Figure 4.15). The results showed band plasmid DNA *L casei* RCEID02. Then, pLC13.9: LDH-PRO1 were transformed into *E. coli* DH5α and extract plasmid (Figure 4.16).

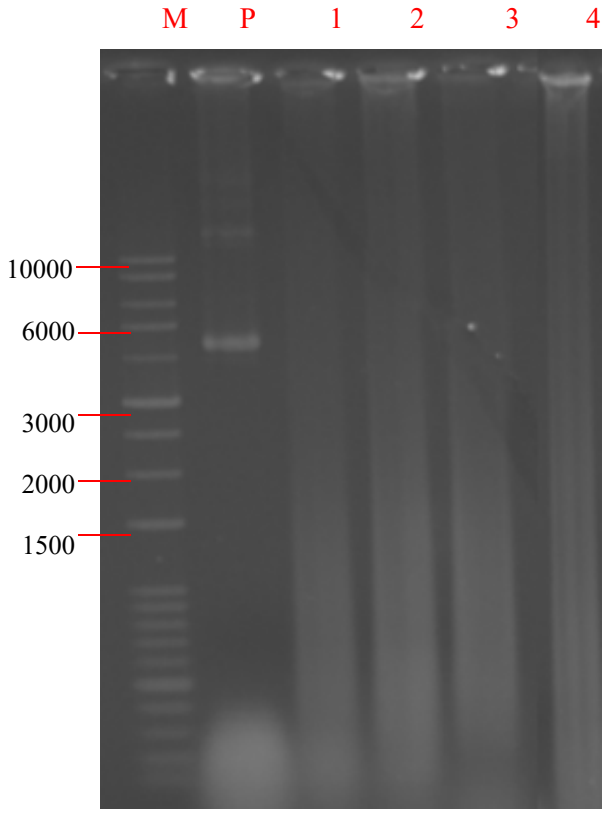

Figure 4.15 The pLC13.9: LDH-PRO1 extraction of *Lactobacillus* species. Lane P = *L casei* RCEID02 (positive control), Lane 1 = *L. rhamnosus* LW01, Lane 2 = *L. rhamnosus* LW02, Lane 3 = *L. rhamnosus* LW09, lane 4 = *L. rhamnosus* LW1, Lane M = 1 kb plus DNA ladder

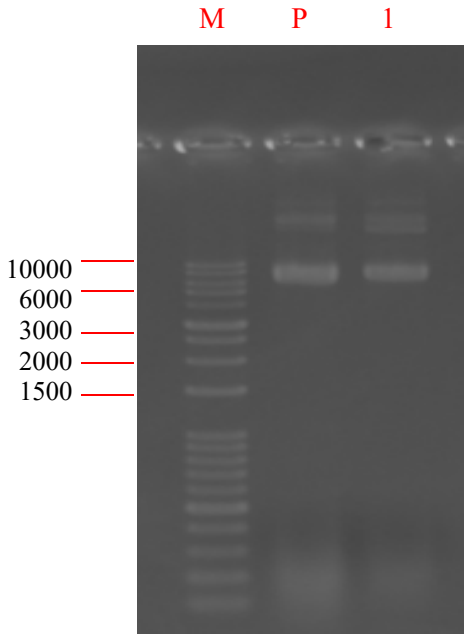

Figure 4.16 The pLC13.9: LDH-PRO1 extraction of *E. coli.* Lane P = plasmid from transformants of *L.casei* RCEID02 (positive control), Lane 1 = plasmid from transformants *of L. rhamnosus* LW10

CHAPTER 5
DISCUSION AND CONCLUSION

Nowadays, probiotic has been interested for developing as an alternative vaccine delivery vehicle for oral mucosal immunization (Trombert, 2014). Several probiotics can be bacteria and fungi, but most of probiotic are member of the lactic acid bacteria (LAB) especially lactobacilli and bifidobacterium species (Harzallah and Belhadj, 2013). These two species bacteria have been reported for the isolation and characterization from different sources including food products (milk, meat, vegetables), mouth, intestine and vagina of the human and mammals (Borriello et al., 2003). In this study, LAB from fecal sample of healthy infants were isolated and investigated of probiotic properties such as hemolysis activity, survival in gastrointestinal conditions, antimicrobial activity, antibiotic susceptibility, cytotoxicity assay, and adhesion assay.

In this study the total 107 bacterial isolates with clear zone around their colonies were selected for determining of bacteria cell morphology by Gram' staining method. The two-hundred and five colonies had gram positive bacilli and Gram-positive branched bacilli, respectively. One of the primitively criteria used to determine the probiotic properties and safety for the human is the lysis of blood by bacteria, termed hemolysis (Abdullah and Ananda, 2018), This study found that all 107 bacterial isolates were not lysed the human blood as indicated by γ-hemolysis, thus these bacterial isolates could be considered as safe and selected for further probiotic characterization.

Survival of bacteria in the harsh condition presented in human gastrointestinal tract including low acid in the stomach and high bile content in the small intestine is important criteria for selection of probiotic to be used as vaccine delivery vehicle (Borriello and Hammes, 2003) (Collado and Sanz, 2007). In this study, ten of thrifty-seven LAB isolates, LW01 to LW10 showed their high resistance to acid solution at pH 3 and to bile salt comparable to that of the reference strain, *Lactobacillus rhamnosus* GG (ATCC53103). The results correlated with previous findings regarding

The variation of bile tolerance among the same species of lactobacilli and bifidobacterium were able to survive the extreme acidic conditions of gastric juice in vitro (Uraipanand Hongpattarakere, 2015).

Antibiotic susceptibility test found that ten LAB isolates showed the resistance vancomycin. Previous studies have also reported high level of resistance to vancomycin. Several species of Lactobacillus are intrinsically resistant to vancomycin such as *L. fermentum, L. acidophilus* and *L. plantarum* (Saarela et al., 2000). The vancomycin resistance in some species of *Lactobacillus* spp. (e.g., *L. rhamnosus, L. casei, L.plantarum, L. brevis, L. curvatus*) has been reported to be chromosomally encoded and not inducible or transferable (Kirtzalidou et al., 2011). Other studies have also reported that some *Lactobacillus* strains are known to be naturally resistant to vancomycin, and such resistances are usually intrinsic, chromosomally encoded, not transmissible and do not usually form a safety concern (Saarela et al., 2000) (Ammor, Florez and Mayo, 2007).

The inhibition of enteropathogenic bacteria appeared to be due to the production of organic acids or hydrogen peroxide produced by the *Lactobacillus* strains. Previously, several studies have reported that a pH-dependent mechanism was involved in the antimicrobial activity of *Lactobacillus* strains (Tomas and et al., 2011). Previous reported that some Lactobacillus strain isolated from healthy infant feces significantly inhibit the growth of pathogenic (Coconnier et al., 1997). In accordance with other reports, in this study, the two LAB isolates of LW01 showed antimicrobial activities against to *Escherichia coli* O157:H7. The four LAB isolates of LW01, LW02, LW09, and LW10 showed antimicrobial activities against to *Vibrio cholerae*.

The ability of probiotics to adhere to the epithelial cells of host intestinal tract is crucial for establishing colonization and exerting health benefit to the host. Therefore, adhesion to epithelial cells has been considered as one of the selection criteria for probiotics strains (Ouwehand et al., 1999). In the present study, the Caco-2 cell line was used as a model for the investigation of lactobacilli adhesion to the intestinal epithelium because it can differentiate and closely resemble enterocytes of the human small intestine. All ten LAB isolates showed high adhesive ability at more than 80 % after incubation for 2 and 4 h. The adhesion of Lactobacillus to epithelial cells may occur through the specific or non-specific adhesion between epithelial cells and

bacterial surface components. Several surface molecules have been reported to contribute to specific and/or non-specific adhesion of lactobacilli to host epithelial cells, for example, mucus-binding proteins, mannose-specific adhesions, S-layer proteins, lipoteichoic acid and exopolysaccharides (Kleerebezem et al., 2010). Adhesion to host epithelial cell is also a key process for pathogenic bacteria since it allows bacteria to release enzymes and toxins to initiate the invasion into host cells and cause diseases (Nantavisai et al., 2018).

In this study, the ten of LAB isolates were devoid of cytotoxic activity on the human intestinal Caco-2 cells line compared to *L. rhamnosus* GG as reference strain. Recent reports supported the safety of lactobacillus as no cytotoxic effect was observed against Caco-2 cells during in vitro studies (Messaoudi et al., 2012). This strengthens the potential probiotic grade of these lactobacilli strains, but these results have to be confirmed in vivo by an acute toxicity study on animal model. Based on molecular identification of LW isolates include LW01, LW02, LW09 and LW10. The four LW isolates belong to the *L. rhamnosus* species.

The plasmid of LW isolates which have the size of approximately of more than 13.0 kb. Lactic acid bacteria generally contain plasmids. the plasmid size was usually 1.9 kb-84.8 kb. Most of the plasmid was less than 20 kb. In the culture process from generation to generation, many plasmids might disappear from the bacterial cell, but most of the plasmids were stable. In the study, the plasmids of the above six strains and the antibiotic susceptive showed no changes after cultivated 30 generations (Charteris et al., 1998). Several plasmids encode some important properties, including antibiotic resistance, exopolysaccharide biosynthesis, chloride or potassium transport, bacteriophage resistance, as well as bacteriocin production (Cui et al., 2015). Previous studies, the three cloning vectors pWCFS104, pWCFS105 and pWCFS106 in rifampin-resistant L. plantarum NZ7109 was determined the plasmid stability. The result found that the stability is segregationally and copy number 0.3-11.9 as low copy (Van Kranenburg and et al., 2005).

pRCEID-LC13.9:LDH:PRO1:GFPuv, the plasmid containing GFPuv gene with L-lactate dehydrogenase (*ldhL*)promote (Panya et al., 2012). Green fluorescent protein expressed under the fluorescent microscopy. In this study, the LW isolates which had been identified as *L. rhamnosus* species. Electrotransformation of plasmid

pLC13.9:pLDH-PRO1:GFPuv to *L. rhamnosus* LW10 isolates found that this plasmid can able to acquire and permissible for the pRCEID-LC13.9 is similar to that of *L. fermentum* 47-7 (Yotpanya et al., 2016). *L. rhamnosus* species isolated from infant feces can be successfully engineered to express GFP. This result indicated that this *L. rhamnosus* species could be use engineered with heterologous replicon-based expression vector for expression other interesting proteins. Therefore, the four *L. rhamnosus* species could be further study in vitro and exploited as protein delivery vehicle for various biotechnological applications.

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APPENDICES

APPENDIX A
CHEMICALS, ENZYMES AND ANTIBIOTICS

1. Chemicals

All chemical used in the study were biomolecular grade. Name and sources of chemicals are listed below.

Chemicals	Sources
Absolute ethanol	VWR BDH Prolabo
Acetic acid glacial	amresco
Agarose	amresco
Ethidium bromide	VWR BDH Prolabo
Glycerol	amresco
Hydrochloric	VWR BDH Prolabo
Phosphate buffer solution	amresco
Phenol Chloroform Isoamyl	Sigma Chemical Co.
Polyethylene glycol 8000	Sigma Chemical Co.
Potassium chloride	Vivantis
Sucrose	Vivantis
Sodium chloride	Vivantis
Sodium dodecyl sulphate (SDS)	Vivantis
Sodium hydroxide	Vivantis
Tris (Hydroxymethyl amino methane)	Vivantis

2. Enzymes

All enzyme used in this study were biomolecular grade. Names and sources of enzymes are listed below

Enzymes	**Sources**
Lysozyme	Vivantis
Mutanolysin	Sigma Chemical Co.
Protease K	Invitrogen
RNase A	RBC Bioscience
Taq polymerase	RBC Bioscience

3. Antibiotic

All enzyme used in this study were biomolecular grade. Names and sources of enzymes are listed below

Antibiotic	**Sources**
Ampicillin	amresco
Chloramphenicol	amresco
Ciprofloxacin	amresco
Erythromycin	amresco
Gentamycin	amresco
Penicillin G	amresco
Tetracycline	amresco
Vancomycin	amresco

APPENDIX B
MEDIA AND BUFFER PREPARATION

1. Media for Lactic acid bacteria

Media	Sources	For 1 liter of preparation	
1.1 MRS	LAB M	55	g
BSM	LAB M	55	g
BHI	HiMedia	52	g

2. Media for *Escherichia coli*

2.1 2xTY medium

Tryptone	16	g
Yeast extract	10	g
NaCl	5	g

Add DW to final volume 1000 ml

2.2 2xTY medium

Tryptone	16	g
Yeast extract	10	g
NaCl	5	g
Bacto agar	15	g

Add DW to final volume 1000 ml

2.3 SOB medium

Bacto tryptone	20	g
Bacto yeast extract	5	g
NaCl	0.5	g
1 M KCl	2.5	ml

Add DW to final volume of 1000 ml and 1 ml of 1 M $MgCl_2$ to each 100 ml aliquot.

2.4 SOC medium

Add 1 ml of 1 M filtered sterile glucose into 100 ml of SOB medium

3. Reagents for agarose gel electrophoresis

Reagent	Sources	For 1 liter of preparation	
TBE	amresco	17	g

3.1 Agarose gel preparation

Agarose gel 1 X of TBE		
1 % agarose	1	g
DW	100	g

3.2 Agarose gel preparation for plasmid preparation

Agarose gel 1 X of TBE		
0.7 % agarose	1	g
DW		
	100	g

4. Reagents for LAB genomic DNA and plasmid preparation

4.1 STE buffer

1 M Tris-HCl pH 8	2.5	ml
0.5 M EDTA	5	ml
Sucrose	5	ml
Add DW to final volume of 100 ml		

4.2 TE buffer

1 M Tris-HCl pH 8	1	ml
0.5 M EDTA	200	µl
Add DW to final volume of 100 ml		

4.3 Alkaline lysis solution

10 % SDS	3	ml
1 N NaOH	2	ml
Add DW to final volume of 10 ml		

4.4 3 M sodium acetate (pH 4.8)

4.5 70 % ethanol

Absolute ethanol	70	ml
Add DW to final volume of 10 ml		

APPENDIX C
RESEARCH PUBLICATION


การประชุมวิชาการเสนอผลงานวิจัย
ระดับบัณฑิตศึกษาแห่งชาติ ครั้งที่ 44
NGRC 44
The 44th National Graduate Research Conference (NGRC 44)

สารบัญ

The 44th National Graduate Research Conference (NGRC 44)

การคัดแยก จำแนก และศึกษาคุณสมบัติของโพรไบโอติกจากอุจจาระเด็กแรกเกิด

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บทคัดย่อ

โพรไบโอติก)Probiotics (เป็น เชื้อจุลินทรีย์ที่มีความปลอดภัยสูง และไม่ก่อโรค ปัจจุบันมีการป้องกันและรักษาโดยใช้โพรไบโอติกเป็นตัวนำแอนติเจนเข้าสู่ร่างกาย เพื่อกระตุ้นระบบภูมิคุ้มกันในร่างกาย การวิจัยในครั้งนี้มีวัตถุประสงค์เพื่อคัดแยก จำแนก และศึกษาคุณสมบัติของโพรไบโอติกที่แยกได้จากอุจจาระเด็กแรกเกิดที่เกิดโดยวิธีธรรมชาติและได้รับน้ำนมแม่ คุณสมบัติโพรไบโอติกที่ศึกษา ได้แก่ การย่อยสลายของเม็ดเลือดแดง การทนต่อกรดและเกลือน้ำดี จากการทดลองการคัดแยกอุจจาระเด็กแรกเกิด จำนวน 7 ตัวอย่าง พบว่าสามารถคัดแยกโพรไบโอติกได้ 10 ไอโซเลต ได้แก่ LW01 LW02 LW03, LW04 LW05 LW06 LW08 LW09 และ LW10 ซึ่งทั้ง 10 ไอโซเลต มีคุณสมบัติเป็นแบคทีเรียแกรมบวก รูปร่างแท่ง ไม่สามารถย่อยเม็ดเลือดแดงคน สามารถทนต่อสภาวะความเป็นกรดและเกลือน้ำดีได้มากกว่า 80 เปอร์เซ็นต์ ดังนั้นเชื้อโพรไบโอติกเหล่านี้จะนำไปทดสอบในขั้นตอนต่อไป ได้แก่ ความไวต่อยาปฏิชีวนะ การสร้างสารยับยั้งเชื้อก่อโรค การเกาะติดเซลล์ลำไส้ของคน และการประยุกต์ใช้เป็นสายพันธุ์โพรไบโอติกในคน เช่น การพัฒนาไปเป็นวัคซีนเชื้อเป็นชนิดกิน หรือ oral live vaccine เพื่อใช้ในการป้องกันและรักษาโรคต่างๆ ในคนต่อไป

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